Lectures

L11.1

Stem cell therapies — should we look for the holy grail?

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In cell therapy the pathway from bench to bedside has shown a quick adoption into clinical trials. Thus preclinical and clinical studies progress along parallel trajectories. A great effort is being undertaken to find out universal tools and strategies, which can be applied to pluripotent stem cells differentiated to distinct cell types. The major unresolved problem is obtaining the sufficient number of autologous pluripotent cells and the inherited association of pluripotency with the risk of teratoma formation. The second obstacle, easier to be solved, is a massive death of progenitor cells after transplantation. It has been convincingly shown that the cell survival can be improved by overexpression of cytoprotective genes. One of the candidates is heme oxygenase-1 (HO-1), the antioxidative, anti-apoptotic and anti-inflammatory enzyme. We have demonstrated that overexpression of HO-1 significantly improved the survival of murine proangiogenic progenitors (PPC, a population enriched in CD45-/Sca-1+/VEGFR2+ cells) after transplantation to the wounded skin of the syngeneic mice. Moreover, expression of HO-1 significantly improved the angiogenic response of PPC and mature endothelial cells to VEGF and SDF-1x, acting mostly through cGMP-dependent pathway and through facilitating the phosphorylation of VASP-1 protein. Accordingly, overexpression of HO-1 after adenoviral or AAV-mediated gene transfer, improved the skin wound healing in diabetic mice and enhanced revascularization of murine ischemic limbs. Thus, HO-1 overexpression can be beneficial in endothelial progenitors. However, the same strategy can lead to unexpected side-effects in muscle precursors. We demonstrated that HO-1-derived carbon monoxide in a cGMP-independent way inhibits the nuclear translocation of cEBPβ, decreases its binding to myoD promoter, and thereby blocks the expression of myoD, the master regulator of myogenesis. In consequence, HO-1 disturbs myoblast maturation and development of myotubes. Furthermore, we showed that transplantation of HO-1 overexpressing myoblasts to the murine gastrocnemius muscle may lead to formation of hyperplastic, undifferentiated tumors.

This example illustrates the importance of choosing the cell-specific approaches and indicates that cell therapies should be based on knowledge on cell-specific regulatory pathways.

L11.2

Pluripotent stem cell-derived cardiac and neural cells for toxicity testing and regenerative medicine

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Induced pluripotent stem cell (iPSC) technology directly reprogramming somatic cells to a pluripotent state allows the generation of patient-derived pluripotent cells for disease modeling, drug screening, toxicology tests and, ultimately, autologous cell-based therapies. Our team has created the first mouse and human iPSC cells in Hungary in 2009/2010 using lentiviral and transposon mediated transgenic methods, and protein-based reprogramming without genetic modifications. Cardiomyocytes (CMCs) derived from embryonic stem cells (ESC) or iPSCs are very valuable for drug testing. To enable large-scale culture of pluripotent cells we developed a scalable bioprocess that directs embryoid body (EB) formation in a fully controlled STLV (slow turning lateral vessel, Synthecon, Inc, Houston, TX, USA) bioreactor following inoculation with a single cell suspension of mouse ESCs. EBs generated by optimized STLV bioreactor were compared to static suspension culture (SSC) and classic hanging drop (HD) condition for efficiency of EB formation, EB yield, homogeneity of EB size, viability and apoptosis of cells. Cardiac specific gene expression was measured by Quantitative RT-PCR and fluorescent immunohistochemistry. Overall, our results demonstrated that EBs culture in STLV provides a superior technological platform for the large-scale generation of ES cell-derived cells and differentiation into CMCs for clinical and industrial applications. Transplantation of CMCs into animal models of ischemic injuries has been also started. Differentiation into neural precursor cells (NPCs) via EB formation resulted in high number and high purity NPCs in vitro by using the STLV system, described above. Further differentiation of NPCs has resulted in various mature neuronal cell types. Overall, the ESC/iPSC based methods are developing rapidly and novel cell based assays and clinical trials with the derived cardiac, neuronal, hepatic and other cell types demonstrate the validity of the approach for further research and product development.

