6th International Meeting

Stem Cell Network North Rhine-Westphalia

_April 5-6, 2011

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_Program

Tuesday, April 5th

8:00 - 9:00 am	_Registration
9:00 - 9:30 am	**_Opening of the Meeting** Svenja Schulze, Minister for Innovation, Science and Research of the State of North Rhine-Westphalia
9:30 - 11:00 am	**_Keynote Lectures, Chair: H. Schöler** John Dick, Toronto: "Stem cells and cancer: are they relevant?" Ludwig Siep, Münster: "Ethical questions concerning research with induced pluripotent stem cells"
11:00 - 11:30 am	_Coffee Break, Poster Session
11:30 - 12:30 pm	**_Session I: Disease Modeling, Chair: O. Brüstle** Kevin Eggan, Cambridge Juan Carlos Izpisúa Belmonte, La Jolla
12:30 - 2:00 pm	_Lunch Break, Poster Session
2:00 - 3:00 pm	**_Panel Discussion, Chair: L. Honnefelder** "International regulation of research translation"
3:00 - 3:30 pm	_Coffee Break, Poster Session
3:30 - 4:30 pm	**_Session II: Homing and Migration, Chair: P. Horn** Francisco Sanchez-Madrid, Madrid Steffen Massberg, München
4:30 - 6:30 pm	**_Poster Session**
7:00 - 10:30 pm	_Networking event at the Zeche Zollverein (bus transport provided)

Wednesday, April 6th

8:00 – 9:00 am _Registration

9:00 - 10:00 am _Session III: Cell Fate Specification in Stem Cells of Model Organisms, Chair: C. Kaltschmidt
Erika Matunis, Baltimore
Elly Tanaka, Dresden

10:00 - 11:00 am _Session IV: Non-human Primate Pluripotent Stem Cells, Chair: S. Schlatt
Erika Sasaki, Kawasaki
Shoukrat Mitalipov, Portland

11:00 - 11:45 am _Coffee Break, Poster Session

11:45 am - 12:45 pm _Session V: Disease Modelling - Cancer Chair: T. Dittmar
Andreas Trumpp, Heidelberg
Eric Holland, New York

12:45 - 2:00 pm _Lunch Break, Poster Session

2:00 - 3:00 pm _Session VI: Transdifferentiation, Chair: M. Zenke
Thomas Braun, Bad Nauheim
Thomas Graf, Barcelona

3:00 - 3:30 pm _Coffee Break

3:30 - 4:30 pm _Session VII: Niche, Hypoxia and Metabolism, Chair: P. Wernet
Norman Iscove, Toronto
Michael Cross, Leipzig

4:30 - 5:30 pm _Session VIII: From Stem Cells to Tissues, Chair: A. Faissner
Arnold Kriegstein, San Francisco
Pierre Vanderhaeghen, Brussels

5:30 pm _Poster Awarding, Closing Remarks

_Register

Bioengineering & Biomaterials

Chromatin & Epigenetics

Development & Regeneration

Disease Modeling

Ethical, Legal & Social Issues

Homing & Migration

Induction & Maintenance of Pluripotency

Metabolism

Niche & Microenvironment

Somatic & Cancer Stem Cells

Transcriptional Regulation & Micro-RNAs

Company Profiles

Contact & Disclaimer

Bioengineering & Biomaterials

Proliferation and differentiation of human mesenchymal stem cells on microstructured polystyrene surfaces

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Recent studies have revealed direct effects of matrix properties, such as chemistry, elasticity or macro-, micro- and nano-structured topography on the proliferation, orientation and differentiation of human mesenchymal stem cells (hMSC). Considering the given potential of matrix structure effects to direct hMSC differentiation, our study was comprised to investigate effects of grooved polystyrene surfaces, developed by the Fraunhofer IPT Aachen, on hMSC behavior. The main focus was to investigate possible effects of the grooves on hMSC adhesion, proliferation and differentiation towards osteoblasts and adipocytes as well as morphological changes in cell shape and cytoskeleton, in comparison to non-grooved controls. Therefore, hMSC were seeded on polystyrene surfaces containing plane control squares as well as structured squares in varying micrometer ranged combinations of width and distance. After 21 days Bodipy staining, Alizarin red staining and immunohistochemistry were conducted and evaluated quantitatively for differences in differentiation and cellular orientation. Our results indicate that proliferation could be enhanced significantly by a polystyrene groove combination of 3 x 1.5 x 5 µm (width, distance, depth). Specific groove combinations promoted differentiation towards the adipogenic or osteogenic lineage while a variety of groove combinations enhanced cell elongation and therefore are considered to induce cytoskeletal stress which is a potential factor to accelerate stem cell differentiation. To further elucidate the impact of polystyrene grooved surfaces and to define the most suitable groove design for increasing or promoting hMSC differentiation, additional experiments with other donors, screening for molecular markers and gene expression analysis on specific groove combinations have to be performed. Microstructured surfaces could improve cell expansion and could contribute to standardized and optimized cell therapies. Moreover, developing microstructured biomaterial surfaces is especially attractive for tissue engineering, because such structures can be used to direct cell function.

Keywords: mesenchymal stem cells, biomaterials, topography
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Towards a 3D biomaterial-based expansion strategy for cord blood-derived CD34+ cells

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In order to use umbilical cord blood (CB) for autologous and allogeneic HSC transplantation in adult patients, several strategies have been studied during the last decade to expand umbilical CB-derived CD34+ cells. *In vivo*, hematopoietic stem cells (HSC) are located in the bone marrow (BM) niche, a very complex 3D microenvironment, which is composed of stromal cells, extracellular matrix molecules and soluble factors, such as growth factors and cytokines. In fact, the role of several cytokines in the context of HSC ex vivo expansion was strongly investigated over the past years. Finding the ideal cytokine-cocktail combination that can achieve good coast-effectiveness rates both in research and clinics remains an outstanding task. In this context, we have previously optimized HSC culture conditions based on the use of a six factor-cocktail including SCF, TPO, Heparin, FGF-1, Angptl5 and IGFBP2 (Ferreira, MV et al., in preparation; Walenda et al., Experimental Hematology 2011). Our current project aims at analyzing the compatibility of several polymers from our established biomaterial bank (Neuss et al., Biomaterials 2008) when used to expand HSC. Basic compatibility testing (live-dead straining) was performed on a panel of different materials comprising seventeen degradable biopolymers and degradable/non-degradable synthetic polymers. Further screenings of compatible materials (Polyvinylidenfluorid (PVDF), Texin, Resomer LR704, Resomer RG503, Polycaprolacton (PCL), Fibrin) include simultaneous analysis of cell viability, cytotoxicity and apoptosis of HSC cultured during 7 days on the different surfaces. Proliferation was also analyzed according to different approaches: i) a method based on the ability of viable cells to reduce resazurin into resorufin was used to perform proliferation curves for HSC cultured over a period of 17 days in each of the materials, either using a standard culture cocktail or the previously described optimized cocktail, ii) a method based on the ability of cells to incorporate, and equally distribute to its progeny, carboxyfluorescein diacetate N-succinimidyl ester (CFSE), was used to monitor cell division of freshly isolated CD34+ cells after an expansion period of 7 days into the materials.Immunophenotypic analysis (flow cytometry), clonogenic analysis (colony-forming unit assay) and morphology (SEM) were also evaluated for a 7-day period of ex vivo expansion of HSC on the compatible materials. Hematopoietic reconstitution of NOD/SCID mice is currently under assessment.Premilinary data show highest expansion potential of HSC expanded in fibrin gels for 7 days, in comparison to other biomaterials. In the future our project aims at developing nano-structured biomaterial scaffolds that can support CB-HSC expansion either directly or in MSC/CB-HSC cocultures.

Keywords: hematopoietic stem cells; biomaterials; expansion; transplantation
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Uptake and intracellular fate of nanosilver within human mesenchymal stem cells

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INTRODUCTION: Silver nanoparticles (Ag-NP) were introduced in different areas, e.g. in the food, electronic industry, and medical applications. Distinct silver compounds are clinically used to reduce skin infections in the treatment of burns and as an antibacterial supplement on implants or bone substitute materials. Depending on the coating technique and during resorption of a biomaterial, Ag-NP may come into close contact to tissue including human mesenchymal stem cells (hMSC). Despite the well known antimicrobial activity of Ag-NP, there is a serious lack of information concerning their biological effects on human cells. One major toxicological concern is a possible uptake of Ag-NP into cells. Therefore, the purpose of this study was to analyze the endocytosis mechanisms into hMSC, the intracellular fate and the exocytosis of Ag-NP. MATERIALS: The Ag-NP (ø < 80nm) were synthesized by reduction with glucose and then stabilized by the polymer polyvinylpyrrolidone. For analyzing the uptake mechanism the cells were treated with inhibitors of endocytosis, for 30 min (chlorpromazine hyrochloride, nystatin, wortmannin). After this pretreatment, Ag-NP were added to the cells and incubated for further 60 min. Focused Ion Beam system (FIB), energy-dispersive X-ray spectroscopy, flow cytometry (FACS) and light microscopy were used to visualize and prove the uptake and exocytosis of Ag-NP. For colocalization studies cells were treated with Ag-NP for 24h and then labeled with specific organelle fluorescent probes. For analyzing the exocytosis, the treated cells were washed and incubated with fresh medium for different time points. RESULTS: Light and FIB/SEM micrographs revealed that Ag-NP were taken up into the cells and occurred as agglomerates in the perinuclear region. The intracellular nature of the silver agglomerates within single hMSC was confirmed by EDX. The quantitative occurrence of intracellular silver agglomerates was correlated (r=0,998) with the amount of the added Ag-NP. Internalized Ag-NP were not detected associated with the Golgi complex, endoplasmic reticulum or nucleus after 24 h incubation but were mainly detected in the endo-lysosomal areas. Quantitative determination of the uptake of Ag-NP by flow cytometry revealed a concentration-dependent uptake of the particles which was significantly inhibited by chlorpromazine and wortmannin but not by nystatin which indicated a clathrin-dependent endocytosis and macropinocytosis as primary uptake mechanisms. Prolonged cell culture periods (72 h) in the absence of extracellular Ag-NP demonstrated that the intracellular occurrence of agglomerates decreased. Interestingly, the decrease in nanoparticles was almost completely inhibited when the medium was depleted of serum. DISCUSSION: Ag-NP enter the hMSC as nanoparticulate material and agglomerate inside the perinuclear region associated with the endo-lysosomal cell compartment. The invasion route is comparable to nanoparticles of similar size but different composition. Due to particle size and intracellular agglomeration it is not likely that particles enter the cell nucleus, the Golgi complex or the endoplasmic reticulum. These intracellular agglomerates were exocytosed after prolonged time points. Interestingly, the decrease

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of exocytosis when the medium was depleted of serum indicating that at least the discharge of particles or ions from vesicles or other pathways at the cell surface membrane requires carrier molecules outside the cells.

These data also indicate that ingested Ag-NP undergo intracellular trafficking and subsequently are released by exocytosis which might lead to further tissue dissemination.

Keywords: human mesenchymal stem cells; endocytosis; silver nanoparticles; intracellular fate; exocytosis
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Rapid manufacturing of porous nickel-titanium as a carrier for human mesenchymal stem cells

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Nickel-titanium shape memory alloys (NiTi-SMA) are of biomedical interest due to an unusual range of pure elastic deformability with an elastic modulus closer to that of bone than any other metallic or ceramic material. Newly developed porous NiTi, produced by Selective Laser Melting (SLM) is currently analysed as carrier material for human mesenchymal stem cells (hMSC). SLM enables the production of highly complex and tailor-made implants for patients on the basis of CT data. Such implants could be used for the reconstruction of skull face or pelvis. HMSC are a promising cell type for regenerative medicine and tissue engineering due to their ability to support the regeneration of critical size bone defects. Loading of porous SLM-NiTi implants with autologous hMSC may enhance bone in growth and healing of critical bone defects. The purpose of the study was to assess whether porous SLM-NiTi is a suitable carrier for hMSC. Therefore, specimens varying in porosity and orientation, were fabricated via SLM using two different laser beam diameters. HMSC were cultured for 8 days on those NiTi specimens. Cell viability was analysed using a two-colour fluorescence staining. Cell morphology and surface topography was analysed by scanning electron microscopy (SEM). Size and number of powder particles, released during cleaning of the specimens, were quantitatively analysed using a laser based particle analyzer. Viable cells were detected on all specimens, after 8 d cell culture. Cell morphology and surface topology was dependent on the orientation of the specimens during SLM production. By reducing the diameter of the laser beam from 128 to 61 µm, the mean particle release could be decreased significantly. The SLM-NiTi samples are suitable carriers for hMSC. Nevertheless, before carrying out *in vivo* studies the manufacturing process must be optimized in order to reduce the particle release.

Keywords: NiTi; Selective Laser Melting; hMSC; biocompatibility
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Germline-derived pluripotent stem cells for tissue engineering

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Tissue engineering represents a particulary attractive approach in regenerative medicine. Stem cells with unlimited differentiation potential, like embryonic stem cells (ESC), induced pluripotent stem (iPS) cells and the recently described germline-derived pluripotent stem (gPS) cells, are an appealing cell source for tissue engineering. gPS cells allow (i) generation of pluripotent cells without ethical concern and (ii) autologous patient-specific applications. Furthermore, gPS cells express pluripotency associated transcription factors and thus are readily generated without transfection of exogenous factors employing specific culture conditions. Biomaterials can inhibit, support or induce proliferation and differentiation of stem cells. Therefore, we intend to identify polymers which maintain self-renewal and differentiation potential of gPS cells. Finding a material which allows feeder-free expansion of gPS cells would be a benefit of this project. A panel of degradable and non-degradable polymers of an established biomaterial bank is used in this study [Neuss et al., Biomaterials, 2008]. Identification of cytocompatible gPS cell/biomaterial-combinations requires analyses of several parameters including morphology, vitality, cytotoxicity, apoptosis, proliferation and differentiation potential. Flow cytometry is used to characterize the survival of gPS cells on biomaterials. gPS cells are Oct4-eGFP and loose eGFP fluorescence upon differentiation [Ko et al., Cell Stem Cell, 2009]. Thus, fluorescence microscopy facilitates the identification of general differentiation on biomaterials. gPS cells are pluripotent as visualized by their Oct4-eGFP fluorescence and shown by expression of the pluripotency factors Nanog and Sox2 by immunhistochemistry. Flow cytometry analysis, viability assay and proliferation assay have shown that gPS cells efficiently adhere to and are viable on synthetic polymers, like LR704 (poly(L-lactic-D,L-lactic acid), PTFE (polytetrafluorethylene) and PVDF (poly(vinylidene fluoride) and on gelatine-coated TCPS (tissue culture polystyrene). Taken together, there are only single materials (no complete material groups) which could be identified as inappropriate surfaces for gPS cells, like polyesteramides and Fibrin. Gelatine-coated TCPS and LR704 could be used as an alternative for feeder-free expansion of gPS cells. Differentiation experiments with embryoid bodies on LR704, PTFE, PVDF and gelatine-coated TCPS were performed. gPS cells on LR704 showed beating areas after 11 days - indicating a cardiomyogenic differentiation – while embryoid bodies of gPS cells on gelatine-coated TCPS and PVDF started beating 1-2 days later. On PTFE, there were no beating areas detectable during a period of 21 days. Cardiomyogenic differentiation of gPS cells on LR704 has to be proved by electrophysiological measurements, transmission electron microscopy and expression of Connexin 43, Troponin T and sarcomeric α-Actinin by immunohistochemistry.

