TRANSLATIONAL RESEARCH IN SERIOUS HUMAN DISEASES

Vydalo Nakladatelství Academia,
Vodičkova 40, Praha 1

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1 Development of the nervous system

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Abstract:
Comparative studies of brain development in several mammalian species confirmed that the prenatal and postnatal development of the minipig brain resembles from many aspects of development of the human CNS. Also basal ganglia of minipigs have similar anatomical structure as human basal nuclei. All included data confirm that minipigs can be considered as the optimal biomedical model for neurodegenerative diseases.

Keywords:
pig, minipig, prenatal and postnatal brain development, telencephalon, basal ganglia

INTRODUCTION

During the last 20 years, pigs and minipigs have become a unique large animal model in translational neuroscience research[1,2]. Therefore, the book "Translational Research in Serious Human Diseases" is prevalently focused to the serious human diseases that affect cells that originate from neuronal precursors and neural crest cells. Therefore, the first chapter elucidates ontogenesis of these cells and tissues to prepare a solid background for chapters dealing with neurodegenerative diseases, eye diseases, spinal cord injury and tumorogenesis of melanoma cells.

Mission of all experiments included in the book is to develop and evaluate new concepts in our laboratories and offer them into clinical trials that can bring hope to change the lives of patients and their families.

The development of the nervous system in mammals involves the studies of neuroscience and developmental biology or embryology to describe the cellular and molecular mechanisms

by which complex nervous systems form and develop during prenatal development and continue to develop after birth to adolescence.

PRENATAL DEVELOPMENT OF THE NERVOUS SYSTEM

The key steps in neural development of embryo include differentiation of neurons from stem cell precursors. The notochord sends chemical signals to the overlying ectoderm, inducing it to become neuroectoderm. This results in a strip of neuronal stem cells that runs along the back of the embryo. This strip is called the neural plate, and it is the origin of the entire nervous system, neurons and glial cells. The neural plate folds to create the neural tube which is filled with cerebrospinal fluid (CSF). Both neurons and neuroglia, non-neuronal cells in the central nervous system, originate from the ventral part of the neural tube. While neuronal cells leave ventricular zone and enter in postmitotic stage, precursors of glial cells keep an ability to divide even after they reach their final position in the neural system.

The paired strips of cells arising from the ectoderm above the neural tube is called the neural crest. These cells migrate in many different locations and differentiate into many cell types within the embryo and they contribute to the peripheral nervous system (both neurons and glia) consisting of sensory ganglia (dorsal root ganglia), sympathetic and parasympathetic ganglia.

As the fetus develops, the anterior part of the neural tube forms three brain vesicles, which become the primary anatomical regions of the brain. The forebrain divides in telencephalon, the future cerebral cortex and basal ganglia, and diencephalon, the future thalamus and hypothalamus. Later the optical vesicle is evident, it will become the optic nerve, retina and iris. The midbrain forms pons and cerebellum and hindbrain represents medulla oblongata. The spinal cord forms from the lower part of the neural tube. The central chamber, which is continuous from the telencephalon to the spinal cord, is filled with CSF and develops into the ventricular system of the central nervous system (CNS).

The further process of neurogenesis is characterized by the migration of immature neurons from their birthplaces in the fetus to their final positions in the brain. Once the neurons have reached their regional positions, they extend axons and dendrites, which allow them to communicate with other neurons via synapses. Synaptic communication between neurons leads to the establishment of functional neural circuits.

Glia, also called glial cells or neuroglia, are non-neuronal cells in the central nervous system. They originate from the ventral part of the neural tube as neuronal cells. The glial cells surround neurons and provide support for them and wedge between them. Glial cells are the most abundant cell types in the CNS. Glial cell types include oligodendrocytes, astrocytes, ependymal cells, Schwann cells, microglia, and satellite cells. Astrocytes account for more than 50 % of the total cells in the brain and they are 10 times more abundant than neurons.

Astrocytes play a pivotal role in the homeostasis, neuronal synaptogenesis and maintenance of myelination[3].

POSTNATAL DEVELOPMENT OF MINIPIG BRAIN

The Göttingen minipig was developed in 1961–1962 at the Institute of Animal Breeding and Genetics of the University of Göttingen (Germany). The present characteristics of the Göttingen minipig, as a small, white, miniature pig with good fertility and stable genetics, were obtained as a result of crossbreeding the Minnesota minipig with the Vietnamese potbelly pig and the German Landrace[4]. Libechov minipigs originate from imports from the University of Göttingen (1968) and the University of Minnesota (1969) and crosses of these two strains with one generation of Lantrace resulted in two different strains:

MeLiM – Melanoma Libechov minipigs of the black color and baring hereditary skin melanoma (see Chapter 21). Similarly, Sinclair minipigs develop a malignant spontaneously regressing melanoma.

The white color Libechov minipigs is characterized with the straight back, the essential feature for spinal cord injury experiments, with 6 to 8 piglets in the litter, optimal viability and growth rate and sexual maturity in 5 months in both sexes[5].

The pig brain, like that of humans, develops perinatally, with the accelerating brain growth extending from midgestation to early postnatal life[6,7].

This is in contrast to other mammalian species, e.g. the brain of guinea pig, sheep and monkey, which has a prenatal growth spurt, or the brain of rat and rabbit, which develops postnatally[8]. The development of the pig brain is also considered more similar to the human brain with respect to myelination, compositions and electrical activity[9,10]. Quantitative studies based on DNA quantification in the human brain have indicated that the major phase of neuronal multiplication occurs during the first half of gestation, prior to the numerically larger but slower phase of glial multiplication, which continues into the first postnatal years[7]. The two-phased growth pattern has similarly been observed in a stereological study on total cell numbers in the developing human fetal forebrain[11].

In an attempt to further evaluate the pig as a potential model for human brain development and to provide a quantitative structural basis for comparative and experimental studies, a number of quantitative examinations on the neonate and adult pig brain have been done at the Research Laboratory for Stereology and Neuroscience in Copenhagen[12]. The total number of neocortical neurons in the Göttingen minipig brain increases from ~253 million at birth (neonate piglet, 1 day old) to ~324 million in adulthood[13]. This significant 28% difference demonstrates a pronounced postnatal development of neurons in the Göttingen minipig brain[14]. A significant postnatal development is also observed for neocortical glial cells (P<0.01), increasing from ~382 million in the neonate to ~714 million glial cells in the

adult Göttingen minipig, which makes 87% difference[15]. The glial-to-neuron ratio changes accordingly from 1.5 to 2.2. The total brain mass increases almost threefold from the mean of 27.8 g at birth to 79.0 g, as adult. It is furthermore interesting that the sulcal pattern of minipig cortex develops with time e.g. primary sulci are established at birth but secondary and tertiary sulci develop and become more prominent during postnatal life (Carsten Bjarkam, personal communication).

All results achieved with neonatal and adult Göttingen minipigs document that the prenatal and postnatal development of the minipig brain resembles from many aspects of development of the human CNS[15]. All above mentioned data confirm that minipigs can be considered as the optimal biomedical model[2].

THE TELENCEPHALON OF THE MINIPIG BRAIN

The surface anatomy and cytoarchitecture of the Göttingen minipig telencephalon based on macrophotos and consecutive high-power microphotographs of 15 µm thick paraffin embedded Nissl-stained coronal sections was described by team headed by C. Bjarkam[12]. In 1-year-old specimens the formalin perfused brain measures approximately 55 × 47 × 36 mm (length, width, height) and weighs around 69 g. The telencephalic part of the Göttingen minipig cerebrum covers a large surface area. The neocortical gyrencephalic part located dorsally is divided into frontal, parietal, temporal, and occipital lobes. The important motoric cortex for Huntington's disease studies is located in the dorsal portion of the frontal lobe. The inner subcortical structure of the minipig telencephalon is dominated by a prominent ventricular system and large basal ganglia, where the putamen and the posterior and dorsal caudate nucleus are separated into two entities by the internal capsule, whereas both structures ventrally fuse into a large accumbens nucleus[16]. The telencephalic coronal sections are presented at http://www.cense.dk/minipig_atlas/index.html. Similarly, cortical sections of the Libechov minipig brain document gyrencephalic structure of Libechov minipigs.

BASAL GANGLIA

The basal ganglia (or basal nuclei) are a group of subcortical nuclei in the brains of vertebrates which are situated at the base of the forebrain and top of the midbrain. They represent a group of highly interconnected nuclei involved in a variety of functions, prevalently movement and cognition[16,17]. The following nuclei are included in the basal nuclei: Caudate nucleus, putamen, globus pallidus, substantia nigra, and subthalamic nucleus. The putamen and globus pallidus are sometimes grouped and called the lentiform or lenticular nucleus. The caudate and the putamen grouped together are called the striatum (or neostriatum).

These three main parts – caudate, putamen, and globus pallidus – when grouped together are referred to as the corpus striatum. The major inputs to the basal ganglia arise in the motoric cortex and thalamus and are carried by the corticostriatal and the thalamostriatal pathways. The basal ganglia then influence behavior by these structures projecting to thalamus and to other subcortical structures involved in movement. The Göttingen minipig nucleus accumbens forms a large ventral striatal structure that can be divided into a core and is in tight connections with other basal ganglia structure[16].

Libechov minipig brains were prepared for histological examination according to ref. 18. The coronal sections were stained with Darp32 antibody for the medium spiny neurons (MSN) in striatum and distribution of wild-type huntingtin with (EPR 5526) in motor cortex.

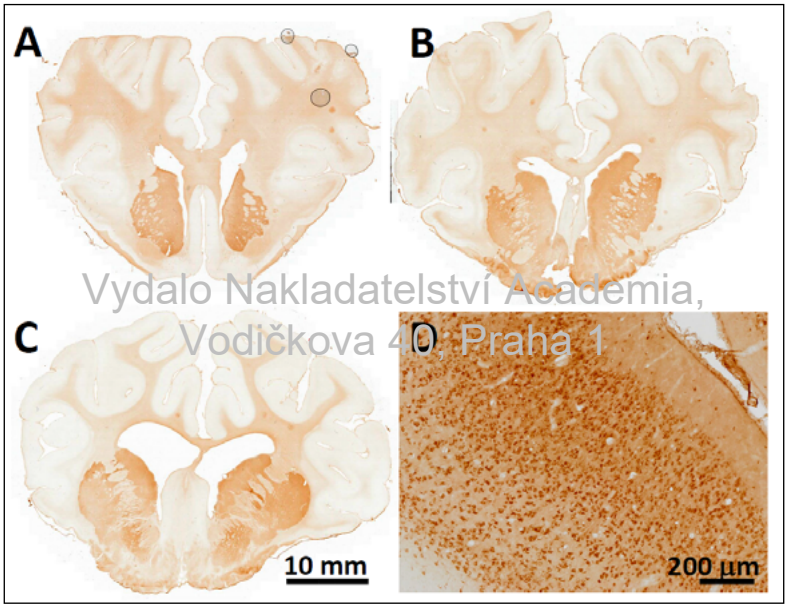

Figure 1. Adult brain transverse corticostriatal sections. Darpp32 IHC staining of transverse corticostriatal sections at 20mm, 24mm and 28mm distance (A, B, C respectively) from rostral apex of minipig prefrontal cortex show Darpp32 positive MSN in NC, Put and their projections to cortical areas. D, motor cortex cytoarchitecture revealed by IHC evaluation of Huntingtin protein expression. Orig. J. Klíma

CONCLUSION

The bulk of recently published data in the well-recognized journals brought several methodological approaches how the glia-to-neuron conversion approach can regenerate new neurons either *in vitro* or inside mouse brain and spinal cord. This approach promises a hope

that lost neurons due to neurodegeneration, brain or spinal cord injuries or stroke could be replaced. Generation of a sufficient number of new neurons for therapeutic applications will require the precise knowledge about CNS morphology and cellular structure because of the heterogeneity of astrocytes throughout the CNS [17].

The team around Carsten Bjarkam prepared the Göttingen minipig brain atlas as a unique tool for each neurosurgical approach to this large animal model[19]. The recently prepared Brain Atlas (www.proteinatlas.org) is a tool that brings the human brain mapping to a new level. It is essential that it provides an overview of all the proteins expressed in the brains of three mammalian species: human, mouse and pig. It underlines again a unique position of pigs and minipigs in biomedical research[2]. Moreover, the knowledge of precise basal ganglia morphology in Göttingen and Libechov minipigs were used for gene transfer of alfa-synuclein[20] and deposition of AAV vectors (AAV5-miHTT) for development of Huntington's disease gene therapy[21].

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2 Huntington's disease: a clinical overview and current therapeutical strategies

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Abstract

Huntington's disease (HD) is a progressive neurodegenerative disorder caused by an unstable CAG triplet expansion within the 1st exon of the IT15 gene encoding huntingtin. The presence of a mutated protein leads to a progressive loss of medium spiny striatal neurons and other neuronal populations. Clinical features of HD include motor symptoms, especially choreatic involuntary movements and gradual loss of voluntary movements control, parkinsonism in later stages and juvenile form of a disease, dysarthria and dysphagia. Non-motor symptoms include psychiatric disturbances such as irritability and depression, progressive cognitive decline and memory loss. So far, the current therapeutic approach is only symptomatic and consists mainly of antipsychotics, antidepressants and supportive care such as nutritional care, physiotherapy and speech therapy.

Keywords

Huntington's disease, neurodegeneration, therapy, motor symptoms, dyskinesia, cognitive deficit, psychiatric symptoms, dysphagia, weight loss

INTRODUCTION

Huntington's disease (HD) is a fatal progressive neurodegenerative disorder inherited in an autosomal dominant pattern. The average worldwide prevalence of HD is 2.71 / 100 000, with lower rates reported in Asian countries (0.40 / 100 000) compared to Europe, North America and Australia, where prevalence is 5.7 / 100 000[1]. The molecular genetic cause of the disease is an unstable CAG triplet expansion within the 1st exon of the IT15 gene (4p16.3), resulting

in the expression of a mutated huntingtin protein (mHtt) with an extended polyglutamine stretch. Wild-type Htt alleles have up to 26 CAG repeats and alleles with more than 36 repeats are considered pathological. Variable penetrance is observed between 36 to 39 repeats while 40 or more repeats show full penetrance. Intermediate alleles with 27 to 35 CAG are highly unstable and have a tendency to expand in future generations. The number of CAG triplets is considered to be the most important contributing factor to the age of onset and the disease course[2]. The presence of mHtt in brain tissue results in progressive loss of GABA-ergic medium spiny striatal neurons as well as other subcortical and cortical neuronal populations[3,4]. In addition to neurodegeneration, a number of primarily metabolic changes in peripheral tissues are also described[5–8].

In clinical practice, we distinguish three forms of HD, classic (adult-onset), juvenile and late-onset form. The classic form of HD typically begins between 30 and 50 years of life and the most prominent clinical manifestations are choreatic involuntary movements. The length of progression of the classical form is variable, but most often around 15–20 years.

The juvenile form is characterized by the development of clinical symptoms before the age of 20, faster progression of the disease compared to the classical form, less pronounced chorea, which is often completely absent with rigidity, akinesia and dystonia as a dominant symptom (Westphal variant). Epileptic seizures are also very common in this form[9]. The rapid development of dementia and behavioural disorders may initially resemble schizophrenia.

At the other end of the spectrum, there is a late-onset HD that affects patients over 60 and usually has a slow and mild course. The dominant symptom is chorea of moderate intensity and patients remain self-sufficient for a long time.

MAIN CLINICAL FEATURES

MOTOR SYMPTOMS

Motor symptoms are usually the most prominent clinical manifestation of HD and include both voluntary movement disturbances and typical choreatic involuntary movements (choreatic dyskinesia). Chorea is defined as involuntary, fast, irregular, and randomly occurring movements of different parts of the body and usually has an acral predilection. Discrete disturbances of voluntary movements develop already in pre-symptomatic stages. Gradually, all levels of voluntary movement control are affected: from planning and running the motor program, through fluidity, targeting, speed and range of movement, adequate trajectory, coordination and persistence (ability to maintain a steady position) to the finishing of a particular movement. In advanced stages of HD, voluntary movements are impeded by increasing akinesia, dystonia and rigidity.

As the disease progresses, severe disturbances of viewing and saccadic eye movements, articulation, swallowing and postural instability are seen. Problems with voluntary movements

control have been found to have a more significant negative impact on self-sufficiency than involuntary movements[10].

PSYCHIATRIC SYMPTOMS

Along with the progression of the neurological condition, there is also a progression of cognitive deficit and psychiatric symptoms, which are often present already in the presymptomatic stage. Anxiety, affective disorders, irritability and aggression are typical; psychotic disorders may also be present[11]. It has been shown that many years before the first clinical symptoms of mutation carriers, emotional recognition disorders have been present[12]. Other manifestations include attention deficit, learning difficulties, short-term memory loss, changes in psychomotor pace and the development of apathy[13]. Alcohol abuse and minor criminal offenses are also common[14]. All these changes have a very negative impact on social interaction and lead to problems in personal and professional life.

COGNITIVE IMPAIRMENT

The dominant cognitive impairment in HD is executive dysfunction with diminished ability to plan, create a concept of certain activity and maintain a mental set-up. At the same time, multi-task, along with the ability to quickly change a specific activity, is severely impaired. This cognitive deficit gradually develops into severe dementia.

WEIGHT LOSS AND METABOLIC CHANGES

Weight loss, despite normal to increased caloric intake, is well described, but the pathophysiology of it is not well-understood. Involuntary movements may contribute to increased energy expenditure, but disruption of energy homeostasis at the cellular and hormonal level is described[5–8].

DIAGNOSTICS

Diagnosis of HD is based on anamnestic data, positive family history, clinical examination (combination of motor and neuropsychiatric symptoms), brain imaging (striatal and cortical atrophy) and genetic confirmation test.

THERAPY

Although there are several research strategies to develop causal therapy for HD (mostly gene silencing by antisense oligonucleotides, RNA intereference or CRISPR-CAS9 technology, the current therapeutic possibilities in everyday clinical practice are only symptomatic.

Well-managed treatment under the guidance of a multidisciplinary team can significantly improve the quality of patients' life and hopefully, with new technologies coming to clinical trials, we will be able to establish causal therapy in the near future.

THERAPY OF MOTOR MANIFESTATIONS OF HD

Antipsychotics are particularly useful in the treatment of chorea because they block dopaminergic transmission. First-generation antipsychotics (haloperidol) have a stronger antichoreatic effect, but also have a higher number of side effects, especially fatigue, psychomotor retardation and worsening of postural instability, dysarthria and dysphagia. Because of this, the second generation of antipsychotics is usually chosen first. Due to good tolerance and lower incidence of side effects, tiapride, risperidone, sulpiride or olanzapine are usually used as a first-line treatment.

Benzodiazepines, in particular clonazepam, are another therapeutic possibility. Although they tend to have a good antidyskinetic effect, their disadvantage is the need to increase doses and the risk of developing dependence. Like antipsychotics, benzodiazepines impair psychomotor speed, attention, memory, orientation, and worsen postural instability, cerebellar symptoms and incontinence.

Tetrabenazine, a presynaptic dopamine depleter, also has a good antidyskinetic effect, but can sometimes induce depression and parkinsonism[15].

As the disease progresses (or from the very onset in the juvenile Westphal form), akinesia, dystonia and rigidity predominate. At this stage, it is worth considering the benefits of continued administration of antipsychotics, which may themselves induce drug-induced parkinsonism, tardive dystonia and akathisia. Parkinsonism, dystonia, and postural instability may, in some cases, improve after amantadine or rarely after levodopa treatment.

THERAPY OF PSYCHIATRIC SYMPTOMS

Irritability, aggression, or psychotic symptoms as hallucinations and delusions can be successfully suppressed by antipsychotics, in case of depression, anxiety, apathy and abulia (lack of initiative), which are very common features of HD, antidepressants from the group of selective serotonin reuptake inhibitors (SSRIs) are usually chosen. SSRIs and valproic acid also suppress irritability and aggression.

SPEECH THERAPY

Regular examination by a clinical speech therapist is crucial in detecting dysphagia and prevention of aspiration. If necessary, the speech therapist should indicate further detailed examinations, in particular videofluoroscopy and flexible endoscopic examination of swallowing (FEES).

NUTRITIONAL CARE

Nutritional care and prevention of malnutrition is an integral part of comprehensive care for HD patients. They are known to have increased energy requirements, not only due to involuntary movements but mainly due to complex metabolic changes in peripheral tissues[5–8,16]. In addition, dysphagia gradually develops, so patients need to start nutritional support sooner or later. Food intake, weight and biochemical nutritional parameters should be monitored regularly for each patient and signs of malnutrition should be detected as soon as possible. Initially, it is sufficient to cover the deficit by sipping, adjusting the diet and consistency of the diet according to the degree of dysphagia. An early indication of percutaneous endoscopic gastrostomy and sufficient nutrition can improve the quality of life, slow the progression of deterioration and reduce the frequency of infectious complications.

OTHER SUPPORTIVE THERAPY

Physiotherapy, occupational therapy and psychological and social intervention including home care or residential facilities (if the patient is already at an advanced stage of the disease) should be an integral part of the comprehensive care.

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3 Huntington's disease modeling in animals

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Abstract

Animal models are valuable tools in the determination of expression and physiologic function of studied genes. Huntington's disease as monogenic autosomal disorder is still enigmatic in mechanism by which mutant gene expression affects molecular, physiological, and neurological functions in patients. Recently many animal genetic models were established in an effort to determine and understand changes in tissues and organs leading to disease onset, its progression and manifestation.

Keywords

Huntington's disease, animal model, mutant huntingtin, CAG repeat, neurodegeneration

INTRODUCTION

Huntington's disease (HD) is a slow and incurable progressive neurodegenerative disorder characterized by motor disturbance, cognitive loss, and psychiatric manifestations[1].

The prevalence of HD in North America, North Western Europe and Australia ranges from 5.96 to 13.7 cases per 100 000 people. Higher HD prevalence in European ancestry populations may be partly explained by the higher average CAG repeat lengths and frequencies of particular HTT gene haplotypes compared to other world populations[2].

HD is caused by mutation in IT15 gene coding for huntingtin protein[3]. Huntingtin gene carries a polymorphic expansion of CAG repeats ranging from 9 to 35 CAGs in the non-HD population, with an average between 17 and 20 repeats[4]. Affected individuals carrying more than 35 CAG repeats are at risk[5]. Higher number of CAG repeats correlates with severity and earlier onset of disease[6].

THE MECHANISM OF HUNTINGTON'S DISEASE

Huntingtin (HTT) is cca 350kDa abundantly expressed protein. Orthologs were found in vertebrates and also invertebrates. Interestingly, polyglutamine repeat (polyQ) is an evolutionary variable in its length. In protostome is not present (Q1) and the expansion of deuterostome Q2 tract could be seen in vertebrates fishes and birds (Q4), rodents (Q7-8) and finally highly polymorphic Q17-Q23 in humans[7].

HTT seems to be involved in maintenance of the balance between cell differentiation, survival and death, and the performance is cell type or tissue specific[7,8]. The mechanism of mutated (polyQ expanded) huntingtin detrimental function is not fully revealed. mHTT gene expression is responsible for selective death of striatal medium spiny neurons and striatal atrophy in humans. But it also affects other neural cells as well as many cells in peripheral tissues[5].

ANIMAL MODELS OF HUNTINGTON'S DISEASE

HD is human specific and there is no animal equivalent disorder. Diversity of genetic background and aging phenomenon accounting for the variability of the onset and progression of syndromes in various animal species and lines raise many additional questions and tasks that impede interpretation and translation of data. Monitoring of cognitive and motor impairment require development of animal model specific motor and behavioural tests. Thus, there is a sustained need to either further develop new or use existing animal models to model and study HD phenotype features.

TOXIN MODELS

Excitatory agonists can promote excitotoxic cell death in susceptible brain nerve cells. Excitotoxin lesions in the monkey and rat, using quinolinic acid, provide an experimental model that simulates the neuropathological and neurochemical features of HD[9,10]. Quinolinic acid, N-methyl-D-aspartate (NMDA) type excitotoxin, is an endogenous intermediate in the kynurenine pathway of tryptophan metabolism. Its elevated levels were reported in HD patients. Possible causative effects of endogenous quinolinic acid could not be simply accepted[11]. Nevertheless, NMDA receptor signalling is affected because these receptors are depleted in human HD putamen[12].

A delayed-onset of nonprogressive dystonia and putaminal necrosis in patients that accidentally ingested 3-NP from contaminated sugar cane demonstrated selective vulnerability of striatum[13]. 3-NP is a mitochondrial inhibitor and irreversibly inhibits succinate dehydrogenase in respiratory chain complex II and the Krebs cycle.

Experimental administration of 3-NP can produce selective striatal lesions in mice, rats and primates[14–16].

There are several administration protocols enabling acute, subacute and chronic experiments that differ in time onset, severity and most importantly in mimicking the HD features. In rodents, the NP3 toxicity is the strain and gender variable and has high detrimental side effects, for example cardiotoxicity must be carefully considered when NP3 model is used. Most accurate chronic models could be generated via systemic administration of 3-NP over 5 days to 4 weeks depending on the protocol[10]. They display more HD specific loss of striatal cells while sparing NADPH-diaphorase neurons.

The great limitation of excitotoxicity and mitochondrial models is being simply a chemically induced phenocopy of the disease. Despite the lack of expanded polyQ, toxin models could still help us to understand the excitotoxicity and changes in mitochondrial dysfunction and adaptation processes occurring in HD vulnerable brain regions.

GENETIC MODELS

Genetic animal models provide the best possible approach to study pathological mechanisms underlying the disease. Compared to toxin models, they are more suitable for therapeutic evaluations as they can model molecular mechanisms leading to disease onset and progression. As mentioned above, HTT gene is ubiquitously expressed throughout the body and not only CNS organs can be severely impaired.

N-TERMINAL TRUNCATED HUNTINGTIN TRANSGENIC MODELS

Identification of accumulated fragments of mHTT in brain post-mortem tissues led to development of several transgenic mouse, porcine and other species models expressing truncated N-terminal human huntingtin protein[17–19].

The very first mouse transgenic lines, R6/1 and R6/2, express mutant human exon 1 HTT[16]. Both lines demonstrated that an N-terminal mutant HTT fragment was sufficient to elicit HD-like neurological phenotypes in the mouse, like tremors, hypokinesis, and abnormal gait. An accelerated phenotype compared to humans and other animal models is accompanied by weight loss and premature death. Even though DNA construct used for transgenesis was identical, onset, progression, and life survival differs. Both lines have 1 coding sequence of exon1 with a part of intron 1. In addition, at both integration sites many insertion and inversion rearrangements are present. The genome integration sites differ, being localized to chr4 and chr3, (R6/2 and R6/1 respectively)[20]. Furthermore, potential molecular consequences of

transgene integration must also be taken into account as *Gm12695* gene was partially deleted by the transgene insertion in R6/2[21].

Early disease onset and rapid disease progression made R6/2 mice the most extensively utilized model. While initial paper defined a mean CAG repeat size of approximately 150, there are now many sublines of R6/2 mice with CAG repeat sizes ranging from 43 to more than 600 as reviewed in *A Field Guide to Working with Mouse Models of Huntington's Disease*[55].

CAG repeat lengths in breeding and experimental animals must be thoroughly monitored to reduce experimental variation. There is an inverse relationship between CAG size and earlier onset of behavioural abnormalities and more severe phenotype in R6/2 mice carrying 50 to 160 CAG repeats[22]. On the other hand, the CAG repeat sizes near or above 200 CAGs show a delayed onset of HD motor symptoms and milder pathology[23,24].

N171-82Q lines express an N-terminally truncated human HTT protein encoded by a cDNA containing the first 171 amino acids and carrying 82 CAGs under the regulation of the mouse prion promoter, which directs expression primarily in the brain[18].

All lines are characterized by the formation of intranuclear inclusions, neuritic aggregates, and diffuse nuclear localization of HTT[19]. There are however high variations in shortened lifespan, as mice from line 77 die at 2.5 months while mice from line 6 die around 8–11 months. All Q82 mice lines show a weight loss. NSL – N171-82Q transgenic mouse lines bearing NLS signal at N-terminal HTT fragment were developed and showed similar phenotype with higher intranuclear aggregate formation.

The hallmark of mouse N-terminal truncated models is rapid and devastating progression of disease syndromes leading to precocious death. This is in agreement with *in vitro* cellular models where mHTT expression of approximately 200 amino acids and less are toxic. It was proposed that cellular toxicity is mediated not only exclusively by expanded polyQ but also by N-terminal fragments of specific size with altered stability or its conformation[25]. This might be further supported by Shortstop HD mouse model expressing the first 117 amino acids of mHTT with no HD phenotype despite polyQ expansion and the presence of neuronal inclusions[26].

Recently, the presence of an aberrantly spliced exon 1 HTT with a short polyadenylated mRNA was reported in mouse models and human patient samples[26,27]. The aberrant transcript is then translated into highly toxic exon 1 HTT protein. Thus R6/1 and R6/2 might be considered as specific models to evaluate the impact of such aberrant splicing events.

The first pig model of Huntington's disease expressed N-terminal (208 amino acids) mutant huntingtin with an expanded polyglutamine tract (105Q)[28]. Involuntary movements were observed in some surviving transgenic piglets together with mHTT expression in brain cells. However, premature postnatal death could not allow further examination of neurodegeneration in adult animals[29].

Recently, a transgenic minipig model of HD (TgHD minipig line) was established. One copy of the human cDNA coding for 548 amino acids long N-terminal part of HTT protein is

integrated into chromosome 1 q24-q25. Transgene carries 124 mixed CAG and CAA repeats. Successful germ line transmission occurred through successive generations (F0, F1, F2 up to F5 generations)[18], and personal communication. Although disease specific phenotype features are mild and their onset is very slow, there are evident progressive neurological and neuroanatomical changes. Of note, the earliest sign of disease specific impairment is lowering reproductive parameters of boar sperm by 1 year of age. The downregulation of DARPP32, a specific marker of medium spiny neurons, is reported from 2 years of age up to 6 years. Neurodegenerative demyelinization and microglia activation occur at 2 years. Increased physical activity of 4.6–6.5 year-old TgHD at particular day periods indicates an altered circadian behaviour. Significant differences in motor and fear-stress test performance were observed in the 6–8 year-old TgHD animals compared to WT controls. Interrupted CAG repeat (mixed CAG and CAA) that inversely affects HD onset in humans or mice may delay or partially diminish phenotype severity in TgHD model[30].

FULL-LENGTH HUNTINGTIN TRANSGENIC MODELS

Transgenic mouse models BACHD and YAC128 express mutant versions of the human full-length gene. In contrast to N-terminal transgenic animals, full-length human gene was introduced via YAC or BAC. Full-Length transgenic models develop disease phenotypes within many months and show normal lifespan.

The YAC128 transgenic mice express mixed 125 CAG repeat expansion where CAG repeat is interrupted by two CAACAACAACAGCAA sequences. Starting at 3 months of age, motor deficits, cognitive impairments, as well as striatal atrophy and neuronal loss are manifested. Cognitive impairments correlate with the appearance of aggregates and neuronal inclusions throughout the brain[31,32].

As early as 4 weeks of age BACHD mice show anxiety-like behavior deficits[33,34]. BACHD mice also display clear motor deficits from 2 months of age and from 12 months of age, cortical and striatal atrophy of BACHD brains are present[35]. Somatic and germ-line instability of CAG repeat that is observed in the HD patient population[35–37] is not recapitulated in YAC128 and BACHD models. The reason is the mixed CAG/CAA repeat in BACHD mice and interspersed CAA codons in YAC128 mice that stabilize the CAG repeat[32,35]. Moreover, both lines contain either 4 or 5, respectively, copies of full-length huntingtin on the background of wild type mouse huntingtin[38].

In addition to slow neurodegeneration development, weight loss seen in HD patients is not observed in both YAC128 and BACHD lines. These two models can not recapitulate many features of HD so the utilization of these two models may be limited. Mild phenotype and normal lifespan make them useful in investigation of therapeutic HTT protein lowering strategies.

OVT73 is a transgenic sheep line expressing a full length cDNA for the human HTT gene. The transgene carries a pure repeat of ~73 CAG[39]. Although a transgene is translated and generates a protein of expected size, several copies of the whole or fragmented gene are present in OVT73.

Aggregates were identified in piriform cortex at 3 years of age and at 5 years of age cortical and putaminal aggregates could be found. However, by 5 years of age no neurodegeneration, no disease-specific atrophy, and little gliosis were present. MRI data did not show any significant structural changes in HD sheep brain before the age of 5 years.

Subtle circadian behavioural disorder was reported only in separately housed genotype-specific groups[40]. Of note up to the age of 8 years there were no signs of motor dysfunction[41].

KNOCK-IN MODELS

To study genetic aspects of HD knock-in animal models are the best genetic models. CAG repeat is introduced in endogenous HTT gene under the natural promoter and localization site. Lines can then be bred as heterozygous or homozygous.

There are two mouse groups of knock-in lines. First group was derived from its ancestry HdhQ150 mice bearing normal mouse allele with 150 pure CAGs that replaced the original mouse polyQ coding sequence[42]. Gametic instability was used for subsequent selection of wide range CAGs repeat line variants from 50 up to over 300 CAGs.

Weight loss, decreased motor activity and clasping behaviour are observed in homozygotic HdhQ150 mice at around 70 weeks of age. Balance and gait abnormalities are manifested later on[43]. Cognitive deficits of HdhQ150 homozygotes were shown to occur at around 24 weeks of age[44]. Within the striatum, nuclear inclusions (NIs) appeared slightly earlier (37 weeks) in homozygous animals as compared to heterozygotes (40–42 weeks). By 70 weeks, HTT immunoreactive NIIs were distributed widely throughout the striatum with persistent diffuse nuclearstaining[45]. By 21 months the neuronal cytoplasm became vacuolated and contained swollen mitochondria with many degenerated cytoplasmic organelles[46].

The second knock-in group harbours chimeric mouse-human exon 1 lines that were produced by replacement of murine exon1 with mutated human exon 1. A series HdhQ20, HdhQ50, HdhQ92, HdhQ111 contains not only human exon 1 but also a part of human intron 1. Moreover, CAG repeat is interrupted by penultimate CAA codon[47].

Other separate series of KI lines in which the mouse exon 1 was replaced with a mutant version of human exon 1 are CAG71, CAG90 and CAG140 lines carrying expanded CAG repeats of 71, 94, and 14[48–50]. Spontaneously developed zQ175 KI line with a highly expanded and unstable 198 CAG repeat[51,52] was generated via germ-line expansion in CAG140 mouse.

Striatal and cortical volumes decreased earlier in homozygous then in heterozygous mice at 3 and 4 months, respectively. In addition, significant decrease of striatal gene markers from 12 weeks of age was detected. Homozygous mice showed motor and grip strength abnormalities with an early onset (8 and 4 weeks of age, respectively). Reduced lifespan in the homozygotes and decreased body weight in both heterozygotes and homozygotes was observed at 100 weeks. Nuclear inclusions were widely distributed throughout the brain and increased with age in both striatum and cortex[53]. Of note is the relative early and robust onset of HD phenotype.

In 2018, the first knock-in porcine model was reported[29]. Porcine exon 1 was replaced by human HTT exon 1 with CAG repeat (150 CAGs) using CRISPR-Cas9 technology. Similar to mice or humans, the germline transmission instability of CAG repeats was observed and pigs with CAG repeats ranging from 113 to 230Q were born.

Contrary to HD KI mice, KI pigs display more severe phenotypes including shortened lifespan. Within 5 to 10 months 90% or 60% mortality of F0 and F1 generation, respectively, was reported. At 5 months a lower weight gain is evident and animals display walking abnormalities. Pig brains revealed cortex thickening and striatal atrophy by MRI. Immunohistochemistry identified selective loss of medium spiny neurons in HD KI pig brains while interneurons were spared.

CONCLUSIONS

mHTT gene function is not fully revealed. Moreover, HTT gene is large and HTT transcripts undergo splicing resulting in several alternative spliced variants. In humans, except CAG expansions there are several CAG polymorphisms, having a striking impact on phenotype penetrance and severity. There is some recent evidence showing the existence of gene modifiers in humans, too. It is obvious that no animal could perfectly model all mechanisms affected by mHTT expression. Nevertheless, each animal model could shed a light on a specific aspect of the disease[54]. Exon 1 expressing models could give us information on toxic and aggregation characteristics of short alternatively spliced HTT transcripts. Models having pure CAG repeats could reveal the contribution of somatic CAG instability in particular tissues to disease phenotype. Large animal models could serve as a tool for translational research like testing of feasibility and safety of mHTT gene expression lowering treatments in preclinical trials.

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4 Motor, cognitive, and behavioural tests conducted in pigs

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Abstract

Pigs have been widely used for modelling various motor, cognitive or behavioural diseases. The more the model simulates the symptoms of the disease, the better it is for the evaluation of the safety and efficacy of potential therapeutic treatments. Therefore, investigation of the tests for monitoring the disease manifestation and evaluation of safety and efficacy of potential therapies arose on the scene. Phenotyping varies in battery of tests that measure behavioural changes as well as changes in motor and cognitive functions. Technological progress enables the use of objective electronic devices and thus eliminates the subjective feature of data collection and evaluation.

Keywords

pig, biomedicine, motor, cognitive and behavioural tests, translational research

INTRODUCTION

Pig (*Sus scrofa*) models are increasingly being utilized in a biomedical research due their body size, physiological, and immunological similarities to humans. Pigs were used in broad range of studies from toxicology to experimental surgery[1,2]. Broad potential of representing the pigs as animals for experimental modelling of human disorders arisen with advanced biotechnology since genetically modified pigs were generated to model human cancers, metabolic diseases, and neurodegenerative disorders[3–6]. Large gyrencephalic brain of pigs permits the identification of cortical and subcortical structures and resembles the human brain more in anatomy, biochemistry, and development than do the brains of commonly used small laboratory animals. Moreover, the size of the pig and its large-sized brain allow the use of the same instruments and techniques commonly

used for humans without the need for scaling instrument dimensions[7–10]. Positron emission tomography (PET)[11], magnetic resonance imaging (MRI)[12–14] or deep brain stimulation[15,16] have been extensively used in pre-clinical work in pigs. The evaluation of the safety and efficacy of any potential therapeutic treatment requires monitoring the disease phenotype. Therefore, monitoring of the disease manifestation in animal models became an indispensable part of preclinical studies. The advantage of testing the pigs is their simple handling and taming (Fig. 1 A, B). Since the different types of disorders manifest in various manners, a spectrum of tests used for investigation and evaluation of the disease progression is very wide.

This chapter presents a short view on motor, cognitive and behavioural tests that have been developed for validation of the disease's symptoms and their progression in pigs.

MODELING HUMAN DISEASES IN PIGS

Pigs have been showed as valuable models for numerous diseases including traumatic brain injuries[17], melanoma skin cancer[18,19], pain[20], *degenerative disorder*s – amyotrophic lateral sclerosis (ALS)[21], osteochondritis[22], atherosclerosis[23], spinal cord injury[24], *psychiatric disorders* – schizophrenia[25], and *neurodegenerative disorders* – Alzheimer's disease[26], Parkinson's disease[27,28], and Huntington's disease[29–31].

The better the model recapitulates the disease phenotype, the more valuable it is in preclinical testing.

TESTS USED FOR MONITORING THE DISEASE SYMPTOMS AND EVALUATION OF THE EFFICACY OF POTENTIAL THERAPEUTIC TREATMENT

MOTOR TESTS

Various ways how to investigate motor functions including gait performance, obstacle walking or re-balancing after unexpected shot were used in different studies. Initially, the gait monitoring was based on methodology described by van Steenbergen[32] and Jørgensen and Vestergaard[33] when swaying hindquarters and general gait movement and pattern were scaled. Descriptive characterization and scoring was applied not only in walking on the straight dry floor[34] but they were also used in obstacle walking[35,36] or Pullback test in transgenic Huntington's disease minipigs[34]. Efforts to assess objectively the measurements brought electronic devices and computer based quantitative gait analysis system on the scene. Video-recording with high speed cameras was used for detection of key gait parameters including swing and stance time of the limb, velocity, step length, maximum step

height and limb support phases in Yucatan miniature pig model simulated ischemic stroke model[37]. Pigs walked through a semi-circular chute measuring 4 m in diameter, where they were video-recorded as they moved perpendicular to two synchronized cameras recording each side of the pig[37]. Video-recording was also applied in a comparative study of motor performance and gait variability among piglets[37]. Next, three dimensional coordinate data were collected by the use of reflective skin markers attached to the head, trunk and limb anatomical landmarks what allowed obtaining the kinematic motion capture in growing pigs reared on different floor types[38] or in breeding sows with lameness[39]. Technical progress allowed monitoring of the gait by GAITFour/GaitRite[36,40,41] pressure mat consisting of thousands of sensors which enable evaluation of stride time, stride length, maximum pressure, activated sensors, and stance time and thus maximize the objectivity of the measurements. Vertical forces produced by the limbs were measured by an embedded microcomputer-based force plate system[42]. Tongue protrusion tests[35,36] demonstrated that not only gait but also fine motor skills can be tested in pigs.

COGNITIVE TESTS

Cognitive testing of pigs has been explored primarily for the study of farm animals welfare[43,44], but it has gained importance for neuroscience. Pigs are curious animals and tend to explore new objects. Their memory for the familiar things can be tested by Spontaneous object recognition test[45]. This test compares how much time an animal spends exploring an unknown versus a familiar object. Pig's inquisitive nature allowed the performance of Skittles and Cover pan tests[34] where the pigs were expected to find a treat hidden under a movable cover. Tests addressing visual discrimination revealed that pigs are capable to identify the box of the determined color[36] similarly like people[46]. An experiment, where the pigs were offered to choose between two different bowls containing unequal amounts of food showed a preference for the bowl containing the larger amount of food[47]. Thus, pigs remember the location, content, and relative value of previously discovered sites that contained stimuli of interest[48]. Pigs eminently use spatial memory, it was confirmed by their abilities to learn to navigate in mazes[49] and other spatial arrangements[50,51]. Spatial learning and memory can be objectively tested by the holeboard developed by Van der Staay[52,53]. Holeboard is an open-field arena with 16 holes where a treat can be hidden.

BEHAVIOURAL TESTS

Changes in behavioural patterns can be monitored by the battery of various tests. Human approach test[54], Tunnel[55], Balance Beam or Seesaw tests[56] (Fig. 1 C, D) were used to assess the animal's ability to cope with stress. In Human approach test, the physical contact with an unknown person was evaluated while in Balance beam and Seesaw tests the pigs were challenged to overcome their fear of crossing the obstacle (Seesaw or Balance Beam). Personality was tested by Dominance test[35], testing two pigs facing each other in a narrow corridor.

Next, Backtest behaviour indicates a coping disposition that is environmentally modulated[57]. Backtest results are also related to other parameters such as immune responses and energy metabolism[58,59].

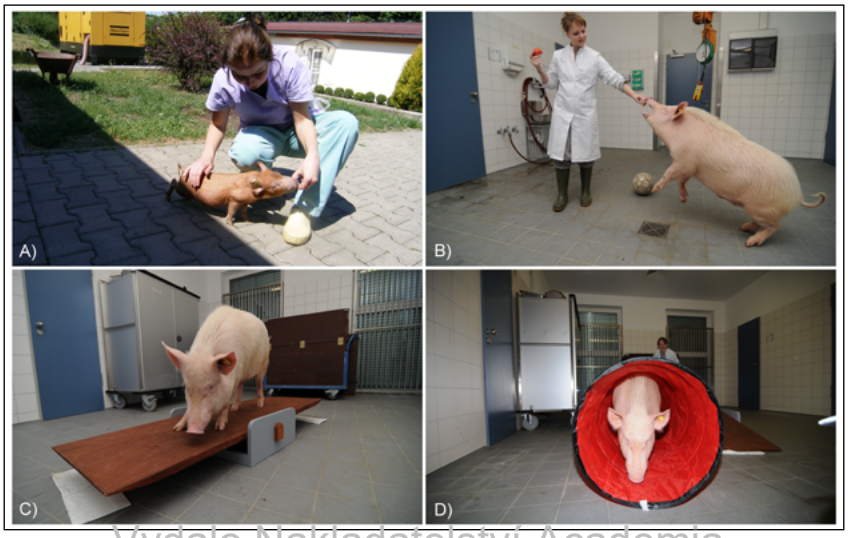

Figure 1. Tests used for monitoring the disease symptoms. A, B) Handling and taming the pigs. C) Seesaw test. D) Tunnel test. Photos A) M.Baxa, B–D) M. Marcegaglia

CONCLUSION

Pigs offer extensive opportunities in evaluation of safety, biodistribution, efficacy and application of novel therapeutic approaches for various human diseases. A spectrum of motor, cognitive and behavioural tests helps to explore the disease progression and the impact of the tested therapy. Quantitative approach used in phenotype monitoring enables more precise evaluation of the treatments which improves translation to human medicine.

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5 Double-strand DNA breaks response and Huntington's disease

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Abstract

Double-strand DNA breaks (DSBs) are considered as the most harmful DNA lesions, as only one unrepaired DSB is sufficient to trigger chromosomal rearrangements, senescence, or cell death. In recent years, it was proposed that alterations in response to DSBs could contribute to pathology of neurodegenerative diseases, including Huntington's disease. Huntington's disease is a neurodegenerative disorder caused by the expansion of CAG trinucleotide repeats in the huntingtin gene giving rise to mutated form of huntingtin protein (mHTT). Recent findings suggest that presence of mHTT may affect one type of the double-strand DNA breaks repair, the non-homologous end joining. It is suggested that impairment of DSBs repair occurs long before clinical diagnostic of Huntington's disease.

Keywords

DNA damage response, double-stranded DNA breaks, homologous recombination, non-homologous end joining, Huntington's disease

INTRODUCTION

Accumulation of DNA damage during long life of neurons may contribute to the pathology of neurodegenerative diseases including Huntington's disease (HD)[1–3]. HD is neurodegenerative disease, which is caused by the expansion of CAG trinucleotide repeats in the huntingtin gene giving rise to mutated form of huntingtin protein (mHTT)[4–6].

Of many types of DNA lesions, double-strand DNA breaks (DSBs) are considered as the most harmful to cells, because only one unrepaired DSB is sufficient to trigger permanent growth arrest and cell death[7,8]. DSBs can arise endogenously during V(D)J recombination, meiotic recombination, replication stress, or can be caused by oxygen radicals produced by metabolism[9,10]. DSBs can also be caused by exogenous factors, such as ionizing radiation or ultraviolet radiation, or experimentally, e.g. by radiomimetic drugs or inhibitors of topoisomerases[10,11]. Thus, cells evolved DSBs response pathway to deal with these threads.

DSBs are recognized by MRN (Mre11-Rad50-NBS1) complex / Ku70, Ku80 heterodimer, which in turn activates downstream kinases ATM (Ataxia telangiectasia mutated), ATR (Ataxia telangiectasia and Rad3-related protein) and DNA-PK (DNA-dependent protein kinase)[12–15]. ATM kinase subsequently phosphorylates histone H2AX in sites of DSBs, and this phosphorylation spreads along DNA up to 2 Mb from the site of DSB[16]. Phosphorylated H2AX (γH2AX) is recognized through BRCT domain of MDC1 protein, which then serves as a platform for proteins required for DNA damage repair[17,18]. As a response to DNA damage other important molecules are phosphorylated by downstream kinases [12,14,19]. For example, protein p53 is responsible for activation of transcription of cell cycle inhibitors. It results in arrest of cell cycle, which provides sufficient time for DNA damage repair mechanisms to repair DSBs[20–22].

DOUBLE-STRAND DNA BREAKS REPAIR PATHWAYS

There are two main DSBs repair pathways: The first one is homologous recombination (HR) and the second one is non-homologous end joining (NHEJ).

HR is initiated by the resection of DNA ends by MRN complex, giving rise to 3' single-stranded DNA overhangs on both sides of the break. These 3' ssDNA overhangs are stabilized by coating with Replication protein A complex, which is subsequently replaced by Rad51 nucleoprotein filament. When the homology is found, both overhangs invade to intact DNA donor, which serves as a template for DNA synthesis. Whole process is finished by resolving Holliday junctions resulting in a crossover or non-crossover product. HR is slow and error-free, as it does not only repair DSBs, but also restores the sequence around DNA break. However it requires sister chromatid as a homologous template, and therefore HR is restricted to S and G2 phase of the cell cycle[23–25].

On the other hand, NHEJ is a very fast repair process, which simply joins the DNA ends together during all phases of cell cycle. NHEJ is initiated by binding Ku heterodimer (Ku70, Ku80) and molecule 53BP1 to the site of DNA break. Subsequently, DNA-PK catalytic subunit is bound to Ku heterodimer, what leads to formation of functional DNA-PK. Free DNA ends are modified by molecules DNA-PK and Artemis to create suitable ends for ligation. DNA ends are afterward ligated by LIG4/XRCC4/XLF complex. As the NHEJ has no build-in potential of restoring original sequence of DNA in site of DSB, it is associated with several sequence alterations, for example addition or deletion of some nucleotides[26–29].

In addition to these two main types of DSBs repair mechanisms, there is another repair pathway called alternative end-joining (A-EJ). Because A-EJ is inhibited by NHEJ and also HR, it is assumed that A-EJ operates only when repair by main repair mechanisms fails or is not functional. In contrast to HR, A-EJ does not require longer homologous template for repair, but it employs 2-25 nucleotides short microhomologies found at the vicinity of DNA

ends. This occurs especially when resection and generation of single stranded strands occurs before end-joining. Ligation of modified ends is executed by ligases LIG1 or LIG3 in complex with XRCC1. Usage of microhomologies in A-EJ results in the repair of DSBs but at the cost of larger deletions in DNA sequence or chromosomal translocations[30–34].

DOUBLE-STRAND DNA BREAKS RESPONSE AND HUNTINGTON'S DISEASE

Recently, several research groups focused on studying DSBs response in connection to HD pathology. It was shown, that the wild-type HTT localizes to the sites of DNA damage and this process is dependent on the kinase activity of ATM[35]. It was proposed that the wild-type HTT is a scaffolding protein in the ATM DNA damage response[35].

It was also shown that presence of mHTT causes accumulation of single-stranded and double-stranded breaks in brain cells in R6/2 mice[36]. Moreover, increased phosphorylation of H2AX and activation of ATM and p53 was described as a result of mHTT expression[36]. Additionally, interaction of mHTT with Ku70 protein compromises ability of Ku70 to activate DNA-PK, what leads to the decrease of DSBs repair by NHEJ[37]. Interestingly, ectopic expression of Ku70 at least partially rescues HD phenotype in mouse and Drosophila model of HD[37,38].

Furthermore, experiments made on confluent dermal fibroblasts isolated from human HD patients showed that mHTT interacts with ATM kinase in cytoplasm, what subsequently decreases the translocalization of ATM kinase into nucleus. These cells exhibit fewer H2AX foci and their longer persistence after induction of new DSBs. Also kinetics of 53BP1 foci formation and disappearance is affected in these cells. It suggests that mHTT compromises both DSBs recognition and also their repair in human patient cells[39].

Defects in DSBs response and accumulation of DSBs precede mHTT aggregates formation and neurological phenotype manifestation in R6/2 mouse model[36]. Experimental manipulations with DSBs response signalling have 'therapeutic' or rescue effects both in cell and animal HD models. The p53 genetic depletion significantly rescues neurobehavioral abnormalities of mHTT transgenic mice[40]. Genetic reduction of ATM partially improves neuropathology in mouse expressing full-length human mHTT and small-molecule inhibitors of ATM reduced mHTT induced cell death of rat striatal neurons and induced pluripotent stem cells derived from HD patients[41].

Moreover, it was shown that wild-type HTT is a part of transcription-coupled DNA repair complex which recognizes DNA lesions in template DNA strand and facilitates the repair of DNA lesions during transcriptional elongation[42]. Presence of mHTT disrupts the functional integrity of this complex and therefore has an adverse impact on transcription and DNA repair[42]. It was also shown, that there is a difference in relative telomeric length between HD patients and healthy individuals.[43] It was proposed, that telomeres shortening can be

related to DNA damage caused by defective DNA repair mechanisms and by reactive oxygen species[43]. Comparison of human embryonic stem cell (hESCs) lines expressing mHTT or wild-type Htt and hESCs with absent HTT showed, that poly-glutamine expansion as well as the loss of HTT can lead to chromosomal instability[44].

CONCLUSION

Huntington's disease is a neurodegenerative disorder caused by the expansion of CAG repeats in the huntingtin gene, giving rise to the mHTT. mHTT is widely expressed in all tissues and causes pathology throughout the brain. It is assumed that HD pathology is mainly caused by fragmentation and aggregation of mHTT in cells, however the exact mechanism of HD pathology remains unclear and there is no cure for HD up to this date. It was proposed that accumulation of DNA damage and impairment of DSBs repair during the long life of neurons may contribute to the pathology of HD and other neurodegenerative diseases and occurs long before clinical diagnostics. Therefore, further investigation of mHTT effects on DSBs repair is very important for the understanding of HD pathology and searching for new therapeutic targets.

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6 Presence of oxidative stress in Huntington's disease pathogenesis

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Abstract

Huntingtin protein (HTT) participates in many molecular processes, which are disrupted by its mutation. Also, mutated huntingtin (mHTT) may gain new functions that are toxic to the cell. An example is the impairment of mitochondria function and the associated redox state. Since mitochondria are a major source of superoxide radicals, their dysfunction leads to energy deficit and oxidative stress. This state exhausts the antioxidant defence, resulting in impairment of cellular integrity and apoptosis.

Keywords

reactive molecules, antioxidants, mutant huntingtin (mHTT), markers

INTRODUCTION

Oxidative stress with other pathophysiological traits such as mitochondrial defects and excitotoxicity caused by increased sensitivity to glutamatergic activation of *N*-methyl-*D*-aspartate receptors participate in the Huntington's disease (HD) progression[1–3]. Naturally, oxidants and prooxidants are produced in cells and their effect is eliminated by the presence of antioxidants. The imbalance between antioxidants and prooxidants in favor of prooxidants leads to oxidative stress, resulting in the disruption of cellular integrity and homeostasis[4–6]. The main prooxidants and oxidants, which among other things have also a positive role in cellular signaling, are low-molecular oxygen and nitrogen compounds, especially their radicals[7,8]. The presence of oxidative stress in HD was first revealed in the brain tissue of the R6/2 mice, a first mouse model of HD[9].

