Session 1. Stem Cells Bioengineering for Therapeutic Translation

Lectures

L1.1

Stem cells and biomaterials for treatment of CNS diseases

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Stem cells have been investigated for their therapeutic potential in spinal cord injury (SCI), stroke and animal models of amyotrophic lateral sclerosis (ALS). We compared human mesenchymal stem cells (MSC) from bone marrow, a conditionally immortalized human stem cell line from fetal spinal cord (SPC-01) and human induced pluripotent stem cell-derived neural precursors (iPS-NPs) for their capacity to migrate towards lesion sites, differentiate and induce better regeneration. Rat models were used; SCI was caused using a balloon-induced compression lesion, stroke was induced using a photochemical cortical lesion, and ALS was observed in transgenic rats. We studied transplantation of MSC, SPC-01 or iPS-NPs labeled in culture with iron-oxide nanoparticles for MRI tracking. Electrophysiology was used to study the properties of stem cell-derived neurons *in vitro*. Animals were tested using motor and sensory tests. We found improved function in animals with a stroke lesion as well as prolongation of lifespan and decreased motorneuronal loss in animals with ALS. Various biocompatible hydrogels (degradable and non-degradable), including those based on non-woven nanofibres, have been developed for bridging tissue defects and for use as 3D stem cell carriers. Animals with chronic SCI were implanted with a PHPMA hydrogel seeded *in vitro* with cells. Ca^{2+} imaging on single SPC-01 cells revealed voltage-activated Ca^{2+} channels, typically observed in neurons. *In vivo* MRI proved that MSC, SPC-01 or iPS-NPs migrated into the lesion and survived for several months. Implanted animals showed functional improvement; MSCs rarely differentiated into neurons, while SPC-01 or iNP-NP implantation resulted in greater improvement, and many implanted cells differentiated into motoneurons. Improved motor and sensory scores in chronic SCI was found after the implantation of biomaterials seeded with MSC or SPC-01. Recently, we also used hydrogels composed of decellularized porcine extracellular matrix (ECM), which can facilitate constructive remodelling of various tissues. After implantation into SCI, we found considerable ingrowth of neurofilaments and blood vessels into this biological scaffold. The ECM biological scaffolds are therefore promising candidates for clinical use not only in the oesophagus, lower urinary tract, muscles, tendons and myocardium, but even in spinal cord repair. Two clinical trials are currently running using autologous MSCs in patients with SCI and ALS.

Acknowledgements

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L1.2

Stem cells for the treatment of ischemic stroke

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Stroke is an acute neurodegenerative disorder and represent one of the leading causes of death and disability in adult humans in developed countries. Stroke cause massive morbidity and mortality and tremendous economic and societal burden, being a leading cause of death and disability in adult humans. In recent years, the recovery phase of stroke attracted much of the attention of researchers and clinicians, and currently is considered as most suitable target for the stroke therapy. This is justified by the long-term therapeutic window and also intrinsic plasticity-based mechanism of recovery which is operating in the brain and represents suitable target of the therapy. These therapeutic approaches would be initiated, many hours, if not days and weeks after stroke onset, with the intention of improving neurological function and not necessarily reducing the burden of the ischemic lesion. The aim of such therapeutic strategy is to enhance and accelerate the spontaneously operating self-repair/recovery mechanism. Stem cells have the capacity to generate neurons and glia cells which are lost in neurodegenerative diseases including stroke. Recently, stem cells of different origin have been tested for their ability to reconstruct the stroke-damaged forebrain and improve function after transplantation in animals models of ischemic stroke. The transplanted cells can survive and partly reverse some behavioral impairments. However, the underlying mechanisms of this improved recovery are unclear and there is little evidence for neuronal replacement. Besides cell replacement, stem cell-based approaches can also improve function by modulating inflammation, preventing neurons from dying, and increasing angiogenesis. These exciting laboratory findings should now be responsibly translated to the clinic. Some initial studies using cell therapy approaches have been performed in patients with stroke. There are several ongoing clinical trials in stroke patients, mainly using autologous bone-marrow derived cells. However, many issues remain before stem cell therapy can advance to full-scale clinical trials. These issues are (i) type of cells suitable for transplantation and their mechanisms of action, (ii) how to control proliferation, survival, migration, differentiation and integration of endogenous and grafted stem cells in stroke-damaged brain, and (iii) procedures for cell delivery, scaling-up, optimum functional recovery, and patient selection and assessment.

L1.3

Application of stem cells for vascular tissue engineering

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Following the generation of the first *in vitro* blood vessel over the 30 years ago, there has been considerable progress in this area in terms of scaffold availability, construction of the vessels and application of stem cells. In this report I will specifically focus on the use of stem cells in the generation of vascular grafts, and highlight the vascular scaffolds available for seeding cells, and the methods used to differentiate stem cells into vascular lineages and their application in generating blood vessels *in vitro*. Recently, we designed a combined protocol of reprogramming and differentiation of human fibroblasts. Four reprogramming factors (OCT4, SOX2, KLF4, c-MYC) were overexpressed in fibroblasts under reprogramming conditions for 4 days with cells defined as partially induced pluripotent stem (PiPS) cells. PiPS cells did not form tumours *in vivo* after subcutaneous transplantation in SCID mice and differentiated into endothelial and smooth muscle cells when seeded on collagen IV and maintained in differentiation media. PiPS-SMCs repopulated decellularised vessel grafts and ultimately gave rise to functional tissue-engineered vessels when combined with previously established PiPS-endothelial cells, leading to increased survival of SCID mice after transplantation of the vessel as a vascular graft. Additional, we have also used c-Kit-cells isolated form stem cells that can differentiate into functional endothelial and smooth muscle cells. When seeded *ex-vivo* on a decellularized vessel, c-Kit+ cell-derived endothelial cells organized in a uniform monolayer, expressing endothelial markers. *In vivo,* implantation of the tissue-engineered vessel in a mouse model led to a markedly reduced neointima formation and mortality, as compared to the non-seeded controls. Thus, we developed a protocol to generate endothelial and smooth muscle cells from stem cells or PiPS cells, useful for generating tissue-engineered vessels.

L1.4

Pluripotent stem cells for vascular repair

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Stem cell (SC) therapy represents hope for treating unmet clinical needs, including in the context of cardiovascular disease. The abilities of embryonic SC (ESCs) and induced pluripotent stem cells (iPSCs) to self-renew indefinitely and to differentiate in all the three germ layers make these SCs very attractive for both basic science investigations and clinical therapies. ESCs/iPSCs can generate vascular endothelial and mural cells to be used for transplantation and to create engineered organs. Moreover ESCs/iPSCs can be used to mimic developmental vasculogenesis and *angiogenesis in vitro*. However, additional studies are needed to understand the mechanisms guiding vascular differentiation of stem cells and hence informing on better protocols for achieving this with high efficiency and reproducibility.
To optimally harness ESCs/iPSCs potential necessitates precise control over biological processes that govern maintenance, pluripotency and cell differentiation including signalling cascades, gene expression profiles and epigenetic modification. Such controls are elicited by several different actors such as growth factors and microRNAs. In our lab, we are working at the role of NT and microRNAs in vascular differentiation of ESCs, showing a preferential effect of BDNF/TRKB during endothelial commitment. We are also developing effective directed differentiation protocol for the differentiation of functional endothelial cells from hESCs, efficient to induce therapeutic neovascularization in ischemic tissue.
I'll give you a general introduction of the research area and describe my cardiovascular scientist journey in the ESCs/iPSCs world.

L1.5

Anaerobic nature of stem cells – an evolutionary paradigm and its impact on stem cell engineering

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Hematopoietic stem cells (HSCs), unlike committed progenitors, can produce the energy for proliferation independently of mitochondrial respiration. "The resistance to hypoxia" increases with the degree of primitiveness of HSCs. HSC pool could be maintained *ex vivo* in parallel with their active proliferation and production of committed progenitors (asymmetric self-renewing cell divisions) relating the stem cell (SC) self-renewal to "hypoxia".
SCs are placed in "hypoxic niche" from embryonic to adult stage exhibiting a very low number of barely active mitochondria and stabilized HIF-1a. They rely mainly on anaerobic glycolysis. While inhibition of oxidative phosphorylation (OxFos) and stimulation of anaerobic glycolysis enhances the stemness regardless the way operating this action and SC type (adult, embryonic, iPSc), commitment/differentiation are paralleled by intensification of OxFos resulting in loss of self-renewal capacity.
So, self-renewing divisions (a simple division "*à l'identique*" i.e. without commitment and differentiation) of SC looks like a basic reproduction of an anaerobic/facultative aerobic ancestral eukaryote. Similarly to the evolution of anaerobic/facultative aerobic eukaryotes into aerobic ones, the SC, with their commitment and differentiation are "evolving" in more and more differentiated and specialized cells (committed progenitors, precursors) with enhanced energy needs and better adapted to O_2.
In addition to the metabolism of glucose, the HIFs system controls more than 200 genes in human cells-including WNT, Notch, Fox, Nanog, Pax, GATA, Oct, Socks, Stat… considered as pluripotency factors; overexpression of some of them results in induction of pluripotency in somatic cells (iPSc), a process still enhanced by low O_2 concentration.
Transcriptome of *Entamoeba histolytica* — an anaerobic unicellular eukaryote — contains GATA, Oct, Socks and Stat, which emphasizes the primitive character of stemness and pertinence of our evolutionary paradigm. Indeed, the main advances in the *ex-vivo* expansion procedures are related to "hypoxic response" (i.e. "ancestral" modality of cellular functioning) leading to the maintenance of stemness. They refer to signaling *via* Notch, HOXB4, Wnt and Hedgehog, stabilization of HIF-a, and attenuation of OxFos in mitochondria.
Thus, energetic metabolism manipulation and related activation of an ancestral part of genome and transcriptome becomes the major tool in SC engineering for regenerative medicine.