Keywords: germline-derived pluripotent stem cells, biomaterials, Resomer LR704, feeder-free expansion, cardiomyogenic differentiation
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Murine induced pluripotent stem cells for cardiac tissue engineering

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Objective - Cardiac cells can be differentiated from stem cells, i.e. embryonic stem cells (ESCs) and induced pluripotent stem (iPS) cells. For future reconstructive therapies, contamination of stem cell-derived artificial cardiac tissue with undifferentiated cells bears the risk of tumor formation after transplantation. Therefore we implemented a cardiac-specific selection system. Material and Methods - iPS cells derived from OG2 mice were used, expressing GFP under control of the Oct 3/4 promotor. Cells were transfected by microporation with vectors conferring Zeocin resistance under control of the cardiac specific alpha myosin heavy chain promoter and a constitutively expressed hygromycin resistance. Integration and resistance gene expression was confirmed by RT-PCR. Cardiac differentiation was induced using the hanging drop method. Zeocin selection was performed starting on d7 of differentiation culture for 10 days. Selected cells were analyzed by immunofluorescence staining, semi-quantitative RT-PCR for pluripotency and cardiac markers, and by multi electrode array (MEA) for drug responsiveness. Selected cells were used for the generation of Bioartificial Cardiac Tissue (BCT) with different amounts of murine embryonic fibroblasts (MEFs, inactivated by irradiation). BCTs were sectioned and stained for cardiac and pluripotency markers. Results and Conclusion - iPS cells were transfected successfully with our vectors and selected for integration of plasmids using hygromycin. Clonal populations were used for further investigations. Cardiac differentiation resulted in up to 100% beating EBs on d7 of differentiation, with the onset of alpha MHC expression on day 6 and considerable numbers of GFP-positive undifferentiated cells (~10%). In contrast, after Zeocin selection no residual GFP-positive undifferentiated cells were observed. Immunofluorescence staining of selected cells seeded as single cells and cultivated for 3 days without selection pressure revealed >99% cTnT+ cells. The calculated cardiomyocyte yield was a ratio of one CM per iPS cell. Electrical activity of selected CMs was drug responsive (lidocain, quinidine, isoproterenol). Using selected CMs for 3D tissue engineering led to spontaneous and synchronous beating of the whole BCT with forces of up to 410 µN. Histology of the tissues showed well aligned CMs connected via Cx43 gap junctions. Neither Oct4-GFP, nor other pluripotency markers were detected in these tissue constructs. In conclusion, iPS cell-derived cardiomyocytes were efficiently selected and used for cardiac tissue engineering purposes.

Keywords: pluripotent stem cells; cardiomyocytes; tissue engineering
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Integrating quality control in the automated generation of induced pluripotent stem cells

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The Standard assays for pluripotency of induced pluripotent stem (iPS) cells, like the differentiation into teratomas in immunodeficient mice, are time consuming and expensive. Therefore quality control in automated iPS cell generation facilities require novel types of significantly faster assays which are suitable for high throughput processing. We recently introduced Pluritest, a bioinformatics assay for pluripotency in human cells, based on gene expression profiles. Recent publications show large variations in pluripotent stem cells derived by heterogeneous protocols even within a single colony, with potential, yet unknown, implications for potential applications . Pluritest assesses pluripotency by looking at the consistent expression of core pluripotency genes as well as on the genome-wide scale. However, even array-based assays are too disruptive and not fast enough for continuous in-line quality control of high throughput production processes. Therefore, we are going to develop novel types of heterogeneous assays for quality of human iPS cells based on fluorescence and phase contrast microscopy. We relate imaging results to Pluritest results in order to identify reliable surrogate markers for genome wide gene expression by a combination of staining for a limited number of surface markers and morphological information automatically extracted from image analysis.

Keywords: stem cell; quality control; biomarker; microscopy; gene expression
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Micro-scale human liver equivalents for chip based biomedical research

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The modeling of human liver tissue has gained much attention recently. Due to increasing rates of discovery of new pharmaceuticals and developments in regulatory requirements, such as the European REACH program on registration, evaluation, authorization and restriction of chemicals, high numbers of experiments on toxicology and pharmacology profiles of chemicals are required. These experiments were performed traditionally in animals, but due to ethic concerns and different metabolic profiles of animal and human cells, new test systems are being developed. Primary human cell culture systems are considered as a promising alternative to those trials. However, current testing protocols using primary human cells in two-dimensional cell culture configurations are not equivalent to animal trials and of limited biological relevance, as they do not adequately mimic the tree-dimensional environment of the liver. Therefore, we are developing a characterized, bioreactor based, human three-dimensional *in vitro* tissue culture test system to overcome these obstacles. The microscope slide-sized reactor system contains micro-fluidic channels that enable a continuous, defined supply of culture medium to two cell culture compartments. As media and waste reservoirs are integrated into the chip, no external feeding is required. Due to physiological flow conditions, long term cultures of micro-scale liver organoids inside the cell culture compartments are possible. The organoids are engineered in a way to represent the smallest functional unit of the human liver, the hepatic lobule. Cell numbers and extracellular matrix components are adjusted to the *in vivo* situation. By providing defined three-dimensional structures within the cell compartments, co-cultures of hepatocytes and non-parenchymal cells organize to emulate specific liver functions. The integration of bioanalytic systems like live cell imaging by two-photon microscopy and the monitoring of metabolic products of the cells results in a multifunctional platform for basic biomedical research.

Keywords: tissue engineernig, liver, micro-scale organoids
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Effect of topography on the differentiation of human embryonic stem cells to neuronal lineage in the nano-micro range

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Human embryonic stem cells (hESC) are pluripotent cells, which have the ability to differentiate into three germ layers. Differentiation of hESC towards neuronal lineage leads great potential for disease modeling and drug screening. As extracellular matrix (ECM) *in vivo* comprises topography in the nanoscale, topography could also influence stem cell differentiation in the nano-micro range *in vitro*. In this work, we use photolithography to fabricate nano/micro structure on PDMS substrates with finely controlled dimensions and different pitches (ridge/groove width: 700, 2000, 10000nm, height: 350nm). hESC-NCL3 were seeded on these structured substrates, and cultured for 10 days. We found that nuclei of hESC-NCL3 were aligned and elongated in the direction of nano/micro structure, while distributing randomly in flat PDMS control. This contact guidance significantly increased when the cells were cultured on the substrates with smaller pitch. Gene expression profiling by RT-PCR and immunostaining showed significant up-regulation of neuronal markers such as β-Tubulin III in structured PDMS. While 700nm and 104nm structures were effective, the 2000nm structure induced highest neuronal marker expression. Even though the elongations of cytoskeleton and nucleus were correlated with such changes in gene and protein expression, the mechanism of topography induced differentiation remains unclear. This study demonstrate the significance of topography, especially the pitch width of structured surfaces in directing differentiation of human embryonic stem cells towards neuronal lineage, suggesting potential application of topography in clinical regenerative medicine.

Keywords: nanostructures, embryonic stem cells, differentiation
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The use of mesenchymal stem cells on collagen/ LMW HA gels for intervertebral disc regeneration

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Intervertebral disc (IVD) degeneration is one of the main causes of lower back pain. Current treatment frequently requires complex surgery, which often does not restore full mobility, or leads to the degeneration of neighbouring IVDs. Development of injectable extracellular matrix-based hydrogels offers an opportunity for minimally invasive treatment of IVD degeneration. These materials should have mechanical properties similar to intact IVD and they can be used in combination with cells to promote the regeneration of IVD. The inner part of IVD, the nucleus pulposus (NP), is populated by chondrocyte-like cells. Here we present the use of bone marrow-derived mesenchymal stem cells (MSCs), stimulated to differentiate along the chondrogenic lineage, for tissue engineering of IVD. Semi-interpenetrating networks made of collagen/ low molecular weight hyaluronic acid (LMW HA) were loaded with gelatin microspheres. MSCs were embedded in this hydrogel and chondrogenesis was induced with TGF-β3. MSCs grown on collagen / LMW HA gel for two weeks produced an increased amount of extracellular matrix (ECM) in the presence of TGF-β3 as demonstrated by histochemical staining (alcian blue, Movat's pentachrome). ECM formation by MSCs was comparable to that of nasal chondrocytes cultured in the same material. In summary, the use of injectable ECM-based hydrogels in combination with autologous or allogenic MSCs and possibly growth factors offers a promising strategy in IVD tissue engineering.

The funding was provided by EU FP7 project Disc Regeneration (Grant No. NMP3-LA-2008-213904).

Keywords: biomaterials, mesenchymal stem cells, chondrocytes, intervertebral disc
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Generation of human hair follicle equivalents *in vitro* for substance testing

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Even though appendages in human skin only cover about 0,1% of the total skin surface area, the hair follicle is an important factor with regard to the penetration of substances through the skin. It further represents a long-term reservoir of topically applied substances. As the hair follicle is highly vascular and surrounded by dendritic cells, it supports penetration of substances through the skin and into the systemic circulation. Since 2009 all cutaneous resorption experiments of the cosmetic industry in the EU have to be performed *in vitro*. Thus animal trials are forbidden and skin equivalents are used to determine the risks, benefits, and consequences of substance penetration into living tissue. However, currently available skin models are lacking important cell types such as Langerhans, Merckel and endothelial cells. Furthermore, most of these models are mere epidermal constructs and even full thickness skin equivalents do not include skin appendages (e.g. sweat glands and hair follicles). Thus, we are aiming at developing procedures to integrate de novo generated human hair follicles into full thickness human skin equivalents. Hair follicles have the ability to grow fast and to quickly regenerate following trauma. Now, our group was able to regenerate hair follicle formation *in vitro*. These microfollicles highly resembled *in vivo* hair follicles and were capable of producing hair shafts. Electron microscopy and histological analysis revealed that follicular keratinocytes, melanocytes, and fibroblasts assembled and created the characteristic hair follicle architecture. We are further optimising the procedure of hair follicle generation and are moreover integrating the *in vitro* generated microfollicles into skin equivalents. This will allow us to evaluate the role of hair follicles in dermal substance transport mechanisms for cosmetic and pharmaceutical products.

Keywords: hair; regeneration; microfollicle; substance testing; de novo
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Chromatin & Epigenetics

RNF12 activates XIST and is essential for X inactivation

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X chromosome inactivation (XCI) in placental mammals ensures dosage compensation of X-linked genes between the sexes(1). During early female development, or upon differentiation of female ES cells, one of the two X chromosomes becomes inactive, and this transcriptional silencing is maintained during cell propagation. Xist, Tsix and Xite are non-coding X-linked regulatory genes, which play crucial roles in XCI. Xist RNA accumulates on the future inactive X chromosome and initiates silencing in cis. Tsix and Xite down-regulate Xist transcription, acting against XCI. In the initial phase of XCI, a counting process determines the number of X chromosomes per diploid genome(2). Each X has a specific probability to be inactivated in a stochastic mechanism. These results predict the presence of an X-linked, trans-acting activator of the XCI process(3). Recently, we identified X-linked Rnf12 to encode an activator of XCI(4). The encoded RNF12 (RLIM) is an E3 ubiquitin protein ligase, known to be involved in down-regulation of LIM homeodomain transcription factors. Transgenic copies of Rnf12 were able to induce XCI of the single X chromosome in male ES cells and XCI of both X chromosomes in an increased number of female ES cells(4). RNF12 activates XCI in a dose-dependent manner, as higher transgenic expression correlated with higher expression of Xist. Here, we demonstrate that X-encoded RNF12 acts as an activator in trans, and activates the Xist promoter. We found that RNF12 does not regulate XCI through Tsix or through the pluripotency factor binding site located in Xist intron 1, which both have been described to be involved in negative regulation of Xist(5). In addition, we found that the Xist intron 1 region is not required for suppression of Xist in undifferentiated ES cells. Analysis of female Rnf12-/- ES cells showed that RNF12 is essential for initiation of XCI, and may exclusively regulate Xist. Ongoing generation and analysis of heterozygous compound mutants of Rnf12 and other regulatory elements of the X inactivation center reinforce the dominant role of RNF12 in the regulation of XCI. We conclude that RNF12 is a key factor in up-regulation of Xist transcription in differentiating mouse ES cells, thereby leading to initiation of XCI.

Keywords: X chromosome inactivation; Rnf12/RLIM; stochastic; epigenetics; ES cells;
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Beating clusters and cardiomyocytes derived from induced pluripotent stem (iPS) cells retain some genetic signature of somatic cells used for reprogramming

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Reprogramming of somatic cells to induced pluripotent stem (iPS) cells offers great potential for *in vitro* disease modeling, drug development and regenerative medicine. Although comparisons between iPS cells and conventional ES cells revealed that they have highly similar growth characteristics, gene expression profiles, epigenetic status and developmental potential, recent comprehensive analyses of various undifferentiated iPS and ES cell lines demonstrated that iPS cells may not be perfectly identical to conventional ES cells at molecular level and carry epigenetic memory of their somatic cell origin. In the present study, we found that microdissected human iPS and ES cell-derived beating clusters (BCs) and pure genetically selected murine ES and iPS cell-derived cardiomyocytes (CMs) have very similar global transcriptional profiles and differ in expression of only few percent of present transcripts. These minor gene expression differences did not appear to detrimentally affect human and murine iPS cell derived CMs because both iPS-CMs and ES-CMs had indistinguishable structural and functional properties. Interestingly, 47% of genes that were upregulated in human undifferentiated iPS cells compared to ES cells remained enriched in human iPS-BCs in iPS-BC versus ES-BC comparison. Similarly, 18 genes that were found to be overexpressed in murine iPS cells versus ES cells also remained overexpressed in iPS cell-derived EBs and pure CMs as compared to their murine ES cell counterparts. Most of these genes were also found to be expressed at similar levels in fibroblasts used for reprogramming and partially overlapped with those reported by others to be differentially expressed between undifferentiated iPS and ES cells. These data suggest that iPS-CMs, although functionally indistinguishable from ES-CMs, may not be perfectly identical to ES-CMs at the molecular level and retain some genetic signature of iPS cells and somatic cells. This signature may represent genes that are refractory to epigenetic modifications not only in the course of reprogramming but also differentiation. Whether these genes may affect physiological properties of iPS-CMs not revealed in this study remains to be elucidated.

Keywords: iPS cells, reprogramming, cardiomyocytes, beating clusters and epigenetic memory
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Specific age-associated DNA methylation changes in human dermal fibroblasts

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Epigenetic modifications of cytosine residues in the DNA play a critical role for cellular differentiation and potentially also for aging. In mesenchymal stromal cells (MSC) from human bone marrow we have previously demonstrated age-associated methylation changes at specific CpG-sites of developmental genes. In continuation of this work, we have now isolated human dermal fibroblasts from young (<23 years) and elderly donors (>60 years) for comparison of their DNA methylation profiles using the Infinium HumanMethylation27 assay. In contrast to MSC, fibroblasts could not be induced towards adipogenic, osteogenic and chondrogenic lineage and this is reflected by highly significant differences between the two cell types: 766 CpG sites were hyper-methylated and 752 CpG sites were hypo-methylated in fibroblasts in comparison to MSC. Strikingly, global DNA methylation profiles of fibroblasts from the same dermal region clustered closely together indicating that fibroblasts maintain positional memory even after *in vitro* culture. 75 CpG sites were more than 15% differentially methylated in fibroblasts upon aging. Remarkable hyper-methylation was observed in the aged group within the INK4A/ARF/INK4b locus and this was validated by pyrosequencing. Age-associated DNA methylation changes were related in fibroblasts and MSC but they were often regulated in opposite directions between the two cell types. In contrast, long-term culture associated changes were very consistent in fibroblasts and MSC. Epigenetic modifications at specific CpG sites support the notion that aging represents a coordinated developmental mechanism that seems to be regulated in a cell type specific manner.