REACTIVE MOLECULES AND ANTIOXIDANTS

Reactive oxygen species (ROS) belong to oxidants in biological systems. Most of them are small molecules produced by oxygen reduction[10].

The key player in the toxic reaction is the radical of superoxide anion ($O_2^{-}\cdot$), which is produced by one-electron reduction of molecular oxygen[10]. It can be protonated, but in cytoplasmatic pH 6.8, the degree of deprotonated one is about one hundred higher. Dismutation reaction of two superoxides generates hydrogen peroxide and molecular oxygen, which is supported by acidification (see scheme 1)[11]. The effect of this reaction is catalysed by the enzyme superoxide dismutase (SOD)(EC 1.15.1.1.) or transition metal ions[12].

$$HO_2\cdot + O_2\cdot + H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

Superoxide can also react with biological molecules as an oxidant and as a reductant[13]. The presence of superoxide also causes formation of hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{-}\cdot$) and other very reactive metabolites or metal ions[10]. Haber and Weiss described the reaction of superoxide with hydrogen peroxide producing very reactive hydroxyl radical (see scheme 2). The reaction is faster in the presence of transition metal ions, for example, Fe^{3+}[14]. Thus, molecules containing iron ions show a greater susceptibility to oxidative damage[11].

$$O_2^{-}\cdot + H_2O_2 \rightarrow O_2 + OH^- + OH^-\cdot \quad (2)$$

Superoxide can also oxidize molecules cofactors or antioxidants. For example, ascorbic acid, NADH in lactate dehydrogenase or glutathione[11].

The most important nitrogen molecule, which acts as reactive radical is nitric oxide (NO), its reactivity is caused by one unpaired electron[10]. NO is produced from L-arginine by NO synthase (EC 1.14.13.39). One of four Ca^{2+} and calmodulin-dependent isoenzyme of NO synthase is present in neurons, where formed NO in low concentration functions like a neurotransmitter[15,16]. However, its accumulation causes oxidative stress[17].

The high toxicity of nitric oxide is caused by the reaction with superoxide, whereby a strong oxidant nitroso peroxyl anion is formed. It can cause lipid peroxidation[18], DNA damage[19], protein oxidation, hydroxylation or nitrosylation and other cell integrity disturbances[20,21].

The level of reactive substances and their products are controlled by antioxidants. It is a group of protein and non-protein molecules that keep the low level of oxidants or remove damaged biomolecules in physiological condition[4,10]. One of the antioxidants is, for example, an enzyme superoxide dismutase (SOD) (EC 1.15.1.1.). SOD has three isoenzymes in eukaryotic cells. Besides different composition, they have specific localization. SOD1 is present in the cytoplasm, SOD2 in the mitochondria and SOD3 in the extracellular matrix. All of them catalyze the conversion of superoxide to hydrogen peroxide, which can be further decomposed into water and oxygen by catalase (CAT) (EC 1.11.1.6). Another enzymatic antioxidant, which can remove hydrogen peroxide, is glutathione peroxidase (GPx) (EC 1.11.1.9). However, it must be first reduced by glutathione reductase (GR) (EC 1.6.4.2) with the cofactor NADPH[10,11] (see fig. 1).

Other protective agents include, for example, metal ions, scavengers like transferrin, ceruloplasmin, hemopexin[23] or vitamins like ascorbic acid[24], α-tocopherol, and other carotenoids[25,26].

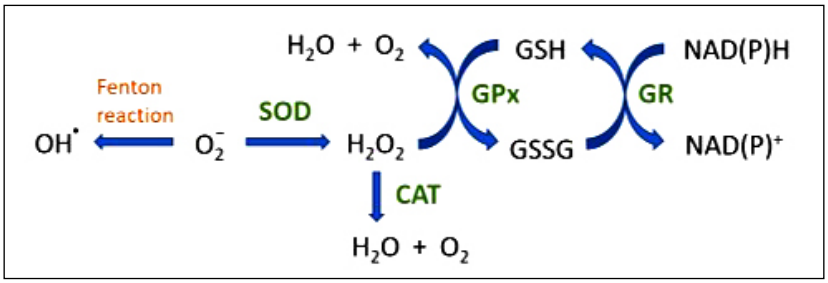

Figure 1. The major endogenous antioxidant pathways. (Adopted from Gil-Mohapel, 2014)[22]

THE EFFECT OF MUTANT HUNTINGTIN ON CELL INTEGRITY

The primary source of reactive oxygen species are mitochondria, which function is impaired in HD patients as well as in animal models[27]. Mutant huntingtin (mHTT) carriers showed reduced activity of the electron transport chain complexes, especially complex II, III and IV in caudate and putamen[3,28]. Presence of free radicals, which oxidize their iron-sulfur cluster, is suggested as a possible reason for mitochondria impairment. In addition to respiratory chain deficiency, the activity of aconitase, another iron-sulfur cluster enzyme, is also decreased by superoxide radical[29–31]. This is supported by the observations that polyQ protein, such as huntingtin, inhibits respiration by increasing ROS[32].

Reduced mitochondrial ATP production leads to energy deficit and failure of ATP-dependent ion pumps and channels. Consequently, mitochondria are unable to restore the membrane potential and Na^+ and Ca^{2+} concentration increases in the intracellular space. The high calcium concentration leads to increased production of hydroxyl radicals[2]. Among other things, mHTT can activate nitric oxide synthase and NO-mediated free radical production[33]. It can also trigger mitochondrial permeability transition pore (mPTP) opening, when pore activated by Ca^{2+} releases cytochrome c and thus induces apoptosis[34,35].

NADPH oxidase (NOX) in the plasma membrane is another source of increased ROS production and its activity is higher in postmortem HD cortex and striatum. A treatment with NOX inhibitors reduced the level of ROS and neuronal mortality[36]. As mentioned above, oxidative stress persists also due to insufficient antioxidant protection as mHTT also influences the production of some antioxidants. For example, mHTT binds directly to PGC-1α protein, which regulates the expression of antioxidants, including SOD1, SOD2, and GPx, or reduces its transcription by binding to its promoter sequence[37]. Moreover, PGC-1α plays a role in mitochondrial biogenesis and respiration. Thus, its reduction leads to antioxidant defence impairment[38] and decreased energy metabolism, which was detected in striatum of HD patients[39].

DETECTION OF OXIDATIVE DAMAGE

Increased presence of reactive oxygen or nitrogen species causes formation of oxidized proteins, lipids, nucleic acids, and their products. Some of them are used as markers of oxidative stress.

Protein carbonylation and also the product of nitration, 3-nitrotyrosine, were detected in striatum and cortex in post mortem HD patients[2,40]. The product of lipoperoxidation, malondialdehyde, can interact with amino groups of proteins or phospholipids to form lipofuscin[41]. Both of them are increased in HD brains and animal models[28]. Malondialdehyde has also been found in patients' blood[42]. Moreover, lipoperoxidation was also detected in fibroblasts isolated from a transgenic HD pig model by direct measurment[43].

Oxidation of DNA produces 8-hydroxy-2'deoxyguanosine (8-OHdG), a biomarker[44] that was also accumulated in brains and serum of HD patients[45]. ROS also induce double-strand breaks of DNA, resulting in an increased presence of DNA fragments in human HD striatal and cortical neurons[2,46].

CONCLUSION

As discussed above, studies confirm the presence of oxidative stress in HD, but the mechanisms leading to its formation are still not well described. In addition to oxidative damage, DNA damage and impaired mitochondria function leading to energy deficit and cell death[31] are observed, too. All these features are detected in humans as well as in animal models.

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7 Innate immunity in Huntington's disease

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Abstract

Huntington's disease (HD) is an inherited progressive neurodegenerative disorder with impairment of motor and cognitive functions. Any preventive or disease-modifying therapies are not available so far. Symptomatic treatment can only affect symptoms and it is not satisfying. Several approaches using RNAi to lower the expression of mutant huntingtin (mHTT) have been developed to date. In order to monitor the success of therapy efficacy in the pre-manifest stages of HD it is important to identify robust biomarkers of the onset and disease progression. The primary pathology of HD results inter alia from massive degeneration of neurons in the basal ganglia and thalamus. However, the expression of mutant huntingtin was detected in all examined tissues. Studies on HD demonstrated altered immune response in HD gene carriers indicating that cytokines may have a significant role in disease development. Therefore, cerebrospinal fluid (CSF) and blood serum may provide insight into pathology of HD, new prospective biomarkers and potential therapeutic targets. Additionally, microglia and monocytes could also serve as possible cellular source of candidate biomarkers to monitor HD progression.

Keywords

innate immunity, immune response, neuroinflammation, central nervous system, microglia, cytokines, biomarker

INTRODUCTION

Huntington's disease (HD) is a fatal inherited monogenic neurodegenerative condition caused by the expansion of trinucleotide CAG repeat within the coding region located in the exon 1 of the *HTT* gene that encodes a protein called huntingtin (HTT)[1]. The prevalence is 1:15 000. The disease onset is in the middle age of the patient (about 35–50 years)[2]. However,

many alterations at the cellular level precedes by decades any clinical symptoms seen in human[3]. HD is a clinically heterogeneous disease with various combinations of cognitive (executive dysfunction, subcortical dementia), psychiatric (personality changes, depression, anxiety, irritability, aggressiveness, psychosis, delirium) and motor symptoms (impairment of voluntary movements, chorea and dystonia, parkinsonian syndrome)[4]. The most significant clinical manifestation is the degeneration within the central nervous system (CNS). It is believed that the primary pathology of HD results from massive degeneration of neurons in the basal ganglia of the striatum and in cortex[5–7]. Moreover, the expression of mutated protein huntingtin (mHTT) was detected ubiquitously in all examined tissues[8]. There is an evidence that multiple non-neuronal abnormalities have been found in the peripheral tissues including the neuroendocrine system, skeletal muscles, cardiovascular system, blood, gastrointestinal tract, skeleton and genital organs[9,10].

Any preventive or disease-modifying therapies are not available by now. A better understanding of the earliest changes in brain as well as peripheral tissues could be critical for development of new therapeutic approaches aimed at elimination or repair of the impairing changes caused by mutant huntingtin. Several recent studies indicated that the immune system could act as a modifier of HD neuropathology[11–13]. In order to monitor the success of any disease-modifying drugs in the pre-manifest stage of HD it is important to identify robust biomarkers of the onset and disease progression.

INNATE IMMUNE SYSTEM ACTIVATION

Although HD is a devastating neurodegenerative illness, the neurodegeneration is also caused by aberrant immune response. Immune activation induced by mutant huntingtin may cause parallel inflammation in the CNS and in the peripheral tissues as well[14,15]. Central and peripheral immune dysfunctions were confirmed in HD patients and in models of HD (mouse, pig).

The immune system represents a network of various hematopoietic cells, molecules, tissues, and organs that operate together and defend the body against entrance of infectious organisms. Myeloid-derived blood monocytes represent peripheral effector cells of the innate immune system and in response to inflammatory signals (cytokines, chemokines) migrate quickly to the site of infection in tissues and differentiate into phagocytic antigen presenting cells – macrophages and dendritic cells. Microglia as the primary cells of innate immune activation in the brain represent resident macrophages of the CNS. Glia are not derived from neuronal progenitors but rather microglia have fetal myeloid precursors as their peripheral counterpart.

The brain is protected from the systemic circulation by the blood-brain barrier (BBB), which strictly regulates the entry of any molecules and blood cells into the CNS[16,17]. In

general, the presence of BBB supported the fact that the CNS is separated from the peripheral immune system and the cross of blood-borne particles is carefully regulated[16,18]. However, a large part of the proteins identified in cerebrospinal fluid (CSF) were also found in peripheral blood plasma of HD patients, therefore there is an assumption that during neuroinflammation and neurodegeneration, the BBB becomes more permeable[19] and some potential biomarkers might be easily transferred from CSF to blood plasma and accessed. Recent study showed that immunomodulatory molecules IL-6 and IL-8 are over-expressed both central and peripheral, in the striatum and in plasma[20]. These molecules may be able to cross the BBB in chronic conditions in both directions[15].

Dysfunctions in peripheral immune system in HD were confirmed. Elevated levels of pro-inflammatory cytokines and chemokines were detected in blood plasma from HD patients with significantly increased levels of IL-6 and IL-8 in pre-manifest stages. These cytokines are involved in the innate immune response. Up-regulation of TNFα was detected later in manifest grade of disease. In addition, anti-inflammatory cytokines IL-4 and IL-10 increased significantly with disease progress and represent components of adaptive immune system[20]. Chemokines MCP-1 and eotaxin are increased across advancing disease stages of HD[19]. Further, proteomic profiling of peripheral plasma and CSF also uncovered the increase of other innate immune protein clusterin, a protein that is associated with the clearance of cellular debris. An acute phase protein α2-macroglobulin (α2M) was released in plasma samples of HD patients and its production is stimulated by IL-6[21]. Detected immune molecules correlate with disease severity and are quantified many years before the onset of any clinical symptoms in carriers of HD. In addition, circulating peripheral blood monocytes from HD subjects expressed mutant huntingtin and were pathologically hyperactive in response to immune stimuli (LPS). Stimulated monocytes from HD carriers and from various mouse models were found to produced higher level of IL-6 compared to control monocytes[20,22,23]. This suggests a cell-autonomous role for mHTT in promoting the activation of myeloid cells[20]. Moreover, mHTT expression compromised cell migration of monocytes and microglia that were derived from mouse models or pre-symptomatic HD patients[13].

INNATE IMMUNITY IN THE CENTRAL NERVOUS SYSTEM – ROLE OF MICROGLIA

Except neurons, there are other cell types in the brain, especially glia (namely astrocytes, oligodendrocytes, and microglia). Glial cells with a non-neuronal origin have physiological functions in maintaining homeostasis of tissues, neuronal integrity and network functioning in the brain[24].

Microglia are immunocompetent cells of the central nervous system and account for about 10 % of all brain cells. Microglia represent the major resident parenchymal myeloid cells of

the brain, the CNS macrophages[25]. Microglia can be activated in response to misfolded proteins (e.g. mHTT). This diverts microglia from their physiological and beneficial functions (M1 phenotype, microglia with a small oval body and fine processes) and leads to their sustained release of pro-inflammatory mediators *in vivo* (M2 phenotype, microglia with swollen amoeboid-like shape with no processes)[14,24–28]. The primary role of active microglia is to phagocyte dying neurons and other particles. Reactive microglia express higher level of their surface antigens and secrete proteases and pro-inflammatory cytokines, which are essential in the innate immune response[18,29]. Microglia appear in the brain during chronic activation (inflammation) and expand rapidly locally causing microgliosis[23,30]. In addition, the sustained exposure of neurons to pro-inflammatory mediators can cause neuronal dysfunction and contribute to cell death[24].

Considering that the neuroinflammation is observed at relatively early stages of neurodegenerative diseases, to find mechanisms that drive this process may be useful for diagnostic and future therapeutic purposes[24].

NEUROINFLAMMATION AND MICROGLIA ACTIVATION IN HUNTINGTON'S DISEASE

Due to the expression of mutant huntingtin in the striatum, the tissue-specific loss of neurons occurs. Neuronal damage is a pathological hallmark of HD and is associated with chronic activation of an immune response in the CNS. Immune activation induced by mutant huntingtin may cause parallel inflammation in the CNS and in the peripheral tissues as well[14,15]. Central and peripheral immune dysfunction was confirmed. Microglia appear in the brain during inflammation and react intense with microgliosis[30]. *Post mortem* studies of HD brains have shown increased microgliosis and astrogliosis[31]. Furthermore, the presence of reactive microglia was significantly identified in regions most damaged in HD, notably striatum and cortex[29]. *In vivo* PET (positron emission tomography) studies supported these findings by indicating that microglia activation appears in the brains of pre-symptomatic HD subjects[32] and that microglial reactivity consistently increases with the disease severity[29,33]. Recent research showed that neuroinflammation is also supported by release of inflammatory cytokines and chemokines from the microglial cells. Elevated levels of interleukins IL-6[20], IL-1β, IL-8 and tumor necrosis factor α (TNFα)[34] were determined. Consequently, increased caspase activation, raised intracellular calcium levels, as well as the production of reactive oxygen species and nitric oxide result in neuronal death and brain structure damage[18,34]. In HD, the complement may be activated upon the presence of pathological protein huntingtin[35]. Compared with non-affected regions of the brain, affected regions also showed evidence of microglial cell production of several complement components (C1q, C4, C3 and C9), complement inhibitors (C1 inhibitor, clusterin) and membrane inhibitors

(MCP – membrane cofactor protein, DAF – decay-accelerating factor and CD59) compared to healthy brain[31].

The activation of microglia has been discovered for over a decade in HD brain and belong to a strong element of neuroinflammation in pathogenesis of Huntington's disease[23].

IMMUNE ACTIVATION IN TRANSGENIC PORCINE MODEL OF HUNTINGTON'S DISEASE

Given evidence that mHTT protein is expressed in central nervous system and peripheral immune cells, it is possible that inflammatory changes detected in peripheral tissues may reflect the inflammatory process in the CNS. Our study was focused on cytokine profiling in microglia secretomes and CSF in order to monitor immune response reflecting HD in the CNS and compare these with peripheral status of cytokines in blood monocytes secretomes and serum[36].

Large animal models are extremely valuable in biomedical research. To identify the mechanisms of inflammation and immune system dysfunction in HD we used transgenic HD porcine (TG-HD) model to better understand disease progression in human[37]. We collected samples from genetically related wild-type and transgenic mutant sibling animals. Biological fluids and cells from the CNS and peripheral system were utilised for detailed cytokine profiling by targeted affinity proteomics approach[37].

Observed dysregulation of cytokine profiles indicated neuroinflammation in the CNS and in peripheral system in transgenic porcine HD model. The most pronounced change was the decline of IFNα in CSF and secretomes of microglia in TG-HD minipigs compared to WT counterparts. In addition, IL-10 was lower in CSF as well as microglia secretomes. On the contrary, pro-inflammatory cytokines IL-1β and IL-8 were significantly increased in non-stimulated microglial cells isolated from TG-HD minipigs compared to WT. IL-8 was also elevated in peripheral system in serum of TG-HD minipigs. Similar change was not observed in blood monocyte of TG-HD animals[36].

Our findings identified molecules in transgenic HD porcine model which were altered (primarily IFNα, IL-10, IL-8). These proteins could serve as promising biomarkers for monitoring of organism's response to treatment against mHTT effect in TG-HD minipigs.

CONCLUSION

In neurodegenerative diseases including Huntington's disease, disability is primarily caused by dysfunction and death of neurons. However, non-neuronal cells have been shown to contribute to the progressive neuronal loss. Mutated protein huntingtin may induce pathological

immune activation in the brain and peripheral tissues of HD carriers as well. Neuroinflammation, characterised by remarkable microglia activation and inflammatory reactions in the CNS, could be revealed in the brains from HD carriers (from pre-symptomatic to advanced stages). There is an evidence that microglial activation is an integral part of HD pathogenesis. Furthermore, abnormal cytokine profile could be detected in the CNS and periphery from HD carriers.

Regarding to the monogenic character of HD and its possibility to be diagnosed before the onset of clinical symptoms, the biomarkers set before symptom onset could be used to track HD progression. Further analysis and testing their efficacy are necessary.

The profile of cytokine alterations showed involvement of innate immune pathways as well as lack of adaptive immune anti-inflammatory response in the CNS in TG-HD porcine model. These finding strongly highlighted IFNα and IL-10 as promising biomarkers in the CNS and IL-8 in blood serum from TG-HD porcine model.

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8 Primary fibroblasts for studying molecular mechanisms in Huntington's disease

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Abstract

Huntington's disease (HD) is dominantly inherited neurodegenerative disorder caused by the mutation in gene encoding huntingtin protein (HTT). HTT has a role in several biological processes and thus many molecular mechanisms are affected by the mutated form of HTT (mHTT). The exact pathogenic mechanism of mHTT action in HD pathology is still not well understood. Several animal models of HD have been created. Minipigs transgenic for the N-terminal part of human mHTT showed up to be suitable large animal model of HD with the onset of locomotor and neurological impairment at the age of 48 months. Primary fibroblasts isolated from this animal model represent well and easily accessible biomaterial. They can be obtained at different ages throughout HD progression thus facilitating the investigation of molecular mechanisms in HD.

Keywords

Huntington's disease, fibroblasts, molecular mechanisms

INTRODUCTION

Huntington's disease (HD) represents an inherited disorder currently lacking any effective treatment. HD is considered as neurodegenerative disease, along with Alzheimer's disease (AD), Parkinson's disease (PD), and others, because neurodegeneration is one of the main symptoms of this devastating disease. Neurodegeneration in HD occurs mainly in the striatum of the basal ganglia[1], and cerebral cortex[1–4].

Besides neurodegeneration, the other symptoms of HD are osteoporosis, testicular degeneration, loss of muscle tissue and heart muscle malfunction, weight loss, metabolic changes, and sleeping disturbances[5–7].

In 1993 single gene mutation, an expansion of CAG (Cytosine – Adenine – Guanine) repeats in the first exon of gene encoding huntingtin (HTT), which causes HD has been discovered. This mutation results in an elongated polyglutamine track (polyQ) of 40 CAG repeats and above near the N-terminal part of the huntingtin protein leading to the development of HD[8]. The mutated form of huntingtin (mHTT) negatively affects not only the central nervous system (CNS) and the brain but it affects also the whole body because huntingtin protein (HTT) including mHTT in HD patients are widely expressed in other peripheral tissues[5–7,9].

IMPORTANCE OF STUDYING MOLECULAR MECHANISMS IN HD

Within the cell, HTT has a role in several biological processes. It stimulates and controls the transcription of BDNF (brain-derived neurotrophic factor) and many other neuronal genes. HTT also regulates fast axonal trafficking, vesicle transport, and synaptic transmission[10]. Thus, many molecular mechanisms are affected by mHTT. However, the exact pathogenic mechanism of mHTT action causing HD is still not well understood.

For many years, links between DNA damage and neurodegenerative diseases including HD have been known[11–13]. Both the detection of DNA damage as well as the repair mechanism of the damaged DNA affected by mHTT have been described[14–18]. Another feature associated with HD pathogenesis is mitochondrial dysfunction which was shown to occur in HD brain, skeletal muscle, and also cultivated skin fibroblasts[19–23]. It has been suggested that mitochondrial dysfunction in HD is directly caused by oxidative stress induced by mHTT[24]. Oxidative stress has been considered as one of the key players in HD disease progression[25,26]. Even though it is still not clear whether oxidative stress is a cause of HD pathogenesis, or a consequence of other mechanisms[27].

In general, the underlying molecular mechanisms and cause of HD progression by mHTT remain under investigation. Identification of the gene responsible for the HD enabled the creation of several HD animal models by genetic manipulation, from invertebrates (*Drosophila melanogaster*, *Caenorhabditis elegans*) to small vertebrates[28]. Animal models of HD represent an important part in HD investigation. Cells isolated from these animal models serve for better understanding of molecular events involved in the disease pathology.

The most used small animal HD models are rodents (R6/2, HdhQ111, and YAC128 mice)[29,30]. But their application for detailed modelling of the pathogenic features of human disease is limited because of their small brain size, differences in neuroanatomy to humans, short lifespan, and fast disease progression (reviewed in[31]). Therefore, large animal models of HD were needed for better modeling and understanding a human disease, and were especially desired for safety

and preclinical tests of potential therapeutics and longitudinal studies of HD. In this consequence, non-human primate[32], sheep[33] and pig[34–37] models of HD, have been generated.

Transgenic minipig model expressing N-terminal fragment of human mutated huntingtin (TgHD) created in Libechov in 2009[36] is a promising large animal model of HD and can be used not only for implementing and assessing future therapeutic interventions but also for studying molecular events occurring in HD. The pre-manifestation stage of HD in these TgHD minipigs refers to the range of 24 months to 36 months of age[38]. The onset of locomotor and neurological impairment in the TgHD minipig model starts at the age of 48 months[39]. TgHD minipig model serves an opportunity to isolate unlimited number of primary cells and unlike primary cells obtained from HD patients, often in the late stages of the disease, the TgHD minipig model allows to monitor molecular changes occurring gradually with age and progression of the disease. Thus, TgHD minipig and primary cells isolated from it may play an important role in investigating and understanding the underlying mechanistic cause of HD.

MOLECULAR MECHANISMS IN PRIMARY FIBROBLASTS FROM TgHD MINIPIG MODEL OF HUNTINGTON'S DISEASE

Primary fibroblasts represent well accessible biomaterial and can be easily isolated from TgHD minipigs at different ages throughout HD progression. The transgene encoding the mHTT fragment in fibroblasts from TgHD animals contains approximately 121 CAG/CAA repeats[39]. Huntingtin protein in TgHD and wild type (WT) primary porcine fibroblasts is stably expressed with age and across generations[40]. Furthermore, compared to other cells, like mesenchymal stem cells (MSCs), primary fibroblasts isolated from TgHD minipigs express significantly higher levels of mHTT[41]. Therefore, TgHD primary porcine fibroblasts represent a suitable model for studying molecular events occurring gradually in HD.

It has been shown, that mHTT expression in neurons causes nuclear DNA damage and further promotes it by impairing components of DNA repair pathway[16]. Assessing the levels of nuclear DNA damage in primary fibroblasts from TgHD and WT minipigs revealed the age-associated increase in nuclear DNA damage. Nuclear DNA damage in fibroblasts from younger TgHD (pre-symptomatic) animals is significantly higher compared to WT. This could be caused by the action of mHTT fragments because of the highest peak of mHTT fragmentation at this age. Later with age, there is gradual decrease in fragmentation of mHTT in primary fibroblasts from TgHD minipigs[40,41]. This correlates with studies of Illuzzi and colleagues[14,15] who reported that DNA damage and response is induced by the expression of mHTT fragment before the appearance of detectable HTT aggregates. Furthermore, Suopanki and colleagues[42] showed that membrane leakage is induced by mutated and WT oligomeric huntingtin fragments (independent of polyQ length) but not by mHTT fibrils which in

turn decrease the membrane permeability. Primary porcine fibroblasts from TgHD minipigs display dramatic decrease in membrane permeability with age (symptomatic stage, at the age of 48 months), while the cell viability remains intact throughout aging, suggesting starting fibrillation process of mHTT in these cells[40].

Despite intact viability, the aging process is characterized by the loss of proliferative capacity[43]. Interestingly, fibroblasts isolated from 48 months old TgHD animals exhibited abnormal higher proliferation and showed significant differences in the normal proliferative pattern[40]. Significantly increased proliferation rate of cells expressing mHTT has also been clearly demonstrated elsewhere[44]. The higher proliferation of fibroblasts from symptomatic TgHD minipigs is further supported by their upregulation of *NEIL3* (nei like DNA glycosylase 3) gene, which is upregulated in highly proliferating cells[45–50], and significantly decreased levels of cyclin B1, regulating G2/M phase transition, and slightly increased levels of cyclin D1, regulating transition from G0/G1 to S[51]. In short, the fibroblasts from symptomatic TgHD animals clearly exhibit significant dysregulation of the cell cycle[40].

Fibroblasts from 48 months old TgHD minipigs also display significant upregulation of *SOD2* (superoxide dismutase 2) gene[40], a key mitochondrial antioxidant enzyme that catalyses the conversion of reactive superoxide to hydrogen peroxide[52] and its activity is mediated by cyclin B1/Cdk1 as a response to oxidative stress initiating cell cycle delay to avoid aberrant proliferation and protect the mitochondrial and nuclear genome integrity[53–55]. Interestingly, despite *SOD2* gene upregulation, the activity of SOD2 protein, which is critical for the maintenance of cell cycle delay, preventing aberrant proliferation, and protecting mitochondrial morphology [54,56], was slightly (non-significantly) decreased in these cells[40], unravelling the compromised (shortened) G2/M cell cycle delay in fibroblasts from symptomatic TgHD minipigs, causing their cell cycle dysregulation – aberrantly shorter G2/M delay.

Mitochondrial dysfunction is a key feature associated with HD pathogenesis[19–23] and is directly caused by oxidative stress induced by mHTT[24]. TgHD primary fibroblasts showed significantly higher levels of oxidative stress clearly demonstrating gradual accumulation of oxidative stress in fibroblasts from TgHD minipigs with age[40]. Oxidative stress has been shown to be increased in HD and highly contributing to the HD disease progression[25,26,57–60]. However, the exact mechanism of how mHTT induces oxidative stress has not been described yet[27].

It has been previously shown that mHTT, but not wild type HTT, binds to and scavenges Ataxia telangiectasia mutated (ATM) in the cytoplasm[17]. ATM has an essential role in G2/M checkpoint where its activity results in preventing activation of cyclin B1/CDK1 complex and thus arresting cell cycle progression, allowing time for genotoxic or oxidative stress repair mechanisms[61]. Disturbed activity of ATM, by the action of mHTT, could potentially lead to the shorter G2/M delay causing gradual accumulation of oxidative stress, as observed in primary fibroblasts isolated from TgHD symptomatic minipigs[40].

Fibroblasts are widely used for studying molecular events occurring in neurodegenerative diseases. Most of such research is done on fibroblast whose cell cycle is arrested in order to recapitulate the postmitotic neurons as much as possible. However, some neurons can re-enter cell cycle under certain circumstances[62]. Using asynchronous population of primary porcine fibroblasts from TgHD minipigs for investigation of gradual molecular changes enabled to detect dysregulation of G2/M delay, one of the possible causes of gradual accumulation of oxidative stress in cells expressing mHTT.

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9 Genetic causes and animal models of basal ganglia related disorders – I. Parkinson's disease

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Abstract

Striatum is the area of basal ganglia known as an important regulator of motor output. Inhibitory GABAergic medium spiny neurons (MSNs) are predominant neuronal population of the striatum. Functional imbalances and disruptions in their function are associated with abnormal function of the striatum and play a role in several movement disorders. Huntington disease is probably the best-known movement disorder affecting striatum. However, secondary striatal dysfunction plays an important role also in Parkinson's disease (PD). In our review, we provide short overview of this neurodegenerative disease, its clinical features and current therapeutical approaches, but mainly focus on summarising current knowledge of underlying genetic causes of PD and available animal models of this serious disease.

Keywords

striatum, Parkinson's disease, medium spiny neurons, animal models

STRIATUM ANATOMY AND PHYSIOLOGY

The basal ganglia play an important role in cognitive and movement control circuits and interconnect with other parts of the brain by multiple parallel loops[1]. The striatum, major

component of basal ganglia, receives excitatory inputs from the thalamus and somatosensory and motor cortex[2]. These inputs differentially innervate MSNs and interneurons in the dorsal part of the striatum. Somatosensory afferents strongly stimulated Parvalbumin interneurons over MSNs, and produced specific behaviour inhibition[3]. The striatum is comprised of a dorsal and ventral part. The dorsal part includes two nuclei, caudate and putamen, in primates separated by an internal capsule. The ventral part consists of most rostroventral caudate and putamen, a ventral extension of putamen, and nucleus accumbens[4]. The whole striatum contains two main neuronal populations, projection medium spiny neurons (MSNs) being the majority part (90–95 %) and interneurons the remaining 5–10 %. Dorsal striatal MSNs contribute to the decision making by integrating emotion, sensorimotor, cognitive and motivation inputs[5]. The ventral part of the striatum plays a role in motivation, reward, and impulsivity. According to their dopamine receptor expression, MSNs can be divided into two distinct groups: D1 (D1R) and D2 (D2R) type expressing MSNs. D1R positive neurons contain the neuropeptide substance P and participate in the so called direct striatal pathway, projecting to the internal segment of the globus pallidus, and substantia nigra pars reticulata. D2R positive neurons contain neuropeptide encephalin and form the indirect pathway, projecting to the external segment of globus pallidus[6].

DOPAMINERGIC SIGNALLING

Dopamine (3,4-Dihydroxytyramine) belongs among catecholamines and acts both as a hormone (neurohormone released by hypothalamus) and a neurotransmitter. As a neurotransmitter, dopamine is produced from tyrosine via L-DOPA by presynaptic dopaminergic neurons at substantia nigra and ventral tegmental area. After production, dopamine is packed into synaptic vesicles and stored until incoming stimulus releases it into the synaptic cleft, where it binds to D1R and D2R receptors of the postsynaptic neurons. Dopamine level is also controlled by reuptake of excess dopamine from extracellular space into presynaptic neurons. This reuptake is mediated by the dopamine transporter protein (DAT) in the presence of chloride and sodium ions[7]. Dopaminergic pathways are involved in maintenance of many functions, including regulation of stress, movement, learning, rewards, and emotions[8].

Both D1R and D2R dopamine receptors belong to G-protein coupled receptor (GPCTs) class. These receptors stimulate/inhibit adenylyl cyclase to convert ATP to cAMP. D1Rs are stimulating receptors, which after their activation increase intracellular levels of cAMP by stimulating adenylyl cyclase. D2Rs are inhibitory and their activation leads to adenylyl cyclase inhibition and decrease in cAMP levels. As second messenger, cAMP then influences many cellular processes via activation of dependent protein kinase A, which phosphorylates

many protein substrates, including DARPP32, phospholipases, tyrosine kinase receptors, ion channels, protein kinases[9]. Dopamine level related abnormalities and defects in cortico-striatal circuits are implicated in many disorders such as Huntington disease (HD) and Parkinson's disease (PD)[1,10].

PARKINSON'S DISEASE

In 1817, James Parkinson published *An Essay on the Shaking Palsy*, reporting six cases with detailed symptoms description. These symptoms include weakness in the hand, trembling, shaking, problems with walking, sudden changes of posture and other non-motor symptoms, e.g. sleep dysregulation[11]. Nowadays, PD is characterized as a complex neurodegenerative disorder manifested by slow and continuous progression of parkinsonian syndrome (bradykinesia, rigidity, postural instability, tremor and other motor symptoms)[12] together with many nonmotor features including cognitive dysfunction, mood and psychiatric disturbances, behavioural changes, autonomic and gastrointestinal dysfunction, sleep dysfunction, fatigue, sensory and olfactory dysfunction, etc.

Parkinson's disease is affecting 1–2 % of world's population over 60. Most cases (85–90 %) are sporadic, with so far unidentified triggers. About 10–15 % of PD cases are familial and some can be linked to mutation in one of the several implicated genes. Median age of onset is 60 years, with mean survival of 15 years from diagnosis[23]. The most common manifestation of PD is considered a classical form PD, while cases of parkinsonism with onset at age 40 or younger are classified as early onset and cases with onset before age of 21 are arbitrary define as juvenile parkinsonism (JP)[24]. Many of the early onset parkinsonism cases are of familial and atypical type.

CLINICAL MANIFESTATIONS OF PARKINSON'S DISEASE

Main motor symptoms of PD include bradykinesia, rigidity, resting tremor, postural instability, gait disturbances, stoop posture, hypomimia, dysarthria and also dysphagia in an advanced stage of the disease. Bradykinesia, tremor and rigidity usually begin unilaterally, and a side asymmetry is often observed even in later stages of PD. *Bradykinesia* is a generalized slowness of movements and is a leading motor symptom of PD together with hypokinesia (reduced range of a movement) and akinesia (problematic start of a movement). It manifests as decreased manual dexterity, slowness of movement and reaction times, reduced arm swing while walking and shortening of steps or difficulties standing up from a chair. Other manifestations include hypomimia (loss of facial expression), hypophonia and hypokinetic dysarthria. *Muscle rigidity* is characterized as an increased resistance to passive movement, accompanied by the "cogwheel" sign. Muscle rigidity is one of the most contributing factors to pain, overall stiffness and slowness. Typical PD

tremor is a resting tremor of a frequency 4–6 Hz, usually asymmetrical and sometimes described as a „pill-rolling" tremor. It is typically present in upper extremities but can also be found in chin, jaw and legs. *Postural instability,* although mostly found in later stages, is another cardinal sign of PD. It results, together with gait disturbances, in frequent falls with a risk of severe injury. Gait difficulties in PD include overall slowness, stoop posture, short steps, reduced arm swings and especially freezing, which is a form of akinesia and is manifested as a sudden inability to move. Freezing is not always present but can be a significant cause of falls.

Among nonmotor symptoms, *cognitive dysfunction and dementia* are very common. Dementia is typically subcortical with overall psychomotor retardation and executive dysfunction (difficulties in creating a concept of a certain activity, impaired multitasking and impaired ability to quickly change specific activity). This is frequently accompanied by mood and behavioural changes. *Apathy, depression and anxiety* are also frequent nonmotor symptoms and are considered to have a severe negative impact on quality of life[13,14]. In addition to mood disorders, many patients show signs of *impulse control disorder*, including gambling, binge eating, hypersexuality, compulsive shopping and punding (repetitive ineffective behaviour such as collecting, disassembling and sorting various objects). Many patients also develop some form of *psychosis*, as the most common psychotic feature in PD are visual hallucinations[15]. Other hallucinations and delusions can also be present but are less frequent. Both impulse control disorder and psychosis can be worsened by dopaminergic therapy, especially by dopamine agonists. *Sleep disorders* are another well described nonmotor symptoms of PD and include insomnia, fragmented nocturnal sleep, excessive daytime sleepiness, restless legs syndrome, periodic limb movement disorder and especially REM sleep behaviour disorder, which is often diagnosed several years before other clinical symptoms[16,17].

Autonomic dysfunction in PD includes typically orthostatic hypotension, sphincter and erectile dysfunction, seborrhoea and excessive sweating. Common *sensory complaints* include olfactory dysfunction, paresthesia and painful sensations. Hyposmia may even precede the onset of motor symptoms[18,19].

NEUROPATHOLOGY OF PARKINSON'S DISEASE

On neuropathology level, PD is characterized by degeneration and loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) with consequent reduction in dopamine input to the dorsal striatum, which leads to motor symptoms. This loss of dopaminergic neurons is accompanied by presence of intraneuronal inclusions – Lewy bodies and Lewy neurites. As the proportion of nigral neurons containing Lewy bodies seems to be constant (3–4 %) irrespective of disease stage, it was proposed that Lewy bodies are causing neuronal death[20]. The main component of these bodies is an aggregated and post-translationally modified form of the presynaptic protein α-synuclein[21]. Ubiquitin

C-terminal hydrolase L1 (UCHL1)[22,23], αB-crystallin, and neurofilament protein are also present in these aggregates. Lewy bodies are present not only in the neurons of substantia nigra, but in late stages also in cortex.

CURRENT THERAPY OF PARKINSON'S DISEASE

The patient‘s age, cognitive status, severity of symptoms and comorbidities should be carefully considered when selecting the right treatment. Current therapeutical strategies can be divided into two groups – treatment of early and middle stages of PD (dopamine agonists, L-DOPA, monoamine oxidase type B inhibitors, Amantadine) and therapy of late stages with several motor and nonmotor complications (L-DOPA, catechol-O-methyl transferase inhibitors, monoamine oxidase type B inhibitors, Duodopa, deep brain stimulation and apomorphine). Dopamine itself cannot be used for PD treatment because it doesn‘t pass the blood-brain barrier, thus, a precursor (L-DOPA) or molecules that mimic dopamine (dopamine agonists) and pass the blood-brain barrier are used to overcome this problem. *Dopamine agonists (DAs)* act directly on dopaminergic receptors in the striatum. They can be used as a first-line treatment for patients under approximately 65 years of age and without any history of cognitive impairment or psychotic symptoms. The effect of DAs is slightly weaker than the effect of L-DOPA and therefore is preferable in the early stages of PD. DAs can have several side effects, including nausea, dizziness, fatigue, peripheral edema, impulse control disorder and psychosis. Because of these side effects, they are not suitable for older patients with cognitive impairment or history of any psychotic features. *L-3,4-dihydroxyphenylalanine (L-DOPA)* is a dopamine precursor, which is taken up by the dopaminergic neurons and converted to dopamine by DOPA decarboxylase. L-DOPA is more potent than DAs and is used (alone or in combination with DAs and other substances) to manage moderate to severe PD symptoms. It is preferred as a first-choice treatment in older patients or in those with cognitive deficit. L-DOPA is administered in combination with DOPA decarboxylase inhibitors (carbidopa, benserazide) that lower L-DOPA decarboxylation in peripheral tissues, thus increasing L-DOPA bioavailability in the brain.

Along with the course of the disease and dopaminergic treatment, late motor and nonmotor complications emerge, especially fluctuations including shortening of the L-DOPA effect, „peak-of-dose“ dyskinesia and sudden worsening of motor state (on-off fluctuations). Administration of *catechol-O-methyl transferase (COMT) inhibitors* increases the bioavailability of L-DOPA and decreases „off“ time in PD patients. *Monoamine oxidase type B (MAO-B) inhibitors* have mild symptomatic effects in early stages and, along with L-DOPA, also reduce overall “off” time[25,26]. *Amantadine*, formerly used as an antiviral treatment, has a mild antiparkinsonian effect by stimulation of dopamine release and inhibition of its reuptake.

In advanced stages of PD, severe motor fluctuations refractory to oral treatment can be indication for *deep brain stimulation (DBS).* Continuous high-frequency stimulation

suppresses neuronal activity in subthalamic nucleus and internal globus pallidus (GP, resulting in decreasing GPi inhibition of the thalamus and improvement of bradykinesia and motor fluctuations[27,28]. *Levodopa/carbidopa intestinal gel (Duodopa)*, administered with a portable pump directly to upper jejunum, or *continuous subcutaneous apomorphine infusion* are possible options for patients who are contraindicated to DBS[28,29].

Management of neuropsychiatric symptoms of PD includes first of all therapy of *depression and anxiety*, where selective serotonin reuptake inhibitors (SSRIs) are a treatment of choice. Episodes of severe anxiety may be a symptom of wearing off in patients with motor and nonmotor fluctuations and can be improved by adjustment of L-DOPA medication.

PD is very often complicated by *psychosis*, which can be triggered by various conditions such as infection or medication, including antiparkinsonian medication itself, especially dopamine agonists. If psychosis cannot be managed by treating underlying conditions or adjustment of medication, atypical antipsychotics tiapride, quetiapine, melperone and clozapine are considered relatively safe to use. Other antipsychotics are contraindicated due to the risk of severe exacerbation of PD symptoms.

GENETIC CAUSES OF PARKINSON'S DISEASE

About 5–10 % of patients suffer from a monogenic form of PD with high penetrance mutations. Some of these disease causing mutations have autosomal dominant inheritance, e.g. mutations in gene for leucine-rich repeat kinase 2 (LRRK2), α-synuclein (SNCA), and VPS35, while others present with autosomal recessive inheritance, including mutations in PTEN-induced kinase 1 (PINK1), Parkin, and DJ-1[30]. Several mutations were described in LRRK2, with G2019S mutation being the most common. This variant was identified in 1 % of sporadic PD cases and up to 4 % of familial PD[31], is autosomal dominant and associated with late disease onset (over 60 years of age)[32]. LRRK2 mutation G2019S increases its kinase activity[33] and has an impact on many pathways including reduced degradation by chaperone mediated autophagy[34]. This could interfere with degradation of α-synuclein and might be the principal cause of mutated LRRK2 neurotoxic effects in PD. LRRK2 kinase domain activity is required for the damaging effects, including formation of inclusions and triggering neuronal cell death[35]. LRRK2 can also interact with several other genes, known as risk factors in PD, including DJ-1, Parkin, PINK1[36], and VSP35[37]. Several point mutations as well as genomic triplications of SNCA gene, coding main Lewy body component α-synuclein protein, cause autosomal dominant familial version of PD[38]. Missense mutations A30P[39], A53T[40], E46K[41], H50Q[38], and G51D[42] lead to protein misfolding, formation of toxic oligomers and fibrils, and finally to cytosolic inclusion of α-synuclein protein. Even normal α-synuclein is prone to aggregation, but how exactly the gene mutations cause the pathology is still largely unclear. Abnormal α-synuclein may be linked to mitochondrial fragmentation and function deficit, oxidative stress, ubiquitin-proteasome system dysfunction and aberrant neurotransmitter release[43].

Familial forms of parkinsonism with recessive inheritance and early/juvenile onset were linked to mutations in Parkin[44], PINK1, and DJ-1 genes[24]. Parkin and PINK1 share the same mitochondrial pathway and mutation in either of these genes is related to defective mitophagy, while DJ-1 plays role in resistance to oxidative stress[45]. Parkin is an E3 ubiquitin ligase with essential role in many cellular proteostatic processes, including the ubiquitin-proteasome system, lysosome degradation, and autophagy. Duplications, exon deletions and missense mutations were identified as PD causing in the parkin gene[46]. PINK1 is a serine threonine-protein kinase localized in the cytosol with a role in neural differentiation and regulation of mitochondrial dynamics. DJ-1 functions as redox-dependent molecular chaperone and inhibits the formation of α-synuclein aggregates. Mutation of DJ-1 leads to oxidative stress and inhibition of proteasome[47].

ANIMAL MODELS OF PARKINSON'S DISEASE

Complex diseases such as neurodegenerations still require use of animal models to recapitulate most of the symptoms. Many animal models were developed to study PD pathogeneses. These models can be divided into genetic models, neurotoxin induced models, and their combination. Selected models are briefly discussed below, for use of these models in biomarker and potential treatment discovery see chapters 12 *Protein biomarkers of neurodegeneration in cerebrospinal fluid* and 13 *Targeted proteomics in translational research of neurodegenerative diseases*.

GENETIC MODELS

The identification of causative genetic mutations can be used to understand the disease pathology and allows the generation of suitable animal models bearing necessary PD hallmarks. SNCA was the first gene to be associated with PD. Transgenic mouse carrying human E46K α-synuclein accumulates cytoplasmic inclusion trough the CNS, e.g. the spinal cord, motor cortex, thalamus, and shows motor impairment[48]. Transgenic mouse model expressing LRRK2 G2019S increases aggregation of α-synuclein protein in neurons and leads to significant decrease in number of neurons in the substantia nigra pars compacta associated with behavioural phenotype[49]. Mouse carrying disruption of parkin exon 3 shows nigrostriatal deficits in the absence of nigral degeneration, reduction of complexes I and IV of striatal mitochondria, and metabolic abnormalities[46]. PINK1 deficient mouse exhibits decreased dopamine levels, mitochondrial dysfunction in ATP-generation and respiration[50]. DJ-1$^{-/-}$ knockout mouse created by an exon 2 deletion show abnormalities in behaviour such as hypoactivity, without any abnormalities in the release of dopamine or dopamine reuptake[51].

NEUROTOXIN BASED MODELS

Several environmental neurotoxins inducing symptoms of parkinsonism were identified. While these represent significant risk to humans, they are useful for modelling the disease in animals. The administration of these neurotoxins causes the loss of neurons in the substantia nigra pars compacta and corresponding changes in motor function but often doesn't create Lewy bodies. Two widely used neurotoxins of this type are 6-hydroxydopamine (6-OHDA) and 1-methyl-1,2,3,6-tetrahydropyridine (MPTP), others being rotenone and paraquat (N,N′-dimethyl-4,4′-bypyridinium). 6-OHDA must be applied directly into the brain as it does not cross the blood-brain barrier. It is toxic to dopaminergic neurons and provokes neuronal loss in substantia nigra pars compacta and ventral tegmental area by inhibition of mitochondrial complexes I and IV[52]. Motor function can be evaluated after apomorphine administration by observing rotational behaviours. Infusion of 6-OHDA into rats striatum reveals lesion of dopaminergic terminals after 24 h and loss of dopaminergic neurons in the substantia nigra pars compacta at the 4th week[53]. MPTP is another dopaminergic toxin which damages dopaminergic pathway and induces loss of dopaminergic neurons. MPTP can cross the blood-brain barrier, and in the brain, it is converted by glial monoamine oxidase B into 1-methyl-4-phenylpyridinium ion (MPP^+). MPP^+ accumulates in neurons and works as a substrate for dopamine transporter. After entering the mitochondria, MPP^+ inhibits the complex I[54]. MPTP induced monkey (Macaca fascicularis) model of PD exhibited bradykinesia, resting tremor, increased rigidity and decreased vocalization. The histochemical study revealed massive loss of neuronal cells in the substantia nigra pars compacta[55]. Rotenone is a pesticide which neurotoxic effects and ability to cross the blood-brain barrier. Rotenone binds to mitochondrial complex I and inhibits its activity, resulting in loss of dopaminergic neurons and dopaminergic degeneration. Rats exposed to rotenone treatment exhibited motor and postural phenotype similar to PD patients, including the formation of Lewy bodies[56]. Paraquat (N,N′-dimethyl-4,4′-bipyridinium) is a herbicide with structure similar to MPTP. Paraquat exposure is linked to production of reactive oxygen species and oxidative stress[57]. Intoxication by fungicide Maneb also induced many features of PD such as postural tremor, bradykinesia, and cerebellar signs[58]. Rats administered intraperitoneally with paraquat/maneb combination exhibited weight loss, respiratory distress, tremor, disturbance of motor coordination and activity. Biochemical assessment revealed a reduction in striatal dopamine[59].

Amphetamine psychostimulants such as methamphetamine (METH) and 3,4-methylenedioxymethamphetamine (MDMA) have neurotoxic effects associated with neurologic events including function deficit and structural changes in dopaminergic pathways[60]. METH generates reactive oxygen species and reduces activity in mitochondrial complex I, leading to depletion of striatal dopamine. METH administered to male C57/B16 mice caused motor impairment, a decline of dopamine level and loss of dopaminergic neurons in the striatum[61].

CONCLUSION

After Alzheimer's disease, Parkinson's disease is the second most prevalent neurodegeneration. Identification of several genes responsible for familial versions of PD and of neurotoxins inducing parkinsonism symptoms allowed for development of multiple animal models. These models are important for improving our understanding of PD pathogenesis and for development of novel therapeutic approaches, including gene and cell replacement therapies.

Table 1. Overview of animal models of PD.

	Gene/Compound	Species	References
Neurotoxin-based models	6-OHDA	Rat	**53**
	MPTP	Macaca fascicularis	**55**
	Rotenone	Lewis rat and Sprague-Dawley	**56**
	Paraquat	Wistar rat	**57**
	Paraquat/maneb	Sprague–Dawley rat	**59**
	MET/MDMA	C57/B16 mouse	**61**
Genetic-based models	SNCA (α-syn)/ E46K	Transgenic mouse	**48**
	Parkin $^{-/-}$	Mouse	**48,46**
	PINK1	Deficient mouse	**50**
	LRRK2^{G2019S}	Transgenic mouse	**49**
	DJ-1$^{-/-}$	Mouse	**51**

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10 Genetic causes and animal models of basal ganglia related disorders – II. Neuropsychiatric disorders

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Abstract

The striatum serves as an integration hub and relay in a complex information exchange among other parts of brain. Cortico-basal ganglia-thalamo-cortico (CBGTC) loops are involved in regulation of behaviour, and thus their disturbances play an important role across a range of neuropsychiatric disorders. This review provides short overview of several of these disorders and how dysfunction of striatal circuits relates to their pathology. Relevant pharmacological, genetic and environmental animal models available for translational research of these diseases are briefly described.

Keywords

striatum, neuropsychiatric disorders, cortico-basal ganglia-thalamo-cortico (CBGTC) loops, animal models

INTRODUCTION

Both genetic and environmental factors play a significant role in the aetiology of neuropsychiatric disorders. Underlying changes in brain circuity and dysfunctions caused by these factors are still poorly understood. Basal ganglia and particularly striatum are part of many affected circuits.

Better understanding mechanisms of these diseases is required to improve diagnosis and treatment but is complicated by often present comorbid conditions. For example, up to 45–60 % of Tourette's syndrome (TS) cases display also clinical symptoms of attention-deficit / hyperactivity disorder (ADHD) and obsessive-compulsive disorder (OCD) and gene *SLITRK1* is implicated in both Tourette's syndrome and OCD spectrum disorders[1].

Bipolar disorder (BD) and major depressive disorder (MDD) express very similar clinical symptoms without clear boundary[2].

Developing animal models for psychiatric disorders is very difficult because most phenotypes are behavioural. Models usually recapitulate only one or several, but rarely all the relevant behaviours. Environmental, pharmacological and genetic models are being actively developed and many exhibit abnormalities in striatal input/output pathways including changes in relevant neurotransmitters, transporters and receptors and consequent impairment in CBGTC circuits. This chapter provides short overview of neuropsychiatric diseases with CBGTC involvement and of relevant animal models.

CORTICO-BASAL GANGLIA-THALAMO-CORTICO LOOPS

The striatum functions as an information hub receiving and integrating various information via excitatory stimulation from the cerebral cortex and thalamus[3]. The system of interactions between striatal complex and other parts of the brain is called cortico-basal ganglia-thalamo-cortical (CBGTC) loop. CBGTC loop is involved in behaviour, e.g. motivation, affect regulation, evaluation and reward based decision making[4] and its disturbances play a key role not only in movement disorders (Parkinson's and Huntington's disease) but also neuropsychiatric diseases.

The dorsal part of the striatum receives inputs from the cortex and thalamus, central part receives inputs from associative cortical regions, and inputs from limbic area terminate in ventral part of the striatum. Neurons from substantia nigra (SN) project to dorsal striatum, and neurons from ventral tegmental area (VTA) to ventral striatum. The striatum receives also inputs from the hippocampus and amygdala. Inputs from cortex and thalamus are glutamatergic, from substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) dopaminergic, and from brainstem (raphe and locus coeruleus) serotogenic and noradrenergic. The output from the striatum has only two projection pathways of medium spiny neurons (MSN) – the globus pallidus interna (GPi) and second pathway via globus pallidus externa (GPe) to SN[5]. Functionally, the orbitofrontal cortex projects into ventromedial part of caudate, which is involved in motivation, reward, and emotion; while the dorsolateral part of caudate is involved in cognitive functions; and the putamen is associated with motor control[6].