Oral presentations

O1.1

Kinetics of DNA methylation during reprogramming of human somatic cells into induced pluripotent stem cells (hiPS)

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Reprogramming somatic cells into induced pluripotent stem (iPS) cells requires complex epigenetic changes. During this process lineage specific genes are progressively silenced and the expression of embryonic transcription factors is restored. KAP1/TRIM28 is a scaffold protein that interacts with zinc finger proteins containing KRAB domain (KRAB-ZFPs). By this interaction KAP1/TRIM28 is tethered to specific DNA sequences and by cooperation with other proteins activates formation of heterochromatin followed by DNA methylation of gene promoters.
The aim of our study was to analyse changes in DNA methylation profiles during reprogramming process. Primary human dermal fibroblasts (PHDFs) were forced to pluripotency by overexpression of four transcription factors (OCT4, KLF4, SOX2 and c-MYC). Cells were cultured in two variants: with or without KAP1/TRIM28 knockdown. DNA was collected on several representative timepoints. To investigate methylation profile of analysed cells we used RRBS (Reduced Representation Bisulfite Sequencing) method. This high-throughput technique allows to analyse the genome-wide methylation profiles on a single nucleotide level. Firstly, DNA was digested by methylation-insensitive enzyme MspI and then converted with sodium bisulfite. Samples were sequenced on Illumina Hiseq1500.
Our results show that the overall level of methylation in the CG context for the tested samples increased about 8% in iPS cells compared to somatic cells from which they were derived. There was also a slight increase in the level of methylation at CHG and CHH context (H-any nucleotide). We identified over a thousand of DMRs (differentially methylated regions) indicating the difference in methylation levels between samples. What is more, we indentified gene promoters that undergo hyper/hypo-methylation during dedifferentiation. We also confirmed a potent role of KAP1/KRAB- dependent mechanism in establishing proper methylation profile of pluripotent cells.

O1.2

Bone tissue engineering with human mesenchymal stromal cells and wide-porous cryogel scaffolds

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Current methods for treatment of bone tissue defects are based on allogenic or autologous grafting, associated with the risk of transplant rejection, painfulness and high cost of procedure. As an attractive alternative the development of tissue engineering constructs based on natural or synthetic porous scaffolds and autologous cells of patient can be considered.

The aim of this study was to evaluate distribution, metabolic and proliferation activity and efficiency of induced osteogenic differentiation of human MSCs during 3D culture within wide-porous collagen, collagen/hydroxyapatite, alginate and gelatin cryogel scaffolds.

3D porous matrices based on collagen, collagen/hydroxyapatite, alginate and gelatin were prepared by the method of cryotropic gelation. MSCs were isolated from human adult adipose tissue, expanded *in vitro* and then seeded into scaffolds by perfusion technique ($2.5–3\times10^5$ cells/scaffold). Cell morphology within scaffolds was estimated by FDA staining. Metabolic and proliferation activity of MSCs was assessed by Alamar blue test on 1st and 7th day of 3D culture. Cell localization and distribution in the scaffolds were assessed using MTT assay. DNA concentration of cells cultured on porous matrices was determined using the Quant-iT PicoGreen dsDNA assay (Invitrogen). Efficiency of induced osteogenic differentiation was evaluated by alkaline phosphatase expression (ALP 2×60 kit, Biolatest Lachema) and calcium content (Ca L 1×250 kit, Biolatest Lachema).

After seeding into all investigated porous matrices MSCs attached to their surface and had flattened fibroblast-like morphology. The MTT assay showed the uniform distribution of viable MSCs within the whole volume of scaffolds. The Alamar blue fluorescence intensity on the 7th day of 3D culture increased for 30–35% compared to 1st day, indicating cell proliferation. MSCs within all investigated scaffolds under the influence of appropriate inductors differentiated into osteogenic direction. The maximal levels of alkaline phosphatase expression were obtained in MSCs grown within collagen-based scaffolds, while cells within alginate or gelatin scaffolds demonstrated less efficiency of induced osteogenic differentiation. The highest calcium deposition level was achieved within collagen/hydroxyapatite scaffods, while lowest – within gelatin scaffolds.

The obtained results show the advantages of collagen and collagen/hydroxyapatite scaffolds for bone tissue engineering applications.

O1.3

Mesenchymal stem/stromal cells (MSCs) transplanted into mouse heart

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Numerous of literature data indicates that the mesenchymal stem/stromal cells (MSCs) can be used in a many therapeutic strategies. MSCs can support wound healing, inhibit abnormal immune response, take part in the regeneration of various tissues, organs, among others, improve the function of post-infarcted heart. The aim of the study was to determine therapeutic abilities of MSCs isolated from human heart on a murine model of myocardial infarction.

The material used in the study consists of 10 hearts removed during heart transplant surgery in Silesian Center for Heart Diseases in Zabrze. Cells were isolated from collected fragment of a heart. After the initial selection on the plastic dishes, cells were sorted on FACS Aria III. Cells with $CD105^+CD34^-$ phenotype were cultured approximately 10 days in IMDM medium supplemented with 20% serum and bFGF. The resulting $CD105^+CD34^-$ cells cultures were characterized by the presence of markers: CD73, CD90, CD29, CD44 and lack of: CD45, LIN, HLA-DR. They differentiated also into adipocytes and osteoblasts. $CD105^+CD34^-$ cells were directly injected into the mouse myocardium seven days after LAD ligation in amount of 500 000 cells on mouse. The control group consisted of mice with injected PBS. 42 days after $CD105^+CD34^-$ cells or PBS injection echocardiographic analysis were performed. Subsequently mouse hearts were removed for histochemistry and immunofluorescence analysis in order to assess the progress of therapy.

Echocardiography showed an increase in left ventricular ejection fraction (26% increase vs. control group). We observed reduction in the size of the post-infarction scar in mice treated (n = 8) compared with the control group (n = 6) by 11.58%. There has also been a reduction in fibrosis in mice treated (n = 8) compared with the control group (n = 6) by 16.34%. Our results indicate that isolated human mesenchymal stem/stromal cells (MSCs) improve functionality of post-infarcted mouse heart.

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O1.4

In vitro and *in vivo* induced myogenic differentiation of Pax7-/- pluripotent stem cells

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Skeletal muscles possess a capacity to regenerate after injury or disease. However, repetitive injuries may lead to the exhaustion of myogenic precursors, i.e. satellite cells population and, in consequence, to the impairment of regeneration and muscle dysfunction. Transplantation of stem cells that could replenish satellite cells population and support muscle regeneration could be considered as possible therapy of such diseases. Many types of cells have been tested *in vivo* and *in vitro*, so far. Among the factors used *in vitro* to induce myogenic differentiation are azacytidine analogues which replace cytosine during DNA replication. Their impact at the epigenetic modifications was shown to be connected to the interference with the function of DNA methylases.

Pax7 protein plays a crucial role in the specification of skeletal muscle precursor cells during myogenesis and maintaining of satellite cells within adult muscle. In this study we focus at the role of Pax7 in myogenic differentiation of pluripotent stem cells. As a tool we use wild type and Pax7-/- embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). We found that azacytidine analogues treatment of pluripotent stem cells impacted at their differentiation leading to the formation of myotube-like structures. Next, we revealed that differentiating *in vitro* Pax7-/- ESCs downregulated Nanog while upregulated Pdgfαr (mesoderm marker) more efficiently than wild type cells. Moreover Pax7+/+ and Pax7-/- ESCs and iPSCs treated with 5-azacytidine express myogenic transcription factors, e.g. MyoD, Myf5, myogenin. Next, we documented that both types of cells, i.e. Pax7+/+ and Pax7-/-, transplanted into damaged mice muscles are able to undergo myogenic differentiation, and participate in the formation of new skeletal muscle fibers. Transplantation of Pax7-/- cells is more efficient. After Pax-/- cells transplantation we observed higher levels of MyoD, Cdh, Myh2, Myh7 transcripts, which coding myogenic regulators and structural proteins.

We conclude that myogenic differentiation of Pax7-/- pluripotent cells occurs more readily than of Pax7+/+ wild type ones.

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O1.5

New strategies in stem cell transplantation

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For last few years we can observe increasing number of ongoing clinical trials with stem cells application. The most commonly used in a clinic are human mesenchymal stem cells. Their regenerative action is thought to be evoked by strong immunomodulatory as well as cell replacement capabilities. Unfortunately among conducted trials substantial differences in treatment protocols and stem cell derivation/cultivation procedures unable positive falsification of the results scattered from the lack of effects to the full recuperation.

Searching for the reasons of divergence in neuroprotection induced by MSC treatment we have focused on the interrelation between environment, phenotypic cell evolution depending from culture conditions and interactions between "therapeutic" WJ-MSC cells and targeted tissue. For this we used the co-culture model of human WJ MSC with intact or injured by oxygen glucose deprivation (OGD) rat organotypic hippocampal slices.

Our experiments showed that the strongest ability for neuroprotection was provided by freshly excised pieces of WJ tissue and the first cohort of migrating MSC cells (passage O). Along further passaging the cells phenotype changed substantially and cell neuroprotective effect declined together with modification of paracrine capabilities of WJ-MSC-secreted cytokines.

These results will be challenged with our previous data gathered in preclinical and clinical experimentations showing that undifferentiated, SRTF* expressing MSC, capable to time-locked proliferation, migration and ultimately to neural differentiation are the most effective in various therapeutic transplantation models.