Keywords: fibroblasts; MSC; DNA methylation; aging; positional memory
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Chromosome centromers positioning during differentiation of human myoblasts in *in vitro* culture

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For several years skeletal muscle stem cells were investigated in order to be applied in post infarction heart. Numerous clinical trials were performed however their results did not meet expectations. Differences in received data drew attention to myoblasts features particularly to inability for skeletal myoblast – cardiomyocyte coupling. Therefore, despite of the number of desired features (like resistance to ischemic conditions or ability to conduct electromechanical signals and muscle contraction), there is a need to investigate myoblast subpopulations with different expression of transcription factors that determine their fate. Moreover, until now there is no defined markers that could diversify among these cell subsets. To provide such information there must be more data about particular genes that drive stem cell differentiation upon the chromosome activation and positioning in the mentioned cells. A great effort to find such characteristics currently is being performed. Moreover, little is known about nuclear architecture and its changes during cell differentiation process, as intranuclear movement of particular chromosome during global expression profile modification is a common phenomena. The aim of the study was to estimate alterations in selected centromers topology during in vitro propagation and differentiation of human skeletal muscle satellite cells. Investigated chromosomes were chosen concerning genomic localization of genes crucial in cell differentiation and in the maintenance of the stemness of satellite cells. Analysis was performed by two- and subsequently three-dimensional fluorescent in situ hybridization (2D & 3D FISH). For classification purposes, nucleus was divided in 5 regions based on radial distance starting from the central point. So far the most crucial chromosomes, including 1, 7, 11, 12, 16 and 17, were examined by 2D FISH in two time points –undifferentiated, newly isolated primary skeletal muscle stem cells and at stage of myotubes after differentiation in vitro. From this comparison the changes of 1, 11 and 12 chromosome centromere positions were observed. In all cases movement was directed towards peripheral side of nucleus. Summarizing, a first step in examination of chromosome territory alterations during skeletal muscle stem cells in vitro propagation and differentiation was performed. Several observed changes will probably be significant at final conclusions, however they must be carefully investigated by 3D FISH analysis. The following step will be an investigation of selected territory by the whole chromosome painting, as well as exact localization of crucial genes by gene-specific probes during observed nuclear architecture repositioning.

Keywords: human myoblasts, chromosome positioning, stem cells differentiation
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EED-dependent global and local chromatin states in pluripotent and differentiating mouse embryonic stem cells

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Polycomb group proteins are repressors of developmental genes in pluripotent embryonic stem (ES) cells. The Polycomb repressive complex 2 (PRC2) consists of the core subunits EZH2, SUZ12 and EED. It catalyzes the methylation of histone H3 at lysine 27 (H3K27me3). The deletion of EED leads to the loss of EZH2 and SUZ12 protein expression and results in a global loss of H3K27 methylation accompanied by derepression of development-associated genes. In contrast, pluripotency-associated genes are expressed at comparable levels in wildtype (wt) and EED knockout (KO) ES cells. We aim to understand the role of EED/PRC2 in the organization of nuclear chromatin architecture. Further we address the question of what function EED/PRC2 has during *in vitro* ES cell differentiation. By micrococcal nuclease digestion we observed similar chromatin accessibility in wt and EED KO ES cells, indicating that the absence of H3K27 methylation does not result in a globally open chromatin configuration. In agreement with this, FRAP analyses revealed similar linker histone dynamics in wt and EED KO ES cell nuclei. However, by fluorescence microscopy we observed that DAPI-/H3K9me3-positive heterochromatic foci were more defined, larger in size and less frequent in EED KO ES cells as compared to wt ES cells. Further, global H3K9me3 levels were slightly decreased, pointing towards a PRC2-mediated crosstalk between the enzymes that set or read H3K9 and H3K27 methylation marks. Currently we perform genome-wide H3K9me3 ChIP-seq analyses using wt and EED KO ES cell chromatin preparations. These data will provide further information on the genomic regions affected by PRC2-dependent H3K9 methylation. To investigate the role of PRC2 during ES cell differentiation, we induced embryoid body (EB) formation of wt and EED KO ES cells in the absence of LIF. The analyses revealed severe defects in EED KO ES cell differentiation, as 1) the cells failed to form contracting cardiomyocytes, 2) cell populations retained undifferentiated cells with colony formation potential after an extendend period of culture under differentiation conditions and 3) expression levels of pluripotency-associated genes remained high in EED KO cells during this period. In addition, ChIP-analyses revealed that in differentiated wt cells the repressive H3K27me3 mark became prevalent at pluripotency-associated genes while in EED KO cells it remained absent. These data further support the notion that PRC2 is involved the silencing of pluripotency-associated genes during ES cell differentiation, besides its well-established function in the repression of development-associated genes in self-renewing pluripotent cells. Together, we report that H3K9me3-enriched constitutive heterochromatin is reorganized in the nuclei of EED KO ES cells and that silencing of pluripotency-associated genes is negatively affected in differentiating EED KO ES cells.

Keywords: Polycomb; pluripotency; ES cells; EED; chromatin
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Germ cell specific gene Stra8 has an impact on the pluripotency network and functions as an epigenetic regulator

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Stra8 gene (Stimulated by retinoic acid gene 8) was first found by Bouillet et al. in 1995 when they identified retinoic acid-responsive genes in P19 embryonal carcinoma cells. It was shown, that the Stra8 expression is restricted to premeiotic spermatogonia in adult male mice and to premeiotic follicles in adult female mice. Very recently it was shown, that Stra8 protein in embryonic stem cells is not exclusively localized in cytoplasm but can shuttle between it and the nucleus. Here, we show that Stra8 has also an impact on the pluripotency network. First, we performed expression analysis with Northern Blot and RT-PCR and showed that the expression of Stra8 is limited to pluripotent cell lines while it is downregulated during differentiation of the cells. Immunhistochemistry showed the presence of Stra8-protein in all embryonic stages until blastocyst. During embryogenesis Stra8 shows both cytoplasmic and nuclear localization. Stra8 protein sequence includes a putative helix-loop-helix DNA binding domain, suggesting a role in transcriptional regulation. siRNA- knockdown of Stra8 caused upregulation of the known pluripotency-regulating genes, while siRNA-knockdown of Oct4 resulted in downregulation of Stra8, providing an indication that Stra8 might play a role in the Oct4 dependent pluripotency pathway. Stable overexpression of Stra8 in ESCs resulted in the expected downregulation of the pluripotency network while the differentiation markers are upregulated. To elucidate the molecular function of Stra8, we performed a yeast two-hybrid screen and identified Setd8 and Arid4b as interacting partners in pluripotent cells and subsequently validated their interaction with Stra8 by in-vitro and in-vivo studies. Since both Setd8 and Arid4b are well-known chromatin remodelling proteins involved in the regulation of genomic imprinting, we performed chromatin immunoprecipitation (ChIP)-PCR assays and real-time RT-PCRs to quantify histone modifications and gene expression at imprinting control regions (ICRs) upon Stra8 down-regulation and Stra8 overexpression in embryonic stem cells (ESCs). Altered Stra8 expression levels were found to strongly affect ICR-associated histone methylation marks and mRNA levels of imprinted genes at ICRs. Finally, we also analyzed the adult Stra8 knockout mouse testis cross-sections and found the loss/depletion of Setd8 mediated histone 4 lysine 20 mono-methylation (H4K20me1) from pre-meiotic germ cells, whereas other histone modifications were unaffected. Collectively, our results demonstrate that Stra8 functions as an epigenetic regulator and play a role in the maintenance and regulation of pluripotency.

Keywords: Stra8, pluripotency, chromatin, imprinting, ES cells
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Sirt1 regulates neural stem cell homeostasis and plays a role in the response to cellular stress

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Our previous work identified Sirt1, a member of the histone deacetylase family regulated by NAD , as a master switch that determines the fate of neural progenitors under mild oxidative stress conditions (Prozorovski et al., Nat Cell Biol 2008; 10: 385). In this context, the association of Sirt1 with the Notch downstream effector molecule Hes1 is required for the suppression of neuronal fate. These findings prompted us to investigate whether Sirt1 may be a candidate molecule regulating the maintenance of neural stem cells. The examination of cultured forebrain neural stem cells isolated from Sirt1-deficient mice revealed a decreased self-renewal capacity. Analyzing gene expression, we found a dysbalance of signalling pathways relevant for "stemness". Furthermore, we show an altered response of Sirt1-deficient cells towards different cellular stress conditions. We propose that Sirt1 plays a role in both the suppression of differentiation and the resistance against metabolic and oxidative stress in cultured neural stem cells.

Keywords: Sirt1; HDAC; self-renewal; neural, stress
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The landscape of cellular aging: long-term culture of mesenchymal stem cells is associated with specific changes in their DNA-methylation profile

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Mesenchymal stem cells (MSC) can only be culture expanded for a limited time until they reach a senescent state, the so called "Hayflick-limit", which is accompanied by growth arrest, cell enlargement and reduced differentiation capacity. Therefore, culture associated changes in MSC may hamper their therapeutic potential. In this study, we have analyzed genetic and epigenetic changes upon long-term culture of MSC from human adipose tissue. The fibroblastoid colony-forming unit (CFU-f) frequency and the differentiation potential were already significantly impaired within the initial passages. Relevant chromosomal aberrations were not detected by karyotyping and SNP-microarrays and this supports the notion that human MSC possess relatively little genomic instability. Subsequently, we have compared DNA-methylation profiles with the Infinium HumanMethylation27 Bead Array and the profiles differed markedly in MSC derived from adipose tissue and bone marrow, indicating that the epigenetic makeup of MSC is highly dependent on the tissue of origin. Highly consistent senescence-associated modifications at specific CpG sites, especially in developmental genes, occurred already within the early expansion phase (between the passages 5 and 10). Remarkably, these DNA-methylation changes are highly enriched in regions with repressive histone modifications such as trimethylation of H3K9, H3K27 and EZH2 targets. These results indicate that cellular aging is not just a random accumulation of cellular defects, but that it is precisely regulated by epigenetic means in the course of culture expansion. Specific modifications in the DNA-methylation profile can be used to track cellular aging of MSC.

Keywords: cellular aging; mesenchymal stem cells; DNA-methylation; long-term culture; senescence; epigenetic
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Positive cofactor 4 coordinates self-renewal and genome stability in mES cells

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Mouse embryonic stem cells (mESC) are derived from inner cell mass of blastocysts. They hold great potential for regenerative medicine and cell replacement based therapy. Recent advances from induced pluripotent stem cells (iPSC) have substantially strengthened our knowledge about celluar reprogamming and shed light on future perspective of tissue formation and transdifferentiation. Despite the sparkling success of iPSC induction, they simultaneously raised several serious concerns. Among those, potential genome instability and certain inbuilt tumorigenecity of the reprogrammed cells have received increasing attentions. Tumor suppressor p53, as the guardian of the genome, plays a significant role in preventing the accumulation of DNA damage from geno- and celluar toxicity, which is detrimental to the cells. Of particular importance for mESC, such damages will be inherited by the daughter cells when ES cells differentiate into different cell types representing individual germ layer, which will finally devastate the fundement of pluripotency and self-renewal. In the current study, we established a doxycycline-inducible and reversible shRNA expression system for specific candidate genes knockdown as well as for a limited screening. We identified Sub1, also known as positive cofactor 4, as one of the major players in mESC proliferation as well as in the maintenance of genome stability of mESC. Although depleting of PC4 is leathal for postimplantation embryos during early development, PC4 knockdown mES cells retain the similar phenotype as wild-type mESC with a much slower self-renewal rate. Downregulation of PC4 enhanced the binding of p53 to its consensus sequences both *in vitro* and *in vivo*. As a consequence of the enhanced binding, p53 program was activated *in vivo*, which can be blocked by several kinase inhibitors, indicating the complex nature of different signalling transduction pathways, which eventually lead to p53 activation. Further investigation at the molecular level unravelled that both proliferation and genome stability depend on the single-stranded DNA binding activity of PC4, which was reveally by the study of its crystal structure, indicating an inherent linkage to global transcription. We hereby demonstrate an interwinding network using mES cells as an ex vivo model, while highlighting PC4 as a coordinator among global transcription, self-renewal, and genome stability.

Keywords: embryonic stem cells; p53; genome stability; postive-cofactor 4; self-renewal
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Development & Regeneration

Parthenogenetic human ES cells form functional neurons

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Pluripotent parthenogenetic embryonic stem cells (ESC) from several species including human have been successfully generated and differentiated into neural and other cell types (Revazova et al., 2007). In addition, successful gene therapy was recently demonstrated in a mouse model of beta-thalassemia by allele selection using parthenogenetic ESCs (Eckardt et al., 2011). This genetic correction strategy without gene targeting is potentially applicable to any dominant disease. Therefore, parthenogenetic human embryonic stem cells (hpESCs) are promising sources for basic research to study the influence of maternal and paternal genomes on organ development, and potentially for future cell replacement strategies. Here, we investigated the *in vitro* differentiation capability of hpESCs into neural stem cells (hpNSCs) and further into neural subtypes including midbrain TH-positive neurons. In parallel we examined functional aspects and the imprinting status of parthenogenetic cells. hpESCs were *in vitro* differentiated into hpNSCs as previously described for normal, biparental human ESCs (Koch et al, 2009). Expression analyses showed that hpESC-derived NSCs expressed the neural stem cell markers Nestin, Pax6, Sox1, Sox2 and Vimentin and lost expression of the pluripotency markers Oct4 and Nanog both at RNA and protein levels. Upon spontaneous *in vitro* differentiation for 28 days hpNSCs generated neural subtypes with specific neural morphology and expression of neural/glial markers such as Tuj1, Map2, Tau, Synapsin and GFAP. hpNSCs were also responsive to instructive regionalization cues known to induce regional phenotypes such as midbrain TH-positive neurons. After 29 days *in vitro*, hpNSC-derived neurons had a mature resting membrane potential, immature spike pattern and showed typical neuronal Na /K currents. Expression analysis of imprinted brain genes in hpESCs and in hpNSCs as compared to biparental human ESCs and NSCs revealed that the maternal-specific imprinting is by and large maintained upon differentiation. In summary, despite the lack of a paternal genome, hpESC-derived NSCs show normal neural differentiation potential and could therefore be therapeutically relevant for future cellular replacement strategies.