NEUROTRANSMITTERS INVOLVED IN CBGTC

Glutamatergic pathways release excitatory neurotransmitter glutamate. Anatomically, they include following descending and ascending projection pathways: I. from cortex to brainstem areas (SN, VTA, raphe and locus coeruleus), II. from cortex to the striatum (cortico-striatal pathway) and nucleus accumbens (cortico-accumbens pathway). These

projection pathways constitute cortico-striatal part of cortico-striatal-thalamic loop, III. Ascending glutamatergic pathway from thalamus projecting to pyramidal neurons in cortex (thalamocortical pathway) and IV. descending pathway from the cortex to the thalamus (corticothalamic pathway), V. glutamatergic communication between cortical neurons (cortico-cortical pathway)[7]. Two types of glutamate transporters, vesicular glutamate transporters (VGLUT1–3)[8] and excitatory amino acid transporters (EAAT) play an important role in glutamate neurotransmission. Glutamate receptors can be divided into N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and Kainate receptors. NMDA receptors are essential for synaptic formation and plasticity, necessary for the memory and learning. Over-activation of the NMDA receptors leads to excessive influx of Ca^{2+}, resulting in excitotoxicity involved in many neurodegenerative diseases. GABA (γ-aminobutyric acid), the main inhibitory neurotransmitter, is synthesized from glutamate by glutamate decarboxylase (GAD) enzymes. There are two classes of GABA receptors, $GABA_A$ (ligand-gated ion channel) and $GABA_B$ (G protein coupled receptors). Medium spiny neurons (MSN), the main neuronal population of the striatum, are GABAergic inhibitory cells. The balance between glutamate and GABA is crucial for normal brain function and physiology, and change in this excitatory-inhibitory ratio contributes to the pathophysiology of psychiatric diseases[9,10]. Excessive glutamate release and decrease of GABA is included in development in schizophrenia (SZ), bipolar disorder (BD), and major depressive disorder (MDD).

Dopamine (DA), serotonin (5-hydroxytryptamine, 5-HT), and norepinephrine (noradrenaline) are additional neurotransmitters involved in CBGTC loop and regulating many functions in the brain. The dopamine pathway regulates reward and motivation, and learning. Abnormalities in dopaminergic pathway lead to behavioural deficits and are apparent in many neuropsychiatric disorders. Dopaminergic receptors are G protein-coupled receptors and have 5 subtypes belonging to two groups: D1-like and D2-like. D1-like receptors stimulate adenylate cyclase, while D2-like receptors are inhibitory[11]. Dopaminergic transmission is partly controlled by dopamine transporter (DAT), which is localized to dendrites and cell bodies of SNc and VTA neurons and reuptakes dopamine from the synaptic cleft (see chapter 9 *Genetic causes and animal models of basal ganglia related disorders – I. Parkinson's disease* for details on dopamine neurotransmission). Raphe nuclei in the brainstem are composed of several nuclei producing the neurotransmitter serotonin (85 % of total brain serotonin is produced in this area). Serotonin signalling controls mood, emotional state, regulates aggressive behaviour and sleep, and is involved in memory and learning. Serotonin is synthesized from tryptophan by tryptophan hydroxylase (TPH)[12]. Serotonin receptors (5-HT receptors) belong mostly to G-protein coupled receptor group, but ion channel serotonin receptor exists as well[13]. Serotonin transporter 5-HTT is important for control of serotoninergic neurotransmission through the reuptake of 5-HT and is main target of selective serotonin reuptake inhibitors (SSRI) antidepressants. Similarly, most of the brain production of norepinephrine is

located to neurons of locus coeruleus, small nucleus in pons. Locus coeruleus and target areas of its norepinephrine production are called locus coeruleus-noradrenergic system (LC-NA) and play an important role in stress response, attention and arousal[14].

STRIATUM RELATED NEUROPSYCHIATRIC DISEASES

SCHIZOPHRENIA

Schizophrenia (SZ) is a chronic neuropsychiatric disorders characterized by a presence of psychotic behaviours. The main positive symptoms include delusions and hallucinations, while common negative symptoms are lack of motivation, speech deficit, and social withdraw. Impaired working memory, attention and executive function are possible cognitive symptoms[15]. Lifetime morbid risk of SZ was estimated 7 and lifetime prevalence 4 per 1,000[16]. Genetics is a strong risk factor for SZ, with 80% heritability estimated by meta-analysis of twin studies and many suspect loci uncovered by genome-wide association studies (GWAS) and other molecular genetic studies[17]. SZ is characterized by dopaminergic dysregulation in the dorsal striatum and changes in D2 receptor binding profiles, where increased dopamine release inhibits D2 expressing neurons[18]. Dopamine and cyclic AMP-regulated phosphoprotein (DARPP-32) is highly expressed in MSNs. Decreased DARPP-32 expression and phosphorylation was described in SZ. DARPP-32 phosphorylation is regulated by neurotransmitters glutamate and dopamine, which are both implicated in the pathology of schizophrenia. Treatment with antipsychotic drugs restores DARPP-32 phosphorylation[19]. The zinc-finger SWIM domain-containing protein 6 (ZSWIM6), expressed during brain development and postnatally localized to the striatum, was implicated in SZ by several GWAS studies. In mouse KO model, the loss of *Zswim6* led to changes in the morphology of MSNs, reduction in striatal volume, and to behavioural symptoms reminding SZ and other neurodevelopmental disorders[20]. Disrupted in schizophrenia 1 (*DISC1*) is a gene locus identified by cytogenetic analysis in Scottish family suffering mental illnesses associated with abnormal chromosomal translocation t(1;11) (q42.1;q14.3)[21]. *DISC1* plays a crucial role in early neurodevelopmental and later during adolescent brain maturation and synaptic regulation[22]. $Disc1_{tr}$ transgenic mice carry two copies of truncated *Disc1*, mimicking the chromosomal translocation and show abnormalities corresponding to severe SZ symptoms, e.g. reduced parvalbumin cells, dilated lateral ventricles, reduced neuronal proliferation in layers II/III, reduced cerebral cortex, and longer immobility in depression test[23]. Neuregulin 1 (NGR1) together with its receptor tyrosine kinase erbB4 regulates activity dependent maturation of glutamatergic synapses[24]. Polymorphisms in NGR1 were linked to SZ susceptibility in Icelandic population[25] and later confirmed in other contexts. Extracellular matrix protein Reelin, expressed by GABAergic interneurons, regulates movement and position of cortical cells during neurodevelopment. Postnatally, it is associated with synaptic plasticity

and lower expression of Reelin leads to cognitive impairment in SZ patients[26]. Heterozygous reeler mice with 50% lowered expression of reelin mRNA and protein show GABAergic defects similar to SZ[27]. Microdeletions at 22q11.2 locus lead to 30-fold increase in risk for development of SZ[28], and associate also with bipolar disorder[29] and other psychiatric disorders[30]. Decreased expression of *DGCR8* gene located in this region and serving in microRNA processing might be responsible for SZ association[28]. MicroRNAs are critical to many neuronal processes and single nucleotide polymorphisms (SNPs) in miRNAs (e.g. miRNA-137) and their target genes were linked to SZ phenotype[31]. Df(16)$A^{+/-}$ mice carry microdeletion in region syntenic with human 22q11.2 locus and model abnormalities related to SZ criteria[32]. Dysbindin-1 (DTNBP1) is widely expressed in the brain, but reduced in cortex and hippocampus of SZ patients[33]. Spontaneous mutation in mouse Dysbindin-1 led to creation of Sandy (Sdy) mouse model, which in homozygous state recapitulates various features of SZ, including changes in pre- and postsynaptic dopaminergic, glutamatergic, and GABAergic transmission[34].

Several pharmacological SZ rodent models are available. The prenatal (E17) administration of neurotoxin methylazoxymethanol acetate (MAM) in rats leads to neurodevelopmental abnormalities similar to SZ symptoms[35]. Another induced model of SZ, using chronic administration of NMDA receptor antagonist MK-801 to Wistar rats, confirmed increased protein levels of NRG1 and erbB4 in the prefrontal cortex, suggesting it recapitulates at least some typical features of schizophrenia[36].

TOURETTE'S SYNDROME

Tourette's syndrome (TS) is a neurodevelopmental disorder with unknown mechanisms and aetiology. George Gilles de la Tourette described a disease that is characterized by vocal and motor tics in 1885. Tics are rapid, sudden, brief, repetitive, and involuntary with a specific wax and wane course. Clinical diagnosis of TS is based on a presence of several motor and at least one phonic tics, persisting for at least 12 months. The onset of TS is in childhood or teenage years, with mean age of onset 5–7 years. The symptoms often decrease and eventually disappear with onset of adulthood. The prevalence of TS is estimated between 0.4–1 %[37]. TS is very often associated with neuropsychiatric comorbidities such as ADHD (60–80 %), OCD (45–60 %), impulse control disorder, anxiety, mood disorders, learning disabilities, acquired maladaptive behaviours and depression[37,38].

Some evidence shows that the input part of the striatum plays an essential role in the pathophysiology of tics, specifically changes in the cortico-basal ganglia circuit. Changes in the sensitivity of dopamine receptors (hypersensitive), increasing density of striatal dopamine transporter (DAT) binding sites[39], an excessive amount of dopamine, and decrease dopamine 2/3 receptor binding in the striatum[40] were all implicated in TS.

The role of the dorsal striatum in tic-like movements was demonstrated in an animal model of juvenile Wistar rats treated with neurotoxin 6-OHDA. This neurotoxin specifically

destroys noradrenergic and dopaminergic neurons. Rats injected with 6-OHDA into anterior and central parts of dorsal striatum showed abnormal movements which are resembling simple and complex tics. Results of mRNA expression revealed an imbalance in dopaminergic regulation in both direct and indirect MSNs pathways[41]. Two other animal models of motor tics were generated by the administration of $GABA_A$ receptor antagonists, bicuculline and picrotoxin (PTX), implicating disruption of striatal GABAergic connectivity in TS and other disorders with tic-like symptoms. In one model, bicuculline was delivered using microinjection into the dorsal putamen of Macaca fascicularis monkeys. Injected monkeys exhibited motor tics and confirmed link between impaired GABAergic transmission and motor tick development[42]. PTX is a poison isolated from plant Anamirta cocculus. PTX injected into dorsal striatum, and sensorimotor cortex of C57B1/6 mouse induced intermittent non-rhythmic tic-like movements, while injection into central striatum didn't show this effect[43]. Blockade of glutamatergic afferent signalling from cortex by infusion of NMDA receptor agonist into striatum abolished this effect of PTX. This confirms involvement of cortico-basal ganglia loops in various studies. This behaviour identified genes linked to TS such as SLIT and TRK-like 1 (*SLITRK1*), dopamine transporter gene (*DAT1*), monoamine oxidase-A (*MOA-O*), and *DRD2*[38]. The *SLITRK1* gene encodes transmembrane protein expressed in fetal and postnatal brain, which plays a crucial role during early brain development in the striatum, where it regulates expression in projecting neurons and determines the cortico-striatal-thalamo-cortical circuit. It is involved in neuronal growth, promotes and controls neurite outgrowth[44], and regulates the formation of synapses and presynaptic differentiation. *Slitrk1*$^{-/-}$ mice exhibit behavioural abnormalities such as elevated anxiety and depression, and decrease in locomotor activity[45]. Neurochemically, these mice show an increase of norepinephrine in prefrontal cortex, which implicates noradrenergic mechanisms in TS pathogenesis.

Another transgenic mouse model often used in TS research was created by expressing intracellular subunit of cholera toxin (CT) under the control of D1 receptor promoter[46]. In this D1CT-7 mouse, CT expression activated stimulatory G-protein (G_s) signalling and elevation of cAMP levels. The model was originally described as displaying mainly OCD symptoms, including grooming-associated repetitive skin biting, and hyperactive locomotor behaviour. Comorbidity with many TS symptoms, including juvenile tick manifestation, was described later[47]. D1CT-7 model showed increase in tic-like behaviour and sensorimotor gating deficit in response to stress (spatial confinement), two important features of human TS pathophysiology[48]. DAT1 controls the level of extracellular dopamine via the reuptake of dopamine. *DAT*$^{-/-}$ mice show two to five-fold increased extracellular dopamine concentration. Mice are hyperactive, indicate several types of stereotypical activity and locomotion, reduction of anxiety phenotype, novelty-seeking[49]. All together both pharmacological and genetic animal models of TS confirm involvement of corticostriatal loops and dopaminergic, GABAergic and glutamatergic neurotransmission.

OBSESSIVE-COMPULSIVE DISORDER

Obsessive-compulsive disorder (OCD) is characterized by obsession or compulsion, or both together, where obsession is defined as repeated thoughts, urges or mental images causing anxiety, and compulsion as repetitive behaviours person with OCD is urged to do in response to intrusive thoughts and impulses. The lifetime incidence is 1–3% in the general population[50]. Compulsive-repetitive behaviour is shared between OCD and autism spectrum disorder[51]. Patients can't control their feelings and thoughts, leading to obsession and anxiety. This behaviour can be followed by compulsions (repetitive, ritualized behaviour) to neutralize the anxiety. Abnormalities in the CBGTC circuity, accompanied by metabolic hyperactivity in the striatum, orbitofrontal cortex, and anterior thalamus were reported. These abnormalities involve dopaminergic activity[40] as well as serotogenic, and glutamate[52] neurotransmissions. Both ASD and OCD show increase in glutamate linked to compulsive and repetitive behaviours[53].

Again, both pharmacological and genetic animal models of OCD were developed. Rat model of OCD used D2 and D3 dopamine receptor agonist Quinpirole to induce compulsive behaviour, similar to checking rituals[54]. Microdeletion at chromosome site 22q11.2 affects 40–50 genes and manifests with a variable impact and wide range of symptoms. Congenital heart defects are among main phenotypes, but anomalies in skeletal development, CNS, and cognitive function are also common[55]. From neuropsychiatric disorders, increased risk of ASD, schizophrenia, ADHD, anxiety and mood disorders was described[56,57]. OCD related genes *Sapap3*, *Slitrk5*, *Hoxb8*, and *Slc1A1* (known also as *EAAC1*; *EAAT3*) have overlapping brain expression patterns and their expression areas are part of the CBGC circuity[51]. Sapap3 protein is highly expressed at excitatory glutamatergic synapses in the striatum. Mice with homozygous deletion of *Sapap3*$^{-/-}$ exhibited a compulsive-like behavior, such as increased anxiety, and less time exploring, which can be decreased by a serotonin reuptake inhibitor. Both AMPAR and NMDAR mediated glutamatergic synaptic transmission is defective in these mice[58]. SLIT and TRK-like protein-5 (Slitrk5) is expressed in neurons of CNS, including striatum and cortex, and regulates neuronal survival and neurite outgrowth. *Slitrk5*$^{-/-}$ mouse model presented with abnormalities in the striatum, e.g. decrease striatal volume, decrease in dendritic complexity and behavioral phenotypes similar to *Sapap3*$^{-/-}$ mice[59]. Mice with disruption of *Hoxb8* gene have fewer microglia and exhibit excessive grooming behavior, which leads to compulsive hair removal and skin lesions[60]. Hoxb8$^{-/-}$ mice show defects in synaptic structures leading to increased excitation of the corticostriatal circuit[61]. SLC1 family proteins protect neurons against glutamate exitocitity[62]. Solute carrier family 1, member 1 (Slc1A1, EAAT3) is a glutamate transporter, expressed in cortex, striatum, and thalamus. *Slc1A1*$^{+/-}$ heterozygous knockout mice don't exhibit OCD phenotypes, although Slc1A1 variants are linked to OCD in human[63].

MAJOR DEPRESSIVE DISORDER

Major depressive disorder (MDD) symptoms include anhedonia, disturbances of motivation, loss of interest, reduced energy, attention, and concentration, disruption of sleep, lowered

self-confidence and self-esteem. Lifetime prevalence is estimated ~15–17 % and over 50 % of patients suffer from some form of impairment in ability to normally function in day to day life. Self-harm and risk of suicide is connected with MDD[64]. Activation of ventral striatum contributes to induction of a positive mood and cortico-striatal pathways are important for sustained positive mood. Disruption in these pathways may be involved in MDD[65]. Some studies show structural changes in striatal volume in paediatric patients, with striatum approximately 7% smaller than in controls[66]. Compared to other neuropsychiatric disorders, heritability of MDD is modest at ~40 %[67]. Many genes with a small impact are involved in MDD, and their combination elevates the risk of the disease[68,69]. Both candidate gene studies and GWAS were performed in attempts to identify particular genes involved in MDD. SNPs associated with MDD were found close to SIRT1 and LHPP, genes associated with regulation of gene expression and cellular metabolism, and DCC, transmembrane receptor involved in axon guidance[69,70]. A decrease in neurotransmitter serotonin levels is involved in the pathophysiology of MDD[71] and Selective Serotonin Reuptake Inhibitors (SSRIs), blockers of 5-HTT serotonin transporter, are commonly used as antidepressants. Imbalance in glutamate and GABA system contribute to the pathophysiology of depression and GABA was reduced by 52 %[9,10] in the occipital cortex of MDD patients. Vesicular glutamate transporter 1 (VGLUT1) is responsible for loading glutamate into synaptic vesicles and regulates the neurotransmission[72]. Reduction of VGLUT1 in heterozygous *VGLUT1*$^{+/-}$ mice led to a depressive-like behaviour, anxiety, anhedonia, and helplessness[73,74]. The most common rodent models of MDD are still created by manipulating environmental factors (e.g. stress exposure, learned helplessness), but genetic and pharmacological (corticosterone administration) models are also available[75,76].

BIPOLAR DISORDER

Bipolar disorder (BP) is a neuropsychiatric disorder with a high heritability of 59–85 %, and a prevalence of 2.4 %[77]. The bipolar spectrum includes bipolar I and II disorders (BD I, II). BD I is characterized by presence of at least one manic episode, while BD II is defined by long depressive periods intermitted with hypomanic episodes. Depressive episodes are more prevalent in autumn or winter[78]. The switch from mania to depression may be influenced by circadian control and seasonal changes associated with a length of photoperiod. Change in neurotransmitters from dopamine to somatostatin expression was observed in rat hippocampus in response to long day, and opposite effect in response to prolonged dark period[79]. Abnormal activation in the cortico-striatal system[80] and failure in striatal activation was described in BD patients[81]. As in many other neuropsychiatric disorders, polymorphism in DAT contributes to BD[82], implicating dopamine transmission abnormalities in disease aetiology. Reduced availability of DAT in striatum[83] might lead to hyperdopaminergic response. Changes in dorsal striatum specific gene expression network, specifically in regulation of energy metabolism and immune response were identified in post-mortem samples of BD[84]. Genetic association study revealed role of phosphodiesterase 10A (PDE10A) in BD I, and

disrupted in schizophrenia 1 (DISC1) and guanine nucleotide-binding protein, α-stimulating polypeptide 1 (GNAS) in BD II, implicating changes in cAMP mediate signalling[85]. PDE10A is highly expressed in the MSNs of the striatum and based on genetic association plays also a role in schizophrenia (SZ).

All these above described mechanisms were employed in creation of animal models of BSD. CLOCK protein affects circadian rhythms, and *ClockΔ19* mice exhibited not only dysregulation of circadian pattern in response to dark and light cycles changes, but also behavioural abnormalities reminiscent of BD manic episodes, such as hyperactivity, decreased anxiety, sensorimotor gating deficit, and increased specific exploration[86]. DAT knock-down (KD) and DAT pharmacological inhibition mouse models revealed abnormal behavioural patterns similar to BD patients[87]. Pharmacologically induced BD model in Wistar rats used Ouabain (OUA), an inhibitor of Na^+/K^+-ATPase enzyme. Decreased activity of Na^+/K^+-ATPase led to oxidative damage and caused mood swings with specific maniac phenotype in early-stage and depression in late-stage[88].

AUTISM SPECTRUM DISORDER

Autism spectrum disorder (ASD) is characterized by repetitive behaviour, stereotyped movements, adherence to routines and resistance to change, impairment in social interaction, language development, and communication[89,90]. The first sign of ASD is usually an elevation in repetitive behaviours detectable at as early as 1 year of age[91]. Anxiety, seizures, motor deficit, higher aggressive behaviour, sleeping disturbances, and gastrointestinal problems are common comorbid traits. Specific phobias and OCD are the two most common comorbid psychiatric disorders, occurring in up to 30–40 % of ASD patients[92]. ASD is highly heritable, and most cases can be attributed to common genetic variants with small individuals effects, while rare variants are responsible for monogenic and syndromic autism[93,94]. Repetitive behaviour, social, and cognitive deficit can postulate striatal dysfunction. MRI showed an increase in the growth rate in caudate nucleus in children with ASD[95]. Genetic abnormalities inducing imbalance between excitatory glutamatergic and inhibitory GABAergic neurotransmission point to connection between abnormalities of striatal function and behavioural phenotypes observed in ASDs patients[96]. Mutation in neuroligin-3 (*NLGN3*) and neuroligin-4 (*NLGN4*), two X-linked genes coding cell adhesion molecules with postsynaptic localization were identified as associated with autism[97]. Both *NLGN3* deletion and point mutation R451C cause synaptic impairment in the ventral striatum (nucleus accumbens) and synaptic inhibition in D1-dopamine receptor MSNs in mouse models of ASD. This leads to increased repetitive behaviours[98]. The R451C mutation also represents additional gain-of-function effects not observed in *NLGN3* deletion models, leading to impaired social behaviours but improved spatial learning abilities[99]. This might be connected to impaired cortico-striatal glutamatergic synapses in the dorsal part of the striatum, with specific role in long term plasticity[100]. Knock-in mouse model of mutated DAT $T356M^{+/+}$ showed impairments in DA neurotransmission and

behaviours resembling phenotype in ASD or ADHD patients such as spontaneous locomotor activity, repetitive behaviours, and loss of social dominance[101]. SH3 and multiple ankyrin repeat domains 3 (Shank3) is postsynaptic density protein of excitatory synapses, necessary for neuronal communication. Its disruption by microdeletions and point mutations leads to rare form of autism with intellectual disability. Shank3e4-9 mice with deletion of exons 4-9 coding the ANK domains exhibit abnormal social behaviour, fewer social interaction, changes in vocalization, repetitive behaviours, memory deficiency and reduced synaptic plasticity[102]. Complete loss of mouse *Shank3* induced by deletion of exons 4-22 (Δe4–22) led to ASD like symptoms. Regionally restricted deletion of the same *Shank3* exons showed Shank3 deficiency in neocortex, was responsible for increase in grooming behaviours, while deletion in striatum induced preservative exploratory behaviour[103].

Changes in distribution and morphology of neurotransmitters receptors were also connected to ASD and pharmacologically active compounds influencing neurotransmission can induce ASD-like symptoms. Valproic acid (VPA) is an antiepileptic drug, which reduces excitability by increasing GABA inhibitory neurotransmission[104], although full mechanism of VPA action is still unclear. Valproate treatment during pregnancy displays an increased risk of autism, neural tube defects, and behavioural impairments. Pregnant Sprague-Dawley rats exposed to VPA at E12 showed essential changes in behaviour, reduced social preference, and animal sociability[105] as well as a reduction of glutamate receptors expression with an associated deficit in synaptic plasticity. Dietary factors and state of gut microbiome were connected to worsening of ASD symptoms. Propionic acid (PPA) is a common intermediate metabolite and a by-product of gut bacteria. Intraventricular administration of PPA to Long-Evans rats led to development of ASD-like phenotype, including abnormal behavioural responses such as repetitive behaviour, and hyperactivity. Increased astrogliosis and activated microglia in brain tissue were on biochemical level accompanied by an increase in oxidative stress, and decrease in glutathione[106].

CONCLUSION

Mental disorders have often comorbid diseases and some are hard to precisely diagnose. Misdiagnosis can lead to incorrect medication with severe impacts on patients and their families. Better understanding of underlying environmental and genetic factors contributing to these diseases is critical for improving therapeutic outcomes. Dysregulation of neurotransmitter pathways and consequent impairment of CBGTC strongly contributes to the pathology of many neuropsychiatric diseases. This article summarizes previous studies of neuropsychiatric disorders, their connection to the striatum, and provides short overview of some of the available animal models. Animal models based on pharmacologic, genetic, or environmental inducers can enrich our knowledge of the disease mechanisms, help with early diagnosis and identify potential therapeutic targets.

Table 1. Overview of animal models of neuropsychiatric diseases.

Model Type	Gene/Compound	Species	References
SCHIZOPHRENIA			
Pharmacological	MAM	rat	**107,35**
	MK-801	Wistar rat	**36**
Genetic-based	Disc1	transgenic mouse	**23**
	Df(16)A$^{+/-}$	mouse	**32**
	Df(h1q21)/+	mouse	**108**
	Dtnbp1(sdy)	mouse	**34**
	Reelin	heterozygous reeler mouse	**27**
TOURETTE SYNDROME			
Pharmacological	6-OHDA	Wistar rat	**41**
	Bicuculline	Macaca fascicularis	**42**
	Picrotoxin	mouse	**43**
Genetic-based	D1CT-7$^{-/-}$	mouse	**46,48**
	DAT1$^{-/-}$	mouse	**49**
	SLITRK1$^{-/-}$	mouse	**45**
OBSESSIVE–COMPULSIVE DISORDER			
Pharmacological	Quinpirole	rat	**54**
Genetic-based	Sapap3$^{-/-}$	mouse	**58**
	Slitrk5$^{-/-}$	mouse	**59**
	Hoxb8$^{-/-}$	mouse	**61**
	Slc1A1$^{-/-}$	mouse	**63**
MAJOR DEPRESSIVE DISORDER			
Genetic-based	5-HTT$^{-/-}$	mouse	**109**
	VGLUT1$^{+/-}$	mouse	**73,74**
BIPOLAR DISORDER			
Pharmacological	Ouabain	Wistar rat	**88**
Genetic-based	ClockΔ19 mutant	mouse	**86**
	DAT KD	mouse	**87**
	BDNF$^{+/-}$	mouse	**110**
AUTISM SPECTRUM DISORDERS			
Pharmacological	VPA	Sprague-Dawley rat	**105**
	PPA	Long-Evans rat	**106**
Genetic-based models	DAT T356M$^{+/+}$	mouse	**101**
	Shank3 (Δe4–22)	mouse	**103**
	Shank3e4-9	mouse	**102**
	Neuroligin-3 R451C	mouse	**99,100**

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11 System wide proteomic approaches – methods and applications in neurodegeneration research

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Abstract

Omics approaches became popular in last decades due to their undeniable asset in providing complex information about system of interest, e.g. *in vitro* cultivated cell population. Their main benefit is a large number of analytes (e.g. RNAs or proteins) monitored in parallel. As proteins are responsible for phenotype, proteomics is the technique of choice. Two main diverse proteomic approaches exist – antibody-based or mass spectrometry-based approach. This review is focused on characterisation of different methods from both approaches, their descriptions and examples of their applications in research of neurodegenerative diseases.

Keywords

proteomics, antibody microarray, flow cytometry, mass spectrometry, neurodegeneration

INTRODUCTION

In last few decades complex omics approaches, e.g. genomics, transcriptomics, proteomics, lipidomics or metabolomics, became widespread in practically all fields of research. Their main advantage is that they provide system wide information about the object of study, which cannot be obtained in affordable and time-effective manner by separate analysis targeting one specific gene or protein. Recently, integration of different omics methods allowed start of the new era of personalized medicine bringing novel directions in early diagnose and successful treatment of even rare and serious diseases (for review see[1]).

Transcriptomics (using RNA microarrays[2] or RNA sequencing techniques[3]) was in the past two decades approach of choice for analysing differential gene expression in biomarker

discovery[4], cell replacement experiments[5] or studies of neurodegenerative diseases[6]. However, phenotype is caused by proteins, not by mRNA expression. Schwanhäusser et al. proved that correlation between gene expression at mRNA and protein level is limited, because protein synthesis is mainly regulated at translation level. Proteins are in general more stable than mRNAs (median half-life of 46 and 9 hours for proteins and mRNAs, respectively) and median number of protein copies per cell is 50,000 compared to 17 copies of mRNA. Similar situation exists in case of average transcription or translation rates per cell – for mRNAs, median transcription rate is about two molecules of particular mRNA per hour, while for protein the estimated median rate is about 140 newly synthesized protein molecules per one mRNA per hour[7]. Taken together with significant progress in development of novel system wide proteomic methods, in particular based on mass spectrometry (MS) techniques, proteomics came to the forefront of interest.

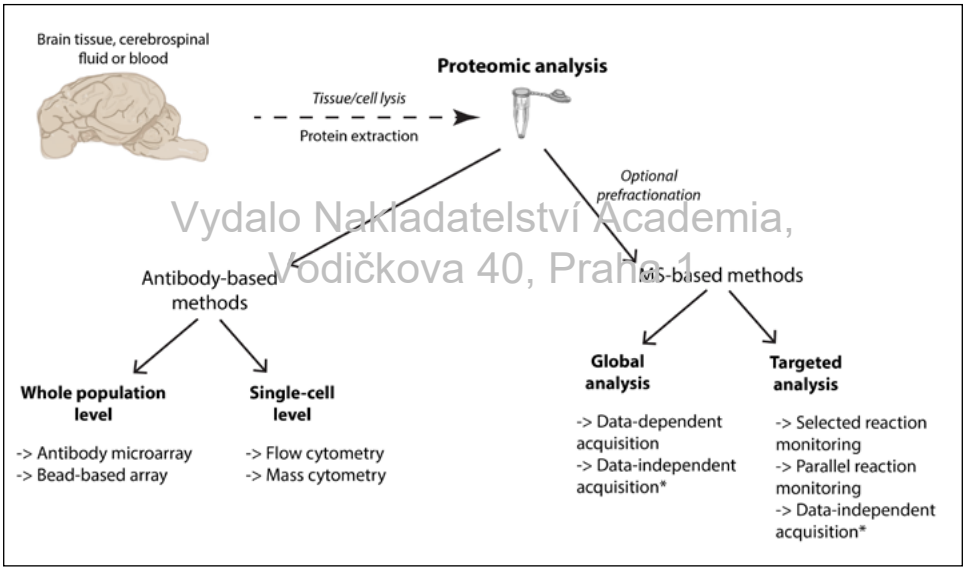

Figure 1. Overview of proteomic methods. Proteomic methods can be divided according to approach used for protein detection and quantification – antibody-based or MS-based. These methods may be further divided based on the level of information provided (antibody-based) or their targeting (MS-based). *Data-independent acquisition method allows both types of targeting according to used protein set – whole proteome (global analysis) or preselected proteins (targeted analysis). Orig. J. Červenka

Basically, two different approaches are used for detection and quantification of proteins in proteomics: a) antibody-based methods (e.g. protein chips or flow cytometry), and b) MS-based methods (Fig. 1). Although several methods combine techniques from both approaches (e.g. mass cytometry, which employs antibodies for protein identification and quantification

and mass spectrometer for signal detection), division of proteomics into antibody or MS-based methods is generally applicable.

Aim of this chapter is to provide general overview of different system wide proteomic methods and technologies with focus on basic explanation of methods principle and their applications in a research of neurodegeneration.

ANTIBODY-BASED METHODS

Antibody-based methods, as their name suggests, employ antibodies for detection and quantification of target proteins. Their advantages are high sensitivity (because antibodies are able to enrich their targets even in complex mixtures), simplicity and quickness together with usually no need for in-house development as many commercial antibodies and kits are commonly available and already tested for particular applications. On the other hand, not all antibodies are extensively tested for their selectivity and specificity, which may cause falsely positive or negative results[8]. Likewise combinations of different antibodies have to be tested for cross-reactivity and usage of high number of antibodies (tens to hundreds) in one analysis may be impossible or at least very expensive.

ANTIBODY MICROARRAY

Antibody microarray (also called antibody chip) is a specific type of protein chip technique, which employs libraries of antibodies printed on a solid support (typically glass slide or nitrocellulose membrane). Immobilized antibodies bind specifically to their targets allowing to wash-out nonspecifically bound molecules and semi-quantitative detection of tens up to thousands of proteins. Two different arrangements of detection are possible – direct labelling of sample or sandwich array using labelled secondary antibody. This method provides high throughput and sensitivity, commercial kits as well as homemade systems are available, whole process from sample preparation to signal detection is relatively fast (less than 24 hours) and only small amount of sample is required[9,10]. On the other hand, antibody microarrays have also several drawbacks – specificity of each antibody has to be vigorously tested and in sandwich array arrangement each antibody pair has to be validated for possible cross-reactivity. Moreover, array scanner for fluorescent microarrays is required, but this service is broadly offered by different companies and institutions[10].

Ray et al. employed antibody microarray to analyse 120 proteins in human blood plasma samples from patients with different stages of Alzheimer's disease (AD) and healthy controls. They identified significant changes in abundances of 18 signalling proteins and this panel of proteins was able to distinguish between healthy control and AD samples with about 90% accuracy[11].

Doecke et al. studied blood plasma samples from patients with AD and healthy participants with antibody chip against 151 different targets and identified 11 possible biomarkers of AD. Validation of these biomarkers together with 7 additional clinical biomarkers on a different cohort of AD patients and healthy participants shown high sensitivity (77%) and specificity (84%) of this assay[12].

Mahlknecht et al. used antibody microarray to analyse 174 cytokines in blood serum from patients with Parkinsonian syndromes – Parkinson's disease (PD), multiple system atrophy, progressive supranuclear palsy, corticobasal syndrome and healthy individuals. They found expression of 12 cytokines changed, however, validation of the results confirmed significant changes of only two cytokines – Platelet-derived growth factor subunit B and prolactin[13].

Skalnikova et al. studied development of muscle spasticity in the rat model of spinal cord injury with focus on spinal parenchyma and dorsal root ganglions (DRGs) two and four weeks after complete spinal cord transection. Using antibody microarray for 626 signalling proteins and their phosphorylated forms they observed crosstalk between proteins acting in cellular processes of angiogenesis and neurodegeneration. Moreover, they also found significantly increased expression of several proteins between DRGs and spinal parenchyma regions suggesting the role of these proteins both in initiation as well as in maintenance of muscle spasticity[14].

BEAD-BASED ARRAY

Bead-based arrays work in principle similarly to antibody microarrays, but antibodies are immobilised on microbeads, instead of a planar surface. Microbeads are mainly polystyrene or magnetic and each microbead has its internal fluorescence label, which is common for all microbeads with the same immobilized antibody. Detection and quantification is performed by binding of secondary antibody (as in case of sandwich array) that is either directly fluorescently labelled or possesses biotin available for detection by a conjugate of streptavidin-phycoerythrin. Specialized instruments (e.g. Luminex platform) exist, but analysis can be performed on a classical flow cytometer capable of at least two fluorescence channel detection – internal microbead fluorescence and streptavidin-phycoerythrin. The main advantages of this method are its high sensitivity (below pg/mL) together with broad dynamic range (3–4 orders of magnitude), reproducibility and low requirements for sample amount. However, bead-based arrays are relatively low throughput (typically tens of protein targets are measured in one analysis) and processing of larger batches of samples may be laborious (especially when polystyrene microbeads are used)[10,15].

Keene et al. performed analysis of amyloid β, hyperphosphorylated Tau, Glial fibrillary acidic protein and Allograft inflammatory factor 1 in formalin fixed, paraffin-embedded brain tissues from patients with AD, using bead-based array protocol (based on Luminex platform). They observed increased expression of amyloid β and hyperphosphorylated Tau in

AD patients compared to healthy controls, but these results require validation on different cohort of individuals[16].

Valekova et al. analysed secretion of seven cytokines in blood serum, cerebrospinal fluid (CSF) and in media from *in vitro* cultivated peripheral blood monocytes or microglial cells from transgenic minipigs bearing N-terminal fragment of human mutant huntingtin and healthy controls. They found significant changes in abundances of Interleukin-1β, Interleukin-8, Interleukin-10 and Interferon α, suggesting that these proteins could serve as biomarkers for Huntington disease (HD) progression, at least in this porcine model[17].

FLOW CYTOMETRY

Flow cytometry is a method, which enables single-cell analysis, thus providing additional level of information in comparison with other antibody or MS-based techniques. Analysed cells (living or fixed) or particles (e.g. microvesicles) are firstly stained by fluorescent dyes or by monoclonal antibodies conjugated to fluorochromes. After injection into the flow cytometer, sample is focused by fluidic system and each cell is analysed separately by combination of lasers and detectors for different wave lengths. Cells can be also sorted based on their different characteristics (signal intensity of fluorochromes and forward/side scatter) with approach called fluorescence-activated cell sorting (FACS), developed in 1965[18] (for review see[19]). Although flow cytometry offers very fast analysis of up to millions cells within minutes at single-cell level, its main drawback is a low throughput as only tens of parameters (e.g. protein abundances, but also cell size and shape or DNA content) can be analysed in instrument-dependent manner (based on number of lasers and detectors in the particular flow cytometer).

Several protocols for flow cytometry analyses applicable in the field of neurodegeneration were recently published. Gylys and Bilousova described method for quantitative analysis of protein Tau in synaptosomes from brains of AD patients[20]. Dukhinova et al. prepared method for characterisation of microglia and macrophage cells involved in neuroinflammation and neurodegeneration in brain and spinal cord[21].

Using flow cytometry, Rangaraju et al. identified sub-populations of neurodegeneration-specific disease-associated microglia in mouse models of AD. Their neuro-immunomodulatory therapy against pro-inflammatory disease-associated microglia led to increased amyloid β disposal in mouse AD model, suggesting that modulation of neuroinflammation might be beneficial for patients with AD[22].

MASS CYTOMETRY

Mass cytometry is a novel technique (developed in 2009 by Bandura et al.) that combines flow cytometry principle with signal detection by mass spectrometer, for why this technique is also called cytometry by time-of-flight (CyTOF). Antibodies are not labelled by fluorochromes, but conjugated with stable isotopes of metals, which are detected by inductively coupled plasma TOF MS. This allows single-cell analysis with high sensitivity and resolution

for higher number of markers than in case of flow cytometry without any limitation caused by laser availability or fluorochrome interferences. Disadvantages of this method are absence of information about shape and size of the measured cells or particles and inability to perform sorting, due to vaporization of sample prior its detection[23].

Comprehensive evaluation of mass cytometry and its comparison to flow cytometry shown different advantages of both techniques and support implementation of mass cytometry into clinics[24]. Moreover, similarly to flow cytometry, protocol for characterisation of immune cells in brain tissue using mass cytometry was recently established[25].

Ajami et al. analysed populations of myeloid cells in mice models of amyotrophic lateral sclerosis (ALS), multiple sclerosis and HD. Using mass cytometry, they identified five populations of monocytes (with distinct expression of cytokines) that were present in brains of mice with multiple sclerosis, but completely missing in brains of mouse models of ALS and HD. These results indicate differences between neurodegeneration and neuroinflammation and authors also suggest promising target for neuroinflammation treatment – α5 integrin[26].

MASS SPECTROMETRY-BASED METHODS

Mass spectrometry-based methods are currently primary approach of choice in proteomic research as they enable identification and quantification of up to thousands of proteins simultaneously in one analysis, even in complex samples. Moreover, some of these methods allow also analyses of posttranslational modifications (PTMs) without prior knowledge about types or positions of modifications at measured proteins. MS methods for global analyses of samples (i.e. shotgun MS), as well as targeted methods, exist. Although it is possible to measure intact proteins in so called top-down approach[27], different approach called bottom-up[28] is widely used. Using this approach, proteins are digested (typically by specific protease, as a trypsin) to peptides that are usually separated by high-performance liquid chromatography (HPLC), ionised (mostly by electrospray ionisation – ESI) and analysed in tandem mass spectrometer. Exact masses and charges of precursor ions (peptides) and their fragment ions are measured together with signal intensities of all detected ions. Using computational analysis, these values can be then assigned to theoretical values of peptides and proteins from databases of protein sequences leading to protein identification and quantification.

Main advantages of MS-based proteomics are high throughput, possibility to analyse PTMs and very high specificity. On the other hand, MS has also several limitations – only ionised peptides are detected and quantified, low abundant proteins (like signalling molecules, which may be the most interesting) in complex samples (e.g. body fluids as a blood plasma) are under detection limit of (global) MS analyses and comprehensive and up-to-date databases of protein sequences are essential, which may be challenging for non-model organisms[10]. Furthermore, processing of large batch of samples is time consuming and purchase of a new

mass spectrometer is relatively expensive, but many institutions provide MS measurements as a paid service.

GLOBAL ANALYSIS – DATA-DEPENDENT ACQUISITION

Data-dependent acquisition, DDA, also called shotgun MS, is a global approach for identification and relative quantification of peptides and belonging proteins. Tandem mass spectrometer performs MS1 scan for precursor ions at first, then precursor ions (typically 10–30) are consecutively selected for fragmentation based on their signal intensity (from the highest to the lowest). Fragment ions are measured in MS2 scans and after that another MS1 scan is performed. Up to thousands of proteins can be identified and quantified using DDA (or even more with implementation of sample prefractionations and combination of analyses[29]) including their PTMs. However, the major issue of this method is a partial stochasticity of precursor selection that may lead to omitting of precursor ions with low signal intensity (often from low abundant proteins), which impairs quantification results. Thus, validation of results by another independent method is required[10].

Several different quantification methods may be implemented to DDA analyses. Besides label-free methods based on counting of spectra for particular peptide or measurements of precursor peak intensity[30], metabolic labelling method (stable isotope labelling by amino acids in cell culture, SILAC)[31], enzymatic labelling method (proteolytic ^{18}O-labeling, when enzymatic digestion is performed in $H_2{}^{18}O$)[32] and chemical labelling methods (e.g. isobaric tags for relative and absolute quantitation – iTRAQ[33] or tandem mass tags – TMTs[34]) exist. Each method has its benefits and drawbacks, for example label-free methods are simple and without additional costs, but as each sample has to be measured alone, instrument time may be relatively long. In contrast, labelling methods allow simultaneous measurements of up to 11 samples in one run, thus lowering effect of DDA stochasticity. However, these methods may be expensive, laborious and require MS instruments with high resolution.

Khoonsari et al. analysed human CSF from patients with AD and healthy controls using shotgun MS to identify potential biomarkers of AD. They discovered significantly decreased expression of eight proteins acting in different biological processes like cell migration or regulation of the synapse and verified the results by bead-based array[35].

For additional information about employment of MS methods in biomarker discovery studies see also chapter 12 *Protein biomarkers of neurodegeneration in cerebrospinal fluid*.

TARGETED MASS SPECTROMETRY METHODS

To overcome limitations of DDA technique and quantification issues, targeted MS methods were developed. These methods enable relative or even absolute quantification of preselected set of proteins with high accuracy and reproducibility. Typically, three different targeted MS techniques are used – Data-independent acquisition (DIA), Selected reaction monitoring (SRM) and Parallel reaction monitoring (PRM). These methods are reviewed in detail

together with their applications in chapter 13 *Targeted proteomics in translational research of neurodegenerative diseases*.

CONCLUSION

Proteomics presently provides variety of techniques for comprehensive analyses at system wide level. Two different detection and quantification approaches are used – antibody-based and MS-based. Antibody-based methods are frequently employed due to their relative simplicity, quick assay processing and availability. However, these methods may suffer from low specificity of antibodies and are difficult to multiplex because of cross-reactivity and cost of higher numbers of different antibodies. MS-based methods offer indeed high throughput (up to thousands of proteins measured simultaneously), high specificity and accuracy, but their sensitivity is limited, especially in highly complex samples (e.g. blood plasma). Taken together, all mentioned methods have their specific applications, in which the particular method is the best possible option. Thus, all the advantages and limitations of particular method should be considered during method selection, as well as appropriate combination of several techniques from both approaches.

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12 Protein biomarkers of neurodegeneration in cerebrospinal fluid

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Abstract

Cerebrospinal fluid (CSF) is a body fluid, which is an attractive biomaterial due to its proximity to the brain and relatively non-invasive and accessible sample collection. Changes in the composition of CSF reflect different aspects of brain pathological states in brain tumours, neurodegenerative, neuro-inflammatory and metabolic disorders. This chapter focuses on up to date knowledge of biomarkers of neurodegenerative disorders in CSF with information about their discovery, potential application and current state of translation into the clinical applications.

Keywords

cerebrospinal fluid, biomarkers, CNS diseases, non-invasive early diagnosis, biomarkers discovery, translation to clinic

CEREBROSPINAL FLUID

The cerebrospinal fluid (CSF) is a physiologically colourless body fluid that flows in central nervous system (CNS) with yet not fully explored circulation patterns. Modern model of CSF circulation suggests that it is a complex dynamic equilibrium of fluids in cranial and spinal subarachnoid compartments[1], where CSF functions are hydromechanical and immunological protection to the brain and transport of nutrients and metabolites[2,3]. The biggest portion of CSF is formed by plasma ultrafiltrate. CSF is renewable material (turnover of around half litre per day in a healthy adult) and its normal parameters are affected by age, sex, weight, physical activity or drug usage. CSF parameters routinely measured in clinical practice are

total protein concentration; albumin, glucose and lactate ratio; oligoclonal intrathecal immunoglobulin synthesis and typical cytological findings for determination of viral and bacterial CNS infections, inflammatory neuropathies and malignant diseases. Such diagnostics has to be performed within hours after puncture[4].

CSF is a material of choice for studies of pathological cerebral conditions in vital patients, in which tissue biopsy is not an option. The main advantage of CSF analysis is the higher concentration of brain-derived analytes in comparison to plasma. Overall, variations in CSF composition show biological state of CNS. Therefore, CSF could be used for diagnostic purposes for cerebropathies and myelopathies[3]. Consequently, quest for biomarkers of both early diagnosis and treatment monitoring became widely popular in neurodegenerative and cancer studies.

BIOMARKERS

In biology, a biomarker is any biomolecule, which could be used for identification of significant endpoints for specific biological state with severity degree[5]. Ideally, biomarkers should be specific, reproducible, easy and cost-effective to determine with low variability in the population. Biomarker could be measured independently or in combination with other biomarkers to improve diagnostic accuracy for either different aspects of pathology or to distinguish overlapping conditions[5].

METHODS FOR BIOMARKERS DISCOVERY AND DETECTION

Complete biomarker discovery studies consist of candidate biomarker identification and verification followed by validation and implementation steps. Modern technologies allowing the simultaneous analysis of many targets in a single measurement are preferred for the biomarker discovery studies. In forefront of these methods stand mass spectrometry (MS) for proteomics, metabolomics and lipidomics and next-generation sequencing (NGS) for transcriptomics and genomics studies, where high sensitivity and massively parallel sequencing allow rapid detection of low-frequency genetic variants and mutations[6,7].

MS is a powerful technique, with many variations applicable at every step of the biomarker discovery pipeline[8,9]. High-throughput data-dependent analysis (DDA) or emerging data independent analysis (DIA) is used in the discovery studies for analysis of thousands of proteins per sample with the aim to quantitatively compare the proteome changes. Those data are filtered by follow-up studies and candidate biomarkers could be verified with targeted measurement like selected reaction monitoring (SRM).

Mass spectrometry for protein analysis has been steadily entering clinical practice. As a result of big multinational studies, several companies developed SRM assays for the combination

of multiple biomarkers in one run. For example, Caprion introduced ProteoCarta assay for the quantification of 142 proteins in human CSF (http://www.caprion.com/en/services/new-ndd-panel.php). Biognosys offers a kit PQ 500 for absolute quantification of 500 human plasma proteins in one measurement (https://biognosys.com/media.ashx/pq500casestudy.pdf).

Multi-omics became a common approach for the novel biomarker discovery and complex characterization of pathophysiology. Besides, obtaining multi-omics data from one sample helps simplify correlation statistics due to eliminated biological heterogeneity. Contrarily, processing big data remains challenging[10].

Challenges in detection of low abundance proteins by MS can be overcome by using more sensitive antibody based techniques such as an enzyme-linked immunoassay (ELISA), but at the cost of expensive assay development. Multiplexed methods such as protein microarrays, electrochemiluminescent (ECL) immunoassays or a bead-based multiplexed immunoassay Luminex allow simultaneous detection of many analytes and are more suitable for biomarker discovery[11,12]. On the other hand, antibody based techniques have often difficulties with cross-reactivity and matrix effects, influencing protein capturing in complex samples[13].

LIMITATIONS OF CSF BIOMARKER DISCOVERY ON MODEL SYSTEMS AND ITS TRANSLATION TO CLINIC

Even with constantly improving technologies, biomarker discovery is not without challenges. Firstly, the protein concentration in CSF is 80 times lower than in plasma, with albumin being the most abundant protein. Secondly, availability of CSF is limited from hundreds of microliters to a few millilitres per sample even in humans. Besides, it is a good practice to pair CSF samples with affected tissue samples and plasma. Thirdly, the biological variance is large, with inflammatory processes affecting biomarker ratios. Tight control and standardization of CSF sampling across studies also represents significant challenge. All of this leads to inconsistencies in results interpretation and difficulties in establishing reliable biomarker cut-offs and guidelines.

Animal models or human cell based models are still irreplaceable in biomedical research, as both bring the possibility to better understand pathogenic mechanisms or to identify target molecules for therapy development. The biggest advantage of human cell model system over animal models is that they carry the genotype of human disease[14].

Unfortunately, even with already existing methods and new protocols for *in vitro* organoids culture, improvements in the cell model system are still required to fully capture the complexity of human neurological diseases. Influence of other organ systems on drug metabolism, complex neural circuits wired in developmentally correct way, and other features of the whole organism are still missing from *in vitro* models, making animal models still indispensable.

Animal models are engineered to mirror hallmark characteristics of studied condition[14]. Few models of neurodegenerative disorders have been used to study mechanisms of disease development and in preclinical studies and many more models are under development with modern techniques such as Crispr/Cas[15]. In general, a heterogeneous and mixed manifestation of neurodegeneration is not fully understood and seldom observed in animals and it is unclear how similar to humans will be neuropathological phenotype developed[14].

Rodents, non-human primates, pigs and canines are often used in preclinical studies. There are several technical details, which should be considered for results interpretation, including physiological differences between species in brain size, CSF volume, and distance between brain and spinal compartments or speed of CSF clearance. Sampling in small animal models is often performed from cisterna magna, compared to big animal models where lumbar puncture like in humans is feasible. Moreover, due to small total amount of CSF, sample collection from small animal models requires catheter application, which affects speed clearance effect. Upright body posture is another advantage for studies on non-human primates[16]. However, number of subjects and limited genetic and epigenetic influence are issues.

In addition to biological and technical issues, there are legislative and ethical questions, which prolong translation from basic research to clinical trials. Also, limits of financial funding during translation process and lack of support by insurance system at final clinical studies stage have influence on so far insufficient translation into clinics[17].

SPECIFIC CSF BIOMARKERS OF NEURODEGENERATION

Neurodegenerative diseases have in common the gradual loss of structure and function of neural cells, resulting in neuronal death. Many neurodegenerations begin with protein misfolding that leads to aggregation – e.g tau protein (neurofibrillary tangles) and amyloid protein (plaques) in Alzheimer's disease (AD) and alpha-synuclein (Lewy bodies) in Parkinson's disease (PD) apart from others[18].

Search for robust protein biomarkers of various CNS disorders with ultimate aim to completely understand their biological function and correlation with other biomarkers continues over decades. Unfortunately, cross-studies of the function of identical biomarkers in different neuropathies are lacking. Further, we describe the most promising biomarkers for translation in neurodegenerative diseases.

HUNTINGTIN

Huntington's disease (HD) is inherited disorder caused by a mutation in huntingtin (HTT) gene leading to expansion of CAG repeat coding for amino acid glutamine near the N-terminus of HTT protein. This results in production of mutated huntingtin (mHTT) protein, with over 36 glutamine repeats. Wild type HTT is a ubiquitously expressed protein with not

completely known function, mHTT protein is toxic mainly for neural cells with striatal neurons being the most affected. Thus mHTT itself is a promising biomarker for the detection of HD at an early stage and evaluation of the effectiveness of mHTT lowering and gene silencing therapies[19,20]. Since concentration of mHTT protein in body fluids is low, CSF represents a promising source for analysis.

Consequently, methods selection is limited due to the sensitivity issues. Few breakthroughs were reported in quantification of soluble mHTT. In 2015, Wild and his team were the first, who developed a single-molecular counting (SMC) immunoassay for mHTT. They were able to quantify soluble mHTT with neuronal origin down to a few femtomoles[21]. Later in 2017 from the same research group Fodale validated SMC as ultrasensitive, reproducible and precise method[22]. In 2015 another group was able to detect mHTT in CSF with immunoprecipitation and flow cytometry[20]. However, the currently used quantification strategies are able to detect only soluble forms of mutated huntingtin. The assays use anti-polyQ antibodies and thus the response is dependent on polyQ length which may vary and thus impair the quantification. Moreover, the quantification of the endogenous non-mutated huntingtin form is so far unfeasible.

AMYLOID BETA

Amyloid beta (Aβ) is a product of proteolytic cleavage of amyloid precursor protein (APP) and the component of amyloid plaques[18,23]. Several Aβ C-terminal fragments are known as Aβ1–40 with 40 amino-acid residues, Aβ1–42 with 42 amino-acid residues and Aβ1–38 with 38 amino-acid residues, where Aβ1–42 has the highest affinity for aggregation and amyloid plaque formation[23,24].

Amyloid beta is a core biomarker of neuronal death for AD diagnosis, where its concentration in CSF is significantly lower when compared to healthy population[25]. However, decreased concentration of Aβ1–42 in CSF was detected in HD, PD, Creutzfeldt-Jakob disease (CJD) and on the spectrum of dementia disorders[26,27]. So, it is advised to use Aβ in combination with other biomarkers. Ratio between Aβ fragments appear to be more accurate for Alzheimer disease diagnosis[23,28]. Also, ratio of Aβ1–42 and tau protein was suggested to be more useful for AD prediction[23,29]. All variants could be detected and distinguished with specific antibody by ELISA method.

TAU PROTEIN

Tau protein is located in the neuronal axons. There are six different isoforms and numerous phosphorylation sites of tau protein in the human brain[30]. Tau in CSF occurs predominantly as a series of N-terminal and mid-domain fragments.

Both total tau (t-tau) protein and phosphorylated tau (p-tau) in CSF are considered as biomarkers of AD[24,29]. Increase in CSF tau concentration in AD patients is associated with faster progression from mild cognitive impairment (MCI) to AD and higher mortality rate

in patients with AD dementia[24]. Furthermore, p-tau corresponds with the formation of neurofibrillary tangles in the brain and correlates with neurodegeneration[24,31].

Methodically, few ELISA based variations are used for t-tau and p-tau detection, but further biomarkers are still needed due to low disease specificity of these two biomarkers. For example, increase of t-tau in CSF reflects neuronal damage also in HD and CJD[29,31,32].

NEUROGRANIN

Neurogranin (Ng) is a postsynaptic protein located in dendrites of neurons, involved in biological processes such as postsynaptic modulation of chemical synaptic transmission and transduction[28,33.]