Recently we have focused on the mechanism of adult type stem cells phenotypic plasticity evoked by culturing WJ MSC in lowered to 5% O_2 atmosphere. Results strongly suggest that induction of the less differentiated, SRTF-expressing, pluripotent-like state of MSCs significantly increase they proliferation, epigenetic stability, survival, and capability of cells to differentiation into neural as well as endothelial directions. Most importantly, intra-ventricular transplantation of the cells with similar phenotypic characteristic were recently find biologically safe in 5 years of prospective clinical observation.

*SRTF — Stemness-Related-Transcription-Factors (Oct4A, Nanog, Rex1, Sox2)

Acknowledgements

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Posters

P1.1

KRAB-ZFPs expression kinetics during reprogramming of human somatic cells to induced pluripotent stem cells

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Zinc finger proteins containing Krüppel associated box (KRAB - ZFPs) comprise the largest family of transcriptional repressors in mammalian genomes. KRAB domain interacts with KAP1, a scaffold protein, which recruits various transcriptional factors and causes epigenetic repression of genes bound by KRAB-ZFPs. Currently, little is known about physiological functions of KRAB-ZFPs and their gene targets. It has been reported, however, that KRAB-ZFPs family have a role in regulating differentiation, cell proliferation, apoptosis, cell cycle regulation and embryonic development. Our previous studies, utilising RNA-seq analysis revealed a set of KRAB-ZFPs that are overexpressed in induced pluripotent stem cells (iPSCs) compared to somatic cells. Thus, we hypothesized that these KRAB-ZFPs might affect reprogramming to iPSCs at various stages of this process. In order to explore the kinetics of those expression changes during reprogramming to iPSCs, we transduced adult primary human dermal fibroblasts (PHDFs) with lentiviral STEMCCA vector carrying four pluripotency transcription factors (OKSM). We collected the cells at 7th, 14th and 21st day of reprogramming, isolated RNA and analyzed expression of KRAB-ZFPs with RT- qPCR method. According to the previous study, we observed increased expression of majority of selected KRAB-ZNFs at the final stage of reprogramming. One of them — ZNF114 — become upregulated at 7th day, much earlier than the other analysed KRAB-ZFPs. Our data suggest that ZNF114 can influence cell reprogramming and pluripotency.

P1.2

Sonic hedgehog and WNT family proteins involvement in differentiation of mouse embryonic stem cells

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Skeletal muscle tissue originates from mesoderm – one of three germ layers forming during early step of embryogenesis. During mammalian embryo development mesoderm is further spatially divided into paraxial, intermediate, and lateral mesoderm. Paraxial mesoderm segments into somites, in that myogenic progenitors that will form trunk and limb muscles arise. These progenitors subsequently migrate to limb buds and undergo further differentiation, leading to the formation of limb skeletal muscles. Each of these processes: gastrulation, somitogenesis, specification of myogenic progenitors as well as their migration and differentiation is controlled by a set of signalling proteins among which Sonic hedgehog (Shh) and WNT family proteins play the key role. Both Shh and WNT proteins induce the expression of MRF (Myogenic Regulatory Factors) in myogenic precursors, therefore, allowing their further commitment and differentiation.
In our study we focus at the role of Shh and WNTs in mouse embryonic stem cells (ES cells) during their *in vitro* differentiation in embryoid bodies. We examined the expression of those proteins as well as their receptors, i.e. Patched and Frizzled at the mRNA and protein levels. Next, we examined how presence of these factors in the culture medium influences differentiation of ES cells.

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P1.3

Neuronal differentiation of induced pluripotent stem cells (iPS) into inhibitory GABA interneurons

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Inhibitory GABA interneurons are neural cells populating the cortical areas of the brain. They play a key role in higher brain processes by modulating neuronal networks activity and plasticity. Disturbed cortical GABA projections may cause diseases such as epilepsy, schizophrenia and autism. These diseases pose a big challenge due to the lack of accurate knowledge of the mechanisms underlying their etiology and pathology. Moreover, there is lack of reliable animal models to study the function of GABA.
In 2006 Yamanaka for the first time established induced pluripotent stem cells (iPS) by introducing exogenous reprogramming factors into adult somatic cells. As pluripotent, iPS cells possess unlimited proliferative potential and can differentiate into almost every cell, including neural cells.
The aim of our study was to evaluate good model for both, *in vitro* research under GABA projections and possible cell therapy. For this purpose we used two protocols for differentiating iPS. The main distinction between them was the way of obtaining neuroectoderm, ready for terminal differentiation. First one, leading consecutively through stages of embryonic bodies (EB), rosettes and neurospheres (NS) formation and the other, through dual SMAD inhibition. Finally, both types of acquired neuroectoderm were differentiated into inhibitory GABA interneurons. On each step cells were characterized for expression of specific markers (embryonic and neuronal Nestin, Tuj-1, FOXG.1, GAD1, GAD2) on the level of mRNA (by RT-PCR) and protein (by immunocytochemistry; Tuj-1, GABA).
We believe that the continuation of our research can deliver a good model for pharmacological studies *in vitro* and *in vivo* and to obtain an excellent tool for research on the mechanisms of neuropathological processes that can lead to serious neurological disorders such as epilepsy, schizophrenia and autism.

P1.4

miRNA in myogenic differentiation of Pax7-/- embryonic stem cells.

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miRNAs are crucial regulators of development and tissue regeneration. Among them: miR1, miR133a, miR133b and miR206 has been shown to regulate myogenic cells differentiation during embryonic development and muscle regeneration. For example, miR133a promotes myoblast proliferation via inhibition of SRF. miR1 and miR206 inhibits HDAC4, allowing expression of MyoD and Mef2, inducers of myoblast differentiation. Next, miR206 inhibits expression of Cx43, which is necessary for myoblast fusion but dispensable during later stages of differentiation. Myogenic miRNA has been also shown to inhibit expression of Pax7, i.e. paired box transcription factor expressed at early stages of muscle development and also responsible for maintaining satellite cell population in adult skeletal muscle. Pax7 also affects myogenic miRNA levels through inhibition of factors regulating myogenesis. In our project we focus at the relationship between Pax7 and miRNA. Using Pax7 deficient embryonic stem cells we analyze the interplay between myogenic miRNA and Pax7 and ask how this "relationship" influence myogenic differentiation of pluripotent cells.

P1.5

Human induced pluripotent stem cells-derived microvesicles transfer RNAs and proteins to recipient cells modulating cell fate and behaviour

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Microvesicles (MVs) are circular membrane fragments shed into the extracellular environment by direct budding from the cell plasma membrane or are derived from the endosomal compartment. MVs originated from a given cell type may act as mediators of cell-to-cell communication by transferring surface determinants and genetic material to target cells.

In this study we investigated the potential of MVs derived from human induced pluripotent stem cells (hiPS) on transferring biologically active components to recipient cells and analyzed subsequent effects on cell fate and behaviour.

Human iPS cells were generated from umbilical cord-derived mesenchymal stem cells (UC-MSC) with Sendai virus in serum-free and feeder-free conditions. Cells were characterized by expression of surface antigens, genotyping, immunohistochemistry and teratoma formation assay, showing their pluripotential nature. hiPS-MVs were harvested by ultracentrifugation at 100000 *g*/twice. Analysis of selected transcripts, miRNome and proteome profile revealed that hiPS-MVs are rich in mRNA, miRNA and proteins, however, the content and concentration of particular components very between parental cells and MVs. Furthermore, we demonstrated that hiPS-MVs can transfer their cargo to recipient cells, which exerted stimulating and protective effects, as well as enhanced cardiac and angiogenic differentiation. By using lentiviral transduction, we modified genetically hiPS cells to express green fluorescent protein (GFP) and we showed, that apart from endogenous transcripts, also an exogenous material can be transferred by hiPS-MVs.

This study suggests that hiPS-MVs may be important mediators of signaling between various cell types and may be useful therapeutic tools for horizontal transfer of RNAs and proteins to target cells.

P1.6

Cells with expression of pluripotency marker SSEA-4 are present in adult human skin

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Different populations of stem cells can be distinguished that maintain skin homeostasis by providing a pool of new cells to replace those lost during skin turnover or after injury. Furthermore, several previous studies demonstrated that cells with multipotent character reside in the bulge region of hair follicles and contribute not only to interfollicular epidermis but also to hair follicle and sebaceous gland. The stage-specific embryonic antigen 4 (SSEA-4) is an epitope on glycosphingolipid widely used as a marker for pluripotent embryonic stem cells, which is down-regulated during their differentiation. Moreover, it was shown that in human, SSEA-4 is expressed by neural cells and by non-neural cells such as erythrocytes.

In the present study, we show that SSEA-4 positive cells are present in adult human skin. While we were looking for a rare population of pluripotent stem cells in adults called very small embryonic stem cells (VSELs) characterized by small size and expression of specific markers (CD133, SSEA-4, Oct-4), we found distinct population of cells which were SSEA-4 positive, but they did not co-express stem/progenitor marker CD133. Using flow cytometry, we estimated that an average number of these cells constitutes 0.06% of analyzed small nuclear cells both in epidermis and dermis of adult humans. With confocal microscopy we searched for SSEA-4 expressing cells in the bulge region of a hair follicle. In our experiments we detected single cells expressing SSEA-4 on their surface (about 20 μm in diameter) localized in the inner root sheath (IRS) of hair follicles. Next, we used immunohistochemical staining to analyze skin tissue sections. We confirmed localization of SSEA-4 positive cells in hair follicles, however, we could also observe these cells within the basal cell layer of the epidermis, with a marked tendency to be localized at the tips of rete ridges, a protective niches of interfollicular epidermal stem cells. Additionally, scattered single SSEA-4 immunoreactive cells were detected within the dermis, especially in the papillary layer.