Acknowledgements

Democratizing pluripotency: using iPS cells to model development, disease and therapy

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Reprogramming of somatic cells into embryonic-like pluripotent stem cells by overexpression of key transcription factors is a novel technology that promise to revolutionize human disease modeling and regenerative medicine. Induced pluripotent stem cells (iPSC) resemble human embryonic stem cells (hESC) in their morphology, growth and differentiation abilities. Both in vitro and in vivo they differentiate into mesoderm, endoderm and ectoderm lineages. iPSC have been already generated from individuals suffering from several human pathologies and their differentiation into relevant tissues allows for recapitulation of some of the disease features. Our laboratory is interested in endodermal-derived organ development, and more specifically in human iPSC in vitro differentiation into liver lineages and their potential application in disease modeling and therapy. We have previously developed a novel lentiviral vector for reprogramming of mouse and human somatic cells, based on a single polycistronic construct expressing all four reprogramming factors that allows for the most efficient reprogramming reported to date. Using this methodology, we have already generated more than a hundred normal and disease-specific human iPSC clones. In particular, we have generated iPSC from patients suffering from two monogenic liver diseases, amylodosis and hemochromatosis, and have shown their ability to differentiate into definitive endoderm followed by liver specification. Using this approach we aim at developing new methods to study the pathophysiology mechanisms underlying these diseases, as well as in the long-term, at correcting their respective mutations and using iPSC-derived liver cells for tissue regeneration.

Isolation, characterization and chondrogenesis of myofibroblasts from human Wharton’s jelly

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Osteoarthritis like other disorders of cartilage, represents crucial clinical problem. New medicinal products associated with gene therapy, cell therapy and tissue engineering offer new ways to treat many diseases, among others cartilage injuries. An important factor for the development of research in the field of tissue engineering is to find sources of cells that would be able to differentiate towards a specific cell type. In recent years, have shown remarkable plasticity myofibroblasts from the Wharton’s jelly of umbilical cord. The applicability of these cells for regenerative medicine, however, will depend on the exact characterization of the mechanisms that control their differentiation, so that it was possible to obtain cells with appropriate phenotype.

The aim of the studies was to isolate myofibroblasts from human Wharton’s jelly of umbilical cord by explant method and in vitro culture. Subsequently characterized parameters such as population doubling time, the survival of cryopreservation. Finally myofibroblasts was differentiated into chondrocytes with special medium containing transforming growth factor-β and dexamethasone in pellet culture system. Additionally examined the effect of time on chondrogenesis. Evaluation of the process of chondrogenesis based on the transcriptional activity of selected extracellular matrix genes. Real-time PCR based on SYBR GREEN methodology was applied to analyze quantitatively the transcript levels of collagen I, II, III and aggrecan.

To date studies have demonstrated that umbilical cord Wharton’s jelly can be a source of significant amounts of mesenchymal stem cells. Derived myofibroblasts showed for stem cell phenotype, show expression of surface markers characteristic of MSC and were characterized by a high proliferation rate in cell culture. Fetal bovine serum is an essential component of culture medium for myofibroblasts. Transforming growth factor-β and dexamethasone in various culture conditions stimulate myofibroblasts to increased gene expression of extracellular matrix characteristic for cartilage.

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Chitosan membranes for applications in ocular surgery

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The tissue engineering approach primarily aims at restoring damaged tissues or organs based on the transplantation of cells in combination with supportive matrices, biological factors and biomaterials. Scaffolds for tissue engineering fabricated using biomaterial technology play an important role in the creation of the cell environment, adhesion and proliferation. They usually should be porous, biocompatible, biodegradable or resorbable, non-toxic and also should have adequate mechanical strength. Degradation products also must be nontoxic and nonimmunogenic. The number of new biomaterials in medicine is steadily growing. The most widely used materials for scaffold preparation are polymers.

The aim of this work was to synthesize and to determine the properties of a polymeric material as a novel corneal epithelium substitute for ophthalmic surgery applications. A hydrogel scaffold in the form of a membrane was obtained from chitosan, a linear polysaccharide derived from chitin. It is recommended as a functional biomaterial because of its anti-bacterial, high adsorption and biocompatibility properties. Genipin, a naturally occurring and non-toxic agent, was used to crosslink chitosan. A membrane based on chitosan cross-linked by genipin containing proteins and other saccharides, seeded with keratinocyte stem cells was prepared as a novel corneal epithelium substitute.