Significantly increased concentration of Ng in CSF was detected in brain and CSF by multiple studies of AD, multiple sclerosis and PD[26,33]. It is believed, that brain synaptic loss/dysfunction happens before neuronal degeneration. Ng thus may be useful as an early biomarker of neurodegeneration when first changes in the brain occur before the plaque formation[28,33]. Moreover, in amyloid-positive cases level of Ng in CSF correlated with t-tau and p-tau and more rapid change in cognition[28].

Detection of neurogranin is possible with ELISA kits, MS and a single molecule array (Simoa)[28,34].

NEUROFILAMENT LIGHT CHAIN

Neurofilament light chain (NfL) is one of three intermediate filament proteins in neurons. During neuronal degeneration, NfL is released from axons[35,36] and thus has been suggested as a biomarker of axonal damage in the CNS. NfL is widely used in studies of neurodegenerative and neuro-inflammatory disorders as one of the most promising biomarkers[37–40].

In case of HD, concentration of NfL in body fluids correlates with the length of CAG repeats in mHTT and may be useful in prediction of neuronal death and grey matter mass loss[39]. Due to blood-CSF barrier, concentration of NfL in CSF is significantly higher when compared to serum[41]. Therefore, CSF is a better choice for prognosis and measurement in early preclinical stages of neurodegeneration[42]. In combination with other biomarkers or as addition to conventional magnetic resonance imaging, NfL is a promising biomarker for making therapeutic decisions[42,43].

Detection is possible with Simoa, modern immunoassays[44] or highly sensitive MS[45].

CHITINASE-3-LIKE PROTEIN 1

Chitinase-3-like protein 1, also known as YKL-40, is a glycoprotein of mammalian chitinase-like protein family. It is suggested, that this protein is expressed in neuroglia, astrocytes, macrophages, neutrophils and in some cancer cells[46,47]. YKL-40 is also secreted by astrocytes and/or cancer cells. It may play role in cell proliferation, differentiation, survival, inflammation and tissue remodelling[46].

YKL-40 in CSF is considered as a biomarker mainly for neuro-inflammation[46,47], but it is suggested to be an early marker for AD as well and showed a correlation with tauopathies. Though, it does not represent specific biomarker and could be used in variety of neurological conditions. Elevated YKL-40 is measurable in early stages of AD, multiple sclerosis and traumatic brain injury. It has also been proposed as a candidate biomarker in tumours, asthma and arthritis[47].

Level of YKL-40 in CSF shows potential to distinguish AD from progressive supranuclear palsy[48]. However, changes in YKL-40 CSF levels do not track in plasma. In this context, YKL-40 has potential to be used as diagnostic and prognostic marker mainly in CSF and in combination with other markers.

ALPHA-SYNUCLEIN

Alpha-synuclein (α-syn) protein is encoded by *SNCA* gene, its mutations are found to be strongly causative for autosomal dominant variant of familiar Parkinson's disease and SNCA polymorphism might be associated with cognitive decline in sporadic PD[26] (more details on PD mechanisms and models in chapter 9 *Basal ganglia related disorders – I. Parkinson's disease*). Fibrillary aggregation, caused by misfolded α-syn protein are hallmarks of synucleinopathies, such as autosomal dominant Parkinson's disease, Parkinson's disease dementia, dementia with Lewy bodies (DLB) or multiple system atrophy[26]. It is generally assumed that in neurodegeneration level of α-syn in CSF should be lowered. However results were not uniform across several studies as reviewed in Waragai et al 2010[49].

DJ-1

DJ-1 protein is coded by *PARK7* gene. Deletion in this gene is associated with recessive inheritance of PD[50]. DJ-1 is expressed in cytoplasm, mitochondria and nucleus of most somatic cells. In brain tissue DJ-1 is expressed in neurons and glia, with noticeable elevation in astrocytes during oxidative stress. Its function is protection of dopaminergic neurons from oxidative stress and protection of neurons against α-syn aggregation[51,52]. Commonly used method for measurement is ELISA, which showed elevated DJ-1 in CSF in HD, DLB and AD as well[49,52].

CONCLUSION

Cerebrospinal fluid represents a relatively easily accessible material with the great promise for early diagnosis of wide spectrum of CNS conditions. Over decades CSF was used mainly in biomarker studies of neurodegenerative disorders and oncogenesis. However, even with modern technologies and development of better study models, list of candidate biomarkers steadily prolongs, but only a few progressed to preclinical and clinical studies and none was approved for routine clinical use. As the disease pathology of individual neurodegenerations is still not fully understood, distinguishing among similar conditions based on biomarkers

alone remains problematic. CSF thus remains promising, but challenging sample material, mainly because of low analyte concentration, questionable sample stability and often non-standardized sampling.

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13 Targeted proteomics in translational research of neurodegenerative diseases

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Abstract

With recent technical improvements in the field of mass spectrometry-based analysis of proteins, the technology is now ready to enter clinical laboratories for routine measurements of protein biomarkers. Targeted MS methods like selected reaction monitoring or recently presented parallel reaction monitoring showed a great potential for sensitive, selective, and robust assays. Ease of development and multiplexing capacity of these methods is especially suitable for verification and validation of proteomics discovery data. The translation of preclinical findings in research of neurodegenerative disorders into practical use now relies on necessary implementation of independent tightly controlled clinical studies on a large cohort of well-defined subjects. In our review we briefly describe the targeted proteomics techniques and their employment in clinical proteomics validation of neurodegeneration biomarkers, with hope to identify disease diagnostic, prognostic or predictive biomarkers.

Keywords

proteomics, targeted mass spectrometry, selected reaction monitoring, parallel reaction monitoring, neurodegeneration, biomarker

INTRODUCTION

Proteins are key effector cell molecules and often targets for therapeutic interventions or even therapeutic drug itself. New systems biology "omics" approaches aim to study functions, structures and dynamics of complex biological systems. Discovery driven genomics, transcriptomics or proteomics studies are producing tremendous amount of data with expectation of revealing new disease molecular mechanism, treatment strategies and biomarkers. The ultimate goal of these studies is translation into clinic in the form

of personalized medicine. However, this discovery and preclinical data must be carefully validated before entering clinical medicine. Great hopes are being put into hunt for new diagnostic, prognostic and therapy monitoring protein biomarkers, yet the outcomes are not fulfilled so far.

Targeted mass spectrometry (MS) proteomics provides a very good solution for selective and sensitive validation of protein of interest or more precisely proteoform of interest[1]. Targeted MS proteomic approaches already possess the sensitivity comparable to immunoassays, especially when protein/peptide pre-enrichment or affinity enrichment step like SISCAPA[2] is applied (detection limits below fM range). In contrast to immunoassays, targeted MS approaches provide high specificity, potential for multiplexing (hundreds of peptides may be measured in one assay), higher through-put and also faster and low cost assay development[3]. However, the sample preparation for MS analysis requires more steps and thus is more laborious and generally requires more sample material. This is usually not an issue in case of body fluid analysis but may be limiting when analysing rare samples. Other challenge is the broad dynamic range of protein expression that approaches seven orders of magnitude in a cell or up to 12 orders of magnitude in biofluids, as well as the high number of proteoforms due to the posttranslational modifications (PTMs), alternative splicing or sequence changes[4]. On the other hand, the ability to distinguish PTMs is one of the strongest advantages of targeted proteomics over DNA/RNA-based techniques as only a PTM may indicate the disease.

TARGETED MASS SPECTROMETRY METHODS

Targeted mass spectrometry (MS) comprises three different methods; selected reaction monitoring (SRM, also known as multiple reaction monitoring MRM), parallel reaction monitoring (PRM) and data independent acquisition (DIA) (Fig. 1). All these methods are quantitative and when used as targeted method the assay development follows targets whose selection is hypothesis driven. The typical workflow is based on enzymatic digestion of proteins, where peptides become protein surrogates bearing the quantitative information. Peptides used for quantification have to be carefully selected and evaluated with respect to their uniqueness and quantotypicity, e.g. peptides with amino acids prone to chemical PTMs like methionine should be avoided.

SELECTED REACTION MONITORING

Selected reaction monitoring (SRM) is a "gold standard" in MS-based targeted methods. SRM requires previous knowledge about the analysed sample and allows relative or absolute quantification (using internal standards) of targeted proteins. For SRM analyses triple quadrupole (QQQ) mass spectrometer is used. Precursor ions with preselected m/z (mass to charge ratio) value are filtered in the first quadrupole (Q1) and send to collision cell

(the second quadrupole – Q2), and fragmented. Fragment ions are then passed to the third quadrupole (Q3), in which fragment ions with preselected m/z value are filtered and send to a detector. Specific pair of precursor and fragment ions of particular m/z values is called transition. Measurements of transitions together with information about peptide retention time in HPLC system guarantee high specificity and selectivity of SRM method. Main drawback of SRM method is quantification only on proteotypic peptides (peptides which are unique in the whole proteome) – this limitation enables reliable results but decreases number of usable peptides from each protein. Detailed guideline for development of quantitative SRM assay was published[5].

Large benchmark study across different laboratories and MS instruments showed high robustness and reproducibility of SRM method, even for such complex sample as an unfractionated blood plasma[6]. Despite the fact that SRM is commonly used in clinical laboratories for analysis of small molecules like metabolites or hormones for almost 40 years, the peptide analysis in clinical labs is still now far from being routine[7,8].

PARALLEL REACTION MONITORING

Parallel reaction monitoring (PRM) technique is in principle very similar to SRM and differs mainly in use of Q-Orbitrap mass spectrometer instead of QQQ. Precursor ions are again selected by first quadrupole, however Q3 is replaced by Orbitrap, thus all product ions are analysed with high resolution and accuracy. This allows very precise identification and quantification of peptide with higher sensitivity and without background interferences. No optimisation of collision energy or preselection for product ions is needed thanks to full MS2 scan. Basics of PRM assay development and data analysis were recently reviewed[9]. The main disadvantages are relatively high price of Q-Orbitrap mass spectrometer and need of previous knowledge about sample and targeted proteins as in case of SRM.

There are numerous MS quantitative methods using stable isotope labelled peptides (SIL). Both SRM and PRM typically use heavy SIL peptides (lysine and/or arginine labelled by ^{13}C and ^{15}N isotopes) that are mixed with their native counterparts as internal standards in known concentration for absolute quantification (AQUA)[10] or for relative quantification. SIL peptides display identical physicochemical properties as native peptides, ensuring identical LC separation and ionisation pattern, but can be easily resolved in mass spectrometer due to the mass shift. The quantity of the endogenous peptide is calculated from the chromatographic ratio between the synthetic reference and the native peptide.

DATA-INDEPENDENT ACQUISITION

DIA is a term for a variety of different techniques developed for distinct MS instruments from different vendors. In this text, only one DIA technique called Sequential Windowed Acquisition of all Theoretical Mass Spectra (SWATH-MS) is explained. In comparison with standard MS data dependent acquisition (DDA), SWATH-MS (and other DIA methods in

general) MS1 scan is virtually divided into windows with specific m/z values and all precursor ions in a particular window are fragmented together. This should theoretically lead to identification and quantification of all detectable peptides. Resulting fragmentation spectra are very complex due to fragment ions originating from different precursor ions. This can be resolved computationally by *in silico* generation of spectral (assay) library[11] or by parallel measurements of DDA of the analysed samples and generation of sample specific spectral library. Unfortunately, parallel measurements prolong instrument time, but use of general spectral library (e.g. Pan-human[12]) may lead to false positive results. Benefit of DIA approach is a possibility to reanalyse data anytime, for example with novel assay library with deeper proteome coverage (e.g. prefractionations or different tissues from the studied organism) to access larger set of quantifiable proteins[12].

DIA method is the only one from the three mentioned MS methods that can be used in untargeted discovery mode as well because thousands of proteins are measured and may be quantified in a single sample.

Multi-laboratory assessment of SWATH-MS method in eleven laboratories worldwide confirmed its high robustness and consistency of results[13]. Recently, a comprehensive guideline for SWATH-MS technique was published[14].

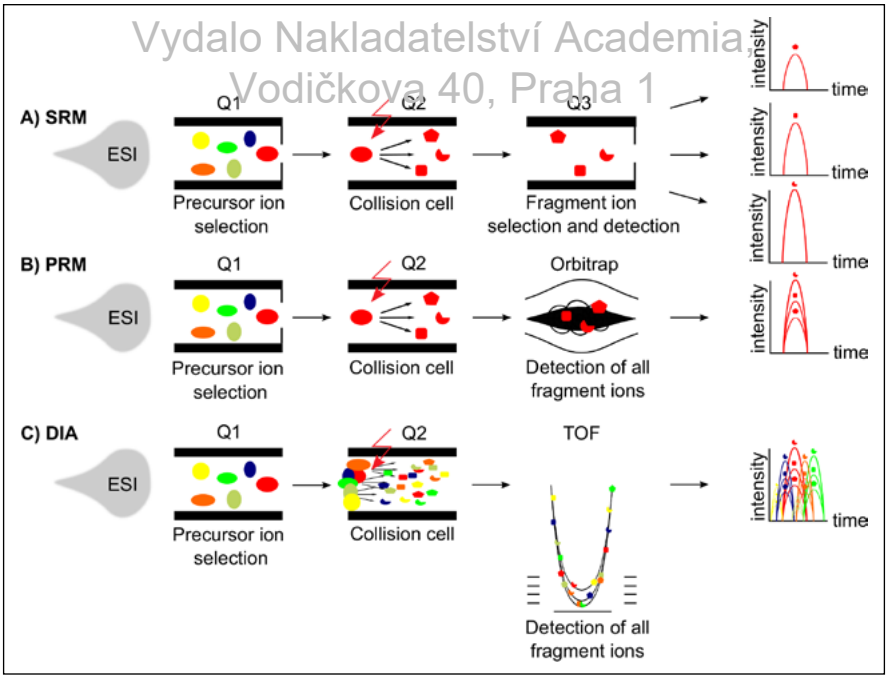

Figure 1. Schematic overview of the targeted MS methods. Orig. J. Tylečková

TARGETED ASSAYS QUALITY GUIDELINES

To translate new discoveries into clinical applications, the targeted assays have to be reproducible, robust and easily adoptable across laboratories. The three tier "fit-for-purpose" approach based on the performance of the measurements was defined in 2013[1] by proteomic community with the aim to develop the guidelines to evaluate the assay quality[15]. Tier 3 assays suit the exploratory studies where proteins or their proteoforms are relatively compared in biological systems, tier 2 assays precisely measure relative proteoform changes and tier 1 assays provide precise absolute concentration of analyte measured. Tier 1 and 2 assays require analytical validation and internal labelled standards for each analyte measured in the sample. Tier 1 assays are closest to clinical grade assays. Clinical Proteomic Tumor Analysis Consortium assay portal offers the open-source repository of well characterized tier 2 assays[16].

TARGETED MS IN NEURODEGENERATION RESEARCH

Neurodegenerative diseases are a heterogeneous group of diseases generally leading to neuronal damage, neurodegeneration and death. Underlying diseases mechanisms are still only partially or poorly understood, but often protein aggregation, protein misfolding or mitochondrial disfunction play major role[17,18]. The social and economic burden of neurodegenerative disorders is huge with expected further increase in next few decades. In the following section we will discuss the application of targeted MS in the research of neurodegeneration in different model systems.

SAMPLES FROM POST-MORTEM BRAINS

In comparison to some other types of disease research, the availability of human sample material represents a major challenge in the studies of neurodegeneration. Often post-mortem brain autopsies of patients are obtained from brain banks but the protein degradation in this type of samples is rapid, especially changes in protein phosphorylation pattern[19]. Moreover, samples from only the late stages of the disease are usually available.

Combination of immunoprecipitation with MS DDA and PRM analysis was used for quantification of α-synuclein forms in soluble, detergent-soluble as well as detergent-insoluble post-mortem brain cortex fractions of Parkinson's disease (PD) patients and controls. Several potentially modified proteoforms of α-synuclein were identified and α-synuclein levels were increased in PD[20]. SWATH-MS analysis of synaptic proteomes in brains of Alzheimer's disease (AD) patients and healthy controls was performed by Chang et al. They were able to quantify 2077 proteins, 30 of them with differential expression in brains affected by AD. These 30 proteins were mainly involved in cellular processes like RhoA signalling and cellular assembly and organization[21].

ANIMAL MODELS

Reliable animal models either chemically induced, transgenic or knock-ins/knock-outs (KO) may be necessary to understand the initial disease stages and to discover disease biomarkers[22].

Potential early PD diagnostic markers were proposed in a longitudinal mitochondrial proteome analysis using SWATH-MS approach in the cortex and striatum of PTEN-induced kinase 1 (PINK1) KO rat model. The analysis revealed changes in mitochondrial pathways like decrease in levels of complex I of the electron transport chain during asymptomatic phase of PD in this rodent model[23]. More discovery oriented targeted assays were developed to quantify protein composition of post-synaptic density from mouse/rat brain cortex samples[24,25]. These assays may be used in further studies of neurological disorders not only in rat and mouse brain tissue.

CELL MODELS

In vitro cell models including patients derived induced pluripotent stem cells (iPSCs) represent another valuable model system for proteomic discovery or validation studies without the ethical issues connected to human samples or animal models[26]. Proteomic analysis of culture media of AD patients derived-iPSC differentiated neurons were searched for very early AD biomarkers but only Alpha-1-acid glycoprotein was confirmed by SRM as decreased in the culture media from AD neurons[27]. Most current cell models of neurodegeneration lack the complexity of the nervous system which may be partially overcome by novel three dimensional brain organoid models[28].

CEREBROSPINAL FLUID

Cerebrospinal fluid (CSF) is in direct contact with brain and spinal cord tissue and its collection is a relatively non-invasive procedure. This makes CSF valuable material available for validation studies of neurodegenerative disorders in human patients.

Currently great effort is given to the early diagnosis of AD as the treatment focused on delaying cognitive decline is believed to be most effective in the early disease stages. The nowadays accepted AD biomarkers are Amyloid beta (Aβ), total Tau and phosphorylated Tau proteins, however other biomarkers apart from mentioned are still needed to distinguish other forms of dementia.

For example measuring free monoubiquitin by SRM in CSF may discriminate AD from other neurodegenerative diseases[29]. Andersson et al. developed PRM assays to verify 20 proteins previously identified by antibody arrays in 94 CSF samples from AD, mild cognitive impairment (MCI), non-AD dementia and healthy controls[30]. Thirteen out of the twenty proteins were validated by PRM, three proteins Neuromodulin, Vascular cell adhesion molecule 1 and Prosaposin were suggested as potential pre-clinical AD markers, yet the correlation between PRM assay and antibody-based measurement was generally low. Another two panel PRM assays including SIL peptides were measured in CSF with the aim to validate 13 novel biomarker candidates, six

of them were lowered in AD[31]. Recently very extensive SRM panel monitoring 320 peptides from 142 candidate biomarker proteins on a large and well characterized cohort of AD patient identified several potential prognostic peptides[32]. Heywood et al. validated a set of almost 30 known and novel markers specific and/or shared between AD, PD and Lewy body dementia by rapid 10 minute UPLC-SRM assay[33]. Several of them have potential to show changes between Lewy body dementia and AD that can be otherwise difficult to discriminate.

Recently, new PRM based method utilizing SIL peptides and digestion with two distinct enzymes named CompTryp was used for direct absolute quantification of Tau in CSF from AD cases and healthy controls with much lower coefficient of variation when compared to Tau ELISA measurement, but interestingly with ten times higher Tau levels[34] which is consistent with other PRM study measuring absolute concentration of Tau peptides in CSF[35].

Nice example of proteomics PD biomarker discovery and verification in CSF is a study by Shi et al.[36], who selected about 300 CSF observable proteins (1400 peptides) out of 4000 proteins from previous experiments and data mining. 126 peptides showed good SRM performance, with 17 of them significantly different in PD vs. healthy controls. Finally, a panel of 6 peptides/proteins biomarkers with good diagnostic sensitivity/selectivity for PD was selected after validation on independent cohort of AD, PD and controls. These potential PD diagnostic markers need to be confirmed in a larger cohort of different disease conditions together with other known potential PD markers like α-synuclein.

Sensitive SRM assay for PD progression monitoring by absolute quantification of α-synuclein in CSF without any enrichment or fractionation was developed by Yang et al. They suggested one diagnostic and prognostic α-synuclein peptide with excellent correlation to immunoassay analysis that can distinguish controls from PD patients and changes in this peptide can follow disease progression[37]. In contrary, SRM quantification of α-, β- and γ-synucleins in CSF reported by Oeckl et al. did not find any changes in levels α-synuclein in PD but they found increased synucleins concentration in AD and Creutzfeld-Jakob disease indicating synuclein may be a more general marker of synaptic degeneration[38].

For more information on AD and PD biomarkers see reviews[39–41] and for biomarkers in CSF see chapter 12 *Protein biomarkers of neurodegeneration in cerebrospinal fluid.*

PLASMA

Cerebrospinal fluid collection by lumbar puncture is considered relatively non-invasive when compared to other tissue biopsies. However, in comparison to blood collection it still represents invasive procedure with associated risks, not suitable for routine checkups of people without any apparent clinical symptoms. For that reason, peripheral biomarkers of neurodegenerative diseases are highly demanded. Extremely low concentrations of brain derived biomolecules in plasma/serum make the measurement technically challenging.

Very sensitive SRM assay was used for detection of Aβ surrogate marker peptides in human plasma samples without immunoaffinity enrichment involving abundant plasma protein

depletion and cation-exchange sample preparation steps that enabled quantification of these low abundant peptides in concentration levels below fmol/mL[42]. Pan et al. targeted plasma peptides originating from brain glycoproteins using N-glycocapture technique for enrichment in a large cohort of PD patients and controls[43]. They suggested a four peptide panel to distinguish PD from controls (peptides derived from Prion protein, Neural cell adhesion molecule 1, Heparan sulphate proteoglycan 2 and Multiple epidermal growth factor-like domains 8) with high sensitivity but the specificity reaching only about 50 %. Moreover, combination of two peptides (peptides derived from Multiple epidermal growth factor-like domains 8 and Intercellular adhesion molecule 1 correlated with severity of PD.

ANALYSIS OF POSTTRANSLATIONAL MODIFICATIONS

Selected reaction monitoring may be also successfully applied in the analysis of different Tau protein PTMs[44–46]. Although Tau phosphorylation is the best estabilished Tau PTM[47], other Tau PTMs like ubiqiutination, acetylation or methylation of lysine[46] have been reported as important for Tau protein aggregation and degradation. Furthermore, changes in histone PTMs including methylation[45] or acetylation[44] may be related to epigenetic regulation in the AD pathology. Changes in several histone methylation and ubiquitination patterns in frontal cortex from AD patients were reported by Anderson and Turko[45]. Another example of MS based quantification of protein isoforms that was not feasible so far with antibody-based methods confirmed decrease in long isoform of C9ORF72 in *post mortem* frontal cortex samples of frontotemporal dementia patients bearing C9ORF72 mutation[48]. It is also known that different polymorphic forms of ApoE (ApoE4 genotype) may increase the risk of late-onset AD significantly (reviewed in[49,50]). Absolute quantification of total ApoE and ApoE isoforms in CSF and plasma was performed by SRM with high sensitivity up to 1 fmol[51].

CONCLUSION

Targeted analysis of potential neurodegeneration biomarkers is a powerful tool to enable translation of discovery data into clinics. However, it is necessary to emphasize the need for independent high quality follow up studies with addition of SIL peptides for absolute quantification on a large cohort of patients with other neurological disorders to make a real impact in the clinical field.

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14 Extracellular vesicles: a potential source of biomarkers in neurodegenerative diseases

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Abstract

Extracellular vesicles (EVs) are mediators of intercellular communication, which can transfer protein, RNA or small molecules into recipient cells and change their phenotype. In the central nervous system, EVs play roles in various physiological functions, including development, synaptic transmission or neuronal protection and regeneration. In neurodegenerative diseases, EVs may participate in transfer of misfolded proteins into healthy cells but possess also diagnostic/prognostic and therapeutic potential. Research of neurodegenerative disease pathophysiology is limited to studies on cellular models, experimental animals or *post-mortem* tissues. EVs offer a valuable alternative to access the diseased nervous system with minimal invasiveness.

Keywords

extracellular vesicles, exosomes, body fluids, neurodegenerative disorders, Huntington disease, pig HD model, proteomics, biomarkers

EXTRACELLULAR VESICLES

Extracellular vesicles (EVs) are small membrane-enveloped particles released by cells to the surrounding extracellular space and body fluids[1]. EVs are capable to transfer molecules and information into the recipient cells and thus influence their physiological and pathological functions. EVs are produced by both prokaryotic and eukaryotic organisms[2]. In mammals, EVs can be secreted by most of cells and their presence was detected practically in all body fluids[3]. EVs participate in various physiological processes, including blood coagulation, angiogenesis,

regulation of immune responses, reproduction, tissue and organ homeostasis maintenance, tissue remodelling and many others[2]. Currently, three main EV types are recognized: apoptotic bodies, microvesicles, and exosomes, differing in size and mechanism of biogenesis[4,5].

Apoptotic bodies reach sizes approximately 1–5 μm in diameter and are released as blebs during cell death by apoptosis. Despite the molecular composition of apoptotic bodies is less explored, they seem to be enriched in phosphatidylserine and contain cellular components including fragmented DNA[4,5].

Microvesicles are extracellular particles with various sizes spanning approximately from 50 to 1000 nm. Microvesicles are formed by budding of the plasma membrane into extracellular space[6]. Presence of cytoskeletal components, adhesion receptors, signalling molecules, as well as mitochondrial and ribosomal proteins has been detected in microvesicles[5].

Exosomes are small spherical vesicles with a diameter around 30–100 nm. Exosomes originate in the inner (intraluminal) vesicles of multivesicular bodies (MVB), which are released by exocytosis into the extracellular space in form of exosomes[6] (Fig. 1). Exosomes are also characterized by a floating density of 1.13–1.19 g/mL in density-gradient ultracentrifugation and have an artificially obtained cup-shaped morphology upon fixation for transmission electron microscopy[6,7] (Fig. 2). Exosomes are surrounded by a lipid bilayer membrane with specific composition, distinct from the plasma membrane of the source cell – enriched in cholesterol, sphingolipids, ceramide and glycerophospholipids[8]. Exosomes carry specific recognition molecules on their surface, which enable their uptake by recipient cells. Exosomes thus play a significant role in intercellular communication[2,9]. The lumina cargo of exosomes comprises of DNA, RNA, proteins, peptides and metabolites, which are incorporated into vesicles during their biogenesis[7,10]. Since exosomes originate from endosomes, proteins involved in MVB formation (e.g. TSG101-protein), proteins of membrane transport, adhesion and fusion (e.g. flotillin, integrins, tetraspannins), heat shock proteins (Hsp70, Hsp90) and lipid-related proteins (e.g. Annexin V) are frequently identified in exosomes irrespective of the cell type of origin[5,11]. On the other hand, exosomes mostly do not contain proteins of mitochondrial, nuclear, ER or Golgi origin[12]. In recent years, it has been shown that exosomes contain a rich repertoire of RNA, including regulatory small RNA transcripts[13–16]. These transcripts enclosed in exosomes are protected from RNases in extracellular space. The RNAs can be transferred into recipient cells, where they can modulate gene expression and biological pathways by repressing or inhibiting translation[17]. The ability of transferred small RNAs to exert a biological effect suggests that they are stable and functional in the recipient cell[16,18,19]. Exosomes are the most explored extracellular vesicles. However, as various types of vesicles exist and it is practically impossible to identify their mechanism of biogenesis once they are secreted, the common term EVs is used in this chapter.

In the nervous system, almost all cell types, including neurons, astrocytes, oligodendrocytes, and microglia release EVs under physiological conditions[20,21]. EVs have been shown to implicate their functional roles in neurophysiology process, including neurogenesis, synaptic transmission, neuronal regeneration, or other pathways in neuronal development[20,22,23].

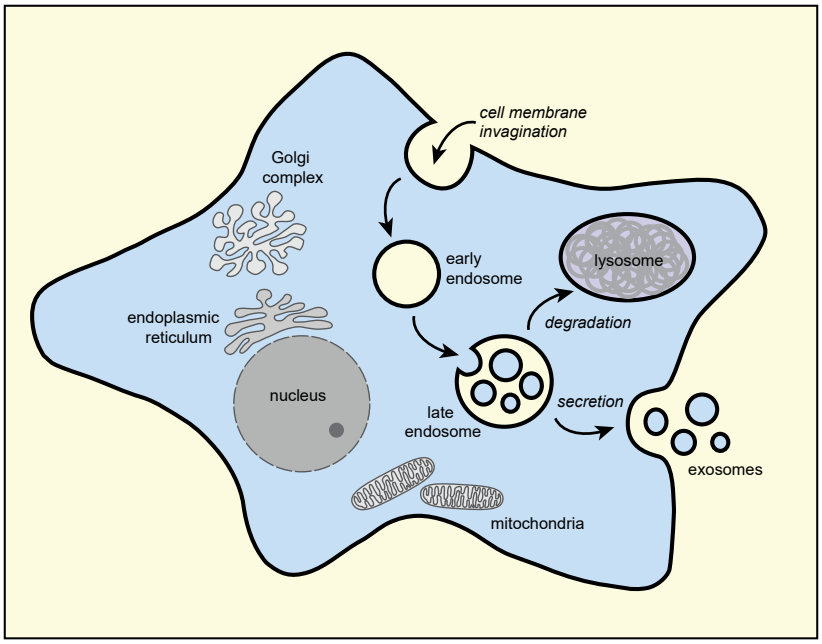

Figure 1. Exosome biogenesis. During endocytosis, cell membrane invagination leads to a formation of intracellular vesicles, called early endosomes. By additional inward budding of endosomal membrane, intraluminal vesicles (ILVs) are formed inside of the late endosomes. Such late endosomes are also called multivesicular bodies (MVB). The ILVs may be released from cell in a form of exosomes. Late endosomes may also fuse with lysosomes, where the endosomal content is degraded. Exosome biogenesis is tightly regulated. Specialized proteins, e.g. ESCRT complexes (endosomal sorting complexes required for transport) and RAB proteins participate in ILV formation and vesicular trafficking, respectively[6,24,25]. Orig. H. Kupcova Skalnikova

EXTRACELLULAR VESICLES IN NEURODEGENERATIVE DISEASES

EVs IN NEURODEGENERATIVE DISEASE DEVELOPMENT

Neurodegenerative diseases, such as Alzheimer's diseases (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS), Huntington's disease (HD) or prion diseases are accompanied by a progressive accumulation of protease-resistant misfolded protein aggregates[26,27].

Misfolded proteins can self-propagate through seeding and spread the pathological abnormalities to other cells. Aggregates of misfolded proteins are toxic for cells, which leads to destruction of neurons in specific areas of the brain or spinal cord[26]. EVs are supposed to participate as possible carriers in spreading of misfolded proteins into healthy cells in neurodegenerative diseases and such a EV-mediated disease propagation was experimentally noticed not only in prion diseases, but also in AD, PD, ALS and HD[28–32].

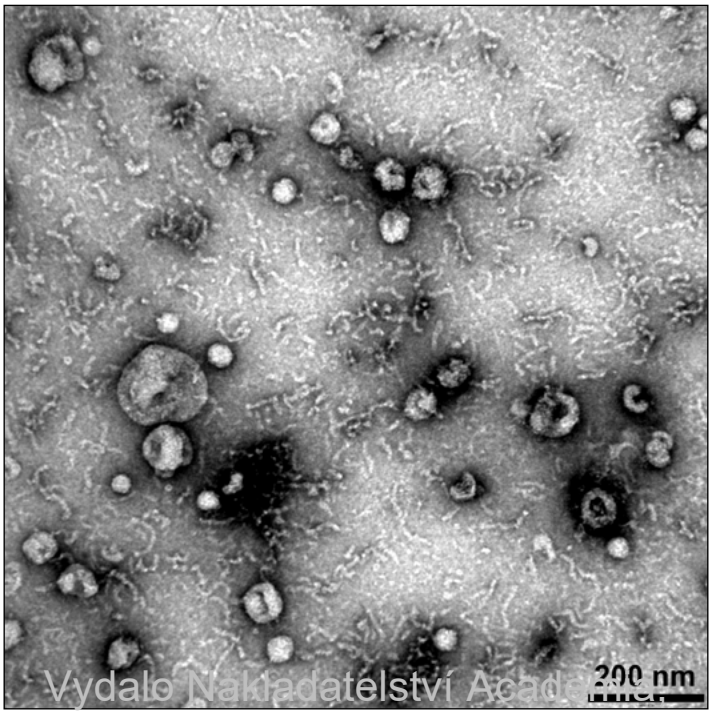

Figure 2. Small extracellular vesicles (corresponding in size to exosomes) visualized by transmission electron microscopy. Originally spherical particles during contrasting and drying obtain typical cup-shaped morphology. Magnification 200 000x, scale bar 200 nm. Orig. H. Kupcova Skalnikova

DIAGNOSTIC AND PROGNOSTIC POTENTIAL OF EVs

Neurodegenerative diseases show large variations in the length of the disease progression period from very few years in most of ALS cases to many (>10) years in AD or HD. In slowly developing diseases, it is difficult to monitor the disease progression due to very little changes detectable by non-invasive techniques (e.g. magnetic resonance imaging), as well as due to inaccessibility of the central nervous system for invasive biopsies, and limited availability of specific measurable biomarkers in the periphery, i.e. in blood or cerebrospinal fluid. The need of disease progression monitoring is particularly important in currently emerging new therapies, particularly cell based and gene therapies, to monitor their efficacy and safety[33–36].

EVs as naturally occurring nanometre sized particles are supposed to travel across endothelial cells of the blood-brain barrier and enter the blood stream. The exact mechanisms of blood-brain-barrier crossing are not fully elucidated, however, the receptor-mediated endocytosis represents one of the candidate mechanisms[29,37]. Body fluid EVs, and particularly

their protein and RNA content, possess a high potential in biomarker discovery and disease monitoring due to their availability by so called "liquid biopsies". The blood represents an easily accessible body fluid, however, as it flows through the whole organism, it contains products of various cell types and EVs originating from the tissue of interest, e.g. nervous system, might represent very minor subpopulation. EVs released by intrinsic blood cells and endothelial cells account for the majority of plasma EVs and release of EVs from platelets upon coagulation activation may result in up to 90 % of blood EV counts[38]. Techniques for selective enrichment of EVs of neural origin from blood have been recently developed by group of E. J. Goetzl[39–41], using a nonspecific precipitation of particles from blood plasma by Exoquick reagent, followed by immunocapturing of neural cell-derived EVs by anti-NCAM (neural cell adhesion molecule) or anti-L1CAM (L1-cell adhesion molecules) antibodies, recognizing surface cell adhesion proteins expressed predominantly (but not exclusively) on neural cells (neurons and glial cells) or neurons, respectively.

Beside the blood plasma, other body fluids are taken into consideration as possible EV sources for neurodegenerative disease monitoring. Obviously, cerebrospinal fluid that is in direct contact with neural cells is the most promising biofluid to search for neurodegenerative disease biomarkers. However, collection of this fluid is an invasive procedure and the EV counts in cerebrospinal fluid are very low[42]. Other body fluids, such as saliva or urine, are also studied as possible EV sources of biomarkers, as such fluids can be collected non-invasively.

The diagnostic/prognostic potential of body fluid EV content was already demonstrated in neurodegenerative diseases with high incidence in population, such as AD and PD. In early stages of AD, toxic forms of proteins (particularly p-tau and Aβ1–42) are detectable in EVs isolated from body fluids. Correlations of phospho-S396-tau, phospho-T181-tau, and Aβ1-42 levels in neural-derived blood EVs with the AD progress were described[39,43]. Interestingly, based on these EV protein levels, development of AD could be predicted up to 10 years prior to clinical onset[39]. Similarly, levels of autolysosomal proteins in neural-derived blood EVs distinguished AD patients from case controls and appear to reflect the pathology of AD up to 10 years before clinical onset[40]. Detection of selected protein forms in peripheral EVs is expected to become a biomarker-based method in the diagnosis of not only early stage AD but also other neurodegenerative diseases[31]. Beside proteins, altered levels of several kinds of miRNAs were also observed in EVs from various sources (brain, blood and cerebrospinal fluid) of AD patients[44].

In PD, substantially higher levels of α-synuclein in neural-derived blood EVs were identified in a large cohort of PD patients compared to healthy controls[45]. Results of recent studies of EVs candidate protein and micro RNA biomarkers in PD were reviewed by Wu et al.[46].

In much less frequent HD, the presence of mutant huntingtin or its fragments has not yet been studied. Nonetheless, our preliminary results on HD minipig model[47] show association of mutant huntingtin with pig body fluid-derived EVs.

Despite several studies showed association of selected protein forms, aggregate-forming proteins or selected RNAs with the neurodegenerative disease progression, other authors have not confirmed such a correlation. Various isolation techniques, e.g. differential centrifugation with ultracentrifugation, density gradient ultracentrifugation, size exclusion chromatography, affinity capturing, or precipitation, give different yields and purities of isolated particles. Co-isolation of contaminants (e.g. cellular debris/fragments, lipoprotein particles, other vesicles) is mostly inevitable using a single isolation technique or even combinations of several techniques and such contaminants may be easily detected especially by highly sensitive methods, such as RT-PCR. In addition, pre-analytical issues, e.g. sample collection and processing, may have key effects on the sample quality, EV content and quantity of contaminants. Due to these facts, comparisons of results of individual studies might be difficult. As the EV research field is quickly evolving and is accompanied by a massive increase in published results, some of them of lower quality, the need of standardization is highly urgent. The International Society for Extracellular Vesicles (ISEV) continuously monitors ongoing research and has published several guidelines for EV research[1,48–51].

POTENTIAL OF EVs IN TREATMENT OF NEURODEGENERATIVE DISEASES

Extracellular vesicles possess high potential in therapy, both as native EVs directly used as therapeutic agents in tissue regeneration and immunomodulation, or in a form of engineered EV-based vehicles for delivery of biologically active material to target cells[52–54]. Vesicles produced by stem cells, particularly mesenchymal stem cells, hold promise in neurodegenerative disease treatment, mainly due to their neuroprotective and immunomodulatory properties[55].

Targeting central nervous system by therapeutic drugs faces difficulties with overcoming of blood-brain barrier. Although the small molecules are expected to cross the barrier, experiments show that ~98 % of small molecule drugs are not transported[56]. Transfer of larger molecules across blood-brain barrier is even more limited. As exosomes are naturally occurring nanoparticles without immunogenic potential and are able to cross the barrier, they have attracted substantial attention as drug delivery vehicles into the central nervous system. Potential therapeutic molecules to be delivered via EVs into central nervous system include small molecule drugs, therapeutic proteins and various therapeutic RNAs[31,46,57,58].

CONCLUSION

Extracellular vesicles are indispensable mediators of intercellular communication in the central nervous system. MicroRNA carried in the EVs may influence gene expression in surrounding or more distant recipient cells. EVs also participate in maintenance of nervous system

homeostasis by possessing functions in neuronal survival, regulation of synaptic transmission, axon regeneration, protection against stress or against excessive neuron excitation.

The prolongation of life span in human population is accompanied by rising incidence of neurodegenerative diseases. EVs have been shown to be involved in promotion of neurodegenerative diseases as they may participate in spreading of misfolded proteins into healthy cells. On the other hand, EVs are very topical and intensively studied entities as carriers of potential protein and RNA biomarkers of various pathological conditions and have been shown to carry potential markers of progression in Alzheimer's and Parkinson's disease. EVs able to cross blood-brain barrier have potential in delivery of therapeutics into the central nervous system. As the research of neurodegenerative disease pathophysiology is limited to studies on animal and cellular models, or *post-mortem* brain tissue, we believe that EVs may offer a valuable alternative to access the diseased nervous system with minimal invasiveness.

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15 Microbiome-gut-brain axis in pathogenesis of neurodegenerative diseases

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Abstract

From the very beginning of our lives, our gut is colonized by a complex of commensal, symbiotic and pathogenic microorganisms. Changes in development and composition of microbial communities can be a potential risk for many diseases, surprisingly including neurodegenerative illnesses. Bidirectional communication between central neural system and gastrointestinal system, called gut-brain axis, plays an important role in this phenomenon. Huntington disease is a hereditary neurodegeneration characterized by motor, cognitive, psychiatric changes, and metabolic dysfunction, e.g. weight loss. This review briefly discusses the gut-brain axis hypothesis and on example of HD gives short overview of possible involvement of gut microbiome in progress of neurodegenerations.

Keywords

microbiome, neurodegenerative diseases, Huntington disease, gut-brain axis

THE GUT MICROBIOME

The human body is the residence of many microbial species and our evolution is linked to a mutualistic partnership with the gut microbiota which includes not only bacteria, but also viruses, archaea and fungi[1–3]. For long time it was believed that these microorganisms outnumber human cells almost 10 : 1. However, Sender et al. recently estimated ratio of bacteria to human cells to be 1.3 : 1[4], with most human cells being red blood cells. Non-pathogenic bacteria that form the key component of microbiome in the narrower sense can be assigned to four major phyla: *Firmicutes* (species such as *Lactobacillus, Clostridium, Enterococcus*),

Bacteroidetes (species such as *Bacteroides*), *Actinobacteria (Bifidobacteria)*, *Proteobacteria (Escherichia-coli)*[5–9], *Fusobacteria, Verrucomicrobia* and *Cyanobacteria*[8,10,11]. By the relative abundance, *Firmicutes* and *Bacteroidetes* represent more than 90 % of the gut microbiome[2,6,9]. Under normal conditions, pathogenic microorganisms are present only in very limited quantities. The variation of phyla is initially obtained from mother. Everything starts already with *in utero* colonization, as the placenta is not the sterile environment as was thought previously[12]. However, most of the infant's microbiota is acquired during the birth and depends on way of delivery, and is continuously developed through feeding and contact with the external environment[12,13]. The infant microbiota evolves very fast in the next 2–3 years to resemble that of the adult person. These years have come to be viewed as a vulnerable period in which perturbations may have far reaching impacts on the brain development and be predictive for later diseases[12–14]. The composition of gut microbiota in humans is from about 1/3 "common" while the other 2/3 are individual, providing our "personal microbial identity"[11,15]. This variation in microbiota composition among individuals in the population is influenced by diet, age, gender, health, family and pet exposure, hygiene habits, use of disinfectants, antibiotic, antimycotic and antiviral drugs, probiotics and possibly by other variables. Changes in development of microbiome and disruption of its homeostasis can be a potential risk factor for many diseases including neurological illnesses[2,6,9].

MICROBIOME GUT-BRAIN AXIS

Our gastrointestinal tract (GI) hosts a complex ecosystem of microbes which work symbiotically with the host and act as key modulators for the bidirectional communication between the GI and the central nervous system (CNS)[16,17]. Although all parts of GI contain microbiota to some extent, this complex GI-CNS interaction is known mostly under the simplified term a gut-brain axis (Fig. 1). The communication between brain and enteric nervous system is mediated by neurons of the sympathetic and parasympathetic nervous systems, as well as by circulating hormones and other neuromodulatory molecules, including neurotransmitters, brain neurotrophic factors, bacterial cell wall and metabolites[13,18,19]. In particular, millions of nerves end in the gastrointestinal tract[15], with the vagus nerve constituting the main direct link from the gut to the brain[19]. All these factors may help us to elucidate the effects of the microbiota on homeostasis and complex CNS disorders.

Bacteria cell walls consist of peptidoglycan that activates both the innate and adaptive arms of the host mucosal immune system. Other bacterial cell wall components include porins, lipopolysaccharide, lipoteichoic acids and mannose-rich sugar chains. Together these bioactive bacteria associated molecules are called pathogen-associated molecular patterns (PAMPs)[19]. The defence cells of innate immune response are stimulated through interaction of pattern-recognition receptors (PRRs) with bacterial PAMPs, leading to the production of

pro-inflammatory cytokines[19,20]. These cytokines, including interleukin 6 (IL-6) and chemokine ligand 2 (CCL2), can influence the brain indirectly through peripheral vagal pathways or directly through permeable regions of the blood-brain barrier (BBB)[19,21]. PAMPs may also stimulate the production of additional molecules involved in neural signalling from intestinal epithelial cells[19,20].

Metabolites from our digestion and microbial fermentation of dietary and nutritional components may have a significant effect on brain processes and responses of immune system. It has been proven that the gut microbiota composition has an influence on the availability of fatty acids and tryptophan, which in turn interact with and regulate the immune system responses[19].

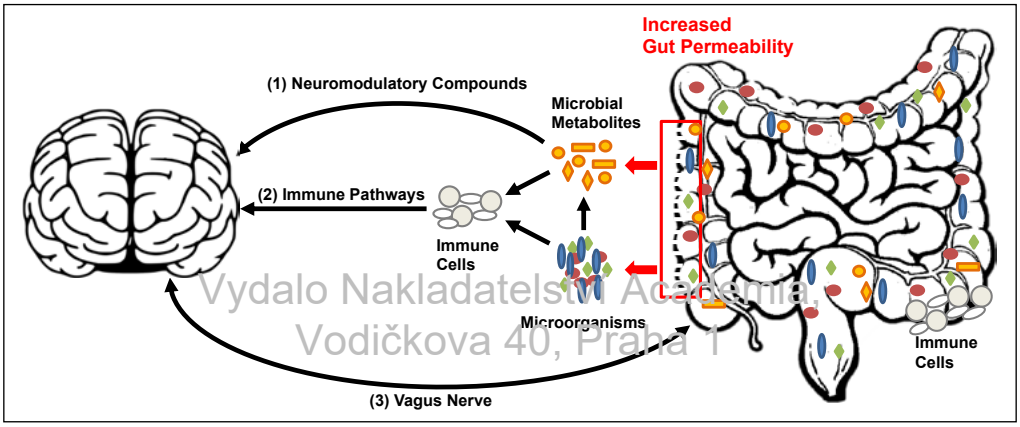

Figure 1. Interactions between gut and brain. Bidirectional communication exists between gut microbiome and brain. This is further enhanced in case of dysbiosis, which increases gut permeability. (1) Some of the microbial metabolites (fatty acids, neurotransmitters and hormones) act directly as neuromodulatory compounds. (2) Immune system is activated by interaction with microbiota and their metabolites and then indirectly influence brain (e.g. cytokines of inflammatory pathways). (3) Direct communication is also mediated by vagus nerve, representing the main connection between CNS and enteric system. Orig. T. Panková

The brain is a tissue with high content of fatty acids, which are both an important structural component of cell membranes as well as metabolically active entities involved in regulation of neurotransmission, cell survival and neuroinflammation[19,22]. Dietary fatty acids participate in the production of eicosanoids, important messenger molecules responsible for regulation of diverse biological processes from membrane composition and function alterations to biosynthesis of cytokines and modulation of gene expression[19,23]. Fatty acids can also directly regulate gene expression through activation of nuclear receptors/transcription factors known as peroxisome proliferator activated receptors (PPARs). While some dietary fatty acids bind specific immune cells such as T-cells, B-cells and macrophages to promote inflammation,

short-chain fatty acids produced as primary bacterial metabolites have potent anti-inflammatory properties[19,24] and play a role in the release of neuropeptides from GI tract L-cells[25].

The gut microbiota is a rich source of bioactive metabolites which are able to specially target G-protein coupled receptors (GPCRs) within the gut-brain axis[26] and by this molecular mechanism are supposed to manipulate with their hosts. The hormone ghrelin plays a crucial role in maintaining energy balance, metabolism, and central modulation of food intake, motivation, reward and mood. Ghrelin secretion is controlled by GPCRs sensing metabolites generated both by host's metabolism directly from dietary nutrients and metabolites produced by gut microbiota[27]. The ghrelin receptor GHSR-1a (growth hormone secretagogue receptor 1a) is expressed in the gut and also in the brain and today it is considered promising therapeutic target for diverse set of disorders such as obesity, diabetes, anxiety and depression, and even cancer[28]. It was shown by Torres-Fuentes *et al.*[29] that bacterial metabolites can attenuate ghrelin-mediated signalling throughout the GHSR-1a.

GUT MICROBIOME DYSBIOSIS

The healthy community of microorganisms in the gastrointestinal tract is characterized by stability, diversity and resilience[30]. Respectively by the richness of the ecosystem and amenability to perturbation and ability to return to the pre-perturbation state[31]. The undesirable shift in microbiota composition, also known as gut dysbiosis has been associated with the development of various gastrointestinal and metabolic diseases, such as inflammatory bowel disease, obesity and diabetes[32–35]. Three possible types of gut dysbiosis were described[31]: bloom of pathobionts (also called bacterial overgrowth), loss of commensals and loss of diversity. Bloom of pathobionts is dysbiosis caused by overgrowth of microorganisms typically present at low relative abundances. They proliferate when aberrations occur in the intestinal ecosystem of the commensal microbiota and have a potential to cause pathology. One typical example is bloom of the *Enterobacteriaceae*, such as *E. coli*, frequently observed in enteric infection and inflammation[36]. Dysbiosis caused by loss of commensals is a situation, in which some of the normally residing microbiota are reduced in numbers or completely absent. This can be the consequence of diminished bacterial proliferation or death of microbial populations[31]. In the last case, dysbiosis in form of reduced alpha diversity as a consequence of abnormal dietary patterns can contribute to many modern lifestyle diseases[37]. The origins of dysbiosis and mechanisms that contribute to the development and maintenance of a dysbiotic state are for example infection, inflammation, diet, xenobiotics, genetics, familial transmission and many other causes such as pregnancy[38] or physical injury[31,39]. The gut dysbiosis can cause the breakdown of the intestinal permeability barrier and lead to generalized inflammatory conditions as the proinflammatory cytokines get into the bloodstream and reach the brain[8,15]. As the intestinal microbiota has been shown to influence neurodevelopment, brain

function and behaviour[16,17,40], dysfunction of gut-brain axis may have broad pathophysiological consequences[41]. While gut dysbiosis is correlated with several neurological and psychiatric illnesses, including autism spectrum disorder, major depression, Parkinson's disease (PD) and Alzheimer disease (AD)[32–35], causal relationship was not established so far.

MICROBIOME IN HUNTINGTON DISEASE

Huntington disease (HD) is a hereditary fatal neurodegenerative disorder characterized by motor, psychiatric, and metabolic dysfunction[42] which is caused by a trinucleotide CAG repeat expansion in the *huntingtin* (*HTT*; *IT15*) gene to over 36 repeats. This repeat codes for extended stretch of glutamines in N-terminal part of huntingtin protein, changing its biochemical properties and causing aggregation and toxicity especially to striatal neurons. Huntingtin is expressed ubiquitously throughout the brain and peripheral tissues including the skeletal muscles, heart, and gut[33,43–46].

HD is not only a brain disorder; there are several indications that peripheral features such as weight loss and skeletal-muscle wasting are not directly associated with neurological dysfunction and contribute to disease progression[47]. These complex brain-body perturbations, including dysregulation of bioenergetics pathways may be related to the gut microbiome. In 2015 Rosas *et al.*[42] identified specific and selective alterations of plasma metabolome that could provide useful markers of prodromal and symptomatic HD patients. They analysed the plasma metabolomics profiles from HD patients without symptoms of disease, and early symptomatic HD patients. They identified alterations in tryptophan, tyrosine, purine, and antioxidant pathways, including many related to energetic and oxidative stress derived from the gut microbiome. They demonstrated mutually distinct metabolomic profiles; it means that the processes that determine onset were likely distinct from those that determine progression. „Gut microbiome-derived metabolites particularly differentiated the HD patients without symptoms of disease metabolome, while symptomatic HD metabolome was increasingly influenced by metabolites that may reflect mutant huntingtin toxicity and neurodegeneration."

Weight loss is one of the abnormalities that affect almost all individuals with HD, usually is very progressive, ending with profound cachexia in advanced stage patients[48,49], and correlates with CAG repeat number[47]. The main cause is an increased metabolic rate[50,51]. HD patients with a higher body mass index at the beginning of symptoms tend to have a slower rate of disease progression[52]. Disruption of the gastrointestinal microbiome (gut dysbiosis) has been recently reported in several neurological and psychiatric disorders, and therefore there is a hypothesis that it might also occur in HD[33,46]. While no data on microbiome in HD patient populations were published so far, studies in HD animal models provide some preliminary support for this hypothesis. Lower production of hormone ghrelin in stomach

of R6/2 mouse HD model caused by reduction of ghrelin-producing neurons was described[51], leading to digestive system dysfunction. Normally ghrelin stimulates food intake and inhibits energy expenditure[47]. It is thus possible that weight loss in HD might be related to bacterial metabolites influencing ghrelin secretion. Ghrelin also represents a potential therapeutic target for intervention focused on mitigating peripheral symptoms of HD[47].

Recent study by Kong *et al*.[53] characterized the gut microbiome of the R6/1 transgenic mouse model of HD in comparison to wild-type (WT) littermate controls. Using 16S rRNA gene sequencing on DNA isolated from faecal samples, authors identified specific changes in bacterial diversity and taxonomic composition. Across all mice there were two dominant phyla *Bacteroidetes* and *Firmicutes*, which together made up approximately 98 % of total abundance. The remaining phyla were made up of *Actinobacteria*, *Proteobacteria*, *Cyanobacteria*, *Deferribacteres* and *Tenericutes*. An increase in alpha-diversity was observed in male HD when compared to WT, which contrasts with studies reporting gut microbiome alterations in some other disorders, including AD and chronic fatigue syndrome[33,46]. An increased abundance of *Bacteroidetes*, with a proportional decrease of *Firmicutes* was found in HD mice, which corresponds to similar findings in AD, type 2 diabetes and chronic fatigue syndrome[33,46]. Some of the observed differences in the HD mice gut microbiome were sex related. Outside of HD context, there are several reports on sex differences in the gut microbiome, which are likely mediated by sex hormones[54,55]. Sexual dimorphism is a common characteristic in a variety of diseases, including metabolic, psychiatric and other neurodegenerative diseases such as PD, AD and HD[53,56–61].

The observed gut microbiome dysbiosis was in male HD mice accompanied by inability to gain weight despite higher food intake. This agrees with independent studies of metabolic disturbances in HD patients and mouse models[53,62–65]. Additionally, higher faecal water content was observed in HD animals at 12 weeks of age.