Keywords: parthenogenetic human embryonic stem cells; in vitro differentiation; neural stem cells
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Reprogramming the embryonic cell cycle after somatic cell nuclear transfer

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Establishment of pluri- or totipotency after somatic cell nuclear transfer (NT) into oocytes does not only require reprogramming of gene expression to an embryo-like pattern but also pacing of the cell cycle from quiescence to an embryonic cleavage sequence, characterized by precisely timed rapid succession of DNA replication and mitotic divisions without significant G1 or G2 phases. Failing adaptation of NT embryos to the embryonic cell cycle may lay the foundation for their reported lower cell counts at the blastocyst stage. We demonstrate that mouse embryos cloned by NT have an extended duration of the second (2- to 4-cell stage; plus two hours) and third (4- to 8-cell stage; plus seven hours) cell cycles. Extended G1 and S phases account for the prolongation of the second cleavage stage; strikingly, extension of the third cleavage stage is due to an average 5.5 additional hours spent in S phase. Additionally, in contrast to the highly synchronous fertilized embryos, time-lapse cinematography showed that cloned counterparts varied substantially in the time taken to complete the third cell cycles. Interestingly, the lengths of the 2- and the 4-cell stage were inversely proportional for many NT embryos. Moreover, time-lapse using embryos expressing a histone-GFP transgene showed similar rates of chaotic mitoses for fertilized and NT embryos, but NT embryos were considerably less tolerant to mitotic chaos than fertilized counterparts. Although NT embryos cleaving to 4-cell stage first formed blastocysts about 1.8-fold as efficiently as slower counterparts, efficiency of ESC derivation or fetal formation was similar, indicating negligible post-blastocyst-stage functional consequences of preimplantation cell cycle pace. We hypothesized that if incomplete reprogramming – i.e. failed activation of essential embryonic genes during embryonic genome activation – delayed NT embryo cleavage, cloned embryos with shorter cell cycles should show a more fertilized-embryo-like gene expression pattern. However, microarray analysis exposed highly similar mRNA patterns of fast versus slow-cleaving NT embryos, suggesting that delayed cell cycle progression is transcription-independent. Because culture environment has considerable impact on developmental success of NT embryos, metabolic restraints may delay their cell cycle progression. To determine whether cloned and fertilized embryos have different demands on the culture medium, we determined their amino acid turnover at different preimplantation stages. Strikingly, NT embryos consume less arginine from the culture medium than fertilized embryos throughout preimplantation development. By supplementing the culture medium with arginine – the only cellular precursor for synthesis of cell-cycle-driving and preimplantation-development-regulating nitric oxide (NO) – progression of NT embryos to the 8-cell stage was accelerated by 4.3 hours, and development to the blastocyst stage was enhanced. We conclude that low cell counts suffered by NT embryos are caused by a cell cycle delay, which is reprogramming-independent and can be partially reverted by increasing arginine supply.

Keywords: cell cycle; embryo; nuclear transfer; reprogramming; SCNT
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A novel and efficient system for the generation of neural progenitor cells from human pluripotent stem cells using STEMdiffTM neural products

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Induction of neuroectoderm from human pluripotent stem cells (hPSCs), which include human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), is the first step in differentiation protocols used to produce neural progenitor cells (NPCs) and differentiated cell types of the CNS such as neurons, astrocytes and oligodendrocytes. High purity and availability of large quantities of these cells is a prerequisite for specialized assays such as drug screening or toxicity testing as well as for therapeutic approaches in neurodegenerative disease. To date, numerous neural induction protocols have been developed using different base media and the addition of cytokines and small molecules. However, variability in the production of pure neural cell populations is still a major hurdle common to all protocols. In order to reduce variability in neural induction protocols used for hPSCs, we have developed two novel reagents: 1) STEMdiffTM Neural Induction Medium (STEMdiffTM NIM) for the rapid and efficient induction of NPCs from hPSCs, and 2) STEMdiffTM Neural Rosette Selection Reagent (STEMdiffTM NRSR) for the selective isolation of "neural rosettes" – an *in vitro* structure commonly accepted as an indicator of early NPC induction. In addition, we have optimized protocols which combined the use of STEMdiffTM NIM and STEMdiffTM NRSR with AggreWell™800, which is a system to generate uniform sized aggregates in the first step of the neural induction protocol. Aggregates containing 10,000 cells each were formed from hPSCs in STEMdiffTM NIM using the AggreWell™800 procedure and the aggregates were maintained within the AggreWell™800 plates for 5 days with daily ½ media changes. Aggregates were then harvested from AggreWell™800 plates and plated onto Poly-L-Ornithine/laminin (PLO/L) coated plates in STEMdiffTM NIM for a total of 6 days to allow aggregate attachment and neural rosette formation within the colonies. Cultures were then treated with STEMdiffTM NRSR to selectively detach rosette structures and these were re-plated onto PLO/L coated plates containing STEMdiffTM NIM to generate adherent cultures. Cells were cultured until they reached ~ 90-100% confluence before they were enzymatically dissociated and the resulting cell suspensions were either sub-cultured multiple times in a proliferation media (under development) or subjected to differentiation conditions. Our results showed that after 2 days in STEMdiffTM NIM, neural aggregates had attached and formed colonies with 90-100% of the colonies containing neural rosettes. Characterization of neural rosette structures by immunocytochemistry revealed that the cells within neural rosettes co-expressed the early neural markers, Pax6, Sox1 and Nestin, thus confirming that the first step in the aggregate-based protocol for neural induction using our STEMdiffTM NIM rapidly produced early-stage NPCs. We found that these early-stage NPCs had the ability to re-form secondary neural rosette structures when sub-cultured in proliferation medium, indicating that these cells maintained development properties of early neuroectoderm. This work describes a highly efficient system for the production of pure populations of early-stage NPCs from hPSCs that is fast and potentially scale-able to achieve higher cell yields. Future work is on-going to further characterize these early-stage NPCs, and to examine their potential to be patterned using developmental cues as well as their ability to differentiate into the three mature cell types of the CNS.

Keywords: pluripotent; stem cells; neural induction; neural progenitor cells; AggreWellTM

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The development of the limbal region in chicken embryos

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Introduction: The transition zone from corneal to conjunctival epithelium is the origin of the putative limbal stem cells (LSC). Beneath the palisades of Vogt a niche for somatic limbal stem cells is assumed. Renewing and repairing of the cornea epithelium starts zentripedal from this limbal region. The aim of this study was to clarify the possibility to compare human and chicken limbal development with the help of typical epithelial differentiation and stem cell markers in order to develop a new model system. Material and Methods: Eyes from chicken embryos from stage HH 25 to HH 41 were stained with a standard protocol (HE and PAS) and by immune fluorescence against epithelial differentiation markers and limbal stem cell markers. Results: The morphology of the anterior segments of a chicken eye is very similar to the developing human eye. Chicken embryos allow a precise definition of the age so that the developmental sage can be classified. The region of the embryonic limbus and fornix represent clusters of epithelial cells in a very early stadium. Whereas the p63 staining is predominant on the epithelial structures is that for Oct4 and Sox2 located sub epithelial. Epithelial differentiation markers (K3/12, K19 and K15) perform an amazing changing of localization during the development. Conclusions: The chicken embryo is an easy to handle, exact definable and ethical model to investigate limbal development. First analysis on differentiation and immunoreactivity of stem cells suggest a well organized and successive transition from general to fate specific regulation in developing limbal epithelium.

Keywords: corneal limbus, chicken embryo, somatic stem cells, limbal stem cells
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A novel xenogenic co-culture system to examine neuronal differentiation capability of various adult human stem cells

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Targeted differentiation of stem cells is nowadays mainly achieved by the sequential administration of defined growth factors, although these approaches are quite artificial as well as cost-intensive and time-consuming. We now present a simple xenogenic rat brain co-culture system which supports neuronal differentiation of adult human stem cells under more *in vivo*-like conditions. This system was applied to well-characterized stem cell populations isolated from human skin, parotid gland and pancreas. Beside a general multi-lineage differentiation potential these cells tend to differentiate spontaneously into neuronal cell types *in vitro* and are, by that, ideal candidates for the introduced co-culture system. Consequently, after two days of co-culture up to 12% of the cells, regardless which stem cell population, showed neuronal morphology and expressed corresponding markers on the mRNA and protein level. Therefore, the here described co-culture system is an ideal tool to test neuronal differentiation capability of any stem cell population. Especially when using human cells it is of clinical relevance for future cell-based therapies.

Keywords: adult stem cells, neuoronal differentiation, co-culture system
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Polycomb group protein Bmi1 promotes hematopoietic cell development from ES cells

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Bmi1 is a component of the Polycomb-repressive complexes (PRC) and essential for maintaining the pool of adult stem cells. PRC are key regulators for embryonic development by modifying chromatin architecture and maintaining gene repression. To assess the role of Bmi1 in pluripotent stem cells and upon exit from pluripotency during differentiation, we studied forced Bmi1 expression in mouse embryonic stem cells (ESC). We found that ESC do not express detectable levels of Bmi1 RNA and protein and that forced Bmi1 expression had no obvious influence on ESC self-renewal. However, upon ESC differentiation Bmi1 effectively enhanced development of hematopoietic cells. Global transcriptional profiling identified a large array of genes that were differentially regulated during ESC differentiation by Bmi1. Importantly, we found that Bmi1 induced a prominent up-regulation of Gata2, a zinc finger transcription factor, which is essential for primitive hematopoietic cell generation from mesoderm. In addition, Bmi1 caused sustained growth and a more than 100-fold expansion of ESC-derived hematopoietic stem/progenitor cells within 2-3 weeks of culture. The enhanced proliferative capacity was associated with reduced Ink4a/Arf expression in Bmi1-transduced cells. Taken together, our experiments demonstrate distinct activities of Bmi1 in ESC and ESC derived-hematopoietic progenitor cells. In addition, Bmi1 enhances the propensity of ESC in differentiating towards the hematopoietic lineage. Thus, Bmi1 could be a candidate gene for engineered adult stem cell derivation from ESC.

Keywords: hematopoietic stem cells, embryonic stem cell, polycomb protein
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Transmission of Venus fluorophore protein to embryonic development by transgenic and non-transgenic boar spermatozoa

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The pig is an important model for biomedical research. Recently, we produced transgenic pigs using a non-autonomous Sleeping Beauty transposon1. The pigs are at the moment one and a half year old and show a persistent expression of the Venus fluorophore in almost all cell types. Here, sperm cells from two boars carrying 3 copies of the Venus-transposon cassette were analyzed. Mature, haploid spermatozoa were uniformly Venus-positive and gave a distinct fluorescence peak in flow-cytometric measurements (n=6). Here, we characterized the prominent fluorophore expression in mature spermatozoa, and assessed, whether it correlated with active transcription, presence of the transcript, or the haploid genotype. In addition, sperm samples were analyzed for general parameters, sorted according to X and Y chromosome bearing sperm fractions, assessed for potential detrimental effects of the reporter, and used for inseminations. An unique cellular compartmentalization of the fluorophore protein was found in spermatozoa. The fluorescent spermatozoa carried only the fluorophore protein, but no fluorophore messenger transcripts. Most likely the phenotypically uniform fluorophore-positive sperm cell population arose from an equal protein distribution between immature spermatids via syncytial bridges, whereas the 3 copies of the transgene segregated according to Mendelian rules during meiosis and a certain fraction of the spermatozoa was non-transgenic. The analysis of 67 F1-offspring confirmed transposon segregation and identified 10 % non-transgenic animals. The carry-over of the transgenic protein by non-transgenic spermatozoa to oocytes represents a kind of non-genetic contribution to an embryo. Even sophisticated analysis by flow cytometry did not reveal any differences in Venus fluorescence in the spermatozoa samples. The data presented here, extend the knowledge about distribution mechanisms of paternally derived proteins in pre-spermatogonia and might be stimulating for research in non-genetic transmission by spermatozoa. The uniform partition of Venus protein suggests that a specific mechanism organizes the equal distribution of several paternal proteins in pachytene spermatids. These non-genetic contributions might gain importance for protocols for the manipulation and cryopreservation of spermatozoa for assisted reproductive technology as necessary components for effective fertilization and subsequent embryo development.

Ref. W Garrels, L Mates, S Holler, H Niemann, Z Izsvak, Z Ivics,WA Kues, 2010: Generation of transgenic pigs by Sleeping Beauty transposition in pig zygotes. Reproduction in Domestic Animals 45, 65 (abstr.)

Keywords: Venus fluorescent protein; germline transmission; meiosis; segregation
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FGF2/ERK signaling inhibits neural induction in human embryonic stem cells

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Human embryonic stem cells (hESCs) can exit the self-renewal program at any given time and differentiate along the three germ layer lineages, which is mediated through the action of signaling molecules. We have investigated the roles of three signaling pathways, TGFß/SMAD2, BMP/SMAD1, and FGF/ERK, in promoting a transition of hESCs into the neuroectoderm lineage. We find that inhibition of SMAD2 and ERK signaling cooperatively promotes an exit from self-renewal through the rapid downregulation of NANOG. Inhibition of SMAD1 signaling maintains SOX2 expression and prevents non-neural differentiation through HAND1. Finally, inhibition –not activation – of ERK induces the key neuroectodermal fate determinant PAX6. Conversely, FGF2 signaling specifically represses PAX6, revealing a novel role for FGF2 in sustaining the undifferentiated state of hESCs. Combined inhibition of the three pathways leads to highly efficient neuroectoderm formation within 4 d, and prolonged treatment, implying an embryoid body intermediate step, yields high-purity hESC or hiPSC-derived sensory neurons within 8 d. Our data clarify the controversial role of FGF signaling in the context of human neural induction. Moreover, our protocol enabling the rapid and high-efficiency generation of sensory neurons will be useful for in-vitro approaches to model human peripheral neuron diseases.

Keywords: human pluripotent stem cells; neural induction; FGF; PAX6; sensory neurons
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A novel fluorescence gene-trap identifies molecular dynamics during early mouse development

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The basic knowledge of the mammalian development is essential for the efficient application of stem cell biology to regenerative medicine. However, the molecular mechanism of mammalian embryogenesis is poorly understood. The primary aim of this project is to understand the molecular program underlying formation of the pluripotent inner cell mass (ICM) in the mouse blastocyst. We have recently developed two complementary screens to identify genes involved in this process and to establish resources for comprehensive studies: i) a fluorescent-based gene-trap: a Venus reporter, expressed under the endogenous control of the genomic locus of integration, will allow us to characterize the molecular dynamics during blastocyst patterning; ii) gene expression profiling of the individual blastomere in the mouse preimplantation embryo. Venus-trap screen has successfully generated 107 founder mice that led us to characterize 63 integrations. Of particular interest are those 9 traps active specifically in one of the initial lineages, including three mouse lines expressing the reporter exclusively in the ICM. This is, to our knowledge, the first fluorescent gene trap successfully applied to mouse development. The trapped lines allow high-resolution 4D-imaging and precise tracking of dynamic lineage segregation during mouse pre-implantation development. Depending on the locus of proviral insertion, homozygous mice will produce a phenotype ranging from wild-type to partial or null. Thus, some of the generated lines will additionally allow a functional analysis. Ongoing studies of the established lines will for the first time (i) visualize the initial lineage segregation between the ICM and TE in developing embryos, and (ii) illustrate the emergence and resolution of molecular heterogeneity in the mouse pre-implantation embryo. Recent findings will be presented.

