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CELL TECHNOLOGIES AT THE EDGE: RESEARCH & PRACTICE

TRANSLATIONAL RESEARCH IN CELL THERAPY

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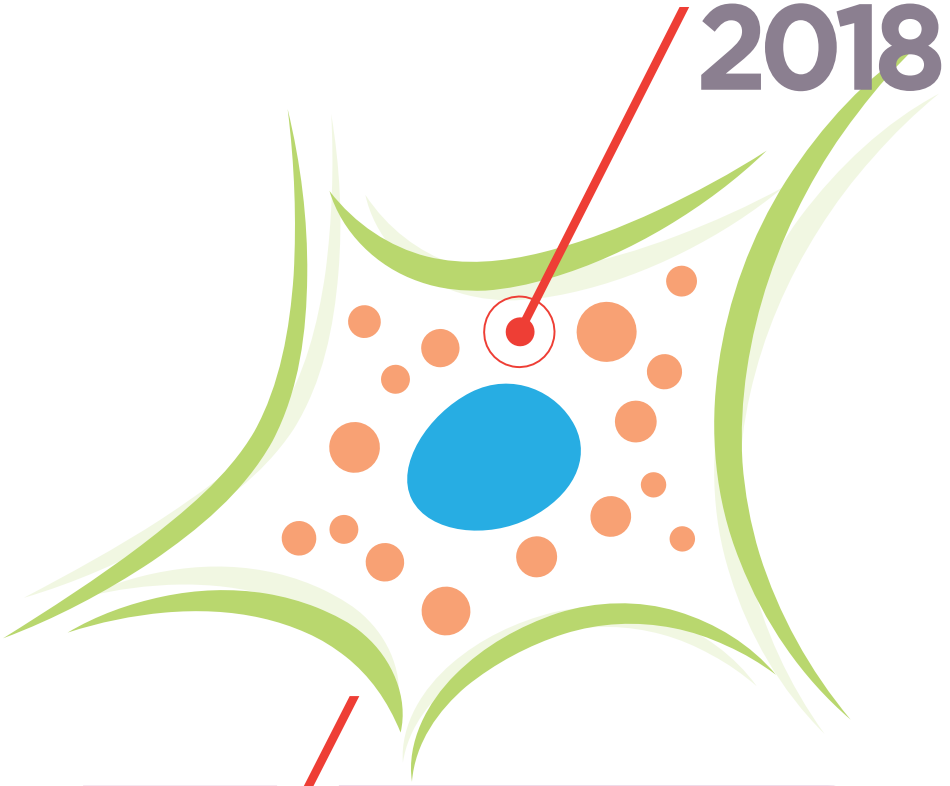

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THE SECOND INTERNATIONAL CONFERENCE

“CELL TECHNOLOGIES AT THE EDGE: FROM RESEARCH TO PRACTICE” (CTERP) “TRANSLATIONAL RESEARCH IN CELL THERAPY”

MOSCOW, APRIL 11–13, 2018

11 APRIL

OPENING SESSION

MAINTENANCE AND DIVISION OF NEURAL STEM CELLS

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Dynamic regulation of adult neurogenesis underlies cognitive function, response to therapies, and brain repair. Continuous age-driven decline in neuronal production may limit plasticity and repair of the nervous system and may underlie age-related cognitive deficits. A plethora of stimuli, including widely used therapeutic drugs and treatments, can modulate neurogenesis and may potentially ameliorate disease- and age-related impairment of the nervous system. Therefore, understanding the basic mechanisms of stem cell maintenance could guide the choice of strategies for stem cell therapies of neurodegenerative and neuropsychiatric disorders.

We developed novel approaches to investigate division and signaling in neural stem cells and have proposed a new model of stem cell maintenance in the adult brain. Our results imply that various modes of division and differentiation of stem and progenitor cells, while leading to seemingly identical outcomes, may be induced by different mechanisms and may have profoundly different long-term consequences, relevant to the prospects of brain rejuvenation.

Key words: neural stem cells, adult hippocampal neurogenesis, models of stem cell maintenance, proliferation analysis.

SIGNIFICANT EVENTS IN GLOBAL ATMP DEVELOPMENT: HOW STRONG IS THE EUROPEAN SECTOR?

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Not since the discovery of recombinant protein technology has the field of medicine seen such a transformation in drug development as we are seeing in the field of advanced therapies (ATMPs).

In contrast to all conventional pharmaceuticals and biologics, these new drugs are developing from academic centres but are now transferring to licensing and global availability. This is a largely untrodden route; no other type of drug has arisen from academic trials in the same way and no other drug type can be manufactured at academic scales yet still address a sizeable clinical need. The supply chains for ATMPs are more aligned with those used for routine haematopoietic stem cell transplants; skills which lie in hospital and academic laboratories and not with conventional pharmaceutical companies.

However, the clinical success associated with some of these treatments and the relative paucity of new conventional pharmaceuticals has encouraged many large drug companies to enter the field and this trend is set to continue for the foreseeable future. The challenges to be met are now well understood for some autologous or patient-directed therapies but more complex tissue engineered products remain challenging.

This presentation will review the history of commercialisation of ATMPs globally and highlight the most recent events in Europe and beyond. It will highlight the challenges still to be met and discuss the controversies of unregulated provision of ATMPs across the EU and globally.

Keywords: ATMP development, drug development.

INDUCTION OF PLURIPOTENT AND MULTIPOTENT STEM CELLS

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The pluripotent and multipotent states of mammalian stem cells are governed by the formation of a highly interconnected regulatory network comprising specifically expressed transcription factors organized together with more widely expressed transcription factors. The transcriptional network exhibits a hierarchical structure, with a small number of transcription factors playing an essential role in maintaining cellular potency and regulating the expression and/or function of the numerous auxiliary transcription factors.

The ultimate goal of cell and developmental biology is to program cells at will. One powerful way to convert any given cell type into another cell type is to achieve a pluripotent stem cell state that resembles that of embryonic stem cells. Somatic cells need exogenous transcription factors to achieve pluripotency. Reprogramming of mouse and human somatic cells into pluripotent stem cells, termed induced pluripotent stem (iPS) cells, was first described by Yamanaka more than 10 years ago using fibroblasts and initially required the virally-expressed transcription factor quartet of Oct4, Sox2, c-Myc, and Klf4. Later, we reported that the POU transcription factor Oct4 alone is sufficient to directly reprogram adult mouse and human fetal neural stem cells (NSCs) into iPS cells, indicating that Oct4 plays a crucial role in the reprogramming process. Brn4 is also a POU transcription factor, but specifically expressed in the neural system. When we replaced Oct4 by Brn4, the network of fibroblasts was converted into that of multipotent NSCs. Oct4 and Brn4 thus appear to play distinct but related roles in remodeling gene expression by influencing the local chromatin status during reprogramming.

Strikingly, our most recent data show that - depending on the expression levels of Sox2, KLF4 and cMyc as well as the expression system - Oct4 is not required to induce pluripotency.

Keywords: pluripotent mammalian stem cells, multipotent mammalian stem cells, transcriptional network.

12 APRIL

BASIC PROBLEMS OF CELL RESEARCH

NEW PLAYERS IN THE FIELD OF PLURIPOTENCY

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Pluripotent stem cells have gained considerable interest due to their unique features and tremendous potential in regenerative medicine. Despite significant and breathtaking progress over the past two decades in understanding molecular mechanism of pluripotency control, many aspects of this cellular state remain little understood. We have recently characterized two teams of players that control cellular pluripotency at different regulatory levels. One team belongs to the family of RNA- and DNA-binding factors of the KH-proteins, and we have identified several members of this family as upstream effectors of the key pluripotency gatekeeper, Oct4 (Pou5f1), differentially involved in transcriptional regulation of this gene in naïve and primed pluripotent stem cells. The second team of the novel players surprisingly involves subunits of proteasome moieties that participate in antigen presentation in the immune system, i.e. immunoproteasome, and we have shown their critical role in the process of reprogramming into pluripotent cell state.

The research has been supported by Russian Science Foundation (grants 17-14-01407, 14-50-00068), and by Russian Foundation for Basic Research (grant 18-04-01199).

Keywords: pluripotent stem cells, molecular mechanism of pluripotency control.

FROM MOLECULAR MECHANISMS TO CELL THERAPY OF FACIOSCAPULOHUMERAL DYSTROPHY

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Facioscapulohumeral dystrophy (FSHD) is an autosomal dominant disease with a prevalence of up to 1:8000. There are approximately 15000 FSHD patients in Russia and no treatment currently exists for this disease. The clinical features of FSHD typically concern the weakness of muscles of the face and upper extremities that can progress caudally with age to affect abdominal muscles and the lower limbs and feet. The major form of facioscapulohumeral dystrophy (FSHD1) results from a combination of three genetic events at 4q35: a reduction in the number of D4Z4 repeats and two polymorphisms, 4qA161 and 4qA. The 4qA161 polymorphic site contains an insulator essential to control the activity of neighboring genes: *DUX4, DUX4c, FRG1, FRG2, ANT1* and others. This function is defective in the 4q35 locus in cells from the FSHD patients. The combination of these three events leads to an increased gene expression in the 4q35 locus. At the same time the role of these genes in pathophysiology of FSHD remains obscure. Our studies have revealed the molecular mechanisms of three important features of FSHD: (1) changes in gene regulation in FSHD muscles, (2) defects in myogenic differentiation, (3) invasion of attained muscle with connective and fat tissue; (4) inflammation, oxidative stress and DNA damage in muscles of FSHD patients. Here we present preliminary data on personalized therapeutic approaches for FSHD using recent biomedical technologies including CRISPR/Cas9.

This work was supported by the MEGAFSHD grant from the Association Française contre les Myopathies (AFM) and by the Grant No. 0108-2018-0008 of the “Fundamental research for biomedical technologies” program of Presidium of RAS to YSV and the grant No. 16-54-16015 from the Russian Foundation for Basic Research to EP.

Keywords: Facioscapulohumeral dystrophy, polymorphism, gene expression.

SKIN TISSUE AND ORGAN REGENERATION

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Skin reconstruction is one of the few examples of successful and long-term application of cell technologies and tissue engineering in medical practice. On the basis of techniques for skin cell cultivation we have developed several novel approaches and specialized products for reconstruction of those tissues which contain mesenchymal and epithelial components. Depending on the purpose, various combinations of cell lineages, matrices, and additional components were used. We are moving towards the preclinical and clinical trials with subsequent state registration of two products intended for treatment of skin defects. Besides, these approaches will be used for the development of full-thickness skin models for drug testing and treatment of severe inherited skin disorders. The perspective goal is to reconstruct not only epithelial-mesenchymal bi-component structure of the skin but regenerate skin appendages such as hair follicles. Basing on the study of specific properties of cells derived from hair follicles and their morphogenetic abilities we were successful in reconstruction of hair germ organoids from postnatal human skin and hair follicle cells. Additionally, induced pluripotent cells can be used for modelling of early events in skin and hair follicle morphogenesis.

The work was performed in accordance with State program of Institute of Developmental Biology № 0108-2018-0004.

Key words: skin, hair follicle, morphogenesis, epidermis, keratinocytes, dermal papilla, organoids

EXTRACELLULAR VESICLES OF MESENCHYMAL STROMAL CELLS IN TISSUE REGENERATION

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Mesenchymal stem/stromal cells (MSCs) are located in the stromal fraction of the most tissues of human body. They participate in tissue repair and regeneration by differentiation into various cell types as well as by secretion of biologically active molecules and extracellular vesicles (EVs). EVs are membrane vesicles, containing cell-type specific proteins, lipids and various types of RNA. Depending on the biogenesis pathway EVs are classified into exosomes, microvesicles and apoptotic bodies. MSC derived from adipose tissue produce EVs with an average size around 110nm, which include both exosomes and microvesicles. These membrane entities appeared to be necessary for a MSCs action on tissue regeneration. MSCs-derived EVs transfer cytokines and membrane receptors, as well as microRNAs into target cells, such as endothelial cells thereby affecting their functional activity. Growth factors such as PDGF-BB and bFGF modulate MSCs ability to produce EVs as well as cause changes of their molecular content. Our recent data suggest that hormones, including catecholamines and angiotensin could also regulate EVs production by MSCs and affect their regenerative potential. Further improvement of EVs purification methods would potentially allow the development of novel cell-free approaches for a clinical usage. The study was supported by RSF grant 14-15-00439.

Keywords: Mesenchymal stem/stromal cells, secretome, extracellular vesicles

UNCOVERING THE MECHANISM OF LIFE/DEATH DECISIONS AT CD95/FAS

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Apoptosis is the program of cell death that is essential for all multicellular organisms. Substantial evidence exists that the de-regulation of apoptosis plays a major role in various diseases, with insufficient apoptosis leading to cell accumulation, resistance to therapy and defective tumor surveillance by the immune system. An apoptotic signal can be induced by DNA damage, growth factor withdrawal or activation of death receptors (DR). CD95/Fas is a member of the DR family that mediates induction of apoptotic and anti-apoptotic pathways, in particular, NF-κB. CD95/Fas signaling is triggered by binding of CD95L/FasL leading to the formation of a death-inducing signaling complex (DISC), which serves as a platform for initiator procaspase-8 activation. Recently, we and others have shown that procaspase-8 is activated *via* the formation of death effector domain (DED) chains/filaments at the DISC leading to apoptosis induction. DISC and DED chain assembly triggers not only apoptotic pathway but also induction of anti-apoptotic pathways. The decision between the induction of the apoptotic and the anti-apoptotic pathway is complex and multifactorial. In this regard, the cutting edge technology of imaging flow cytometry (IFC) a great asset. It combines microscopy and flow cytometry in one measurement circuit, thereby providing a major breakthrough methodology in contemporary cell research. IFC enables label-free quantitative analysis of single cell images estimated over a large number of cells simultaneously. Using IFC and Amnis FlowSight we can distinquish apoptotic vs. necroptotic cell death programmes as well as a quantitatively detect induction of both apoptotic and anti-apoptotic signaling pathways in a single cell. We shall discuss how this technology could provide new insights into the quantitative characterization of apoptosis, necroptosis and NF-κB. Furthermore, using a combination of biochemical analysis of the DED chains, IFC analysis and computational modeling, we have uncovered how several competing pathways control the fate of a cell, which in turn plays an important role for development of anti-cancer therapies.

Keywords: apoptosis, CD95/Fas, DED chains.

CAR-T THERAPY PERSPECTIVE: DEVELOPMENT OF BIOMEDICAL CELL-BASED PRODUCTS UTILIZING GENETICALLY MODIFIED IMMUNE CELLS

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One of the most promising methods of adoptive cellular immunotherapy for the treatment of oncohematological diseases is a CAR-T therapy. CAR-T is a T cell genetically modified to express a chimeric antigen receptor (CAR). These engineered receptors allow the T cells to recognize and attach to a specific protein, or antigen, on tumor cells. CAR is comprised of an extracellular domain specific for a tumor cell surface molecule, and linked to an intracellular signaling module for signaling transduction.

In this study, human T cells were isolated from the whole blood and transduced with a lentiviral recombinant vector caring the anti-CD19-CAR and green fluorescent protein (GFP) genes. Flow cytometry was used to characterize cell number, viability and cytokine expression levels. The results of the experiments showed that the anti-CD19-CAR-T cells maintained high viability (more than 90% of CD3+ viable cells) along with lentiviral transduction efficiency of at least 40%. During direct co-cultivation experiments (48 and 120 hours), CAR-T cells actively secrete cytokines (IFN-γ, IL-2, TNF) and demonstrated high cytolytic activity against CD19-positive targets.

Thus, we demonstrated that genetically modified T cells could be used in the manufacturing of biomedical cell-based products for the treatment of hematologic malignancies. Further pharmaceutical development and marketing authorization of CAR-T therapy rely on the elaboration of a comprehensive quality management system to control safety and efficacy of this type of innovative drugs.

Keywords: CAR-T therapy, chimeric antigenic receptor, adaptive cellular immunotherapy, lentiviral transduction

INHIBITION OF WIP1 DURING CELLULAR REPROGRAMMING LEADS TO INCREASE OF IPS COLONIES OUTCOME

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Process of cellular reprogramming is associated with genome-wide epigenetic changes. Major number of small compounds, able to facilitate reprogramming (e.g. derivatives of valproic acid) acts as epigenetic modulators, switching repressive histone marks to activating ones. Previously, it was reported that hyperosmosis significantly increased the efficiency of generation of induced pluripotent stem cell (iPSC) colonies in a p38-dependent manner and accompanied by genome-wide chromatin demethylation. Earlier our group has identified that Wip1 phosphatase, negative regulator of the number of stress-induced signaling pathways, is activated upon hyperosmosis. We hypothesized that precise modulation of Wip1 activity during various stages of reprogramming could enhance the iPSC generation efficiency.

Wip1 gene activation was evaluated using luciferase reporter under the control of Wip1 promoter in SaoS2 cell line. Mouse embryonic fibroblasts (MEFs) transduced with lentiviruses carrying OKSM reprogramming set were used as a cell reprogramming model. Osmotic stress was generated by addition of NaCl to the culture medium (100 mM final concentration). Reprogramming efficiency was assessed by counting of alkaline phosphatase-positive colonies at day 15 post-infection.

Using SaoS2 luciferase reporter model, we confirmed that NaCl treatment leads to significant Wip1 overexpression. Consistently with previous reports osmotic stress significantly increased the efficiency of MEFs reprogramming (218±34) in comparison to control conditions (22±3). Treatment of MEFs with Wip1 inhibitor GSK2830371 during osmotic stress was able to increase the reprogramming efficiency even more (365±69) confirming our assumption.

Our data suggest that Wip1 activity during osmotic stress-mediated reactivation of p38 negatively regulates the reprogramming most likely through the p38 inhibition. To decipher Wip1-dependent effects on cellular reprogramming with high accuracy, we plan to take advantage of our Wip1-deficient mouse model. Wip1 heterozygous mice do not show haploinsufficiency, spontaneous loss of heterozygosity and have close to wild type mice phenotype. A beta-galactosidase reporter under the regulation of Wip1 promoter integrated into the mouse genome allows us to track Wip1 expression easily. Moreover, with the flow cytometry techniques we were able to distinguish between MEF with a high and low expression of Wip1. Reprogramming efficiency would be evaluated with high accuracy using SSEA1-positive cell counting using flow cytometry.

In conclusion, the obtained data would provide a comprehensive view of Wip1-dependent modulation of signaling pathways and chromatin remodeling in the cellular reprogramming.

This work was supported by RSF grant #14-50-00068

Key words: iPS, reprogramming, Wip1, osmotic stress

EFFECT OF THE INHIBITOR OF DEUBIQUITYLATING ENZYMES ON COFILIN LEVEL IN THE NERVE CELLS

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Cofilin (CFL) is a regulator of actin dynamics involved in various physiological processes, including embryonic development, wound healing, tumor metastasis, and neural network formation. The changes in the CFL activity and level were shown in different pathological states. A key role in the regulation of intracellular concentration of different proteins belongs to the ubiquitin-proteasome system (UPS). Inhibition of the UPS can affect different signaling pathways in the cell. At the same time ubiquitylation is a reversible process. Upon termination, ubiquitin is removed and recycled by the action of deubiquitylating enzymes (DUBs). The mechanism of action of the UPS inhibitors on the level and activity of various proteins remains poorly understood.

The aim of this research was to study the effect of PR-619, the inhibitor of deubiquitylating enzymes, on cofilin level in the nerve cells. For the study, hippocampus from the brain of C57BL/6 mice was used. Hippocampal cells were treated for 2 h with 0.5 % DMSO (Sigma) and 5 μM PR-619 (Abcam). After treatment cells were lysed using RIPA buffer. Cell lysates were analyzed by a western blot assay. Detection and quantification of band intensities was conducted using Gel Analyzer 2010a software.

The treatment of the nerve cells with the inhibitor of DUBs with broad specificity PR-619 (5 μM) was accompanied with accumulation of middle-weight molecular forms (proteoforms) of cofilin in comparison with intact hippocampal cells and control (0.5% DMSO). Thus, it was demonstrated that deubiquitylation catalyzed by intracellular DUBs is involved in control of CFL proteoforms. The sensitivity of CFL proteoform levels to inhibition of DUBs suggests a non-catabolic mechanism of cofilin regulation in the nerve cells. This mechanism may be involved in the regulation of cellular functions in normal and pathological conditions.

This work was supported by the Russian Science Foundation (grant number 17-75-10202)

Keywords: cofilin; nerve cell, deubiquitylating enzyme; PR-619

CELLULAR MODELS OF HUMAN DISEASES

HUMANIZED CELLS AND MICE FOR MODELLING THERAPIES OF HUMAN DISEASES

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Proinflammatory cytokines TNF, IL-6 and IL-1 contribute to pathogenesis of several autoimmune diseases providing a basis for successful anti-cytokine therapies. However, many antibody-based drugs used in humans are not active in mice. We have generated multiple mouse strains for cytokine research, including lines producing human cytokines, such as TNF and IL-6. From these mice primary cells can be used for *in vitro* experimentation and both mice and cells would respond to clinically used drugs. Using conditional gene targeting we established that only limited number of cell types in experimental diseases is producing “pathogenic” cytokine, while the same cytokine from other cellular sources may be protective. Based on these findings we are developing an approach to cell type-restricted cytokine neutralization by utilizing bispecific antibodies that would attach to the cell surface of a particular type of immune cells, and capture the cytokine released by these cells, preventing its dissemination. Our constructs are based on single domain antibodies (V_HH) specific for human TNF and IL6 and for cell type-specific markers, such as F4/80 and CD11b. We find that such antibodies can effectively attach to the cell surface, capture and retain released cytokines. Our findings may serve as a basis for bioengineering of a new type of cytokine inhibitors.

Additionally, our laboratory is developing prototypes of artificial lymph nodes based on 3D fibroin scaffolds with various cell compositions that can be transplanted under renal capsule.

Supported by Russian Science Foundation grant 14-25-00160 and by the Program of fundamental research for State Academies for 2013-2020 (№ 01201363822).

Keywords: human cytokines, single domain antibodies.

POSSIBILITIES OF USING PLURIPOTENT STEM CELLS FOR RESTORING THE DAMAGED RETINAL PIGMENT EPITHELIUM

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The retinal pigment epithelium (RPE) is a monolayer of pigmented, hexagonal cells connected by tight junctions. These cells form part of the outer blood-retina barrier, protect the eye from excessive light, have important secretory functions, and support the function of photoreceptors, ensuring the coordination of a variety of regulatory mechanisms. It is the degeneration of the pigment epithelium that is the root cause of many retinal degenerative diseases. The search for reliable cell sources for the transplantation of retinal pigment epithelium is extremely urgent. Pluripotent stem cells (embryonic stem or induced pluripotent) can be differentiated with high efficiency into the pigment epithelium of the retina. Pioneering clinical trials on transplantation of RPE cells differentiated from pluripotent stem cells in the United States and Japan confirmed the need to develop and optimize such approaches to cell therapy. For effective use, RPE differentiated from pluripotent stem cells should have a set of functional properties characteristic of pigment epithelial cells in vivo. We developed a robust protocol of differentiation and expansion of RPE cells from integration-free iPSCs from healthy donors. Resulting RPE cells accumulated pigment, expressed markers of pigment epithelium and had the ability of phagocytosis. We also tested the ability of a set of scaffolds prepared by electrospinning to support the maturation and polarization of IPSC-derived RPE cells. RPE cells cultured on the modified polyurethane showed good adhesion, formed hexagonal honeycomb morphology, tight junctions and developed apical microvilli.

Different differentiation protocols based on literature data and our own data, and the problems on the way to the therapeutic use of RPE differentiated from pluripotent stem cells will be discussed.