The obtained data indicate that there is a rare population of cells deposited in human skin demonstrating one of characteristic features of embryonic stem cells. However, further characterization of these cells is needed to establish whether expression of SSEA-4 marker in adult human skin is associated with pluripotency, unipotent epidermal stem cells or neither of them.

Acknowledgements

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P1.7

Molecular relationship between genes involved in neuronal differentiation, hypoxia and epigenetic modifications in HUCB-NSC under different oxygen conditions

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The previous studies of our group have indicated thatlowering oxygen concentration to 5% was beneficial for human neural stem cell reprogramming. Therefore, we asked the question whether low oxygen concentration influences the neuronal differentiation of Human Umbilical Cord Blood Neural Stem Cells (HUCB-NSC) and how does it correlate to the hypoxia inducible factors (HIF's) and epigenetic regulation.
To reveal the possible molecular relationship between neural differentiation, hypoxia and epigenetic regulation, expression of genes *MAP2, NANOG, HDAC1, HDAC2, DNMT3a, DNMT3b* as well as *HIF1a*, *HIF2a*and *HIF3a* were examined. Changing cellular accumulation of HIF's by DMOG (prolyl hydroxylase inhibitor) in cells cultured at different developmental stages (induced by serum free, low serum and 100 μm dBcAMP conditions) at 21% and 5% oxygen concentration, we looked at mutual dependence of this pathways.
HUCB-NSC express *HIF1a* and *HIF2a*, but not *HIF3a* gene in both tested oxygen concentrations. Low oxygen was beneficial for the cells proliferation. Addition of DMOG increased significantly the expression of *HIF1* and *HIF2* genes in cells cultured in low serum (2%) medium, but not serum free conditions in both tested oxygen concentrations. However, *HIF1a* and *HIF2a* accumulation associated with the DMOG treatment was higher in cells cultured in low oxygen concentration.
Induced by dBcAMP expression of MAP2 was further increased by the presence of serum and HIF accumulation, however this was not correlated to different oxygen conditions. Spectacular increase in the expression of MAP2 was observed only in one combination of tested conditions: low serum and low oxygen in DMOG treated cells, but not pre-differentiated by dBcAMP.
Expression of genes involved in regulation of epigenetic processes (*HDAC1, HDAC2, DNMT3A, DNMT3B*) was higher in HUCB-NSCs growing in low serum conditions in comparison to serum free culture. Moreover the highest expression of *HDAC1, HDAC2, DNMT3A* and *DNMT3B* genes was observed in cells cultured in low serum/low oxygen conditions with presence of DMOG.
The studies revealed strong correlation between *HIF1a* and *HIF2a* accumulation and expression of *HDAC1, HDAC2, DNMT3A, DNMT3B*. Moreover, the impact of oxygen on proliferation rate and the differentiation process has been demonstrated. That may indicate that oxygen level affects these processes by epigenetic modifications.

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P1.8

Cell cycle control by *Pax7* — a new role of the old myogenic gene?

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Postnatal growth and regeneration of the skeletal muscles depends on stem cells, i.e. satellite cells. Some pathological conditions, such as muscular dystrophies, lead to the exhaustion of satellite cells pool resulting in the failure of skeletal muscle regeneration. Among the therapies aiming to improve regeneration of such affected muscles are transplantation of exogenous stem cells. Next, among the stem cells considered to be the most promising are pluripotent stem cells, such as embryonic stem (ES) or induced pluripotent (iPS) cells. However, their myogenic potential as well as factors and mechanisms regulating myogenic differentiation are still not fully characterized. Pax-7 transcription factor is the key marker and regulator of embryonic myogenesis as well as skeletal muscle satellite cells maintenance. Pax-7 was shown to be involved in regulation of proliferation, differentiation of myogenic cells, as well as prevention of their apoptosis.
In the current study we use cells lacking *Pax7* functional gene to investigate the role of *Pax7* in the regulation of proliferation of differentiating ES cells as well as mouse embryonic fibroblasts (MEF). We show that proliferation rate and cell cycle profile are not altered in *Pax7*-null cells. Next, we analyzed expression of genes coding cell cycle regulating proteins. We observed differences in expression levels of some of those transcripts between *Pax7*-null and wild-type cells. Finally, lack of *Pax7* also impacts at the percentage of apoptotic cells. Altogether, this data demonstrates the involvement of *Pax7* gene in regulation of cell cycle machinery and maintenance of differentiating ES cells and MEF.

Acknowledgements

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P1.9

BMP-2 increases RO/NS levels in serum-free cultures of human bone-marrow derived mesenchymal progenitor cell line

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Adult bone marrow-derived mesenchymal stem cells are potent source of osteoprogenitors and they can be differentiated into osteoblasts in culture with different growth factors, including bone morphogenetic protein 2 (BMP-2). Osyczka & Leboy (*Endocrinology* 2005) showed that in serum-free insulin-containing media (SF+I), differentiation of these cells in response to BMP-2 was more efficient. Since reactive oxygen/nitrogen species (RO/NS) are proposed to play pivotal role in early osteoblastogenesis, we have tested the level of ROS in cultures of bone marrow-derived mesenchymal progenitor cells line (hMPC 32F, Osyczka *et al*., 2001, *Calcif Tiss Int*). Cells were cultured for 4 days in serum-containing medium (SCM) and than transferred to fresh SCM or SF+I media with or without BMP-2. After 15 minutes of changing culture conditions, RO/NS levels cells were analyzed by flow cytometry and we found significantly lower levels of RO/NS than in cells maintained in SCM. However, the cells stimulated with BMP-2 in SF+I increased RO/NS production, whereas cells treated with BMP-2 in SCM decreased ROS levels. To determine a source of RO/NS, we have examined nitric oxide (NO) production in hMPC 32F cultures using Griess approach. NO was detected at low levels in all cell culture conditions. The mitochondrial membrane potential (MMP) was assessed using JC-1 fluorescent probe and flow cytometry. No evident changes in MMP in hMPC 32F was noted in either SCM or SF+I with and without BMP-2. This suggests that the mitochondrial activity might not be the source of reactive species in BMP-treated cultures of hMPC in SF+I. Other factors seem to be responsible for changes in cellular RO/NS levels regulating hMPC osteogenic differentiation. Further studies are aimed at RO/NS sources and their potential role in BMP-mediated hMPC osteogenesis.

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P1.10

Human Mesenchymal Stem Cells osteogenesis in 3D cultures stimulated with media perfusion flow and/or rhBMP-2

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Bone morphogenetic protein 2 (BMP-2) is a potent osteoinductive growth factor stimulating osteoblasts differentiation *in vitro* and promoting bone formation *in vivo*. Although BMP-2 have been approved for spinal fusion or tibia non-unions therapies in humans (Lo KW *et al*., 2012, *Adv Drug Deliv Rev*), the use of BMP-2 still requires optimization regarding a dosage, delivery systems and release control. It is known that recombinant human BMP-2 (rhBMP-2) is a poor osteogenic inducer in human bone marrow mesenchymal stem cell (hMSC) cultures. Osyczka & Leboy (*Endocrinology* 2005) indicated culture strategies to increase rhBMP-2 osteogenic potential in hMSC. We now test the hypothesis that culturing hMSC in 3D scaffolds and perfusing culture media through the scaffold pores may also increase osteogenic effect of BMP-2 in hMSC cultures.

We seeded hMSC cells on gelatine-coated polyurethane scaffolds in serum-containing or serum-free medium with or without rhBMP-2 and applied a single perfusion flow session at day 7 (2.5 ml/min; 2 h). After 1 or 6hrs mRNA levels of osteogenic markers were assessed with Q-RT PCR followed by ALP activity assay. Static control (no perfusion in hMSC 3D cultures) was used as a reference. Our results indicate that in 3D hMSC cultures rhBMP-2 induces *BMP-2* mRNA expression more effectively when cells are maintained in serum-containing rather than in serum-free media. Perfusion of culture media in the presence of serum increases *C-FOS*, *RUNX-2* and *BMP-2* mRNA levels as well as ALP activity in hMSC compared to static cultures. In serum-free media we observed a slight increase in *BMP-2* mRNA expression under perfusion flow, which was less significant compared to serum-containing medium. However, when BMP treatment and perfusion are combined, increased mRNA levels of *BMP-2* are observed only in serum-free cultures compared to serum-containing ones. Furthermore, in serum-free cultures rhBMP-2 applied together with a single perfusion flow exhibits pro-osteoblastic activity upregulating osteoprotegrin, *OPG* mRNA contrary to serum-containing medium. RhBMP-2 applied in the presence of serum exhibits pro-osteoclastic activity, causing *RANK-L* mRNA upregulation.

P1.11

Endothelial cells derived from murine induced pluripotent stem cells – proof of functionality

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Induced pluripotent stem cells (iPSCs) can be useful for studies of patient-specific endothelial dysfunctions. Our aim was to characterize the cells obtained after endothelial differentiation of murine iPSCs.

Tail-tip fibroblasts were isolated from the wild type C57Bl6 mice and then used for generation of iPS cells by delivery of polycystronic lentiviral vector containing four reprogramming factors (Sox2, Klf4, c-Myc and Oct4) in a single stem cell cassette (kind gift from prof. Gustavo Mostoslavsky). Expression of pluripotency markers - Oct4, Sox2, SSEA1 was confirmed in iPSCs and pluripotency was proven by teratoma formation. Pluripotent iPS cells were subsequently differentiated toward endothelium. Shear stress culture was performed using Fiber Cell Systems technology.