Keywords: mouse embryo; pluripotency; embryo patterning; ES cells; gene-trap
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Investigating conditions for the induction of cultivatable neural progenitor populations from induced pluripotent stem cells

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Previous works show that neural stem cell (NSC) progenitors can be cultivated *in vitro* and are capable of differentiating into various region-specific neuronal and glial cell types in response to appropriate conditions. The broad differentiation potential is unique to early neural progenitors. Optimizing protocols for stable and clonal proliferation of neural progenitors could pave the way towards a therapeutically viable and biomedical application. In the present study, we used patient-specific induced pluripotent stem cells (iPSCs) generated from bone marrow cells (BM-iPSCs) and monitored the induction of neural progenitors under different culture conditions. Based on our protocol described for neural differentiation from mouse pluripotent stem cells, we induced neural differentiation of BM-iPSCs by culturing BM-iPSCs as cell aggregates in NSC medium and subsequently plated them to different substrates in defined media. After 3 days of plating (d3 3) BM-iPSCs underwent morphogenetic changes by the formation of radially organized epithelial cells referred to as "neural rosettes". These rosette structures stained progressively positive for PAX-6, SOX-1, SOX-2, Nestin and ßIII-Tubulin, suggesting that they indeed converted to populations of neural progenitors. Gene expression analysis exhibited the progressive loss of pluripotency markers, and an increase in the expression of markers defining early neural fate. It turned out that a combination of Matrigel and NSC-Medium is optimal for the generation of a neural progenitor population in our culture system. Furthermore, our data demonstrate that extended mass culture of BM-iPSCs in NSC-Medium was not beneficial to the formation of neural rosettes. To enhance formation and maintenance of neural progenitors, inhibition of BMP and TGF-ß (dual SMAD inhibition) was also evaluated through usage of Noggin and the small molecule inhibitor SB43154. However, dual SMAD inhibition increased the production of cells with neuronal morphology which expressed the early neuronal marker ßIII-Tubulin, suggesting that it was beneficial to induction of neuronal differentiation, but not to induction and maintenance of neural progenitors. Currently we are establishing an optimized condition to cultivate and maintain the neural progenitor population *in vitro*.

Keywords: PSCs; differentiation; neuronal; NSC; progenitor
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Regulation of astrocyte generation by the extracellular matrix molecule Tenascin C

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During the development of the central nervous system neural precursor cells give rise to three major cells types, namely neurons, oligodendrocytes and astrocytes. While extensive information has been gained with regard to the regulation of both oligodendroglial and neuronal lineages in the developing spinal cord of the CNS, the situation is far less clear for the radial glia and especially for the astrocytes derived therefrom. Here, we analysed the role of the extracellular glycoprotein Tenascin-C (Tnc) in spinal cord development with particular focus on the astrocyte lineage progression. To gain first insights into possible roles of Tnc in spinal cord development we initially undertook a systematic expression analysis of Tnc both on mRNA and protein level and found that Tnc was expressed by Nestin-, GLAST-, and Vimentin-positive glial precursor cells during the late embryonic development as well as by GFAP-positive astrocytes at the end of embryogenesis. The loss of Tnc led to a sustained generation of Fgfr3 expressing immature astrocytes *in vivo* during early gliogenesis as revealed by BrdU incorporation analysis. Additionally, the migration of these cells into the prospective ventral white matter was severely delayed in the Tnc mutant spinal cord. Consistent with an increased generation of astrocytes we also documented an increase number of GFAP-positive cell processes in the ventral white matter at the end of embryogenesis. In order to get a mechanistic insight into these phenotypes we performed a micro array analysis of E15.5 Tnc mutant spinal cords in comparison to wildtype tissue. Strikingly, we observed gene expression changes of a variety of key patterning transcription factors, whose expression depends on graded sonic hedgehog signalling in the ventral spinal cord. Moreover, we found a dorsal expansion of Nkx2.2 expressing cells in the Tnc mutant spinal cord. This finding represents the first example of the regulation of transcription factor expression territories by a component of the extracellular matrix and thus adds a novel and hitherto unsuspected function to ECM-constituents in general and to Tnc in particular. Beyond these observations, we also provide evidence that the expression of the heparan sulphate specific Sulfatase 1, which is known to negatively regulate FGF signalling and a well known target gene of Nkx2.2, is upregulated as well. It has been shown, that FGF signalling fosters EGF responsiveness at later embryonic ages. Strikingly, we found a delayed EGF responsiveness of Tnc -/- mutant neural precursor cells, indicating a reduced FGF signalling. These observations offer a mechanistic insight into the phenotype of the glial precursors of the spinal cord in the mutant. Taken together our study revealed that Tnc promotes the timely astrocyte lineage progression during spinal cord development.

Keywords: Gliogenesis; Tenascin C; extracellular matrix; growth factor responsiveness; neural patterning
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Mitochondrial morphology affects cardiomyocyte differentiation of mouse embryonic stem cells through Calcineurin and Notch signaling pathways

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During cell differentiation, mitochondria are mostly considered as a power house of the cells, supplying most of cellular ATP via the conversion of biological energy through oxidative phosphorylation (OXPHOS) to meet energy demand. It has been reported that the bienergetic switch from glycolysis to OXPHOS occurs during mouse embryonic stem cell differentiation into cardiomycyte; mitochondria also play essential and versatile roles in pathophysiology of cardiac cells, such as controlling Ca2 homeostasis, and Ca2 signaling, permeability transition pore, and apoptosis impacting on almost any process, like outcome of ischemia/reperfusion and electrical synchronization during EC coupling. Mounting evidence is supporting a role for mitochondrial shape in determining organellar function. Mitochondrial morphology is controlled by a growing family of "mitochondria-shaping" proteins that includes the pro-fusion large dynamin-like GTPases optic atrophy 1 (OPA1) and mitofusin (MFN) 1 and 2 and the pro-fission dynamin-related protein 1 and its mitochondrial receptor FIS1. In addition, these mitochondria-shaping proteins not only control mitochondrial morphology, but also have crucial roles in key cellular processes, such as apoptosis, tethering of organelles and Ca2 homeostasis. However, if mitochondria-shaping proteins regulate differentiation into functional and specific cell lineages is largely unknown, in particular in the cardiomyocyte. To understand the role of mitochondria-shaping proteins in cardiomyocyte differentiation, we used mouse embryonic stem (ES) cells lines (Opa1gt and Mfn2gt), which are heterozygous for a gene trap in Opa1 or Mfn2 gene respectively, and performed *in vitro* differentiation into cardiomyocyte. Down-regulation of OPA1 or MFN2 did not affect stemness and self-renewal of ES cells nor mitochondrial bioenergetic parameters, but displayed shorter mitochondria, and abnormal Ca2 signaling, with sustained capacitative Ca2 entry. Hanging-drop differentiation showed that appropriate levels of OPA1 and MFN2 are required for differentiation into beating cardiomycytes. The differentiation defect was corrected by the calcineurin inhibitor FK506, as well as dominant negative form of calcineurin. Accordingly, Opa1gt and Mfn2gt cells showed higher levels of calcineurin activity, suggesting that mitochondrial shape plays a role in the regulation of the activity of this phosphatase. Calcineurin in turn triggered higher activity of the inhibitor of cardiomycyte differentiation Notch. Ablation of OPA1 and MFN2 in ES cells shows that mitochondrial shape regulates cardiomyocyte differentiation by impinging on a pathway involving the phosphatase calcineurin and Notch signaling.

Keywords: embryonic stem cell; mitochondrial dynamics; cardiomyocyte; calcineurin; Notch signaling
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Differentiation of murine embryonic (mESCs) and induced pluripotent stem cells (miPSCs) into definitive endoderm and airway epithelial cells

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Introduction: The ability to specifically produce respiratory progenitor and airway epithelial cells *in vitro* would offer new therapeutic options to treat pulmonary injuries and diseases. Pluripotent stem cells may represent a suitable source for the production of airway epithelial cells. Aim of this study was the establishment of a monolayer-based differentiation protocol enabling the generation of respiratory epithelial cells from mESCs/miPSCs via enhanced definitive endoderm formation. Material and Methods: A double transgenic mESC line expressing eGFP under the control of the brachyury (Bry) promoter and a truncated human CD4 under the Foxa2 promoter (Gadue et al. 2006) was used to optimize generation of definitive endoderm. Activin A, or the recently described small molecules (IDE-1 and IDE-2, Borowiak et al. 2009) were evaluated as inductors of definitive endoderm. Transgenic mice expressing tetracycline-regulated GFP and lacZ under control of the rat Clara cell secretory protein promoter were bred, and miPSC clones were generated from fibroblasts of these mice. Established miPSC clones were then differentiated *in vitro* and airway differentiation was analysed. Results: Bry and Foxa2 promoter dependent reporter expression revealed an increased but delayed differentiation on mESC into mesendoderm / definitive endoderm progenitor cells in ML differentiation cultures compared to EBs. Furthermore, the use of RPMI with 0.2 % FCS was superior to IMDM with 15 % FCS resulting in up 40 % FoxA2pos endoderm cells when combined with Activin A induction. In contrast to Activin A, IDE-1 and IDE-2 did not show significant effects regarding mesendoderm / definitive endoderm formation. The established protocols were applied to differentiate miPS cells and resulted in the appearance of CCSP-lacZ expressing airway epithelial cells after 26 days. A more detailed characterisation of miPS-derived airway cells is ongoing. Conclusions: The generation of miPSC derived CCSPpos airway cells represents a first step towards the development of cellular therapies for different pulmonary disorders including hereditary ones such as cystic fibrosis.

Keywords: murine iPS, murine ES, serum free monolayer differentiation, airway epithelial cells
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Endothelial-like cells derived from adult murine germline-derived pluripotent stem cells

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Functional endothelial cells and their progenitor cells are required for basic physiological and pharmacological vascular research as well as for cell-based therapies of ischemic cardiovascular diseases including approaches based on tissue engineered vascular grafts or single cell preparations, respectively. Currently, such applications are predominantly relying on endothelial progenitor cells (EPCs). However, their purification from peripheral blood or bone marrow is laborious. Therapeutic applications based on autologous EPCs are additionally compromised by the fact, that patient-derived EPCs are numerically reduced and mostly functionally impaired. Therefore, the evaluation of alternative sources of cells suitable for basic research as well as therapeutic applications is required. We have recently obtained germline-derived pluripotent stem (gPS) cells from adult mouse unipotent germline stem cells. The objective of our study is to reveal whether functional endothelial-like cells (ECs) can be derived from this new type of pluripotent stem cells. Briefly, PECAM-1 (CD31)-positive cells were isolated from single cell suspensions of gPS-derived embryoid bodies (EBs) by fluorescence-activated cell sorting (FACS) and subcultivated on OP9 stromal cells. Subsequently, EC-like colonies were mechanically isolated and expanded on collagen IV-coated cell culture dishes. Using FACS analysis it was demonstrated that the cells expressed the endothelial cell–specific markers PECAM-1, von Willebrand Factor, Tie2, Flk1, and vascular endothelial-cadherin. The cells were successfully maintained in in-vitro culture for many passages without a significant loss in expression of the endothelial cell markers. Furthermore, the cells were capable of forming capillary-like structures when cultured on Matrigel. Dil-conjugated acetylated low-density lipoprotein uptake confirmed the functionality of the gPS-derived ECs. To allow for an assessment of their potential suitability in various fields of applications their phenotype with respect to arterial, venous or lymphatic properties is currently being analyzed.

Keywords: endothelial progenitor cells ;endothelial cells;germline-derived pluripotent stem cells
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TOX3 is a neuronal transcription factor expressed in neuronal precursor cells

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TOX3 is a nuclear protein containing a high mobility group (HMG)-box domain, which regulates Ca(2)-dependent transcription in neurons through interaction with the cAMP-response-element-binding protein (CREB). TOX3 is expressed downstream of a cytoprotective cascade together with CITED1, a transcriptional regulator that does not bind directly to DNA. In previous work, we showed that TOX3 is a neuronal survival factor that induces transcription depending on the presence of CITED1 or phosphorylated CREB in the transcriptionally active complex. A thorough analysis of the expression pattern of TOX3 showed that TOX3 is mainly expressed in the stem cell niche of the adult and developing mouse brain. Its expression peaks at E14 in the ganglionic eminence, where neuronal precursor cells (NPCs) are the predominant cell type. NPCs can proliferate and differentiate into neurons, astrocytes and oligodendrocytes. In the adult animal, TOX3 is mainly expressed in the subventricular zone, where also neuronal stem cells prevail. TOX3 expression is downregulated by differentiation of NPCs and by treatment with TGFβ. Activation of the NOTCH pathway with the soluble NOTCH ligand Jagged, in contrast, induced TOX3 expression. These data speak in favor of a predominant role of TOX3 in the undifferentiated state of NPCs. Over-expression of TOX3 in NPCs induced the expression of several anti-apoptotic transcripts similar to results already shown in Neuro2a cells. We therefore assume a function for TOX3 in the survival of NPCs. We hypothesize that it is involved in the early stage of development, because of its high expression at the beginning of differentiation and its induction through the NOTCH pathway, which plays an important role in embryonic mouse development. We are currently in the process of generating a TOX3-deficient mouse model.

Keywords: NPC, TOX3, transcription
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Paracrine signals restrict neuronal migration from human neural precursor cell containing grafts

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An intriguing question in neural replacement therapy is how cells from neural grafts can be enabled to migrate and integrate into the adult brain parenchyma. Neural precursor transplants typically form dense clusters with limited neuronal integration into the recipient CNS, a phenomenon, which has generally been attributed to inhibitory interactions between graft and host tissue. Contrary to this view, we found that an important mechanism underlying cluster formation and limited integration is a chemotactic interaction between the grafted neural precursors and their own neuronal progeny. Receptor tyrosine kinase ligands such as FGF2 and VEGF play an important role in this interaction. Both factors are expressed by neural progenitors and stimulate chemotaxis of neurons. Conversely, treatment with the receptor tyrosine kinase inhibitor BIBF1120 results in enhanced migration and tissue integration. Thus, "auto-attraction" between neural precursors and their neuronal progeny provides a new target for improving migration and integration of neural grafts.

Keywords: neural stem cells; neuronal migration
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Putative tissue resident stem cells in the testes of adult Axolotl (Ambystoma mexicanum)

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The Mexican Axolotl, Ambystoma mexicanum, is one of the most interesting animal models in regenerative biology, due to its ability to regenerate entire limbs after amputation. Although regeneration of limbs and nerves has been extensively studied, little is known about the regeneration of inner organs. Therefore, in our current study we addressed the regenerative potential of the Axolotl's testis and focused on the role of putative tissue resident stem cells in this process. In the first part of the study, six adult Axolotls (wild type, male, length 20-22 cm, 2 years old) were used. The hind end of the fully sexually mature right testis (approx. ¼ of the total size) was excised through a ventrolateral abdominal incision under general anaesthesia, and animals were allowed to recover. Testes were harvested 1 (n=3) or 3 (n=3) weeks later. In a second part of the study, healthy testes from early larval Axolotls (n=4, 2.5 -3.0 cm total length), late larval axolotls (n=4, 9.8-10.5 cm total length), and Axolotls during partial metamorphosis (n=7, 13.0 – 16.4 cm total length) were harvested. All testis tissues were fixed, embedded, and sectioned. Sections of the adult tissues were stained histochemically for the detection of connective tissue and neutrophile granulocytes, and immunohistochemically to detect α-smooth muscle actin, TGF-β and stem cell markers such as OCT3/4 and PGP 9.5. Tissues of the younger animals were stained immunohistochemically to detect OCT3/4 and PGP9.5. In the adult animals 1 and 3 weeks after surgery, the injured testis appeared smaller than the healthy testis. Histochemically, an increased amount of connective tissue, smooth muscle actin and neutrophil granulocytes were detected at the wound. But no increased TGFβ-1 signalling was apparent, and no putative tissue resident stem cells (OCT3/4 or PGP9.5 cells) were present in the wound. However, we observed a thin anterior projection of the testis (APT), originating at the front end of the testis, which contained single putative primordial germ cells (PGCs) showing polylobulated nuclei and expressing OCT3/4. Thus, the APT shows a striking resemblance with the appearance of the larval gonad, because in the gonads of the early larvae, the late larvae, and the animals that were in partial metamorphosis, we also observed PGCs in all testicular tissues, as determined by their characteristic nuclear appearance. Also, these PGCs expressed OCT3/4 and PGP 9. In conclusion, we have shown that a) the testis of the adult Axolotl does not show immediate regeneration in response to injury and b) that the testis of Axolotl's of many different developmental stages presents an anterior projection which, throughout live, maintains a larval phenotype and contains putative PGCs expressing OCT3/4 and PGP 9.5. We postulate that this larval part of the testis might serve as a putative stem cell hub potentially allowing testis regeneration even after major injuring. So far, only PGC-like putative germ line stem cells have been encountered, and we are currently addressing the question, whether also somatic stem cells (e.g. undifferentiated Sertoli cell precursors) are present in the anterior projection of the testis.