Key words: Retinal pigment epithelium, differentiation, embryonic stem cells, induced pluripotent stem cells, cell therapy

DECIPHERING MECHANISMS OF CONGENITAL HEART DISEASES USING CARDIAC MESENCHYMAL CELLS

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Recently fine-tuned sequential activation of Notch genes has been shown to be responsible for the proper heart chamber development. In addition, mutations in several genes of the Notch pathway have been shown to be associated with Tetralogy of Fallot (TOF) and hypoplastic left heart syndrome (HLHS). To elucidate more precisely the role of Notch pathway in these congenital defects we aimed to estimate expression of Notch related genes in cardiac mesenchymal cells in the patients with TOF, HLHS and ventricular septal defect (VSD). Cardiac mesenchymal cells were isolated from leftovers after surgical intervention by collagenase digestion and were cultured for several passages in vitro. Gene expression of Notch family genes (*NOTCH1, NOTCH2, NOTCH3, NOTCH4, HEY1, HEY2, JAG1, DLL4, BMP2, BMP4*) was assessed by qPCR. Notch receptors and ligands surface expression was also estimated by flow cytometry. Our data on cardiac mesenchymal cells derived from VSD, HLHS and TOF patients suggest direct involvement of Notch pathway dysregulation in the pathogenesis of the HLHS. Regarding tetralogy of Fallot, the role of Notch pathway remains questionable and requires further research.

Key words: Notch, congenital heart disease, cardiac mesenchymal cells

CELL THERAPY OF ISCHEMIC STROKE: STUDIES USING THE RAT MIDDLE CEREBRAL ARTERY OCCLUSION MODEL

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We re-assessed the effects of cell therapy in experimental ischemic stroke using modified rat middle cerebral artery occlusion (MCAO) model. Mesenchymal stem cells (MSCs) isolated from human term placenta or neural progenitors (NPs) differentiated from human induced pluripotent stem cells were injected into rats intravenously or intra-arterially 24 hours after transient MCAO or sham operation. The volumes of cerebral infarction zone were evaluated by magnetic resonance imaging (MRI) and the level of neurological deficit estimated by neurological scoring. Double superparamagnetic iron oxide (SPIO) and fluorescent labeling allowed tracing of transplanted cells in live brain by MRI and histological verification. Cell proliferation and additional verification of the identity of transplanted cells were assessed by immunohistochemistry. Cell transplantation enhanced neurological recovery after MCAO and induced faster and more complete reduction of the infarction zone. Beneficial effects were more pronounced if transplantation was carried out under optimum conditions with regard to the dilution of cell suspension and injection rate. Neurological deficits were better restored after transplantation of NPs, while transplantation of MSCs resulted in faster reduction of vasogenic edema and increased overall survival rate. Transplanted cells penetrated the blood-brain barrier and either underwent homing in the immediate proximity of blood vessels or migrated along blood vessels concentrating around the infarction zone. Transplantation of NPs boosted the numbers of proliferating cells in the neurogenic zones of the host's brain. Our data further substantiate the therapeutic efficacy of cell transplantation in brain ischemia and show that specific curative goals can be addressed by varying transplanted cell type and transplantation route.

Key words: ischemic stroke, animal model, cell therapy, mesenchymal stem cell, iPS-derived neural progenitor cells

UNDERSTANDING PATHOGENESIS OF HUMAN CHROMOSOMAL DISORDERS RELATED TO INTELLECTUAL DISABILITY BY CELL REPROGRAMMING TECHNOLOGIES

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Introduction: Current molecular cytogenetic techniques provide a great opportunity to detect small submicroscopic chromosomal changes related to idiopathic forms of congenital malformations and developmental delay. Sometimes these abnormalities affect a copy number variations (CNVs) of a single gene only making it a strong candidate for major clinical features of microdeletion or microduplication syndromes. Here, we report about gene's expression analysis in neuronal cells derived by *in vitro* differentiation of induced pluripotent stem cells (IPSC) from patients with intellectual disability and novel reciprocal microdeletion and microduplication of 3p26.3 affecting a single gene *CNTN6*, which involved in the formation of axon connections in the developing nervous system (Kashevarova et al., 2014).

Materials and Methods: Cortical neurons were obtained by *NGN2* differentiation of IPSCs derived from skin fibroblasts of two patients with intellectual disability and dysmorphic features with reciprocal CNVs at 3p26.3 as well as from three healthy donors with normal karyotype. The neuronal RNA was examined using SurePrint G3 Human Gene Expression 8×60K Microarray Kit (Agilent Technologies, USA) and droplet-digital PCR. The enrichment analysis for identification of functional groups of genes was performed.

Results: For the first time, the preferential expression of maternal allele of *CNTN6* was observed in donor's neuronal cells as well as in patient's neurons with microduplication. It was found also, that expression of duplicated allele of paternal origin was significantly reduced in spite of the *CNTN6* copy number increasing due to chromosomal microduplication. The comparison of gene expression in a wild type neurons and neurons with *CNTN6* microdeletion revealed 756 differentially expressed genes

(142 downregulated and 614 upregulated genes). The enrichment analysis of the downregulated genes revealed their involvement in responses to glucocorticoid and corticosteroid, astrocyte development, basic amino acid transport and transmembrane transport. Upregulated genes were involved in regulation of synaptic signaling, trans-synaptic signaling, anterograde trans-synaptic signaling, chemical synaptic transmission, cell-cell signaling, nervous system and neuron development, regulation of postsynaptic membrane potential.

Conclusions: The transcriptome analysis of iPSC-derived neurons revealed the common affected neuronal pathways and signaling mechanisms which can explain the complexity on neuronal phenotype in patients with chromosomal disorders. Allele-specific biased expression of *CNTN6* may contribute to incomplete penetrance of CNVs depending on parental origin of preferentially expressed allele.

This study was supported by Russian Science Foundation, grant 14-15-00772.

Keywords: iPSC-derived neurons, gene's expression analysis, chromosomal disorders.

MOUSE MODELS OF DEVELOPMENTAL NEUROLOGICAL DISORDERS

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The cerebral cortex, is the seat of higher cognitive capacity that distinguish humans from other species. The development of the cerebral cortex is a complex and highly orchestrated process whose disruption can result in a wide range of developmental disorders that are recognized as malformations of cortical development (MCD). Malformations of the cerebral cortex can frequently cause epilepsy, developmental delay, neurological deficits, and mental retardation in humans. Intellectual disability and epilepsy caused by neurodevelopmental disorders has a high prevalence of about 2% in human population. Humans and rodents share the basic structural organization and developmental principles in the cortex. Therefore, mice can be used as good animal models to investigate molecular basis of MCDs. One of the models that we developed is a mouse model of Mowat-Wilson Syndrome (MWS). MWS patients suffer from intellectual disability, microcephaly, epilepsy and delayed motor development. Another model that we generated is the mouse line mimicking Satb2 Associated Syndrome (SAS). SAS in humans is characterized by to severely impaired speech & intellectual disability. Both model allowed us to identify and characterize molecular targets of both Satb2 and Sip1 genes that cause developmental brain malformations.

This work was supported by RSF grant to VT.

Keywords: malformations of cortical development (MCD), neurodevelopmental disorders, Mowat-Wilson Syndrome (MWS), Satb2 Associated Syndrome (SAS).

ORGANOIDS AND TISSUE ENGINEERING

ADVANCED MODELLING OF NEURODEGENERATION WITH THREE-DIMENSIONAL HUMAN MIDBRAIN ORGANOIDS

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Models of the human midbrain are critical for understanding human development as well as for identifying and testing novel therapeutics for neurodegenerative diseases such as Parkinson's disease. However, the human midbrain is a complex 3-dimensional structure composed of multiple cell types, including neurons, astrocytes and oligodendrocytes, which has been difficult to model *in vitro*, particularly in a scalable manner. Here, we describe a novel approach to obtain large numbers of high quality midbrain organoids, derived from our unique midbrain floor plate neural progenitor cells (mfNPCs) that can be differentiated with high efficiency in classical 2D cultures. Using these cells, we report the generation of midbrain-specific organoids containing astrocytes, oligodendrocytes, and midbrain dopaminergic neurons, which can be used for disease modeling. 3D organoid cultures of mfNPCs form functional neuronal networks and secrete dopamine. Importantly, midbrain organoids derived from Parkinson's disease specific stem cells reveal disease relevant phenotypes. mfNPCs, enable research to study human midbrain development, modeling of region-specific neurodegenerative diseases, and high throughput drug screening using organoids.

Keywords: iPSC, neural stem cells, midbrain dopaminergic neurons, midbrain-specific organoids, Parkinson's disease

STEM CELLS AS ORGANELLES DONORS: INTRACELLULAR TRANSFER OF MITOCHONDRIA

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Transport of mitochondria in cells was firstly discovered in neurons with long axons, where mitochondria are able to move from the cell body to the periphery, toward the region of the synaptic terminals (anterograde transport) and back from the periphery to the perinuclear zone (retrograde transport). It is believed that such movement of the mitochondria is to provide ATP to places with high energy consumption. Recently, it was open the possibility of intercellular transport of mitochondria. Active study of this transfer began with the discovering of the tunneling nanotubes (TNT) – intercellular structures most widely described as mechanism of organelles transport between cells. Moreover, some researchers suggest that the presence of TNT is a necessary and sufficient condition for transfer of mitochondria. Interestingly, most of the works describing the transfer of mitochondria between cells, refer to stem cells, although there was evidence of intercellular mitochondria transport for other cell types, e.g., tumor cells. Since there is number of diseases related to the mitochondrial dysfunctions, it looks very attractive to replace malfunctioning organelles with healthy donor cells mitochondria from stem cells.

In the past few years, we studied the mitochondrial transfer between mesenchymal multipotent stromal cells (MMSC), and various types of differentiated somatic cells: neurons, cardiomyocytes, renal epithelium cells.

The transfer of mitochondria was studied on the model of cocultivation of MMSC with differentiated cells, transfer of mitochondria was assessed using confocal microscopy and flow cytometry. To discriminate the mitochondria in different types of cells, the organelles were stained with the specific mitochondrial fluorescent probes or genetically encoded fluorescent proteins with mitochondrial address.

In the heterologous cell cultures, we observed transfer of mitochondria from MMSC to neurons, renal epithelium and cardiomyocytes. For example, after 24 h of cocultivation MMSC and neurons, MMSC-derived mitochondria with red fluorescent protein Mito-DsRed, was observed in neurons, whose own mitochondria contain the green fluorescent protein Mito-GFP, however, the reverse transport was not observed. Similar observations were made for cardiomyocytes and renal epithelium. In the case of renal epithelium xenogenic system were used, in which human MMSC were cocultured with rat renal cells. In this case, the transfer of mitochondria was confirmed by immunofluorescence with specific antibodies against human cytochrome oxidase. Rat cells mitochondria were not stained by these antibodies, whereas after cocultivation we observed the appearance of mitochondria positive for human cytochrome oxidase, that was transferred from human cells.

Noteworthy, simultaneously with the transport of mitochondria the transfer of low molecular weight components of the cytoplasm was observed between cells. The direction of transport of the cytoplasm was different in various cell systems. During cocultivation of MMSC and cardiomyocytes the cytoplasm was transferred in both directions: toward MMSC and backward to cardiomyocytes. When MMSC were cocultivated with renal epithelium the transport of the cytoplasm from the epithelial cells toward MMSC was predominant, but in coculture with neurons, the transfer of the MMSC's cytoplasm into neurons was not observed at all. However, in all systems the direction of transfer of mitochondria was the same – from the MMSC to the recipient cell, although the efficiency of such transport was quite low: not more than 10% of the cells in the culture received mitochondria from the MMSC.

We found that the level of Miro1 protein (Rho-GTPase responsible for transport of mitochondria) was doubled in MMSC, cocultivated with neurons. Based on this observation, we developed MMSC, overexpressed Miro1. During cocultivation of such MMSC with neural cells the ratio of mitochondria transfer was doubled.

Very important was the observation of the recovery of bioenergetic functions after the transfer of mitochondria. Neuron-like PC12 cells were investigated, in which mitochondrial DNA was damaged by ethidium bromide. In such cells due to disturbed respiration, the main part of ATP is produced by glycolysis, resulting in increased production of lactate. After cocultivation of these cells with MMSC the amount of lactate in the medium was decreased, indicating the recovery of oxidative phosphorylation.

Thus, we have shown that MMSC can serve as donors of functionally active mitochondria, transferring them into different somatic cells. Such transfer of the mitochondria possibly affects the restoration of mitochondrial function in recipient cells damaged by oxidative stress. Perhaps the transport of mitochondria from MMSCs into the damaged cells provides some positive effects of MMSCs transplantation during cell therapy against the various pathologies associated with mitochondrial damage.

This work was supported by grants of the President (MD-2065.2018.4) and RSF (14-15-00107).

Keywords: intercellular transport of mitochondria, model of cocultivation.

FIBROBLASTS: CHRONICALLY BAD BUT NOT SO DOWN IN THE MOUTH

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Chronic non-healing wounds are a major health problem within our ageing society. Such wounds are characterized by persistent inflammation, failed re-epithelialisation, a lack of granulation tissue formation and they are exacerbated by high levels of bacterial infection. Despite some clinical advances, 10-20% of chronic wounds still do not heal and thus novel therapeutic approaches are required. Our investigations have focussed on whether age-related functional changes in dermal fibroblasts may contribute to this dysfunctional healing phenotype. Microarray analysis of chronic wound fibroblasts (CWFs) and normal skin fibroblasts (NFs) demonstrated altered regulation of numerous genes including molecules involved in the protection against oxidative stress. CWFs proliferate more slowly than NFs and premature senescence of CWFs was confirmed by increased SA X-Gal activity and their larger, polygonal morphology. Analysis of telomere lengths revealed that whilst senescence in some CWFs was telomere-dependent in others it was telomere-independent. This premature senescence significantly decreased their abilities to carry out key wound healing activities. This is however, in direct contrast to fibroblasts isolated from wounds that heal in a scarless manner (oral mucosal fibroblasts; OMFs) that demonstrate differential gene analysis, increased proliferation, lower levels of senescence (longer telomeres) and accelerated wound healing responses. From our microarray data we have now described a transcriptional signature for this 'wound healing continuum' which may assist in the therapeutic assessment/treatment of a patient's wound. Our recent findings however, suggest it is the presence of a progenitor cell sub-population resident within the heterogeneous oral stromal population that is key to the preferential healing. Such oral mucosal lamina propria-progenitor cells (OMLP-PCs) can be rapidly and clonally expanded *in vitro*, are neural crest-derived and are multipotent. Furthermore, they are potently immunosuppressive and have distinct antibacterial activities. Such activities may be delivered by a distinct population of OMLP-PC exosomes which are currently under investigation. We postulate that OMLP-PCs could be an efficacious cell-based therapy for the future amelioration of chronic wounds.

Keywords: chronic non-healing wounds, dermal fibroblasts.

MULTIMODAL IMAGING FOR CELL TECHNOLOGIES AND TISSUE ENGINEERING APPLICATIONS

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Currently the cell technologies and tissue engineering take a special niche in the biomedicine. The urgent task in this area is the pre-clinical testing of the bioengineering constructs and the biomedical cellular products using multimodal imaging and genetic markers. Such unique techniques allow to visualize the individual cells embedded in the constructs, their migration, proliferation, and also to study the cell differentiation processes. The study of the epigenetic mechanisms of stem cell (mesenchymal stromal cells (MSC), induced pluripotent stem cells (iPS)) differentiation is an actual problem. So using fluorescence lifetime imaging microscopy (FLIM) the metabolic switch from glycolysis to oxidative phosphorylation was shown during MSC differentiations by the lifetimes changing of NAD(P)H. Also we study the involvement of seeded allogeneic MSCs in bone formation using the model of transgenic mice expressing fluorescent protein GFP and genetically labeled cells. Allogeneic MSCs were found on the scaffolds 6 and 12 weeks post-implantation. By week 12, a newly formed bone tissue and blood vessels from the seeded cells were observed. Despite the significant progress in developing of skin equivalents (SEs) a problem of non-invasively assessing the quality of the cell components and the collagen structure of living SEs both before and after transplantation remains. Using the methods of optical coherence tomography (OCT), multiphoton tomography (MPT) and FLIM, the structure and quality of dermal SEs before transplantation, and remodeling of collagen matrix and microcirculation in the wound healing after dermal SEs transplantation were studied. Thus, the methods of optical imaging and genetic labeling are a powerful tool for the solving a huge number of problems in both the tissue engineering and the biomedicine in general.

This work has been financially supported by Russian Science Foundation (grants No. 17-75-20178).

Key words: mesenchymal stem cells, iPS, multimodal imaging

NEW APPROACHES TO TRACHEAL EPITHELIUM REPAIR: ELECTROSPUN SCAFFOLD FOR MUCOSAL BIOENGINEERING AND AN ANIMAL MODEL TO ASSESS ITS THERAPEUTIC EFFICACY

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Tracheal epithelium has a limited ability for regeneration. Therefore, its full-thickness damage due to inflammation, trauma, surgical intervention or tumors often leads to tracheal stenosis which represents a significant clinical problem. If the cartilaginous carcass of the trachea remains intact, the mucosal integrity can be restored using epithelial equivalents comprised of either natural or artificial polymer scaffolds impregnated with autologous cells. Nevertheless, such therapeutically sound equivalents of the respiratory epithelium still do not exist. There are two primary reasons for that. Firstly, tracheal epithelium bioengineering is hampered by the lack of adequate polymeric scaffolds combining therapeutically adequate mechanical properties and the ability to support growth and differentiation of the functional respiratory epithelium (formation of both ciliated and Goblet cells). Secondly, the search for the most efficient composition and structure of the scaffold requires an efficient experimental *in vivo* model of tracheal epithelium damage and repair that would allow to test a series of constructs differing in composition and properties.

In order to fill these technological gaps, we developed an original biodegradable co-polymer comprising chitosan, gelatin and poly-L-lactide, which appeared to be highly biocompatible with epithelial progenitor cells (obtained from nasal mucosa) supporting their active growth and differentiation into ciliated and Goblet cells (as tested on polymeric films). This co-polymer was also highly efficient in supporting the growth of primary tracheal fibroblasts. Using this co-polymer and electrospinning technology we obtained a bi-layered non-woven scaffold possessing adequate mechanical properties and sufficient for generation of a full-thickness tracheal equivalent, comprising functional epithelium and an analog of tracheal submucosa.

Then, we developed an experimental *in vivo* model for assessment of tracheal epithelium regeneration through application of either polymeric scaffolds of different types or epithelial equivalents. Using laboratory rabbits we have established several new techniques which include: 1) prevascularization of the future zone of tracheal intervention using a fascial flap transfer technique in order to improve blood supply to the trachea; 2) formation of a critical (not self-healing) full-thickness anterolateral mucosal defect by tracheal mucosa excision; 3) fixation of the scaffold or equivalents at the site of the tracheal epithelium defect using two different approaches - suturing or a vascular stent (which appeared to be much more efficient). In addition to these techniques, we outlined a set of polymeric scaffold features essential for efficiency of their therapeutic use and showed that non-woven materials are most prospective for tracheal regenerative therapy.

Altogether, we have developed an experimental model for routine assessment of efficiency of bioengineered tissue equivalents in treatment of tracheal epithelial defects. These techniques allow the use of autologous cells, thus providing a possibility for therapeutic application of developed approaches.

Keywords: tracheal epithelium, biodegradable co-polymer, epithelial progenitor cells.

THE USE OF PHOTOCURABLE BIODEGRADABLE IMPLANTS BASED ON METHACRYLATED SILK FIBROIN FOR THE PREVENTION AND TREATMENT OF INSOLVENCY OF THE GASTROINTESTINAL TRACT ANASTOMOSIS

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Relevance: despite the significant success of surgery, the results of surgical treatment are not always satisfactory. One of the leading factors is the failure of anastomosis of hollow organs, which can reach 20%, with lethality of up to 80%. We propose the use of tubular biodegradable implants based on methacrylated silk fibroin, as an alternative way of reducing anastomotic dehiscence incidents.

The aim of the study is to evaluate the effect of a biodegradable implant based on methacrylated silk fibroin on the process of gut wall regeneration on the experimental model of intestinal anastomotic dehiscence.

Methods of investigation: experimental study on Wistar rats. In the course of the study, the animals were divided into 3 groups: 1st - circular bowel defect was corrected with a tubular biodegradable implant, 2nd - correction with the biodegradable implant vitalized with bone marrow cells, 3rd - circular defect of the small intestine was bridged with a single-node interrupted suture, after preliminary ligation of the feeding vessel. On the 30th day after the operation, the animals were removed from the experiment. At the autopsy, the state of the abdominal organs was assessed. The site of the intestine with the implant was examined histologically and immunohistochemically.

The results of autopsy were as follows: no expressed adhesion process or anastomotic dehiscence was observed in the animals of the 1st and 2nd groups. In the third group, 50% of the animals died from peritonitis as a result of intestinal anastomotic dehiscence.

Histological examination determined the complete restoration of the intestine.

Conclusions: the current method of using photocurable biodegradable implants based on methacrylated silk fibroin can help to reduce the number of anastomotic dehiscence in hollow organs.

Key words: biodegradation, implants, fibroin, cells, anastomoses.

OBTAINING MIXED CULTURES OF NEURONS AND GLIA FROM HUMAN INDUCED PLURIPOTENT STEM CELLS IN THE 3D SYSTEM

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The mortality from cardiovascular diseases of the brain in our country takes a second place, slightly yielding to mortality from heart diseases. These diseases lead to the loss of neurons in certain areas of the brain. Existing methods of treatment are based on the use of neuropeptides isolated from the brain of mammals. The xenogeneic origin of the preparations significantly limits their efficiency. The perspective direction is the obtaining drugs based on human induced pluripotent cells (iPSC) differentiated in the neural direction. The use of autologous or allogeneic cells allows obtaining a drug enriched with neurotrophic factors, devoid of immunogenic properties for apply in nootropic and neuroprotective therapy.

The aim of the work is to develop a method of effective directed neural differentiation of iPSC in a 3D system.

We have developed a protocol for obtaining neuronal / glial cultures in a 3D system without using specific neural inductors. iPSC was cultured for 7 days on low adhesion plastic in DMEM / F12 medium with addition of reagents B27, N2 and EGF to form embryoid bodies. Further 28 days were cultured in medium supplemented with FGF-2. The resulting neural stem cells were grown and then differentiated in medium without growth factors 14-28 days. In the formed neurospheres were identified mainly neuronal progenitor, mature neurons and glial cells. Methods of PCR and immunocytochemistry were showed the presence of markers characteristic for different stages of neural differentiation: Pax6, Foxp2, Nestin, Tubb-βIII, Map2, NSE, GFAP.

The developed protocol allows obtaining neuronal / glial cell cultures in unlimited quantities using a bioreactor without expensive specific inducers and components of animal origin.

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Keywords: neuronal / glial cultures in a 3D system, neurospheres, neural differentiation.

13 APRIL

GENOME EDITING

A CRISPR/CAS9-BASED GENE EDITING FRAMEWORK ALLOWS MODELING EARLY-ONSET ALZHEIMER'S DISEASE IN GENETICALLY DEFINED HUMAN IPSC LINES

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Our aging society is confronted with a dramatic increase in patients suffering from Alzheimer's disease (AD), for which no mechanism-based cure is available. Animal models, although very useful for understanding some the molecular mechanisms resulting in AD pathology, do not capture key disease aspects and have limited use in developing treatments.

The development of induced pluripotent stem cells (iPSCs) and their differentiation into human brain neurons now allows generating human *in vitro* AD models that could enhance functional studies and improve drug screening. Although first studies have shown promising results, the field still lacks an efficient technology for generating genetically defined lines that faithfully display disease-relevant phenotypes.

We developed a highly efficient CRISPR/Cas9-based genome-editing framework that allows selective introduction of homo- and heterozygous mutations into human iPSCs. By systemically interrogating gene editing events at the APP and PSEN1 loci, we identified experimental conditions that allow for efficient insertion of mutations at only one allele, which is crucial for direct replication of the mutation zygosity found in early-onset AD (EOAD) patients.

Using this approach, we generated the first human iPSCs with heterozygous and homozygous dominant EOAD APPSwe or PSEN1M146V mutations. When differentiating iPSCs into disease-relevant cortical neurons we found genotype-dependent disease phenotypes, particularly in the pathological amyloidogenic processing of APP.

Taken together, our findings will allow the field to apply a new generation of genetically defined human *in vitro* models for uniquely human neurodegenerative or other diseases, which can shed new light on underlying molecular mechanisms.