CONCLUSION

Our body is colonized by many microorganisms from the very beginning of our lives. Changes in microbial development and disruption of homeostasis (dysbiosis) leads to increased release of bacterial metabolites and activation of immune system. This can be a potential risk for many diseases, from gastrointestinal and autoimmune to neurodegenerative and psychiatric illnesses. Dysregulation of bioenergetics pathways and of the gut microbiome was identified in HD, showing even hereditary diseases can be influenced by microbiome changes. Additional studies are needed to confirm importance of metabolic dysfunction and gut microbiome role in HD pathogenesis. If confirmed, these could represent interesting model for studying microbiome influence on homeostasis of CNS and its disruption in neurodegenerative diseases.

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16 Reprogramming – a promise of regenerative medicine

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Abstract

Reprogramming cells into induced pluripotent stem cells (iPSc) is a part of regenerative medicine that can provide specific cell types to replace lost or damaged tissue in degenerative diseases or injury. In this review, we provide a new strategies and advances of *in vivo* reprogramming in case of four disease model and also discuss the future challenges of these preclinical studies for the clinical settings.

Keywords

regenerative medicine, *in vivo* reprogramming, transcription factors.

INTRODUCTION

The reprogramming story begins in 1962 with Gurdon's research. He demonstrated for the first time that the adult cells can be reprogrammed back into embryonic state and become pluripotent by turning the somatic cell nucleus into an enucleated oocyte in Xenopus[1]. Similar research was then successful in mammals resulting in cloned Dolly the sheep[2]. These pioneering studies provided evidence that reprogramming factors in the oocyte's cytoplasm can overwrite the cellular identity encoded in the nucleus of a completely different cell[3]. But how many transcription factors are needed? This question was answered in 2006 by Yamanaka and his group. They demonstrated that a cocktail of four transcription factors (Oct4, Sox2, c-Myc, and Klf4) is sufficient to reprogram skin fibroblasts into induced pluripotent stem (iPS) cells that resemble primitive embryonic state[4]. Therefore, the Nobel Prize of Medicine in 2012 was awarded to Gurdon and Yamanaka for these groundbreaking discoveries that have completely changed our view of the developmental biology and opened the revolution of regenerative medicine.

The main aim of regenerative medicine is to replace lost or damaged cells/tissues. Here, we focus on *in vivo* reprogramming. In this process, the patient's endogenous cells can be turned into a different cell/tissue type for regenerative purposes. This therapy is very attractive for tissue engineering and regenerative medicine because it eliminates the risk of immune rejection of the reprogrammed cells after transplantation. People in the 21st century have a big chance to use this therapy. This is not just an illusory dream. Based on the different studies published in this field, we believe that the success of this therapy approach is on the horizon.

SPINAL CORD

Spinal cord injury (SCI) is a damage of any part of the spinal cord or nerves at the end of the spinal canal (cauda equina). The damage of the spinal cord causes paralysis and neurological dysfunction. Paralysis negatively affects the psychological state of the patient, reducing the quality of their life. Modern advances in surgical and traumatic interventions involving the spinal column and underlying cord have significantly reduced mortality rates and extended the lifespan of SCI patients. SCI has experienced great improvements in health care since the middle of the 20th century[5]. Although this improved survival of SCI patients, the incidence of SCI is still significant. *In vivo* reprogramming could provide a new treatment for unconverted neuronal loss and glial scar formation in SCI (Table 1).

Table 1. Different studies involving *in vivo* reprogramming for spinal cord injury.

	Disease model	Reprogrammed cells	Reprogrammed protocol	References
In vivo reprogramming for spinal cord	SCI in NSG mice	Endogenous astrocytes to neurons	-Lentiviral vector -Transcription factor (SOX2)	**6**
	SCI	Astrocytes to neurons	-Lentiviral vector -Transcription factor (SOX2) -Neurotrophic factor (GDNF, FGF2, BDNF, NOG)	**7**

BRAIN

Brain damage including traumatic and acute brain injuries can happen due to accidents or biological complications. Traumatic brain injury (TBI) can have wide-ranging physical and psychological effects. Some signs or symptoms may appear immediately after the traumatic damper, while others may appear days or weeks later. Young children with brain injuries might not be able to communicate headaches, sensory problems, confusion and similar symptoms.

Table 2. Different studies involving *in vivo* reprogramming for brain injury.

	Disease model	Reprogrammed cells	Reprogrammed protocol	References
In vivo reprogramming for brain	Damaged brain	Non-neuronal cells to generate new neurons	-Retroviral vector -Combination of growth factors and transcription factor (Neurog 2)	**8**
	Neural conversion	Fibroblasts to functional neurons	-Lentiviral vector -Neural conversion factor (Asc11, Brn2a, Myt11)	**9**
	Brain injury or Alzheimer' Disease model	Reactive glial cells (reactive astrocytes) to functional neurons	-Retroviral vector -Transcription factor (Neuro D1)	**10**
	Traumatic brain injury (TBI)	Reprogramming reactive glia into iPSCs	-Retroviral vector -Transcription factor (Oct4, Sox2, Klf4, c-Myc)	**11**

Furthermore, because of the considerable damage and limited therapeutic approaches, TBI is a serious public health problem. The majority of brain cells are formed prenatally. This means the brain has limited capacity to regenerate cells, therefore brain injury often results in irreversible damage. With the recent advances in reprogramming therapy, these irreversible damages are likely to be treated, as suggested in various regenerative reprogramming. Through this approach, somatic cells can be transformed into functional neurons. Non-neural cells (such as astrocytes, glial cells) can be converted into new neurons by addition of exogenous factors. This has been applied in various neurodegenerative disease models (Table 2).

LIVER

The liver is the only internal organ in the human body capable of regeneration. In fact, people can lose up to 75 percent of liver size, and the remaining parts can regenerate itself into a new liver again. However, in chronic liver disease with damage in over long periods of time, the accumulation of scar tissue can inhibit the liver's ability to function and repair itself[12]. There is enough evidence that adult hepatocytes retain their ability to proliferate *in vivo*. With the use of *in vivo* reprogramming technology, the damaged liver can be repopulated with the reprogrammed hepatic cells. As summarized in Table 3, *in vivo* reprogramming has been applied by reprogramming hepatic cells towards hepatocytes or pluripotent stage.

Table 3. Different studies involving *in vivo* reprogramming for liver.

	Disease model	Reprogrammed cells	Reprogrammed protocol	References
In vivo reprogramming for liver	Diabetic	Neo-islet to hepatic progenitor cells	-Adenoviral vector -Transcription factor (Ngn3)	**13**
	Healthy mouse liver	Hepatic cells to pluripotent stem cells	-Naked plasmid injection -Transcription factor (Oct3/4, Sox2, Klf4, c-Myc)	**14**
	Chronic liver disease	Hepatic myofibroblasts into induced hepatocyte-like cell (iHEP)	-Adenoviral vector -Transcription factor (FOXA3, GATA4, HNF1A, HNF4A)	**15**
	Liver fibrosis	Myofibroblasts into hepatocyte	-Adeno-associated virus vector (AAV) -Transcription factor (Foxa1, Foxa2, Foxa3, Gata4, Hnf1a, Hnf4a)	**16**

HEART

Heart is a vital organ and there are many external factors affecting it. Stress, unhealthy diet, smoking, and genetic predisposition can cause heart attacks. After myocardial infarction, death of myocardial cells leads to heart failure. Adult cardiomyocytes have limited regenerative capacity and unfortunately current therapeutic strategies cannot restore the loss of myocardium after injury. Recent studies have successfully shown that endogenous fibroblasts residing in the heart can be reprogrammed to cardiomyocyte-like cells after myocardial infarction and heart function can be improved subsequently. Therefore, *in vivo* reprogramming technology has also strong impact on the treatment of heart failure (Table 4).

Table 4. Different studies involving *in vivo* reprogramming for heart.

	Disease model	Reprogrammed cells	Reprogrammed protocol	References
In vivo reprogramming for heart	Myocardial infarction (MI)	Cardiac fibroblasts to cardiomyocyte-like cells	-Retroviral vector -Transcription factor (Gata4, Mef2c, Tbx5)	**17**
	Cardiac injury	Cardiac fibroblasts to cardiomyocyte	-Lentiviral vector -Mature miRNAs (1, 133, 208, 499)	**18**
	Cardiac injury	Cardiac fibroblasts to cardiomyocyte-like cells	-Retroviral vector -Transcription factor (Gata4, Mef2c, Tbx5, dsRed)	**19**

	Disease model	Reprogrammed cells	Reprogrammed protocol	References
In vivo reprogramming for heart	Cardiac injury	Non-cardiac myocytes to cardiac myocytes	-Lentiviral vector -Combination of miRNAs (miRNAs 1, 133, 208, 499; miR combo	**20**
	Complete heart block	Cardiomyocytes into pacemaker cells	-Adenoviral vector -Transcription factor (TBX18)	**21**

CHALLENGES FOR FUTURE

In vivo reprogramming is a young field and many problems still need to be solved. Reprogramming factors that control the transformation to the target cell type are still the major limitation. Over 200 differentiated cell types with genes encoding more than 30 000 proteins are generated from a single zygote. This is a very complex and poorly understood process. Using screening strategy, scientists search for a list of important transcription factors for the development of certain cell types. However this is a very demanding and time consuming task. To address this issue, computational algorithm and artificial intelligence (AI) could help to predict the transcription factors that promote cellular reprogramming.

Gene delivery system for reprogramming factors is another obstacle. The delivery should be safe, effective and cell specific. As seen in the tables, there are numerous methods used to deliver genes, including lentivirus, retrovirus, adenovirus and AVV. Lentivirus and retrovirus can deliver larger inserts, but these are hard to integrate into the host genome. AVV is a good gene delivery system showing safety and effectivity in preclinical and clinical treatment[22]. However, AVV has a limit of insert gene size (about 5 kb), this is not suitable in the case of more than one transgene[23]. Therefore, a modification of AVV will be a favorable direction to improve the potent gene therapy vector capable of superior gene expression levels. The way we are doing with Anc-80 now is an example[24].

Finally, safety and efficacy trials in large animals will be necessary, particularly for organs such as the heart, because exponentially more cells will be needed for regeneration in humans and large animal models than in small animals. Safety issues will involve not only those related to delivery, but also the potentially detrimental consequences of partially reprogrammed cells. By the similarity of many body parts (nerve system, circulatory system and digestive system)[25], pig will be an attractive model for testing *in vivo* reprogramming and also organ transplantation.

In summary, a promising technique for regenerative medicine has formed from advances in developmental biology and cellular reprogramming. The application of reprogramming *in vivo* provides an exciting regenerative strategy to reprogram and repurpose endogenous cells within damaged tissues, which overcomes the limitations associated with transplantation of donor cells. Although we have many challenges to improve this technology and bring

it to approval for clinical treatments, considering the rapidly changing field of biotechnology, the replacement of our organs can become reality.

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17 Gene Therapy of Monogenic Diseases

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Abstract

Gene therapy repairs or modifies the expression of individual gene in targeted cells by transfer of nucleic material to the cells. The development of the gene therapy has been impressive over the last decade. It can reduce the production of mutated proteins causing the disease, elevate the production of proteins curing the disease, or produce modified or new proteins. Here we briefly review the main alternatives of gene targeting together with the options of delivery to the cells.

Keywords

RNA interference, antisense oligonucleotides, therapy by translational read-through-inducing drugs, programmable endonucleases, viral delivery, nanoparticles

INTRODUCTION

Monogenic disorders caused by only one gene are the most susceptible for gene therapy. Particularly recessive single gene diseases could be easily treated because the disease causing mutation usually leads to protein functional insufficiency, loss of function. Here, gene replacement could very likely correct the lost function. For example, Leber's congenital amaurosis type II (LCA2), undergoes a clinical trial for replacement of RPE65 function in the eye[1]. On the other hand, autosomal dominant diseases are more challenging for gene therapy since only one copy of the gene is defective, therefore an allele specific abnormal gain of function needs to be targeted by therapy. This is the case of Huntington's disease (HD), where allele specific mutant huntingtin downregulation is needed.

Several relatively safe clinical studies of gene therapy of different diseases are ongoing but with variable outcomes. A privilege in the gene therapy have eye diseases. The eye is

easily accessible, and immune privileged due to the blood-ocular barrier. A small amount of vector is needed because of the small size of the eye. The non-invasive monitoring of phenotype rescue is easily done. And importantly many inherited retinal degenerations (IRD) are monogenic[2].

The gene therapy approaches are naturally limited to the restoration of a specific single genetic function. Therefore, preclinical animal testing is very important. Pre-clinical examinations can address many aspects of the treatment including biodistribution, transduction efficacy, longevity of the treatment as well as the immunogenicity of the mostly viral vectors.

RNA TARGETING STRATEGIES

One of the most common RNA targeting strategy to reduce unwanted gene expression is RNA interference (RNAi). RNAi silences gene expression by targeting specific mRNA. Double strand 21–28 nucleotide long RNA (dsRNA) is upon delivery to the cell cleaved by DICER (approximately 200 kDa large RNase III) producing small-interfering RNA (siRNA). siRNA binds to an effector complex called RISC (RNA-induced silencing complex), where it is separated into sense and anti-sense strand. The anti-sense strand binds to a specific mRNA transcript inducing its degradation and inhibition of translation of the specific gene[3]. Many clinical trials test RNAi approach, and some already entered phase III[4]. At PIGMOD Center in the Czech Republic we pre-clinically tested the safety, biodistribution, transduction efficiency and huntingtin (HTT) lowering effect of miHTT-AAV5 gene therapy on minipig model for HD in cooperation with the pharmaceutical company uniQure[5]. The miHTT-AAV5 vector was injected into striatum by magnetic resonance imaging (MRI) guided convection enhanced delivery (Fig. 1). Due to this testing, FDA and EMA approved this therapeutic approach for fast track clinical trial in January 2019.

Another strategy to silence gene expression uses antisense oligonucleotides (ASOs). ASO is 18–30 bases long single strand DNA (ssDNA) that is designed to target complementary specific pre-mRNA. Upon binding to ASO the mRNA is modified and subsequently degraded. The disadvantage is that the ssDNA is easily degraded by nucleases in the cell, which lowers its efficiency. The advantage is that ssDNA can be taken up by cells and wide spreads in the tissue without any enhanced delivery[6]. Therefore, ASOs were the first strategy to enter clinical trial for HD gene therapy. Anti-HTT-ASO is applied into CSF by lumbar intrathecal injection[7]. In contrast to RNAi miHTT-AAV5, which single administration should last for couple of years or the rest of the life, the ASOs need to be injected repeatedly, about every two months.

Both ASOs and RNAi gene therapies for HD have been first developed as allele unspecific treatment with a theoretical risk of the effect of healthy gene reduction. Nowadays, also

more challenging allele specific treatment is tested. Nevertheless, it targets patients' specific snips (SNP), which differ in different groups of patients, and therefore different specific drugs would be needed to cure all the patients. Also due to the SNP targeting the selection of RNA binding sequences is limited and increases the risk of side effects. Another allele specific silencing possibilities in monogenic diseases caused by CAG elongation such as in HD is to target the CAG repeat. In this case all patients could be treated with one drug but the off-target effects and a risk of down-regulating other important genes with elongated CAGs needs to be carefully addressed.

Figure 1. Pre-clinically testing of miHTT-AAV5 gene therapy on minipig model for HD, cooperation between the pharmaceutical company uniQure (Netherlands) and the PIGMOD Center (Czech Republic). A) Surgery B) MRI during application, convection enhanced delivery of miHTT-AAV5 C) MRI image of delivery of the drug with a tracer D) Minipigs shortly after the surgery. Orig. Z. Ellederova

RNA can be targeted not just to be cleaved or inhibited but also to be corrected. A treatment option for genetic diseases caused by a nonsense mutation can be therapy by translational read-through-inducing drugs (TRIDs). TRIDs suppress the action of release factors on the translation machinery, thereby, suppressing premature STOP codons and facilitate the translation of full length proteins. Ataluren (PTC124, Translarna)[8], the front-runner of TRIDs, has already been approved for variants of Cystic Fibrosis or Duchenne Muscular Dystrophy.

DNA TARGETING STRATEGIES

Artificially prepared programmable restriction endonucleases enable to manipulate DNA in a sequence specific way. They can cleave DNA at a particular sequence specific site and initiate double-stranded DNA break (DSB). DSB can be repaired by the cell either by non-homologous end-joining (NHEJ) or homology directed repair (HDR). NHEJ usually leads to insertion of a few nucleotides or deletion of a sequence leading to a knock-out (KO). An incorporation of a new transgene or correction of a gene can be achieved by HDR which requires the addition of DNA template with homologous sequence. The nucleases used for gene editing are: Zinc finger nucleases (ZFNs)[9], transcription activator-like effector nucleases (TALENs)[10], meganucleases[11], and clustered regularly interspersed palindromic repeats associated nuclease (CRISPR/Cas9)[12]. They all are able to introduce specific DSBs but under different conditions. This influence their choice for a certain situation. However, since CRISPR/Cas9 requires only simple relatively cheap molecular cloning techniques to be engineered, compared to the other programmable nucleases, it has become the most common choice. But CRISPR/Cas9 also has its disadvantages. One of them is the size of the Cas9 coding sequence (around 4kbp) which together with a larger cargo (template of interest) might have difficulty to be loaded in the most common delivery vector, adeno-associated-virus.

Even though CRISPR/Cas9 is a very powerful tool for gene therapy, the transition to bedside needs to be done with great caution. Unlike RNA targeting strategies CRISPR/Cas9 aims for a permanent nonreversible changes in the genome. Off-target issue needs to be addressed with maximum confidence[13].

DELIVERY

VIRAL DELIVERY

Viral vectors are the most common vehicles for gene therapy because they are able to transduce animal cells easily. Their natural mechanism is based on insertion of their own DNA or RNA into the host genome and enabling subsequent replication. Retroviruses, particularly lentivirus, adenoviruses, and adeno-associated viruses (AAV) are used for gene therapy. Lentiviruses can transfer a large cargo and randomly integrate into the host genome[14]. Adenoviruses also have a large packing ability, they do not integrate into the host genome but they can induce unwanted immune response[15]. Therefore, AAVs are the most popular, although their packing ability is smaller (about 4.7 kb), they do not integrate into the host genome, they provide efficient transduction, and do not initiate considerable immune response[16].

NON-VIRAL DELIVERY

Even though the viral delivery still plays the prime, the non-viral technologies improve their efficiency and gain importance[17]. Non-viral vehicles can use different force such as electroporation, injection of DNA, or ultrasound to increase the permeability of the cell membrane or employ nanoparticles (NPs) as carriers of DNA/RNA or protein[18]. These NPs form with their cargo a positively charged complex which is attracted by the negatively charged membrane enabling them to enter the cell by endocytosis. In order to be effective, they need to be released from the endosomes and enter the cell nucleus. One of the main advantages is that NPs can load a large cargo, do not initiate a significant immune response, can cross the blood brain barrier, and also NPs complexed with Cas9 allow Cas9 to be in action right after entering the cell, consequently following its degradation, and thus reducing the off-target effects. Still, their efficiency is quite low. However, peptide-modified lipid nanoparticles showed the ability to replace *Rpe65* by a single subretinal injection in a RPE65 deficient mouse in the same efficiency as AAV-2[19]. Also lipid NPs encapsulating CRISPR/Cas9 showed a high efficiency of gene editing *in vivo*[20].

CONCLUSION

Genome editing offers promising solutions to genetic disorders by editing DNA sequences or modifying gene expression. CRISPR/Cas9 technology can be used to edit single or multiple genes widely for *in vitro* and *in vivo* of cell types and organisms. Herein, the RNA and DNA targeting strategies which have been developed in order to deal with genetic diseases are explained in detail. We highlighted viral and non-viral delivery systems for future clinical trials.

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18 Age-related macular degeneration and modern aspects of therapy

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Abstract

Age-related macular degeneration (AMD) is a widespread worldwide disease in population over 60 years old. The macula and its retinal pigment epithelial cells (RPE) are influenced by AMD and patients can lose central vision. It can limit reading, driving a car, recognizing faces – living normal life. AMD consists of two types – wet and dry form. The wet form is treatable by injection of anti-VEGF. On the other hand, the dry form is still not treatable and it is subject of research. It includes RPE or stem cell transplantation, macular translocation or gene therapy. The cell transplantation includes injecting suspensions of RPE cells, transplanting RPE cell sheets or inserting membranes with cultivated RPE cells. Primary cells, embryonic stem cells or induced pluripotent stem cells can be used for RPE cell transplantation. Another surgical procedure can be macular translocation, which describes delivering healthy macular mass into an area with healthy RPE cells. But this procedure has been largely abandoned due to poor outcomes. The last described treatment is gene therapy, which can turn the cells of the inner retina into "replacement photoreceptors".

Keywords

retinal pigment epithelium, age-related macular degeneration, cell therapy, transplantation

INTRODUCTION

AGE-RELATED MACULAR DEGENERATION

Age-related macular degeneration (AMD) is a leading cause of blindness in Europe, the USA, and Australia[1]. AMD affects two-thirds of the population over 60 years old[2,3]. It represents a major clinical problem with 20–25 million individuals currently affected worldwide[4]. AMD influences a critical area of the retina known as the macula at the initial stage of the disease.

The retina consists of two layers: the inner neurosensory layer and the outer retinal pigment epithelial (RPE) cell layer. Between RPE monolayer and the vascular choroid layer is Bruch's membrane. RPE layer is an essential supporting tissue involved in retinol cycling, nutrient transport, growth factor production and phagocytosis of the fragile photoreceptor outer segments[5].

AMD encompasses two types of disease: dry and wet forms. With both forms, patients may lose central vision, which limits their ability to read, drive a car, and recognize faces. The beginning of the disease is often asymptomatic, but it could be detectable.

THE DRY FORM OF AMD

Dry-type of AMD is described with the presence of drusen. Drusens are yellowish deposits, which are under retina[6]. These days, dry form is still untreatable. Nevertheless, the progression of dry form could be reduced by antioxidants[7,8].

THE WET FORM OF AMD

The second type is the wet form. Wet AMD affects only 10–15 % of patients[9]. It consists of aberrant choroidal neovascularisation[10], where the RPE is broken with new vessels growing from choroidea. It contains leaking fluid, lipids, and blood. This type of AMD form can be treated by anti-VEGF intravitreal injections, which are based on the suppression of vascular endothelial growth factor (vEGF). Injections are applied to the vitreous cavity via sclera[11]. Monthly intravitreal injections of such drugs as ranibizumab (Lucentis, Genentech/Novartis) or aflibercept[12] (Eylea, Bayer) can prevent vision loss in almost 95 % of patients and can improve vision at 40 %. This was shown by landmarks clinical trials in 2006[13].

RISK FACTORS

More than 10 % of people older than 80 years have age-related macular degeneration. Female sex is shown as a risk factor as well[14]. Also darker iris pigmentation, previous cataract surgery, and hyperopic refraction rank are among the risk factors[15,16,17]. Cigarette smoking, obesity, sunlight exposure, and cardiovascular disease are included among other risk factors[18,19,20].

POSSIBILITIES OF TREATMENT

As written above, wet form is treatable by injections with anti-VEGF drugs. On the other hand, there is no cure for the dry form of the disease. For the future, the best option to save the vision can be RPE cell transplantation. RPE replacement is only possible if the rods and cones are healthy. For transplantation purposes, primary RPE cells, RPE cells derived from embryonic stem cells or induced pluripotent stem cells can be used. Another surgical treatment could be macular translocation or gene therapy.

CELL TRANSPLANTATION

Cell implant can be delivered into an eye in many ways. Three most published procedures of transplantation are injecting cell suspensions, inserting cell sheets and cell cultivation on membranes. Cell suspensions are cultured as a monolayer and they are dissociated to single-cell suspension before transplantation. The suspension is injected under the neuroretina[21]. This procedure seems to be not so effective because RPE cells are polarized which makes the incorporation under the retina difficult. The second option is the implantation of a cell sheet. M'Barek et al. (2017) used the human amniotic membrane for RPE cells[22] as a biocompatible substrate and manufactured injectors for delivering sheet to the right place. The last procedure is culturing cells on membranes and subsequent implantation using an injector under the retina. Membranes can be produced from non-degradable material such as polyester (Corning Inc., USA). More physiological for eyes is employing degradable and biocompatible materials such as polylactide-based ones[23]. These membranes are transferred to the subretinal space using the manufactured injector. Polylactide membranes can combine the advantages of high porosity, large pore size, and low thickness in comparison with polyester ones.

PRIMARY CELLS

For the first time, human retinal pigment epithelium was isolated and characterized 40 years ago[24]. RPE replacement with primary RPE cells would prevent photoreceptor degeneration and may stabilize visual function[25]. Li and Turner (1988)[26] showed that the injection of healthy RPE cells into the subretinal space preserves the outer nuclear, outer plexiform, and photoreceptor layers. Afterward, there was a successful RPE transplantation in RPE65 knockout mice. These mice were not able to isomerize all-trans-retinal to 11-cis-retinal[27].

EMBRYONIC STEM CELLS

Embryonic stem cells (ESCs) represent a potential source of therapeutic cells for tissue loss or cell dysfunction. Human ESCs are isolated from the inner cell mass of the 5-day old blastocyst. These cells are pluripotent[28]. They have great therapeutic potential, because of plasticity and unlimited capacity for self-renewal. Unfortunately, there is a risk of the ability to form teratomas and other tumors, sometimes immune response development, and the risk of differentiating into undesirable cell types[29]. Nevertheless, in most cases, ESCs tolerate foreign antigens or non-histocompatible cells without developing an immune response. For this reason they are a good candidate for transplantation[30]. Lund et al. (2006)[31] published that subretinal transplantation of human ESC-derived retinal pigment epithelium may rescue photoreceptors and prevent visual loss in preclinical models of macular degeneration.

INDUCED PLURIPOTENT STEM CELLS

In contrast to ESCs, deriving the induced pluripotent cells (iPSC) may be done from adult somatic cells. First experiments, 50 years ago, with reprogramming vertebrate somatic cells

by Gurdon[32], opened the way for further investigation. In Japan 2006, experiments culminated in the discovery of the essential transcription factors that were necessary and sufficient to convert a somatic cell to a pluripotent embryonic stem cell in mice[33]. Shortly afterward, there were a lot of studies of reprogramming somatic cells into iPSC. Namely, Takahashi et al. (2007)[34] used a combination of four genes OCT4, SOX2, KLF4 and cMYC to reprogram human fibroblasts to a pluripotent state of cells. Another laboratory used another four genes – OCT4, SOX2, NANOG, and LIN28[35]. It can be a unique, powerful and patient-specific tool for developing cell-based treatments for retinal degenerative diseases[36], in our case for AMD. The ability for gene repairing in human iPSC rises their potential to be used in autologous cell transplantation. The best option is to generate pluripotent lines from an individual patient, repair any genetic defects outside the body and transplant differentiated cells to the same patient[36].

MACULAR TRANSLOCATION

It is a procedure, where the neurosensory retina is detached and reattached from an area of damaged RPE to an area of healthy RPE[37]. Because it is a surgical procedure it ranks among the transplantations. This translocation can improve long-term visual stability and quality of life[38]. All patients after macular translocation had to undergo other surgical revision, as reposition extraocular muscles. Because of these complications, this type of transplantation has been abandoned by clinicians[39].

GENE THERAPY

Even if some photoreceptors are lost, it was shown that inner retinal neurons can survive for an extended time period. These neurons are still able to send visual information to the brain. Nowadays, we can use microchips to restore some degree of vision. However, the patient recognizes only objects with high contrast[40]. Kleinlogel (2011) described optogenetic therapy, which can turn the cells of the inner retina into "replacement photoreceptors" by the introduction of a gene encoding a light-sensitive membrane protein. This therapy recovered light sensitivity in mice. Optogenetic therapy to restore the vision holds therapeutic potential. Recovery of light sensitivity of surviving cells is more physiological than retinal transplants[41].

CONCLUSION

We can conclude, that there are a few promising possibilities on how to reduce the progression of AMD. The wet form of AMD can be treated by injection of anti-VEGF to prevent the proliferation of new vessels into the retina. The procedures described above bring us an overview of suitable cell transplantation treatment for the dry form of AMD. This is the best choice on how to save a vision.

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19 The role of metalloproteinases in eye wounding and healing

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Abstract

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that play a significant role in extracellular matrix (ECM) remodelling, signal transduction, proliferation, migration, and contraction. MMPs are involved in tissue inflammation and leukocyte infiltration, and engaged in embryogenesis, morphogenesis, angiogenesis, and wound repair. Additionally, MMPs take part in the various processes during pathological conditions, including osteoarthritis, cancer, and neovascularization. All structures of the eye consist of a large amount of ECM that provides normal functioning of the ocular system. Therefore, even a little misbalance of MMPs and the tissue inhibitors of metalloproteases (TIMPs) in the eye tissue can lead to the disorders. Consequently, investigation of MMPs with TIMPs and elucidating molecular mechanisms is significant to further development of new therapies for eye disease treatment.

Keywords

metalloproteinases, cornea, retina, iris, lens, eye diseases

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that take part in remodelling of the extracellular matrix (ECM) by cleavage various proteins[1]. MMPs have a conserved structural topology, the catalytic domain that contains a consensus motif with three histidines and provides a zinc-binding site, and a conserved Met-turn motif that resides beneath the active site zinc ion[2]. MMPs have a propeptide sequence, a hinge region or linker peptide, and a hemopexin domain[1,3,4]. The classification of MMPs is based on their domain combination and substrates. There are gelatinases, collagenases, matrilysins, stromelysins, membrane-type MMPs (MT-MMPs) and other MMPs[1].

Cells secrete MMPs in an inactive form with propeptide, which has to be cleaved for MMP activation by different proteinases, for instance, plasmin and other MMPs[1,5]. MMPs cleave proteins such as elastin and collagen causing ECM remodelling. MMPs take part in cell signalling, proliferation, migration, and contraction. MMPs also play a key role in tissue inflammation and leukocyte infiltration. Additionally, MMPs are engaged in the process of tissue remodelling during embryogenesis, morphogenesis, angiogenesis, and wound repair[1]. MMPs also play a role in pathological conditions such as osteoarthritis, cancer, infarction, fibrotic disorders, corneal and retinal neovascularization, etc[1,3]. Increasing of specific MMPs is noticed during lower extremity venous disorders, venous dilation, arterial remodelling, and aneurysm formation[1].

MMPs are regulated at the level of mRNA expression and by activation of their pro-MMP form. Several endogenous inhibitors tightly control MMP activity. Endogenous tissue inhibitors of metalloproteinases (TIMPs) take part in the MMP inhibition and even activation in some cases. The MMP/TIMP ratio can be used for assessment ECM protein degradation level and tissue remodelling[1,4].

MATRIX METALLOPROTEINASES IN THE CORNEA

The cornea is a transparent curved structure in the anterior part of the eye that covers the pupil, iris, and the anterior chamber. The cornea consists of five layers, an overlying epithelium with a below fibrous structure called Bowman's layer. The medium layer of the tissue is a collagen-rich formation called the stroma that occupies approximately 90 % of the cornea. Beneath this lies Descemet's membrane with the following single layer of endothelial cells. The cornea takes part in the initial focusing of the light image into the eye, and defenses the following ocular structures from the harmful influence of the environment, for instance, UV-radiation[6,7].

Infections, physical objects or chemical agents can permanently damage the cornea with further opacification and worsening of visual acuity. Therefore, the fast healing of the cornea is very important to prevent inflammation, which can lead to loss of vision[8].

Inflammation is a key process in corneal wound healing as well as in skin wound healing[8]. There is a stable response to MMP expression during healing. Firstly, local stromal fibroblasts secrete collagenases for remodelling the wound area. Secondly, it was noticed the subsequent activity of gelatinase B in the healing of epithelial wounds[3].

The corneal stroma is a stable tissue, undergoing hardly ever remodelling during its lifetime. However, corneal damaging causes the stromal cell activation and general protein synthesis upregulation, cytoskeletal stress fibers formation, proliferation, and new matrix molecules secretion. Additionally, increasing of the stromal cells activation markers occurs, including the alpha5 integrin subunit, actin, and MMP-1 (collagenase) and MMP-3 (stromelysin).

Accordingly, fibroblasts can reconstruct fibronectin matrix, secrete and remodel a new collagen reticulum to regain optical clarity[3].

Basement membrane remodelling and cell migration are necessary for corneal reepithelialization. Constant corneal epithelial cell erosion and incorrect surface remodelling can lead to the formation of stromal ulcers. Sivak et al.[3] have shown that MMP-9 (gelatinase B) is the primary MMP synthesized and secreted by basal corneal epithelial cells migrating to resurface a wound[9,10]. The MMP-9 expression correlates with the timing of basement membrane degradation[11]. The expression of other MMPs is different. MMP-2 (gelatinase A), stromelysin, and collagenase are located in the stroma and increase gradually over a long period of matrix remodelling[9]. Fini et al.[12] have shown that overexpression of gelatinase B causes the failure of reepithelialization and chronic corneal ulcerations commonly observed after thermal injury. Inhibition of MMP activity in this model leads to recovered basement membrane integrity[12].

Investigation of MMP-9 in corneal epithelial cell culture on the promoter of **MMP-9** revealed the existence of activator protein 1 (AP-1), activator protein 2 (AP-2) and nuclear factor kappa B (NF-kB) – key transcriptional response elements, which confer responsiveness to repair and stress stimuli, such as phorbol esters, cytokines and growth factors[13,14]. Additionally, in comparison with collagenase, the interleukin 1alpha (IL-1alpha) autocrine loop does not affect the MMP-9 expression[15]. Overall, AP-1 is necessary for basal MMP-9, however, only with other transcription factors or their combinations, the expression of MMP-9 occurs properly[16].

Besides that, Sivak et al.[17] noticed the increase of Pax-6 protein during corneal epithelial wounding that correlates with MMP-9 induction. They also have shown the direct interaction of gelatinase B promoter elements in corneal epithelial cells by Pax-6[17].

In normal conditions, the cornea does not contain any blood vessels. Stromal capillaries stop at the limbus – the boundary between the sclera and cornea. However, injuries subsequently cause angiogenesis in the cornea. This phenomenon can radically reduce visual clarity[18]. MMP-2, MMP-9, and MMP-14 (MT-1 MMP) can facilitate neovascularization in the cornea[3]. Additionally, fibroblast growth factor 2 (FGF-2) induces the expression of MMP-9 that also correlates with AP-1 transcription factor binding and angiogenic activity[19].

MATRIX METALLOPROTEINASES IN THE RETINA

The vertebrate retina is a multilayered structure with a large diversity of component cells that form morphologically and functionally distinct circuits that work in parallel, and in combination, to produce a complex visual output[20]. The retina transmits light signals into chemical signals that are sent to the brain. This process requires the ability to sense the stimulus of light and transmit that signal from cell to cell[21].

Six major cell types form the various layers within the human retina: the outer nuclear layer consists of photoreceptors (rods and cones), the inner nuclear layer is formed by bipolar

cells, Amacrine cells, retinal ganglion cells make up form the ganglion cell layer (innermost layer, furthest from the photoreceptors), horizontal cells and Muller cells. Axons tract towards the back of the eye and form the optic nerve[21].

Matrix metalloproteinases (MMPs) and related metalloproteinases with a disintegrin domain (ADAMs) have become interesting probes and targets in eye diseases, including diabetic retinopathy[22]. Retinopathy is a hallmark of diabetes and a common cause of blindness. It comes as two entities: vasculopathy and neuropathy. Proliferative diabetic retinopathy (PDR) evolves in the long term as a microangiopathic complication of diabetes. The principal feature of PDR is retinal angiogenesis[22].

High blood glucose levels cause a chemical reaction of glucose and amino groups of plenty of proteins, for example, with basement membrane molecules. The molecular products of this reaction are named advanced glycation end products (AGE)[23]. Because of their accumulation, the basement membranes thicken and the small vessels become weaker, which results in decreasing oxygen and nutrients exchanging. Additionally, AGE induces changes in the vessel walls and makes them more fragile. Therefore, small breaks occur in the vessel walls that lead to intraretinal hemorrhages. The local hypoxia and gradual ischemia stimulate the local production of angiogenic factors, including vascular endothelial growth factor (VEGF). VEGF forms a major stimulus for the formation of new blood vessels at the optic disc or the periphery. The new vessels are thick and fragile; hence, they are inclined to bleeding[5]. The bleeding and the macula obscuring lead to the sudden significant worsening of vision[22].

Specific chemokines, VEGF and MMPs determine the angiogenesis in diabetes. The significant high level of MMP-1, MMP-7, MMP-9, and VEGF were observed in proliferative diabetic retinopathy patients in comparison with non-diabetic control ones. Meanwhile, MMP-2 and MMP-3 levels were similar in both groups[24].

Remarkably that among the collagenases (MMP-1, MMP-8, and MMP-13), only MMP-1 (interstitial collagenase) was increased in PDR. MMP-1 cleaves the tissue collagens into gelatins[25,26] with subsequent activation of gelatinases and liquefying of the extracellular matrix. There are two mammalian gelatinases: MMP-2 (gelatinase A) and MMP-9 (gelatinase B). Only increasing of MMP-9 was confirmed in diabetic vitreous samples. Both MMP-1 and MMP-9 are angiogenic molecules[27,28], MMP-7 also has angiogenic properties[29].

There is a positive response regulation between VEGF and MMP-9. VEGF treatment induces MMP-9, but not MMP-2. Conversely, exogenous MMP-9 increases the activity of VEGF, whereas MMP-2 decreases the VEGF secretion[30]. Interestingly, the activation of MMP-9 happens locally in the eye, not by MMPs or autocatalytically, but instead by serine proteases such as plasmin, which is produced after blood coagulation *in situ*. The activated MMP-9 takes part in the vitreous hemorrhagic transformation of PDR[5].

MMP-14 (MT1-MMP) is the transmembrane type MMP involved in eye development and disease. El-Arsar et al.[31] observed MMP-14 in human epiretinal membranes of PDR patients in vascular endothelial cells, leukocytes, and myofibroblasts. Increasing MMP-14 is noticed in

the vitreous of PDR in comparison with non-diabetic control patients and increased levels of MMP-14 protein and mRNA were found in the retinas of diabetic rats versus controls. Therefore, MMP-14 may be a biomarker of angiogenic activity in PDR[31].

MATRIX METALLOPROTEINASES IN THE IRIS

Iris consists of two layers. The first layer is stromal and made of pigmented, fibrovascular tissue, the second one made of pigmented epithelial cells under the stroma. The pigmented layer of iris limits the light transmission and provides its passaging through the pupil to the retina[32].

Lan et al.[33] showed the localization of MMP-1, MMP-2, MMP-3, and MMP-9 in the anterior part of the iris. The high level of immunoreactivity was in the anterior epithelium and stromal cells. Meanwhile, immunoreactivity in the posterior epithelium was weak. Additionally, the staining intensity of TIMP-1, TIMP-2, TIMP-3, and TIMP-4 correlated with MMPs distribution. Therefore, according to the stoichiometric inhibition, one molecule of TIMP inhibits one molecule of MMP. Overall, the MMPs/TIMPs ratio is balanced in the iris that is important for the homeostasis because of the extracellular matrix synthesis and degradation, cell migration, proliferation, and apoptosis in the normal iris functioning[33].

MATRIX METALLOPROTEINASES IN THE LENS

The lens consists of an outer capsule, a middle layer called the cortex, and an inner layer called the nucleus. The capsule is the basement membrane of the lens epithelium, which lies beneath. Lens separates the aqueous and vitreous chambers[32].

There was evidence about MMP and TIMP participation in the lens differentiating and cataract formation. There is a report about a low level of MMP-1, MMP-2, MMP-3, MMP-9 and TIMP-1, TIMP-2, TIMP-3 in healthy human donor lenses[34]. Additionally, MT-MMPs play a significant role in the lens of adult people. It was noticed the high expression of MT1-, 2-, 3-, and 5-MMP in the anterior part of the lens. MT4-MMP and MT6-MMP were observed in all three compartments of the lens, however, on the low level. Metalloproteinases take part in the maintaining of lens homeostasis providing stability and integrity. MT-MMPs play a major role in the activation of pro-MMPs[35,36].

On the other hand, MMPs also can be a reason for pathological conditions. Crystallin is the protein of the lens cells that provides transparency of the lens. Crystallin proteolysis and precipitation of its fragments are general key mechanisms of cataract[37]. Descamps et al.[37] showed that even weak activity of MMP-9 can lead to the lens penetration and crystallin cleavage[37].

CONCLUSION

All structures of the eye contain a vast amount of extracellular matrix (ECM), whose natural structure provides the normal functioning of the entire optical system. MMPs are zinc-dependent endopeptidases that play a significant role in ECM remodelling, inflammation, wound healing, etc. Hence, even a little misbalance of MMPs and their endogenous inhibitors TIMPs in the eye tissue can lead to pathological conditions such as cataract, corneal and retinal neovascularization. Therefore, investigation of MMPs with TIMPs and elucidating molecular mechanisms is important for further development of new therapies for eye injuries or diseases.

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20 Pig as a model for spinal cord injury study

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Abstract

Spinal cord injury (SCI) in humans as well as animals represents an incurable CNS disorder until now. There are many of ischemic as well as traumatic animal models of spinal cord injury which serve as an important tool for therapeutic strategies testing. Many recent studies have shown that the pigs but mainly minipigs look very promising as a large animal model for SCI pathology process study and healing interventions. Due to the economical and ethical issues as well as anatomical proportions the pigs but mainly minipigs will be constantly exploited in neurosciences.

Keywords

spinal cord injury, pigs, rodents, animal models of spinal cord injury

SPINAL CORD INJURY IN MEN

Spinal cord injury (SCI) is a devastating neurological condition associated with significant morbidity and chronic disturbances in motor, sensory, and autonomic function. The prevalence of SCI has been increasing globally over the past decades and ranges of 236–1298 affected individuals per million with an estimated 768,737 new cases annually worldwide[1–3]. Males in young adulthood (20–29 years) and older age (70+) are most at risk with male-to-female ratios of at least 2:1 among adults, sometimes much higher. Spinal cord injuries are most often traumatic, caused by lateral bending, dislocation, rotation, axial loading, and hyperflexion or hyperextension of the cord or *cauda equina*. Motor vehicle accidents (~38 % of new spinal cord injuries each year) are the most common cause of SCIs, while other causes include falls (most often after age of 65, ~30.5 %), work-related accidents, sports injuries like football, gymnastics, diving into shallow water etc. (~9 %), and penetrating trauma such

as stab or gunshot wounds (~13.5 %). SCIs can also be of a non-traumatic origin (~9 %), as in the case of cancer, infection, intervertebral disc disease, vertebral injury, and spinal cord vascular disease[4–6]. While a cure that could repair the injured spinal cord is unforeseeable, recent advances in biological and engineering strategies, biological biomarkers monitoring (microRNAs in serum samples) as well as spine surgical approaches have opened promising avenues for improving function after SCI[7–13].

PIG AS A BIOMEDICAL MODEL

The pig represents one of the large animal models currently used in human disease-related translational research. The pig body size, organ(s) physiology and anatomical dimensions are similar to humans, thus providing a readily accessible large animal system for pre-clinical translational modeling. Moreover, in comparison to non-human primates, the use of pigs offers several specific advantages including: 1) short gestation interval (120 days), 2) generation of multiple piglets from a single sow (up to 6–12 piglets per litter), and 3) cost-effectiveness. Pigs have already become important in modeling a number of human diseases, such as diabetes, cardiovascular disease (myocardial ischemia) and multiple neurodegenerative disorders such as stroke, spinal ischemic and traumatic injury[14].

Moreover, minipigs had been successfully used as a model for preclinical testing of new biomaterials, growth factors and preparation of stem cell derived cells for the treatment of diseases affecting tissues derived from mesoderm: cartilage, bone, ligament and meniscus[15–19]. Similarly, pigs and cells isolated from them play an important role in eye biomedical research. Recent studies have shown that retinal epithelium cells transplantation in pigs represent an animal model most closely resembling retinal epithelium cells grafting in humans, and offers possibilities for testing various treatments[20–22] and there was also developed a pre-clinical model of corneal graft rejection in the semi-inbred minipig as a model of human rejection[23].

The first transgenic pigs were generated for agricultural purposes about three decades ago, but today, the major application of genetically engineered pigs is found in the field of biomedical disease modeling. Progress in reproductive techniques and gene transfer methods nowadays allow targeted modifications of the porcine genome, although the overall success rates are still low. A bottleneck for porcine transgenesis is the lack of authentic embryonic stem (ES) cells, which are suitable for blastocyst complementation experiments. Recently, the first attempts to generate porcine induced pluripotent stem (iPS) cells have been published; however, the potential of current porcine iPS cells to contribute to chimera formation in blastocyst complementation, seems to be limited. Despite these bottlenecks, the recent employment of active transgenesis techniques based on ectopic enzymes, such as transposases, recombinases, and programmable nucleases, allows the generation of pig models at

unprecedented pace[24]. Gene editing by CRISPR/Cas has revolutionized many aspects of biotechnology within a short period of time and guidelines for CRISPRing primary cells in pig and cattle, with a specific focus on testing gRNA *in vitro*, on generating single cell clones, and on identifying modifications in single cell clones were presented recently[25]. The breeding of minipigs as a biomedical model has a long tradition at the Institute of Animal Physiology and Genetics in Libechov. The first miniature pigs were imported in 1967 from the Hormel Institute, University of Minnesota (two boars and three sows) and from the Institute for Animal Breeding and Genetics, University of Gottingen, Germany (two boars and four sows). These animals were crossbred for porcine blood group studies with several other breeds or strains: Landrace, Large White, Cornwall, Vietnamese pigs and miniature pigs of the Gottingen origin. During the next 50 years of breeding, animal health and body shape were thoroughly controlled and outbreeding conditions were maintained by imports of several additional boars from Göttingen. The continuous selection made it possible to increase the average litter size (now about 8–10 piglets) and to fix the white color. The animals reach sexual maturity at about 4 months of age, when they weigh about 12–15 kg[26]. Different cross-breeding produced more than 2000 descendants without any signs of melanoma. Nevertheless, a few black piglets with melanoma had occurred in this genetically heterogeneous population by 1989. They originated from mating two male brothers with four related sows. These parents had no visible skin tumors. The MeLiM strain was established using selective breeding for melanoma and is used for melanoma research until now[27–31]. In 2009 we also successfully created the first transgenic minipig carrying the first 548 amino acids of human huntingtin with 124 glutamines under the control of human huntingtin promoter by lentiviral transgenesis, at the Institute of Animal Physiology and Genetics in Libechov[32]. At present time we possess four generation of these transgenes which represent a suitable large animal model for evaluating potential Huntington disease therapeutics, preclinical markers or pathology[33–37].

RODENT MODELS OF SPINAL CORD INJURIES

Spinal cord injuries (SCI) in rodents have been created by laceration, contusion, compression, or intramedullar injection of toxic agents[38]. Adult rodents represent the most frequent animal models for spinal cord injury studies accompanied by many advantages as well as disadvantages. Rats were selected because these animals are easy to handle and the functional, biochemical, and morphological changes of SCI were thought to be similar to those of humans[39–41]. Using rat models of spinal contusion, weight-drop, or epidural balloon-compression induced injury, a correlation between the degree of neurological deficit (as measured by the Basso-Beattie-Bresnahan [BBB] or Combined Behavioral Score [CBS]) and the extent of local tissue degeneration at the injury epicenter has been reported in numerous studies and independently validated in several laboratories[39,42–44]. However, interestingly,

even after severe spinal cord injury characterized by 85–95 % of axonal loss or overall tissue degeneration, progressive recovery is seen over a 3- to 6-week period after injury, with the average BBB score ranging between 6 and 11 in both T10 contusion and weight-drop models in rats[45–47]. In addition, the fact that the rodent's response to different experimental models of SCI is strain-dependent was recently observed. In three substrains of Sprague-Dawley rats purchased from three different European breeders, Harlan rats regained significantly more hind limb function than Charles River and Scanbur rats using mild contusion spinal cord injury. Authors also observed substrain differences in the recovery of the ability to empty the bladder and development of hypersensitivity to mechanical stimulation[48]. The mechanism of this spontaneous recovery is not clear but progressive remyelination and axonal sprouting from the remaining brainstem-derived descending motor axons below the injury may in part account for this effect[49,50]. More importantly, such a high degree of spontaneous recovery represents a significant challenge in detecting relevant clinical benefit from the various therapeutic strategies examined. Preclinical and clinical data demonstrated that so far none of the therapies with promising outcomes in mouse or rat models was effective in human patients[4,51,52]. Such a differential responsivity between rodents and humans to a given treatment may reflect differences in the pathophysiology of spinal cord injury, and/or our inadequate understanding of the interventional limitations when directly translated from rodents to man[52–54]. Moreover, differences between the injury response of humans and rodent models could be because the cerebrospinal fluid (CSF) layer of the human spine is relatively large, while that of rodents is extremely thin[55]. Furthermore, re-growing of axons seems to be critical because one millimeter of growth may be significant in rodents but insufficient in larger animals or humans[56]. A recent survey of 324 members of the SCI community reported a strong support for demonstrating first the efficacy of various therapies in large-animal models (in addition to rodent models), as well as independent replication of promising results before moving forward to human clinical trials[57,58].

PIG AS A LARGE MODEL FOR SPINAL CORD INJURIES STUDIES

WHY MINIPIGS

While many promising advancements have been made in recent years, such as interventions promoting axonal regeneration and sprouting, effective therapies for SCI have not yet been attained. One of the biggest challenges in developing successful therapies for SCI is modeling the human spine and spine pathology in a relevant and clinically translatable manner. High translatability requires a preclinical research platform that accurately models the anatomy of human spine and spinal cord, and the complexities of human neurophysiology and pathophysiology induced by injury. Relevant SCI animal models can enhance understanding of complex mechanisms involved in the pathophysiologic response, enable the identification of

relevant therapeutic targets, and validate therapies and devices for use in the clinical setting. To start human clinical trials based solely on studies in models that have notable limitations (rodents) increases the risk for failure in a number of respects[14,59]. The genetic, anatomical, vascular, immunological, physiological, and pathophysiological proximity of the (mini)pigs (non-human primates too) to humans, and the similar spinal cord anisotropy, surface to volume ratios, and nerve tract organization, make (mini)pigs an ideal model for preclinical studies of SCI. Miniature pigs can also maintain adult human weight (68–91 kg), while conventional pig breeds grow at a rate that renders them impractical for use in studies longer than a month duration and for studies using clinical imaging modalities such as MRI, CT, and PET[60,61]. Importantly, corticospinal tract in pigs demonstrates anatomical similarity to humans, suggesting that the porcine model has importance as a translational intermediary pre-clinical model [62]. Recently, several acute or chronic spinal trauma injury models have been developed in pigs or piglets. In these models, whole or segmental spinal cord injury were induced after local irradiation, diving decompression illness, full or partial transection, minimally invasive approach by epidural balloon-compression or surgical exposure of trauma-targeted spinal segment(s) using weight drop, computer-controlled contusion/compression devices, calibrated vascular clips, thermal damage or separation/root stump pull/torsion[14,39,68–76,40,56,59,63–67].

CONTUSION, COMPRESSION, TRANSECTION, THERMALLY-INDUCED, DECOMPRESSION SICKNESS, ALLERGIC MODELS

One of the first minipig spinal cord injury models was documented in 1985. The authors used a dropping 25 g weight from a height of 20 cm upon the exposed spinal cord for studying phospholipids, fatty acids and cholesterol changes closely after SCI. By this minipig model they revealed that edema following spinal cord trauma is much more extensive than previously assumed without any effect of trauma on the phospholipids composition of whole spinal cord and myelin. The data also suggested that peroxidation of myelin or other membrane lipids is not a significant factor in the pathologic changes occurring 3 hours after experimental spinal cord injury[77]. The new rediscovering of minipig for SCI modulation or spine surgical studies had been appearing after 2000 again. Interestingly and similarly to study of Segler-Stahl et al. from 1985, farm piglets (18–22kg) were used for measurement of excitatory amino-acid release, nitric oxide generation, PGE2 synthesis, and myeloperoxidase content after weight drop model of SCI. The steel impactor measuring 1 cm in diameter and 3 cm in length and weighed 25 grams was dropped from a height of 45 cm onto the spinal cord at thoracic level (T13) and 30 minutes after SCI the authors evaluated the effects of systemic or intrathecal administration of methylprednisolone. Contrary to earlier animal studies intravenous or intrathecal methylprednisolone had no effect on any of the measured parameters[78].

In 2009, Skinner et al.[79] developed several porcine models of SCI to better define possible relationships between mechanical spinal cord stimulation (injury) and spinal cord motor

conduction block. The authors created spinal cord sectioning of transaxial compression at thoracic level while recording muscle-derived electrically stimulated transcranial motor evoked potentials (TcMEPs) and electromyography (EMG) readings from the same electrode derivations. Results of this study confirmed that intraoperative neuromonitoring of high-risk spinal surgeries at the spinal cord level may benefit from the addition of EMG recording to tests of spinal cord motor conduction such as TcMEP. The scientists also used adult pigs for the study of neurologic and functional changes that occur in the central nervous system after high-velocity behind armor blunt trauma (BABT) of the spine describing a nonpenetrating injury to the organs of individual wearing body armor. The results from this animal model indicate that high-velocity BABT of the spine generates high pressure and acceleration in the spine, induces varying degrees of paralysis of hind limbs, and disturbs cerebral function. The authors suggested that neuronal degeneration caused by the pressure wave may be one of the important pathologic events involved in the development of trauma-related complications[80].

Similarly, deep tissue injury (DTI) in combination with SCI (hemisection at L2 level) have been studied in Yucatan minipigs, an animal model that resembles humans. DTI represents a severe medical complication that commonly affects patients with spinal cord injury and the studies demonstrated that intermittent electrical stimulation IES may be an effective technique for preventing the formation of DTI in loaded muscles after spinal cord injury[81]. Interestingly, the white matter segments of spinal cords isolated from Yorkshire pigs (6 months old, ~80 lb.) were also used for characterization of unconfined compression behavior and determination of constitutive model which best captured the stress–strain behavior. This study showed spinal cord white matter to be less stiff than previously estimated by inverse finite element methods, which will have a significant effect on finite element model predictions of the magnitude and distribution of stresses and strains in the spinal cord[82].