Keywords: Axolotl; regeneration; testis; primordial germ cells;
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Alternative pluripotent stem cells as a source of hepatic cells

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Recently there have been scientific reports that have described the generation of pluripotent stem cells from the mammalian germ-line. Parthenogenetic activation of female oocytes can give rise to blastocysts, whose inner cell mass can be isolated and propagated as parthenogenetic embryonic stem cells (pESCs). Murine testis-derived pluripotent germ-line stem (gPSCs) cells, obtained from adult mouse testes through culture of unipotent spermatogonial stem cells under ES cell conditions, has also been recently described. The pESCs and gPSCs show expression of pluripotency markers, at levels comparable to embryonic stem cells (ESCs). In our present study we investigate, the hepatic differentiation potential of these alternative sources of pluripotent stem cells. We have previously reported the use of an embryoid body formation-based protocol to derive hepatic progenitors from the gPSCs and the pESCs. However, this protocol lacked efficiency in generating higher number of hepatic cells with a mature phenotype that were suitable for further transplantation experiments. We have also previously reported a cytokine based differentiation protocol which mimics the *in vivo* embryonic development of the liver. Modification of this cytokine-based protocol was able to generate higher numbers of hepatocyte-like cells, with expression of hepatic markers comparable to Hepa 1-6 cells. Activin A treatment of pESCs and gPSCs grown as a monolayer in serum-free differentiation (SFD) medium, initiated the induction of early endoderm. Further treatment of the cells with Bone Morphogenic Protein 4 (BMP4) and basic Fibroblast Growth Factor (bFGF) directed the cells towards the definitive endoderm. The cells were then cultured in Hepatocyte Culture Medium (HCM) along with Oncostatin M (OSM) and human Hepatocyte Growth Factor (hHGF), in order to induce hepatic cell maturation. An Albumin-Neomycin vector was used for further selection of Albumin producing cells in the differentiated population. Our study hence provides an insight into the use of germ-line derived hepatocytes for cell therapy in treatment of liver disorders.

Keywords: germ-line derived pluripotent stem cells; parthenogenetic ES cells; hepatocytes; differentiation
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Efficient generation of oligodendrocytes from human ESC- und iPSC-derived gliogenic neural stem cells

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Efficient generation of human oligodendrocytes provides interesting prospects for basic research, screening applications for oligodendroglial-specific compounds and eventually the development of stem cell based therapies for diseases requiring oligodendroglial replacement. In recent years, human embryonic stem cells (ESC) have emerged as particularly versatile and unlimited source of neural donor cells. In addition, reprogramming of somatic cells into induced pluripotent stem cells (iPSC) provides fascinating prospects to generate patient-specific pluripotent stem cells. However, while the generation of neural precursors and differentiated neurons from human ESC and iPSC is well established, oligodendrocyte differentiation, so far, requires complex run-through *in vitro* differentiation protocols. In this study we used a retinoic acid-based approach to generate an adherent and clonogenic population of gliogenic neural stem cells (glioNSC) from human ESC and iPSC, which is amenable to efficient oligodendroglial differentiation and can thus serve as a direct and stable source of human oligodendrocytes. GlioNSC are positive for markers typically associated with a neural stem/radial glia cell fate including nestin, musashi, PAX6, SOX2, BLBP, GLAST, 3CB2 and vimentin. They express transcription factors compatible with a posterior hindbrain and anterior spinal cord identity (e,g. GBX2, EGR2, HOXA2, HOXB6, HOXD9). With respect to dorsoventral patterning, glioNSC express transcription factors of dorsal (PAX3, PAX7), intermediate (IRX3, PAX6) and ventral (NKX6.1, NKX6.2) domains but are negative for the most ventrally expressed transcription factors OLIG2 and NKX2.2. Upon growth factor withdrawal glioNSC exhibit tripotential differentiation into neurons of different subtypes, astrocytes and oligodendrocytes. Notably, using defined differentiation conditions promoting the expression of OLIG1/2, NKX6.2 and SOX10, human ESC- and iPSC-derived glioNSC can be efficiently converted into NG2-positive oligodendroglial progenitors, which further differentiate into O4-, GalC- und MBP-positive oligodendrocytes. Translation of our differentiation paradigm to disease-specific hiPSC will offer interesting prospects to study cell-autonomous pathomechanisms underlying myelin-related disorders directly in affected human cells.

Keywords: human pluripotent stem cells; neural stem cells; oligodendrocyte differentiation
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Mesenchymal stromal cells immobilized in an oriented 3D scaffold accelerate functional recovery after acute spinal cord injury

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Background: Human mesenchymal stromal cells (hMSC) have important features that make them of high interest in the repair of the injured spinal cord: (1) hMSC express and release a variety of growth factors important for an enhanced neurite outgrowth, and (2) the extracellular matrix produced by hMSC is capable of influencing the direction of neurite outgrowth. In an attempt to combine both of these important features, hMSC were seeded onto 3D collagen scaffolds with oriented micro-pores and implanted into acute rat spinal cord lesions to investigate the regenerative properties. Methods: GFP-positive hMSC were seeded onto the porous collagen scaffold and implanted into an acute T10 lateral funiculotomy of the adult rat spinal cord. Non-seeded scaffolds and human fibroblast (hFbl)-seeded scaffolds served as controls. Motor and sensory function was investigated weekly for up to eight weeks using gridwalk and Catwalk analysis. Animals were perfused with 4% paraformaldehyde and the spinal cord processed for immunohistological staining to evaluate host axonal regeneration, astrocytic scarring and inflammatory responses. Results: Four weeks after transplantation, animals receiving hMSC-seeded scaffolds demonstrated significantly better recovery in motor function as compared to controls. This difference, however, was only transient and decreased by the end of the experiments. Although only few donor hMSC could be identified within the scaffold by 8 weeks, animals receiving hMSC-seeded scaffolds demonstrated elevated numbers of regenerating axons, as well as reduced astrogliosis and inflammatory response as compared to hFbl- and non-seeded scaffolds. The donor hMSC that could be detected 8 weeks were found to express chondroitin sulphate proteoglycans (CSPG) and, although only few cells were present, the scaffold was full of CSPG equally to empty control scaffolds. Conclusion: The combination of an oriented 3D collagen scaffold and donor hMSC demonstrated a transient enhancement of functional recovery in a model of acute spinal cord injury. It is possible that this effect might be associated with the recently reported positive function of CSPG expression as well as growth factor and cytokine release.

Keywords: extracellular matrix, growth factors, mesenchymal stromal cells, spinal cord injury, scaffold
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Large-scale immunoscreening of planarian cell plasma membrane proteome reveals heterogeneity of the S/G2 fraction of stem cells

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The planarian adult stem cell (pASC) population has a specific molecular signature and can easily be visualized and isolated by flow cytometry. However, the lack of antibodies against specific surface markers for planarian cells prevents a deeper analysis of specific cell populations. Here we describe the results of large-scale immunoscreening of pASCs plasma membrane proteins. A novel papain-based method for planarian cell dissociation enabling high yield and cell viability was used to isolate proteins of the microsomal fraction. Microsomal proteins were used for intraperitoneal immunization of mice and thus more than 1000 hybridoma clones were generated and screened. Supernatants collected from the clones positive to the ELISA test stained the membranes of living planarian cells. The majority of these stained all the cells, whereas some labeled specifically a subfraction of planarian cells. A detailed analysis of three hybridoma supernatants revealed that large subfractions of the X1, X2 and Xin populations differentially express specific membrane markers. Moreover, qPCR analysis of the positive versus the negative cell fractions demonstrated enrichment in the expression of markers of either early or late differentiation. Thus, cycling planarian stem cells in the S/G2 phase of their cell cycle showed a specific membrane signature coupled with expression of markers hitherto considered to be restricted to differentiating, non-dividing cells. In summary, a library of monoclonal antibodies against the planarian plasma membrane proteome was generated. The analysis of some of the clones generated revealed an unexpected heterogeneity in the X1 (S/G2) fraction of pASCs, casting doubt on the homogeneity and the bona fide pluripotency of individual pASCs and demonstrating the usefulness of this library for planarian research.

Keywords: stem cells, planarian, regeneration, cell surface antibodies
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Expression of accessory proteins of glutamate receptors during neural differentiation of embryonic stem cells

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Ionotropic glutamate receptors (iGluRs) mediate the majority of excitatory neurotransmission in the adult vertebrate CNS. Recent studies gave reason to expect iGluRs to be involved in the process of neural differentiation of embryonic stem cells. Since there is evidence that a group of proteins, namely transmembrane AMPA receptor regulatory proteins (TARPs), alter electrophysiological properties and trafficking of AMPA receptors, interaction of accessory proteins with iGluRs could also influence neural differentiation of embryonic stem cells and their descendants. To examine the expression levels of iGluRs and their related accessory proteins during neural differentiation on mRNA and protein level we used an *in vitro* system of different murine cell types, namely 46C embryonic stem cells (ESCs), neuroepithelial cells (NEPs), early neurons, neural stem cells (NSCs) and astrocytes. Following the neuronal differentiation of ESCs into early neurons an increase in expression levels on mRNA level of accessory proteins correlating to an increase in expression of iGluRs was observed. Furthermore, protein expression, except for PSD-95, was first detectable after differentiation of the cells into early neurons. PSD-95 instead was already detected on protein level in the stage of NEPs. During glial differentiation of NSCs into astrocytes there was no general trend of increasing or decreasing expression levels of the analyzed proteins observed. Additionally detection of protein expression was only possible for NR2A in NSCs. A second part of the work focused on the electrophysiological characterization of an N-methyl-D-aspartate receptor (NMDAR) composed of the subunits NR1-1a and NR2A coexpressed with cRNA of the putative NMDAR accessory protein NETO1. These measurements were performed in the heterologous expression system of Xenopus laevis oocytes. The obtained results showed that coexpression of NETO1 alone had no effect on the agonist-induced currents of the analyzed NMDAR. However a combined coexpression of NETO1 and total RNA isolated from adult mouse whole brain resulted in increased agonist-induced currents. Additionally, a lowered ion conductance at negative membrane potentials and an inhibition of the block by MK801 on the analyzed NMDARs was observed. These results lead to the conclusion that an additional protein is probably needed for modulation of NMDARs by NETO1.

Keywords: TARP 46C, accessory, NETO
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ATOH8, a novel regulator of skeletal myogenesis and muscle regeneration

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Purpose: ATOH8 was found to be severely deregulated in a patient who suffered from a severe myopathy. ATOH8 is expressed in the somites during chicken embryo development. To study its role in myogenesis, we have silenced ATOH8 in the developing somites and analysed its expression profile in satellite cells and myogenic progenitors on muscle fibres. Methods: The ATOH8 gene expression pattern has been investigated by in situ hybridization in chicken embryos. Vector-based RNAi was used to knock-down ATOH8 in chicken embryos. Using ATOH8, Pax7 and Myogenin specific antibodies, the expression of ATOH8 in satellite cells was studied on freshly isolated muscle myofibers obtained from the EDL of mice and on cultured muscle fibres in growth and differentiation medium. Real time PCR was performed on C2C12 myoblast. Results: Knock-down of cATOH8 in the somites by RNAi resulted in an effective silencing of ATOH8 and a down-regulated expression of MyoD, Myf5, accompanied with a decrease in myosin heavy chain expression and an up-regulated expression of Pax3. IHC on muscle fibres revealed the existence of Pax7 /ATOH8- and Pax7 /ATOH8 sub populations of satellite cells. Similarly, in the progenitor cells arising from the satellite cells we observed two subpopulations grouped as Myogenin /ATOH8 and Myogenin /ATOH8-. Real time PCR results show a rise in ATOH8 expression levels at the onset of myoblast differentiation. Conclusions: We conclude that ATOH8 is involved in myogenesis and is expressed during satellite cell differentiation. Its expression levels increase at the onset of satellite cells differentiation and decreases towards terminal differentiation. This is in line with the decrease of expression observed during maturation of muscles during chicken embryogenesis.

Keywords: Myogenesis, satellite cells, regeneration
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Human unrestricted somatic stem cells (USSC) in spinal cord injury: Characterization of cell migration, paracrine neurotrophic support, axon regeneration, and functional improvement

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Spinal cord injury (SCI) results in permanent loss of axons, scar formation and consequently functional disability. One approach to promote axonal regeneration is to transplant cells with the capacity to protect the endogenous tissue and/or to stimulate axon growth. Actually, different stem cell types have been grafted into animal models and humans with SCI. Due to inconsistent results, it is still an open question which stem cell type will prove to be therapeutically effective. Thus far, stem cells of human sources grafted into spinal cord mostly included barely defined heterogeneous mesenchymal stem cell populations derived from bone marrow or umbilical cord blood. Here, we have used a well-defined unrestricted somatic stem cell (USSC) population isolated from human umbilical cord blood and transplanted into an acute traumatic SCI of adult immunosuppressed rat. Stereotactical grafting of native USSC into the vicinity of a dorsal hemisection injury at thoracic level Th8 yielded massive migration of these stem cells to the lesion center. *In vitro* under-agarose chemotaxis assays revealed that the attraction of USSC to the injured spinal cord tissue is hepatocyte growth factor-mediated. After transplantation into the injured spinal cord, USSC accumulated within the lesion area where they survived for at least three weeks without neural differentiation. Histological analysis showed significantly enhanced axon ingrowth into the lesion site five weeks after grafting as assessed by anterograde tracing. In addition, USSC conditioned medium efficiently increased neurite outgrowth of rat embryonic dorsal root ganglion explants and primary cortical neurons comparable with the capacity of astrocyte conditioned medium. Importantly, long-term behavioral studies (16 weeks) including three different locomotor tests (open field BBB locomotor score, horizontal ladder walking test, CatWalk gait analysis) demonstrated significant functional benefits following USSC transplantation. The observed functional improvement correlated well with reduced tissue loss or augmented tissue sparing and stimulation of regenerative axon growth. To accomplish the beneficial effects, neither neural differentiation nor long-lasting persistence of the grafted human stem cell appears to be required. The secretion of neurite outgrowth promoting factors *in vitro* further suggests a paracrine mechanism underlying the beneficial effects of USSC in SCI. Given the highly supportive functional characteristics in SCI, production in virtually unlimited quantities at GMP-grade and lack of ethical concerns, USSC appear to be a suitable human stem cell source for clinical application in CNS injuries.