Keywords: CRISPR/Cas9, genome editing, induced pluripotent stem cells, Alzheimer's disease

GENE EDITING FIDELITY: WHAT MAKES A TARGET DIFFERENT FROM A NON-TARGET

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Modern genome editing makes great progress in the laboratory, yet its advance into the wide domain of public health is hampered by one single factor: the possibility of off-target changes in the genome. For genome editing to be considered generally safe and reliable without full genome sequencing to confirm the absence of off-target mutations, its fidelity should be comparable with the fidelity of replication, which, in human cells, amounts to ~0.3 mutations per cell division per diploid genome. To be sure that this goal is ever achievable, we have to understand the sources of errors made by genome editing systems. Here we address the question whether DNA damage, which is persistently generated by a multitude of intracellular and environmental factors, could affect the recognition of protospacers and protospacer-adjacent motifs (PAM) by Cas9 endonuclease. We show that Cas9 can efficiently recognize some sequences that carry non-coding, miscoding or dual-coding damaged nucleotides. Moreover, introduction of an abasic site, one of the most abundant types of DNA damage, into PAM does not interfere with PAM recognition in the supercoiled DNA, despite the loss of absolutely conserved interactions with Gua bases in PAM, but makes this recognition dependent on the topologic properties of DNA. Thus, DNA damage can be a source of off-target cleavage by Cas9. We also report on the effects of chemical modifications in single-guide RNA on the activity and fidelity of Cas9.

Supported by Russian State funded 2013–2020 budget project VI.62.1.5 0309-2016-0003.

Keywords: genome editing, Cas9, off-target effects, DNA damage

CRISPR-CAS9 MEDIATED KNOCKOUT OF HOST RESTRICTION FACTOR GENES ENHANCES INFLUENZA A VIRUS REPLICATION IN 293FT CELL LINE

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The propagation of influenza virus in cell cultures of human origin is an attractive way to more accurately reproduce the original human isolate, preserve its glycosylation profile and antigenic properties. However only a few cell lines are highly permissive to influenza virus and none of them are of human origin. The barrier is believed to be associated with host restriction factors inhibiting influenza growth. The aim of presented work was exploring the CRISPR-Cas9 mediated knockout of several genes as a way to overcome the host restriction barrier and generate human cell line permissive to influenza infection.

The first results are dedicated to CRISPR-Cas9-mediated editing of ANXA6 gene and analyzing of several 293FT-ANXA6$^{-/-}$ clones. It was found that, 293FT-ANXA6$^{-/-}$ cells demonstrated increased virus production. Depletion the ANXA6 factor, normally counteracting influenza infection, can be one of the steps in the development of a highly permissive cell culture of human origin, intended for the isolation and characterization of influenza viruses and production of influenza vaccines. We propose that multiple gene knockout could provide the desired level of influenza virus replication in adherent 293FT to use this cell line for influenza virus isolation and characterization.

The study was supported partially under State funded budget project (VI.62.1.5, 0309-2018-0003).

Keywords: Influenza virus, CRISPR-Cas9-mediated knockout, permissive cell line, genome editing

GENES KNOCKOUT IN INDUCED PLURIPOTENT STEM CELLS WITH CRISPR/CAS9 AND CLONES SELECTION USING DROPLET DIGITAL PCR

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Induced pluripotent stem cells (IPSC) are promising resource for regenerative medicine, since IPSC can be differentiated into a wide range of adult cells. With the rapid development of genome editing systems CRISPR/Cas9, have become actual genes knockout methods in cell cultures, including IPSC. In this way, genes knockout in IPSC can help us to identify the role of these genes in development or differentiation. However, due to a number of IPSC features, for example, a low transfection efficiency, low cloning ability, and their property to grow in dense colonies, obtaining separate cloned mutant IPSC lines poses a certain problem. We tried to summarize several methods (electroporation, cell sorting, screening of clones, including droplet digital PCR) into one protocol for obtaining IPSC lines with genes knockout.

Research was funded by the IDB RAS government program of basic research № 0108-2018-0004.

Keywords: hIPSC, CRISPR/Cas9, gene knockout, electroporation, cell sorting, droplet digital PCR.

THE OBTAINING OF CRISPR/CAS9-MODIFIED HUMAN INDUCED PLURIPOTENT STEM CELL LINE WITH UPREGULATED HYPOXIA INDUCIBLE FACTOR: A CONTRIBUTION TO PLURIPOTENCY AND ANGIOGENESIS

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Pluripotent stem cells (PSC) are the model object that allows to obtain any cells of an adult organism. The transition from the normoxia to the hypoxia state causes changes in gene expression, affects the pluripotency and differentiation of PSC. Cellular responses to hypoxia are mainly regulated by the activation of transcription factors known as hypoxia-inducible factors (HIFs). HIFs influence stress response signaling pathways that regulate development and metabolism, inflammation and the other processes, which play a crucial role in angiogenesis regulation. Nowadays, HIF-pathway modulation is a superior therapeutic strategy for various disease control. The aim of the work is to obtain genetically modified human induced pluripotent stem cell line with stabilized HIF-2α expression in normoxia conditions, realized by CRISPR/Cas9-mediated silencing of *eIF3e/INT6* that is inhibitor of HIF-2α. The sgRNA sequences were designed for *eIF3e/INT6* gene to obtain a deletion of 200 b.p. including the promoter and the beginning of the 1st exon. Obtained vectors was delivered to human iPSC by nucleofection. The presence of required deletion was confirmed by sequencing. By using qRT-PCR it was found out that the level of *eIF3e/INT6* expression is significantly reduced by more than 3 times in obtained genetically modified subclones of iPSC. The expression level of HIF-2α was significantly elevated in comparison with the control line of iPSC. The presence of HIF-1α and HIF-2α proteins was confirmed by Western blotting. Obtained genetically modified iPSCs will be used for the research of HIF-2α activation influence in normoxia conditions on angiogenic properties of endothelial derivatives.

The work is supported by The Russian Science Foundation (№ 17-75-10047)

Keywords: hypoxia inducible factor, human pluripotent stem cells, angiogenesis, CRISPR/Cas9 system

DEVELOPMENT OF A MODEL SYSTEM FOR EPIDERMOLYSIS BULLOSA SIMPLEX IN HACAT CELLS BY MUTAGENESIS OF KERATIN 5 WITH CRISPR/CAS9 TECHNOLOGY

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Epidermolysis bullosa is a heritable skin disease with various clinical heterogeneity. EBS form of disease is caused by defective assembly of keratin intermediate filaments in basal keratinocytes.

In EBS form of disease, mutations arise in keratins genes: KRT5 and KRT14. In the present work, we have applied CRISPR/Cas9 technology to modify krt5 gene and develop a model system of EBS on the base of HaCaT cells. Here, we analyzed and compare on-target mutation frequency in HaCaT cells using Cas9 nuclease (spCas9) or Cas9 nickase (spCas9n) genome editing with paired gRNAs specific to exon 7 of krt5 gene. Recent sequence data of patients revealed that there are more than 20 different mutations in this particular domain, among these mutations there are at least two with premature stop codon in conserved 2B rod domain of KRT5, which are involved in pathogenesis of skin in patients showing the characteristic clinical and ultrastructural features of EBS-Dowling Meara - the most severe subtype of EBS. Study of particular clones with impaired krt5 gene permits us to observe structural abnormalities in IFs and formation of IF aggregates in keratinocytes upon stress. We hope, that development of such models allows testing new approaches to the genome editing of the patients to create a cell-based therapy against EBS. Also, such models can help in screening of new drugs for treatment of EBS.

This work was funded by the program of Presidium of RAS “Fundamental Research for Biomedical technologies” №0108-2018-0009.

Key words: keratin 5, filaments, CRISPR/Cas9, genome editing, mutation.

BEST PRACTICE IN CELL THERAPY AND CELL PRODUCTS DEVELOPMENT

REGENERATIVE TREATMENT OF SPINAL CORD INJURY

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We have designed a two-component matrix (TCM) including 1) a solid anisotropic complex scaffold prepared by electrospinning from a mixture of recombinant analogues of the spider silk proteins – spidroin 1 and spidroin 2, 2) a “liquid matrix” based on platelet-rich plasma (PRP) and a neural medium containing growth factors. In experiments in vitro, we have studied biocompatibility and applicability of TCM for neural tissue engineering. It was previously shown that encapsulation of human directly reprogrammed neural precursor cells (drNPC) in PRP dramatically activated neurogenesis and promoted formation of neural tissue organoids connected by long processes of neurons. drNPC-01, obtained by direct reprogramming of bone marrow-derived mesenchymal cells, were mixed with freshly prepared liquid PRP, with the addition of Ca2+ and neuronal differentiation factors, and were placed into the anisotropic complex spidroin scaffold, in which PRP formed a clot. Neuronal differentiation was activated in drNPC embedded in such a two-component matrix, which was confirmed by the cascade expression of βIII-tubulin and MAP2. drNPC adhered well to the spidroin scaffold, and the processes of differentiating neurons interacted with its microfibrils and oriented parallel to them. At the same time, glial progenitor cells differentiated into GFAP-positive astrocytes, with localization on the periphery of neurons and having a probable supporting function. As a result, tissue engineered constructs consisting of neurons with unidirectionally oriented processes were prepared in two weeks. Implantation of the prepared tissue engineered construct containing human drNPC into the brain and spinal cord of two healthy Macaca mulatta monkeys has shown good biocompatibility with the neural tissue: no astro- and microglial reaction was present around the implanted construct. At the same time, human drNPC within the construct

composition grafted effectively, survived during 3 months of observation and differentiated with the formation of MAP2 positive neurons. By varying the shape and length of the TCM scaffold, tissue engineered constructs may be prepared by this technique for regeneration of both peripheral nerves and an injured section of the spinal cord.

Keywords: neural tissue engineering, neural stem cells, neural precursor cells, recombinant spidroin, PRP, spinal injury.

A NOVEL INJECTABLE BIOCOMPOSITE HYDROGEL FOR REGENERATION OF OSTEOCHONDRAL DEFECTS

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Restoration of the vast and deep osteochondral defects of the knee and hip joints remains one of the most difficult and still unsolved problems in traumatology and orthopedics. Currently, in clinical practice the need for cartilage grafts occurs primarily in the osteochondral defects, which is characterized by destruction of the articular cartilage and bone caused by trauma or disease. Herein, we describe the features of a novel injectable biocomposite hydrogel designed to simultaneously induce the endogenous regeneration of hyaline cartilage and subchondral bone. Developed biocomposite hydrogel was produced by gelation, using modified heparin-conjugated fibrinogen and thrombin. As active components, synovium-derived mesenchymal stem cells (SDMSCs), human recombinant transforming growth factor (TGF-β1) and bone morphogenic protein (BMP-4) were added in the hydrogel. *In vitro* study showed that developed biocomposite hydrogel has high biocompatibility and able to control a release TGF-β1 and BMP-4 into microenvironment. In order to evaluate therapeutic efficiency of the developed biocomposite hydrogel, a rabbit articular osteochondral defect model was used. Our preclinical study showed that biocomposite hydrogel containing SDMSCs and growth factors can completely regenerate damaged hyaline cartilage and subchondral bone during 3 months after implantation into osteochondral defect. This data suggest that our injectable biocomposite hydrogel can be a good therapeutic candidate for effective regeneration of damaged joints.

Keywords: Biocomposite hydrogel, Osteochondral defect, Regeneration, Mesenchymal stem cells, Growth factors.

NEW BIOMATERIALS BASED ON MODIFIED POLYGALACTURONIDES PRESENT A VARIETY OF PROSPECTIVE APPLICATIONS IN REGENERATIVE MEDICINE

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Biocompatible materials characterized by the controllable degradation rate are promising tools for the tissue engineering and regenerative medicine. We have generated a series of standardized preparations of plant polygalacturonides by means of chemical engineering of the food-grade pectin. Using these preparations hydrogels, films, and sponges can be easily formed in the physiological conditions. Depending on the degree of the esterification they demonstrate different properties and exhibit an opposite cell behavior in adherent and 3D cell cultures. Biocompatibility and degradation rate were determined by the histological techniques and biochemical monitoring of the blood after subcutaneous sample implantations into rodents. Polygalacturonides supplemented with two types of collagens were able to form stable hydrogels with prolonged degradation rates and stimulated neurite outgrowth in cell cultures and experimentally provoked spinal cord injury in rats. Low and high esterification degrees were more suitable for the cell protrusion formation. Another type of materials distinguished by intermediate esterification degree showed the ability to support an undifferentiated state of neural stem cells culture and round–shaped phenotype of glioma and neuroblastoma cells with high viability even in the low concentrations of the growth factors. We analyzed the viscoelastic properties of the hydrogels with different amounts of the anionic groups and found no correlations with these findings. To grasp the mechanism of cell proliferation control we analyzed Ras-Raf-MEK-ERK pathway components and observed their inhibition in cells cultivated on polygalacturonide hydrogels. These data promise a broad range of applications of newly developed materials in biotechnology and regenerative medicine – from drug and cell delivery to stem cells cultivation, differentiation, and regeneration techniques.

Keywords: biocompatible materials, polysaccharide hydrogels, neuroregeneration, cell technology.

THE MODEL OF BIOMEDICAL CELL PRODUCTS FOR PRECLINICAL STUDIES

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Currently, the main areas of regenerative medicine are cell technology and tissue engineering. Their intensive development is based on the need to create new modern technologies for treatment of various diseases and eliminating the consequences of trauma. The so-called scaffold technologies are rapidly developing in tissue engineering. They are aimed at creating new materials, which would perform the function of a mechanical frame for cells and provide optimal conditions for their normal metabolism (J. A. Stella et al., 2010; Baolin G. et al., 2015). Biomedical cell products obtained by combination of cellular and scaffold technologies have great potential in regenerative medicine. The most important stage in the development of biomedical cell products (BMCP) according to Federal law # 180 “On Biomedical Cell Products”, which came into effect on 01.01.2017, is organizing and carrying out preclinical studies on homologous models of laboratory animals.

The aim of the study was to develop protocols for the preparation of matrix-carrier, and cellular component for the creation of model BMCP intended for preclinical studies on a large laboratory animal (pig).

Materials and methods. Experimental animals were 5 piglets-females of Landras breed (age 8 weeks, 13-15 kg). Subcutaneous fat was used as a source of MSC. The cells were cultivated under standard conditions: 5% CO2, 37°C, and absolute humidity. Dulbecco’s Modified Eagle Medium (DMEM, Gibco, USA), antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin), 2% glutamine and 20% TPP (Gibco, USA). For induction of differentiation of MSC of passage 3 Hyman Mesenchymal Stem Cell Functional Identification Kit (R and D systems, USA) was used. Adipocytes were revealed by staining with Oil Red O. Calcium deposition was revealed by staining for 5 min with Alizarin Red. Chondrocytes were revealed by staining with antibodies S-100 (Sigma,USA). The immunnophenotype was

determined by flow cytometry with specific antibodies CD 44 FITC, CD 90 PerCP-Cy5.5, CD 10 Pe-Cy7, CD 45 PE (Flow cytometer FACS CANTO II, BD).

Scaffolds (priority certificate No. 2017112424 dated 11.04.2017) are formed on the basis of cryoprecipitate of blood plasma obtained from healthy donors. The technology includes PEGylation of the protein part of cryoprecipitate (PEG-NHS, Sigma-Aldrich, Germany) with subsequent addition of a solution of bovine collagen type I (Sigma-Aldrich, Germany). The next stage is the introduction of the suspension of MSC in the phosphate buffer ($1.2*10^5$ per 1 ml of composite). Polymerization of the composite thrombin-calcium mixture (80 IU/ml thrombin, Sigma-Aldrich, Germany, in 1% CaCL2 solution) completes the process. When preparing the scaffold for preclinical studies on a large laboratory animal (pig) cryoprecipitate of pig blood plasma was used as the basis of the scaffold composite.

Results. 24 hours after isolation, cell adhesion to plastic was observed, 72 hours later the medium was changed. By 6 - 10 days the cells had reached 60% of confluence, they were detached from the culture dish using 0.25% trypsin containing 1 mM EDTA (Gibco BRL), and they were washed with PBS, counted and plated again. During the whole period of observation, the cells had fibroblast-like morphology, were well decomposed, and differentiated in three directions. Cell phenotype was typical for MSC (CD 90+, CD 44+, CD 10+, CD 45-).

The scaffolds formed on the basis of cryoprecipitate of pig's blood plasma had the characteristics which did not differ from the scaffolds formed on the basis of cryoprecipitate of blood plasma of donors. They were a form-stable, elastic, transparent, dense hydrogel structure, preserving stability during long-term (14 days) incubation in growth medium. MSC were distributed relatively evenly throughout the entire scaffold structure. It is shown that scaffold based on the components of pig's blood plasma showed no cytotoxicity and provided the spreading and subsequent three-dimensional growth of cells with the formation of multiple processes and intercellular contacts. Subsequently, the formation of a dense cell network was recorded in the same time periods as in scaffold based on blood plasma of healthy donors.

Thus, cells isolated from adipose tissue of a pig, possess the characteristics of MSC and can be used to generate model BMCP and study their properties in preclinical studies. Pork cryoprecipitate of blood plasma allows to form scaffolds with the same characteristics as cryoprecipitate of blood plasma donors and can be used as the basis for the formation of a scaffold in preclinical studies on a large laboratory animal (pig).

Keywords: scaffold technologies, regenerative medicine, adipose tissue, biomedical cell products.

ROUND TABLE: PERSPECTIVES AND CHALLENGES IN BIOMEDICAL CELL TECHNOLOGY DEVELOPMENT

OPTIMISING BENCH-TO-BEDSIDE RESEARCH TO BE FIT FOR FACTORY-TO-PHARMACY IN THE FUTURE

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In contrast to all conventional pharmaceuticals and biologics, ATMPs are still mostly developed in academic centres and these are performing their own early phase clinical trials. In fact there is a significant trend for academic trials to move to randomised phase II and, in some cases, trial data can be so remarkable that these can lead to a marketing authorisation.

This is good for academic trialists and can be good for the developing biopharma industry but it carries significant risks to both. Academic ATMP development usually focusses on an unmet clinical need; the products are conceived by the clinician who sees the clinical need. The pre-clinical work is performed in research labs; rarely to the standard of GLP which would be the case for pharmaceutical company research. The data arising are suitable for phase I trial submission but the trials are never conventional phase I since the first subjects treated are always patients; not healthy volunteers. The probability of adverse events is thus much greater yet the pre-clinical models are unlikely to predict them reliably.

A phase I trial in patients is actually a phase I/II since it would be unethical to allow a study in patients if there was no intention to assess potential efficacy. No academic trialist wants to show that his/her ATMP is just "safe" – the publication being sought will only have impact if the ATMP "works". If the phase I/II trial shows any efficacy then it will lead to a phase II, possibly with randomisation. Indeed many regulatory agencies are encouraging a seemless transition from phase I/II into randomised phase II within a single trial protocol. This generates rapid clinical data but gives very little time to improve the manufacturing process and develop the assays to better define the product. New ATMPs which show dramatic clinical efficacy such as CAR-T can then rapidly advance to commercialisation while the product is poorly defined and the process almost amateur. To quote Phil Vanek of GE Healthcare "a 2017 product by a 2010 process".

This presentation will demonstrate how academic developers of ATMPs can make intelligent decisions early in the process which greatly facilitate clinical trials, commercialisation and clinical adoption by presenting real-world examples from my own facility.

Keywords: ATMP development, drug development.

THE EXPERIENCE IN DEVELOPMENT OF GMP PRODUCTION OF BIOMEDICAL CELL PRODUCTS AS A PART OF GXP QUALITY GUIDELINES IN RUSSIA

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Regenerative medicine is a relatively young sector of medicine. Federal Law No. 180 of June 23, 2016 “On Biomedical Cell Products” has been in force in Russia, since 2017. The law regulates all aspects related to the turnover of biomedical cellular products (BMCP). State regulation is aimed at ensuring the safety and effectiveness of the application of cellular technologies in medicine. The implementation of quality assurance systems GxP (good practice) at all BMCP life stages, from preclinical research (GLP) to clinical use (GCP), allows guaranteeing its quality. At the same time, the obligatory task is the development and implementation of rules and guidelines, which will address the related issues. A key document for the field of cellular technology should be good practice rules for working with BMCP, which are GxP guide for this industry. That rules are developing in Russian Ministry of Health.

The most important stage, responsible for the quality of the product, is its production. Good Manufacturing Practice (GMP) guides are the standard in the pharmaceutical industry of the entire developed countries of the world and are also used in regenerative medicine.

The success of the Institute of Cytology RAS (INC RAS) staff in the development and introduction of cellular products in medicine required the creation of a local pilot production of BMCP for clinical trials and post-registration stages. The key requirement was the compliance of such production with strict GMP standards. Such a pilot production was created on the territory of the INC RAS and put into operation in 2017.

The most important stage in the creation of a production was the development of a technical task for reconstruction. This document is based on the implemented technologies and contains information on the equipment usage, the microclimate parameters, “critical points” and processes. The DQ (design qualification) validation of project was completed with help of GMP-project Ltd (Czech Republic). Reconstruction was completed in 2017. Insulator technologies were introduced and it makes possible to conduct aseptic stages in class D in accordance with GMP. Accredited laboratory certified the cleanroom complex for compliance with classes D (ISO8) and C (ISO7).

An important part of GMP production is the standardization of all processes. It is achieved through the introduction of a multilevel system of quality control and assurance, the regulation of all processes and the automation or robotization of the processes. The automatic system of cells processing Compact Select (TAP-Sartorius) allows us to robotize the basic stages of cellular processing and it’s a part of a technological chain. At the moment, the pilot production of INC RAS is equipped with all the necessary equipment that has been installed and commissioned in accordance with the recommendations of the GMP. Work is under way to adaptation the production technologies of BMCP, developed both in the INC RAS, and in other organizations into a new infrastructure. The immediate task is to create cell lines, using the new infrastructure, in accordance with the requirements of the 180-FL and GMP recommendations and their banking. Pilot production of BMCP became part of Center of cell technologies, that is competency center in regenerative medicine in INC RAS.

The work was carried out with the financial support of the Russian Science Foundation project 14-50-00068.

Keywords: GxP, producing of biomedical cellular products, regenerative medicine.

POSTER SESSION

TRANSFROMATION DEFENCE IN HUMAN ENDOMETRIAL MESENCHYMAL STEM CELLS WITH GENETIC INSTABILITY

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Human endometrial mesenchymal stem cells show high anti-inflammatory and vasculogenic potential. These valuable features are associated with a special role of endometrial mesenchymal stem cell (eMSC) in endometrium growth. Cultured eMSC were successfully applied in heart disease treatment and encouraging long-term results were reported. At the same time, cell cultivation increases genome instability that may promote malignisation. The aim of the study was to investigate transformation potential of sublethal heat shock (SHS) induced genome instability in human eMSC that escaped stress induced senescence (SIPS) on the 6th passage after the STS. Genetic changes and transformation potential were evaluated by the methods of classic karyotyping, mRNA sequencing and bioinformatic analysis. The results of transcriptome analysis confirmed high genetic instability that exerts global effect on transcriptome activity. The evaluation of Hanahan-Weinberg hallmarks of cancer (Hanahan-Weinberg, 2011) did not reveal features of transformation. This result implies that SHS survived eMSC possess particular defense against transformation. The investigation of functional distribution of gene modules demonstrating statistically significant difference of activity between intact and SHS survived cells revealed several lines of defense. The first line is provided by the ability of eMSC to switch off the expression of MYC, hTERT and AKT1 oncogenes. The second line is based on the ability to impair the activity of pro- oncogenic pathways in response to DNA damage and aneuploidy. The third line of defense is provided by the induction of tumor suppressors including TP53, p21 (CDKN1A)and p16 (CDKN2A), as well as DNA repair pathways. In general, our data show that despite severe genetic instability, SHS-surviving cells show replicative aging, confirming their cancer safety.

Funding for the study: the grant from the RNF No. 14-50-00068

Keywords: Human endometrial mesenchymal stem cells, genetic instability, cancer protection, transcriptome analysis, heat shock.