Two distinct cell subpopulations were present in cultures of iPSC, defined by expression of either CD31 or CD34. Over three month differentiation period they shifted towards a CD34+CD31- phenotype with increasing percentage of $CD34^{+}Tie\text{-}2^{+}Sca\text{-}1^{+}$ cells, and with less frequent expression of c-Kit, CD133, and CD146. Tie-2 was found up-regulated also at RNA level, whereas KDR starts to be up-regulated at late time-points of differentiation. Importantly, immunocytochemical stainings revealed, that the cultured cells are positive for activated phospho-eNOS (at Ser1177) and von Willebrand factor. Moreover, eNOS functionality was confirmed by detection of nitric oxide production. The pro-inflammatory activation of iPS-derived cells was tested after TNF-α stimulation and increased cytokine production was measured with Luminex assay after 24 h. Direct angiogenic capacity of generated cells was tested *in vitro* and *in vivo*. No tube formation was found on matrigel *in vitro*, unless the iPS-derived cells were cultured together with mature endothelial cells (ECs). iPS-derived cells also incorporated in pre-existing structures formed by primary ECs. In the other angiogenic assay spheroid formation was delayed (6 days) in early time-points of differentiation, whereas after almost 4 months in culture the spheres were formed within 24h as in primary ECs. After embedding of spheroids in collagen the pronounced outgrowth of capillary network was observed, confirming the endothelial activity of the iPS-derived cells. *In vivo* the cells had rather paracrine effect than directly building blood vessels. Additionally, iPS-derived cells were cultured under shear stress conditions and up-regulation of shear-specific miRNAs was found, thus confirming iPS-ECs functionality.

To sum up, murine iPSCs can be differentiated into endothelial progenitor-like cells, with some immunophenotype features and angiogenic activities typical for endothelium.

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P1.12

Epigenetic regulator TRIM28/KAP1 controls reprogramming and self-renewal of mouse induced pluripotent stem cells

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Induced pluripotent stem (iPS) cells are highly similar to their respective embryo-derived embryonic stem (ES) cell counterparts with reference to morphology, function and gene expression pattern. However, iPS cells offer advantages over ES cells – they can be derived from somatic cells, such as fibroblasts, thus avoiding the ethical difficulties regarding the use of embryos as well as the problem of tissue rejection following transplantation in patients. Therefore, iPS cells provide a promising source of pluripotent cells for not only basic stem cell biology but also clinical cell-based therapies and disease modeling. Somatic cells can be reprogrammed to an embryonic-like state by forced over-expression of embryonic transcription factors that commonly involve: Oct4, Sox2, Klf4 and c-Myc. The major goal of this study is to determine the role of KAP1 protein in reprograming and self-renewal of mouse iPS cells. TRIM28/KAP1 is a cofactor of KRAB-containing zinc finger proteins (KRAB-ZFPs). These are known as the transcriptional repressors which act by formation of heterochromatin through histone modifications, HP1 binding and DNA methylation. In the first step, TRIM28/KAP1 expression in primary mouse embryonic fibroblasts (MEF) was silenced by a mean of lentiviral vectors carrying short hairpin RNA (shRNA) specific for TRIM28/KAP1. Next, the cells were reprogrammed to iPS cells using lentiviral vector carrying Oct4, Sox2, Klf4 and c-Myc transgenes. Immunofluorescence analysis of pluripotency markers and alkaline phosphatase staining revealed that TRIM28/KAP1 knockdown results in reduced number of emerging iPS colonies. Moreover, knockdown of TRIM28/KAP1 significantly abrogates the self-renewal of iPS cells. Therefore, we speculate that knockdown of TRIM28/KAP1 can also promote reprogramming of somatic cells, however emerging cells quickly lose the ability to self-renew and differentiate immediately. Our preliminary results suggests that repressive chromatin state imposed by TRIM28/KAP1 prohibits the reprogramming of somatic cells and self-renewal of iPS cells thus it helps to maintain identity of differentiated cells.

P1.13

Sdf-1 promotes migration of embryonic stem cells

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Muscle injury leads to the induction of the regeneration that occurs in two overlapping stages: myolysis and reconstruction. Myolysis phase includes active muscle degeneration and inflammation process which take place in the first few days post-injury. The reconstruction of damaged muscle relies on the pool of satellite cells (unipotent stem cells), which activated start to proliferate and differentiate into myoblasts that fuse to form myotubes and finally muscle fibers. Importantly, in patients suffering from dystrophies, reoccurring muscle degeneration and reconstruction leads to the exhaustion of satellite cells population. Thus, the search for the "replacement" stem cells is one of the crucial strategies in the therapy of such diseases as muscular dystrophies. Promising source of exogenous stem cells characterized by the myogenic potential are embryonic stem cells (ESCs). Previously, we showed that Sdf-1 (Stromal derived factor -1) induced migration of ESCs *in vitro*. In response to Sdf-1 treatment ESCs migrate and fuse with myoblasts more effectively than control cells. Thus, such treatment gives a chance to increase their myogenic potential, and to facilitate application of these cells in therapy. Presently, we examined which signal transduction pathways and adhesion as well as migration related mechanisms are activated in ESCs in response to Sdf-1 treatment *in vitro*. In our study we focused at focal adhesion kinase (FAK), Rac1, and Cdc42, and actin cytoskeleton. By silencing the Cxcr4 or Cxcr7 expression we also dissect the role of these two Sdf-1 receptor in the ESCs differentiation and migration.

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P1.14

Difficulties in evaluation of multidrug resistance proteins in endothelial progenitor cells differentiation

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Endothelial progenitor cells (EPCs) are a population of cells able to differentiate into mature endothelial cells and/or to support the processes of new blood vessels formation. Besides the contribution in physiological processes EPCs participate also in pathological angiogenesis that takes place in e.g. retinopathy or tumor neoangiogenesis.
Multidrug resistance proteins (mdr) expression is one of the main causes of tumor treatment failure. However, the main role played by these proteins (members of the ABC transporters superfamily) is the protection of cells from various toxic or harmful agents entering the cell; these proteins form a barrier in intestine, are part of the blood-brain barrier in brain endothelium, as well as participate in the hepatobiliary secretion and renal elimination of many drugs. In recent years there is growing evidence that mdr proteins are important for growth and survival of EPCs and play a key role in hematopoietic stem cell differentiation as well as maturation and differentiation of dendritic cells. Our preliminary data show that mdr proteins are present both in EPC isolated from human cord blood and in human immortalized EPC cell line HEPC-CB.1 (established by our team).
It seems possible that the expression of mdr proteins is changing during the EPCs differentiation process. To verify this hypothesis we have tested mdr expression on both cells isolated from umbilical cord blood and cells from the EPC cell line, before and after their differentiation. The protein evaluation with the help of flow cytometry and Western blotting techniques turned out to be insufficient because of very low amount of mdr proteins in EPCs. Therefore, mRNA for mdr proteins was tested in RT-PCR and real-time RT-PCR.
Expression of mRNA for different mdr proteins - MDR1, BCRP, MRP1, MDR3 and LRP was found at the beginning of differentiation and after differentiation process in EPCs isolated from cord blood. Similarly, mRNA for MDR1, BCRP, MRP1, MRP4 and MRP5 proteins were detected in EPC cell line HEPC-CB.1. During the differentiation process the expression of MDR1 and BCRP decreased, whereas the expression of MRP1 and MRP5 did not change or even increased for MRP4. The hypoxic conditions (1% O_2) slightly compensated the mRNA level for MDR1 and BCRP and generally did not change MRPs expression. mRNA for the examined mdr proteins were also detected in some of mature endothelial cell lines, both in normoxic and in hypoxic conditions.
Our results suggest that at least three of the examined mdr proteins (MDR1, BCRP and probably MRP4) are involved in the EPCs differentiation. We expect that conclusive results will be obtained after blocking the expression of these proteins.

Acknowledgements

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P1.15

TRIM28 is a barrienr during human somatic cells reprogramming

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Induced pluripotent stem cells (iPS) are derived from somatic cells through ectopic expression of few embryonic transcription factors. They have two unique properties: self-renewal and pluripotency, i.e. ability to differentiate to almost every cell of the adult organism. The broad potential of the iPS cells technology can be applied in regenerative medicine and/or basic research to study fundamental mechanisms of mammalian development. Results of the recent studies of human iPS cells revealed that epigenetic mechanisms are involved in the process of reprogramming of somatic cells into pluripotent state. KAP1/TRIM28 protein is one of the epigenetic regulators that regulate heterochromatin formation through histone modifications and DNA methylation. Here, we probed a potential role for KAP1/TRIM28 protein during reprogramming of human primary fibroblasts to iPS cells. In the first series of experiments, we achieved efficient and stable knockdown of KAP1/TRIM28 gene expression in human primary fibroblasts using lentiviral vectors carrying specific shRNAs. Next, the modified fibroblasts were transduced with lentiviral vector carrying Oct4, Sox2, Klf4 and c-Myc transgenes and reprogrammed to iPS cells. Pluripotent phenotype of the obtained iPS colonies was confirmed by analysis of surface embryonic markers expression using immunofluorescence and FACS. The number of emerging iPS colonies was significantly higher when KAP1/TRIM28 gene expression was specifically silenced during the middle stages of reprogramming process. Importantly, the colonies of pluripotent cells appeared much faster in the absence of KAP1/TRIM28 expression. Our preliminary results strongly suggest that knockdown of KAP1/TRIM28 gene in human primary fibroblasts facilitates formation of human iPS cells, suggesting a novel role for KAP1/TRIM28 during reprogramming of human somatic cells.