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PPAR-gamma modulation influences PPC functions

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PPARγ nuclear receptor is a target for thiazolidinediones (TZD), the commonly used insulin sensitizers. Besides improving insulin action and normalising glycemia in diabetic patients TZD can also improve some functions of proangiogenic progenitor cells (PPC). Although activation of PPARγ was shown to be beneficial for PPC both in vitro and in vivo more experiments must be performed to demonstrate pathways involved in this actions. Our aim was to find expression changes in diabetic and healthy PPC and check how modulation of PPARγ activity affects PPC biology.

Experiments were performed on the 12-weeks old wild type (wt) and diabetic (db/db) mice. For in vivo tests PPC were characterised as the CD45-/KDR+/Sca-1+ cells, whereas in vitro studies bone marrow population enriched in PPC (approx. 600 times) was used. Importantly, percentage of CD45-/KDR+/Sca-1+ cells was significantly reduced by 40–80% in the bone marrow of diabetic mice in comparison to wt (in db/db only 0.0003% of all cells were PPC). Reduction related to blood glucose levels suggested that diabetes influences the number of PPC according to its severity. These defects was partially or fully reversed by an oral application of rosiglitazone 10 mg/kg, for 14 or 28 days, respectively. We observed a similar trend for CXCR4+ cells in bone marrow: a reduction in db/db animals and stimulation upon rosilitazone treatment. Although PPC number was reduced by 40% in blood of db/db mice the oralrosiglitazone administration did not change it. Furthermore, in in vitro assays, PPC isolated from db/db mice displayed impaired migratory and angiogenic potential. Migration of db/db cells was decreased by 50%, whereas ability to form tubes on matrigel by 40%. Importantly, in both tests functions of diabetic PPC were restored by rosiglitazone (10 μmol/L, 24 h) in a PPARγ-dependent manner.

Next, based on the PPC transcriptome analysis we found that proteoglycan 4 (PRG4) was one of the strongest downregulated gene in db/db cells (7-fold decrease). Since PRG4 stimulates proliferation and survival of hemangioblasts we tried to induce its expression in vitro by rosilitazone (10 mmol/L, 24 h) stimulation. We observed 2.5 fold PPARγ dependent induction in PRG4 expression both in wt and diabetic PPC. Thus, PPARγ is an important regulator of PPC biology and its induction may be used to improve the PPC functions.
P11.4
Orchestrating myogenesis: deregulation of heme oxygenase-1 leads to development of muscle derived tumors

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Myoblasts are precursor cells in myogenesis. Pathologies in their maturation may lead to development of skeletal muscles derived tumor called rhabdomyosarcoma. We investigated the role of heme oxygenase-1 (HO-1), the cytoprotective enzyme, on microRNAs biogenesis including class of myocyte specific miRNAs (miy-miRs) in terms of myoblast maturation and proliferation.

Heme oxygenase-1 (HO-1) is a heme degrading enzyme, which via products of its catalytic activity may stimulate many biologically important pathways. Via carbon monoxide, biliverdin, bilirubin and iron ions it influences apoptosis, cell survival, oxidative stress and angiogenesis. By influencing carbon monoxide it can silence EBPd nuclear translocation which leads to lower activity of Myod important factor influencing process of myogenesis.