SIGNIFICANCE OF POLY(C)-BINDING PROTEINS FOR PLURIPOTENT STEM CELLS

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Pluripotent stem cells (PSCs) are characterized by their ability to self-renew and differentiate into all types of somatic cells. In the past decade there are a number of evidences that divide pluripotency to early (naïve) and late pluripotency (primed) respective to their presence in embryonic development, culture conditions, etc. Oct4 is a key transcription factor of PSCs and is encoded by *Pou5f1* gene. This gene is regulated by its promoter, and also by distal (DE) and proximal (PE) enhancers. All of these elements are targets for methylation in differentiated cells and for regulatory proteins in PSCs. It was also shown that DE is active in naïve PSCs, and PE is active in primed PSCs. There are two essential elements present in the DE – site 2A and site 2B. Site 2B is a specific target for Oct4/Sox2 heterodimer, while the identity of protein(s) binding to the site 2A (CCCCTCCCCCC) has not been established; evidences suggest that these proteins are present not only in pluripotent, but also in differentiated cells.

Using EMSA, affinity chromatography and mass-spectrometry approaches, we identified two members of poly(C)-binding proteins that can bind to the site 2A *in vitro* – hnRNPK and Pcbp1. We have confirmed the occupancy of the site 2A by hnRNPK in naïve PSCs by chromatin immunoprecipitation method, while Pcbp1 was detected at this site only in primed PSCs. Notably, the 1A site (GGGGGAGGGGTG) from the PE, which is similar to the site 2A present in the DE, shows a strong *in vivo* binding to the both hnRNP-K and Pcbp1 in primed PSCs.

To carry out functional analysis we chose method of gene knock-out with CRISPR-Cas9 system for receiving of hnRNPK$^{-/-}$ and Pcbp1$^{-/-}$ PSCs. While hnRNPK-deficient naive PSCs lost their viability, Pcbp1-deficient naive PSCs were morphologically nor-

mal, except that they were relatively slow in growth rate. However, differentiation of Pcbp1-deficient naïve PSCs into primed PSCs was accompanied by massive cell death.

Our data imply that hnRNPK and Pcbp1 both play significant roles in cellular pluripotency – one being critical for the maintenance of viability of naïve PSCs and the other – for cell viability during the transition from naïve to primed pluripotent states, respectively.

The work was supported by the Russian Science Foundation (grant № 17-14-01407).

Keywords: KH-domain, Pluripotency, Oct4, Stem Cells, Enhancer

IMMUNOSUPPRESSIVE POTENTIAL OF PERIPHERAL REGULATORY T-LYMPHOCYTES IN THE PROCESS OF TUMOR PROGRESSION IN PATIENTS WITH METASTATIC SOFT TISSUE SARCOMAS

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Introduction. Tregs are considered to be crucial for the maintenance of self-tolerance and for maintaining immune balance to prevent damage of healthy cells from excessive immune reactions in cooperation with the immune checkpoint system. It is reported that Tregs are recruited and accumulate in tumor tissues by chemokine-induced chemotaxis via interaction between CCR4 and its ligands produced by cells of the tumor microenvironment. The self-maintenance conditions of T-regulatory lymphocytes (Treg) are created by tumor cells due to the production of vascular endothelial growth factor VEGF and chemokine CCL2.

Background. It was proposed that Treg-mediated immunosuppression was a crucial tumor immune-evasion mechanism in ovarian, gastric, breast, and pancreatic cancers and might be one of the main obstacles to successful tumor immunotherapy. Evidence addressing the efficacy of monoclonal antibodies against immune checkpoints began to emerge as recent studies have yielded both promising and disappointing results across different histologies. The purpose of this project was to scare up the new targets for immunotherapy in patients with soft tissue sarcomas.

Method. In present work there was evaluated the quantitative content of chemokine proteins in cultured cell supernatants of metastatic soft tissue sarcomas (STS) as well as characterized the immunophenotype of peripheral blood Treg by flow cytometry. For the study samples of metastatic tumor were taken to obtain sarcoma cell culture and samples of peripheral blood of patients in the absence of tumor growth (stable disease-SD) or disease progression (PD).

Results. The statistically significant differences were found in the quantitative content of CCR10+Treg (9.1% and 4.5%, respectively, p=0.001), CCR4+Treg (10% and 3.3%, respectively, p=0.001), neuropilin-1 (Nrp1+) Treg (6% and 4.5%, respectively, p=0.021) in patients with PD and SD. A direct correlation of high strength was found between production of VEGF, CCL2 by metastatic STS cells and expression of Nrp1 (r=0.93, p=0.001), VEGFR-2(r=0.88, p=0.007), CCR4(r=0.81, p=0.024) by Treg cells. Statistically significant differences in the CCR10+Treg (9.5% and 2.93%, respectively, p=0.012) and CCR4+Treg (68, 3% and 3.95%, respectively, p=0.007) were detected in the group of patients with liposarcoma and synovial sarcoma.

Conclusion. Thus in patients with metastatic STS there is directional chemotaxis of Treg into tumor microenvironment providing the creation of tumor-induced tolerance, which could be associated with DP. Therefore Tregs are a potential target for novel tumor therapy by inhibition of immunosuppression in the tumor site. The revealed regularities could be used to plan adjuvant and palliative treatment of STS patients.

Key words: metastatic soft tissue sarcomas, regulatory T-lymphocytes, immunosuppression, CCL2, CCR4, CCR10, Nrp1.

THE TGFB-INDUCED DIFFERENTIATION OF FIBROBLASTS INTO MYOFIBROBLASTS MAY REGULATE BY COMPONENTS OF MESENSHYMAL STROMAL CELL (MSC) SECRETOME

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One of the most important types of cells involved in the development of fibrosis are myofibroblasts - cells that produce an excessive quantity of extracellular matrix proteins (ECM) and are able to contract it. Recent research has shown that mesenchymal stromal cells (MSC) exert a strong impact on the development of fibrosis, but the mechanisms of this influence are still unclear. Therefore, the aim of this work was to explore the role of paracrine secretion of MSC, especially extracellular vesicles (EV) and soluble factors (SF), in fibrogenesis.

The EV and fraction that contains SF were isolated from 2 days conditioned medium (MSC-CM) of human MSC (hTERT MSC) by ultrafiltration. The model of transforming growth factor beta(TGFb) - induced differentiation of human dermal fibroblasts was use to study the modulation of myofibroblasts activity. The effect of MSC-CM on the differentiation of fibroblasts was analyzed through 4 days therapy with EV and SF using the immunoblotting, QT-RTPCR, immunocytochemistry, and collagen gel contraction assay.

Our results demonstrated that EV and SF from MSC-CM fractions suppressed differentiation of fibroblasts resulting in the reduction of alpha smooth muscle actin expression ($p<0.05$). We showed that the addition of MSC-CM components reduced collagen I and fibronectin mRNA content ($p<0.05$) and decreased the contractility ($p<0.05$). Importantly, we also showed that EV and SF were able to induce reverse differentiation of myofibroblasts into fibroblasts.

Taken everything into consideration, these results demonstrated that MSC secretome affect fibroblasts into myofibroblasts differentiation at least in vitro. Moreover, we showed the important role of both fractions, soluble factors and extracellular vesicles,

in the suppression of fibrosis. Our results provide an opportunity to search for new approaches to the treatment of fibrosis using stem-cell-free therapeutic strategies.

The study was conducted using biomaterials from project “Scientific basis for national bank-depositary of living systems” (RSF agreement #14-50-00029) and supported by Russian Foundation for Basic Research (#18-015-00525).

*Keywords:*M SC, fibrosis, myofibroblasts, MSC secretome

OBTAINING INSULIN-PRODUCING CELLS FROM A NONPANCREATIC SOURCE

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One of the widely studied approaches for the treatment of insulin-dependent diabetes mellitus, at the moment, is cell therapy, which consists in the development of methods for obtaining insulin-producing cells. A number of papers were devoted to the search for a nonpancreatic source of cells capable of expressing insulin.

The aim of this work was to obtain and study the insulin-producing cells of mouse and human from a nonpancreatic source (IPCNS). As a result, methods of cultivation and differentiation of cells were developed, which were further investigated using methods of flow cytometry, immunocytochemistry, as well as enzyme immunoassay and qPCR.

The data obtained by qPCR analysis showed an increase in expression of transcription factors genes affecting the development of endocrine cells of the pancreas in embryogenesis. The analysis of expression of genes typical for different lineages of pancreatic cells showed that differentiation mainly leads towards the formation of β-like cells.

It was demonstrated that the cultivation in three-dimensional conditions increases the efficiency of human IPCNS differentiation. ELISA results confirmed a glucose-dependent manner of insulin secretion, which indicates the proximity of the phenotype of the studied cells to the endocrine cells of the pancreas.

With the help of experimental models it was found out that when introduced to the organism intraperitoneally, the studied cells possess tropism to the pancreas. In the diabetes experimental model, it was shown that after transplantation of a suspension of

murine IPCNS, restoration of the amount and total area of islets of Langerhans occurs and these parameters approximate to those in healthy animals.

Thus, we have obtained insulin-producing cells from a nonpancreatic source, that possess affinity to the pancreas, the ability for glucose-dependent insulin secretion and potential for regeneration of damaged islets of Langerhans.

The study was executed within the state assignment of Ministry of Health of the Russian Federation.

Keywords: diabetes, insulin-producing cells, differentiation, pancreas.

NORADRENALINE SWITCHES MESENCHYMAL STEM/STROMAL CELLS TO PROINFLAMMATORY PHENOTYPE

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Mesenchymal stem/stromal cells (MSCs) are located in the stromal fraction of the most tissues of an organism. They participate in tissue reparation and regeneration by differentiation into various cell types as well as by secretion of biologically active molecules. MSCs can inhibit or promote the inflammation by producing distinct cytokine arrays, which target immune cells. Functional activity of MSCs is controlled by many factors including hormones and neuromediators. Recently, we showed that noradrenaline induces heterologous sensitization of alpha1A-adrenergic receptors on MSCs by acting on beta-adrenergic receptors. However, the physiological significance of observed switching of the intracellular signaling in response to noradrenaline was not elucidated. In this study we hypothesized that noradrenaline-induced switch could affect the expression of pro- or anti-inflammatory cytokines in MSCs.

We addressed this hypothesis by NanoString technology using PanCancer Immune Profiling Panel. Human MSCs (hTERT-MSC line) were treated by single or repeated addition of noradrenaline as in previous study [Tyurin-Kuzmin et al., *SciRep*, 2016]. We showed that noradrenaline induces the elevation of RNA level of many proinflammatory cytokines, as well as receptors and members of signaling pathways, activated by these cytokines. We have also explored changes in secretion of 17 proinflammatory cytokines using BioRad BioPlex at the protein level. We observed that stimulation by noradrenaline leads to an increased production of proinflammatory cytokines, including IL-6, IL-8, G-CSF, IFN-G and MCP-1. Repeated stimulation of MSCs by noradrenaline did not further increase the expression of cytokines.

Taken together, our data suggest that MSCs acquire proinflammatory phenotype under noradrenaline action, which consisted in the elevated pro-inflammatory cytokines secretion and increased sensitivity to these molecules. The study was supported by RSF grant 14-15-00439.

Keywords: MSC, inflammation, noradrenaline, cytokines, adrenergic receptors

RECONSTITUTION OF HUMAN HAIR FOLLICLE MORPHOGENESIS IN HUMANIZED MOUSE

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Regeneration of fully functional hair is difficult due to the requirement to reconstitute its complex three-dimensional organization. Xenografting of human skin to the immunodeficient mice is one of the most invaluable approaches for the *in vivo* investigation of human hair follicle morphogenesis. In our study we have developed a model of human skin transplantation to nude mice to study the morphogenesis of human hair follicle. The foreskin was used as human skin which allowed us to detect trichogenic properties of cells, since it does not contain endogenous hair follicles. Human dermal papilla cells and keratinocytes were employed to reconstruct hair follicle morphogenesis in xenograft model.

The human foreskin was engrafted to mouse skin, covered by the mouse skin flap to prevent graft drying. Two weeks following transplantation, visual examination of the grafts revealed live, intact and slightly contracted human skin. Histological examination 2 months after transplantation of human skin onto Nude mice showed that the graft well integrated with mouse skin, the epidermis had human origin, as confirmed by its morphology and hNuclei staining. The cellular suspension of human dermal papilla cells and human keratinocytes introduced into the transplant were tested for the ability to induce the formation of hair follicles. 2 months following transplantation we observed follicle formation within a skin graft injected with human dermal papilla cells and human keratinocytes.

In summary, we confirm that Nude mice provide an adequate model ensuring human skin graft integrity and engraftment for at least two months. Human donor cells can induce hair morphogenesis when injected to glabrous human foreskin. This model provides the opportunity to investigate mechanisms orchestrating human hair follicle morphogenesis.

The work was funded by the Russian Science Foundation (Project No 16-14-00204).

Keywords: xenografting, hair follicle, dermal papilla, keratinocytes, immunodeficient mice

3D SCAFFOLDS BASED ON MICROSPHERES FROM POLY (3-HYDROXYBUTYRATE) FOR BONE TISSUE ENGINEERING

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Poly(3-hydroxybutyrate) (PHB) is a biocompatible and biodegradable polymer produced by a microbiological way. The devices from this biomaterial open up great possibilities for application in regenerative medicine to restore the integrity of damaged organs and tissues. PHB is particularly suitable for the regeneration of bone tissue due to its properties: mechanical strength, slow biodegradation, etc. Mesenchymal stem cells (MSCs) are commonly used in regenerative medicine because they have a potential to proliferate to different cell types, e.g. osteoblasts. The shape and microstructure of the polymer substrate have a great effect on growth and differentiation of MSCs, which imposes special requirements on the design of 3D scaffolds. The aim of this work was to study growth of MSCs on 3D scaffolds based on porous microspheres of different diameter from PHB.

PHB was produced by controlled bacterial biosynthesis using strain-producer Azotobacter chroococcum 7B. The obtained PHB (with molecular weight 250 kDa) was isolated and purified to medical grade. To produce porous microspheres the double water-oil-water emulsification technique using ammonium carbonate as a blowing agent was used. The obtained microspheres have different diameter 600±85 and 170±50 mkm with pore size 2,5±0,5 and 4,2±1,8 mkm accordingly. To produce 3D scaffolds the obtained porous microspheres were immobilized on the PHB film by gluing with chloroform. MSCs were isolated from bone marrow of newborn rats. The growth pattern of MSCs on produced 3D-scaffolds after three weeks of cells cultivation was analyzed by scanning electron microscopy. The activity of alkaline phosphatase was studied to test possible MSCs osteogenic differentiation. In osteogenic media the activity of alkaline phosphatase in MSCs isolated from bone marrow was elevated 330-times in comparison with control. The produced 3D scaffolds were able to support growth of MSCs on scaffolds, on scaffolds from small microspheres cell grew on the surface of microspheres and between them; on scaffolds from large microspheres MSCs grew preliminary on the surface of microspheres. At the next stage we plan to investigate the differentiation of MSCs cultured on 3D scaffolds.

Keywords: poly(3-hydroxybutyrate), biodegradable polymer, 3D-scaffold, microspheres, mesenchymal stem cells.

POSSIBILITY OF OBTAINING THREE-DIMENSIONAL DESIGN OF MUSCLE FROM MULTIPOTENT MESENCHIMAL STORMAL CELLS (MMSC)

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Defects of intervertebral discs constitute a significant part of the pathology of the human population. One possible approach to solving the problem may be the integration into the cavity of the gelatinous core of a meniscus-like volumetric tissue engineering construct based on the directed differentiation of multipotent mesenchymal stromal cells (MMSC) grown on a non-matrix basis and corresponding to the volume of the cavity formed in the patient's core region.

The purpose of the study is to study the possibility of obtaining a three-dimensional design of the cartilaginous tissue on the basis of directed differentiation of human MMSC, with a volume of up to one cubic centimeter and corresponding to the shape of the disc.

In the work, MMSC was used, isolated from adipose tissue of the anterior abdominal wall of patients obtained with liposuction in a hardware way with their informed consent.

Fabrication of the fabric structure was carried out by the author's method with the stage of cultivation, which makes it possible to give the structure a given shape and structure. The latter provided for the modeling of a structure consisting of layers with different degree of maturity of cells-more mature and differentiated-in the center, young cells at the periphery.

The resulting cartilaginous design fully corresponds to the given shape of a disk measuring 2.5-3 mm in height and 19 mm in diameter, contains cells of round, oval or triangular shape at various stages of differentiation: on the periphery there are precursors of chondroblasts and young chondroblasts with a multitude of outgrowths and small size. In the central part the cells are more differentiated, have a larger size, an ovoid shape and are compactly arranged in 2-4 cells, forming the so-called isogenic groups that appear after the last division, with a lacunar structure. The gradation of the degree of differentiation from the periphery to the center, as a whole, resembles the structure of the cartilaginous tissue that forms in vivo.

Keywords: defects of intervertebral discs*, cellular therapy.*

DISTRIBUTION OF A TANDEM REPEATS BINDING PROTEIN DDX 5 (RNA-HELICASE P68), IN OOGENESIS AND EARLY EMBRYOGENESIS IN MOUSE

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Oogenesis of mammals is a complex process that includes the accumulation of non-coding RNA transcripts of tandem and dispersed repeats, as well as proteins that regulate their transcription. Accumulated transcripts and regulatory proteins are essential in the early embryogenesis. In our laboratory, the protein DDX5 (RNA-helicase p68) was isolated and characterized as a protein binding to tandem repeats of the mouse and involved in the regulation of the transcription of such repeats. The protein is multifunctional and acts not only as an RNA helicase but also as a transcription factor binding insulator sequences. Its role in embryo- and oogenesis is unknown yet.

The aim of this work was to determine the localization of DDX5 in mouse oogenesis and early embryogenesis. Oogonia were received from embryos at 10-15 days of development. To obtain preovulatory oocytes of mice (germinal vesicle oocyte, GV), a puncture of antral follicles of sexually mature, non-stimulated mice was performed. For the production of postovulatory mouse oocytes (MII), females aged 6-10 weeks were stimulated and cells were washed out from oviducts. Zygotes, 2- and 4-cell embryos were obtained from stimulated females, caged with males after a proper period of time. All oocytes and embryos were fixed, permeabilized, stained with an antibody (AT) against DDX5 protein.

In oogonia, stained with the DDX5 AT, brightly colored granules were found in the cytoplasm and nucleus. In the preovulatory oocyte (GV), DDX5 appeared in the nucleolus-like-body (NLB). When the NLB disassembled and cells switched to the stage of MII oocyte, DDX5 AT stained small granules in the ooplasm. During the oogenesis, DDX5 was not associated with either pericentromeric or centromeric heterochromatin. In zygotes, DDX5 was distributed in the close proximity of the pronuclei while tandem repeats DNA were positioned at the pronuclei periphery. In 2 and 4 cell embryos, DDX5 was associated with tandem repeats DNA that started to form chromocenters. We assume that during the process of oogenesis primary accumulation of DDX5 protein takes place in the form of granules of various sizes in the nucleus and in the cytoplasm of the oocyte. But this pool of DDX5 is not associated with heterochromatin. The association of DDX5 with tandem repeats DNA was detected only in 2 and 4 cell embryos.

Keywords: mouse oogenesis and embryogenesis, DDX5, tandemic repeats.

HETEROGENEITY OF CELL MATERIAL AS A CHALLENGE FOR THE DEVELOPMENT OF REGENERATIVE MEDICINE PRODUCTS

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Postnatal stem and progenitor cells are considered as a powerful and multifunctional tool for regenerative medicine. They could be harvested from multiple tissue types and expanded ex vivo in special conditions conserving their regenerative properties. However, high variability of isolated cells, both phenotypical and functional, reflects dynamics and heterogeneity of stem and progenitor cells within tissue and could be a critical issue for the development of therapeutically effective and reproducible cell-based products. It dictates the necessity to elaborate both single-cell analysis methods and the prudent approaches for cell-based product standardization. These problems are explored in part under the scope of the project working on scientific basis of living systems depository “Noah’s ark”. Thus, we have shown the diversity of cultured multipotent mesenchymal stromal cells (MSC) population and revealed some mechanisms of their functional heterogeneity based on differential expression of hormone receptors and cell response to the regulatory signals. Recent studies indicated that the benefits of MSC therapy was mediated mostly by producing multiple bioactive components, including soluble factors and extracellular vesicles, stimulating angiogenesis, neurogenesis, activating tissue-resident stem cells and modulating immune reactions. We have extensively studied the secretome of cultured MSC from many donors and showed the pronounced variability in the secretion of several proteins suggesting that these cells existed as a heterogeneous population containing functionally distinct subtypes, which differ in numbers between donors. Prediction computational models were used to overcome this variability. Taken together, the development of comprehensive and well-reasoned approaches for the standardization of cell-based regenerative medicine products providing the reproducible pattern of cell heterogeneity is crucial for their effective translation into clinical practice.

The study was conducted using biomaterials collected and preserved in the frame of RSF grant #14-50-00029 using the equipment purchased as a part of Lomonosov MSU Program of Development and supported by RSF grant #14-15-00439.

Keywords: regenerative medicine, cell-based products, heterogeneity, stem and progenitor cells, multipotent mesenchymal stromal cells, receptors, secretome.

NOVEL APPROACHES FOR GENERATING CLONAL CELL LINES PRODUCING THERAPEUTIC PROTEINS USING THE CELL METRIC HIGH RESOLUTION IMAGING SYSTEM

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In recent decades, therapeutic monoclonal antibody (mAb) products have become an inseparable part of modern medicine. Process of generating those products consists of many stages, and establishing a clonal cell line with stable expression of the target protein is among the key stages of the process. Product quality is directly associated with quality of the cell line. Clonality of producer cell lines is crucial at the stage of developing a pharmaceutical product. Our studies demonstrated that along with significant decrease in antibody production during cell division, polyclonality of cell populations also leads to variation in critical quality attributes (CQA) of the target product over time. Changes in these attributes are critical and may affect product immunogenicity, ADCC and CDC in a varying degree, which makes it impossible to scale up the technology to industry-scale production.

Establishment of clonal cell lines using different approaches including picking producing cell lines from a semi-solid medium or standard method of limiting dilution does not ensure clonality of SCLs and usually requires multiple rounds of sub-cloning. Additional rounds of cloning make the process of therapeutic product generation significantly longer (up to 3 months per round). Generation of SCLs by the limiting dilution method with subsequent identification of single cell population growth using the Cell Metric imaging system provided us with fast high-throughput clonal selection of SCLs with good growth characteristics, as well as confirmed clonality of cell lines, which provided higher antibody titers (up to 30% higher) and stable protein expression through 60 cell generations. Target product quality assessment confirmed minimal variation of CQA (within the standard deviation), which makes it possible to scale up the technology to manufacturing scale volumes. Therefore, new approach in SCLs development using the Cell Metric imaging system enabled us to select the most promising clonal cell lines for the production of pharmaceutical antibodies in a one-step cloning process.

Key words: Monoclonal antibody (mAb) product development, stable cell lines (SCLs), clonality, Cell Metric

HALLOYSITE\BIOPOLYMER COMPOSITES AS NANOVEHICLES FOR ENZYME-ACTIVATED INTRACELLULAR DRUG DELIVERY

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Here we demonstrate the selective drug delivery into human cells using biocompatible 50-nm diameter and alumen diameter of 12-15 nm clay nanotube carriers. We have developed a new platform for intracellular delivery of several drugs loaded into halloysite lumen, while dextrin coating was employed as nanotube ends capping material. Halloysite biocompatibility allows for its safe use for intracellular delivery at concentrations up to 0.5 mg/mL. Intercellular glycosyl hydrolases decompose the dextrin tube-end stoppers triggering the release of the lumen-loaded drug, facilitating the preferable elimination of rapidly proliferating cells. We encapsulated anti-cancer substances (brilliant green, binase and paclitaxel) in halloysite and evaluated the drug release kinetics in simulated gastric and intestinal conditions. To enable the efficient drug release in intestines, clay nanovehicles were coated with pH-sensitive polymer poly (methacrylic acid-co-methyl methacrylate), which triggered drug release at higher pH corresponding to digestive tract environment. In vitro anticancer effects of paclitaxel-loaded halloysite nanotubes were evaluated on human cancer cells, polyploid nuclei of different sizes and fragmented chromatin were observed. The viability of colon adenocarcinoma cells incubated with halloysite clay nanotubes loaded with cytotoxic enzyme binase was investigated. Incubation of colon adenocarcinoma cells with binase loaded halloysite resulted in 60% decrease of cell viability. The enhanced antitumor activity of binase-halloysite composites indicates for a promising anticancer nanoformulations development. This study was performed according to the Russian Government Program of Competitive Growth of Kazan Federal University and and by the subsidy allocated to Kazan Federal University for the state assignment in the sphere of scientific activities (16.2822.2017/4.6).