P1.16

Activation of bone marrow-derived very small embryonic-like stem cells by acute tissue injury and their regenerative potential *in vivo*

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Very small embryonic-like stem cells (VSELs) were identified as rare quiescent population of nonhematopoietic ($CD45^-/Lin^-$) cells sharing several pluripotency features, present in adult murine and human tissues including bone marrow (BM). It has been shown that murine BM-derived VSELs are mobilized into peripheral blood (PB) in tissue injury. Moreover, these primitive stem cells can participate in heart repair after injection into infarcted myocardium by enhancing tissue perfusion and angiogenesis.

In this study, we examined if acute tissue injury such as ischemic limb injury may stimulate both proliferation of quiescent VSELs in BM and their mobilization into PB. The other goal was to investigate regenerative potential of VSELs injected into ischemic tissue.

Thus, 4-6 week old C57BL/6 mice underwent a hind limb ischemia (LI) by permanent proximal femoral artery occlusion. Mice were administrated with bromodeoxyuridine (BrdU) and scarified at 2, 7, 14 and 28 days following ischemia. PB and BM were collected from individual animals including controls. The presence of proliferating ($BrdU^+$): VSELs ($Sca\text{-}1^+/Lin^-/CD45^-$), endothelial progenitor cells (EPCs; $Flk\text{-}1^+/Sca\text{-}1^+/Lin^-/CD45^{-/dim}$) and hematopoietic stem/progenitor cells (HSPCs; $Sca\text{-}1^+/Lin^-/CD45^+$) in PB and BM was evaluated by flow cytometry and ImageStream system. The expression of genes related to the presence of VSELs and EPCs was examined by real-time PCR. Moreover, we examined the change in expression of 53 angiogenesis-related proteins. Then, eGFP VSELs sorted from ischemic and non-ischemic mice by employing MoFlo XDP were injected at 2 days following LI into ischemic tissues. At 2, 7, 14 and 28 days post transplantation, blood flow were measured by Laser Doppler System. Paraffin-embedded ischemic tissue sections were analyzed for eGFP and PCNA *(Proliferating cell nuclear antigen)* co-expression by immunohistochemistry.

We found that the number of proliferating VSELs was significantly increased in BM of ischemic mice at 7d post injury. Similar results were obtained for EPCs. Elevated number of $BrdU^+$ VSELs was accompanied with change in expression of genes guiding their proliferation. Moreover, VSELs injected into ischemic tissues enhance tissue perfusion and angiogenesis. The data indicates vast impact of acute injury on activation of VSELs proliferation *in vivo* and possibility of their application in regenerative medicine.

P1.17

Osteogenic effects of media perfusion in 3D hMSC cultures depends on the stimulation regime and cell differentiation stage

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Human bone marrow-derived mesenchymal stem cells (hMSC) cultures are a major source of osteoblast progenitors and these cells have a great potential for bone tissue engineering applications. Modern strategies of bone tissue engineering require osteogenic cells, growth and differentiation factors, bone-imitating scaffolds and mechanical stimulation. The latter can be generated by perfusion of culture media through the scaffold pores in vitro and such mechanical stimulation can influence hMSC osteogenesis increasing their ALP activity, collagen deposition and extracellular matrix mineralization (Tanaka *et al.*, 2005, *Calcif Tissue Int* **76**: 261–271; Liu *et al.,* 2011, *Int J Biochem Cell Biol* **43**: 1591–1601; Mai *et al.*, 2013, *PLoS One* **11**: e61600). The aim of this work was to determine the osteogenic effects of a single perfusion flow session in 3D cultures of hMPC 32F cell line (Osyczka *et al.*, 2002, *Calcif Tissue Int* **71**: 447–458), depending on the stimulation regime and the cells differentiation stage.

HMSC were seeded on commercially available polyurethane 3D scaffolds coated with gelatine. Cells were treated with dexamethasone from day 1 culture and stimulated with media perfusion (2.5 ml/min. 2-h session). Perfusion was either repeated at culture days: 3, 5 and 7, or applied only once at either day 5, 7, 15 or 19. One hour after perfusion cells were analysed for gene expression (*C-FOS, BMP-2, OCN, OPN, COL1, BSP, FAK*), ALP activity, collagen deposition and matrix mineralization. Static conditions (no perfusion) were used as a reference.

We have also tested the effects of hMSC predifferentiation on their osteogenic response under media perfusion. To do so, hMSC were pretreated in 2D culture with dexamethasone (Dex) for 7 days and next transferred to the 3D scaffolds for a further 14-day culture. The effects of media perfusion in cells pretreated with Dex in 2D culture were compared to cells treated with Dex for 2 weeks in 3D culture.

HMSC stimulated with perfusion flow at the early culture stages (culture days 3 and 5) responded to perfusion flow by a higher expression of mechanoresponsive gene *C-FOS*, but at culture day 8 exhibited significantly lower ALP activity compared to either static control or cells stimulated at day 7. Perfusion flow applied at later culture stages (culture days 15 and 19) enhanced both collagen production and matrix mineralization. Pre-differentiation of cells in 2D culture enhanced their osteogenic response to perfusion flow compared to cells treated with Dex only in 3D culture, as shown by increased gene expression (*BMP-2, OPN, OCN, FAK*), collagen deposition and matrix mineralization.

We suggest that osteogenic differentiation of hMSC cultured on 3D scaffolds and stimulated with a short session of media perfusion can be enhanced depending on cells osteogenic differentiation stage and the application of media perfusion seems more effective in more differentiated osteoblasts.

P1.18

Characterization of mesenchymal stem/stromal cells (MSCs) isolated from human heart

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Mesenchymal stem/stromal cells (MSCs) have been among the most intensively studied cells in recent years. The main source of these cells is bone marrow or adipose tissue. Also, heart has its own pool of MSCs. The aim of the study was to characterize MSCs isolated from human hearts.

The material used in the study was obtained from human hearts removed during heart transplant surgery performed at the Silesian Center for Heart Diseases in Zabrze, Poland. Cells were isolated from hearts fragment and were then cultured in plastic dishes. Subpopulation $CD105^{+}CD34^{-}$ cells was sorted on FACS Aria III. Cells were cultured in IMDM medium supplemented with 20% serum and bFGF. Afterwards, cells were characterized by the presence of various surface antigens, proliferation rate, speed of senescence and ability to differentiate. Also, cytokines and growth factors released by these cells were analyzed.

The results suggest that, *in vitro,* MSCs isolated from human heart change phenotype. Expression of CD105 surface marker becomes increased whereas that of CD34 drops down. The CD105+CD34- population shows features characteristic for mesenchymal cells such as: presence of CD105, CD73, CD29 and lack of CD45, HLA-DR, Lin antigens and ability to differentiation into adipocytes and osteoblasts. MSCs release numerous proangiogenic and anti-inflammatory cytokines and growth factors, including a large amount of IL-6.

Our results confirm the assumption that human heart can be a novel source of specific MSCs. We isolated a population of heterogenous, plasticity cells from human hearts. Our results suggest that MSCs can be used to test various therapeutic strategies.

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P1.19

TRIM28 protein influences self-renewal of induced pluripotent stem cells (iPS cells) in a phosphorylation dependent manner

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Triptate motif protein 28 (TRIM28), also known as transcription intermediary factor-1β (TIF1β) or KRAB-associated protein 1 (KAP1), triggers the formation of heterochromatin by recruiting heterochromatin protein 1 (HP1), nucleosome remodeling complexes, histone modifying enzymes: histone deacetylase-containing complex NuRD and histone methyltransferase SETDB1. TRIM28 function is crucial in early development stages in mammals and influences reprogramming of somatic cells into pluripotent stem cells, by promoting pluripotency in a phosphorylation-dependent manner.

To investigate the role of TRIM28 phosphorylation in self-renewal of iPS cells and identify specific signaling pathway we constructed lentiviral vectors expressing different variants of TRIM28. Starting from vector carrying exogenous TRIM28 sequence and hairpin, silencing expression of endogenous TRIM28 gene (hFT28R-shT28) we obtained vector without hairpin (hFT28R-ctrl) enabling TRIM28 exo- and endogenous expression. We also constructed an produced vectors carrying hairpin and mutations in TRIM28 gene: Ser473D and Ser824D which mimic TRIM28 phosphorylation and Ser473A and Ser824A which are nonphosphorylable mutants.

To probe the role of TRIM28 phosphorylation in self renewal process iPS cells were transduced with obtained lentiviral vectors. IPS cells were cultured for at least 14 days under standard culture conditions. To obtain pure and homogenous population of cells expressing given TRIM28 variant, iPS cells were subjected to puromycin selection. Analysis of transduced iPS cells confirmed silencing of endogenous TRIM28 and expression of exogenous TRIM28 modifications.

Differences between cells expressing variations of TRIM28 were observed and analysed during experiment. We performed FACS analysis of surface pluripotency markers - SSEA4, Tra1-60, Tra1-81. Expression of pluripotency and differentiation markers was assayed by RT-qPCR analysis and IF staining.

The results of our studies suggest that TRIM28 provides a phosphorylation-dependent regulation of iPS cells self-renewal.