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Influence of defined growth factors on the differentiation of murine embryonic stem cells into alveolar type II epithelial cells

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Type II alveolar epithelial cells (AT2 cells) have important functions, including the production of surfactant and regeneration of lost type I pneumocytes. The ability to produce AT2 cells would offer a variety of new therapeutic options to treat pulmonary injuries and diseases, including genetic surfactant deficiencies and pulmonary fibrosis. Recently, we demonstrated that AT2-like cells can be generated from murine embryonic stem cells (mESCs). However, with respect to the poor efficiency of existing differentiation protocols we aimed at the identification of key factors of lineage specification thereby enabling a more efficient differentiation.Based on published data demonstrating an effect of keratinocyte growth factor (KGF, FGF-7) on primary AT2 cells, we investigated the influence of KGF on the differentiation of mESCs in embryoid bodies. In addition, the effect of dexamethasone, 8-bromoadenosine-cAMP and isobutylmethylxanthine (DCI) either alone or together with KGF was evaluated. Effects on the respiratory differentiation of ESCs were quantified through qRT-PCR specific for TTF-1, surfactant proteins C (SP-C), B (SP-B) and Clara Cell Specific Protein (CCSP). Microarray analyses have been performed in order to identify molecular pathways of differentiation. Electron microscopy was applied to search for ultrastructural features of respiratory epithelium. Finally, ESC clones expressing SP-C promoter dependent reporter genes were generated to enable visualization and enrichment of ESC-derived AT2 cells. Whereas qPCR analyses revealed an increase in the expression of SP-B and SP-C after application of either KGF or DCI, a strong synergistic effect with significantly increased expression of SP-B, SP-C, TTF-1 and CCSP could be shown after combined application of KGF and DCI. Surprisingly, the most profound effect was detected after early KGF application starting at d0 of differentiation, suggesting an additional, and so far unknown effect of KGF during early differentiation. Ultrastructural analysis confirmed the presence of AT2-like cells with a more mature phenotype than without application of KGF/DCI. Finally, microarray analyses revealed upregulation of a series of genes known to be associated with lung development. Analysis of microarray data is currently ongoing in order to identify underlying molecular pathways, and reporter lines with SP-C promoter dependent expression of selection genes are currently analyzed in detail. In conclusion, were were able to demonstrate a synergistic effect of KGF and DCI on the differentiation of mESCs resulting in increased expression of respiratory marker genes and more mature AT2-like cellular ultrastructure. Results of microarray analyses and characterization of the generated reporter cell lines will be presented at the meeting.

Keywords: differentiation; embryonic stem cells; alveolar type 2 epithelial cells; lung, SP-C
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Influence of glucose on definitive endoderm and early pancreatic progenitor formation

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Current studies provide an important proof-of-principle that *in vivo* development of insulin-producing beta cells can be recapitulated *in vitro* using embryonic stem (ES) cells and the appropriate growth and transcription factor stimulus. The challenge remains though to generate specific and functional pancreatic cells in sufficient quantities to be considered for cell replacement strategies or as *in vitro* test systems. Little attention has been paid to the analysis and manipulation of the nutritional environment and its influence on the differentiation of ES cells into the pancreatic lineage. However, a large panel of intrauterine growth retardation animal models has shown that under-nutrition during prenatal life gives rise to a decrease in the number of beta cells that will form. It is known, that glucose controls beta cell development by activating the insulin gene promoter and supporting nuclear translocation of the key transcription factor Pdx-1 during mid and late pancreatic differentiation. Yet, the influence of glucose on the formation of definitive endoderm (DE) and early pancreatic progeny has not been studied in detail. DE formation is a prerequisite for successful generation of Pdx-1-positive pancreatic progenitors and subsequently insulin-producing cells. Therefore, we differentiated murine ES cells in the presence of activin A to generate definitive endoderm followed by treatment with cyclopamine and indolactam V to obtain early pancreatic progenitors. We analyzed the effects of low and high glucose concentrations within different time frames of the differentiation process. Our studies demonstrate that low concentrations of glucose cause massive cell death and do not support pancreatic differentiation. However, high glucose concentrations during early ES cell differentiation significantly increased the transcript and protein levels of Sox17 while markers of the extraembryonic endoderm such as Sdf1 remained low. This points to an inducing effect of high glucose concentrations on DE formation via activin A. Likewise, expression of Pdx1 and Ngn3 was significantly induced by high glucose while ectodermal markers like NeuroD6 were not detected. We therefore conclude that applying high glucose conditions in tightly controlled time frames can support early pancreatic differentiation of ES cells.

Keywords: mES; definitive endoderm; glucose
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A unique surface glycan demarcates FGF-2-responsive self-renewing neural precursors from EGF-dependent symmetrically expanding intermediate progenitor cells

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At the peak of neurogenesis, the majority of neurons are generated directly from radial glial cells or indirectly by dividing intermediate progenitor cells. A specific complex chondroitin sulfate epitope selectively recognized on the cell surface of neurogenic radial glia by the monoclonal antibody 473HD allows for selective enrichment by immunopanning. Since growth factors are critical for the proliferation of neural stem/progenitor cells during cortical neurogenesis, we investigated in this study the effects of FGF-2 and EGF on the properties of dividing 473HD-epitope carrying radial glia from mouse embryonic day 13 mouse telencephalon. The cell fate of their immediate progeny was analyzed using cell pair assays. Among these selectively isolated FGF-receptor-positive radial glial cells we identified a subpopulation of EGF receptor-positive, Tbr-2 expressing intermediate progenitor cells. FGF-2 maintained radial glia by sustaining self-renewing cell divisions that were characterized by the preferential distribution of the 473HD-epitope to one or both daughter cells. Treatment with EGF *in vitro* had three distinguishable activities: it increased the cell pool of intermediate progenitors, raised proliferation and augmented the fraction of symmetric neurogenic cell divisions within this population, and finally promoted neurogenesis. Infusion of EGF into the lateral ventricle of mouse embryos at E13 selectively increased the number of Tbr2-positive intermediate progenitor cells in the cortical subventricular zone *in vivo*. We conclude that the dynamic interplay between EGF and FGF-2 orchestrates the temporal order of birthdates of progenitor subpopulations by coordinating cell division modes, revealing the importance of the microenvironment of neural stem and progenitor cells.

Keywords: Neural stem cells, radial glia, cell division, EGF, FGF, intermediate progenitors, ECM, proteoglycans
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Cytokine directed differentiation and *in vitro* selection of hepatic hiPSC- and hESC-derivatives

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Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hIPSCs) hold great promise in regenerative medicine. Due to their merely unlimited self-renewal capacity and their ability to differentiate into derivatives of all three germ layers, these cells might serve as hepatic transplants for metabolic or acute liver diseases. So far, hepatic differentiation efficiencies of hiPSCs and hESCs are well studied, but enrichment of functional active hepatic derivatives for transplantation purposes need further attention. In our study, we aimed for an efficient hepatic differentiation protocol that is applicable for both, hESCs and hiPSCs. We were using a cytokine and small molecule based protocol for direct differentiation of hESC and hiPS cells into hepatic cells. Furthermore, we select the hepatic differentiated cells during differentiation using a hepatic promoter driven G418 resistance. Due to an IRES dependent eGFP reporter expression we were able to track the hepatic differentiated cells and evaluated the most efficient time-frame for G418 selection. The status of the hepatogenic differentiation was determined by qRT-PCR comparing the expression of hepatic markers as AFP, ALB, PCK, SOX17, Cyp1A1 and HNF4 to hepatic cell lines and the loss of pluripotency markers (Oct4, Sox2 and Nanog) to undifferentiated parental cells. Functional analyses of the hepatic phenotype were obtained by measurements of secreted albumin and by analyses of cytochrome P450 type 1A1 activity (EROD). The percentages of differentiated cells were quantified by FACS analyses. Finally, hESC-derived hepatic cells were transplanted into immunodeficient FAH-/- mice. Preliminary data indicate engraftment of transplanted cells into the host liver parenchyma and functional contribution to albumin synthesis. In conclusion, the modified protocol shows that both pluripotent cell sources (hESC and hiPSC) can be differentiated *in vitro* into hepatic cells with similar efficiencies. Further analyses need to reveal the repopulation efficiency of stem cell derived hepatic cells in relation to different pluripotent cell sources and improved differentiation protocols.

Keywords: Liver, regeneration, hiPS, ES cell
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Teratoma formation of murine embryonic stem cells as method for the characterization of pluripotency and differentiation

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Objective: Embryonic stem cell (ESC) sources and induced pluripotent stem cells have to be characterized concerning their pluripotency and differentiation potential *in vitro* and *in vivo*. Teratoma formation is a standard method for characterizing ESC *in vivo*, but for each stem cell source a suitable mouse model has to be chosen and to be standardized. Methods: In this study, mouse ESC line 7AC5/EYFP (derived from a 129S strain) was cultivated under feeder- and serum-free conditions. Teratoma formation was tested by subcutaneous transplantation of 7AC5/EYFP cells into immunocompetent 129S6 mice and immunodeficient NOD/SCID mice. To optimize the model different cell numbers (1x10*3 to 1x10*7) were injected and teratomas were investigated at different points of time. RT-PCR, immunofluorescence and FACS analysis were used to establish a marker profile which characterizes pluripotency and the differentiation status of original ESC *in vitro* and of derived teratomas *in vivo*. Furthermore, teratomas were histopathologically assessed to analyze the three germ layers (ectoderm, endoderm and mesoderm). Results: *In vitro* cultivated 7AC5/EYFP cells expressed typical markers of pluripotency. High RNA expression levels of Oct-3,4 and Sox2 and moderate level for Nanog were found. They stained positive for alkaline phosphatase. Expression of Lin28, Oct-3,4, Sox2 and Nanog was detected by immunofluorescence. Differentiation markers of the three germ layers were very low (AFP, aSMA and Pax6) or moderately (GATA4, Nodal and beta-3-tubulin) detected. After subcutaneous transplantation of 7AC5/EYFP cells into 129S6 mice, no teratoma growth was seen. However, in NOD/SCID mice rapid teratoma formation was observed within 21 days. Teratomas showed structures of the three germ layers and were comparable to undifferentiated ESC *in vitro*. They expressed lower levels of pluripotency markers and increased expression of markers of ectoderm (Pax6), endoderm (AFP) and mesoderm (aSMA). The expression profile and growth rate were independent of the injected cell number down to 1x10*4 cells. The growth rate of teratomas after injecting cell numbers lower than 1x10*4 was delayed. Passaging of teratomas in NOD/SCID mice resulted in growth of new teratomas. Expression profiles of primary teratomas were comparable to the derived secondary teratomas. No histopathological differences were observed. Conclusion: A teratoma formation assay, which enables the assessment of pluripotency of ESC *in vivo*, was established. A panel of markers was selected to measure developmental status and differentiation capacity of ESC *in vitro* and *in vivo*. This is a suitable tool for the characterization of ESC and teratomas.

Keywords: teratoma formation assay; murine embryonic stem cells (mESC); 7AC5/EYFP; pluripotency and differentiation markers

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Systemic mesenchymal stem cell transplantation for the treatment of skeletal muscle trauma

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Introduction Skeletal muscle trauma leads to severe functional deficits. Present therapeutic treatments are unsatisfying and insufficient posttraumatic regeneration is a problem in trauma and orthopaedic surgery. Mesenchymal stem cell (MSC) therapy is a promising tool in the regeneration of muscle function after severe trauma. Our group showed increased contraction forces compared to a non-treated control group 3 weeks after MSC local transplantation into an injured skeletal muscle. In addition we demonstrated a dose-response relationship between the amount of injected MSC and force enhancement. In this study we want to investigate the enhancement of muscle strenght after intra-arterial transplantation of MSCs and migration of the cells via *in vivo* imaging. Materials and Methods 18 female SD-rats received an open crush injury of the left soleus muscles. One week after trauma 2.5 x 10^6 autologous MSCs, harvested from tibial biopsies and transduced with dscopGFP-luciferase, were transplanted intraarterially (n=9). Control animals received saline (n=9). Migration of cells was followed for 7 days via *in vivo*-imaging with an IVIS® Lumina Imaging System and biomechanical evaluation by *in vivo* muscle force measurement was performed 3 weeks after transplantation. Results Twitch stimulation of the healthy right soleus muscles resulted in a contraction force of 0.52±0.14 N. Forces of tetanic contraction in the uninjured muscles reached 0.98±0.27 N. The intra-arterial. MSC-transplantation improved the muscle force of the injured soleus significantly compared to control (fast twitch: 82.4%, p=0.02, tetany: 61.6%, p=0.02). DscopGFP-luciferase marked MSCs could be detected *in vivo* with decreasing intensity over time. Discussion and Conclusions The presented study demonstrates the effect of systemic MSC-transplantation in the treatment of severe skeletal muscle injuries. The functional regeneration could be increased by intra-arterial application of MSCs. Furthermore we could follow the cells *in vivo*. For possible future therapeutic approaches a systemic application is considered to be favourable compared to local injections due to the better distribution of the cells in the target muscle.

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Epidermal differentiation of germline-derived pluripotent stem cells and embryonic stem cells on organotypic skin equivalents

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A general goal in stem cell-based tissue engineering is to generate complex three-dimensional structures mimicking the complexity of the developmental cues *in vitro*. During embryonic development, the skin is formed as a result of reciprocal interactions between mesoderm and ectoderm under the influence of different signaling pathways, such as the Wnt, Nodal and bone morphogenetic protein-4 (BMP-4) pathways. Embryonic stem cells (ES cells) can recapitulate such developmental steps *in vitro*. The generation of pluripotent cells from explanted murine testis without the introduction of exogenous transcription factors with tumorigenic potential allows converting somatic cells into ESC-like, germline-derived pluripotent stem cells (gPS cells). Thus, gPS cells represent a promising and not yet fully investigated novel cell type for tissue engineering. In the current study, we combined mesenchymal-epithelial interactions in an air-exposed, organotypic culture system of skin based on collagen I and III, and analyzed the differentiation capacity as well as the epidermal cell fate of ES cells and gPS cells in the presence and absence of BMP-4. Quantitative RT-PCR data of the pluripotency gene Oct-4 showed that gPS cells have a strong tendency to differentiate and are characterized by an accelerated differentiation capacity compared to ES cells. When subjected to organotypic culture conditions of skin, gPS cells formed complex tubulocystic structures, that were lined by a multilayered, stratified (CK5/6 , CK8/18—) epidermis in ≥ 50%. In contrast, ES cells revealed apoptosis and cell death under the air-exposed epidermal differentiation conditions and only formed small tubulocystic structures lined by simple, CK8/18 epithelia. BMP-4 neither enhanced the epithelial nor the epidermal differentiation of gPS cells. As monolayer cultures induced an epithelial, but not an epidermal differentiation of both pluripotent cell types, the air-liquid interphase seems to be a strong stimulus for epidermal differentiation of gPS cells. Further, gPS cells produced a dense eosinophilic basal membrane surrounding the epithelial structures and spontaneously differentiated into mesodermal and endodermal, but only in low levels into neuroectodermal cells. Concluding, we successfully differentiated gPS cells into keratinocytes generating a multilayered, stratified epidermis on organotypic skin equivalents and demonstrated their extensive differentiation potential resembling early embryogenesis. Among others, two challenges in the future will be the derivation of a pure population of keratinocytes excluding the risk of tumor/teratoma formation as well as the achievement of a plane growth of differentiating keratinocytes to form an anatomically correct epidermis.