Keywords: nanotube carriers, antitumor activity, anti-cancer substances.

KNOCK-OUT OF IMMUNOPROTEASOME SUBUNIT BETA2-I AND REGULATORY PARTICLE PA28 REDUCES THE EFFICIENCY OF MOUSE EMBRYONIC FIBROBLASTS REPROGRAMMING INTO INDUCED PLURIPOTENT STEM CELLS

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Cellular reprogramming is a new and promising technology. There is growing evidence about the use of induced pluripotent cells as a material for substitution therapy for transplantation in model animals. This process was made possible due to the discovery of Oct4, Klf4, Sox2, c-Myc (OKSM) capable to reprogram somatic cells into pluripotent state. Subsequent cultivation of such cells with different factors allows to obtain the cells of any specialization. However, mechanisms of reprogramming into pluripotent state have not been sufficiently explored. It is known that ubiquitin-proteasome system – multi-enzyme proteolytic complex – is one of the main regulators of pluripotency and self-renewal. The main component of the system is the 26S proteasome consisting of a 20S core particle and 19S regulatory particles. Under certain conditions, catalytic subunits of the 20S – beta1, beta2 and beta5 – can be replaced by inducible subunits beta1-i, beta2-i and beta5-i, and such proteasome is designated as immunoproteasome (IP). Until recently, it has been considered that the main function of the IPs is to participate in the MHCI-mediated antigen presentation, but there are literature data reporting an increased expression of IP subunits, as well as regulatory particles, in embryonic stem cells, which confirms the involvement of IPs in maintaining of pluripotency. Despite this, the role of IPs in reprogramming remains unclear. In this work, we induced the pluripotent state of mouse embryonic fibroblasts (MEF) derived from beta2-i/Mecl-1 and the regulatory particle PA28-deficient embryos, using a doxycycline-activated OKSM construct. Subsequent immunocytochemistry with antibodies against pluripotency markers showed inability of the IP-deficient MEFs to form induced pluripotent stem cells.

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Keynotes: ubiquitin-proteasome system, immunoproteasome, Mecl-1, PA28, induced pluripotent stem cells

RECIPROCAL EFFECTS OF MSCS AND PBMCS UNDER PHYSIOLOGICAL HYPOXIA

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MSCs are a promising tool for cell therapy due to its immunomodulation and low immunogenicity. The effect of MSCs on immune cells has been studied in sufficient detail. Meanwhile, the interaction of above cells is a bidirectional with mutual influence on each other. In addition, the importance of microenvironment factors as O_2 concentration could not be underestimated. Here we described the effects of adipose tissue MSC and PHA-activated human peripheral blood PBMC interaction at tissue related O_2 level (5%).

After 72 h of co-culture, the ratio of PBMC up/downregulated genes comprised 69/47 at 20% O_2 vs 42/53 at 5% O_2 as revealed with gene expression microarray analysis. Expression of PBMC late activation marker (HLA-DR) was reduced. T cell division was suppressed, the effect was twice more pronounced at 5% O_2. The levels of TNF-a, IL-6, IL-8 were decreased, whereas IFN-g and IL-10 were increased.

The MSC transcriptome also was significantly changed after interaction: the ratio of up/down regulated genes was 230/74 under 20% O_2 and 108/34 at 5% O_2. HLA-ABC and ICAM expression were significantly increased, a few MSCs started to express MHC II molecules. It was observed an increase in transmembrane mitochondrial potential, lysosomal activity, ROS level, and decrease in endoplasmic reticulum activity. MSC proliferation and osteo-, adipodifferentiation were slowed down. The production of LIF, VEGF by MSCs was increased, while IL-8, IL-6 – decreased. The decrease in the osteodifferentiation and ER activity was more prominent athypoxia. MSCs and PBMCs maintained high viability.

Thus, both PBMCs and MSCs significantly change their functional state after interaction. According to the microarray analysis the interaction provoked a reciprocal priming of cells, the intensity of which was attenuated at 5% O_2. Herewith, the immuno-

modulatory properties of MSCs were enhanced under hypoxia. These data are of great importance for regenerative medicine and cell therapy demonstrating the peculiarities of MSC/PBMCs interaction under tissue related O_2 concentration.

The work was supported by grants RFBR 18-015-00461, 17-04-00942 , grant of RF President MK-2976.2018.4.

Keywords: MSC, PBMCs, interaction, hypoxia

SEROTONIN INDUCED HETEROLOGOUS SENSITIZATION OF ALPHA1A-ADRENERGIC RECEPTORS IN MESENCHYMAL STEM/STROMAL CELLS

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Mesenchymal stem/stromal cells (MSC) are identified in many tissues of an organism and play an important role in reparation, regeneration and homeostasis. MSC themselves are regulated by hormones and neurotransmitters and noradrenaline is one of their key regulators. Recently, we showed that, although all the main isoforms of adrenergic receptors are expressed in MSC, most of them are not coupled with calcium-dependent system of intracellular signaling. Noradrenaline increases this coupling and induces heterologous sensitization of alpha1A-adrenergic receptors on MSCs by acting on beta-adrenergic receptors and adenylyl cyclase (Tyurin-Kuzmin et al., *SciRep*, 2016). In this work we investigated alternative to noradrenaline adenylyl cyclase inducers that are capable of increasing MSC sensitivity to catecholamines. We selected hormones and neurotransmitters whose receptors activate adenylyl cyclase, such as dopamine (DRD1, DRD5), histamine (HRH2), serotonin (HTR4, HTR6, HTR7) and adenosine (A2b, A2a). Using PCR we showed that MSC express mRNA of receptors A2a, A2b, DRD1, DRD5, HRH2, HTR6, and HTR7. We analyzed how these neurotransmitters affect the functional activity of MSC. We stimulated MSC with one of these neurotransmitters and 6 hours later we registered Ca^{2+}-dependent responses to noradrenaline. We found that serotonin increased sensitivity of MSC to noradrenaline. Histamine, adenosine and dopamine did not change it. In order to establish mechanism of this phenomenon we showed that stimulation of MSC with serotonin leads to an increase in alpha1A-adrenergic receptors protein level. Using an inhibitor analysis of signaling pathways we showed that serotonin regulated sensitivity of MSC to noradrenaline with adenylyl cyclase/cAMP/proteinkinase A-dependent manner.

Thus, we defined that serotonin regulated coupling of adrenergic receptors and calcium signaling in MSC. This work was supported by the Russian President Grant for young scientists MK-3167.2017.7.

Keywords: MSC, serotonin, noradrenaline, alpha1A-adrenergic receptors

ULTRASTRUCTURAL FEATURES OF CELLS AND INTERCELLULAR MATRIX IN THE CULTURE OF MESENCHYMAL STROMAL CELLS

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The study aimed at studying the ultrastructural features of cells and intercellular matrix in the culture of mesenchymal stromal cells (MSCs) isolated from an adipose tissue of a rabbit. Cells of the 4th passage were used, incubated for 25 days in DMEM supplemented with 10% *Fetal Bovine Serum* (FBS) in the collagen coating tissue culture dishes. Electron microscopic examination of the cell monolayer was carried out according to standard protocols for sample preparation.

The presence of three cellular subpopulations, probably determined by a contact induction of differentiation, were revealed. In the predominant cellular subpopulation the cells are arranged in groups among the enlightened intercellular space, in which single fibrils are located. In the cytoplasm of these cells, large lipid droplets of different diameters are identified, which tend to merge and are pushed back from the nucleus to the periphery. In the perinuclear space there are channels of granular type, ribosomes and vesicles, but fibrillar structures are not detected. The cells of this subpopulation can be defined as cells of adipocyte specialization. Organization of the cytolemma, nucleus and organelles of cells of the second subpopulation testifies to their active functional state. Simultaneously the cytoplasm lacks the intracellular fibrillar structures, which are, however, large quantities are found in the extracellular environment. The cells of the third subpopulation are fibroblast-like with a few very long processes. Morphology of organelles (ribosomes, granular reticulum, vesicular apparatus) indicates active protein-synthetic function. In the peripheral parts of the cytoplasm, orderly oriented fibrous structures resembling myofilaments are found in a considerable amount, sometimes they end in the region of desmosomes. In the extracellular space, long fibrillar structures are identified that are identical in diameter and density to intracellular ones and are often collected in loose fibrous bunches. The fibrillar protein of these fibers is released by exocytosis outside the cell.

Thus, ultrastructural analysis of the MSC culture of adipose tissue of rabbit showed heterogeneity of the cell population, which may be a consequence of the heterogeneity of the initial population or indicate the differentiation processes spontaneously occurring in the aging culture. It has been found that the secretion of extracellular matrix for 25 days of cultivation remains localized around the producer cells, whereas cells of adipocyte specialization support their adhesion mainly by means an artificial (pre-coated) substrate. This feature is useful to consider when planning long-term cultivation of differentiated cells.

Keywords: intercellular matrix, mesenchymal stromal cells, heterogeneity of the cell population.

DEVELOPMENT OF TISSUE ENGINEERING VESSEL OF SMALL DIAMETER

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The main cause of disability and mortality of the population according to WHO, are diseases of the cardiovascular system. The main way to treat this diseases associated with blood vessel obliteration is by using auto-auto/auto-arteries. However, the absence of autologous material leads to the need to use alternative vascular prostheses. Despite its availability and ease of use, synthetic and biological small diameter prostheses (<6 mm) have such a serious disadvantage as a high risk of break-in as a result of extensive hyperplasia of neointima or thrombus formation. Thus, the aim of the work is to create a tissue-engineering vessel (TEV) of small diameter based on a polymeric scaffold populated by cells. Two polymers were used to create the TEV: lactic acid-based poly-L-lacticacid (PLL) and poly-ε-caprolactone (PC), which are a number of properties. In the PLL-vessels, a suspension of MSC was added and cultured for 7 days under standard conditions. Assessments of the presence and condition of cells in the vessel was performed using the immunofluorescence method. The cell-free PC-vessel was sewed into the abdominal aorta of the rat, intraoperatively the blood flow was restored. The animal was withdrawn from the experiment on the 7th day. Evaluation of the presence and condition of cells in the vessel was performed by histological examination. Cell-free PC-vessel caused the development of non-specific productive inflammation and was accompanied by phagocytosis of its material by histiocytes. Endothelialization on the 7th day reached about 17% of the length of the prosthesis. The formation of neointima in these terms was absent. The scaffold material did not cause noticeable formation of wall microthrombi in the lumen of the main artery. It seems promising to further use the PC-vessel as a scaffold in the creation of TEV.

Keywords: tissue engineering, small vessels, atherosclerosis, poly-ε-caprolactone, poly-L-lacticacid.

This work was supported by a grant of the Russian Science Foundation №14-50-00068.

CARDIAC SODIUM CHANNEL NAV1.5 – GLYCOGEN SYNTHASE KINASE 3 BETA CROSSTALK IN IPSC MODEL OF DESMOSOMAL-RELATED CARDIOMYOPATHIES

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Arrhythmogenic cardiomyopathy (ACM), the most common form of desmosomal-related cardiomyopathies, is an inherited disease with complex phenotype combining electrical activity abnormalities with fibrosis and adipogenesis progressing in myocardium. Molecular abnormalities of desmosomal proteins were shown to result in the disorganization of connexome within intercalated discs leading to dysregulation of electrical activity, particularly voltage-dependent sodium current, and action potential propagation. Recently the glycogen synthase kinase 3 beta (GSK3B) inhibition was reported to restore the cardiac electrical activity, cease fibrosis development, and improve heart function during ACM. Nevertheless the interplay between desmosomal proteins, GSK3B and ACM phenotype development remains largely unexplored.

The aim of this study was to create and characterize the in vitro model of desmosomal-related cardiomyopathy based on iPSC-derived cardiomyocytes, to test the transgene-based approach for restoration of impaired cardiomyocyte function and to study molecular mechanisms of GSK3B inhibition.

Two iPSC lines from patient carried two mutations in *PKP2* gene coding for desmosomal protein plakophilin 2 (NM_004572.3: c.354delT, p.Lys859Arg) and two control iPSC lines were generated and characterized. iPSCs were differentiated into cardiomyocytes using Wnt modulation-based protocol. Sodium currents were measured using whole-cell patch clamp configuration. The activity of canonical Wnt signaling was determined using TopFlash assay.

The obtained patient-specific cardiomyocytes recapitulated ACM phenotype: lack of *PKP2* within cell junctions, decreased canonical Wnt signaling activity and reduced sodium current density. Transduction of cardiomyocytes from ACM patient with wild type *PKP2* as well as application of GSK3B inhibitor CHIR99021 was able to restore

sodium current to the control level, and to promote canonical Wnt signaling. Transduction of cardiomyocytes with *CTNNB1*-S33A gene coding for stabilized beta-catenin had similar effect. Short-term application of inhibitor CHIR99021 (two hours incubation) did not have a significant effect.

Our data indicate that PKP2 deficiency cause both electrophysiological and signaling abnormalities. The beneficial effect of GSK3B inhibitors along with the similar effect of *CTNNB1*-S33A transduction and absence of short term GSK3B inhibition effect indicates that regulation of sodium current is transcriptional-dependent and is associated with canonical Wnt signaling activation.

This work was supported by Russian Science Foundation grant number 14-15-00745-П.

Keywords: desmosomal-related cardiomyopathy, arrhythmogenic cardiomyopathy, plakophilin 2, sodium channel, glycogen synthase kinase 3 beta, canonical Wnt signaling

THE ROLE OF EXTRACELLULAR CA^{2+} AND ROCK INHIBITOR IN CELL-CELL INTERACTIONS IN HUMAN EMBRYONIC STEM CELLS

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Human pluripotent embryonic stem cells (hESCs) have the potential to be an important source of any cell type for basic research, drug development and clinical cell therapies. Unlike other types of embryonic stem cells, hESCs are technically much harder to culture also because of induced by cell dissociation apoptosis. hESCs grow as monolayer colonies that have epithelial structure. E-cadherin is a responsible for Ca^{2+}-dependent homophilic cell-cell adhesion surface glycoprotein. It regulates various cellular processes and functions. Disruption of E-cadherin contacts in hESCs stimulates activation of Rho-associated coiled coil protein kinase (Rho-kinase), which eventually leads to caspase signaling cascade activation and cellular apoptosis. It has been shown that ROCK inhibitor could stop this process and prevent cell death. The study of dissociation-induced apoptosis mechanism and interrelation of its members is an important task to developing the efficient manipulation techniques required for stem cell research.

The aim of this study was to find out the role of extracellular Ca^{2+} on cell-cell interactions in hESC and whether the ROCK inhibitor affects on contact stabilization.

Materials and methods. The hESC line SC5 (from Center of collective using “Cultures collection of cells of vertebrates”) was used for all experiments. hESC colonies were cultured on layer of feeder cells (human fibroblasts). Cells were treated by PBS without Ca^{+2} and Mg^{+2} for 20 min. and by dissolved in culture medium EGTA (5 mM) for 10 min. with or without ROCK inhibitor (Y-27632, 10μM). Cells were fixed and stained for E-cadherin and cytoskeleton.

Results. Passive (PBS) or active (EGTA) elimination of Ca^{+2} from medium lead to cell-cell contact disruption and cell dissociation. ROCK inhibitor does not stabilize cell contacts, but it prevents blebb formation - an important sign of apoptosis. It has been shown by lazer confocal microscopy, that Ca^{+2} elimination leads to an increase of

E-cadherin on surface of hESC. There were no similar effect in the presence of ROCK inhibitor. There were no visible changes of E-cadherin expression in feeder cells after the same treatment.

Conclusions. Ca^{+2} is a key component of cell-cell contacts in hESC and its elimination lead to complete dissociation. Inhibition of Rho-kinase pathway prevent cell blebbing and apoptosis but do not promote recovery of cell contacts. E-cadherin expression regulated by Ca^{+2} and this regulation connected with the signaling pathway in which the Rho-kinase is involved.

The study was supported by the Russian Science Foundation grant № 14-50-00068.

Keywords: human pluripotent stem cells, cell-cell interactions, dissociation-induced apoptosis.

SHORT TANDEM REPEAT PROFILING IS INDISPENSABLE AND USEFUL PART OF QUALITY CONTROL IN STEM CELL BANK

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Cell culture misidentification is one of the most serious and persistent problems which often originates from cross-contamination. For products based on human cells and tissues, it is necessary to confirm that the cells originated from the intended donor and were not mixed with or replaced by cells from other donors. Short tandem repeat (STR) profiling is the standard authentication method that is capable of rapidly and unambiguously identifying human cell tissues and cell preparations to the individual donor level. The American National Standards Institute (ANSI) and the American Type Culture Collection (ATCC) provide a documentary standard (ASN-0002) for human cell lines based on the use of short tandem repeats (STR)-profiling. Since 2017, in Pokrovsky Stem Cell Bank (PBSC), STR-profiling used for umbilical cord mesenchymal stem cell (UC-MSC) identification is the one part of the quality control. However, a growing number of studies examining human cancers and inherited diseases have found that STRs can be unstable (Filoglu et al., 2014).

The purpose of the study was to evaluate the stability of the STR-profile during the standard period of ex vivo expansion. The analysis of short tandem repeats (STR) was carried out using the “COrDIS Plus” (Moscow, Russia) kit for DNA identification of 19 STR markers and a human amelogenin locus. The analysis included identification of 20 loci, including 13 of the combined DNA index–CODIS (D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, CSF1PO, FGA, TH01, TPOX, VWA), 5 –ENFCI (D1S1656, D2S441, D10S1248, D12S391, D22S1045), and also SE33 and amelogenin.

A reference STR profile was established on the primary material like DNA obtained directly from umbilical cord inner tissues. Once established, the reference STR profile was used to assess the identity of cell products and intermediates during production.

The UC-MSC for banking were expanded for 1 passage and the further cryopreserved according the company standards. The cells were collected for recurrent STR profiling in order to compare the reference profile and the profile after expansion and ensure that donor cell cultures are authentic. A total of 197 samples were analyzed. The STR loci combination did not change during the process of cultivation. So, UC-MSC STR-profile is genetically stable in early passages and can be used for MSC identification.

Keywords: short tandem repeat, STR-profile stability, stem cell bank.

MAXIMAL LIFE SPAN AND BONE MARROW CHIMERISM AFTER NONMYELOABLATIVE SYNGENEIC TRANSPLANTATION OF BONE MARROW FROM YOUNG TO OLD MICE

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At old age, the content of stem cells in the bone marrow falls by more than 10 times, and their substitution by the transplanted material can occur without the myeloablative conditioning of the recipients. The aim of this work was to determine the effect of nonablative syngeneic transplantation of young bone marrow (BM) to laboratory animals (mice) of advanced age upon maximum duration of their life. To do this, total of 100 million BM nucleated cells of young syngeneic donors were injected to each of old nonablated animals at the time when half of the population had already died.

As a result, the maximum life span (MLS) defined as the average life span of 10% of the longest-living mice, increased by 31+-3% in the experiment comparably to control group, and the survival time from the beginning of the experiment increased 3+-0.3-fold. This significant effect on MLS indicates that BM transplantation affects the intrinsic aging mechanism. The life-extending effect was significantly stronger than in earlier works with similar design (no irradiation or chemotherapy, no hereditary pathologies in recipients, advanced age at the start of the BM administration) because of (i) the larger amount of transplanted material and (ii) a close relation of donors and recipients.

The BM chimerism 6 months after the transplantation was 28%, as we determined using fluorescent donors of age 3-15 weeks, heterozygous for the green protein transgene (expressing GFP), of the same B10-GFP line and the same mice family as non-fluorescent old mice recipients. This high and permanent chimerism indicates, that rejuvenation is caused not only by paracrine effect, but also by direct cell replacement.

The observed life span extension is accompanied with the extension of active and healthy life period. Transplanted mice were active, had an even spine and shiny even hair at the same age of 19 months when the last mouse of the control group died, sedentary almost immobile and hunchback with poor hair. The result is encouraging for clinical adaptation for aged humans (70-80-years old).

Keywords: bone marrow transplantation; mesenchymal stem cells; longevity; life extension; cryobank.

CREATION OF A SET OF DNA CONSTRUCTS FOR GENOME EDITING OF OCT4 LOCUS WITH APPLICATIONS IN STEM CELL BIOLOGY

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Stem cell research has become one of the most studying fields in life sciences because of its promising applications for tissue replacement therapy, disease modeling, and developmental biology. But for proper cell fate manipulation, we need to understand better mechanisms underlying the regulation of stemness. Besides this, already is shown that despite its potential in therapy, stem cells have some critical dangers, such tumor formation. Therefore we need additional tools for stem cells research and usage.

In the last decade, there was a tremendous rise in interest for genome editing techniques. It is not surprising because manipulation on genomic scale allowed more detailed and specific collection of information for research needs and more accurate cell fate operation for therapeutic needs.

In our case, we created a set of constructs for genome editing of Oct4 locus. It is shown that Oct4 is the master regulator of pluripotency of the stem cells. Therefore we choose this gene for our genome editing strategy. We have created vectors for simultaneous expression of puromycin resistance gene, thymidine kinase gene and EGFP gene in various combinations along with Oct4. Together with CRISPR/Cas9 tool, as the main method, we can easily use them for different purposes.

These purposes include enrichment of pluripotent cells, which can be used for further efficient differentiation. Puromycin and EGFP expression can serve as a sensor for in vitro studies of maintaining and induction of pluripotency. On the other hand, thymidine kinase can serve as a suicidal cassette for the elimination of the cells that potentially can show tumorigenic properties. As a conclusion, we can claim, that stem cell research together with genome editing techniques will continuously rise with new synthetic instruments that will expand our understanding of biological systems and our abilities to affect them.

The work was supported by the Russian Science Foundation (grant 14-50-00068).

Keywords: stem cells, genom editing, Oct4, plurypotency

CYTOPROTECTIVE EFFECT OF HIGHLY DISPERSED SILICA NANOPARTICLES ON THE VIABILITY OF GRANULOSA FROM PORCINE FOLLICLES

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Products of nanotechnology revolutionize medicine, as evidenced by scientific advances and global initiatives to support nanotechnology and nanomedicine research. The aim of the present study was to determine the effect of *highly dispersed silica nanoparticles* (HDSn, Chuiko Institute of Surface Chemistry, Ukraine) on frequency of naked nuclei and DNA-fragmentation ("apoptotic ladder") in granulose cells (GC) cultured in vitro. GC were obtained from the healthy follicles (d= 3-6mm) and were centrifuged at 500xg for 10 min thrice. GC in TC-199 with 5% FBS and 50μg gentamycin [without HDSn (control) or with HDSn(experimental group)] were cultured at 38.5°C, 5% CO_2 for 24 h. DNA-fragmentation ("apoptotic ladder") was performed according standard protocol. The extracted DNA was quantified by a spectrophotometer, NanoDrop 1000 (USA). The DNA samples were electrophoresed on a 1.0% agarose gel containing 1μL/100mL SYBR-Safe DNA gel stain (Invitrogen) under 80V. The gel was examined and photographed by an ultraviolet gel documentation system (INTAS, Goettingen, Germany). Base liquid cytology technique with the aspirates were used for cytological slides preparation. Slides were stained with azur-eosine (May-GrunwaldGiemsa stain, "REACHIM", Russia). Parameters "frequency of naked nuclei" and "frequency of apoptotic cells" were determined by 500 cells (in 5 replicates) scoring under microscope "Leica"4000, Germany. The addition of 0.001% HDSn to culture medium decreased the frequency of naked nuclei from 28 ±4,93% to 3±1,7%, $P<0.01$, χ^2 test. We have not found apoptotic bodies in GC cultured with HDSn while level of apoptotic bodies in control group amounted to 13±3,36%. We have not found DNA fragmentation in GC cultured with 0.001% HDSn. In conclusion, HDSn positive affect on the survival of porcine granulosa cells. However the mechanism of the influence of HDSn on other cellular organelles remains to be explored. Funded by FASO Russia, project #18-118021590132-9.