P1.20

Radiobiological effects of radiation on embryoid bodies

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Introduction: The main goal of this study was to investigate the influence of X-ray radiation on survival and differentiation capacity of embryoid bodies (EB) derived from human embryonic stem cells (hESC). Aim of study: The formation of EB has already been proven as a reliable and effective method used to induce hESC differentiation. However, the efficiency of EB formation is still inadequate to be implemented as important procedure of tissue engineering. One of the disadvantages during the process of EB differentiation is insufficient permeability of medium components through the exterior layer of cells, and unsynchronized formation of germ layers, what impairs further differentiation and diminishes its efficiency.

Materials and Methods: The methods of EB formation was described previously. After 10 days of culture, EB was irradiated in Gamma Cell 1000 at dose: 0.5 Gy, 1.0 Gy, and 2.0 Gy. The clonogenic assay was performed. Morphology of irradiated EB was observed every two days. To assess the influence of X-ray irradiation on the differentiation process of EB in the three germ layers immunofluorescence assay was performed.

Results: IR has changed differentiation process of EB in the three germ layers and clonogenic capacity.

Conclusions: There is no information about studies investigating the influence of X-ray radiation on embryoid bodies differentiation. Thus far, only the first mentioned analysis of project has been accomplished, giving still unexplained, but intriguing results. Irradiation may become a useful tool in differentiation of EB, however the influence of X-ray on differentiation process needs further studies.

P1.21

KRAB-ZFPs/KAP1 contribution to epigenetic mechanisms implicated in the maintenance of pluripotency and self-renewal in stem cells

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Zinc finger proteins containing Krüppel-associated box domain (KRAB-ZFPs) comprise the largest family of transcriptional repressors in mammalian genomes. KRAB domain interacts with KRAB-associated protein 1 (KAP1/TRIM28), a universal mediator of KRAB-ZFP function. KAP1/TRIM28 is an epigenetic modifier that mediates deposition of inactivating marks: trimethylation of lysine 9 at histone 3 and DNA methylation, which subsequently leads to heterochromatization of a target region. KAP1 has been shown to be involved in the maintenance of pluripotency and self-renewal of mouse embryonic stem cells (ESC). Also, our previous studies indicate that KAP1 depletion facilitates reprogramming of human somatic cells to induced pluripotent stem cells (iPSC). While the exact molecular mechanisms behind these phenomena remain unclear, we hypothesized that certain KRAB-ZFPs might be implicated in the KAP1/TRIM28-dependent regulatory network responsible for the maintenance of pluripotent state. In order to test this hypothesis, we first aimed to identify KRAB-ZFPs overexpressed in pluripotent stem cells. We took advantage of RNA-seq expression profiling that was performed on human fibroblasts (N=4) and derived from them iPS cell lines (N=8) that were generated in our lab. The differential expression data was ranked according to the p-value adjusted for multiple testing and a significance level was set at $p<0.05$. This analysis allowed identification of eight KRAB-ZFPs that became upregulated in the pluripotent stem cells in comparison to the parental fibroblasts. We further performed qRT-PCR analysis for selected KRAB-ZFPs in the same panel of fibroblasts and iPS cells and validated their overexpression in iPS cell lines. Importantly, we verified that the same KRAB-ZFPs are highly expressed in pluripotent stem cells using available data reported in the Gene Expression Atlas (EMBL-EBI). In conclusion, our results show that a number of KRAB-ZFPs is overexpressed in pluripotent stem cells compared to somatic cells. It is likely that these KRAB-ZFPs may afford an epigenetic switch that facilitates repression of differentiation markers, thus supporting pluripotency and self-renewal in stem cells and reprogrammed fibroblasts.

P1.22

Factors produced by adipose tissue mesenchymal stem cells as evaluated by Custom C Series Human Cytokine Antibody Array

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The aim of the study was to evaluate factors released by Mesenchymal Stem Cells (MSC), which may be partially responsible for their therapeutic effect, exerted by these cells after transplantation.

Introduction. MSC have been experimentally applied in different human pathologies to obtain regeneration of bone, cartilage, adipose tissue, myocardium and other tissues.

Adipose tissue-derived mesenchymal stem cells (atMSC), applied as autotransplant in experimental therapy of patients with chronic wounds, caused accelerated healing in 75% patients. We hypothesize that MSC, releasing soluble factors, may induce repair and regeneration by cytoprotection and/or neovascularization.

Methods. Cells isolated from patients fat tissue using CELLUTION 800 instrument were cultured in Dulbecco medium supplemented with 15% FCS, for 7–10 days. Factors secreted to culture medium were evaluated using protein membrane (Custom C-Series Human Cytokine Antibody Array, RayBiotech Inc.). Phenotype of cells was evaluated by flow cytometry (FACS Calibur).

Results. Cells prepared to autotransplantation were a mixture of leukocytes ($CD45^+$) ~ 20%, endothelial cells ($CD31^+$) ~ 20% and MSC ($CD73^+CD90^+CD34^+$) ~ 60%., After 7–10 days culture, more than 90% expressed MSC phenotype. In supernatants both directly (IL-8) and indirectly acting proangiogenic factors (GRO, MCP-1, G-CSF) were found.

Conclusion. During *in vitro* cultureatMSC secrete several soluble factors that may potentially enhance wound vascularization and healing. Presence of factors, selected from about 1000 proposed by manufacturer, may be demonstrated in culture supernatants using custom made Antibody Array.

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P1.23

The effect of human platelet lysate on proliferative and differentiation potential of mesenchymal stromal cells

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Clinical application of multipotent mesenchymal stromal cells (MSCs) requires the development of serum-free culture media for optimal cell growth and differentiation. Recently, human platelet lysate (PL) was proposed as a substitute alternative for xenogeneic fetal bovine serum (FBS) for expansion of MSCs.The aim of the present study was to evaluate proliferative and differentiation potential of MSCs when cultured in media containing fetal bovine serum (FBS) or human platelet lysate (PL).

Human adipose tissue-derived MSCs of the 4–6th passages were used in this study. For PL preparation whole blood from adult donors was fractionated by two-step centrifugation. After removal of the upper platelet poor plasma fraction obtained preparation (~ 3×10^{10} platelets/ml) was frozen and stored at –20°C. To align individual differences in content of growth factors PL aliquots (n=5) were mixed, centrifuged and filtered before use. Cell expansion was carried out in α-MEM, supplemented with either 10% FBS or 10% PL. Proliferative activity was determined by the increase in the number of cells within 7 days of culture. To evaluate colony-forming efficiency the number of colonies, their size and cellularity was determined. Induced adipogenic and osteogenic differentiation was determined by the accumulation of lipids or by expression of alkaline phosphatase.

When cultured for 7 days in the presence of 10% FBS the number of cells increased by 1.5–2.2 times. The proliferative activity of MSCs in media containing 10% PL was 3 times higher in comparison with the standard culture conditions. Assessment of colony-forming efficiency has shown the presence of 3 colony types with different packing density (dense, mixed and diffuse) and morphological characteristics of cells. In PL-containing media lager colonies of the same type were identified, but their ratio changed to dense and mixed types. In addition, the total number of colonies formed in PL was 1.6 times higher compared to FBS. MSCs after expansion in media with PL retained their ability for differentiation into adipogenic and osteogenic lineages. Moreover, the efficiency of their osteogenic differentiation was significantly (on 15%) higher than that of MSCs grown in FBS. Spontaneous differentiation in both groups was less than 4%.

The results demonstrate that platelet lysate is a promising natural alternative to xenogeneic serum, which can be used in the development of mesenchymal stromal cells culture technologies for regenerative medicine and tissue engineering.

P1.24

Change of surface markers in population of human mesenchymal stromal cell during differentiation to osteoblasts

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International guidelines for human mesenchymal stromal cells require that almost all cells in the population should have following surface markers: CD105 (endoglin), CD90 (Thy-1) and CD73 (ecto 5'-nucleotidase). These cells should also not express markers specific for hematopoetic stem cells. Mesenchymal stromal cell have a capacity to differentiate to osteoblasts and other cell lines too [1]. Therefore, it is important to be able to discriminate well between osteoblasts and mesenchymal stromal cells derived from bone marrow. The method widely accepted for separating two populations of cells is flow cytometry. However, for proper separation appropriate surface antigen should be selected. These antigens should allow to discriminate between cells of the two populations. Additionally, mesenchymal stromal cell obtained from a patient should be cultured in animal serum-free media in order to be used for transplantation.

In this work, the change of surface antigens characteristic for mesenchymal stromal cells cultured on commercial serum-free medium (MesenCult ™-XF, StemCell Technologies, Canada) was tested at various stages of osteoblast differentiation. For this purpose, the cells were labeled with specific antibodies and then analyzed by flow cytometry. Percentage of mesenchymal stromal cell containing CD90, CD105 and CD73 was compared with cells differentiated for 4, 7, 14 and 28 days to osteoblasts.

Results of the study revealed that vast majority of cells obtained from human bone marrow expressed surface markers specific for mesenchymal stromal cells following culture in serum-free medium and did not have surface markers specific for hematopoietic stem cells. During the process of osteogenic differentiation, there was a gradual reduction in the number of cells with BMSCs markers. The greatest decrease was noticed for the CD105 marker, which after 28 days of differentiation was found on less than 30% of the cells. For other markers the decline in their number was lower. After 28 days 95% and 85% of the cells were positive for CD73 and CD90, respectively. Thus, none of the tested markers characteristic for mesenchymal stromal cells cannot be used to fully discriminate these cells from osteoblasts.