Experiments were performed on murine immortalized myoblast cell line C2C12, stably transduced with retroviral vectors to express luciferase/GFP reporter genes and overexpress HO-1 and on human rhabdomyosarcoma cell lines of embryonal and alveolar origin. High level of HO-1 activity in C2C12 cells potently inhibited the myoblast differentiation, as indicated by reduced formation of myotubes and decreased expression of myogenic regulatory factors, such as Myod, myogenin and miy-miRs (miR-1, miR-133a, miR-133b, and miR-206). Accordingly, rhabdomyosarcoma cell lines of more aggressive phenotype were characterized by higher amounts of HO-1. Interestingly SDF-1, which levels are dependent on HO-1 exhibits effect on miy-miRs, similar to that of HO-1 overexpression. Also, we showed decreased levels of EBPd in HO-1 overexpressing cells or after CO stimulation. Overexpression of this protein leads to restoration of proper myogenesis. Higher amounts of HO-1 driven by viral vectors or from endogenous pathways lead to decreased levels of enzymes indispensable for miRNA processing such as DGCRI, Dicer or lin28. Following this finding we find that levels of total mature and precursor miRNAs are decreased in HO-1 overexpression. Finally, transfection with miy-miRs leads to reversal of HO-1 effect on myocytes differentiation. Thus, HO-1 is an important factor regulating microRNA biogenesis and influencing myocytes differentiation in possibly SDF-1 and miy-miRs dependent pathway.

P11.5
Reprogramming of somatic cells in embryonic environment

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Development is a process during which cells differentiate from a totipotent zygote to over 220 specialized cell types. For many years it was considered to be an irreversible process, but recent findings have shown, that it is possible to change or reverse cell fate. The first success in reprogramming was the creation Dolly the sheep, by somatic cell nuclear transfer. Recently other methods of reprogramming have been developed, however until now they are very inefficient and have limitations to be challenged before they could be used in medicine or industry.

Here we show a new method of reprogramming somatic cells by the influence of embryonic environment. Fibroblasts expressing RFP were introduced to morula stage embryo and then cultured for 2 days, until blastocyst stage. In about 30% of embryos introduced cells, integrated with the embryo. Blastocysts with integrated cells were fixed and stained for: Cdx2, Gata4 and Nanog, markers of blastocyst lineages. About 60% of cells, that were integrated, expressed these markers, which means that they were reprogrammed.

Some of the reprogrammed cells also expressed GFP, which was a marker of the host embryo, and that means, that they were a product of fusion of fibroblast and recipient blastomere. Further studies were carried out to discriminate the two mechanisms of reprogramming: by the influence of embryonic environment and by the fusion with host blastomeres. We show that both mechanisms are possible and almost equally probable (45 vs 55%).

To summarize we show, that it is possible to reprogram somatic cells both by the influence of embryonic environment and by fusion with blastomeres. Cells introduced to morula stage embryo can integrate with the embryo and become a part of chimaera as early as at the blastocyst stage.

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Adipose-derived stem cells (ADSCs) are the stem cells residing in the adipose tissue (AT). Two different models of ADSC medical applications have been exploited: (1) correction of tissue repair after mastectomy and/or radiotherapy in breast cancer patients, and (2) construction of bioimplants made of polycaprolactone scaffolds seeded with ADSC stimulated into osteogenic differentiation.

**Methods:** ADSCs were enzymatically (collagenase) isolated from the liposuction material. (1) Cells for the breast reconstruction (0.7–13.1 × 10^6) and/or AT were transplanted into breast region of 36 patients following surgery and radiotherapy. Patients were examined for early effects of AT or AT plus ADSC transplantation. (2) Cells for bioimplant construction were expanded in vitro until 3rd passage and loaded into honeycomb-like, 4 × 6 mm scaffolds (1.5 × 10^5 cells per scaffold). Results were measured by the extent of scaffold colonisation by ADSCs and the presence of osteogenic marker (Alizarin Red).

**Results:** ADSCs may be isolated from autologous AT (up to 2 × 10^6 cells per patient) without medical complications. Cells express CD29+, CD44+, CD71+, CD90+, CD105+, SH3+, CD31-, CD34-, CD45- markers, the minority of cells (up to 13%) express CD31+, CD34+, CD105+, CD45- phenotype, characteristic for the endothelial stem cells (ESC).

(1) Application of ADSC with AT for breast repair resulted in elimination of the transient inflammation episodes, occurring in 25% patients after AT — only treatment. (2) Analysis of ADSC-colonized scaffolds revealed scaffold colonization and the osteogenic differentiation of cells residing inside the scaffold structure. It may be concluded, that ADSCs are the promising candidates for stem cell treatment for the regenerative medicine purposes.

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