Keywords: apoptosis, granulosa, highly dispersed silica

DECELLULARIZED EXTRACELLULAR MATRIX OF HUMAN MESENCHYMAL STROMAL CELLS AS A NOVEL BIOMATERIAL FOR REGENERATIVE MEDICINE

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Objective. The rapid development of cell-based technologies leads to the transition from artificial biomaterials to modulation of regenerative processes involving the endogenous resources and mimicking the natural healing processes. One of the perspective approach is to use a complex of bioactive products secreted by stem and progenitor cells, in particular extracellular matrix (ECM) proteins. ECM is an important component in the determining of cell behavior, including cell adhesion, proliferation and differentiation. Mesenchymal stromal cells (MSC) represent a promising source of multipotent adult stem and progenitor cells for cell therapy and tissue engineering. Accumulating evidence indicated that MSC effects on regeneration are mostly mediated by their ability to produce a wide range of bioactive molecules such as growth factors and cytokines as well as ECM. ECM production by MSC is enhanced if cell are cultured as cell sheets. The aim of the current research was develop a novel biomaterial based on decellularized ECM produced by human MSC cell sheets.

Material and methods. The protocol of decellularization was carried out using immortalized human MSC (hTERT MSC, ATCC). Based on the recent investigations focused on development of optimal protocols for decellularization and conservation of ECM structure and biological functionality we selected the following agents for decellularization: TritonX-100, Trypsin-EDTA, CHAPS, and sodium deoxycholate. To enhance the efficiency of decellularization some cell sheets were also treated with biological agents such as DNAse I and apoptosis inductor (rotenone). The structure of obtained

samples was characterized by Coomassie staining, scanning electron microscopy (SEM), Two-photon excitation microscopy.

Detection of residual DNA was assessed by Hoechst staining. In addition, the stability of ECM proteins was measured by immunohistochemistry. The evaluation of proliferation and cytotoxicity of the obtained biomaterial was studied by cultivation of hTERT MSC on decellularized sheets using automated live-cell analysis system IncuCyte.

Results and conclusions. Optimal protocols for decellularization of ECM produced by MSC cell sheets were developed. Apoptosis of MSC was efficiently induced by rotenone in 24-72 hours, but it didn't improve the decellularization of ECM produced by these cells. It has been shown by different methods that the structure of obtained biomaterials were meshy and branched, it also mimicked the ECM protein orientation in MSC cell sheets. In addition, short treatment with DNAse I was necessary to minimize the amount of residual DNA. Main ECM proteins, such as collagen I type, fibronectin and laminin, were preserved in biomaterial after decellularization. The viability and proliferation of hTERT MSC cultured on biomaterial obtained by different methods of decellularization were retained. The obtained results will be used for further studies investigating the impact of ECM proteins produced by MSC on regeneration processes as well as developing of a biomedical cell-free product for regenerative medicine.

Financial statement: The study was conducted using biomaterials collected and preserved in the frame of the project "Scientific basis for national bank-depositary of living systems" (RSF agreement #14-50-00029) using the equipment purchased as a part of Lomonosov Moscow State University Program of Development and supported by Russian Ministry of Science and Education (grant #MK-2422.2017.7).

Keywords: decellularization, mesenchymal stromal cell, extracellular matrix, regenerative medicine

THE TOTAL METHYLATION LEVEL OF THE GENOME IN THE CELLS OF MICE' TESTES AND ITS REGULATION AFTER SIMULATED MICROGRAVITY

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Methylation of the genome is one of the main epigenetic factors involved in the establishment of the gene expression patterns in mammalian cells. At the same time, the change of some environmental conditions, in particular, the cells' orientation in the gravitational field, also leads to a change in the expression of a number of genes, for example, encoding cytoskeletal proteins. Nevertheless, until now it remains unclear what causes this effect.

The aim of this study was to determine the level of total methylation and its regulation in the testes' cells of the mice that were under antiorthostatic suspension for 30 days (group 30HS, n=7), followed by a 12-hour recovery (group 30HS+12h, n=7) and the corresponding control group C (n=7).

The total methylation level of the genome (determined by the EpiJET DNA Methylation Kit, Thermo Scientific) was lower than in the control after suspension and was not restored after 12 hours of readaptation: reduced compared to the control by 13% in the 30HS group ($p<0.05$), 15% group 30HSE+12h ($p<0.05$). At the same time, the relative content of DNA methylase DNMT1 was below the control level by 35% in the 30HS group ($p<0.05$) and by 26% in the 30HS+12h group ($p<0.05$), as well as DNMT3a – by 15% ($p<0.05$) and 16% ($p<0.05$), respectively.

At the same time, the content of 5-hydroxymethylcytosine (determined using dot-blotting with specific antibodies, Abcam) remained at the same level in all study groups. Nevertheless, the relative content of demethylases TET1 and TET2 was significantly reduced ($p<0.05$) in the suspension group 30HS (decreased by 48% and 23%, respectively) and suspension group with subsequent recovery 30HS+12h (decreased by 54% and 39%, respectively).

The obtained data may indicate that a change of the external mechanical conditions leads to a change of the methylation level in the testes cells, in particular, under the conditions of modeling the effects of weightlessness - towards reducing it, completely demethylated patterns are established without accumulation of the intermediate product 5hmC.

The study was supported by program of the fundamental research SSC RF – IBMP RAS and program of RAS presidium “Molecular and cell biology”.

Keywords: methylation, weightlessness, sperm, testis

EFFECT OF OVEREXPRESSION OF THE KLOTHO GENE ON THE GROWTH OF TUMOR CELLS

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Oncological diseases have a relatively wide prevalence and can often be characterized by the severity of the course and outcome. This is the reason for the interest in finding new diagnostic and therapeutic approaches in this area.

The aim of the present studies was to evaluate the effect of induced overexpression of the klotho gene on the growth characteristics of tumor cells in vitro.

The studies were carried out on cultures of tumor cells of various origins of A-172, Rd, and Caco-2 lines (provided by the RAS IC, St. Petersburg). Hyper expression of klotho was modeled using a plasmid vector (Howard Hughes Medical Institute, USA), which includes the klotho gene. The increase in gene expression was confirmed by real-time PCR. Cell proliferation was assessed by means of the MTT test, incorporation of neutral red dye, and the incorporation of total 3H-thymidine into the cell DNA. The clonogenic activity of cell cultures was analyzed. An attempt was made to study the activity of apoptotic mechanisms with the help of fluorescent dyes on initiator and effector caspases.

The methods used to study the proliferative activity of cultured cells demonstrated inhibition of cell proliferation up to 60%. Cell cultures also showed a decrease in clonogenic activity. In addition, for cells of human glioblastoma cell line A-172, an increase in the proportion of cells with caspase activity was noted.

Thus, the present work shows the high prospect of further detailed study of the role of the klotho gene in the development of a wide spectrum of oncological diseases. Perhaps understanding the involvement of klotho in carcinogenesis and its impact on the course of oncology will open the way to the formation of new methods for diagnosing and treating such diseases.

Key words: klotho, gene therapy, signaling pathways, carcinogenesis, oncology

MONITORING OF VISCOELASTIC PARAMETRS IN MESENCHYMAL STROMAL CELLS AND IPS DURING DIFFERENTIATION USING FLIM AND HIGH-RESOLUTION MICROSCOPY

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The effective control of structural and functional changes during stem cell (mesenchymal stromal cells (MSC), induced pluripotent stem cells (iPS)) differentiation is a big problem because of the complex relationship between different signaling pathways, extracellular microenvironment and metabolic cell requirements. Fluorescence lifetime imaging microscopy (FLIM) and high-resolution microscopy in combination with exogenous markers allow non-invasive and long-term study of the changes underlying the viscosity and the cytoskeleton.

In this study we investigated viscoelastic changes in MSCs undergoing differentiation in two directions (chondrogenic, osteogenic) and in iPS undergoing neuronal and endothelial differentiation. The plasma membrane viscosity was studied using the staining of cells with the molecular rotor BODIPY 2 and the FLIM method. The cytoskeleton structure was analyzed using the staining of cells with Sir-actin or transient transfection of cells with TagRFP and stochastic optical reconstruction microscopy (STORM).

The fluorescence lifetime analysis of BODIPY2 showed an increase in membrane viscosity in chondrogenically differentiated MSC and its decrease in osteogenically differentiated MSCs. The cytoskeleton ultrastructure assessment in osteogenically and chondrogenically differentiated cells showed the change in the actin fibers orientation and an increase in their terminal parts when compared with undifferentiated cells.

Preliminary data about structural and functional parameters in iPS during neuronal and endothelial differentiation showed a similar trend.

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Keywords: mesenchymal stromal cells, induced pluripotent stem cells, viscosity, cytoskeleton.

MULTIMODAL IMAGING FOR CELL TECHNOLOGIES AND TISSUE ENGINEERING APPLICATIONS

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Currently the cell technologies and tissue engineering take a special niche in the biomedicine. The urgent task in this area is the pre-clinical testing of the bioengineering constructs and the biomedical cellular products using multimodal imaging and genetic markers. Such unique techniques allow to visualize the individual cells embedded in the constructs, their migration, proliferation, and also to study the cell differentiation processes. The study of the epigenetic mechanisms of stem cell (mesenchymal stromal cells (MSC), induced pluripotent stem cells (iPS)) differentiation is an actual problem. So using fluorescence lifetime imaging microscopy (FLIM) the metabolic switch from glycolysis to oxidative phosphorylation was shown during MSC differentiations by the lifetimes changing of NAD(P)H. Also we study the involvement of seeded allogeneic MSCs in bone formation using the model of transgenic mice expressing fluorescent protein GFP and genetically labeled cells. Allogeneic MSCs were found on the scaffolds 6 and 12 weeks post-implantation. By week 12, a newly formed bone tissue and blood vessels from the seeded cells were observed. Despite the significant progress in developing of skin equivalents (SEs) a problem of non-invasively assessing the quality of the cell components and the collagen structure of living SEs both before and after transplantation remains. Using the methods of optical coherence tomography (OCT), multiphoton tomography (MPT) and FLIM, the structure and quality of dermal SEs before transplantation, and remodeling of collagen matrix and microcirculation in the wound healing after dermal SEs transplantation were studied. Thus, the methods of optical imaging and genetic labeling are a powerful tool for the solving a huge number of problems in both the tissue engineering and the biomedicine in general.

This work has been financially supported by Russian Science Foundation (grants No. 17-75-20178).

Keywords: mesenchymal stem cells, iPS, multimodal imaging

IN VITRO MODELING OF PARATHYROID GLAND DEVELOPMENT USING PATIENT'S IPSCS TO UNCOVER PARATHYROID ORGANOGENESIS FOR HYPOPARATHYROIDISM TREATMENT.

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Background: Hypoparathyroidism is the most frequent permanent complication after a thyroid surgery and total parathyroidectomy. The standard treatment with active metabolites of vitamin D and calcium preparations cannot compensate all the effects of the parathyroid hormone (PTH) deficiency in the body and is associated with many complications. The substitution therapy of hypoparathyroidism with recombinant human PTH (1-84) demonstrates good results and benefits over standard treatment. At the same time, this drug has several limitations: the potential risk of osteosarcoma, the inconvenience of daily subcutaneous injections, as well as its high cost.

The simplicity of the parathyroid glands (PG) makes hypoparathyroidism an ideal candidate for *in vitro* organogenesis and treatment by cellular replacement. Modern reprogramming technologies make possible to generate patients own parathyroid-like cells via differentiation of pluripotent stem cells into PTH secreting cells.

Purpose: The aim of our study is to establish an effective protocol of generation patients induced pluripotent stem cells (iPSCs) and their differentiation into parathyroid cells *in vitro* for cellular replacement therapy of hypoparathyroidism by autotransplantation.

Methods: Skin fibroblasts were isolated from biopsies and used for integration free reprogramming with Oct3/4, Sox2, Klf4, and cMyc transcription factors. Selected colonies were characterized using immunocytochemistry and RT-PCR. Differentiation of iPSCs was performed by Activin A and Sonic hedgehog exposure.

Results: We successfully generated a number of iPSC lines from patient's skin fibroblasts that demonstrated pluripotency markers expression such as Oct3/4, TRA1-60, and others. iPSCs formed embryonic bodies that were forced to differentiate into parathyroid cells by Activine A and Sonic hedgehog. Differentiated cells were analyzed for calcium-sensing receptor (CaSR) and glial cells missing gene 2 (GCM2) expression by RT-PCR and ICC. The cells also secreted PTH.

Conclusion. Our studies have established an in vitro model to investigate the regulatory mechanisms that orchestrate early parathyroid organogenesis and provide a significant step towards cell-based regenerative therapy for hypoparathyroidism.

Keywords: induced pluripotent stem cells; hypoparathyroidism; parathyroid glands; parathyroid hormone.

REGENERATION IN A MODEL OF THE NONHEALING SKIN WOUND IN MICE

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Development of the nonhealing wound model for investigation of potential therapy by the biomedical cell product is actual because there isn't any unified small rodent model of this pathology.

The purpose of our research was morphological description of the nonhealing wound model in mice.

We used balb/c mice weight 18-20 g in our experiment. Manipulations with mice were done under an anesthetics and with acceptance of the Ethic committee. The H-shaped 10×30 mm skin flap and full-thickness circular wound 5-7 mm in diameter in the middle of the flap were inflicted onto the back of an animal. Induced skin flap ischemia provoked transition of the wound status to nonhealing one.

On the day 5 after an operation, the wound bed was fully or partially filled with immature granulation tissue that was characterized by absence of vessels and epitelium, abundance of fibroblasts and inflammation cells, prevalence of the cell component on fibrils and chaotic position of fibrils. The granulation tissue of a part of animals was less representative and was incorporated into subcutaneous tissue infiltrated by inflammatory cells. On the day 7, the granulation tissue was represented in wounds of majority of mice, in wound beds of some animals, we observed cell death and evidence of pus. Margins of the wound were characterized by edema, degradation of cells and cell death. On the day 14, wounds were epithelized, however such abnormalities as death and degradation of cells in the wound bed and areas of inflammatory infiltration were observed. These pathological changes persisted to the day 21 in the majority of mice and this was the result of ischemia. Wound healing during 21 days is abnormally long term for mouse organism. Therefore, we conclude that the model we developed corresponds to nonhealing wound. The work was fulfilled for Research Project "Development of technology for manufacturing, storage and application of biomedical cellular products for wound healing" in accordance to grant agreement with Ministry of Education and Science of Russia No. 14.610.21.0012, Unique identifier RFMEFI61017X0012.

Keywords: skin, nonhealing wound, ischemia, regeneration.

INDUCED PLURIPOTENT STEM CELL-DERIVED NEURAL PROGENITOR CELLS IN TREATMENT OF ISCHEMIC STROKE IN RATS

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Introduction. Induced pluripotent stem cell-derived progenitor cells (iNPCs) according to the results of the recent research may be a promising therapeutic strategy for an autologous cell therapy for stroke without ethical concerns and no risk of immune rejection. The aim of this study was to assess therapeutic effects after intra-arterial and intravenous transplantation of iNPCs in rats after MCAO.

Research methods. Male Wistar rats 24h after 90 minutes MCAO were randomly attributed to the following groups: intra-arterial(IA) (n=10) and intravenous(IV) (n=15) infusion of iNPCs ($5x10^5$ in 1 ml), control group (n=12). For evaluation of therapeutic effects and infarct volume behavioral test and 7T-MRI and were performed at 1d, 7d, 14d after MCAO (before histology).

Results. IA administration of iNPCs improves survival, stroke volume and functional recovery after 7d from stroke onset, while IV administration of iNPCs significantly reduces neurological deficit only 14d after MCAO. In case of both administration routes iNPCs are capable to migrate through the blood–brain barrier into the brain parenchyma.

Conclusions. We have shown that systemic (IV and IA) transplantation of iNPCs are save and improves recovery after stroke. However, intra-arterial transplantation has more prominent therapeutic effects.

This work was supported by The Ministry of Education and Science of the Russian Federation (project № 14.604.21.0184 RFMEFI60417X0184).

Key word: ischemic stroke, animal model, cell therapy, iPS-derived neural progenitor cells

DEVELOPMENT OF BIODEGRADABLE POLYMER VASCULAR GRAFT FOR CARDIOVASCULAR SURGERY

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Cardiovascular diseases remain the leading cause of highmortality in the world. To treat peripheral vascular diseases, large volume of blood vessel substitutes is necessary. Autologous vascular grafts are the best available clinical option but they often have limited length and poor quality. Until now, synthetic materials, such as polytetrafluoroethylene, have not corresponded to the effectiveness of native blood vessels, particularly in the case of medium or small diameter (≤6 mm). In the past decade, significant progress in the field of vascular tissue engineering has demonstrated the possibility of using polycaprolactone vascular grafts as a substitute for blood vessel. However properties of polycaprolactone vascular grafts are insufficiently studied. The aim of this study was to develop a method of manufacturing polycaprolactone vascular grafts of different thickness and diameters. The structural characteristics of polymer grafts were investigated by scanning electron microscopy (SEM). It was shown that thickness of different graft can vary from 10 to 100 μm. The rate of *in vitro* graft degradation depends on its thickness, which was confirmed by chromatographic methods. It was shown that mechanical properties of polycaprolactone grafts after 1 week degradation at physiological condition varied slightly. The biocompatibility of vascular graft was demonstrated by seeding of endothelial cells of ECV-304. Histological analysis of polycaprolactone grafts after implantation in the rat aorta confirmed the efficiency of regenerative processes.

This work was supported by Russian Science Foundation (project №14-50-00068) and the Federal Agency of Scientific Organizations (Russia).

Keywords: vascular graft, polycaprolactone, endothelial cells of ECV-304

DRUG DISCOVERY: A NEW CELL-BASED ASSAY FOR SCREENING OF TNF-ALPHA INHIBITORS

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The use of TNF-alpha inhibitors in clinical practice is considered as one of the largest achievements of medicine in recent decades. TNF-alpha causes inflammatory reactions in the body, which leads to such serious diseases as rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, psoriasis, refractory asthma and others. These diseases are successfully treated with monoclonal antibodies against TNF-alpha. The success of TNF-alpha-inhibitory drugs has sparked a boom in research for finding new molecules with TNF-alpha inhibiting activity. We developed a new, ready-to-use in vitro assay IEMB TNFScreen for screening of TNF-alpha inhibitors. IEMB TNFScreen assay is performed using the primary culture of human chondroblasts. Primary human cells are more physiologically and clinically relevant for drug discovery than the use of non-human cells or immortalized (oncogenic) human cell cultures. TNF-alpha dependent expression of cytokine interleukin-6 by human primary chondroblasts from different donors is demonstrated. It has been shown that both cryopreservation and prolonged passages of cells do not change chondroblast's ability to respond to both TNF-alpha induction and TNF-alpha inhibition by commercially available TNF-alpha inhibitor Enbrel®. 96-well plate format of the test is achieved. Thus, a fast, convenient and reliable in vitro method for screening of compounds with TNF-alpha inhibitory activity is developed. The other applications are discussed.

Keywords: Cell-based assay in vitro, Primary human chondroblasts, TNF-alpha, Interleukin-6.

AN INCREASE IN NUMBER OF BLOOD VESSELS IN THE RAT REGENERATING NERVE AFTER THE INJECTION OF MESENCHYMAL STEM CELLS

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In recent years, the experimental elaboration of different cellular technologies to stimulate regeneration of the damaged nerve is carried out actively. There is evidence that the mesenchymal stem cells **(**MSCs) derived from bone marrow, adipose tissue, umbilical cord stroma, amniotic fluid, and other tissues, promote the growth of the recipient regenerating axons after transplantation into the damaged nerve or a conduit. The purpose of this study is to investigate the vascularization of the nerve after injury and transplantation of MSCs. MSCs derived from bone marrow of Wistar-Kyoto rats (provided by "Trans-Technologies", St. Petersburg, Russia (D.G.Polyntsev, Head)) were cultured for 7 days and labeled with BrdU 3 days prior to usage. The sciatic nerve of adult Wistar-Kyoto rats was pinched and a suspension of BrdU-labeled cultured MSCs (5×10^4 in 5 µl culture medium) was immediately transplanted into the damaged sciatic nerve. 21 days later, the nerve segments containing transplant were fixed in a zinc-ethanol-formaldehyde.Using immunohistochemical detection of von Willebrand factor (vWF), a marker of blood vessel endothelial cells, a quantitative analysis of the blood vessels of the distal segment of the sciatic nerve of the rat was performed. It was found that 21 days after injury and injection of MSCs, the number of blood vessels in the nerve increases by more than 1.5 times in comparison with the control (damaged nerve without transplantation). It is assumed that MSCs have a stimulating effect on the growth of the vessels of the damaged nerve by secretion of angiogenic factors. Our results demonstrated a stimulating effect of MSCs on the growth of the vessels in the damaged nerve, proposedly, by secretion of angiogenic factors.

Keywords: mesenchymal stem cells, nerve vascularization, angiogenic factors.

MULTIPOTENT MESENCHYMAL STROMAL CELLS AS A MODEL FOR STUDYING OF OSSEOINTEGRATION PROPERTIES IMPLANTS

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Multipotency, ability for specifically migration in the foci of damage determines of the repaired functions mesenchemical stromal cells (MSC). Currently, the prevailing view about the therapeutic effect of MSC transplantation is due not only to their ability to differentiate, but also mostly to their regulatory function, which associated with their paracrine activity. The aim of the investigation the functional state of MSC in contact with new anti-corrosion osteogenerating coatings deposited on magnesium and titanium.

The architectonics of surface, morphology, metabolism and expression of CD44, CD29, CD71, CD106, CD120a and CD124 by MSC in contact with metal implants without coating and with the coated on them calcium phosphate coated formed by plasma electrolytic oxidation (PEO) and PEO with ultradisperse polytetrafluoroethylene composite coated (PEO UDPFE) were studied.

It was established that the most active cells adhered to the surface of the composite PEO UDPFE coated on implants, while for magnesium and titanium without and with calcium-phosphate PEO coated the number of these cells was significantly lower. In the study of cytotoxicity it was found that the percentage of viable cells was higher after their contact to composite PEO UDPFE coated than in contact with the magnesium or the calcium phosphate coating on it. The maximum of stimulation cellular metabolism were observed during the first 3 days of contact with the coatings, further, indicators of enzyme activity decreased in contrast to MSC contacted with implants. The architectonics of the MSC surface reflected the stimulating effect of the coatings, whereas no similar changes were observed on the surface of the implants. Upon contact with the

composite PEO UDPFE coated the high expression of CD106 receptor was revealed, which indicated on the differentiation of MSCs in the direction the osteocytes.

So the study of morphology and functional status of MSC showed the promise of further engineering of composite PEO UDPFE-coated for protection of implants as an osteoinductive material for the regeneration of bone tissue.

The research is funded by the Russian Science Foundation (project no. 14-33-00009, 2014-2016).

Keywords: mesenchemical stromal cells, plasma electrolytic oxidation, PEO-ultradisperse polytetrafluoroethylene, bone tissue regeneration.

DEVELOPING OF DERMAL-EPIDERMAL EQUIVALENTS ON THE 3D BIOPOLIMERIC SCAFFOLDS

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The aim of our study was to find the most suitable components for developing of dermal-epidermal equivalents. We compared cell culture conditions for different cell types (keratinocytes, dermal papilla cells, and multipotent stromal cells (MSC) on two different scaffolds (biopolimeric film G-derm and Sponge matrix(SM)) and investigated the effect of scaffold structure on the dermal-epidermal equivalent morphology.

We applied mouse and human cells. We examined 6 models of dermal-epidermal equivalents: dermal papilla fibroblasts from mouse hair follicle and keratinocytes with G-derm (1) and SM (2) as scaffolds; mouse MSC and keratinocytes with G-derm (3) and SM (4); human MSC and keratinocytes with G-derm (5) and SM (6). Time of cultivation for all models was the same – 16 days. Models 1-4 were studied in hypoxia conditions (O_2=5%, CO_2=5%). We used human and mouse skin sections as positive control. On the 7 and 14 days we examined the state of equivalents with the help of membrane tracer DiO and Hoechst33342. To elucidate specific stromal and epithelial cell markers we performed immunofluorescent array, which revealed the presence of keratinocyte-specific markers (CK14, CK19, desmoglein 1), stromal-specific marker (vimentin), skin-specific markers of extracellular matrix and basement membrane (collagen IV, laminin 5). Also routine histological examination was performed. We confirmed that in all dermal-epidermal models performed on G-derm there were 2-6 keratinocyte layers. Keratinocyted formed cell-aggregates on SM, at the same time MSC and DP fibroblasts formed smooth cell layers. It may be due to the matrix structure: keratinocytes prefer flat structure to adhere.