Reference

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P1.25

Induced pluripotent stem cells as a model for diabetes investigation

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AIM: Mouse and human induced pluripotent stem cells (iPSCs) may represent a novel approach for modeling diabetes. Taking this into consideration, the aim of this study was to generate and evaluate differentiation potential of iPSCs from *lepdb/db* (db/db) mice, the model of diabetes type 2 as well as from patients with Maturity Onset Diabetes in the Young 3 (MODY3), which results from mutations in hepatocyte nucluear factor 1 alpha (*HNF1A*) gene.
METHODS AND RESULTS: Murine iPSCs were obtained by reprogramming of tail-tip fibroblasts from control as well as *db/db*animals whereas human iPSCs were generated from skin fibroblasts derived from healthy subjects or MODY3 patients. In both cases, STEMCCA lentiviral vectors with Oct3/4, Klf4, Sox2 and c-Myc within one expression cassette have been used to induce reprogramming process. Mature murine iPSCs colonies from both wild type and db/db mice were positive for markers of pluripotency: Oct3/4, Nanog, SSEA1, CDy1 and alkaline phosphatase. No differences in expression of Oct3/4, Nanog and Sox2 were detected between the genotypes, neither differences in formation of embryoid bodies (EBs), differentiation into cells of three germ layers *in vitro* nor in formation of teratomas in immunocompromised mice. Thus, the functional leptin signaling is not essential for establishing iPSCs. However, expression of heme oxygenase-1, angiopoietin-1 and proteoglycan 4 was lower in db/db iPSCs in comparison to control counterparts which may influence the differentiation capacity of iPSCs. Indeed, our preliminary results indicated impaired differentiation of db/db cells into endothelial progenitor-like cells expressing CD34 and Tie2 markers. Additionally, endothelial sprouting assay demonstrated decreased angiogenic potential of differentiated db/db iPSCs.
Human control and MODY3 iPSCs also expressed pluripotency markers: Oct3/4, SSEA4, TRA-1-60, TRA-1-81, formed EBs and differentiated into cells of three germ layers. Additionally, insulin expressing cells were obtained from those iPSCs with direct differentiation as well as embryoid bodies-mediated differentiation method.
CONCLUSIONS: Both db/db mouse and human MODY3 iPSCs are unique tools for modeling different types of diabetes. Differentiation of those cells into pancreatic β cells or endothelial cells may help to better understand the mechanisms responsible for defective insulin production or vascular dysfunction.

P1.26

Cartilage regeneration using human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) differentiated into chondrocytes

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The main objective of the study is to establish the efficient protocol for chondrogenic differentiation from pluripotent stem cells: human embryonic stem cells (hESCs) and human induced pluripotent stem cells (iPSCs) in order to create mature and fully functionalized chondrocytes, which will be able to repair of "full thickness" cartilage damage *in vivo*. During the differentiation of pluripotent stem cells into chondrocytes *in vitro* was considered a lot of factors such as the presence of growth factors (GFs) and their combination in medium, composition of basal medium and type of culture. The combination of the growth factors determines the direction of differentiation. In chondrogenic differentiation the mesodermal germ layer is crucial.In our study, several protocols with different GFs were performed. The most significant factors seems be TGF-β3 (Transforming Growth Factor – β3) and BMP-2 (Bone Morphogenetic Protein – 2) with regard to the required low concentration, the repetitive, and quick course of differentiation as well as the facility in scale-up culture. At first stage, the differentiation mainly *via* embryoid bodies was conducted. However, differentiation in high-density culture and pellet culture was also successful. After differentiation, cells were examined through set of analyses: morphologic assessment of cells, the flow cytometry analyses of surface antigens, immunohistochemistry and immunofluorescence analyses as well as molecular studies (RT-PCR, genome sequencing). Our results confirmed that the obtained cells reveal chondrogenic features. The expression of collagen type II, Sox9, Sox6, chondroitin sulfate proteoglycan, CXCR4, CD44, heparin sulfate proteoglycan was shown in differentiated cells. The next step was the confirmation of functionality of these cells in rabbit animal model *in vivo*. In preliminary animal studies the hES cells – derived chondrocytes were used in repair of "full thickness" cartilage damage, penetrating subchondral bone.Currently osteodegenerative diseases constitute a serious problem, basically efficient lifelong treatment is not available. The results of this study will provide a novel knowledge about culture and application of pluripotent stem cells in regenerative medicine. Our future plans includes the examination of several sort of available components as the most suitable scaffolds for differentiated cells for application in clinical practice.

P1.27

Effects of stable huntingtin silencing on Huntington disease phenotype in HD YAC128 iPSCs

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Huntington disease (HD) is an incurable brain disorder where neurons degenerate and die. In HD, a treatment option is a cell therapy using autologous genetically-corrected cells generated from induced pluripotent stem cells (iPSCs). However it is unclear if cells derived from iPSCs can be used as source of auto transplantation since the HD phenotype in iPSCs is not well-defined.
We established and characterized iPSCs lines derived from adult dermal fibroblasts of HD mouse model YAC128 by reprogramming with a piggyBac transposon OSKML vector. Resulting YAC128 iPS cells showed pluripotency both in *in vitro* and *in vivo* assays, expressed mutant huntingtin, and in pluripotent state developed deficits in oxidative stress and signaling pathways (Wnt/β-catenin and MAPK), known to be affected in HD. We then genetically modified the iPSCs lines by piggyBac transposon-based insertion of therapeutic constructs expressing anti-huntingtin siRNAs in miR-30 backbone. The stably expressed construct efficiently silenced mutant huntingtin expression in iPSCs-derived neurons.
In addition, we describe the effects of huntingtin silencing on the HD molecular phenotype in the iPSCs and, upon their differentiation towards neuronal lineage, in neural stem cells (NSCs) and derivative neurons. The generated mouse sh-iPSCs lines can be used for evaluation of autologous cell therapy approaches *in vivo*.

P1.28

Kinetics of retroelements activity during reprogramming of human somatic cells to induced pluripotent stem cells (iPSc)

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Nearly half of the human genome is composed of retroelements (REs). These mobile sequences are able to amplified themselves and move through the genome from one location to another. Such an acitivity may have a significant role in many diseases and affect genome evolution contributing to its diversity. Cells developed various defence mechanisms to protect genome integrity. Expression of retroelements is regulated mainly through epigenetic mechanisms, including histone modifications and DNA methylation, and in normal somatic cells the mobility of REs is blocked. During cellular reprogramming and formation of induced pluripotent stem cells (iPSc) through forced expression of selected transcription factors, chromatin structure undergoes intensive epigenetic remodelling. Here, we investigated the kinetics of retroelement activity during generation of iPSc. The study involved various molecular analyses, including high-throughput sequencing technology. Our results demonstrated that cellular reprogramming induces activation of a number of various subclasses of retroelements in human cells. In order to obtain insight into epigenetic mechanisms of the observed phenomena, ongoing research is focused on different epigenetic factors potentially engaged in mobile elements repression. Our research will contribute to better understanding of the molecular mechanisms that control REs during cellular reprogramming and are involved in host defence against these genomic parasites.

P1.29

α2β1 integrin-mediated mechanical signals during osteodifferentiation of cells isolated from umbilical cord

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Tissue formation and maintenance is regulated by various factors, including biological, physiological and physical signals transmitted between cells as well as originating from cell-matrix interactions. To gain a better understanding of the regulation of cellular differentiation by mechanical cues, we studied the influence of matrix stiffness on osteodifferentiation of two cell lineages characterized by different response: mesenchymal stromal/stem cells isolated from umbilical cord Wharton's jelly (UC-MSCs) with strong stiffness-depending response and bone-derived cells (BDCs) which do not react to the changes of support rigidity. We focused on the role of α2, α5 and β1 integrin subunits to address the question if there are any changes in integrins level and function during *in vitro* osteogenic differentiation of cells on different stiffness materials surfaces. Our results provide evidence that matrix rigidity affects osteogenic outcome of cells through mechanotransduction events that are mediated by α2 integrin subunit.

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P1.30

An in vitro interplay between mesenchymal stem cells and myoblasts - an introduction to co-transplantation.

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Urinary incontinence (IU) is a serious socio-medical problem which affects 4–14% of younger women and 12–35% of elder women. Cell therapy is emerging as an alternative method for the treatment of IU. Both, myoblasts and mesenchymal stem cells (MSCs) have been proposed as a material for transplantations into the urethral sphincter. Myoblasts possess high myogenic potential, but the survival of this population after transplantation is very limited (what was shown to be associated with the presence of oxidative stress). On the other hand, MSCs poorly differentiate into muscle tissue, but are believed to possess immunomodulatory properties. Therefore, our hypothesis is that the co-injection of mentioned populations can improve the efficacy of transplantation. Herein we evaluate an interaction between MSCs and myoblasts using in vitro assays. The experiments were performed on primary myoblasts and MSCs isolated from adult goats (n=8). Both populations were characterized in term of proliferative and differentiation potential. The susceptibility to oxidative stress was assessed using MTT assay. Mesenchymal stem cells appeared to be more resistant to oxidative stress than myoblasts - the significant decrease of metabolic activity comparing to the untreated control was achieved in the presence of 800 and 600 uM of hydrogen peroxide respectively. Whereas, to significantly impair the viability of both populations cultured together - 900 uM of hydrogen peroxide was needed. Tests performed on inserts revealed that the presence of MSCs affects significantly neither migration nor differentiation of myoblasts. To visualize direct interaction between MSCs and myoblasts, cells were labeled with different colors using membrane linker fluorochromes (PKH26 and PKH67). These experiments demonstrated that the cells from both populations communicate with each other by direct contact. Moreover, it was shown that MSCs are able to join myotubes formed by myoblasts and therefore can contribute muscle formation. In conclusion, obtained results support the hypothesis that co-transplantation of myoblasts and MSCs may be an attractive alternative for intrasphincteric injections.