Our scientific team is also focused on spinal cord injury therapy and we needed the standard and well quantifiable model of SCI in miniature pigs closely resembling the most situations after SCI in men, which could be used for next stem cell therapy or other therapeutic approaches. Thus, dorso-ventral compression of the Th12 segment was induced using a computer-controlled spinal compression apparatus consisting of a stepping motor, and a vertically-oriented digital force gauge (0–5 kg range) bridged to an aluminum rod 5 mm in diameter. Important is that compression device-controlling software FORCE permits pre-programming of variable compression parameters, including maximum compression pressure, velocity of compression, and duration of compression. The compression curves of each animal were also recorded to assure consistency of compression parameters across animals. The dura mater was left intact. Compression pressure peak force was set at 1.5 kg, 2 kg or 2.5 kg with 6–8 animals for each group. Following 2.5kg spinal cord compression the animals demonstrated a near complete loss of motor and sensory function with no recovery over the next 4–9 months. Those that underwent spinal cord compression with 2 kg force

developed an incomplete injury with progressive partial neurological recovery characterized by a restricted ability to stand and walk. Animals injured with a spinal compression force of 1.5 kg showed near normal ambulation 10 days after injury. In fully paralyzed animals (2.5 kg), MRI analysis demonstrated a loss of spinal white matter integrity and extensive septal cavitations. A significant correlation between the magnitude of loss of small and medium-sized myelinated axons in the ventral funiculus and neurological deficits was identified. These data demonstrating stable neurological deficits in severely injured animals, similarities of spinal pathology to humans, and relatively good post-injury tolerance of our strain of minipigs to spinal trauma, suggest that this model can successfully be used to study therapeutic interventions targeting both acute and chronic stages of SCI[39].

In 2013, a novel thoracic SCI model was established by weight drop device at T10/11 level in Yucatan minipigs. Varying degrees of injury severity were induced by altering the height of the weight drop (5, 10, 20, 30, 40, and 50 cm). Behavioral recovery over 12 weeks was measured using a newly developed Porcine Thoracic Injury Behavior Scale (PTIBS). This scale distinguished locomotor recovery among animals of different injury severities, with strong intra-observer and inter-observer reliability. Similar to our minipig model of SCI, this model of SCI may also represent a useful intermediary in the testing of novel pharmacological treatments and cell transplantation strategies[67].

One potentially important difference between the rodent and the human spinal cord is the presence of a significant CSF volume within the intrathecal space around the human cord. While the CSF may "cushion" the spinal cord, pressure waves within the CSF at the time of injury may contribute to the extent and severity of the primary injury. For that reason, the same scientific team from University of British Columbia had characterized this novel minipig SCI model in detail. One of the objectives was to establish the feasibility of measuring spinal CSF pressure during injury. For this purpose, the authors used fiber optic pressure transducers implanted in the thecal sac and they declared that the Yucatan miniature pig is an appropriate model for studying CSF, spinal cord, and dura interactions during injury[68]. By using the similar weight drop (T11) minipig SCI was determined quantitative *in vivo* ultrasound imaging of spinal cord and dura morphology and how the CSF pressure (CSFP) and CSF pulse pressure amplitude (CSFPPA) cranial and caudal to the injury site was changed after an acute SCI with subsequent thecal occlusion and decompression. The authors released the 20g weight from the height of 32 cm (moderate severity) or 125 cm (high severity) and after 8 hours of sustained compression. CSFP has been measured cranial and caudal to the injury site, using miniature pressure transducers, during compression and for 6 hours after decompression. Although extradural compression exists at the site of injury, lumbar CSFP may not accurately indicate CSFP cranial to the injury and CSFPPA was not a consistent indicator of decompression in this animal model. Importantly, decompression of an acute SCI may result in residual cord deformation followed by gradual swelling or immediate swelling leading to subarachnoid occlusion. The response is dependent on initial injury severity. These

observations may partly explain the lack of benefit of decompression in some patients and suggest a need to reduce cord swelling to optimize the clinical outcome after acute SCI[83,84]. In the next study the same scientific team from University of British Columbia had also measured the pressure impulse (mmHg.ms), apparent wave speed, and apparent attenuation factor by using the weight drop SCI model in pigs. The data indicates that the fluid pressure wave may be sufficient to affect the severity and extent of primary tissue damage close to the injury site. However, the CSF pressure was close to normal physiologic values at 100 mm from the injury. On the other side, the high injury severity animals had less tissue sparing than the moderate injury severity animals but this difference was statistically significant only within 1.6 mm of the epicenter. The authors characterized the pig's models of SCI more appropriately for basic and preclinical SCI research due to its similarities to human scale, including the existence of a human-like CSF fluid layer[55].

Similarly, understanding how the combination of contusion and compression influences the early pathophysiology of SCI has been studied in female Yorkshire pigs. Authors used T10 weight drop contusion (50 g from a height of 50 cm for 5 minutes) followed by sustained compression (added an additional weight of 100 g onto the 50 g impactor) for 4 h. Disturbances in the metabolism of energy-related substrates such as lactate, pyruvate, and glucose, which are important aspects of secondary damage, was estimated using the microdialysis technique. Following the contusion injury, both lactate and pyruvate levels near the epicenter increased, while glucose remained quite stable. On the contrary, when the contusion injury was followed by sustained compression, authors observed a transient raise in lactate, while pyruvate and glucose levels dropped rapidly, which may reflect the decreased regional spinal cord blood flow. Furthermore, contusion with sustained compression produced a prolonged and dramatic increase in the lactate-pyruvate (L/P) ratio as a marker of tissue hypoxia, whereas after contusion injury alone, a transient and less significant elevation of the L/P ratio was observed[85]. Interestingly, the pig thoracic spinal cord contusion model at T10/11 level induced with a modified weight-drop impactor (50g weight, dropped from a height of 15 cm) was also used for quantitative assessment of spinal cord perfusion using contrast-enhanced ultrasound (CEUS) managed by an intravenous bolus injection of 2.0 ml of SonoVue. Using juvenile domestic pigs (3 months old, 15–16 kg) and conventional ultrasound, the authors revealed that the spinal cord was hypoechoic and homogeneous, whereas the dura mater, pia mater, and cerebral aqueduct were hyperechoic. Intramedullary blood vessels were displayed as segmental and columnar on conventional ultrasound (US) and Color Doppler US (CDFI). They were homogeneous on CEUS. After spinal cord contusion, the injured region on gray scale of US was hyperechoic. CDFI demonstrated that intramedullary blood vessels of adjacent region (~1.5 cm rostrally and caudally from the injured site) had increased and dilated during the observation period. On CEUS the epicenter of contusion site was hypoperfused, whereas its adjacent region was hyperperfused compared to the distant region. Quantitative analysis showed that peak intensity decreased in epicenters

of contusion but increased in adjacent regions significantly at all time points (before and 0 min, 30 min, 120 min after SCI). Furthermore, CEUS technique proved to be practical because it provides overall views for evaluating microcirculatory pattern in spinal cord injury[86]. Although quasistatic and quasi-linear viscoelastic properties of the spinal cord was reported previously, there were no published studies that investigated the fully (strain-dependent) nonlinear viscoelastic properties of the spinal cord. The data published in 2013 indicated that the porcine spinal cord exhibited fully nonlinear viscoelastic properties. These parameters obtained from pig experiments should be important for future studies investigating various damage mechanisms of the spinal cord and studies developing high-resolution finite elements models of the spine[87].

Pigs with chronic compression spinal cord injury at Th12–L1 level were also successfully used for high intensity spinal cord stimulation (SCS) testing to restore an effective cough mechanism using epidural surface mounted wire leads, whereas this method may be a safe and useful technique to restore a functional cough in spinal cord injured subjects[88].

Complete SCI invariably leads to alterations of sublesional functions and literature concerned with SCI patient's expectation and quality of life highlighted a critical need for bladder and bowel functions restoration in all patients. Preclinical studies using non-injured pigs showed, for the first time, that epispinal stimulation causes significant detrusor and rectal responses and allows considering further studies with the objective of treating urinary and rectal disorders in spinal cord injury patients[89,90]. The role of ischemia in the secondary injury cascade has been well studied in animal models. The mechanisms of secondary injury after SCI have been well defined; loss of spinal cord microcirculation, loss of autoregulation, and ischemia are all important in the pathogenesis of secondary spinal cord damage. One of the basic tenets of management of acute SCI is the prevention of spinal cord ischemia by avoiding systemic hypotension and hypoxia. Rapid decompression of the compressed spinal cord with stabilization of the spine to prevent further injury and aggressive medical resuscitation with mean arterial pressure (MAP) elevation have become common practice. Although no class I evidence supports its effectiveness, MAP elevation is now recommended for routine use after cervical SCI. Other potential nonpharmacological interventions include cerebrospinal fluid drainage (CSFD) and hypothermia. The neuroprotective strategy of CSFD has been used in patients undergoing abdominal aortic aneurysm surgery, decreasing the neurological dysfunction after aortic occlusion. The effectiveness of this strategy was recently successfully demonstrated in a porcine model of mild contusion SCI whereas the combination of MAP elevation and CSFD significantly and sustainably improved spinal cord blood flow and spinal cord perfusion pressure[91,92].

Modern spine surgery techniques, including pedicle subtraction osteotomy, vertebral column resection, and other methods, involve using electrocautery (EC) in close proximity to the spinal cord and nerve roots. Effects of EC on spinal cord function, particularly as measured by routine neuromonitoring modalities, wasn't reported. Using these techinques, three

domestic pigs (36, 43 and 44 kg) were used for development of thermally generated SCI model in the next study. EC was delivered to thoracic level dural root sleeves within 6–8 mm of the spinal cord (n = 6). Temperature recordings were made near the spinal cord and similarly to previous study EMG and MEP were recorded by multiple gluteobiceps intramuscular electrodes before, during, and after EC. In all roots, a minimum of 20s EC and a temperature maximum of at least 57 ºC at the dural root sleeve were required to induce MEP loss. This porcine study may help spine surgeons and their monitoring teams to recognize and reduce EC risk to neural structures including spinal motor tracts[69]. Also, the effectiveness of the soft coagulation system for stopping bleeding from the epidural vein using different outputs and the safety in terms of tissue damage including spinal cord injury was clarified using 3 months old pigs (Landrace/Large White/Duroc). The authors used soft coagulation monopolar output (SCM), soft coagulation bipolar output (SCB), and conventional bipolar output (CB) as the coagulators, whereas the neurological assessment by somatosensory evoked potential and histological analysis to determine the area of thermal damage were evaluated. Results of the study assessing the potential risk of severe neural tissue damage including spinal cord injury strongly suggest to use a bipolar soft coagulation in spine surgeries[93].

Yorkshire males were also used as an important preclinical model of SCI connected with decompression sickness (DCS). Animals received emulsified perfluorocarbon Oxycyte intravenously at dose 3 or 5 cm^3/kg with concurrent 100% O_2 for 1h during decompression and the onset of DCS after non-linear compression to 200 fsw (feet of sea water). However, Oxycyte at 5 cm^3/kg provides both spinal cord protection and statistically significant survival benefit, Oxycyte at dose 3 cm^3/kg produced no significant detectable survival benefit but had reduced spinal cord lesion area[94].

In addition, piglets (50–60kg) of domestic pigs have been used as model of acute experimental allergic encephalomyelitis (EAE) for estimation the ability of diffusion tensor imaging (DTI) to detect and monitor acute axonal injury at cervical level C2/3. Results of this study showed that axial diffusivity (AD) may be a useful noninvasive tool to investigate the underlying pathogenic processes of multiple sclerosis and to monitor the effects of experimental treatments for axonal injury[95].

JUVENILE SCI MODELING

The pediatric spinal cord and spinal column have anatomic and biomechanical differences compared with adults that predispose children to different mechanisms of injury and result in a unique injury profile. The one of the first contusive SCI in piglets (infant piglets 3–5 weeks old) was performed by controlled cortical impactor at T7 level. By this approach the authors characterized a reproducible model of traumatic pediatric SCI in a large animal with chronic survival and utilizing multiple outcome measures, including evoked potentials, magnetic resonance imaging, functional outcome scores, and histopathology[96]. Piglets (5–9 kg) were also used as a model for simulation of cervical SCI in children. The authors

created a complete SCI by compression with controlled cortical impact (CCI) device at C3–C4 level. As they hypothesized, complete cervical SCI in piglets produced hemodynamic alterations consistent with the withdrawal of sympathetic tone similarly reported in adult human patients[97].

SPINE SURGERY MODELS

In 2001 the scientists used piglets of domestic pigs for safe pedicle screws placement at thoracic level without unexpected SCI[98]. Similarly, swine animal model was used for demonstration of the da Vinci Surgical System to perform an anterior spinal procedure as a safe and effective example of robotic surgical system in spine surgery[99]. Interestingly, pigs were used to estimate the amount of the spine that can be shortened without injury. The data of these studies showed that spinal shortening of 104.2 % of one vertebral body height at the thoracolumbar level caused spinal cord injury, but shortening of 73.8 % did not result in injury and the shortening procedure was performed in three phases[100]. The same scientific team of Department of Orthopedics from Guro Hospital also studied the opposite situation connected with surgical procedures for correction of scoliosis and kyphosis, as these procedures produce lengthening of the vertebral column. The authors used the porcine model in which they caused spinal cord injury by vertebral column distraction and evaluated the histological changes in the spinal cord in relationship to the pattern of recovery from the spinal cord injury. SCI was defined when TcMEPs signals disappeared or decreased by > 80 % compared with the baseline amplitude caused by the distraction of osteomized vertebra. Spinal cord injury was developed at a distraction distance of 20.2 ± 4.7 mm, equivalent to 3.6 % of the thoracolumbar spinal length, and the distraction distance was correlated with the thoracolumbar spinal length[101]. Using porcine model, they also found that a continuous 74.3% segmental vertebral height (SVH) distraction over an average of 10.7 min caused a delayed SCI, which was indicated by mild histologic changes in the spinal cord. Recovery patterns from SCI after distraction release were compatible with the degree of histological change; however, these patterns differed from the previously investigated prompt type of SCI[102].

ISCHEMIC MODELS

Spinal ischemia model in pigs by thoracic aorta clamping at the distal arch was demonstrated in 1987. Moreover, the authors showed that the hypothermic regional perfusion of thoracic aorta after clamping prevented spinal cord ischemia pathology[103]. In 2004, an ischemia model was described in juvenile pigs by cross-clamping of aorta at thoracic level as well as promising strategy dramatically to increase the tolerance of the spinal cord to ischemia based on mild hypothermia (32 ºC) for simulation of SCI during thoracoabdominal aortic aneurysm repair in men[104]. Spinal cord injury due to prolonged thoracoabdominal aortic occlusion was also simulated using pigs 2 years later. The ischemia was accomplished by two balloon occlusion catheters inserted through the carotid and femoral artery under

fluoroscopic guidance[105]. Using minipig ischemia SCI model created by clamping of a thoracic aorta, it was also demonstrated that left-heart bypass (LHB) may provide superior spinal protection than simple clamping and monitoring of spinal-cord ischemia using the cortical somatosensory evoked potential (CSEP) was rapid and feasible[106]. Porcine ischemia SCI model induced by aorta clamping for 60 minutes was also used to demonstrate the protective effect of retrograde venous perfusion of cryogenic liquid via accessory hemiazygos vein as well as treatment with resveratrol[107]. Similarly and recently, the minipigs were used to test a strategy for minimizing ischemic spinal cord injury after extensive thoracoabdominal aneurysm (TAAA) repair. Authors occluded a small number of segmental arteries (SAs) endovascularly 1 week before simulated aneurysm repair in Yorkshire pigs. The study showed that endovascular coiling of 2 to 4 SAs prevented paraplegia in this experimental model of extensive hybrid TAAA repair, and helped protect the spinal cord from ischemic histopathologic injury[108]. Similarly, porcine model of aortic balloon occlusion-induced spinal cord ischemia/reperfusion injury was successfully used for comparison of carbamylated EPO-FC fusion protein (cEPO-FC) with recombinant human erythropoietin (rhEPO). Both of these proteins comparably protected pigs against ischemic spinal cord dysfunction and neuronal damage[109]. Juvenile Yorkshire pigs also helped investigate the impact of sudden stent graft occlusion of thoracic intercostal arteries after open lumbar segmental artery (SA) ligation. Authors underlined the potential of the staged approach in hybrid procedures as well as highlighted the need for established adjuncts for preventing paraplegia in hybrid and pure stent-graft protocols in which sudden occlusion of multiple SAs occurs[110].

CELL, GENE AND NANO THERAPY IN (MINI)PIG MODEL

In 2008 as well as 2013, Spanish scientists used adult minipigs for estimation of autologous bone marrow stromal cells (BMSC) effectiveness in SCI. These experimental SCIs were created by the application of two surgical Heifetz's clips for 30 min at Th12–Th13 (L2–L3) vertebral level of spinal cord. Interestingly, similarly to previous rodent studies, BMSC transplantation showed progressive functional recovery in transplanted (intraparenchymally, subarachnoidally and intrathecally) minipigs at chronic stage of disease – 3 months after injury[40,111,112]. In 2010, the development of sacrocaudal spinal cord injury model in cloned Yucatan minipigs usable for cellular transplantation research was reported. The authors demonstrated that transection of the sacrocaudal spinal cord (at the junction of the last sacral and first caudal spinal cord segment) in Yucatan SCNT clones produces profound, quantifiable neurological deficits restricted to the tail. They also confirmed survival of transplanted porcine GFP-expressing neural stem cells (isolated from brain tissue of green fluorescent protein transfected SCNT clones) in the SCI lesion and their differentiation into glial and neuronal lineages for up to 4 weeks without immunosuppression[113]. Our scientific team is also interested in study of spinal cord injury and its potential in cell and gene therapy using minipig, as a large animal model. Therefore, in our first study we characterized the optimal dosing regimen and safety profile

of human spinal stem cells (HSSC) when grafted into the lumbar spinal cord (L2–L5) segments of naive immunosuppressed minipigs. Based on the data from this study the safe total number of injected cells and volume of injections was determined to be 30,000 cells delivered in ≤ 6 µl of media[114]. Spinal cord's tolerance to increasing volumes and numbers of human neural progenitor cells in minipigs was also tested by other scientific team 5 years later[115]. Similarly, we transplanted 10 bilateral injections of human embryonic stem cell line-derived neural precursors (ES-NPCs) into the L2–L5 gray matter of naive immunosuppressed Gottingen–Minnesota minipigs. The animals survived for 23–49 days after transplantation whereas using human-specific axonal neurofilament antibody (HO14), which does not cross-react with the porcine tissue, extensive axonal sprouting was detected in hNUMA-positive grafted regions. Moreover, numerous HO14+ axons projecting towards CHAT-immunoreactive host a-motoneurons were identified. These data show that ES-derived, FACS-sorted NPCs can represent an effective source of human NPCs to be used in CNS cell replacement therapies[116]. Next, we performed similar experiment with other multipotent human neural stem cells (hNSCs) derived from embryonic stem cells (hESCs) and injected them just above and just below the injury epicenter (L3 spinal segment) in chronic spinally injured adult minipigs resulting in viable and functional engraftment of hNSCs with terminals apposed with host neurons in ventral horn and in intermediate zone[117]. At this time, induced pluripotent stem cells (iPSCs) represent promising and ethically acceptable cell source for cell therapy of SCI and our scientific team was also able to demonstrate that the transplantation of syngeneic porcine iPSC-derived neural precursor cell (NPC) into the spinal cord in the absence of immunosuppression is associated with long-term survival and neuronal and glial differentiation. No tumor formation was noted. Similar cell engraftment and differentiation were shown in spinally injured transiently immunosuppressed swine leukocyte antigen (SLA)-mismatched allogeneic pigs[118].

The advance in gene engineering and cell therapy was successfully demonstrated by Russian scientists with the minipig's model of contusion SCI. Intrathecal application of genetically engineered umbilical cord blood mononuclear cells simultaneously transduced with three recombinant adenoviruses Ad5-VEGF, Ad5-GDNF and Ad5-NCAM (delivery of neurotrophic factors producing cells) 10 days after SCI significantly improved histological, electrophysiological, and clinical evaluation in treated animals[119]. Effective *in vivo* use of adeno-associated virus (AAV)-based vectors to achieve gene-specific silencing or upregulation in the central nervous system was limited by the inability to provide more than limited deep parenchymal expression in adult animals using delivery routes with the most clinical relevance (intravenous or intrathecal). To overcome this disadvantage, our scientific team has recently developed the new subpial delivery route for (AAV)-based vectors gene specifically using our porcine model. Subpialy injected pigs showed (i) potent spinal parenchymal transgene expression in white and gray matter including neurons, glial, and endothelial cells after single bolus subpial AAV9 delivery; (ii) delivery to almost all apparent descending motor axons throughout the length of the spinal cord after cervical or thoracic subpial AAV9

injection; (iii) potent retrograde transgene expression in brain motor centers (motor cortex and brainstem); and (iv) the relative safety of this approach by defining normal neurological function for up to 6 months after the AAV9 delivery[120].

Minipigs and rats were successfully used for preclinical testing of determination the accessibility of intravenously administered biodegradable nanoparticles (NPs) as a drug delivery system to the lesion site in contusion models of SCI. Results from this study showed that NPs, which could potentially be explored as a carrier for delivery of therapeutics to the lesion site to minimize the impact of post-SCI response, were dose-dependently increased and significantly greater localized at the lesion site than in the remaining uninjured segment of the spinal cord[121].

CONCLUSION

Spinal cord injury represents a disorder with major socioeconomical impact in human population. Significant costs are incurred throughout the life of a person with SCI, including initial hospitalization and acute rehabilitation, home and vehicle modifications, and recurring costs for durable medical equipment, medications, supplies, and personal assistance[122]. Using appropriate large animal models for spinal cord injury may be valuable for obtaining information about diseases progressing as well as represent perspective tool for finding potential therapeutic methods. Spinal cord injury represents a CNS disease, in which the cause is known but the cure is still missing. Therefore, it is modelled by many rodents as well as large animal models of different origin. The most frequent animal models for SCI study are rodents. Nevertheless, the promising SCI therapies functioning in rodent models failed to work in men. The physicians request testing the new SCI therapies in large animal models before clinical studies. Recent studies have shown that pigs and mainly minipigs seem to be an ideal large animal model for SCI study due to the ethical, economical, and anatomical parameters. There is a hope for early SCI therapy finding considering the actual extensive SCI research using large animal models.

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21 Melanoma progression and spontaneous regression in the Melanoma-bearing Libechov Minipig (MeLiM) model

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Abstract

Cutaneous melanoma is a malignant tumour arising by neoplastic transformation of melanocytes, normal skin melanin-containing cells. It is the most dangerous skin cancer with worldwide incidence in Caucasian population increasing during the last decades. Despite present successes in the melanoma treatment using immune checkpoint inhibitors, its therapy in metastatic stage is still unsatisfactory and new approaches need to be sought. The Melanoma-bearing Libechov Minipig (MeLiM) is a unique model of hereditary metastatic melanoma which shows histopathological, immunohistochemical, biochemical, haematological, and genetic similarities with the human melanoma. Long-term monitoring and detailed analyses of affected animals allow identifying genes and understanding the biological processes associated with the melanoma development and progression as well as its spontaneous regression. In addition, new techniques for melanoma treatment can be tested using MeLiM piglets with progressing melanoma. Tumour devitalization (surgical ischaemization) was successfully utilized in animals with metastatic melanoma and multiple cutaneous tumours. Increased expression of heat shock proteins HSP70 and gp96 in the devitalized melanoma followed by increased population of tumour infiltrating lymphocytes in all untreated melanomas and generalized destruction of melanoma cells in all cancer deposits including organ metastases were observed in the treated pigs. They were in long-term remission without relapse. MeLiM represents a valuable large animal model to study melanoma regression and new methods for melanoma treatment.

Keywords

melanoma, swine MeLiM model, melanoma progression, spontaneous regression of melanoma, devitalization of cutaneous melanoma

INTRODUCTION

Incidence of all skin cancers is rising worldwide[1]. Non-melanoma cancers (e.g. basal cell carcinoma, squamous cell carcinoma), which are more frequent than melanoma, affect mainly the elderly population and are relatively well treatable[2]. Cutaneous melanoma represents less frequently occurring but the most aggressive skin cancer[1]. In fair-skinned population in Europe, North America, Australia, and New Zealand, the incidence of the cutaneous melanoma has steadily increased over the last 50 years[3] and rising incidence is occurring in ever younger individuals[4]. Epidemiological data for melanoma in the Czech Republic, collected by the National Oncological Register (NOR) and analysed by software SVOD (System for Visualizing of Oncological Data, www.svod.cz), clearly document the same trend in melanoma incidence that increased 5-times from 1977 to 2017. On the contrary, mortality showed stabilized level in the last ten years (being around 4–5 cases per 100 000 population), which might be caused by the earlier detection of the disease and/or improved therapeutic approaches.

Melanocytes, pigmented cells originating from melanoblasts, are dispersed in hair follicles and the basal layer of epidermis, but also elsewhere throughout the body. Neoplastic transformation of these cells leads to melanoma development[5]. Sun exposure and number of sunburns (especially in childhood) were confirmed as the main "external" risk factor of melanoma development, but other environmental factors may play role in the disease development (e.g. exposure to cosmic radiation, heavy metals, benzene and other chemicals)[6]. The most important "internal" risk factors are type and number of melanocytic nevi. Additional host factors affecting melanoma risk are fair skin, hair, and eye colour and ability to tan[4]. In approximately 10 % of cases, familial history was recorded and certain variants of melanoma predisposition genes (such as *CDKN2A*, *CDK4*, *MITF*, *BAP1*, and *POT1*) were found to increase the risk of development of this deleterious disease[7,8].

Spontaneous regression is a rare biological phenomenon that appears in all types of human cancers. It is defined as the partial or complete disappearance of a malignant tumour in the absence of any treatment or in the presence of inadequate therapy, which cannot significantly influence the cancer. Among the 130 well documented cases of spontaneous regression of various tumours summarized since 1900 to 1964 by Everson[9], regression of melanoma was observed most frequently. Partial spontaneous regression of primary cutaneous melanoma is relatively common event. In collection of 563 cases of melanoma diagnosed between 1971 and 1990, incidence of the partial regression ranged from 9 to 46 % depending on Breslow thickness, with higher incidence in thinner lesions[10]. On the contrary, complete spontaneous regression of primary melanoma occurs very rarely. High with co-workers[11] found only 34 cases published in the English literature between 1963 and 1985 and they presented another four cases of completely regressed advanced melanoma with metastases. In another newer and detailed review of literature data since 1866, 76 cases of complete spontaneous regression of metastatic melanoma were revealed that represent the incidence of only

about 0.23 %[12]. Spontaneous regression of primary melanoma seems to be associated with a poorer prognosis[13], while spontaneous regression of metastatic melanoma can indicate improved outcome[12]. The precise biological mechanisms responsible for spontaneous regression of melanoma are still unknown. A dense infiltrates of lymphocytes surrounding melanoma cells (which demonstrate tissue destruction) and numerous pigment-laden macrophages (melanophages) were histologically found in regressing lesions[10,14], suggesting activation of host immune system against melanoma cells. Various immune cells and melanoma antigens seem to be the most important players in this process[15]. Infection (postoperative or systemic inflammation from other causes) and operative trauma (such as surgical disruption of the blood supply to the residual tumour)[12] could also initiate spontaneous regression.

Because of ethical reasons, long term monitoring of growing tumours in human patients is not possible. That is why various animal models have been developed (for example via chemically induced carcinogenesis, genetic manipulations or breeding of affected parental animals) for detailed research of cancer diseases including melanoma[16].

MELANOMA IN SWINE

Melanoma appears in swine spontaneously (similarly as in other domestic animals, such as dog, cat, horse, cow, and sheep)[17] with very low frequency. Monitoring 747 014 slaughter pigs revealed only 220 cases (i.e. 0.03 %) with melanoma lesions[18]. Melanomas were found in pigmented meat breeds such as Duroc and Iberian pig[19–22]. Unlike Caucasian population which is highly sensitive to melanoma, the white-coloured pig breeds do not show melanoma development. It can be explained by the absence of melanocytes in the white pig skin[23]. Analysis of progeny from mating of affected parents revealed inherited predisposition to melanoma in Duroc pigs[24]. In miniature pigs, three models with spontaneous hereditary melanoma has been established: the Sinclair Miniature Swine, the Munich Miniature Swine Troll and the Melanoma-bearing Libechov Minipig.

The Minnesota Miniature Swine (also called Hormel Miniature Swine) was developed at the Hormel Institute (University of Minnesota, Austin, USA) since 1949 by crossbreeding of four smaller and feral pig sources (Alabama's Guinea hogs, Catalina Island's wild pigs, Louisiana's Piney Woods pigs, and Guam's Ras-n-Lansa pigs) and selection for small size. One herd of the Minnesota Miniature Swine was acquired with breeding rights by the Sinclair Comparative Medicine Research Farm (University of Missouri, Columbia, USA) in 1965 and used for biomedical research as the *Sinclair Miniature Swine*[25]. Cutaneous melanoma was firstly observed in this strain in 1967[26]. Selective breeding greatly increased its incidence. The highest level was found in offspring from mating of both affected parents. It gave 54% incidence in new-born piglets, which grew to 85% in 1-year old pigs because most of the tumours developed postnatally (mainly during the first four months after birth)[27].

Cutaneous pigmented lesions were classified as benign nevi, superficial spreading melanoma or nodular melanoma. Almost 90 % of the tumours penetrated deeply into the dermis and subcutaneous fat (corresponding to the level IV and V according to the Clark's classification for human melanoma[28]) and metastases to lymph nodes and visceral organs were observed[29]. Most of affected Sinclair swine undergo spontaneous regression of melanoma. It is connected with activation of both cellular (γδ T lymphocytes, macrophages)[30,31] and humoral (anti-melanoma antibodies)[32] immune response to melanoma. This process is usually accompanied with partial or generalized skin and bristle depigmentation[33] suggesting immune response to antigens, which are common in normal (melanocytes) and malignant (melanoma cells) pigmented cells[32]. A lineage of the Sinclair Miniature Swine with hereditary predisposition to melanoma is now produced by Sinclair Bio-Resources, LLC (Auxvasse, Missouri, USA) as a suitable animal model of spontaneous regression of this cancer[34]. Chromosomal abnormalities were detected in three cell lines derived from Sinclair melanoma[35] and two-locus model explaining expression of cutaneous melanoma at birth was suggested[36]. However, exact predisposition genes responsible for melanoma development in the Sinclair Miniature Swine were not yet identified.

Literature data about another swine hereditary melanoma model, the *Munich Miniature Swine Troll*, are very limited. This model was created at the University of Munich (Germany) in 1986 using parental animals (one melanoma-bearing boar and two melanoma-free sows) from a herd produced by mating the Hanford and the Columbian miniature swine at the Medical Service Munich. Cutaneous tumours appeared at birth or during the first two months of postnatal life. Selective breeding of affected pigs increased the incidence of melanoma to 70 %. A three-locus model (with two recessive alleles per locus) was suggested for development of cutaneous melanoma in this swine model. However, no melanoma predisposition genes were found. Complete spontaneous melanoma regression together with skin and bristle depigmentation was also observed, but frequency of this phenomenon was not mentioned[37,38]. Elevated expression of porcine endogenous retroviruses (which can transform benign cells to malignant ones and promote immunosuppression leading to cancer formation and spreading[39]) was demonstrated in melanomas and cell cultures from pulmonary metastases isolated from affected animals of this model[40]. This article published in 2007 is the last one that was found through the PubMed about the Munich Miniature Swine Troll. Thus, it is not clear if this melanoma model still exists.

MELANOMA-BEARING LIBECHOV MINIPIG

ESTABLISHMENT AND CHARACTERIZATION OF THE MeLiM STRAIN

Pigs are bred in the Institute of Animal Physiology and Genetics (IAPG) of the Czech Academy of Sciences in Libechov since 1966. They were originally used for the study of blood groups.

For this purpose, six Goettingen miniature pigs (University of Goettingen, Institute of Animal Breeding and Genetics, Germany) and seven Minnesota miniature pigs (Hormel Foundation, Austin, USA) were firstly imported. Then, pigs of various commercial meet breeds (Canadian Landrace, Cornwall, and Large White) and Vietnamese pigs were also purchased and used for breeding to cover a wide range of pig blood groups. The first few darkly pigmented piglets with cutaneous melanomas appeared in this genetically heterogeneous population in 1989. Using selective breeding of affected parental animals for several generations, the incidence of cutaneous melanoma has stabilized around 40–60 %[41,42] but it fluctuates year-to-year (up to roughly 80 % in 2018) depending on the degree of the parental animal affection. This pig model with hereditary melanoma has been designated by the acronym MeLiM (*Me*lanoma-bearing *Li*bechov *M*inipig; originally Melanoblastoma-bearing Libechov Minipig[41]).

Cutaneous melanomas are found usually as multiple skin lesions of nodular type but superficial spreading melanomas and nevi (according to the human melanoma classification) also appear. They are ascertained already at birth or they develop during two months thereafter. Nodular melanomas grow exophytically reaching very variable size (roughly from 5 mm to 120 mm). The most affected animals are black-pigmented MeLiM piglets. Several melanomas are usually found also in piglets with the brown/red coat colour[41,43] contrary to the Sinclair Miniature Swine[33]. White coloured piglets with black spots are born very rarely and they do not develop any skin lesions. This colour coat variability reflects multi-breed character of the MeLiM strain.

Melanoma cells in nodular melanomas show vertical spreading from the basal layer of epidermis into the dermis and the hypodermis corresponding to the Clark's level V of human melanoma. Numerous metastases in the lymph nodes and various visceral organs (mainly in the lungs and spleen) are detected at autopsy of melanoma-bearing animals, confirming malignant character of this cancer in the MeLiM strain[41,43,44]. Very high concentration of melanosomes and melanin (corresponding to dark melanoma pigmentation) and three typical melanoma enzymes (tyrosinase, α-mannosidase, γ-glutamyltransferase) were demonstrated biochemically in the MeLiM melanoma tissue[23]. Immunohistochemical staining of MeLiM melanoma revealed expression of RACK1 (Receptor for activated C kinase 1), a potential marker protein of malignancy in human[45], horse[46] and canine melanoma[47]. In addition, MITF (Microphthalmia-associated transcriptional factor), a specific protein of the melanocytic cell lineage, was also revealed in melanomas of these four species[45,46,48].

Melanoma predisposition genes have not yet been revealed in the MeLiM model. A three-gene inheritance of nodular melanoma was suggested on the basis of segregation ratios obtained from various mating types[42]. Nine boars and eighteen sows of the MeLiM strain were gradually exported from the IAPG to the INRA/CEA (Institute National de la Recherche Agronomic/Commissariat à l'Energie Atomique, Jouy-en-Josas, France) in 1997-2008. These animals were crossed there with each other and with healthy Duroc producing MeLiM, F1 and also backcross families, which were used for detailed genetic analyses. The *CDKN2A,*

CDK4 and *BRAF* genes (three of predisposition genes for human familial melanoma) were excluded as candidates for melanoma susceptibility in the MeLiM strain and revealed that this swine melanoma was inherited as an autosomal dominant trait with incomplete penetrance[49,50]. Moreover, five chromosomal regions involved in susceptibility to this swine melanoma (three of them corresponding to human regions with melanoma candidate loci)[50], a specific variant (*MC1R*2*) of *MC1R* gene (associated with melanoma development in black pigs)[51] and of *KIT* gene (associated with melanoma development, ulceration, and cutaneous invasion)[52], and potential involvement of *MITF* gene in the swine melanoma biology (but not to melanoma predisposition)[53] were ascertained.

SPONTANEOUS REGRESSION OF MeLiM MELANOMA

The MeLiM strain is unique animal model allowing particular and time-lapse studies of melanoma progression and spontaneous regression (Fig. 1) from various viewpoints. Spontaneous regression of melanoma appears in MeLiM animals similarly as in the Sinclair Miniature Swine and the Munich Miniature Swine Troll. Roughly 70 % of affected MeLiM piglets exhibit this phenomenon[41]. Some clinical scientists consider the MeLiM unsuitable to model human disease, because of the high incidence of spontaneous regression, which is in contradiction with its lower frequency in human melanoma. In fact, spontaneous melanoma regression in humans may be higher than reported in the literature, because: 1) not all melanoma patients come to the physician and thus some cases of spontaneous regression may not be recorded; 2) melanoma patients who come to the physician are immediately treated with appropriate anticancer treatment. For obvious ethical reasons, there is no delay in therapy and no waiting whether or not spontaneous melanoma regression will occur.

In MeLiM pigs, developing cutaneous tumours are black and initially show growth phase and overall size increase. The first macroscopic changes associated with their spontaneous regression occur since the 10 weeks of age[54]. The tumours begin to flatten, their size gradually decreases and the colour changes from black to grey. Then, discoloration of skin and bristles is observed that is the best detectable in black animals (Fig. 1B, 1D). First, white bristles (sparsely scattered across the body or concentrated around some melanomas) and skin depigmentation appear (often as halo around some cutaneous tumours). These changes in coloration gradually spread during spontaneous regression and in some individuals eventually affect almost the entire body. Black pigmentation of the skin and bristles is maintained without any colour changes in some MeLiM animals with spontaneous melanoma regression[41,43,54,55].

Histological observation of regressing tumours documented dense infiltrates of lymphocytes and melanin-laden histiocytes, gradual destruction of melanoma cells and replacement of melanoma tissue with the fibrous tissue[43,54] (Fig. 2B). These morphological changes were found already at 6–8 weeks of age, i.e. when cutaneous tumours were still growing, exophytic and black[54]. The tumour tissue remodelling was in accordance with changes in the expression

Figure 1. Progression and spontaneous regression of melanoma in the MeLiM model. A) A piglet with melanoma progression (age of 10 weeks). Multiple cutaneous growing melanomas and cachexia are noticeable. B) A piglet with spontaneous regression of melanoma (age of 4 months) shows almost normal body weight, flat and grey larger cutaneous tumours (arrows) and considerable skin and bristle depigmentation. C) Macroscopic appearance of the progressing nodular melanoma and several nevi on black skin. D) Macroscopic appearance of spontaneously regressing (originally nodular) melanoma with depigmentation of skin and some bristles. E) Occurrence of a huge number of melanoma metastases (black spots) in both lung lobes. F) Multiple metastases in the spleen (*), one in the stomach (**) and three very small in the liver (***) together with metastatic lung (on the right side) are seen in piglet with melanoma progression. Orig. V. Horak

of extracellular matrix proteins (collagen IV, laminin, fibronectin, and tenascin C) and matrix metalloproteinase-2 (an enzyme which is involved in the degradation of extracellular matrix) demonstrated immunohistochemically[54,56]. Spontaneous regression is not synchronous in all cutaneous lesions and is completed around 6–10 months of age depending on the overall extent of melanoma deposits. Flow cytometry results of spontaneously regressing MeLiM melanomas showed increased infiltration with cytotoxic T-lymphocytes ($CD3^+CD8^+$)[57] and a specific population of recirculating effector/memory $CD4^+CD8^{hi}$ αβ T-lymphocytes that are

involved in this process[58]. Taken together, these results suggest that the regression phenomenon results from activation of the immune system against swine melanoma antigens, which may be melanoma-specific or common to melanoma cells and normal melanocytes, leading to depigmentation of skin and bristles. Genome-wide time-dependent gene expression profiling identified a large scale of genes, which were up-regulated and down-regulated during spontaneous regression[57] and suppression subtractive hybridization showed *RARRES1* (the retinoic acid responder 1) gene as a main actor in the regression process[59]. In addition, microRNA analysis revealed significant up-regulation of five miRNAs with the highest up-regulation of the tumour suppressor miR-193b suggesting that this miRNA can down-regulate its downstream cell-cycle-related genes during the early step of spontaneous regression of MeLiM cutaneous melanoma[60]. However, it is not yet clear, what impulses are triggering this biological phenomenon.

PROGRESSION OF MeLiM MELANOMA

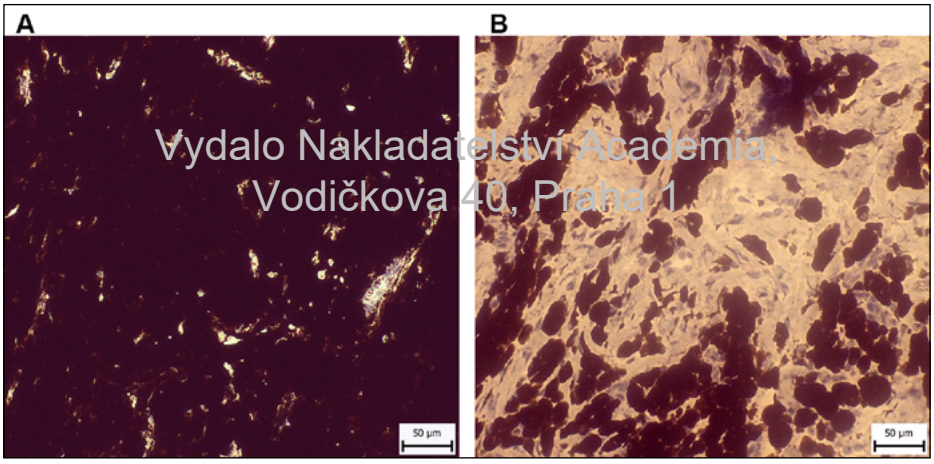

Figure 2. Histological appearance of progressing and spontaneously regressing cutaneous melanomas (cryosections stained with Weigert's hematoxylin-eosin). A) **Progressing melanoma** (MeLiM piglet, age 10 weeks). Tumour tissue is composed of melanoma cells (completely filled with black-brown melanin), which are in a close contact with diminutive extracellular spaces only. B) **Spontaneously regressing melanoma** (MeLiM piglet, age 10 weeks). Melanoma cells (filled with melanin) are dispersed forming small clumps. Lymphocyte infiltration, melanoma cell destruction, and rebuilding of the original tumour tissue into the fibrous tissue are noticeable. Orig. V. Horak

Melanoma progression is observed in about 30 % of affected MeLiM pigs[41]. It enables to study development of cutaneous lesions and organ metastasis for several months that would not be possible in human patients from ethical reasons. This property of the MeLiM strain represents a great advantage in comparison with other swine melanoma models (Sinclair

Miniature Swine, Munich Miniature Swine Troll), which demonstrate only spontaneous regression in all (or almost all) individuals. Body weight of piglets with melanoma progression is very similar to that of piglets with spontaneous regression since birth to the fifth week of age. Cutaneous lesions are present at birth or they develop shortly thereafter. They are dark black and prevailingly of nodular type with exophytic growth (Fig. 1C). They grow rapidly, showing sometimes ulceration, local necrosis, and bleeding. Melanoma cells extensively metastasize from cutaneous melanomas into lymph nodes (which greatly increase in size) and various visceral organs (mainly into the lungs and spleen, often also into various pars of the digestive tract, liver, and kidney) (Fig. 1 E, F), resulting in growth retardation and cachexia (Fig. 1A) (that deepens with age of animals) and death of animals within two to three months of age. Depigmentation of skin and bristles does not occur in these piglets[41,44,55].

Histological evaluation of progressing melanomas showed a dense aggregation of smaller, black-brown pigmented cells (due to completely filling with melanin), with diminutive extracellular spaces (Fig. 2A). Detection of haematological parameters and iron concentration in peripheral blood revealed that all MeLiM pigs (with progression as well as spontaneous regression of melanoma) were affected by iron deficiency and cancer-related microcytic hypochromic anaemia. Greatly reduced values of red blood cell count, haematocrit, and haemoglobin concentration, together with highly increased number of platelets were characteristics of pigs with melanoma progression. On the contrary, pigs with spontaneous regression of melanoma demonstrated higher values of the red blood cell parameters and lower number of platelets. Thus, monitoring haematological parameters (together with detection of body weight, macroscopic changes of cutaneous melanoma and colour coat, histological, and immunological analyses) makes possible to distinguish MeLiM piglets with progression and spontaneous regression of melanoma in early stages of postnatal development[55]. Similar changes (thrombocytosis, iron-deficiency, and anaemia) were also observed in melanoma patients predicting metastatic disease and poor prognosis[61–63].

SWINE MELANOMA REGRESSION INDUCED BY TUMOUR DEVITALIZATION

Devitalization (also called devascularisation) is an original surgical method, which has been developed by the Czech surgeon Karel Fortýn (1930–2001) for treatment of solid tumours. Devitalization consists in ischaemization of tumour by ligation of all vessels – arteries and veins – with non-absorbable suture material and leaving the treated tissue *in situ* (without any excision). After its promising experimental application in healthy miniature pigs in the IAPG[64], devitalization technique was successfully utilized in several patients with inoperable colorectal carcinoma[65]. The devitalized tumour tissue was destroyed, changed into a small fibrous residue and no visceral metastases (observed before devitalization) were found at revision operation. Recently, a new case report of a patient with invasive metastatic colorectal carcinoma, who is surviving more than 14 years after devitalization with no sign of malignancy, has been published, demonstrating curative potential of devitalization[66]. Despite

such positive outcomes, there is no valid recommendation for this method in the current human medicine.

Effect of devitalization on melanoma has been experimentally tested using MeLiM piglets of both sexes with metastatic melanoma and progressively growing multiple cutaneous nodular tumours. In this cancer, devitalization is relatively simple and quick surgical procedure. Partially overlapping mattress stitches are conducted around the base of one of multiple cutaneous tumours, strongly tightened and the tumour is left *in situ*. Thereafter, gradual destruction of melanoma cells in the treated melanoma as well as in all other untreated melanomas and in organ metastases and replacement of the original malignant tissue by the fibrous tissue was observed during 4–6 months. No health complications or side-effects were detected. Depigmentation of skin and bristle (similarly as during spontaneous melanoma regression) were only ascertained[41,67]. Reduction of α-mannosidase and tyrosinase activities in untreated melanomas observed 6 months after devitalization of another melanoma lesion corresponded with the melanoma cell destruction after devitalization[23]. Immunohistochemistry and Western blotting demonstrated increased expression of two heat shock proteins – HSP70 and gp96 – in the devitalized melanoma since the 1st day after the treatment and persisting for next two weeks. Then, proportion of tumour infiltrating lymphocytes (cytotoxic $CD3^+CD4^-CD8^+$ T-lymphocytes and double positive $CD3^+CD4^+CD8^+$ T-lymphocytes) gradually grew in untreated cutaneous melanomas that correlated with melanoma cell destruction. Effectiveness of melanoma devitalization was around 80% and no relapses were ascertained[68]. These results show that melanoma devitalization in the MeLiM model is capable of eliciting a cell-mediated anti-tumour immune response that leads to the healing of animals with metastatic cancer. They correspond with knowledge about immunostimulatory function of both detected heat shock proteins that form complexes with cancer cell-derived immunogenic peptides and through antigen-presenting cells activating cytotoxic T-lymphocytes[69,70]. Vitespen (formerly Oncophage) is the first personalized gp96-peptide cancer vaccine based on this property of heat shock proteins, derived from autologous tumour lysate and tested in melanoma patients[71,72].

CONCLUSIONS

Great progress has been made over the past years in the identification of human melanoma risk genes and also in melanoma treatment. However, the revealed genes explain only a smaller part of familiar melanomas and new, more efficient therapeutic procedure are still needed. Swine seems to be a proper animal species for melanoma studies due to some histological and immunohistochemical similarities with human skin[73]. Genetic engineering could be very helpful in this respect because its current technologies can make precise genetic modifications providing models that mimic human cancer. Several swine cancer models have been produced

by this technology[74], but no transgenic melanoma swine model has been developed by this way up to now. Thus, the Sinclair miniature swine and the Melanoma-bearing Libechov Minipig, two swine hereditary melanoma models with similarities to human melanoma – are still very useful for detailed genetic, histopathologic, and immunologic analyses of melanoma.

Spontaneous regression of melanoma appears in both of these swine models. It is found in all (or in almost all) Sinclair swine (so that they are used as a spontaneously regressing melanoma model) and in about two thirds of melanoma-bearing animals in the MeLiM model. The high incidence of spontaneous regression may appear to be a significant disadvantage of these models because complete regression of metastatic melanoma is very rare in human. However, its detailed long-term monitoring from various aspects can reveal new knowledge, applicable in development of immunotherapeutic methods, that cannot be ascertain in human melanoma due to ethical reasons and low incidence of this biological phenomenon. Compared to the Sinclair miniature swine, the MeLiM model is unique because about 30 % of affected pigs show melanoma progression causing death. On the basis of health status, macroscopic and microscopic analyses of cutaneous tumours, and haematological parameters, MeLiM pigs with melanoma progression can be distinguished from those with spontaneous regression and used for development of new methods for melanoma therapy or for the study of regression phenomenon, respectively.

Acknowledgements

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22 MicroRNA biogenesis, function, and its role in cutaneous melanoma pathogenesis

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Abstract

Cutaneous melanoma is a skin cancer arising from transformed melanocytes. Melanoma is a leading cause of death among skin cancers and its incidence is rapidly rising worldwide. When diagnosed at later stages, metastases are frequently developed and the disease is refractory to current therapies. It is supposed that microRNAs, which are small non-coding RNAs, might be useful as biomarkers, therapeutic targets or even therapeutic agents in the treatment of this malignant disease.

Keywords

melanoma, miRNA, cancer, MeLiM, regression

MELANOMA

Cutaneous melanoma (CM) is a skin cancer with rapidly rising incidence. In the Czech Republic a four-fold increase in the incidence of melanoma was observed over the last 4 decades[1]. CM arises from the transformed pigmented skin cells melanocytes. In a healthy skin, melanocytes produce melanin, pigment protecting DNA of skin cells from ultraviolet (UV) radiation[2]. Melanoma is considered to be one of the most aggressive human cancers, because even a relatively small tumor is likely to develop metastases. Early diagnosed tumours (thickness below 1 mm, no local or distant metastases) can be treated by surgical excision with a wide protective rim resulting in a very good prognosis for the patient. However, melanomas that penetrate the basal membrane and form distant metastases are often refractory to therapies and have an unfavourable prognosis[3]. Thus, despite its relatively

rare incidence, CM is a leading cause of death among skin cancers. In both hereditary and sporadic melanomas, various mutations have been identified, affecting mainly regulation of signalling pathways, cell cycle, proliferation, as well as pigmented cell development and migration or senescence and apoptosis[4,5]. Beside such gene mutations leading to disfunctional proteins, deregulation of gene expression may also participate in melanoma development and spreading. One of the key molecules involved in regulation of gene expression are short (21–23 nucleotides) single stranded non-coding RNA molecules, called micro RNAs (miRNAs)[6]. The miRNAs recognize and bind to complementary sequences of target mRNA molecules, which leads to target mRNA degradation or its less efficient translation into protein.

BIOGENESIS OF miRNAs

The first miRNA was discovered in 1993 by the team of V. Ambros in a study of larval development of microscopic worm *Caenorhabditis elegans*[7]. This miRNA was named lin-4 and was found to influence the LIN-14 protein levels in the first larval stage. The loss of lin-4 function disrupts timing of larval developmental events and leads to the absence of adult structures (such as cuticle and vulva).

The biogenesis of ~22 nt long RNA structure (Fig. 1) was described in more details in 2002[8]. Sequences coding miRNAs are mostly located within exons or introns of non-coding RNAs or in introns of pre-mRNA. They are transcribed from DNA as more than 1000 nt long precursor molecule (primary transcript, pri-miRNA) by RNA polymerase II. This pri-miRNAs have one or more stem-loop structures that are recognized by Drosha enzyme, which in complex with other proteins cleaves pri-miRNA between stem-loop and single strand structure. Resulting molecule of ~60–100 nt length is called pre-miRNA and is transported from nucleus to cytoplasm, where is processed by enzyme Dicer. This enzyme cleaves pre-miRNA into two parts, one of them is generally degraded while the other becomes part of RISC complex (RNA-induced silencing complex). This complex binds to the 3'UTR (untranslated region) of target mRNA and based on accuracy of binding in "seed" sequence of miRNA, the target mRNA is degraded or protein synthesis is prevented. Such a pathway is called canonical. Several alternative mechanisms of miRNA function have been discovered, including pathway independent on Drosha enzyme (rev. in Saliminejad, K. et al., 2019)[9]. It is estimated that miRNAs target approximately one–third of human mRNAs, and about 200 transcripts could be targeted simultaneously by a single miRNA due to its differential target binding patterns (rev. in Thyagarajan, A. et al., 2019)[10].

RNA sequencing, widely used for study of miRNA expression, led to an unexpected discovery that pre-miRNAs often give rise to more than one mature miRNA molecules. Such variants are called "isomiRs"[11]. IsomiRs were primarily considered as sequencing mistake or

biological trash, however, results of Cloonan et al. demonstrate that isomiRs are functional and have their own biological role[12]. Hansen et al. found that some miRNAs (derived from pseudogenes or paralogs) can function as miRNA sponges. This suggests that they can bind to complementary miRNAs and prevent their binding to target mRNA molecules[13]. Understanding of miRNA biology is still in the beginning and study of miRNA/isomiR levels will extend our understanding of miRNAs[14].

While miRNAs play important roles inside all cells of multicellular organism, they are also present in body fluids. These miRNAs seem to play roles in intercellular communication. It is expected that circulating miRNAs could be used as disease biomarkers and in the treatment of various diseases, including cancer, in the future (rev. in Hruštincová, A. et al., 2015)[15].