Thereby it preferable to use biopolimeric film G-derm for development of dermal-epidermal equivalents with multilayered epithelial structure. At the same time, SMs allows one to obtain greater cell mass of stromal cells. The work was fulfilled for Research Project “Development of technology for manufacturing, storage and application of biomedical cellular products for wound healing” in accordance to grant agreement with Ministry of Education and Science of Russia No. 14.610.21.0012, Unique identifier RFMEFI61017X0012.

Keywords: dermal-epidermal equivalent, scaffolds, MSC, keratinocytes, DP cells

PROLIFERATION OF MESENCHYMAL STROMAL CELLS DURING LONG-TERM CULTIVATION UNDER LOW OXYGEN TENSION

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Mesenchymal stem cells (MSCs) are an important member of the stem cell family and can be found in most postnatal organs and tissues. Due to multilineage differentiation potential, broad range of bioactive molecules secretion and other properties, these cells are being studied for their potential use in different modes of therapy. However, there is still no consensus on certain properties depending on the age of the donor or the duration of the culture. At the same time, there are the evidences on the slowdown of aging and accelerated proliferation of MSCs under hypoxic conditions. The aim of this study was to investigate the proliferation activity of MSCs along prolonged subcultivations under low oxygen tension.

MSCs were isolated from human adipose tissue. Cells were cultured in αMEM with antibiotics-antimycotics (1%) and 10% fetal calf serum at 5% or 20% O_2 up to 18-26 passages. Senescence of MSCs was identified by assessing the activity of β-galactosidase. Viability was estimated by with Annexin V–FITC kit (Immunotech, France). Proliferative activity was assessed as population doubling (PD). Cell cycle estimation was performed by flow cytometry analysis of cellular DNA content. To estimate number of fibroblast colony forming units (CFU), MSCs was seeded at low density and was stained by crystal violet.

Attenuation of proliferative activity is one of the main indicators of the cell senescence *in vitro*. The data demonstrated that PD was decreased significantly after 18 passages. Cell senescence was confirmed via β-galactosidase staining. At late passages percentage of cells in G_2/M phase and CFU number was decreased both at 20 and 5% O_2 level. The share of damaged cells was enhanced.

MSCs constantly propagated at low oxygen display enhanced proliferative potential at early passages. It was shown enhanced CFU number and percentage of cells in G_2/M phase, the share of SA-β-gal-positive ASCs was significantly lower at 5% O_2. The cell viability elevation was detected.

It was confirmed during the long-term passaging that MSCs propagated at 5% O_2 had higher proliferation, viability and exhibited less cells with an active β-galactosidase than at 20% O_2.

This work was supported by RFBR № 16-04-01244.

Key words: MSCs, replicative senescence, hypoxia, proliferation.

DIFFERENTIATION OF HUMAN PLURIPOTENT CELLS INTO THE EPIDERMAL AND DERMAL LINEAGES FOR SKIN EQUIVALENTS CONSTRUCTION

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The problem of technologies development for cultivation and transplantation of 3d-cell structures is significant in modern cell biology and regenerative medicine. These structures can be constructed using embryonic stem cells or cells derived from reprogramming of adult cells, for example, from induced pluripotent human cells.

In this research we concentrated on obtaining hair follicle-like cells: the equivalents of dermal papilla cells and epidermal keratinocytes, from pluripotent cells to provide the sources for future 3D human hair follicle reconstruction.

For epidermal keratinocytes differentiation we investigated different protocols: classical variant, with BMP4 and retinoic acid, modified variant with Src-inhibitor addition and the novel one with sodium butyrate as differentiation inductor. Dermal papilla cells were obtained through neural progenitor cells stage with their further differentiation with bovine fetal serum as differentiation inductor. The expression of specific markers were analyzed using qPCR and immunocytochemistry methods.

The results demonstrate positive expression of keratinocite's markers, cytokeratin8/18, 5, and 14 in obtained epidermal cells and positive expression of dermal papilla markers: versican, smooth muscle actin, S100A4, fibronektin and vimentin. The PCR results indicates the expression of sox9, vimentin and alkaline phosphatase in obtained dermal papilla lines. All obtained result is planned to use for folliculogenesis in vitro and in vivo induction.

Directed differentiation of pluripotent stem cells makes it possible to generate certain cell lines and current results precede the creation of hair follicle in vitro.

The work was funded by the Russian Science Foundation (Project No16-14-00204)

Keywords: induced pluripotent human cells, epidermal cells, dermal cells.

DEVELOPMENT OF A TISSUE EQUIVALENT FOR THE RECONSTRUCTION OF THE URETHRA SITE IN THE PROXIMAL FORMS OF HYPOSPADIA

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The proximal forms of hypospadias are characterized by the absence of the anterior wall of the urethra along the entire length of the trunk of the penis. The main sources of epithelial tissue in the reconstruction of the urethra are skin from the penis or in case of its shortage, the skin from the non-scalp part of the patient's body is used. A significant number of reconstructive surgery failures are associated with a deficiency of the patient's own tissues, or with the awakening of sleeping hair follicles in the transplanted skin and the urethral viremia in the patient at puberty. The purpose of this work was to develop a tissue equivalent for the reconstruction of the epithelium of the urethral tube based on skin keratinocytes and to study its behavior in the model of the epithelial defect of the rabbit urethra after transplantation. Experiments on transplantation of tissue equivalent into the urethral defect were carried out on adult male rabbits, in whom an experimental urethral defect was inflicted, creating a cut through all the layers of the anterior wall of the urethra and completely removing the epithelium in the middle and terminal sections of the urethra. Equivalent with autologous keratinocytes was created from skin cells taken from the animal's ear. For subsequent identification, skin keratinocytes were labeled with EGFP. Histological and immunohistochemical study of the rabbit urethra in the experimental group revealed that 21 days after the operation, the entire closure of the defect with a flat epithelium occurred, and by 45 and 90 days the epithelium in the neo -urethra corresponded to the norm, was represented by a multilayer epithelium of the transitional type. Co-expression of EGFP and differentiating markers of urothelium (K 7, K18, UPIII) was observed at all times of observation. Thus, when transplantated into the urothelium defect, autologous skin keratinocytes are incorporated into the urethra structure, restoring its integrity and functionality and acquiring phenotypic signs of urothelial cells. Thus, the tissue equivalent developed by us can be recommended as an alternative source of plastic material for reconstruction of the urethra with proximal forms of hypospadia.

The work is supported by the Government Program of the N.I. Pirogov Russian National Research University.

Keywords: hypospadias, autologous skin keratinocytes, urethra reconstruction.

THE INHIBITION OF IMMUNOPROTEASOME SUBUNIT LMP7 REDUCES THE EFFICIENCY OF REPROGRAMMING SOMATIC CELLS INTO PLURIPOTENT STEM CELLS

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The ubiquitin-proteasome system plays an important role in maintaining protein homeostasis and in the regulation of many cellular processes. The proteasome is a multi-subunit protease complex, consisting of a core 20S particle and 19S regulatory particles. Under certain conditions, the constitutive catalytic subunits of the 20S particle beta1, beta2 and beta5 can be replaced by special subunits – beta1i/LMP2, beta2i/Mecl-1 and beta5i/LMP7. In this case the proteasomes, designated as immunoproteasomes (IPs), are involved in antigen presentation. On the other hand, it has been shown that there is an increased expression of the IP subunits in murine embryonic stem (ES) cells during their differentiation. Besides, there is an increased expression of IP subunits in human ES cells, while the expression of these subunits drops during their differentiation. These observations imply that IPs are involved in regulation of pluripotency and differentiation of pluripotent cells. Another intriguing issue regards a possible role of IPs in the induction of cellular pluripotency. To address the latter question, we have performed reprogramming of mouse embryonic fibroblasts (MEF) into induced pluripotent stem cells (iPSCs), using a doxycycline-activated transgene construct OSKM (polycistronic sequence of factors Oct4, Sox2, Klf4 and c-Myc). Throughout the process of reprogramming the cells were treated with a selective inhibitor of beta5i/LMP7 subunit - PR-957, and the inhibitors of the beta5, beta1i/LMP2 and beta5i/LMP7 subunits of the proteasome - MG-132. Staining for alkaline phosphatase - a known marker of undifferentiated pluripotent cells - showed a significant decrease of the efficiency of reprogramming MEF treated in the presence of the inhibitors. Our results indicate the important functions of both proteasomes and IPs in the process of cellular reprogramming.

This work was supported by RSF 16-14-10343

Keywords: ubiquitin-proteasome system, immunoproteasome, PR-957, induced pluripotent stem cells

IL-6 ORCHESTRATES THE MIGRATION OF UROKINASE RECEPTOR-DEFICIENT NEUROBLASTOMA CELLS

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Relevance. Neuroblastoma is a extracranial tumor of the nervous system with a highly invasive phenotype. Previously it was shown that activation of urokinase and its receptor (uPAR) has a stimulating effect on migration and invasion of most cancer cells. Here we show a new mechanism of neuroblastoma migration involving IL-6 up-regulation.

Purpose of the research. To determine the role of the uPAR in the migration of neuroblastoma cells.

Research methods. Downregulation of uPAR (Neuro2a-ΔuPAR) in Neuro2a murine neuroblastoma cell line was carried out using CRISPR/Cas9. Overexpression of uPAR (Neuro2a-uPAR) was performed using plasmid transfection with cDNA of murine uPAR. uPAR and IL-6 content was evaluated by Western blot. Wound scratch assay was performed to evaluate the migration capacity of cells. Results were analyzed within 24 h using Leica AF6000LX detection system and data counting by Wound Healing Tool (ImageJ software).

Results. We investigated the effect of uPAR downregulation on migration of Neuro2a cells in the presence of its genuine ligand uPA. We demonstrated that the uPA-mediated Neuro2a migration inversely depend on uPAR expression: administration of uPA within 12 h of the experiment resulted in a 1.5-fold increase in Neuro2a-ΔuPAR migration to the scratch wound compared to control and Neuro2a-uPAR cells. After 24 h Neuro2a-ΔuPAR cells almost completely (up to 79%, $p<0.05$) recolonized the scratch wound, while control and Neuro2a-uPAR cells recolonized it to a significantly less ex-

tent (9 and 12%, correspondingly). Furthermore in Neuro2a-ΔuPAR cell we detected a significant upregulation of IL-6 expression - a highly potent migratory and metastatic cytokine for neuroblastomas.

*Conclusions.*These results allow us to propose a novel mechanism of uPAR-dependent neuroblastoma cell migration involving IL-6.

Source of funding: Grant №14-24-00086 of the Russian Science Foundation and Grant №14-50-00029 of the Russian Foundation of Basic Research.

Keywords: uPAR, IL-6, neuroblastoma, cell migration, CRISPR/Cas9

EVALUATION OF THE INFLUENCE OF SUPERPARAMAGNETIC IRON OXIDE NANOPARTICLES ON THE PROPERTIES OF BONE MARROW MESENCHYMAL CELLS UNDER IN VITRO CONDITIONS

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Integration of nanoparticles into medicine as therapeutic or diagnostic tools is a rapidly developing field, with a great potential for use in regenerative medicine. Currently, superparamagnetic iron oxide nanoparticles (SPIONs) are the most used and approved by the FDA for clinical use (enhanced contrast in MRI, targeted drug delivery, tumor therapy). The use of nanoparticles in cell therapy makes it possible to trace the further fate of cells implanted in the body. Despite the fact that SPIONs are the only metal oxides approved for medical use, their influence on cell functions is still being discussed.

Objective: to investigate the effects of SPIONs on the functions of cultured cells, in particular on the expression of cytoplasmic proteins under in vitro conditions.

Materials and methods: human mesenchymal bone marrow cells (MSCs FET) were used in the work. The cells were first incubated with SPIONs (less than 50 nm in size) at a concentration of 150 μg/ml for 24 hours, then the cells were re-introduced in a ratio of 1:3, after which they were incubated at different times (1, 3, 6, 10, 24 and 48 hours) and fixed by freezing. Using the methods of protein electrophoresis and immunoblotting, a cytoplasmic cell extract was analyzed.

Results and conclusions: quantitative differences in the content of cytoplasmic proteins in the experimental variant and in control cells that did not contain nanoparticles were found. The inclusion of SPIONs by cells effectively influences the expression of cytoplasmic proteins with molecular masses of 55 kDa and 70 kDa (presumably, tubulin and HTS-70) at terms of 3, 6, 10, 24 h. In addition, immunoblotting at the same time

was the difference in the content of cytoplasmic G-actin is revealed, which implies the influence of nanoparticles on the nature of the organization of the cytoskeleton and the ability of cells to migrate.

The work was carried out with the financial support of the RNF Grant No. 14-50-00068.

Keywords: nanoparticles, regenerative medicine, human mesenchymal bone marrow cells

THE CONSUMPTION OF GLUCOSE BY CHONDROCYTES FROM THE MINI-PIG'S EAR IN VITRO

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Glucose is an important plastic and energy substrate for all types of cells. In the metabolism of chondrocytes, glucose plays a key role in energy metabolism, since the role of oxidative phosphorylation in chondrocytes is reduced due to a low concentration of oxygen inside the cartilage due to its avascular structure. At the same time, glucose is a precursor of amino sugars, which are part of carbohydrate chains of proteoglycans - components of the extracellular matrix of chondrocytes. The ability of chondrocytes to synthesize proteoglycans and type 2 collagen determines the chondrogenic ability of chondrocytes. To obtain the necessary amount of chondrocytes for a tissue engineering design, cultivation technology is needed, one of the most important parameters of which is the consumption of glucose by chondrocytes. The chondrocytes of the mini-pig's ear have fast proliferation and therefore are a convenient model for studying the consumption of glucose by chondrocytes.

The aim of the study was to evaluate the change in the processes of glucose consumption by cells during the cultivation of chondrocytes of the mini-pig ear.

The change in the glucose concentration in the colorless medium DMEM / F12 (Gibco, USA) culture medium with 15% FBS (Gibco, USA) in the growth dynamics of the culture of the auricular chondrocytes of the mini-pig was studied depending on the mode of culture medium change. The culture growns in 48 well plates (TPP, Switzerland). Three modes of culture medium change were tested: 1- the medium was changed after 3 days, 2 - after 2 days, 3 - daily. Glucose was determined by a set of glucose-UV-Novo (Vector-Best, Russia) spectrophotometric method. The cells were counted in haemocytometer.

It was shown that the active growth phase, regardless of the culture regime, lasted two days in the culture of chondrocytes P0 and P1 passages. At the same time, the sat-

urating concentration of cells did not depend on the mode of environmental change in the 0 passage culture. The mode of environmental change influenced the chondrocytes of the P1 passage: the maximum saturating concentration of the culture of P1 was observed with a medium change regime 1 time in the 3rd day. Moreover, it exceeded the saturating concentrations of cells cultivated when the medium changed after 1 day and daily 2 times.

The environment change mode affects the consumption of glucose by chondrocytes: with the same number of cells at 0 passage at the end of the active proliferation phase, glucose consumption depends on the mode of the environmental change. Despite the same number of cells, glucose was consumed faster when the medium changed after 1 day. Perhaps, in this mode of cultivation, glucose is consumed by chondrocytes not for proliferation, but for the synthesis of matrix components, for example, proteoglycans. But for culture 1 passage this regime is not suitable, since it delays the onset of the phase of active proliferation. For culture 1 passage, the optimal mode of cultivation is a medium change every 3 days. In this mode, the largest population of cells is observed and their number decreases smoothly.

Key words: auricular cartilage, culture of chondrocytes, glucose consumption.

REPROGRAMMING FACTORS OF THE HUMAN RETINAL PIGMENT EPITHELIAL CELLS

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Traumatic eye damage and neurodegenerative processes often lead to visual impairment and blindness. The low regenerative potential of the tissues of the central nervous system and the retina, in particular, raises the issue of choosing ways for functional restoration, including: cell substitution therapy, cell reprogramming and induction of regeneration. The main sources of CNS regeneration are permanently multipotent neural stem cells and latent progenitors, capable of dedifferentiating and reprogramming under the influence of pathological signals. In the neural retina of the eye, latent progenitors include Muller glia cells. We assume that pigment epithelium cells (PE), which normally play a key role in the life support of photoreceptors and neurons, have the same properties, and in human pathology (for example, proliferative vitreoretinopathy), PE is reprogrammed into fibroblast-like cells that cause visual impairment and blindness. We studied these processes on an in vitro model and showed that human PE cells dedifferentiate (reduce the expression of *RPE65, CRALBP*), activate pluripotency genes (*SOX2, OCT4, NANOG*) and acquire the ability to differentiate not only in mesenchymal but also in mesenchymal on the neural path (*Musashi, PAX6*). To activate neural differentiation, the effect of bFGF on the reprogramming of PE cells in ARPE-19 cells was studied. After the addition of bFGF, an increase in the expression of KLF4 mRNA and a decrease in mRNA expression of *PAX6, MITF* and *OTX2*-specific PE cell markers were noted. The highest expression of KLF4 mRNA was observed 72 hours after the action of bFGF, whereupon it dropped sharply, which was accompanied by a threefold increase in mRNA expression of *βIII tubulin*, a neural marker. Immunocytochemically it was shown that under the influence of bFGF, some cells retained epithelial properties and stained with connexin 43, while the other had long axon-like processes and stained for βIII tubulin, which indicates a transdifferentiation along the neural pathway. Thus, despite the dominance of epithelial features, ARPE-19 cells demonstrate multipotency and, under the influence of bFGF, modulate *KLF4* and show proneural properties, suggesting that they are likely to belong to latent CNS progenitors.

The work was carried out within the framework of the Program of Fundamental Scientific Research of Koltzov Institute of Developmental Biology of Russian Academy of Sciences (0108-2018-0004)

Key words: Cell reprogramming, retinal pigment epithelium, neural differentiation

EFFECT OF CHRONIC BLOCKADE OF TYROSINE-KINASE RECEPTOR B (TRKB) ON SYNAPTOGENESIS OF PRIMARY HIPPOCAMPAL CULRURES *IN VITRO*

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Brain-derived neurotrophic factor (BDNF) is one of the key signaling molecule participating in neuronal differentiation, synaptogenesis and regulates cell metabolism in stress conditions. The main functions of BDNF realize via interactions with high-affinity tyrosine-kinase receptor B (TrkB). The aim of this study was to investigate the influence of blockade of TrkB receptors on neuronal synaptogenesis *in vitro*.

The experiments were carried out on primary hippocampal cultures obtained from 18-day mouse embryos (C57Bl/6). BDNF (1 ng/ml), selective blocker of TrkB-receptors - ANA-12 (1mkM) and their combination BDNF (1 ng/ml)+ ANA-12 (1mkM) were daily added into culture medium from the 3rd day of culture development *in vitro* (DIV). Ultrastructural studies were performed on 10DIV and 14DIV according to classical method of transmission electron microscopy. Ultrastructural slices were obtained on a Leica UC7 ultrasound (Leica, Germany) and then were analyzed on an Morgagni 268D electron microscope (FIE, Netherlands).

Preliminary received data revealed the decrease in the amount of mature synaptic contacts on 10DIV in the group of cultures with chronic blockade of TrkB receptors. The observed differences were negated by 14 DIV. Moreover, in this experimental group we observed the osmyophilicity decrease in the postsynaptic densification on 14DIV. Daily application of BDNF + ANA-12 did not change the number of mature synaptic contacts in comparison with intact cultures.

We assume that exogenous BDNF affects on primary hippocampal cultures bypassing TrkB signaling pathway.

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Keywords: brain-derived neurotrophic factor, primary hippocampal cultures.

CORRECTION OF PATHOGENETIC MECHANISMS OF CORONARY INSUFFICIENCY BY INTRODUCTION INTO THE MYOCARDIUM OF MULTIPOTENT MESENCHYMAL STROMAL CELLS TRANSFECTED WITH THE VEGF-165 GENE

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Despite the success of pharmacology and surgery, coronary heart disease remains the leading cause of death. This is due to the genetically determined absence of neoplasm of vessels in response to hypoxia in the tissues of the heart. The aim of the study was to find ways to correct these violations.

The experiment was carried out on male rabbits of the Chinchilla breed. In order to ensure incomplete occlusion of the anterior descending artery of the heart, the ligation of its proximal segment on the mandrel was performed, narrowing the lumen of the vessel by 80%. Group of animals No. 1 (n = 10) was injected with physiological solution, including all components except stem cells. The experimental group of animals No. 2 (n = 10) immediately after the ligation was injected intra myocardial with MMSC, in the amount of 1.0 x 10^6 cells per cm^2 transfected with the VEGF-165 gene.

MMSC were obtained by explanting subcutaneous fat from the anterior abdominal wall in 10 laboratory animals. According to the protocol of cell isolation:

Transfection was performed with a plasmid with the VEGF165 gene (pWZL Blast VEGF165).

The level of angiogenesis was assessed on the 30th day after the operation on microscopic sections of the myocardium stained with hematoxylin-eosin.

The obtained results indicate that a single intramyocardial injection of MMSC transfected with the VEGF-165 gene under simulated ischemia results in a statistically

significant increase in the total number of capillaries compared to the control group by 8.93%, an increase in the diameter of open capillaries by 7.54 %, an increase in the length of functioning capillaries by 30.53%, an increase in the area of the capillary exchange surface by 35.41%, and an increase in the oxygen partial pressure by 42.12%. In the insertion group of MMCs transfected with the VEGF-165 gene, there is significant neoangiogenesis, but after 1 month, ischemia is inadequate and less than in the VEGF-165 administration group, which may be due to the formation of fewer arterioles.

Keywords: coronary heart disease*, cellular therapy, gene therapy.*

CORRECTION OF CYRROTHOTIC CHANGES IN THE LIVER BY INTRODUCING MULTIPOTENT MESENCHIMAL STROMAL CELLS (MMSC), TRANSMITTED BY THE GENE OF THE HEPATOCYTES GROWTH FACTOR.

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Progressing liver failure is a pathology, for the therapy of which organ transplantation remains practically uncontested. Despite the obvious success of transplantology, the effectiveness of liver transplantation remains low. In view of the foregoing, the purpose of this study was to study the possibility of correcting cirrhotic changes in the liver by administering MMSC after transfection with their episomal vector containing hepatocyte growth factor (HGF).

On the experimental model of chronic fibrosing liver damage (Wistar male rats (n = 120), CCl4 primer, duration of the experiment 90 days), the effectiveness of cell therapy in correcting chronic liver failure was studied. Three groups of experiments were performed: group I (n = 30) - control (introduction of physiological solution); II group (n = 30) - introduction of a suspension of donor multipotent mesenchymal stromal cells into the liver at a dose of 8x106 cells; III group (n = 30) - introduction into the liver transfected by transfection with a plasmid containing the hepatocyte growth factor gene, donor multipotent mesenchymal stromal cells in a total dose of 8x106 cells; IV group (n = 30) - administration to animals of a plasmid containing as a useful gene the growth factor gene of hepatocytes at a dose of 0.03 mg by DNA. It was established that cell therapy in groups II and III of the experiments contributed to reliably accelerated normalization of impaired liver function: to the 40th (group II) and 20 (III group) days instead of 90 days in the control group (group). When histological analysis showed that after 90 days the rate of defibratization of liver tissue in group III was significantly more pronounced than in group II. The obtained effect can be explained by the fact that the developed cellular engineering structures provide adequate conditions for the prolonged vital activity of the transplanted cells. The indices of the IV group of animals did not differ from those recorded in the control group.

Key words: hepatic insufficiency, cellular therapy, gene therapy.

MURINE FIBROBLAST CELL LINES, NIH-3T3 AND STO, CANNOT BE REPROGRAMMED TO PLURIPOTENT STATE

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To date different cell types of various mammalian species have been reprogrammed by Yamanaka's cocktail of transcription factors, including OCT4, KLF4, SOX2, and C-MYC. It has been also shown that several primary human cancer cell lines were reprogrammed to induced pluripotent stem cells (iPSCs). We sought if widely used mouse fibroblast cell lines could be reprogrammed to iPSCs. This approach of generating iPSCs from stable murine cell lines assumed to be valuable for different experimental settings as it allows generating CRISPER/CAS9 base mutant cell lines that can be assayed in reprogramming. At the other hand only single study has reported reprogramming murine carcinoma cell line. To this regard we investigated reprogramming two fibroblast-originated cell lines, NIH-3T3 and STO, with use of highly effective lentiviral polycistronic OKSM expression system. The reprogramming of wild type murine embryonic fibroblasts and NIH-3T3 and STO cells were performed with the conventional method with use of feeder embryonic fibroblasts and N2B27 2i media. Two independent reprogramming experiments have shown that in contrast to control wild-type fibroblasts, NIH-3T3 and STO cells could not be reprogrammed to pluripotent state. While in all groups has been seen generation of cell clones, NIH-3T3 and STO clones were developed much later then wild type ones, and they contained round and larger cells, which are not typical for iPSCs colonies. Alkaline phosphatase and immunostaining on Nanog and SSEA1 confirmed that the cell colonies derived from NIH-3T3 and STO did not express these pluripotency markers. Furthermore in contrast to wild-type fibroblast derived iPSC clones, NIH-3T3 and STO clones could not be maintained by multiple cell passaging in embryonic cell media. Thus this data suggest that probably genetic abnormalities or abnormal cell signaling or both could make these immortalized cell lines be refractory to reprogramming to pluripotent state.

The work was carried out with financial support of the Russian Foundation (14-50-00068).

Keywords: Induced pluripotent stem cell, NIH-3T3, STO, lentiviral polycistronic expression system

MTDNA AS A NOVEL PLAYER IN PATHOGENESIS OF DESMIN-RELATED MYOPATHY

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Desmin is an intermediate filament of muscle cells. Desmin-related myopathy (DMP) is a disorder caused by mutations in desmin gene (Des). Mitochondrial dysfunction is a common pathological trait of DMP, where mtDNA intracellular content and extracellular release used as markers of mitochondrial dysfunction. Expression of mutant Des resulted in large-scale mtDNA deletions and reduced copy numbers. Nonetheless, the molecular pathway depicting mtDNA role in DMP pathogenesis need to be described. For instance, immunogenicity of mtDNA, which depends on its extracellular carriers, might underlie the progression of DMP. Thus, the purpose of the present project was to measure mtDNA extracellular release from cells expressing Des mutations and to analyze its carriers and immunogenic properties. Satellite cells transduced with lentiviruses encoded Des mutations were used as a cell model. mtDNA was isolated from the conditioned media collected in 4 time points: before the differentiation, on 3 d, 7 d, and 11 d of differentiation. Conditioned media underwent successive differential centrifugation steps: at 3000 g for 5 min to sediment cells and cell debris; at 16000 g for 15 min to separate large extracellular vesicles (lEVs) in sediment and lEVs-depleted supernatant. mtDNA copy numbers in samples of lEVs and lEVs-depleted supernatant were quantified using qPCR with SYBR Green was performed. To visualize extracellular vesicles secreted by cells, low voltage scanning electron microscopy (LV-SEM) was applied. To quantify immunogenicity of mtDNA, TLR9/SEAP reporter HEK cells were utilized. Experiments on defining the mtDNA release mode confirmed that lEVs fraction contained 10-fold higher number of mtDNA molecules compared to lEVs–depleted fraction. LV-SEM confirmed the presence of vesicles of expected sizes in both fractions. Examination of mtDNA extracellular carriers revealed that in both fractions majority of mtDNA was confined in membranous structures. Along with the time of differentiation, total mtDNA content increased 3.3-fold in control group and 6-fold in group with Des mutations. Analysis of immunogenicity revealed that TLR9 could be activated by lEVs fraction, but not by lEVs-depleted fraction or purified mtDNA. To sum up, given its immunogenic properties, mtDNA becomes a plausible player in desmin-related myopathy pathogenesis. Importantly, observed immunogenicity cannot be attributed to mtDNA per se, but rather to membrane–confined mtDNA.

The project has been supported by RSCF, agreement №14-15-00745П.

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Keywords: mtDNA, extracellular vesicles, desmin, muscle cells

THE ROLE OF “LINKER” HMGB1 / HMGB2 PROTEINS IN CHROMATIN OF MOUSE STEM CELLS

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Proliferation and differentiation of ESCs depend on the level of expression of non-histone chromatin proteins HmgB1/2 of the High Mobility Group family, their oxidation-reduction state and, presumably, the modification status. The aim of the study was to investigate the post-translational modifications of HmgB1/HmgB2 in mES cells and the role of these proteins in the structural and functional parameters of ESC chromatin during the differentiation. Using FT (ICR) MS, we have revealed functionally significant differences in the modification status of HmgB1 of mES and differentiated mouse cells. According to our data, the HmgB1 of mES cells show post-translational modifications that may result in the loss its ability to flex the DNA. This should probably lead to a partial reversible loss of its functionality in the chromatin of the mES cells. The last statement can explain the fact that CRISPR/Cas9-mediated knockout of the genes HmgB1 and HmgB2, according to our data, does not lead to significant changes in the phenotype of mES cells and does not affect their “ability” to differentiate into epiblast-like (EpiLCs) cells. The main differences between phenotypes HmgB1 + / + / HmgB2 - / - and HmgB1 - / - / HmgB2 - / - mES cells from wild type resides in a reduced rate of cell proliferation. In our future studies we will further address roles of post-translational modification of HmgB1 in the formation of chro-matin landscape of mES cells.

The work was carried out with financial support of the Russian Foundation for Basic Researches (18-04-01199) and support of the Russian Science Foundation (14-50-00068). The MALDI-mass spectrometry was carried out using scientific equipment of the Center of Shared Usage «The ana-lytical center of nano- and biotechnologies of SPbSPU» with financial support of the Ministry of Education and Science of the Russian Federation.

Keywords: HMGB1/2 proteins, posttranslational modifications, CRISPR / Cas9-mediated knockout, mutagenesis of HMGB1

AGE-SPECIFIC FEATURES OF ENDOGENOUS CARDIAC STEM CELLS ACTIVITY

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Relevance: In patients with cardiovascular pathology endogenous c-kit^{pos} cardiac stem cells (eCSCs) capable of generating cardiomyocytes. However, compensatory remodeling of the myocardium is limited and possibly depends on the age activity of eCSCs.

Purpose of the research: analysis of the eCSCs in patients of different ages with cardiovascular pathology.

Research methods: Biopsy specimens of the right ventricular (RV) myocardium of children with the tetralogy of Fallot (TF; n=61; 8,8±5,3 months) and adult patients with hypertrophic cardiomyopathy (HCMP; n=67; 39,5±13,1 years) were studied. Confocal microscopy using double immunofluorescent staining revealed C-kit^{+}/Sarc α-actin^{+} and Ki67^{+}/Sarc α-actin^{+} eCSCs, the relative content of these cells was calculated. The data were correlated with myocardial morphology and results of clinical examination of patients using Spearman coefficient at significance level of p<0,05.

Results: C-kit^{pos} eCSCs were found in the myocardium of children with TF and adults with HCMP in a very small amount of 3 – 43 cells/10^6 cardiomyocytes. These small cells (diameter 5-7μm), with a thin layer of cytoplasm containing sarcomeric α-actin were located in the interstitium. Apparently, c-kit^{pos} and Ki67^{pos} eCSCs represented different states of the same progenitor cells.

C-kit^{pos} eCSCs were detected only in 19,7% of children with TF in an amount of 4,1 - 66,4 cells/10^6 cardiomyocytes and decreased as the diameter of cardiomyocytes increased (r = -0,78; p=0,003). The amount of eCSCs decreased in patients with high pressure gradient between the RV and the pulmonary artery (r=-0,33; p=0,014) and low hemoglobin content in the blood (r=0,51; p=0,007).

In adult patients with HCMP C-kit^{pos} eCSCs were detected in 64,9% of cases, their content varies considerably from 4 to 8224 cells/10^6 cardiomyocytes. The number of eCSCs increased in the myocardium with hypertrophied cardiomyocytes (r=0,54; p=0,00003).

Conclusions: In children with cardiovascular pathology residual eCSCs activity was detected, and myocardial remodeling was carried out mainly due to the accelerated cardiomyocytes growth. In adult myocardium eCSCs activation was one of the forms of compensatory myocardial remodeling along with cardiomyocyte hypertrophy.

Keywords: cardiovascular pathology, compensatory remodeling of myocardium, endogenous cardiac stem cells; cardiomyocyte hypertrophy

3D BIODEGRADABLE SCAFFOLDS PRODUCED BY MODIFIED HIGH MOLECULAR WEIGHT HYALURONIC ACID FOR NEURAL TISSUE ENGINEERING IN TRAUMATIC BRAIN INJURY

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Traumatic brain injury (TBI) occurs, as a result, of direct mechanical insult to the brain, and induces cell degeneration and death in the central nervous system (CNS). Neuro- and cell transplantation have become widely recognized as powerful experimental tools for studying structure-function relationships, development, neuroplasticity, and regeneration in the adult central nervous system and have recently shown promise in the repair of brain injury and in the restoration of function after traumatic brain injury. The most relevant is the development of adequate carriers for transplanted cells that would create a certain microenvironment during the long process of neural networks restoration, as well as maintain the structure of brain tissue before its recovery. Currently, it is believed that such carriers are porous hydrogels that allow cells and nutrients to penetrate the matrix, and the products of life activity are excreted in the body volume, optimal in terms of mechanical compatibility with the tissues of the brain and spinal cord.

In the presented work 3D multifunctional hydrogel scaffolds-carriers of cells produced from modified high molecular weight hyaluronic acid were formed, with the use of which a complex of tasks related both to the biocompatibility and toxicity for neural cells was solved.

During the interaction of scaffold and dissociated brain cells derived from mouse embryos (E18), the absence of toxicity for neural cells, biocompatibility and adhesive properties of the material were proved. With the help of calcium imaging and multielectrode systems in vitro, the possibility of formation of complex neural networks inside the hyaluronic scaffold was revealed.

Modified hyaluronic acid scaffolds populated with neural stem cells from nasal olfactory lamina propria improve learning and sensorimotor function, reduce the lesion volume, and provide the migration of stem cells into the lesion boundary zone after TBI in mice, reduce the formation of glial scar without activation of autoimmune processes after the reconstructive surgery.

Keywords: traumatic brain injury, porous hyaluronic acid hydrogel, biocompatibility, neural stem cells

SENSITIVITY OF HUMAN ADIPOSE DERIVED MESENCHYMAL STROMAL/STEM CELLS TO CATECHOLAMINES IS ASSOCIATED WITH THEIR ADIPOGENIC POTENTIAL

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Relevance. Mesenchymal stromal/stem cells (MSC) are identified in the stromal-vascular compartment within the most of adult tissues including bone marrow, adipose tissue and skeletal muscles. MSC mediate physiological renewal of connective tissues by differentiation into multiple directions such as fat, bone and cartilage. Another important function of MSC, including adipose-derived MSCs, is paracrine regulation of tissue homeostasis, reparation and regeneration. MSC functions are under tight hormonal control, and noradrenaline is one of their most important regulators.

Purpose. The purpose of this study was to define how sensitivity to noradrenaline of adipose derived MSC is regulated and is linked to differentiation properties of these cells.

Methods. Flow cytometry, calcium imaging, Western blotting, adipogenic differentiation on-line under the microscope.

Results. At first, we examined the mechanisms of regulation of MSC sensitivity to noradrenaline. Using flow cytometry and calcium imaging in single cells, we demonstrated that more than 80% of MSC expressed alpha1-adrenoceptors at their surface. However only 6.9±0.8% of MSC responded to noradrenaline by intracellular calcium release, therefore in the most of the cells alpha1-adrenoceptors were not coupled with Ca^{2+}-dependent signaling. We showed that noradrenaline itself regulated MSC sensitivity to that hormone by inducing the down-regulation of beta-adrenoceptors and heterologic sensitization of alpha1A-adrenoceptors. Intracellular signaling pathways triggered by noradrenaline in MSC were switched from cAMP-mediated to Ca^{2+}-mediated ones. To evaluate how MSC responsiveness to noradrenaline is associated with differentiation properties of these cells we used live single cell imaging. First, we detected MSC

responding to noradrenaline by calcium release. Then we induced adipogenic differentiation of these cells and used live single cell imaging to track the fate of noradrenaline responding cells. We showed that although the most of MSC were differentiated into adipocytes, the cells responding to noradrenaline by calcium release never did.

Conclusions. Noradrenaline itself regulated responsiveness of individual cells to that hormone and calcium response to noradrenaline was linked to adipogenic potential of individual cells.

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Keywords: MSC, calcium signaling, noradrenaline, adipogenic differentiation

ANGIOGENIC ACTIVITY AND MAPK-SIGNALING I N MESENCHYMAL STROMAL CELLS AFTER LOW-FLUENCE PHOTODYNAMIC TREATMENT

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Mesenchymal stromal cells (MSCs) are actively used as tissue engineering tool for regenerative medicine due to their high proliferative and regenerative potential and paracrine activity. The same properties make them the key players in the pathogenesis of different diseases. Most of these physiological and pathological processes involving MSCs are accompanied by reactive oxygen species (ROS) induction. However, the ROS effects on MSC functional activity are still poorly investigated. In present study we demonstrated that ROS can modify MSC angiogenic and paracrine activity.

MSCs were obtained from stromal vascular fraction of human adipose tissue. Generation of intracellular ROS was induced by low fluence (0.25 J/cm2) photodynamic treatment (PDT) with Al-phthalocynine. The angiogenic activity was assessed using chorioallantoic membrane assay *in ovo*. Human phospho-MAPK Protein Array Kit was used to evaluate the relative phosphorylation of kinases involved in the MAPK signaling pathway.

Low-fluence PDT resulted in intracellular ROS production that was accompanied by enhanced ERK2, p38a, JNK phosphorylation. Analysis of paracrine activity and MSC-mediated vascularization (formation of blood vessels in chorioallantoic membrane) revealed that intracellular ROS induction can promote angiogenic activity of MSCs through stimulation of IL-8 and VEGFa secretion. Our data showed that ROS can regulate the angiogenic potential of MSC, and MAPK signaling pathway is involved in ROS-mediated modulation of MSC functional activity.

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Keywords: mesenchymal stromal cells, photodynamic treatment, angiogenic activity, MAPK signaling

THE INFLUENCE OF MILLIMETER WAVE ELECTROMAGNETIC RADIATION ON THE GROWTH OF MSC, HACAT AND HELA CELL LINES

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Electromagnetic radiation of millimeter range (also extremely high frequency, EHF 30-300 GHz) was reproduced in artificial conditions in 1965 in Soviet Union. A number of interesting effects were found during the study of the effect of EHF on biological objects and this range of radiation began to be used in medicine. Currently of interest is the difference in the action of different modes of millimeter wave radiation, as well as the specific reaction of cell cultures to the radiation of this range and mechanism underlying the observed effects.

Research methods. We expose to EHF radiation three cell cultures: HeLa, HaCaT, MSC. Radiation with a wavelength of 7.1 mm (42.2 GHz) in continuous mode (power density of 4 mW/cm^2) and pulse mode (power density of 2 mW/cm^2, pulse repetition rate of 8 Hz) were used, exposures of 30, 150 and 300 seconds were used. 24 hours after irradiation cell count and cytochemical DAPI staining were performed.

Results. A statistically significant increase (to 120%) in the number of cells in irradiated samples compared to the control group was found in the cell culture of MSC irradiated in a continuous mode at an exposure of 30 seconds. After irradiation of the HaCaT cell culture in pulse mode with exposures of 30, 150 and 300 seconds, a statistically significant increase in the number of cells was observed. The greatest difference was observed at the exposure of 30 seconds, and amounted to 154% compared to the control group. After irradiation of HeLa cell culture in both continuous and pulsed mode, there was a decrease in the number of cells (to 59%) in irradiated samples compared to the control group.

Conclusions. According to the obtained data, it can be argued that, firstly, the continuous and pulsed millimeter range electromagnetic radiation has different effects on cell cultures. Secondly, the reactions of the studied cell cultures to the same irradiation mode also differ significantly. However, the mechanisms underlying the observed changes in the growth of cell cultures in response to the irradiation of EHF is still not quite clear and need to be studied.

Keywords: cell grown, bioelectromagnetics, millimeter waves, EHF

APPLICATION OF THE TISSUE-ENGINEERING GRAFT FOR THE URETHRA RECONSTRUCTION

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The most significant pathologies of the urethra are the anomalies of development and acquired defects. Currently the oral mucosa has usually used for urethral reconstruction and demonstrate best results. However, despite the good results, there are some problems using of native tissues such as insufficient donor tissues and donor site morbidity. The rapid development of tissue engineering techniques provides an effective way to treat urethral defects. At present, urethral reconstruction, using the tissue-engineering graft (TEG) may be performed with both naturally derived and synthetic materials in the presence or absence of cell seeding. The purpose of the research is an assessment of the efficiency of the application of TEG seeded with different cell types for urethra full-thickness repairing. We used TEG that is a bilayer synthetic scaffold based on Poly-L-lactic acid (PL) and a Poly-ε-caprolactone (PC). The inner porous layer of the scaffold was designed from PL and the second outer layer was prepared from PC. For cells tracking in the graft, rabbit mesenchymal stem cells (MSCs) were labeled with superparamagnetic iron oxide nanoparticles (SPIONs) that can be applied as contrast agents for MRI. Dorsal surface of the rabbit urethra was reconstructed using TEG. The animals were divided into two experimental groups: (i) an experimental group with a PL-PC scaffold seeded with MSCs; (ii) an experimental group with using of the oral mucosa. The follow-up period constituted four-time points of 4, 8, 12 and 24 weeks. Our preliminary results have demonstrated that using of the TEG with MSCs enhanced the reconstruction of urethra defects and reduced the possibility of development of long-term negative obstacles.

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Keywords: tissue-engineering graft, urethral reconstruction, mesenchymal stem cell, superparamagnetic iron oxide nanoparticles (SPIONs)

IDENTIFICATION OF INTRINSICALLY DISORDERED PROTEINS BASP1 AND GAP-43 IN MOUSE OOCYTES AND PREIMPLANTATION EMBRYOS

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Two related, fully unstructured proteins BASP1 and GAP-43 (neuromodulin) are highly expressed in brain neurons where they participate in axon guidance and synaptic plasticity. Recently, BASP1 was also found to be a critical target of the Sox2 and Myc transcription factors and an inhibitor of iPSCs generation from mouse embryonic fibroblasts. In this study, we have revealed that both BASP1 and GAP-43 proteins are present in mouse oocytes and preimplantation embryos. Using immunocytochemical techniques, BASP1 was found to be localized in the cytoplasm of the oocytes, zygotes and blastomeres, with a more pronounced staining of the plasma membrane and actin cortex. Microinjection of bovine BASP1 into the cytoplasm of the metaphase II (MII) oocytes induced their exit from the MII arrest followed by parthenogenetic embryo development. A possible mechanism of BASP1 participation in oocyte activation may consist in sequestration of the plasma membrane polyphosphoinositides, as studied previously in neurons. GAP-43, which is generally regarded as a marker of the postmitotic neurons, was shown to reside at the meiotic spindle microtubules in the MII oocytes. GAP-43 is also colocalized with γ-tubulin at the spindle poles (centrosomes) and at the discrete microtubule-organizing centers (MTOCs) in the cytoplasm. This is reminiscent of GAP-43 centrosomal association found recently in asymmetrically dividing neuronal progenitors. By analogy to the neuronal progenitors, we suggest that GAP-43 may be involved in regulation of oocyte polarity. GAP-43 was also revealed in blastomere nuclei in preimplantation mouse embryos. Interestingly, distinct blastomeres displayed different expression of GAP-43. Using antibodies to Ser41-phosphorylated form of the protein, GAP-43 was shown to be subject to phosphorylation by protein kinase C in oocytes and blastomeres. The presence of BASP1 and GAP-43 in oocytes was independently confirmed by western blotting and RT-PCR. Our results indicate that the role of BASP1 and GAP-43 in early embryogenesis requires further investigation. Supported by RFBR grant 18-04-01357.

Keywords: oocytes, mouse embryo, intrinsically disordered proteins, pluripotency

PARACRINE ACTIVITY OF TNF-A-STIMULATED ENDOTHELIAL CELLS IS ENHANCED UPON INTERACTION WITH MESENCHYMAL STROMAL CELLS

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Mesenchymal stromal cells (MSCs) are a promising tool for regenerative therapy. These cells secrete soluble factors, which play an important role in intercellular signaling, as well as in the regulation of many physiological and pathological processes. The experimental results show that MSCs could affect not only immune cells, but also the endothelial cells (ECs). The goal of this study was to examine MSCs effects on ECs paracrine activity.

MSCs were obtained from human adipose tissue and ECs were isolated from the human umbilical vein. Cells were cultured according to standard protocols. Monolayered ECs were activated with TNF-α (10 ng/ml). Next day, MSCs were added to the ECs in a ratio of 1:1 and co-cultivated for 24 hours. Then, the co-cultured cells were separated by magnetic immunoseparation. Expression of EC *IL6, IL8, CCL2* was assessed using RT PCR. The conditioned medium (CM) was collected after 24 hours of co-cultivation and the concentration of cytokines IL-6, IL-8, MCP-1, TGF-beta, VEGF was determined by enzyme immunoassay.

The interaction between ECs and MSCs led to an increase of IL-6, IL-8 and MCP-1 concentration in CM, level of TGF-beta and VEGF was not affected. The most pronounced IL-6, IL-8 elevation was detected in case of TNF-α activated ECs in comparison with ECs and MSCs in monoculture. MCP-1 level in the ECs/MSCs CM did not depend on ECs activation. To elucidate the contribution of ECs in joint cytokine production in co-culture the expression of *IL6, IL8, MCP-1* was estimated. Co-cultivation did not affect the transcription of *CCL2* in ECs and led to up-regulation of *IL6* and *IL8*. Elevation of transcription was more pronounced in TNF-α stimulated ECs vs nonactivated cells. Thus, MSCs are able to modulate the secretion of pleiotropic cytokines in the ECs during the interaction. This effect is more pronounced in activated ECs, that probably may play an important role in tissue repair and remodeling processes.

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Keywords: mesenchymal stromal cells, endothelial cells, pleiotropic cytokines.

SYNM AS A POTENTIAL CANDIDATE GENE FOR HEART-HAND SYNDROME: WHOLE EXOME SEQUENCING DATA AND SYNEMIN EXPRESSION PROFILE IN MESENCHYMAL STROMAL CELLS

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Relevance and purpose: «Heart-hand» syndromes represent a group of rare congenital conditions where patients in addition to cardiac pathology demonstrate various abnormalities of limb skeleton. To date, pathogenesis of such disorders remains poorly understood. In the present study, we report on a 30-year-old woman with a clinical picture closely resembling ulnar-mammary syndrome with symmetrical fifth finger anomalies, breast hypoplasia with inverted nipples and frequent episodes of non-sustained ventricular tachycardia. Here we aimed to determine a genetic basis of the described phenotype to extend knowledge on causative genes and molecular mechanisms underlying the complex heart-hand disorder.

Methods: Genome analysis was carried out by whole-exome sequencing (WES), target Sanger sequencing and array-based comparative genome hybridization. Multipotent mesenchymal stromal cells (MSCs) were induced to adipogenic and osteogenic differentiation with subsequent analysis of synemin expression using real-time PCR and immunofluorescent staining.

Results: No genetic defects were revealed in *TBX3* gene, the only gene associated with ulnar-mammary syndrome to date, as well as in other genes responsible for common heart-hand syndromes. Based on WES data analysis, we took notice of a novel missense variant in *SYNM* gene (exon 1, p.A58V), encoding an intermediate filament protein synemin. In experimental cell studies, we for the first time characterized synemin expression in mesenchymal stromal cells during tissue-specific differentiation. Taking into account mesenchymal origin of tissues involved in the clinical phenotype, this cell model seems relevant and promising for further investigation of heart-hand syndrome pathogenesis.

Conclusions: Here we present a nontypical clinical case of ulnar-mammary syndrome with no association with *TBX3* mutation. We suggest that the *SYNM* genetic variant could contribute to the complex heart-hand phenotype, possibly by affecting proper MSCs differentiation. Further functional cell studies are planned to confirm the hypothesis.

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Keywords: heart-hand syndromes, whole-exome sequencing, *TBX3*, synemin, mesenchymal stromal cells.