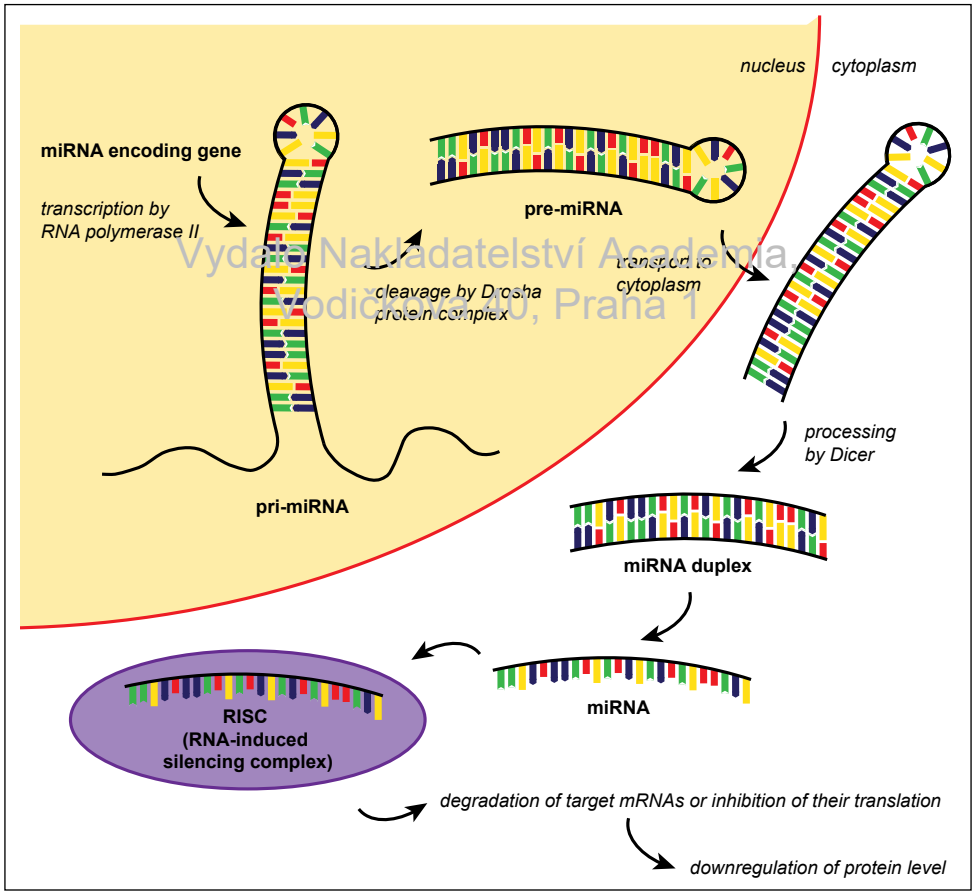

Figure 1. Biogenesis of miRNA. Orig. H. Kupcova Skalnikova

ROLE OF miRNAs IN MELANOMA PATHOGENESIS

miRNAs may show both pro-oncogenic as well as anti-oncogenic functions. One of the first miRNAs, of which deregulated expression was linked to melanoma tumorigenesis, was miR-137. This miRNA regulates Micropthalmia-associated transcription factor (MITF) – the master regulator of melanocyte development, function, and survival. Gene encoding miR-137 is located in 1p22 chromosomal region, previously determined to harbor melanoma susceptibility allele[16].

Additional miRNAs with protumorigenic effects are miR-221 and miR-222. Felliceti et al. performed a detailed study on melanoma cell lines transfected with lentiviral vector carrying miRNA of interest. Cells that overexpressed miR-221 and miR-222 showed an increase in the proliferative rate. Tumorigenicity of these miRNAs was confirmed also in an *in vivo* model (athymic nude mice), where transduced melanoma cell lines showed significantly increased tumor volumes compared to empty vector cells[17]. The overexpression of miR-222 supports melanoma cell invasivity and shortens survival of human patients[18].

miR-21 is supposed to be a key oncogene, which is expressed in many types of cancer[19]. Targets of this miRNA are mRNAs of tumor-suppressor proteins, regulators of cell cycle, and intrinsic and extrinsic pathways of apoptosis[20]. Saldanha and collegues found that plasma levels of miR-21 reflect tumor burden. This suggests that miR-21 can serve as marker for disease monitoring[21].

Highly conserved miRNA across species, miR-7, plays key roles in both normal development and disease. In humans, it is expressed from three genomic loci; each miR-7 gene is transcribed as a unique primary transcript but all these transcripts give rise to the same mature miRNA. Target sites in various mRNAs, which are recognized by miR-7, are also highly conserved across species. In cancer, the miR-7 acts as a tumor suppressor, but in some cases it has been associated with oncogenic promotion (rev. in Horsham, J. L. et al., 2015)[22]. In melanoma, Giles et al. found that miR-7-5p suppresses growth, migration and invasion of melanoma cells and inhibits formation of lung metastases. Loss of this miRNA in melanoma promotes its growth and metastasis formation, which results in worse clinical outcome[23]. miR-7 is the most significantly down-regulated miRNA in selected melanoma cell lines. Re-establishment of miR-7 expression can reverse the resistance to vemurafenib (clinically used inhibitor of V600E mutated BRAF) and inhibit resistant melanoma cell growth (both in xenograft tumor model and *in vitro*)[24].

On the other hand, expression of miR-204 seems to have protective effects against cancer development. The miR-204-5p is decreased in melanoma cell line compared to melanocytic nevi. Alterations in expression of this miRNA caused by application of mimics or inhibitors results in decreased viability and proliferation. This suggests that miR-204-5p has tumor suppressor role in melanoma[25]. Loss of expression of miR-204 in melanoma patients with specific somatic mutations have negative effect on survival rates[26].

Mueller and colleagues compared miRNA expression patterns in normal human epidermal melanocytes and melanoma cell lines (derived from primary tumors and metastatic melanomas). They found that some miRNAs were upregulated from melanocytes to primary melanoma and again from primary melanoma to metastatic melanoma cell lines (miR-133a, miR-199b, miR-453, miR-520f, miR-521, miR-551b), while one miRNA (miR-190) was downregulated in malignant cells. Some of these deregulated miRNAs were not previously described to be of importance in tumor development[27]. Many other miRNAs upregulated (e.g. miR-155, miR-30b-5p, miR-374-5p) and downregulated (e.g. miR-211, miR-193, MiR-126, miR-145, miR-365) in melanoma suggest that miRNAs are important players in disease formation and spreading (rev. in Thyagarajan, A. et al., 2019)[10].

miRNAs are also engaged in the epithelial to mesenchymal transition (EMT) process which has been proposed as one of the key mechanisms of cancer resistance and invasiveness. miRNAs can also regulate immune dynamics in melanoma growth and development (rev. in Romano, G. & Kwong, 2017)[28].

miRNA IN MELANOMA-BEARING LIBECHOV MINIPIG MODEL

The Melanoma-bearing Libechov Minipig (MeLiM) model represents hereditary melanoma pig strain. Majority of MeLiM piglets show spontaneous melanoma regression and long-term survival, while in 10–30 % of piglets, melanoma progression develops leading to metastasis formation and death. Comparison of regressive and progressive MeLiM tumors revealed significant changes in expression of several miRNAs. In regressive melanoma, miR-92a showed a down-regulation, whereas five miRNAs (miR-21, miR-221, miR-222, miR-193b and let-7b) were up-regulated compared to progressive tumors. Importantly, regressive swine tumors showed opposite expression of miR-92a, let-7b, and miR-193b compared to progressive human melanomas. The highest up-regulation in regressive MeLiM melanomas showed miR-193b, recognized as tumor suppressor, which could regulate cell-cycle-related genes during CM regression[29]. Such miRNAs could be potential actors in the regression process of melanoma in the MeLiM model. Future studies are desirable to reveal their potential value in human CM.

CONCLUSION

Research focused on miRNAs is very perspective and fast growing area. Number of anotated miRNAs is rising and due to a high conservation of these structures among species, it is possible to compare results from model organisms to studies on human patients. Nonetheless, our knowledge of miRNA roles in melanoma development and spreading is still very limited

and additional research is needed to understand deeper to functions of single miRNA and their organisation into regulatory networks. The aims of miRNA research are not only the recognition of their biological roles but also their possible use in the diagnosis, treatment and monitoring the course and success of treatment of malignant diseases.

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23 Cytokines in malignant melanoma

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Abstract

Cutaneous melanoma is a malignant skin disease. Melanoma is a highly immunogenic tumour and patient's own immunity plays an important role in the control of melanoma growth and spreading. Cytokines are small secreted proteins that are not only key regulators of immune reactions but also mediators of intercellular interactions in the melanoma microenvironment. Cytokines have diagnostic, prognostic and therapeutic potential, however additional studies are required to fully understand their regulatory networks. A pig MeLiM model has a potential to help our understanding of immune reactions in melanoma regression.

Keywords

cytokines, melanoma, regression, cancer, immunotherapy, pig model, MeLiM

INTRODUCTION

Cutaneous melanoma is an aggressive skin cancer originating from melanocytes. Dysregulation of the microenvironment in the epidermis may lead to disruption of the homeostatic balance and trigger uncontrolled proliferation of melanocytes, which may culminate in melanoma development[1]. The tumour is a complex tissue consisting not only of intrinsic malignant melanocytes but also of stromal cells, such as fibroblasts, endothelial and immune cells (Fig. 1). Interactions among malignant cells and stromal cells, surrounding keratinocytes, secreted factors as well as with extracellular matrix, may all participate in regulation of melanoma growth and spreading and/or response to therapy[2–5]. The intercellular crosstalk is very lively and is mediated mainly by cytokines, chemokines and growth factors.

IMMUNOTHERAPY OF MELANOMA

Melanoma is referred to as an immunogenic tumour, mainly due to high T-cell infiltration[6,7] and frequently observed partial regression of melanocytic lesions[8]. Thanks to this unique immunogenic microenvironment, melanoma represents a suitable candidate for immunotherapy[9]. The aim of immunotherapy is to boost patient's own immune system to fight cancer. Cytokines are key immune system regulatory molecules and interleukin-2 (IL-2) was approved by FDA for such a purpose in 1998[10,11]. Currently, interferon α (IFNα) is approved as an adjuvant agent in melanoma therapy[10].

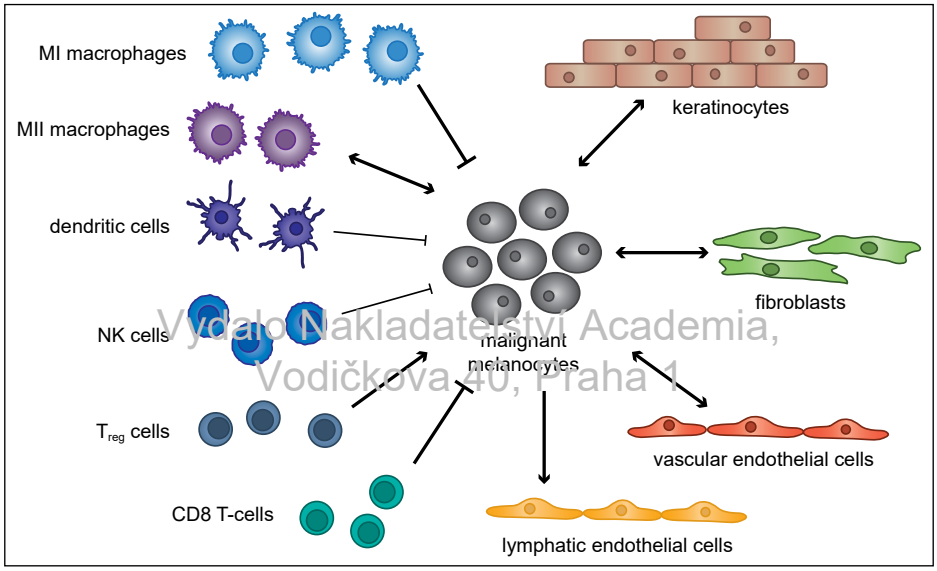

Figure 1. Cells in melanoma microenvironment and their interactions with malignant melanocytes (adapted from ref. 3–5). Factors secreted by keratinocytes, fibroblasts, MII macrophages and regulatory T-cells may stimulate melanocyte proliferation and migration. On the other hand, MI macrophages and CD8 T-cells may inhibit melanoma growth. Dendritic cells and natural killer (NK) cells stimulate recruitment of cytotoxic CD8 T-cells into the tumour and their anti-tumour response. Additional interactions occur among fibroblasts, keratinocytes, endothelial and immune cells (not shown in the picture). Such interactions participate in the regulation of melanoma growth and spreading and have diagnostic/therapeutic potential. Cytokines are key molecules mediating intercellular cross-talk in the tumour microenvironment. Orig. H. Kupcova Skalnikova

Based on new biological knowledge of melanoma and constant efforts to improve efficacy and reduce toxicity of therapy, new treatment strategies have been developed based on the blocking of immune checkpoints[12]. Such inhibited control molecules include cytotoxic T-lymphocyte-associated antigen (CTLA-4)[4] and programmed death 1 (PD-1) receptor or corresponding

ligand PD-L1[13]. Ipilimumab (an anti-CTLA-4 antibody) acts to upregulate antitumor immunity and improves prognostic prospects in patients with metastatic melanoma[14,15]. Nivolumab and pembrolizumab (both anti PD-1 antibodies) are used in patients with metastatic melanoma and with BRAF-mutant melanoma[16]. Efforts are being made to combine ipilimumab and nivolumab treatment, as these two different pathways could complement each other and increase the percentage of advanced melanoma patients responding to the treatment[17].

CYTOKINES – MAIN PLAYERS

Cytokines are secreted regulatory proteins, produced by immune as well as non-immune cell types. Cytokines have multiple functions, including regulatory, pro-inflammatory or anti-inflammatory, and may also trigger proliferation or differentiation of cells. Cytokines regulate and determine the immune response[18] and may also demonstrate antitumor activities[19].

Cytokines often act on several cell types and, together with other cytokines, form cascades, where they interact and exchange information. This regulatory system is referred to as the cytokine network. The great benefit of cytokines is that they can act very quickly and at very low concentrations. Sensitive immunological techniques are required for determination of cytokine levels[20,21].

In cancer, cytokines act as immuno-modulators, influence cell migration, infiltration, proliferation and intercellular interactions in the tumour microenvironment, and thus affect tumour growth and spread. The transformed cells themselves often produce pro-inflammatory cytokines, chemokines, and growth factors attracting immune cells into the tumour stroma. Mediators secreted in tumour stroma further promote cell proliferation and angiogenesis, extracellular matrix remodelling, change in adhesion molecule expression, and increased capillary permeability, leading to the formation of a tumour microenvironment promoting metastasis[22,21]. Interestingly, initial studies show that the melanoma-associated changes in cytokine levels may be reflected also on a system level in patient's blood serum[23–25]. The most explored cytokines with significant roles in melanoma are interleukin 2 (IL-2), interleukin 6 (IL-6), interleukin 8 (IL-8), tumour necrosis factor α (TNFα) and IFNα.

INTERLEUKIN 2

IL-2 belongs to a group of γ_c cytokines, signalling through the same receptor subunit CD132[26]. This cytokine is mainly produced by activated T-lymphocytes (predominantly CD4+ T-cell population). IL-2 is essential for massive T-cell growth, proliferation and differentiation[27]. It also stimulates proliferation and differentiation of natural killer (NK) cells.

IL-2 was one of the first candidates for metastatic cancer research and subsequently first cytokine used in clinical therapy[10,28]. IL-2 was shown to induce tumour regression. However, the IL-2 monotherapy was not optimal due to the activation of both T-effector and

T-regulatory (T_{reg})[29] cell populations, leading to adverse side effects associated with high IL-2 doses[11]. Therefore, lowering of IL-2 dose and combination with other drugs has been considered. Combination with IFNα was unable to reduce the IL-2 toxicity and improve patient survival[30]. Combination of IL-2 with the newly used anti-CTLA-4 antibodies was suggested to help to prevent T-effector cell inhibition and thus to activate the immune response. Nonetheless, such a therapy also seems to offer no significant benefits for melanoma patients[11,31]. A combination of IL-2 with anti-PD-1 or anti-PD-L1 antibodies needs more research.

INTERLEUKIN 6

IL-6 is generally considered a pro-inflammatory cytokine but several anti-inflammatory functions of this cytokine have also been described. IL-6 is a mediator of acute phase reactions and fever, e.g. during infection or traumatic intervention (burns, surgery). In the healing wound, IL-6 stimulates keratinocyte proliferation and migration during reepithelization[32].

Elevation of the IL-6 levels in blood serum of cancer patients in comparison to healthy control groups was observed in many cancers, including malignant melanoma, pancreatic, colorectal, gastric, breast, ovarian, lung and renal cancers[32,33]. In several studies, correlations of IL-6 levels with tumour stage, metastases and prognosis were observed[33]. Together with IL-1β and TNFα, the IL-6 stimulates initiation of cancer cachexia[34]. Higher plasma levels of IL-6 in patients in terminal stages of cancer correlate not only with weight loss but and also associated with anaemia, anorexia and depression[35].

In the tumour microenvironment IL-6 may be produced not only by the malignant cells, but also by non-cancer cells, such as fibroblasts, keratinocytes, endothelial cells, macrophages, T-cells and mast cells[36,37,38]. It is evident from *in vitro* experiments that cancer-associated fibroblasts produce IL-6 and IL-8, particularly when cultured in medium previously conditioned by melanoma cells. Similarly, co-culturing of such fibroblasts with melanoma cells increases production of IL-6 and IL-8, and is accompanied by increased invasion and migration of melanoma cells[37]. Simultaneous blocking of IL-6 and IL-8 by neutralising antibodies inhibits fibroblast-induced malignant melanocyte invasivity[37]. Anti-IL-6 therapy has been developed to prevent cell migration into surrounding tissue and thereby prevent the formation of new metastases. This therapy had a very strong effect on cell migration in experimental studies and can therefore be classified as a migrastatics, which is a novel category of anti-cancer therapeutics[32,39].

INTERLEUKIN 8

IL-8, alternatively known as CXCL8, is a pro-inflammatory CXC chemokine that stimulates CXCR1 and/or CXCR2 receptors. It is produced mainly by macrophages, epithelial and endothelial cells. IL-8 is a chemoattractant for neutrophils[40].

The IL-8 chemokine is overexpressed by tumour cells exposed to stressful situations – chemotherapy or hypoxia[41]. It has been shown that the interaction between IL-8 and its receptor

plays an important role in development of tumour, which includes tumour growth, progression, angiogenesis and metastasis[42,43]. In the melanoma microenvironment, IL-8 is produced predominantly by melanoma cells and influences both fibroblasts and surrounding keratinocytes. *In vitro* cultured cancer associated fibroblasts have been shown to secrete only a negligible amount of this cytokine, however, when co-cultured with melanoma cells, the production of IL-8 by fibroblasts increases[44]. In both *in vitro* and *in vivo* studies, melanoma cells are able to influence locally the differentiation pattern of keratinocytes and involvement of secreted factors including IL-8 is evident[45]. Moreover, IL-8 is a candidate for accurate tumour cell counting. According to a study by Sanmamed et al.[23], serum levels of IL-8 correlate with tumour stage and therefore IL-8 was suggested as a circulating (serum) biomarker of melanoma. Hepatocyte growth factor (HGF) may be another candidate biomarker, as its elevated blood levels indicated melanoma metastases and were associated with worse prognosis of the disease[46].

TUMOUR NECROSIS FACTOR α

TNFα is a member of the TNF/TNFR cytokine superfamily and acts as an acute inflammatory cytokine. TNFα can be produced by many immune cells (e.g. macrophages and monocytes)[47].

Anti-tumour activities of TNFα have been observed already 4 decades ago. While the potent pro-inflammatory activity of TNFα prevents its systemic administration to cancer patients[48], a local application in melanoma localized on extremities shows acceptable toxicity[49]. The local TNFα application by isolated limb perfusion, frequently performed in combination with hyperthermia and melphalan chemotherapy, represents an effective treatment modality for limb melanoma patients with multiple in-transit metastases[50–52].

Currently, there is a growing evidence of pleiotropic functions of TNFα, showing effects on both inhibition and promotion of tumour growth[53]. TNFα participates in development of cachexia in cancer patients[54] and in melanoma, TNFα production is associated with poorer prognosis. TNFα levels were suggested as another biomarker to diagnose cutaneous melanoma[55]. Inhibitors of TNFα are used for treatment of several auto-immune diseases such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease. Such TNFα inhibitor therapy may increase risk of cancer development, including melanoma[56].

INTERFERON α

IFNα is classified as interferon type I and is mainly involved in innate immunity reactions. This protein is secreted by immune and non-immune (e.g. fibroblasts, endothelial) cells in response to infection.

IFNα may enhance the expression of MHC I molecules on the surface of melanoma cells and up-regulation of other cell surface molecules, such as intercellular adhesion molecule-1 (ICAM-1). It may also induce polyclonal proliferation of CD8+ T-cells[57]. Besides the antiviral effect, IFNα shows also anticancer effects, which are possibly derived from activation of signal transducer and activator of transcription (STAT) proteins[58]. IFNα at high doses is

approved adjuvant drug for treatment metastatic melanoma[59,60]. The effects of IFNα adjuvant therapy on patient survival are currently under discussion[61,62], as well as the timing and dosing of such therapies[63,64]. The importance of adjuvant therapy with IFNα should be of predominant importance in patients with high risk of relapse after surgical removal of the primary lesions and eventually a lymph node dissection (stage II and III)[65].

CYTOKINES IN PORCINE MELANOMA MODEL

A unique porcine model Melanoma-bearing Libechov Minipig (MeLiM), in which spontaneous regression of melanoma occurs, was developed at our institute[66–68]. Our initial studies focused on melanoma histopathology and subsequently on immunohistochemical, biochemical and molecular characterization of melanoma regression and progression in MeLiM[69]. Result from MeLiM minipig show several parallels with human melanomas[67,70]. Recently, we have described alteration in haematological profiles of MeLiM pigs[71] and the presence of double positive (DP) T lymphocytes[72]. In addition to the T-cell receptor (TCR), DP cells express on their surface simultaneously both major co-receptors CD4 and CD8[73]. Currently we study roles of cytokines in melanoma regression or progression in pigs as well as their possible predictive values in human patients[74].

CONCLUSION

Cytokines are of immense importance in inter-cellular communication in the cutaneous melanoma microenvironment and in regulation of the patient‘s immune response to melanoma. Levels of selected cytokines correlate with the disease stage and might have prognostic potential. Cytokine application in melanoma therapy to boost patients' immune system against the cancer is studied, however their systemic and pleiotropic effects appear to be an instant problem, which may result in serious side effects of the treatment. Neither the interactions between cytokines and different cell types, nor all the interactions between individual cytokines in melanoma have been fully elucidated up to now. The pig MeLiM model can help us to understand molecular and immune mechanisms of melanoma spontaneous regression and to deepen general knowledge about interactions in the melanoma microenvironment.

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24 Embryotransfer

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Abstract

Embryotransfer is one of the most used techniques in assisted reproduction. During embryo transfer, oocytes and sperms are obtained from donors, zygotes (one-cell stage embryos) are produced using *in vitro* fertilization, and created embryos are placed into the uterus to establish a pregnancy. Alternatively, in animals, zygotes are obtained from female donors already after natural fertilization, and genetic modification is done before embryotransfer. In human medicine, the purpose of embryo transfer is to help give birth to couples, which are subfertile or infertile. With the discovery of gene editing, embryotransfer become a useful technique to obtain model organisms with knock-out or knock-in genes. In this chapter, we summarize the embryotransfer procedure in mice and pigs, two important animal models for biomedical research.

Keywords

embryotransfer, IVF, hormonal stimulation, gene editing

MICE

HORMONAL STIMULATION AND WHITTEN EFFECT

For successful embryotransfer, it is important to have donors and recipient females with the synchronized ovarian and estrous cycles, respectively[1]. The synchronization is acquired by hormonal stimulation. Another reason for the hormonal stimulation of donors is ovarian hyperstimulation to obtain a higher number of zygotes. Mice are stimulated with gonadotropins: 5 IU Pregnant mare's serum gonadotropin (PMSG) and 5 IU human chorionic gonadotropin (hCG) intraperitoneally[2]. Physiologically, follicle-stimulating hormone (FSH) is a gonadotropin secreted from the anterior pituitary gland, and it supports follicular growth. For hormonal stimulation, FSH is replaced by its analog – PMSG[3]. The *PMSG* is a placental glycoprotein from the serum of pregnant mates, and it artificially induces

estrus by inducing the growth of follicles in ovaries[4]. As a result of PMSG stimulation, fully-grown antral follicles contain meiotically component prophase I oocytes, and follicles are responsive to hormonal induction of meiotic maturation and ovulation. Forty-four hours after PMSG stimulation, hCG is applied, followed by natural mating with male mice5. The hCG is used as a replacement for luteinization hormone (LH) that binds on receptors expressed on mural granulosa cells of antral follicles. The LH triggers signalling that leads to meiotic maturation and cumulus expansion. As a result of LH action, matured metaphase II oocytes with expanded cumulus care released to the ampulla of the oviduct[6]. Female mice are mated with males 2 hours after hCG application. Natural fertilization of metaphase II oocytes oocurs 12 hours after hCG administration[5]. The presence of a vaginal plug is a sign of successful mating[7].

After estrus induction, PMSG binds to LCHG receptors in ovaries and promotes the maintenance of the *corpus luteum*, important for the beginning of pregnancy. *Corpus luteum* is secreting progesterone during the first trimester[8]. In hormonal stimulation protocol, hCG is replacing LH hormone for the final maturation of oocytes.

Mice are boxed with 12/12 hours of the light-dark cycle. Females must be stimulated by *PMSG* in time that allows further hCG administration and male adding before night period because it is important to have dark in the room during mating[9].

Another approach for stimulating estrus is the so-called "whitten effect". Male pheromones can cause short and more regular cycles in females. Collecting the bedding from males and place it to the cages with females synchronize their hormonal cycle, so after three days, all females are in estrus. Whitten effect is working well on mice and rats[10]. Estrus in mice can be detected by a visual method when we detect the wide, moist vaginal opening and pink vulva. However, it is better to do the vaginal lavage method and the cytology of lavage. Estrus is characterized by the presence of abundant cornified epithelial cells and granular cytoplasm[11].

EMBRYO COLLECTION

Mice are killed by cervical dislocation for embryo collections. After that, they are placed on a dorsal position, and the abdomen cavity is opened using scissors. Ovaries with oviducts are cut and placed in Petri's dish and poured with isolation medium. With a soft needle, we tear apart oviduct ampulla with embryos surrounded with cumulus cells. The collection of embryos is performed in isolation medium – Human Tubal Fluid (HTF) medium warmed to 37 ºC. Cumulus cells surrounding embryos are removed by adding hyaluronidase to HTF (5 µl hyaluronidase per 100 µl HTF) for 5 minutes. Embryos are cultivated in cultivation medium KSOM Mouse Embryo Media in an incubator with 5% CO_2 atmosphere and 37 ºC temperature[12]. On the second day, 35 hours after *hCG* administration, embryos divide into 2-cell stage[5]. After another two days of cultivation, at embryonic day E3.5, the formation of the blastocoel occurs, and the embryo is considered as blastocyst[13]. For embryo transfer to

oviduct 2-cell stage embryos are needed, but blastocysts are required for intrauterine embryotransfer. CRISPR/Cas9 technology become a worldwide used technique for precise genome editing[14]. Efficient mouse genome engineering in zygotes and creating targeted mouse mutants highly contribute to biomedical research[15].

EMBRYO TRANSFER

Donors and recipients are hormonally stimulated at the same time. Donors are mated with fertile males to obtain zygotes naturally. Recipients are mated with sterile males, on which vasectomy was performed, and females with vaginal plugs are collected and put in isolated cages. Recipients should be younger than donors.

10–15 zygotes are placed towards the ampulla of the oviduct of an anesthetized mouse. The same transfer is performed in the second ampulla[16]. Recipients are anesthetized with Isofluran (1000 mg/g) injected into small cotton in a syringe (Fig. 1) in the volume of 3 ml per mouse for around 10 minutes.

In the case of blastocysts transfer, around 15 blastocysts are chosen with the highest quality and put them separately into a small drop of the medium. Blastocysts are taken with a long pipette from medium and placed intrauterinally up to the cervix[17]. Anesthesia lasts for around 3 minutes, because surgery is not required. Pregnancy in mice can be detected visually after 15th gestation day[18].

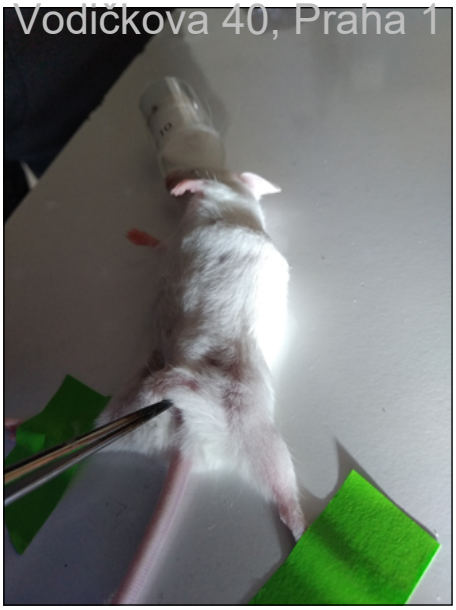

Figure 1. Female mouse anesthetized for embryotransfer. Orig. T. Duricek

PIGS

HORMONAL STIMULATION

Hormonal stimulation of pigs is a little bit different from mice. Pigs after estrus are preferred for estrous cycle synchronization. The first estrus occurs in 170–210 days old pigs. Pigs are treated with gestagens (Regumate, 5 ml per pig, added in food) for 15 days to block estrus induction. One day after gestagens removal from food, pig females are treated with 500 IU PMSG followed by 500 IU hCG 72 hours later. The 42–44 hours post hCG should estrus occur[19]. For the diagnosis of estrus in pigs, we use the visualization method. The vulva is swollen and red; pigs are discomforted with grunt with the presence of standing reflex. We are using probe male that is guided to females and determine estrus. Donors should be conceived with healthy males in the 2nd half of estrus. Same as in mice, the recipient should be younger than the donor.

EMBRYO COLLECTION

8–10 hours after ovulation, oocytes are naturally fertilized forming zygotes. Pigs are killed and ovaries with oviducts are placed in the Petri's dish. Ovaries are cut from ampulla that is washed out with phosphate-buffered saline (PBS) warmed to 37 ºC. Under the stereomicroscope, we search for zygotes and place these into Porcine Zygotic Medium 5 and culture at 38.5 ºC, 5% CO_2, 90% N_2 and 5% O_2 (reduced O_2 tension). After 3 hours, zygotes are microinjected with the proper mix of Cas9, gRNA, and template for gene editing[20-22].

EMBRYO TRANSFER

Recipients are anesthetized and placed in dorsal position (Fig. 2, 3). Using the abdominal laparoscopy technique, about 30 microinjected zygotes are placed into both oviducts (15 zygotes per one oviduct). The estrous cycle in pigs lasts 21 days, so the absence of the next estrus indicates successful embryo transfer and embryo implantation to the uterus. Gravidity can be confirmed after 28 days of gestations with ultrasonography.

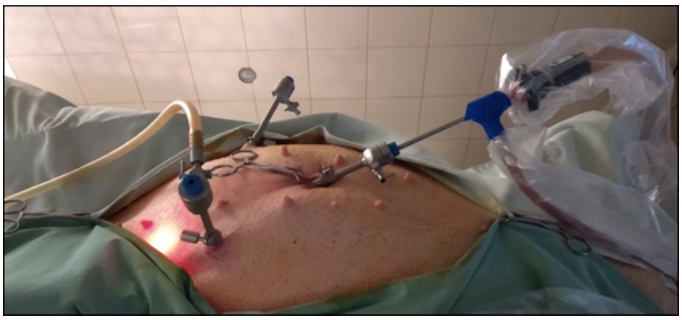

Figure 2. Anesthetized pig with laparoscopic instruments. Orig. T. Duricek

Figure 3. Embryos are transferred into oviduct trough veress needle (needle used for laparoscopic surgery). Orig. T. Duricek

CONCLUSIONS

Embryotransfer, as it is done today, was used for the first time in 1974, when the first foal was born using this approach[23]. Modern reproductive medicine is using IVF for human reproduction on a daily basis. From hormonal stimulation, up to the care of IVF patients, everything must be well performed. Embryo transfer must be professionally done because every intervention on embryo can bring serious problems. CRISPR/Cas9 microinjection is a modern technique to obtain gene-edited organisms, which is now commonly used in biomedical research.

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25 Micronucleus as a mark of genome integrity problems

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Abstract

Micronucleus (MN) is a small round and DNA containing structure located in the cytoplasm outside of the main nucleus. The presence of MN is considered as a sign of chromosomal instability. MN contains a part of an entire chromosome that was incorrectly segregated during anaphase and fail to incorporate into the daughter's cell nuclei correctly. Replication stress, un-replicated DNA, mitotic spindle defects, DNA damage, and non-functional DNA damage checkpoints are contributing to MN formation. Since zygote is a fundament of a whole new organism, the presence of MN in this cell can cause embryonal mortality, infertility or serious congenital disorders.

Keywords

micronucleus, DNA damage, DNA damage checkpoints, double-stranded breaks, replication stress, mitotic spindle defects

INTRODUCTION

Micronucleus (MN) is extra-nuclear body, which is formed during mitosis or meiosis as a reaction to incorrect segregation of chromosomes during anaphase[1]. It can arise from genotoxic chemicals, radiation, random mutations, replication stress or inability to attach spindle microtubules to centromeres on chromosomes during previous cell cycle[2]. The presence of MN is involved in pathogenesis of many diseases, but mostly, MN is presented in cancerous cells[3]. Besides cancer, cardiovascular patients have a predisposition for MN in erythrocytes, where MN are called Howell-Jolly bodies. The number of Howell-Jolly bodies arises in several spleen diseases, splenectomy, hemolytic anemia, idiopathic thrombocytopenia purpura or myelodysplastic syndrome[4]. The higher number of MN also occurs in peripheral

lymphocytes in patients with Alzheimer's and Parkinson's disease[5]. DNA damage from free radicals produced by lung cells is associated with chronic obstructive pulmonary disease, giving these patients predisposition for MN occurrence in blood cells[6]. Higher frequency of MN formation also occurs in serious congenital disorders like Ataxia telangiectasia or Falconi anemia, where patients have a mutation in genes regulating cell cycle or DNA repair[7].

FORMATION OF MICRONUCLEUS

MNs are mostly presented in the pre-cancerous cells. Understanding the mechanism of their formation is essential for the next research in cancer biology. There are several factors involved in the development of MN. Combination of DNA damage, especially double-stranded breaks (DSBs), defects in the mitotic spindle, replication stress, and un-replicated chromatin gives predisposition for mistakes in DNA segregation during mitosis linked with MN formation[8].

DEFECTS IN MITOTIC SPINDLE

physiologically, condensation of chromosomes occurs after nuclear envelope breakdown following lining up of chromosomes into the metaphase plate. Microtubules, polymers of tubulin that construct bipolar spindle, locate and attach to the kinetochores on the centromeres of chromosomes. It results in the tension of two sister chromatids, pulling them back into the opposite spindle poles during anaphase[9]. The result of this process is separation (segregation) of sister chromatids. Defects in mitotic spindle can lead to the inability to correctly attach microtubules to the centromere and results in the missegregation of the chromosome[10]. For example, inhibition of specific microtubule motors in chronic neurodegenerative Alzheimer's disease results in the failure of microtubule to locate and attach to kinetochore and fall-out of the unattached chromosome from a metaphase plate. It increases aneuploidy and MN incidences in daughter cells. Patients with Alzheimer's disease have up to 30 % of neurons containing aneuploidy[5,11].

REPLICATION STRESS AND UNREPLICATED DNA

DNA replication stress is characterized by slowing or stalling of fork progression[12]. Replication stress can be induced by unrepaired DNA lesions, over-expression of oncogenes, reduced density of replication origins, or misincorporation of ribonucleotides[13]. ATR kinase is a transducer molecule that is activated after replication stress. ATR kinase phosphorylates signalling molecules as histone protein H2AX at serine 139 (γH2AX), replication protein A (RPA), and checkpoint kinase 1 (Chk1), which helps to slow down cell cycle progression and suppress late origin firing. If the source of stress is removed, replication forks that were stabilized by the ATR pathway can restart the replication[14]. Also, chromatin contains sites that are more difficult to be replicated, known as replication barriers. Examples of replication barriers are

telomeres, repetitive sequences, or DNA lesions[15]. Since these sites are harder to replicate, they also create replication stress[12]. If the replication stress persists, or replication stress response components (ATR, RPA) are lost, fork fails to restart and collapse. When the cell enters mitosis with erroneous replication, where sister chromatid was not correctly replicated at centromere, tension of microtubules can disrupt chromosome and creates chromosome breakage, which again results in MN[12].

DNA DAMAGE

DNA is constantly under attack by exogenous and endogenous agents. DSBs occur when both sides of DNA helix break apart after the action of the serious DNA damage inductors like some chemical agents or UV irradiation. DSBs are the most critical DNA lesions because non-repaired breaks can lead to the chromosomal rearrangement or up to the loss of genetic information[16]. Accumulation of DSBs after serious DNA damage can lead to replication stress or mitotic spindle defects following incorrect segregation of DNA into daughter cells and MN formation. DNA damage response is a pathway that monitors DNA integrity. It plays a role in sustaining DNA integrity by detection of DSBs, delaying cell cycle progression to provide time to repair DNA, and eventually restart cell cycle progression after DNA repair[17]. Loss of capacity of DNA damage response is caused mostly by aging or congenital diseases like Ataxia telangiectasia or Xeroderma pigmentosum, which result in failure of genomic integrity, accumulation of DNA damage and formation of MN in somatic cells during mitosis[18].

The phenomenon, when thousands of clustered chromosomes rearrangements occur resulting in a massive loss of chromosome fragments, is called chromothripsis. Chromosome fragments formed during mitosis creates anaphase bridges and MN. Chromothripsis is a process, when chromosome fragments are stick together back to nucleus causing mutated genome[19].

DIAGNOSTIC DETECTION OF MICRONUCLEI

Diagnosis of the cells containing MN is mostly based on DNA fluorescent dyers, like 4',6-diamidino-2-phenylindole (DAPI) (Fig. 1), Propidium Iodide or Hoechst. It can be detected under the fluorescent microscopes using immunofluorescence[20,21] on fixed cells or "live cell imaging"[22] or by flow cytometry[23]. Flow cytometry is an automated technique for quantitative analysis used to analyze efficient MN scoring on the high number of cells within minutes[24]. The presence of whole chromosomes in MN can be detected by immunochemical labeling of kinetochore proteins or fluorescence *in situ* hybridization (FISH) using a combination of centromeric and telomeric probes[25].

Measuring the amount of chromatin in MN is determined by MN: nucleus ratio. The amount of DNA in MN is proportional to the genotoxic mechanism[26]. However, it is also very

important to determine the characteristics of MN, which are dynamically changing during cell cycle progression. Visualization of kinetochores by immunofluorescence in MN provides the information that MN was most probably caused by the failure of spindle assembly checkpoint (SAC is constitutively active in every mitosis)[27].

DNA damage detected by γH2AX signal, which is a common marker for DSBs, revealed that γH2AX signal in MN is increasing in the S phase and reaches a maximum in the G2 phase; what is deferent in the main nucleus where γH2AX signal decreases in G2. The presence of DNA damage in MN, but not in the nucleus, does not trigger apoptosis, and cells divide into daughter cells[26]. MN can replicate their DNA and incorporate itself later again into the nucleus. DNA synthesis detected by BrdU staining showed asynchronous DNA replication in MN in comparison to the primary nucleus[26]. Up to 38 % of cells with MN reincorporate MN during mitosis what causes chromosomal rearrangements[28]. Furthermore, MN positive in Lamin A/C antibody showed nuclear envelope around micronuclear chromatin[29].

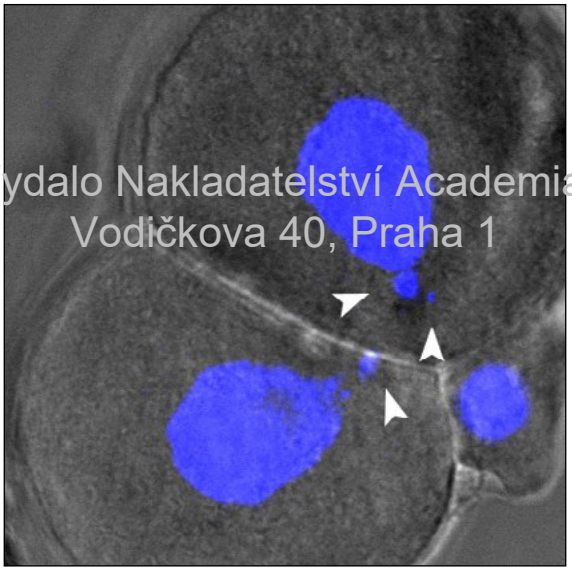

Figure 1. Chromatin in 2-cell stage embryo stained by DAPI (blue). Arrowheads marks MN. Orig. T. Duricek

FAITH OF MICRONUCLEI

As mentioned above, the presence of MN is a sign of rough violation of DNA integrity and gives cell predisposition for the pre-cancerous state. The fate of the cell is determined by the level of the loss of genetic information, and the status of chromosome rearrangements.

There are 4 main outcomes of MN in cells: degradation, reincorporation, extrusion, and persistence in cell[30]. Degradation of chromatin in MN can be done by enzymatic degradation of DNA, degradation by autophagy, or apoptosis-like processes restricted to MN. However, the percentage of cells with MNs, when only MNs are degraded is very small. More cells with MN are found to be apoptotic than viable[30]. Secondly, MN can be reincorporated into the main nucleus. This can happen rather during mitosis, than during interphase. Reincorporation of MN can cause chromosomal abnormalities, like chromosomal rearrangements[30]. Extracellular extrusion of MN (with the main nucleus) occurs physiologically in mammals in erythrocytes during maturation from erythroblasts[31]. Extrusion of MN is mediated by the blebbing (bulge) of a cytoplasmic membrane without DNA degradation[32]. When cells are not able to eliminate MN, it persists and is inherited for several generations. This phenomenon occurs in early embryonic stages, where a higher number of MN occurs with each division because chromosomes repeatedly fail to be segregated[33].

MICRONUCLEUS IN EMBRYOS

Embryonic aneuploidy is a dominant factor of *in vitro* fertilisation (IVF) failure. Understanding the formation of MN in germ cells is essential for reproductive medicine and successful IVF rate[34]. Zygote with its 2 pronuclei is a very specific cell in comparison to the somatic cell. Male pronuclei must undergo major chromatin changes, first cell divisions occur without transcription, and first cell cycles serve to multiply cell mass for further development and cell differentiation[35]. Around 5 % of human and mice 2-cell embryos contain MN. The number of MN arises in mice embryos with the number of divisions and are inherited by mother cells[33]. For example, 75 % of embryos in the morula stage contained MN[33]. Inheritance of MN doesn't seem to stop the embryo from developing but contributes to mosaicism. Still, it depends on which cell stage MN occurs. The presence of MN in first mitotic divisions is more critical to the embryo and will more likely undergo mitotic arrest[36]. During advanced embryonal development, cell competition and blastomere exclusion serve as a mechanism that compares the condition of cells and eliminates cells with worsen condition[37]. It can be one of the mechanisms that ensure DNA integrity during embryo development. Still, unrepaired and non-eliminated cells can give predisposition for congenital disorders.

CONCLUSIONS

The presence of MN is a sign of chromosomal instability and pre-cancerous state of the cell. MN is a consequence of disturbed DNA integrity and is also involved in the pathogenesis of several diseases. DNA damage, replication stress, and spindle defects contribute to MN

formation. The diagnosis of MN depends on fluorescent dyes detected by fluorescence microscopes or flow cytometry. There are four possibilities of MN faith – degradation, extrusion, reincorporation, and persistence in cells. Degradation and extrusion occur less frequently under specific conditions, reincorporation mostly during the next mitosis, and cells, where MN persists, can undergo tumor transformation. MN can be persisting in cells over several generations and distributed into daughter cells nuclei. Detection of MN faith is important for the evaluation of genotoxicity screening and monitoring of chromosomal instability in time. MN in germ cells is especially dangerous because MN in spermatids may lead to infertility, and early embryo containing MN can cause embryonal mortality or serious genetic disorders.

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26 Cell cycle regulation in early embryonic development

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Abstract

Events leading from DNA replication to cell division are called cell cycle progression. The proper coordination of these processes is necessary for ensuring the viability of newly developed cells. This necessity is very notable in early embryonic divisions, where cell cycle deregulation can lead to serious problems, such as developmental disorders or infertility. This chapter is concerned with cell cycle regulation in general and in the early development of model organisms such as *D. melanogaster*, *X. laevis*, *D. rerio,* and mammals. It focuses on different developmental strategies and the adaptation mechanisms of coordinating cell cycle progression with the genome activation among these species.

Keywords

cell cycle progression, CDK, genome activation, *Drosophila melanogaster*, *Xenopus laevis*, *Danio rerio*, mammals

INTRODUCTION

The sequence of events leading to cell division is called the cell cycle. Irregular cell cycle progression can lead to serious diseases with the most negative impact during early embryonic development. A faulty cell cycle regulation in early embryonic development can cause, in extreme cases, abortion, developmental disorders, or infertility.

CELL CYCLE REGULATION

Cell cycle progression divides into five successive phases: First gap phase (G1), DNA synthesis phase (S), second gap phase (G2), mitosis (M), and cytokinesis. Chromosomes are replicated in the S phase, divided in the M phase, and the cell divides during cytokinesis. Gap phases function as preparation for the following phases. G1, S, and G2 are called together as interphase. Quiescence or G0 is a state when a cell exits cell cycle progression but it can resume it. If a cell permanently exits cell cycle progression because of, e.g. extensive DNA damage and is unable to resume it, it is called cellular senescence.

Cell cycle progression is driven by the family of cyclin-dependent kinases (CDKs). CDKs are negatively regulated by Wee1 and Myt1 kinases, which phosphorylate CDKs on threonine 14 (Thr 14) and tyrosine 15 (Tyr15). CDKs are positively regulated by Cdc25 phosphatase family, which antagonizes Wee1/Myt1-dependent phosphorylations[1,2]. Another positive regulator of CDKs is CDK-activating kinase (CAK)[3]. The activity of CDKs is also dependent on association with their cofactors cyclins. Cyclins exhibit various affinities to various CDKs, and their regulation of CDKs also lies in different spatial and temporary expression and degradation during cell cycle progression and development[4]. CDKs are inhibited by binding of a group of small proteins called protein inhibitors of CDKs (e.g. p16, p21)[5]. Apart from the above-mentioned regulatory mechanisms, CDKs are also regulated by their localization. The primary localization regulatory mechanism is a translocation between cytoplasm and nucleus. It ensures spatial and temporary separation or association of reaction partners[6].

The most important CDKs are CDK1, CDK2, CDK4, and CDK6. Cell cycle progression through the G1 phase is ensured by the combined activity of CDK4 and CDK6 with distinct cyclin D isoforms (D1, D2 or D3). CDK2 with cyclin isoforms E1, E2 induces the transition from G1 to S phase. The S phase progression is stimulated mainly by CDK2 with cyclin A (CDK2/A). CDK2 and CDK1 with cyclin A or B regulate G2 and M phase progression[7,8]. CDK1/B1 complex with Greatwall kinase is called mitosis promoting factor (MPF). It is the main regulator of mitosis and cytokinesis progression[9–11].

EARLY EMBRYONIC DEVELOPMENT IN METAZOA

The general cell cycle progression mentioned above is typical for somatic cells. This chapter is concerned with the differences in cell cycle progression in early embryonic development in metazoa.

At the beginning of life, oocytes are arrested in the prophase I of meiotic division and wait for the signals to resume meiosis. After meiotic resumption, oocytes mature into metaphase II where fertilization can occur. Once fertilized, the zygotes develop to form an embryo. Early embryonic development is regulated by stored maternal factors (mRNAs, proteins)

until the genome is activated. Embryonic genome activation consists of epigenetic genome reprogramming, transcription initiation, and maternal factors degradation. Genome activation is a continuous process with the main part referred to as "a major phase" when all the above-mentioned processes are in full progress[12].

Metazoa adopted various strategies to coordinate cell cycle progression in early embryonic development with the genome activation. Early embryonic development differs significantly between flies, frogs, fish, and mammals. The embryos of *Drosophila melanogaster* divide rapidly in 13 cycles alternating only S and M phase without cytokinesis[13]. As a result, the cells form a syncytium, which is called a syncytial blastoderm. The cellular membrane is created during the 11th cell cycle and the embryos form so-called cellular blastoderm[14]. The embryonic genome is activated in the 13th cell cycle and the cells resume cell cycle progression also containing both gap phases[15,16]. The embryos of *Xenopus laevis* also divide during successive rapid divisions containing only S and M phases, but the cytokinesis is present. During the 13th cell cycle, the rapid divisions slow down and the major phase of genome activation begins. The embryos of *Danio rerio* embryos also experience rapid successive cell cycle divisions with genome activation taking place in the 10th cell cycle[17]. The average length of the rapid cell cycles in *D. melanosgater* is 8 min, in *X. laevis* 35 min (after the first cell cycle lasting for 85 min) and in *D. rerio* 15 min. First gastrulation movements of these species take place a few hours after fertilization[18–20]. Mammalian cell cycle progression differs significantly from either *D. melanogaster*, *X. laevis* or *D. rerio*. It contains all cell cycle phases including gap phases, and cell cycle length is almost ten times longer in comparison to either *D. melanogaster*, *X. laevis* or *D. rerio*. The first cleavage in mammals occurs approximately 20 hours after fertilization, and first gastrulation movements occur days after fertilization[21]. In mouse, the estimated time of the first G1 phase is 5 h, of both S phase and G2 phase 5 h and M phase 2 hours. In the 2-cell stage embryo, the length of the G1 phase is estimated for 1 h, S phase for 5 h, G2 for 12 h and M phase for 1 h[22]. Mammalian cell cycle progression also differs between species. The major phase of genome activation in mouse takes place in the G2 phase of the 2-cell stage including massive transcription progression and large chromatin remodelling. In other mammalian species, the major phase of genome activation takes place also in the G2 phase but at later stages – in 4- and 8-cell stage in pigs and humans and in 8- and 16-cell stage in cows[12]. The diversity in cell cycle length among *D. melanogaster*, *X. laevis*, *D. rerio* and mammals is evident. *D. melanogaster*, *X. laevis,* and *D. rerio* develop externally from the mother in contrast to mammals, which develop internally. This is probably the reason where the different evolutionary strategies originate.

The difference of mammalian early embryonic development in comparison to *D. melanogaster*, *X. laevis* and *D. rerio* also lies in the activation of DNA damage and repair signalling pathways. DNA damage signalling pathways are almost non-functional during rapid cell cycles in either of these species. In *D. melanogaster*, DNA damage checkpoint mechanism becomes massively activated with cell cycle lengthening in the 13th cell cycle. The underlying

mechanism has been shown to be dependent on DNA damage created by the collision of replication and transcription machinery, which occurs during genome activation[23]. In *X. laevis*, DNA damage response signalling is actively suppressed by Rad18 ubiquitin ligase. Rad18 ubiquitin ligase is associated with DNA translesion polymerase during replication and prevents the formation of single-stranded DNA. As a result, Rad 18 suppresses in this way DNA damage response signalling and also cell cycle checkpoint activation[24]. Cell cycle checkpoint activation occurs with genome activation also in *D. rerio*, although the underlying mechanism is currently not known. Zhang et al. recently found that one of the key players in *D. rerio* is checkpoint kinase 1 (Chk1)[25].

On the other hand, the mammalian DNA damage response signalling pathway is activated from early cell cycle stages in the zygotes. In mouse, the paternal genome is demethylated in the G1 phase in zygotes. The demethylation processes cause DNA single or double-stranded breaks, DNA damage response signalling and DNA repair[26,27]. Also, S and G2 DNA damage checkpoint are already in function in the zygote[28,29]. However, although p53 is used as a mediator in DNA damage response signalling pathway, cell cycle arrest mediated by p21 protein is not activated until morula stage[30]. As p53 functions as a transcription factor for p21, the delayed activation of cell cycle arrest mediated by p21 is caused probably by genome activation, which takes place in the 2-cell stage in mouse embryos. The signalling pathway leading to apoptosis is not activated until the blastocyst stage in mouse embryos[30].

In zygote, DNA damage response signalling dependent on p53 is initiated in both pronuclei also in the presence of DNA damage only on paternal chromatin from an irradiated sperm[31,32]. This finding suggests a signalling crosstalk between both pronuclei[31]. As shown above, early embryonic cell cycle progression in mammals is quite different from *D. melanogaster, X. laevis* and *D. rerio*. Notably, mammalian zygotic genome activation occurs earlier in terms of cell cycle numbers, but later in the term of time[12]. Coordination of cell cycle progression with genome activation is necessary to ensure proper development. Uncoordinated genome activation with cell cycle progression can lead to DNA damage caused by replication-transcription collision or DNA damage caused by unsuccessful histone modification. If this damage remained unrepaired also through mitotic progression, it could lead to serious diseases and infertility.

CONCLUSION

Proper regulation of cell cycle progression at the beginning of life is crucial. Cell cycle progression regulation depends on the right spatial and temporary translation of cell cycle progression factors. Erroneous cell cycle regulation can cause DNA damage. The DNA damage caused in first divisions is inherited by most of the cells in an organism and if the DNA damage is inefficiently repaired, it could lead to serious diseases or in extreme cases, to abortions and infertility. Cell cycle regulation in early embryonic development differs from

somatic cells in many aspects. DNA replication and cell division have to be coordinated with genome activation, including genome reprogramming and remodelling, transcription initiation, and clearance of maternal products. Various species developed different mechanisms to cope with all these procedures and to protect their genome. The embryos of *D. melanogaster*, *X. laevis* and *D. rerio* develop externally from the mother, and their survival strategy is based on fast embryonic development with many progenies, where some progeny may not survive if DNA damage is extensive. On the other hand, mammals develop internally in the mother and their survival strategy is based on slower development with lesser amounts of progeny but ensuring enough time to correct arising damage. These are the basic strategies which various species developed to ensure efficient cell cycle regulation and thus the development of the disease-free individual.

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27 DNA damage response signalling and cell cycle checkpoint activation in regular and aberrant cell cycle progression

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Abstract

DNA damage response signalling and cell cycle checkpoint activation guard against genome instability, which can be detrimental for cellular development. The purpose of the DNA damage response signalling pathway is to slow down cell cycle progression by the activation of cell cycle checkpoint signalling to provide time for repairing of DNA damage. This chapter is concerned with the molecular characterization of these pathways.

Keywords

DNA damage response, S-phase checkpoint, G2/M checkpoint, ATM, ATR

Many human diseases originate from unrepaired DNA damage. Among such diseases belong, for example, cancer susceptibility syndromes, e.g. Louis–Bar syndrome (Ataxia–telangiectasia)[1–3]. Unrepaired DNA damage can be detrimental in mitosis, where it can result in defects in chromosome segregation or micronuclei formation.

There are different types of DNA damage (lesions), e.g. single or double-stranded DNA breaks, bases modification, nucleotide analogs incorporation, and interstrand cross-links[4]. Among the most serious DNA damage belong double-stranded DNA (dsDNA) breaks (DSBs). DSBs arise from exogenous sources such as ionizing radiation or from unprotected single-stranded DNA (ssDNA), which is submitted to pressure or distortions. Such ssDNA can emerge during replication, e.g. from nucleotide exhaustion or transcription-replication collision, or it can be caused by other chemical agents and mechanical events (reviewed in[4–6]).

DNA damage response signalling pathway is activated in response to DNA damage. The main purpose of this signalling pathway is to stop cell cycle progression and repair the DNA damage. DNA damage response signalling pathway coordinates DNA repair with other cellular processes, such as cell cycle progression, replication, or cellular division (reviewed in[7]). Successful DNA repair results in resuming cell cycle progression. Unsuccessful repair results in cell cycle exit and entering into the cellular senescence (reviewed in[8]), or in programmed cell death (e.g. apoptosis, reviewed in[9]).

DNA damage response signalling pathway consists of many signalling partners that interact among themselves in a linear fashion or positive or negative feedback loops. Firstly, DSBs are detected by the MRE11-RAD50-NBS1 (MRN) sensor complex. MRN complex activates transducing kinase ataxia telangiectasia mutated (ATM)[10], which in turn phosphorylates histone variant H2AX on serine 139. This phosphorylation is further referred to as γH2AX[11]. The γH2AX and ATM signalling recruit other DNA damage mediators such as ATR kinase, MDC1, tumor suppressor p53-binding protein 1 (53BP1), TOPBP1, and BRCA1[12,13]. MDC1 also activates ATM in a feedback loop. This amplification loop ensures the spreading of γH2AX signal on both sides of DSBs and the formation of spots called γH2AX foci. The γH2AX foci can be easily detected by immunofluorescence labeling and confocal microscopy as a proxy of DSBs amount. MDC1 further interacts with RNF168 ubiquitin ligase which then recruits DNA repair signalling proteins 53BP1, BRCA1, BRCA2, and CTIP[14]. Activated ATM kinase also phosphorylates downstream kinases checkpoint kinase 1 (CHK1) and checkpoint kinase 2 (CHK2). CHK1 and CHK2 kinases activate downstream effectors tumor suppressor protein p53 (p53) and inhibit CDC25 phosphatases. p53 is a transcription factor that is also stabilized by ATM phosphorylation[15] and as an effector activate other factors, e.g. Wee1 kinase to antagonize activity of cyclin-dependent kinases (CDKs) or as a transcription factor for CDKs inhibitor $p21^{CIP1/WAF1}$ to stop cell cycle progression[16]. Additionally, DNA damage response and DNA repair proteins are coordinated at DNA damage sites through various posttranslational modifications such as poly(ADP-ribosyl)ation, ubiquitination, SUMOylation, and acetylation (reviewed in[17]).

DNA damage cell cycle checkpoint is operational during S phase to ensure error-free replication completion[18]. The purpose of S-phase checkpoint signalling is replication fork stabilization, origin firing inhibition, and CDK inhibition after replication stress. The essential replication checkpoint kinases are ATR and CHK1[19–21]. S-phase checkpoint is activated after replication stress by ssDNA strands, which arise from replication helicases and are coated by RPA protein[22]. RPA is considered to be a rate-limiting factor[5]. RPA protein recruits ATR/ATRIP complex, which with the help of ETAA1, TOPBP1 and claspin, activates CHK1 kinase[23]. Activated CHK1 is cleaved by SPRTN protease and released into the nucleus[24]. ATR and CHK1 kinases than together operate to delay cell cycle progression and enable to repair DNA damage[5,13]. ATR kinase also prevents global exhaustion of RPA to protect ssDNA strands. Apart from that, ATR kinase activates recombination regulators such as BRCA1, WRN

and BLM helicases[23,25]. CHK1 kinase signalling restrains the activity of CDKs and slows cell cycle progression. Specifically, CHK1 kinase activates Wee1 kinase and inhibits phosphatases from CDC25 family[21]. Activated CHK1 kinase targets CDC25A phosphatase for degradation or restrains mitotic activity of CDC25A phosphatase by binding of 14-3-3 protein[26,27]. During interphase or after DNA damage, CHK1 kinase phosphorylates CDC25C phosphatase, which is sequestrated in cytoplasm by 14-3-3 protein binding[28]. During G2/M cell cycle transition, CD25C phosphatase is phosphorylated by PLK3 and targeted to nucleus[29,30]. During interphase (independent on DNA damage signalling) CHK1 kinase also restrains CDC25B activity on centrosomes[31,32]. Apart from that, CHK1 kinase also phosphorylates PCNA to recruit translesion DNA polymerase to stalled replication forks[13].

DNA damage cell cycle checkpoint signalling also works during G2 phase to detect new or residual DSBs[33]. The DNA damage response pathway acts mostly via ATM activation (as mentioned above), although also ATR activation is present. The purpose of the G2 cell cycle checkpoint is to stop cell cycle progression to mitosis via CDK1/B inhibition. To ensure CDK1/B inhibition, CHK1 kinase targets CDC25A phosphatase for degradation and activates Wee1 kinase[21,27]. It was taught that the final decision of commitment to mitosis is taken by CDK1/B1 activaton of cyclin B1 and its translocation into nucleus[34]. However, it was proposed recently that the duration of the G2/M checkpoint is determined by the activation of mitotic kinase PLK1, which in turn activates CDKs and other mitotic factors[35,36]. PLK1 kinase is activated by WIP1 phosphatase, which antagonizes ATM- and ATR-dependent phosphorylations. It was concluded that the final commitment to mitosis is decided by the balance of ATM and WIP1 activities[35]. This hypothesis implies that commitment to mitosis is not dependent on DNA repair completion, and some DNA damage can remain unrepaired. Potential residual DNA damage in mitosis can generate anaphase bridges. Most anaphase bridges are resolved during anaphase or telophase. However, unresolved anaphase bridges can result in severe genomic instability[6,37–39].

Cell cycle checkpoint, which ensures that the cell enters mitosis only when DNA replication was finished, is called S/M checkpoint. The S/M checkpoint is permanently active in each S phase. The ETAA1 factor activates ATR, which activates CHK1, which inhibits CDK1 and also FOXM1 transcription factor. FOXM1 is activated after replication completion and triggers mitotic program[40].

DSBs are repaired by non-homologues end joining (NHEJ), homologues recombination (HR) or alternative NHEJ[14,41]. HR is active in S and G2 phase, when the cell can use sister chromatid as a template for DNA repair. NHEJ is active in all cell cycle phases, but is predominant in G1, where HR is not functional. In HR, MRN senses the DSB, recruits CTIP for DNA end resection to produce ssDNA strand overhand. ssDNA strand is coated by RPA and Rad51. It forms the Holiday junction. The sister chromatid is used as a template for DNA polymerization of the resected DNA strand[14]. The signalling network is more complex and also depends on ATR and Chk1 activation of Rad51[42].

The decision to repair DNA by NHEJ mechanism is directed by 53BP1 protein[12]. In NHEJ, Ku70-Ku80 helicases sense DSBs and recruit DNA-PK. DNA-PK recruits Artemis for DNA end resection to blunt ends. Then DNA ligase ligates both DNA ends together[4,41].

Incomplete DNA repair or non-functional DNA damage checkpoint can lead to uncontrolled proliferation and cancer. The classic hallmarks of cancer are sustained proliferation, growth suppressor evasion, apoptotic signals resistance, cellular motility acquisition, infinite replicative potential, and angiogenesis induction[43]. Many of these hallmarks are triggered by non-functional or incomplete transcription or translation of DNA damage response and repair proteins[44].

In recent years, some studies showed that there is not a clear threshold distinction between S and G2 phase[45]. The phase which we call the G2 phase may serve to finish the replication of difficult sites and can be considered as a very late S phase. The reason could be that in the past, there were no tools sensitive enough to detect replication outside of S-phase. This would also lead back to the first hypothesis that cell cycle checkpoints are mechanisms to prevent cell division until replication is finished[46]. However, it has not yet been proven that replication intermediates could directly inhibit mitosis by permanent activation of cell cycle checkpoint[45–47].

Also, it seems that it is not stringent ON and OFF state of the checkpoints. More studies show that cell cycle progression is made after crossing a threshold of negative and positive amplification signalling loops when cell cycle signalling machinery countervail from a "non-go" to a "go" state[48–50]. This was proposed, e.g. for mitosis commitment, when some cells divide even when not all DNA damage is successfully repaired.

DNA damage response signalling pathway and cell cycle checkpoints guard against severe DNA damage, which can result in genome integrity defects and cancer development. Continually, our understanding of checkpoint function is shifting from a binomial state (ON and OFF) to rather a continuous process, which at some point results in irreversible mitosis commitment.

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28 The role of protein kinases during mammalian oocyte meiosis

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Abstract

Proper formation of the spindle during the first meiotic division is crucial for correct chromosome segregation and formation of a functional oocyte. Aurora kinases and Polo-like kinases are involved in the mechanisms regulating proper spindle assembly. Both types of these kinases have their specific role to play independently and in cooperation. If anything goes wrong in meiotic spindle assembly, it can lead to infertility or birth defects, as incorrect chromosome segregation causes aneuploidies. Some of these defective chromosome segregations are incompatible with life, some cause birth defects such as Down´s syndrome. Humans are especially prone to these aneuploidies, making the research of Aurora kinases and Polo-like kinases particularly appealing.

Keywords

oocyte, spindle, Aurora kinases, Polo-like kinases

INTRODUCTION

Mitosis and meiosis are both a type of cell division. The major difference between them is the number of cells that are formed as a product of these cell divisions, and the number of chromosomes found in these newly formed cells.[1] Mitosis is a type of cell division, where a single cell divides into two cells.[2] During mitosis, chromosomes align along the equator in the middle of the cell and microtubules coming from centrosomes get attached to centromeres. The identical chromatids of each chromosome are then pulled apart, each one to the opposite pole of the cell. The results are two identical nuclei, with the original number of chromosomes.[1] During meiosis, one cell divides twice into four daughter cells. Meiosis is a kind of cell division that is necessary for sexual reproduction, as it produces

gametes – haploid sex cells (oocytes and sperm). To keep genetic diversity in species, two processes are at play: meiosis, through which the number of chromosomes is reduced from diploid to haploid, and fertilization, which combines two haploid cells (oocyte and sperm) into a new diploid cell.[3–5]

Any defects in chromosome segregation that occur during meiosis can lead to aneuploidy, which humans are particularly prone to, with over 20 % of all eggs being aneuploid.[5] The majority of these defects are formed through missegregation, and most of them are incompatible with life and are a leading cause in miscarriages and infertility in humans. Other possible outcomes are birth defects, one of the most common being Down's syndrome.[3]

For non-defective chromosome segregation, the assembly of a bipolar spindle is crucial.[6] There are some key differences between meiotic and mitotic spindle. Meiotic division of mammalian oocytes is asymmetric – the oocyte preserves its ooplasm while excluding small polar bodies.[7] Because of the oocyte size and its asymmetrical spindle positioning, the oocyte needs a customized mechanism to form the meiotic spindle. One from the mechanism is acentriolar spindle assembly, in which spindle formation is dependent on the clustering of many microtubule organizing centers (MTOCs) into functional spindle poles. The major difference between mitotic centrosomes and meiotic MTOCs is the absence of centrioles in MTOCs.[8] However, these MTOCs consist of pericentriolar material[9] similar to mitotic centrosomes, like CEP192[6], pericentrin[10], and γ-tubulin.[9] Defective fragmentation of MTOCs causes further errors in spindle assembly and may lead to aneuploidy[6]. Defects in MTOCs clustering, despite the presence of bipolar spindle, were also found to be accompanied by segregation errors.[11]

The oocyte spindle formation must be strictly regulated by numerous factors, to correctly reduce the number of chromosomes from diploid to haploid, and retain as much of the original cytoplasm as possible. The mechanism which regulates oocyte meiosis must cooperate, to form specifically sized and positioned spindle.[12] The precise molecular mechanisms that regulate spindle assembly and MTOCs behaviour in mammalian oocytes remain unknown.[13] In these processes, identifying the role of specific protein kinases, most notably Aurora kinases and Polo-like kinases, is crucial for the understanding of the underlying mechanisms.

AURORA KINASES

The cell cycle, including the cell division, is strictly regulated by many different factors, one of which are Aurora kinases (AURKs), a group of serine/threonine kinases.[14] There are three known AURKs encoded in mammalian cells. Most somatic cells express two of these kinases, AURKA and AURKB, while mammalian germ cells are known to also express a third one, AURKC.[15] These AURKs have an important role in regulating the spindle formation, chromosome alignment, cytokinesis and other key processes in mitosis as well as meiosis.[16]

AURKA is in metaphase of meiosis I and II (Met I and Met II) localized to spindle poles, where it regulates spindle mechanism and spindle organization.[16] An important binding partner to AURKA is Bora. It is responsible for activating AURKA as well as its ability to phosphorylate other kinases, e.g. Polo-like kinase 1. Depletion of this protein causes a deficit in AURKA localization, resulting in the defective spindle and chromosome misalignment.[17] AURKA is also responsible for recruiting γ-tubulin to MTOCs if it is not present (due to a knockout), γ-tubulin, as well as pericentrin, are not recruited and MTOCs cannot cluster correctly and gather at the spindle poles, resulting in spindle disintegration.[18] Overexpression of AURKA will in this case also lead to the defective and abnormal spindle, therefore it has to be strictly regulated, as too much and too little of it negatively affects spindle formation.[14]

AURKB is in mitosis first localised on chromosomes and later in anaphase, it is distributed on the spindle midzone. First, it regulates chromatid condensation, chromosome alignment, attachment of microtubules to kinetochores, cohesion and then cytokinesis.[19] In meiosis, the specific role of AURKB is not as well explored as in mitosis. Antibodies detecting AURKB in somatic cells are probably not sensitive enough to detect AURKB in mammalian oocytes, making it difficult to actually localize AURKB.[19] Even though during Met I and Met II AURKB is absent from chromosomes and is localized to the spindle[20], it still probably plays an important role in regulating chromosome alignment. Treatment of mouse oocytes with a low concentration of dual AURKB/C inhibitor resulted in a chromosome misalignment during meiosis I (MI). Overexpression of AURKB, but not AURKC, partially rescued these defects.[19]

AURKB and AURKC have some specific functions, and some similar (overlapping) functions, and in some cases, one is able to compensate for the other. It is difficult to distinguish the specific functions of these two kinases that do not overlap. Even though AURKC seems to be the dominant kinase of chromosomal passenger complex (CPC) over AURKB in mouse oocytes, AURKB compensates for AURKC in case it is absent.[20]

AURKC is exclusively expressed in mammalian germ cells (apart from a few somatic tissue types and human cancer cells).[14] During meiosis, AURKC has a similar localization and function on the chromosomes as mitotic AURKB, but unlike AURKB, AURKC also localizes to the spindle poles. On the chromosome, it is localized on the CPC, which is a multi-protein complex composed of proteins INCENP, Borealin and survivin, in conjunction with AURKC (in mitosis AURKB).[21] AURKC acts as a catalytic subunit in CPC.[21] This complex is changing its location during meiosis. First, it is localized to centromeres and the interchromatid axes in metaphase I, then its localization is changed to the spindle during anaphase.[22] Even though the absence of AURKC leads to chromosome misalignment and MI arrest, some oocytes are able to complete meiosis. As it turns out when AURKC is not present in the oocyte, AURKB can compensate for its loss and take over its function and localization.[14]

Because of the lack of centrosomes in oocytes, many MTOCs are involved in bipolar spindle assembly.[8] Recently it was published that AURKC is regulated by the kinase Haspin and colocalizes with γ-tubulin, which revealed AURKC localized with MTOCs. Haspin ensures, that

AURKC is localized at chromosomes and MTOCs in mouse oocytes. It is also required for the correct clustering of MTOCs and its inhibition destabilizes MTOCs. MTOC clustering defect after Haspin inhibition can be rescued by overexpression of AURKC, which also confirmed its importance in regulating MTOC function.[11]

New functions for three AURKs was recently described by Nguyen at al[14]. The authors found that in mouse oocytes AURKA can compensate for the loss of AURKB and AURKC and thus support meiotic progression. They described that AURKB negatively regulates AURKC activity and AURKC prevents AURKA chromosome localization. Though some roles that the AURKs play in chromosome segregation and spindle assembly are known, the details of their activity and potential cooperation still remain to be studied.

POLO-LIKE KINASES

Another kind of kinases that play a significant role in cell division are Polo-like kinases (PLKs). PLKs (just as AURKs) are serine/threonine kinases that regulate the cell cycle and cell division.[23] Many different PLKs are found in the mammalian genome and their function has been well studied in mitotic cells, most notably PLK1 and PLK4.[24,25]

PLK1 controls the timing of mitotic entry, centrosome maturation, kinetochore-microtubule attachment, chromosome cohesion, and cytokinesis.[24] In meiotic cells, it plays a role in spindle formation, resumption of meiosis and regulating the MTOC fragmentation.[8] Once the cell enters meiosis, PLK1 cause the MTOCs to decondense. After decondensation, MTOCs are stretched along the nuclear envelope by BicD2-anchored dynein. Following the nuclear envelope breakdown, MTOCs are fragmented by kinesin KIF11 and evenly distributed, creating the two spindle poles. If PLK1 is inhibited, MTOC decondensation is completely blocked and the spindle polarization cannot continue.[6]

Even though some roles of PLK1 are known, the specifics of how it works or what exactly it does in meiosis (specifically in oocytes) remains relatively unknown. In recent years, many advances were made, one of them being the study on mouse oocytes by Solc et al.,[26] where the results revealed the multiple functions of PLK1 in oocyte meiosis, like promotion of the meiotic spindle assembly, chromosome segregation at anaphase I, resumption of meiosis and even the onset of nuclear envelope permeabilization.[26]

The meiotic spindle is very sensitive to stimuli from outside and inside. The main factor responding to these signals is PLK1. Many of these signals affect the quality and fertility of the oocyte.[27] PLK1, aside from responding to external and internal signals, is a crucial regulator of the spindle formation in both MI and MII, as it has an important role in regulating DNA repair system.[28] In contrast, pig oocytes with inhibited PLK1 showed no signs of spindle assembly defects, however, a significantly higher number of oocytes showed defects in chromosome segregation. In pig oocytes, PLK1 has been shown to be

very important for an oocyte to transition from MI to MII. This remains to be proven in mouse oocytes.[27]

In the early mouse embryo, polo-like kinase 4 (PLK4) is required for centriole duplication.[29] It is also needed in the acentriolar oocyte for dynamic microtubule nucleation. PLK4 localizes with MTOCs and is one of the factors that activate microtubule nucleation during the resumption of meiosis. If PLK4 is absent in an early embryo, it results into the monopolar spindle, however, in oocytes, the absence of PLK4 still results in a bipolar spindle, showing that other factors are able to trigger microtubule nucleation without the presence of PLK4.[18]

PLK AND AURK CASCADE

In addition to AURKs and PLKs individual roles in spindle assembly and centrosome maturation, they are both important for entering mitosis on time. Research conducted on this topic showed a link of functions of PLK1 and AURKA, and the dependence of timely mitotic entry on PLK1's need to be activated by AURKA. Above mentioned AURKA binding partner Bora has a role in this process as it binds the two kinases together and stimulates PLK1 phosphorylation by AURKA, which activates a signal cascade causing phosphorylation of CDK1, that into mitosis.[30]

Both AURKA and PLK1 are a part of the formation of the bipolar spindle in meiosis I, as mentioned above. In addition, AURKA cooperates with PLK4 to jointly contribute to microtubule nucleation. Both PLK4 and AURKA have some independence when it comes to initiating microtubule nucleation but also work together with RanGTP (guanosine triphosphate-bound Ran protein) gradient to produce non-defective acentrosomal spindle. MTOCs require AURKA to be properly distributed, and AURKA might also be able to regulate the activity of PLK4.[18]

CONCLUSIONS

For most organisms, aneuploidy is rare. Humans are prone to defective chromosome segregation, which frequently leads to miscarriages and even infertility.[14] In humans, about 20 % of oocytes and only 1–4 % of sperm are aneuploid. This might be due to the different timing of meiosis in the two genders. While males continuously undergo spermatogenesis, females are born with a complete set of oocytes arrested in metaphase I, that go through the first prophase of meiosis during prenatal development. After ovulation, oocyte re-enters meiosis and is arrested in metaphase II until fertilization, after which it completes the second meiotic division.[19]

All factors regulating meiotic division (including AURKs and PLKs) must perform accurately, otherwise, chromosomes might not be segregated evenly, thus leading to aneuploidy. Research has shown, that some AURKC and AURKB defects (loss-of-function alleles etc.) lower male fertility and some can even cause male sterility, and presumably somehow affects female fertility, but given the ability of AURKs to compensate for each other in mouse oocytes, human oocytes might be able to form viable eggs.[14]

Human oocytes express AURKA, and both AURKB and AURKC. Healthy somatic cells express only AURKA and AURKB, but in many cancer lines, all three AURKs are overexpressed. Therefore, Aurora kinases have been an interesting subject of cancer studies.[31] Today, the roles of Aurora kinases and Polo-like kinases in mitosis are well known. Moreover, AURKs have been shown to affect male fertility. However, further specifics of AURKs and PLKs in human oocytes and how they affect female fertility still remain to be discovered.[14]

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29 DNA damage in oocytes during reproductive aging

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Abstract

With the increasing age of a woman, her reproductive capacity decreases, which is mainly due to a loss in an oocyte store in the ovaries and an increasing amount of double-stranded DNA breaks accumulating in the remaining oocytes. Age-related increase in DNA damage in oocytes correlates with decreased expression of DNA repair genes.

Keywords

Oocytes, ovarian aging, DNA double-strand breaks, DNA repair genes

POSTPONING OF MOTHERHOOD VS. FERTILITY PERIOD OF A WOMAN

Over the past years, more women are delaying marriages and gestation, while focusing more on career and personal goals[1]. Infertility associated with age is a growing common problem[2]. The length of a woman's reproductive period and ability to reproduce depends primarily on the number of oocytes stored in the ovaries. This predetermined amount of oocytes decreases more rapidly in older women. The whole process is ended by loss of reproduction and onset of so-called menopause[3]. The average age of menopause onset is very different partially because each woman is born with a different number of oocytes – but it is likely to happen sometime between woman's 40's and 50's[4]. Women with an initial follicle number lesser than than 5 million are at greater risk of premature menopause[5]. Most follicles undergo atresia through apoptosis[3].

It is obvious that with the age of a woman supply of the oocytes decreases. It is important to realize, that fertility is not the same throughout the reproductive period, but that it

decreases significantly after the age of 35, i.e. at a time when the menstrual and ovulatory cycle normally works[2]. However, this phenomenon is not limited just to the reduction of fertility and oocyte loss. It includes, for example, unsuccessful pregnancy attempts, errors in meiotic division leading to chromosome abnormalities[3], spontaneous abortions, reduced embryo implantation success rate[1], ectopic pregnancy or a higher risk of stillbirth. For example, specific observations have shown that the risk of spontaneous abortion in women aged 20–24 years is 8.9 %, while the risk increases in women over 45 years old to 74.7 % [6].

LOSS OF REPRODUCTIVE ABILITY ON AN EVOLUTIONARY BACKGROUND

It is interesting to look at the fact that women lose their reproductive capacity at a certain age, unlike men whose potential to reproduce is preserved throughout their lives. It is due to the fact that men, unlike women, have germ stem cells that can be renewed[4]. But what is the reason that led to this loss of oocytes in women, and why is it better than keeping the ability to reproduce? In an evolutionary way of look in long-living species, it was important to decrease the risks of pregnancy, possiblitiy of spontaneous abortion, and childbirth at an older age. That was because not only the mother's life was endangered, but also her already born offspring[1]. This risk, of course, increased with the woman's age. Until the 20th century, maternal mortality rates above 40 years were ten times higher than those of mothers below 20 years[2]. So, when the maternal mortality rate at birth was high, there was an increased risk of her offspring becoming orphans. And precisely to prevent this, the individual reproductive success of a woman has been delayed in evolution to increase the reproductive success of her offspring who carry her genes. Thus, in the middle years, a woman could invest her energy in already-born children, raise and protect them, rather than risk her life by becoming pregnant[2].

At the same time, it is important to realize that loss of reproductive capacity and reproductive aging is not only an achievement of women in the Western world but also occurs in women of traditional cultures, female mammals living in captive and many long-living mammal species in the wild[2].

DOUBLE-STRANDED BREAKS (DSBs) ARE INCREASING WITH AGE

Since DNA is a key molecule in a cell, any damage to DNA poses a certain risk and a threat to its proper functioning. Yet, mutations of DNA are very common and in vast majority they are being repaired by several mechanisms. However, failure of repair mechanisms might occur and in case of severe damage, apoptotic pathway is usually activated. These processes are involved

in the aging of organism. Since aging affects the whole organism, and thus the ability to reproduce, we can predict that unrepaired DNA damage can lead to decreased fertility[7].

Quality of oocytes decreases with age. One reason why oocyte dysfunction occurs is that DSBs accumulate over time. These breaks are repaired throughout life; however, as the amount of DSBs increases, the ability to repair these breaks is impaired. Thus, the oocyte function fails to cope with DNA damage and to activate apoptosis[1].

Number of DSBs is a suitable indicator of DNA damage level in oocytes. DSBs are more likely to be found in older subjects[8]. In an experiment 4 to 5-weeks old mice and 11 to 12-months old mice were compared. DSBs were visualized by staining histone γH2AX. The results say that the number of γH2AX positive follicles increased – (58.5 % in the old mice) vs. (32.6 % in the young mice)[12].

The same result was also reached in humans, where oocytes collected during the IVF process from women aged 21 to 43 years were compared. Again, it was found that oocytes from older women had a greater tendency to accumulate DNA DSBs[7]. In research conducted on vervet animals, *rhesus macaques*, not only the occurrence of DNA damage but also the number of follicles were examined. The number of primordial, primary, and secondary follicles decreased with age – in the oldest individuals only a few follicles were found, almost none of which were antral. The result of the amount of DNA damage is not surprising – the number of DSBs measured (in this case together with the damage on telomeres) in cumulative oocyte cells was increased with the *macaque's* age[4].

EFFICIENCY OF DNA REPAIR GENES

As mentioned above, the amount of double-stranded DNA breaks increases with increasing age[1]. The reason why these DSBs accumulate is the decrease in the expression of genes involved in DNA repair by homologous recombination[7]. If a double-strand break occurs on the DNA, it is first detected by the MRN complex (which consists of the MRE11, RAD50, and NBS1 proteins). This complex activates ATM protein kinase, which phosphorylates histone γH2AX and activates the pathway leading to DNA repair. If the repair fails, either cell cycle arrest or cell apoptosis occurs.

In the experiment performed on oocytes from IVF donations, increased amount of DSBs in oocytes was demonstrated with decreased mRNA expression of DNA repair genes, i.e. BRCA1, BRCA2, ATM, MRE11, and Rad51. It was confirmed that the expression of corresponding proteins, except for the BRCA2, was decreased with age (Fig. 1). This finding significantly supports the claim of the importance of DNA repair during reproductive aging[9].

Furthermore, DSBs and apoptosis increased after siRNA-knockout of BRCA1, MRE 11, Rad51 or ATM. It has been also published that BRCA1 mutants show an accelerated oocyte loss over their controls. And induction of DNA damage led to apoptosis of oocytes more in

old mice than in young animals[1]. These findings support the hypothesis of DNA repair being crucial to control and maintain oocyte functionality.

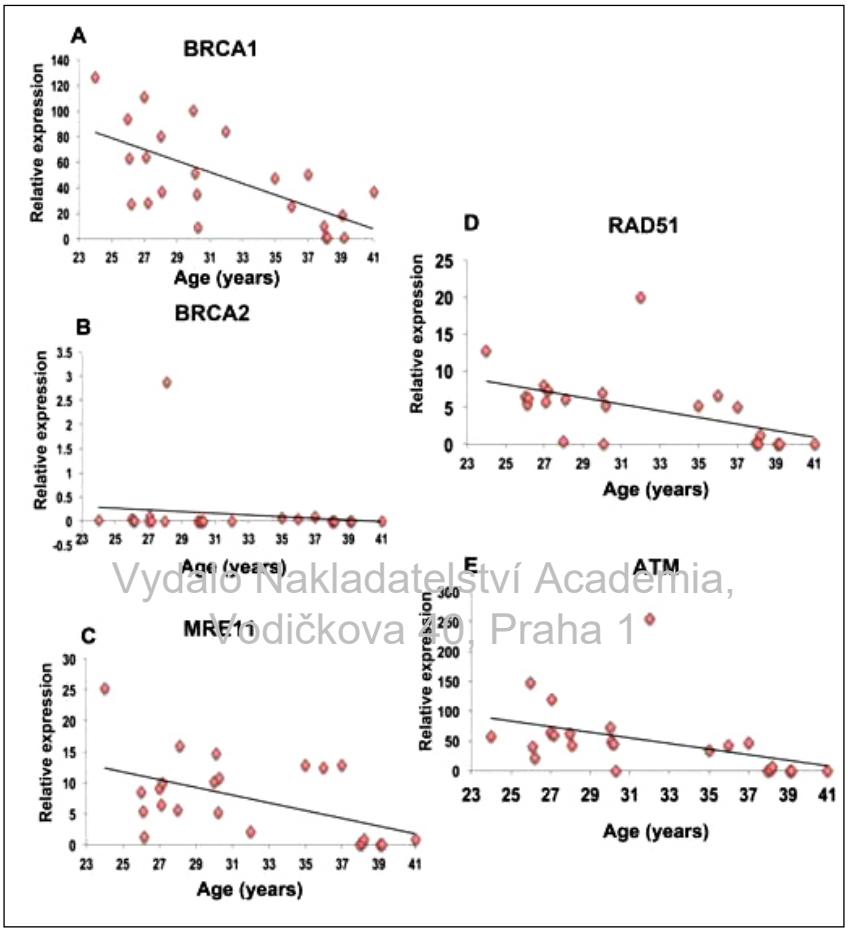

Figure 1. Expression of DNA repair genes in human oocytes. (Adapted from ref. 9)

BRCA1 AS A KEY PLAYER

BRCA1 protein is an integral part of the DSBs repair pathway mediated by ATM kinase. It is not only involved in DNA repair but also regulates the response to DNA damage alone[7], participates in the assembly of the spindle[9], and provides genome protection by maintaining chromosome stability[7]. It also plays an important role in the activation of cell cycle checkpoints to ensure cell cycle arrest or apoptosis, if the repair is not successful.[10].

As already mentioned, the amount of BRCA1 protein, like other proteins involved in DNA repair, decreases with age. Immunofluorescence performed again on rhesus monkeys, where the ovaries of young (3–4 years) and medium-aged monkeys (7–8 years) contained a large amount of BRCA1 in oocytes. In contrast, the ovaries of the elderly (18–19 years) showed a rapid decline. After immuno-histochemical staining, most of the primordial and primary follicles of the young and middle-aged individuals showed the presence of BRCA1, but not the elderly individuals. The same result was achieved by observation under a confocal microscope where granulosa cells of primordial and primary follicles of young and middle-aged monkeys showed a large amount of BRCA1 foci, namely 23.8 ± 1.2 and 22.9 ± 1.4. In the elderly, this amount was reduced to 9.8 ± 0.7[4].

The mutation in BRCA1 brings interesting, but quite disadvantageous consequences. If this mutation occurs in mice, they are born with fewer oocytes that have a higher tendency with age to accumulate DSBs than BRCA1-intact mice[11]. At the same time, they have fewer offspring in the litter. In women with the BRCA1 mutation, earlier onset of menopause can be observed compared to healthy women and their reservoir of primordial follicles is naturally reduced, thus producing lesser amounts of oocytes[7,9]. The response is significantly lower in these women after ovarian hormone stimulation[8]. It is important to note that the mutation in BRCA1 does not have the same manifestation as the mutation in BRCA2. For example, women having a BRCA1 mutation have a significantly lower number of oocytes in the ovaries than women of the same age having a BRCA2 mutation[9]. However, the most prominent consequence of BRCA1 mutants is the predisposition to breast and ovarian cancer[8]. It is because the limited function of BRCA1 leads to erroneous repair of DNA damage, leading to the accumulation of mutations. If the proliferation of breast and ovarian epithelial cells continues having such errors, transformations can occur and potentially lead to tumor formation[1]. Again, this risk varies between BRCA1 and BRCA2 mutants. With BRCA1 mutation, the risk rate of tumor formation is 40–65 %, while for BRCA2 mutants it is "only" 20 %[10]. Lower amount of oocytes in women with BRCA1 mutation can be quantified using anti-Müllerian hormone. Level of AMH decreases with age and it can predict the timing of menopause. Taking into account factors such as age and body mass index (BMI), it was concluded that women with a BRCA1 mutation had a lower level of AMH (0.53 ng/ml) compared to women without a mutation (1.05 ng/ml)[10].

CONCLUSIONS

Delaying maternity and associated infertility due to an insufficient number of oocytes or a high rate of damage in their DNA is a trend research nowadays. To maintain the integrity and functionality of the DNA in the cell, DNA repair pathway function must be sufficient – in particular with DNA repair genes.

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30 Laser microirradiation as a tool for DNA damage response studies

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Abstract

Double-strand DNA breaks (DSBs) are considered as the most hazardous type of DNA lesions. Recognition of DSBs, temporal cell cycle arrest and activation of repair pathways are crucial steps in response to DNA damage. Errors in any of these steps can lead to adverse consequences affecting genome integrity, e.g. chromosomal aberrations or generation of mutations. The genomic instability caused by severe endpoints of DSBs processing can lead to cell death or development of cancer. Therefore, it is crucial to study all aspects of DNA damage response in cells.

In the context of studying DNA damage response in cells, DSBs are typically induced either by γ-irradiation or by UV light. DSBs can be also induced by radiomimetic drugs, such as Neocarzinostatin. However, these techniques have several limitations, e.g. inability to induced DSBs only in a specific region of nucleus. Therefore, the laser UV microirradiation technique combined with confocal microscopy was developed to study the DNA damage response in living cells.

Keywords

laser microirradiation, DNA damage response, double-stranded DNA breaks

INTRODUCTION

Every day, approximately 10 to 50 double-stranded DNA breaks (DSBs) arise in every cell of the human body[1,2]. The number of DSBs depends on the cell cycle phase and tissue. DSBs are randomly generated in the genome by exogenous agents, for example by ionizing radiation[3,4]. DSBs are also generated during cellular processes, for example during meiotic recombination or V(D)J recombination[1,5,6]. Endogenous events leading to DSBs include collapse of replication fork, oxidative stress, or telomere erosion[5,6]. Moreover, DSBs can also be

generated experimentally by radiomimetic drugs or inhibitors of topoisomerases[6–8]. DSBs are considered as the most hazardous type of DNA lesions because only one unrepaired DSB is sufficient to trigger chromosomal rearrangements, growth arrest, senescence or cell death[9,10]. The best possible result of DSBs processing is the successful repair and restoration of intact DNA. However, the pathway choice, which determines the outcome, is dependent on several factors, including the severity of damage, chromatin status, cell type, and cell cycle stage[11]. Several severe outcomes of DSBs processing, like DNA mutations, apoptosis, and cellular senescence are relevant to human diseases and aging phenotype[2].

DSBs REPAIR PATHWAYS

Cells evolved an extensive network of DNA damage response. DDR includes the recognition of DNA damage, the cell cycle delay and the DNA repair[5,12–14].

There are two main DSBs repair pathways: Homologous recombination (HR) and non-homologous end-joining (NHEJ). HR is slow and error-free type of DSBs repair. HR does not only repair DSB but also restores the sequence around DSB. The sister chromatid is required as a homologous template for repair, and therefore HR is functional only in S and G2 phase of cell cycle. On the other hand, NHEJ is very fast process, where DNA ends are simply joined together. NHEJ is functional in G1, S and G2 phases[12,15].

Within NHEJ, Ku heterodimer (Ku70, Ku80) quickly recognizes DSBs and molecule 53BP1 comes to the site of DNA break. The catalytic subunit of DNA-dependent protein kinase (DNA-PK) is bound to Ku heterodimer leading to the formation of functional DNA-PK. DNA-PK together with Artemis modify free DNA ends to create suitable ends for ligation. Complex LIG4/XRCC4/XLF subsequently ligates DNA ends[16]. During DNA ends processing, addition or deletion of several nucleotides may occur, and therefore NHEJ is considered as error-prone repair mechanism[16–19].

Within HR, 3' single-stranded DNA overhangs on both sides of the break are created by the MRN complex. Subsequently, coating by Replication protein A stabilizes these 3' ssDNA overhangs. Later, Replication protein A is replaced by Rad51 nucleoprotein filament. After finding of sequence homology, both overhangs invade to DNA donor. This DNA donor serves as a template for DNA synthesis. The last step in HR is resolving of the Holliday junctions, which can result in crossover or non-crossover products[20–22].

Recent studies demonstrate that there is additional DSBs repair mechanism called alternative end-joining (A-EJ). It is assumed that A-EJ is functional when other repair mechanisms fail or are not functional. A-EJ uses simple end-joining principles; however, the repair by A-EJ is slower in comparison to classical NHEJ. A-EJ does not require sister chromatid as a homologous template, but it uses 2-25 nucleotide long microhomologies found in the vicinity of DSBs. Usage of the microhomologies may lead to large deletions in DNA sequence[15,23–25].

CELL CYCLE SIGNALLING

To enable cells to respond to DSBs, the signal transduction cascade, which induces cell cycle arrest, is activated upon DSBs recognition. MRN complex, which recognizes DSBs, activates downstream kinases ATM (Ataxia telangiectasia mutated), ATR (Ataxia telangiectasia and Rad3-related protein) and DNA-PK (DNA-dependent protein kinase)[13,26,27]. Histone H2AX is phosphorylated in the sites of DSBs by ATM kinase. Phosphorylated H2AX (γH2AX) is recognized by MDC1 protein, which in turn serves as a platform for DNA damage repair proteins[28–30]. ATM and ATR kinase phosphorylate also checkpoint kinase 2 (CHK2) and checkpoint kinase 1 (CHK1), which in turn phosphorylate downstream targets including CDC25 phosphatases, which are responsible for dephosphorylating and activating cyclin-dependent kinases (CDKs)[26,27]. Through inhibition of CDC25 phosphatases by CHK2 and CHK1 cell can be arrested at multiple time points including G1/S transition, S phase and G2/M transition[31,32]. Furthermore, CHK2 stabilizes protein p53, which is responsible for transcriptional activation of cell cycle inhibitors[33,34].

CONFOCAL LASER MICROIRRADIATION

In the context of studying DDR in cells, several agents are used for induction of new DSBs. DSBs are typically induced by UV light or by γ-irradiation[3,4,35]. Another possibility to induce DSBs is usage of radiomimetic drugs, for example Neocarzinostatin or inhibitors of topoisomerases[7,36]. However, the usage of these agents has several limitations, for example inability to induce new DSBs in a specific region of nucleus and at a specific time[35,37,38]. Therefore the development of laser UV microirradiation which allows local and timely controlled induction of DSBs brought irreplaceable experimental technique for DDR studies.

Laser UV microirradiation was used for studying effects of radiation on genome stability already 40 years ago[39,40]. From that time, this technique was improved, broad spectrum of lasers was tested and development of new detection techniques allowed to widely use this method for DDR studies in living cells. Laser UV microirradiation is based on irradiation of a small region of nucleus with high-intensity UV laser. It is commonly used in combination with confocal microscopy[35].

Irradiation with UV light causes the generation of several types of DNA lesions, e.g. cyclobutane pyrimidine dimers, 8-oxo-7,8-dihydro-2'-deoxyguanosine or single-stranded breaks and DSBs[35,37].

Several authors used UVA laser in combination with pre-sensitizing by halogenated nucleotides (e.g. 5-Bromo-2'-Deoxyuridine (BrdU)), or by DNA intercalating agent (Hoechst 33258, Hoechst 33342)[41,42]. However, later it was shown that the pre-treatment is not

necessary step, as DDR proteins are recruited into sites of DNA damage also without pre-treatment[43]. Moreover, it was shown, that after 405-nm UVA laser microirradiation, DNA lesions were characterized by decreased number of pyrimidine dimers[43,44]. Therefore, by choice of UV laser and area of microirradiation, it is possible to regulate the extent of DNA damage and study the kinetics of molecules involved in DDR in physiological conditions. Moreover, it is possible to apply this type of experiment also during particular phase of cell cycle[35].

In practice, such laser UV microirradiation experiment is divided into two phases. The first phase is induction of DSBs using high-intensity laser pulse (100% laser power is normally used) in the selected area of nucleus. The second phase is analysis of DDR, which can be done by detection of endogenous proteins by immunofluorescence assay or by detection of DNA repair factor fused with fluorescent protein using live-cell imaging.

IMMUNOFLUORESCENCE ASSAY

After induction of DSBs, cells can be fixed and stained using specific antibodies against proteins involved in DDR. γH2AX is a widely used marker of DSBs, as the phosphorylation of H2AX occurs specifically at the damage site and spreads from the sites of DSB[30,45,46]. Also components of MRN complex, NBS1, Mre11 and Rad50 can be detected[46–48]. Any protein of interest can be studied if there is available specific antibody.

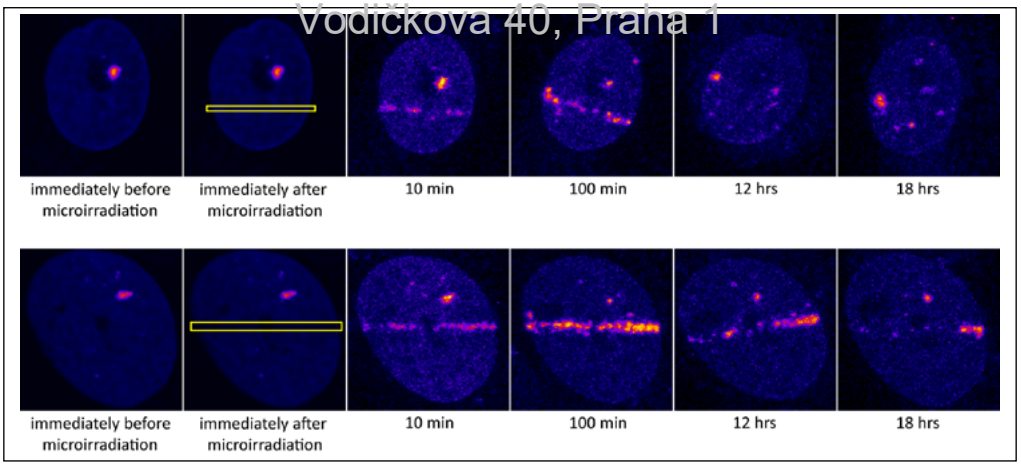

Figure 1. Accumulation of molecule 53BP1-mCherry after laser UV microirradiation in porcine primary dermal fibroblasts. The yellow rectangle represents area of microirradiation. Upper panel shows lower DNA damage achieved by smaller area of microirradiation (height: 0.2 μm, length 15 μm). Lower panel shows higher DNA damage achieved by larger area of microirradiation (height: 1 μm, length 15 μm). Primary fibroblasts were transfected by 53BP1-mCherry mRNA (500 ng/ml) and microirradiated (100% UV laser, 200 ms) 24 hours after the transfection. Orig. M. Vaskovicova

LIVE-CELL ANALYSIS

Instead of detection of endogenous protein by antibody, fusion of DNA repair factor of interest with fluorescent protein can be done. The protein of interest fused with fluorescent protein can be expressed in cells by transient transfection or by creating a stable cell line[49–52]. In the case of primary cells, where the creation of stable cell line is very complicated as well as the transfection using expression plasmid, it was shown that transfection with mRNA is more functional[53]. This method allows studying recruitment of DDR factors to the sites of DSBs in real-time, even very shortly after induction of DSBs, and can be followed for several hours or days (Fig. 1).

CONCLUSION

DSBs are considered as the most hazardous type of DNA lesions. Recognition of DSBs, signalling of DSBs and activation of DNA repair pathways are important steps in DDR. The best result of DDR is successful repair of DSBs and re-entering cell cycle. If the cell is not able to repair the DNA damage, it can permanently stop its cell cycle or undergo programmed cell death. Therefore, alterations in DDR response and DNA repair pathways can lead to genomic instability of the cells, which can, in turn, cause development of cancer or other diseases. Therefore, it is necessary to study all aspects of DDR in cells.

In the context of studying DDR in living cells, laser UV microirradiation technique became very useful. Laser UV microirradiation in combination with confocal microscopy allows us to visualize accumulation of DDR factors, their activation, and kinetics after induction of DSBs.

Acknowledgements

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31 The role of chromosomal signalling gradients in spindle assembly and chromosome segregation during oocyte meiosis

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Abstract

Vertebrate reproduction biology is very specific especially when talking about mammals, including humans. In this short review, we are concerned about the oocyte-specific features of meiotic division. Further, we are describing the function of two chromosomal signalling gradients, RanGTP (guanosine triphosphate-bound Ran)-importin β and chromosomal passenger complex during meiosis in different species, their effect on spindle assembly in oocytes and summarizing the latest speculations about more possible functions.

Keywords

oocyte, spindle, RanGTP, chromosomal passenger complex, Aurora kinases

INTRODUCTION

To understand upper mentioned themes, we need to explore the description of germ cells and how they differ from somatic cells. It is known that germ cells stand behind reproduction process, they include female sex cells called oocytes and male sex cells called sperm. The main difference between germ cells and somatic cells is that when germ cells complete the division, their genome is reduced in half. This kind of cell division into haploid cells is called meiosis. Sex cells which finish meiotic division are known as gametes[1,2].

However, it is important to point out that this review is concerned with the development of female sex cells before complete segregation into haploid cells. Meiosis is essential for correct

sexual reproduction and foetus development[3,4]. Most of the human embryo defects are caused by the improper division of eggs, not sperm[5]. Previous studies suggest that the majority of segregation errors happen during meiosis I (MI). Aneuploidies are not common in most of the vertebrates, however, up to 1/3 of human oocytes are affected by aneuploidy, which can lead to incorrect embryo development including trisomy or spontaneous abortion[4]. It has been also proved that the occurrence of aneuploidies in human oocytes increases with age[6].

PHASES OF MEIOSIS AND MEIOTIC SPINDLE FORMATION

Meiosis is spread in two main phases, called meiosis I and meiosis II (MII)[1]. New somatic cells are made during the whole life of an organism, which does not apply on oocytes. The MI division of oocytes is highly processed during foetus development, then stops in prophase I after DNA replication and homologous recombination[7]. During MII, the chromosome segregation now differs as haploid cells are arisen by separating the sister chromatids of homologous chromosomes[1,4].

For the correct chromosome segregation, the formation of the proper bipolar spindle is essential. The exact molecular mechanisms of bipolar spindle assembly remain unknown[8]. However, different studies have revealed that at least two pathways, chromatin-dependent pathway and microtubule-organizing centers (MTOCs)-dependent pathway are involved in this process[9]. Oocytes of many mammalian species lack centriole-containing centrosomes, which also makes them different from somatic cells. In mouse oocytes, acentriolar MTOCs are involved in bipolar spindle assembly[8,10,11]. Acentriolar MTOCs are built of a complex including pericentriolar matrix proteins containing pericentrin, γ-tubulin and many other proteins in oocytes[8,12–15]. The mechanism of spindle assembly is conditioned by MTOC decondensation following fragmentation[15]. Defective fragmentations and clustering of MTOCs may cause aneuploidies[15,16]. Human oocytes, on the other hand, lack the MTOCs and chromatin-dependent pathways, especially RanGTP, act as a crucial regulator of spindle formation[17].

Many factors are playing an important role in chromosome segregation. In this case, we are going to concentrate on two kinds of cell signalling. We call them chromosomal passenger complex (CPC) and RanGTP-importin β pathway. Active forms of both CPC and RanGTP are localized around condensed chromatin in high concentration and with further distance, their activity decreases[18].

RanGTP-IMPORTIN β SIGNALLING

Ran is a member of the Ras family, defined as a GTP-binding protein[19] which plays a role in nucleocytoplasmic transport and spindle assembly[20]. The role of Ran molecule in cellular

transport is connected with cargoes that include NLS, defined as nuclear localisation sequence. Ran also plays it's role during nuclear export as it binds the cargo including NES (nuclear export signal)[20,21]. Ran has two defined forms. They are active and inactive. Active Ran is bound with GTP and is found around chromosomes after nuclear envelope disassembly in the highest concentration, as previously mentioned. An inactive form of Ran is bound with GDP and localized further from chromosomes[22]. The transformation of Ran is processed via proteins named RCC1 and RanBP2/RanGAP1. Inactive RanGDP is activated by RCC1 after the nuclear envelope breakdown. RCC1 then binds to chromosomes and activates Ran, therefore Ran creates RanGTP active form and binds with importin complex composed of importin α and importin β together with NLS cargoes. The importin complex is responsible for binding the NLS molecules and after interaction with RanGTP, the cargo with NLS is being released. The opposite effect then occurs as after the NLS protein release, active Ran binds the exportin together with NES proteins. After the export, RanBP2/RanGAP1 cause Ran inactivation by GTP hydrolysis to RanGDP, leading to the NES proteins and exportin release[23].

The connection between Ran function and spindle assembly possibly leans on the fact that during cell division, the concentration of RanGTP increases and SAF (spindle assembly factors) are carried to the nucleus via nuclear import mechanism. RanGTP binding to importins alpha and beta dissociates their cargos and induce spatial activity gradients of different NLS-containing spindle assembly factors (SAFs) after nuclear envelope disassembly that are inhibited by importin binding[20,21]. Even though the mechanism of spindle assembly connected with Ran is not fully understood, the studies have revealed that mouse oocytes assembled functional spindles and segregated chromosomes when using the RanT24N dominant-negative mutant for inhibiting Ran function. In *Drosophila* oocytes, RanGTP is not required for the initiation of spindle formation[22,24,25]. Ran inhibition using RanT24N in these studies is caused by the weaker affinity of mutated Ran to guanine nucleotide. Therefore, the mutant is less likely to bind GTP and so the Ran activity decreases[26]. The gradient of RanGTP is required for the correct spindle assembly in *Xenopus laevis* eggs[27]. It has been proved that the microinjection of RanT24N to *Xenopus* eggs causes defective spindle assembly together with spindle formation delay, while the microinjection of constitutively active mutant RanQ69L[28] caused formation of abnormally long spindles and pole defects[24]. Study of mice and *Xenopus* oocytes also suggests a close relationship between spindle assembly and RanGTP pathway as meiotic MII division and spindle formation in *Xenopus* oocytes is defective with a knockout of RCC1[24]. Talking about humans, the process of spindle assembly is affected when using RanT24N[17]. As the human oocytes do not contain MTOCs, they are fully dependent od RanGTP-importin β pathway. Recently, authors found that RanT24N mutant in mouse oocytes increases the overall concentration of free importin beta cargoes because of its direct interactions with importins through the T42 site and thus not act as a specific RCC1 inhibitor. Using chemical inhibition of RanGTP-importin beta interaction and new Ran

double mutant (without detectable interference with importins interaction; RanT24N, T42A), authors confirm the essential role of RanGTP pathway for spindle assembly and MTOCs formation in mouse oocytes[21].

CHROMOSOMAL PASSENGER COMPLEX

There is even more to mention when talking about mechanisms of spindle assembly and chromosome alignment during mitosis and meiosis in mammals. A very important role is played by three Aurora Kinase (AURKs) isoforms known as Aurora A, B and C, which is found in mammalian germ cells only[9]. While the function of AURKA and AURKB is pretty well explained in mitosis, there are still speculations about exact mechanisms in meiosis, linked to the chromosome passenger complex (CPC) which is one of the chromosomal signalling gradients.

Chromosomal passenger complex (CPC) is defined as a structure composed of three main protein subunits and Aurora kinase catalytic subunits. The protein subunits are called INCENP (inner centromere protein), Survivin and Borealin[29]. The exact function of CPC remains unknown. However, recent studies refer to the close relationship between serine-threonine AURKs, linked possibly to the process of spindle assembly and chromosome segregation[9]. There are three types of AURKs. The first is called Aurora A and together with Aurora B is expressed in somatic cells and plays an important role during mitosis. Unlike the Aurora C, which is expressed mainly in germ cells and binds in mammalian oocytes to the C-terminal IN box region of INCENP as a catalytic subunit[30], probably instead of AURKB, which binds to INCENP during mitosis[9]. The experiment in mouse oocytes expressing dominant-negative AURKC suggests that AURKC has a prominent role in chromosome alignment during meiosis I[31]. Another study refers to the usage of AURKC ATP-binding site mutant microinjection causing the meiosis I arrest and disruption, although some of these mouse oocytes completed cytokinesis successfully[32]. Therefore, it is assumed that AURKB might also bind to INCENP of CPC during meiosis to complete the segregation[31]. These oocytes with lack of AURKC however, have finally shown up as aneuploid[33].

Comparing the CPC function in different species, it has been proved that in some cases spindle assembly is not affected by the CPC attenuation, for example by knocking down an INCENP component. This applies to mouse oocytes[34]. The difference though has been spotted in *Drosophila* and *Xenopus* oocytes, which supports the hypothesis that CPC might be a key to the correct process of spindle assembly in some species. Talking about *Drosophila*, two studies refer to spindle assembly delay after partial loss of INCENP as well as to complete prevention of spindle assembly during INCENP or AURKB absence[35]. An important role might be also played by a protein called kinesin-6 Subito, which is described as a protein with microtubule bundling function. The bundling ability of Subito is exposed after nuclear envelope breakdown only and

the protein is localized on the metaphase spindle together with CPC[36]. Focusing on *Xenopus*, the spindle assembly depends on INCENP centromere-targeting and microtubule-targeting domains[37]. Another study points out that CPC is not necessary for spindle assembly in *C. elegans*, however, without CPC, the spindle is still organized incorrectly[38,39].

CONCLUSIONS

The importance of signalling gradients leans on the fact that defective spindle formations can cause aneuploidy, subfertility or complete infertility. Therefore, knowing the connection between molecular pathways, spindle assembly and chromosome segregation might help us understand the negative consequences of incorrect cell division and possibly discover new medical treatments.

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