Keywords: embryonic stem cells; germline-derived pluripotent stem cells; BMP-4; organotypic skin equivalents; epidermal differentiation
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Disease Modeling

Patient-specific induced pluripotent stem cells to model Dravet Syndrome *in vitro*

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Dravet Syndrome is a form of epilepsy known as severe myoclonic epilepsy in infancy (SMEI) usually starting with seizures within the first year of life and finally leading to a status epilepticus. Further characteristics are psychomotoric delay, ataxia and cognitive impairment. Unfortunately, common antiepileptic drug therapy is ineffective. The molecular cause for Dravet Syndrome is a variety of loss-of-function mutations in one allele of the SCN1a gene (including missense, nonsense and splice-site mutations). SCN1a codes for the pore-forming alpha subunit of voltage-gated sodium channel 1.1 (Nav 1.1), which is the major sodium channel of GABAergic interneurons localized in diverse brain regions including prefrontal cortex, hippocampus and spinal cord. In the mouse, Nav 1.1 haploinsufficiency causes reduced sodium currents and impaired action potential firing. In humans, the study of Dravet Syndrome is hampered by poor access to patient-specific primary neurons. To overcome this limitation, we aim at the derivation of primary Dravet-specific neurons from human induced pluripotent stem cells (hiPSCs). Fibroblasts of Dravet patients co-transduced with OCT4, SOX2, KLF4 and c-MYC gave rise to hiPSC lines with typical hESC-like morphology and pluripotency marker expression. Pluripotency of the cells was further confirmed by multilineage differentiation *in vitro* as well as teratoma formation *in vivo*. Since prominent Nav 1.1 expression has been observed in GABAergic neurons, we used an *in vitro* differentiation protocol, which yields a long-term self-renewing neuroepithelial stem cell (lt-NESC) population with a strong preponderance for GABAergic differentiation. Dravet-specific lt-NESCs express the neural stem cell markers PAX6, SOX2 and NESTIN, exhibit a rosette-like morphology and can be proliferated extensively without losing their neurogenic potential. Differentiated lt-NESCs derived from patient-specific and control hiPSCs show robust expression of mutated and wild-type SCN1a alleles. RT-qPCR analyses of Dravet-specific neuronal cultures differentiated from lt-NESCs revealed a 50% reduction in wild-type SCN1a expression. Neuronal maturation could be confirmed also on a functional level with lt-NESC-derived neurons exhibiting voltage-dependent inward and outward currents, action potential generation and spontaneous synaptic activity. Thus, lt-NESCs should provide a versatile and, in principle, unlimited source of Dravet-specific neurons for downstream analyses focusing at the functional consequences of the various Nav 1.1 mutations associated with this disease.

Keywords: Dravet syndrome; severe myoclonic epilepsy in infancy; voltage gated sodium channels; induced pluripotent stem cells; somatic cell reprogramming
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Induced pluripotent stem cell-based *in vitro* modelling of Metachromatic Leukodystrophy

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Metachromatic leukodystrophy (MLD) is a lysosomal storage disorder caused by a deficiency of the enzyme arylsulfatase A (ASA), which in turn leads to an intralysosomal accumulation of the sphingolipid cerebroside sulfate (sulfatide). In MLD patients, sulfatide accumulates mainly in the myelin-forming cells, oligodendrocytes in the central and Schwann cells in the peripheral nervous system, resulting in demyelination and severe neurological symptoms. The precise pathomechanisms leading to cell degeneration are still largely unknown. A human *in vitro* model based on disease-specific oligodendrocytes could provide a tool for studying the pathogenesis of the disease. In this work, induced pluripotent stem cells (iPSC) derived from MLD-specific fibroblasts were converted into multipotent radial glia-like neural stem cells (NSC) with strong gliogenic potential (glioNSC) expressing nestin, PAX6, SOX2, musashi, BLBP, RC2, 3CB2 and vimentin. ASA enzyme activity was significantly decreased in MLD-glioNSC compared to healthy control cells, confirming a disease-related phenotype. In addition, immunofluorescence using an antibody to sulfatide and lipid extraction with subsequent thin layer chromatography revealed disease-relevant accumulation of sulfatide in MLD-glioNSC-derived oligodendrocytes. We expect this iPSC-based model system to provide insight into the molecular pathogenesis of MLD and to facilitate the identification of pharmaceutical compounds for the treatment of this disease.

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Keywords: metachromatic leukodystrophy, induced pluripotent stem cells, oligodendrocytes
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Derivation of induced pluripotent stem (iPS) cells from a patient with catecholaminergic polymorphic ventricular tachycardia (CPVT)

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Human iPS cells are very similar to human embryonic stem (ES) cells but do not require an embryo for their derivation. They can be derived from patients with complex genetic defects to create *in vitro* disease models and thus represent an opportunity to study disease pathophysiology, develop new drugs and test methods for delaying disease progression or reversing its phenotype. We have generated iPS cells from a patient suffering from catecholaminergic polymorphic ventricular tachycardia (CPVT) carrying heterozygous autosomal dominant mutations (p.S408P and p.A1136V), which affect the calcium channel in the sarcoplasmic reticulum, the Ryanodine Receptor 2 (RyR2), bringing about stress-induced arrhythmias and syncope. Retroviral overexpression of four transcription factors Oct4, Sox2, c-Myc and Klf4 was used to induce pluripotency in the patient-derived dermal fibroblasts. These iPS cells show a human ES cell-like colony morphology, express pluripotency markers at the protein (alkaline phosphatase, Tra-1-81, Tra-1-60, OCT4, NANOG, and SSEA4) and transcript level (OCT4, SOX2, NANOG, REX1), and exhibit the methylation pattern in promoter regions of OCT4 and NANOG genes, which is undistinguishable from that of conventional ES cells. In addition, CPVT-iPS cells carry the same genotype and disease-specific mutation as parental somatic cells, possess normal karyotype, form teratomas in immunodeficient animals and differentiate to spontaneously beating cardiomyocytes *in vitro*. Further analyses are being carried out to assess the functional properties of CPVT-specific cardiomyocytes so as to determine whether they recapitulate the patient's disease phenotype *in vitro*. Specifically, electrophysiological analyses elucidating function of the calcium sensing mechanism aided by recordings of calcium transients and calcium sparks is being performed. The CPVT-specific iPS cells generated in this study may serve as a replenishable source of cardiomyocytes for disease modelling and drug discovery.

Note: The following persons also contributed to this work but due to the limitations in the allowed number of listed authors they could not be included in the main list under the title: Martin Farr (Heart and Diabetes Center NRW, University Hospital of the Ruhr, University of Bochum), Martin Lehmann (Institute for Neurophysiology, University of Cologne), Matthias Linke, Ulrich Zechner, Vera Beyer (Institute for Human Genetics, Johannes Gutenberg University, Mainz) and Hans-Christian Hennies (Cologne Center for Genomics, University of Cologne).

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Cardiomyocytes from LQTS 3 specific iPS cells and from LQTS 3 mice have a similar disease related phenotype

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Induced pluripotent stem (iPS) cells can be generated from somatic cells and can be differentiated in to all cell types including cardiomyocytes. This method opens the possibility to investigate cardiac disorders *in vitro*. The long QT syndrome 3 (LQTS 3) is a severe inheritable cardiac disease characterized by prolonged action potential durations, early after depolarizations (EADs) in cardiomyocytes especially at low heart rates and eventually leads to sudden death. LQTS 3 in humans is mainly caused by the DeltaKPQ deletion of the cardiac sodium channel (Scn5a). Therefore we have used a mouse model with this mutation (Scn5aDelta/+) to generate iPS cells and embryonic cardiomyocytes. IPS cells were obtained by retroviral transduction of murine embryonic fibroblasts from wild-type and Scn5aDelta/+ mice either with the three factors Oct4, Sox-2, and Klf4, or additionally with the fourth factor c-Myc. Pluripotency was proven by positive staining for Oct4 and SSEA1 as well as by teratoma formation in SCID mice. Wild-type and Scn5aDelta/+ iPS cells could be differentiated *in vitro* into all three germ layers including spontaneously beating cardiomyocytes. Gene expression analysis showed a decrease of stem cell markers (Nanog and Oct4) and an increase of cardiac specific markers (Myh6, Scn5a, Hcn4, Kcnb1, Cacna1c, Acnt2) during differentiation of wild-type and Scn5aDelta/+ iPS cells. For phenotypical characterization single cardiomyocytes from *in vitro* differentiated wild-type and Scn5aDelta/+ iPS cells were obtained by enzymatic dissociation and investigated using patch clamp experiments. For comparison to murine cardiomyocytes, embryonic hearts form Scn5aDelta/+ matings were individually dissociated and used for genotyping to identify Scn5aDelta/+ and wild-type preparations retrospectively. Action potentials were evoked by current injection at various frequencies and the action potential duration at 90% of repolarization (APD90) was analyzed. Both cardiomyocytes from Scn5aDelta/+ iPS cells and Scn5aDelta/+ embryos showed a prolongation APD90 at low pacing rates (<0.5 Hz). For quantification the APD90 at various pacing periods were plotted for each individual cell and the slope of this relationship was determined by a linear regression. This yielded a similar flat slope in wild-type iPS cell-derived (-2.5 ± 1.4 ms/s, n =8) and embryonic-derived (-2.7 ± 1.5 ms/s, n =8) cardiomyocytes. In contrast the slope was significantly ($p<0.05$) different and steeply positive in Scn5aDelta/+ iPS cell-derived (9.9 ± 3.7 ms/s, n =11) and Scn5aDelta/+ embryonic-derived (5.7 ± 2.2 ms/s, n =16) cardiomyocytes. Furthermore, EADs were observed at low pacing rates in 53.8% of iPS cell-derived and in 50% of embryonic-derived Scn5aDelta/+ cardiomyocytes but never in wild-type. We conclude that cardiomyocytes derived from LQTS 3 iPS cells preserve the electrophysiological phenotype of the disease *in vitro* and are comparable to the LQTS 3 embryonic-derived cardiomyocytes. We therefore suggest that disease-specific iPS cells could be used for patient-specific drug screening.

Keywords: iPS, Long QT 3 syndrome, cardiac, differentiation, murine
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Modified mesenchymal stem cells attenuate bleomycin induced lung injury in the rat

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Hepatocyte growth factor (HGF) gene transfer to the bleomycin rat lung has shown to attenuate fibrosis. Bone marrow derived mesenchymal stem cells (BMSCs) were shown to localize in the fibrotic areas in the injured lung & may be utilized as carriers for novel therapies. In the present study we hypothesize that HGF modified BMSCs exhibit potent antifibrotic and regenerative effects in the bleomycin induced lung injury model. BMSCs were isolated from adult rats and transfected by in vitro electroporation. Adult rats were instilled with bleomycin intratracheally at day 0; 7 days later HGF transfected BMSCs were instilled intratracheally, & animals sacrificed at day 7 & 14 post BMSC. Other group with only BMSC instillation after bleomycin served as control. The instillation of the HGF transfected BMSCs markedly attenuated bleomycin induced fibrosis in the rat lung; the hydroxyl proline content of the rat lung was 2446 ± 277.5ug/gm vs 3066 ± 251.4 ug/gm at day 7 ($p<0.05$) and 1487 ± 110.1 ug/mg at day 14 post HGF-BMSCs instillation. The Ashcroft score in the HGF modified BMSCs was 3.9±0.2 vs 4.42±0.36 in the control group at day 7, at day 14 further improvement was seen. Stereological analysis showed decreased septal thickness after 1 week (11.89 ± 0.91µm vs 8.82 ± 9.485 µm), and increased alveolar surface area (2.29 ± 0.71m² vs 1.40 ± 0,18m²) after 2 weeks in the treated group. The volume fraction (9.32 ± 1.02% vs 4.13 ± 2.81%) and total volume per lung (0.40 ± 0.10cm³ vs. 0.20 ± 0.10cm³) of destructed/ fibrotic lung tissue was further reduced 2 weeks after therapy. HGF modified BMSCs markedly attenuate bleomycin induced lung injury. Modified BMSCs may serve as a promising, novel therapeutic strategy to improve lung fibrosis.

Keywords: lung regeneration, homing, disease modeling
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Modelling protein aggregation in Machado-Joseph Disease using patient-specific iPS cells

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Machado-Joseph Disease (MJD; syn. spinocerebellar ataxia type 3; SCA3) is a dominantly inherited late-onset neurodegenerative disorder caused by expansion of polyglutamine (polyQ)-encoding CAG repeats in the MJD1 gene. Proteolytic cleavage of the MJD1 gene product, ataxin-3 (ATXN3), is believed to trigger the formation of ATXN3-containing aggregates, the neuropathological hallmark of MJD. Here we report the generation of induced pluripotent stem (iPS) cell-derived neurons from a patient with MJD and his non-affected sibling. We demonstrate that L-glutamate-induced excitation of MJD neurons is sufficient to initiate cleavage of ATXN3 and the formation of SDS-insoluble aggregates. Aggregate formation was efficiently blocked by the calpain inhibitors N-acetyl-Leu-Leu-norleucinal (ALLN) and calpeptin but not by caspase inhibition, indicating that excitation-induced activation of calpains is sufficient to generate aggregation-competent proteolytic polyQ fragments. Thus, our study illustrates the powerful potential of iPSC-derived cellular models to identify fundamental pathophysiological processes in human neurodegenerative disorders.

Keywords: iPS cells, disease modelling, neural differentiation, ataxia
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Mesenchymal stem cells - Key players in enhanced vascular calcification in chronic kidney disease patients?

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Once considered as a passive process, vascular calcification has emerged as a tightly regulated, coordinated and osteoblastic process resembling bone morphogenesis. Executive cell types familiar to bone biology are seen in calcified vasculature. As osteoblasts, smooth muscle cells, adipocytes, fibroblasts and chondrocytes all share a common mesenchymal progenitor stem cell, recent studies suggest that mesenchymal stem cells (MSC) contribute to the ectopic osteogenic program of vascular calcification. Uremic patients exhibit an excessive cardiovascular morbidity and calcifications. We analysed the effect of uremic serum on mesenchymal stem cells (MSC) regarding key events in vascular calcification, i.e. proliferation, apoptosis, osteogenic differentiation and procalcific extracellular matrix (ECM) remodelling. Human MSC were cultured in media supplemented with pooled sera from either healthy or uremic patients (20%). Results were compared to MSC cultured in growth medium as well as osteogenic and adipogenic differentiation medium. Using collagen scaffolds, we analysed the influence of uremic serum on ECM synthesis and osteogenic differentiation in a three-dimensional microenvironment. Exposure to uremic serum enhanced the proliferation of MSC (cell counting, BrdU incorporation) whereas apoptosis and necrosis were not affected (annexin V and 7-AAD staining). MSC exposed to uremic serum, did not terminally differentiate into osteoblasts after 21 days, but showed a pre-osteoblast phenotype characterized by significant BMP2-receptor up-regulation followed by alkaline phosphatase and collagen I expression as well as extensive matrix synthesis. After a prolonged culture period of 35 days exposed to uremic serum, the dense ECM surrounding MSC contained crystalline calcium and phosphate as shown by von Kossa staining, energy-dispersive X-ray spectroscopy and calcium measurements. The calcification was accompanied by significant up-regulation of osteoblast-specific genes. Interestingly, BMP 2/4 blockade via a blocking antibody prevented the osteogenic differentiation and matrix calcification under uremic and osteogenic cultivation conditions. In conclusion, MSC exposed to uremic serum recapitulate osteogenesis characterized by extensive ECM remodelling and matrix calcification. Our data support the hypothesis of MSC as critical cells for pro-calcific ECM remodelling in chronic kidney disease patients.

Keywords: vascular calcification; mesenchymal stem cells; chronic kidney disease; uremia; ECM remodelling; osteogenic differentiation
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Generation of retinal pigment epithelial cells from human iPS cells reprogrammed using two exogenous transcription factors and small molecules

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The recent development of technologies capable of producing induced pluripotent stem (iPS) cells from adult somatic tissues may permit their use to generate autologous retinal pigment epithelium (RPE) grafts for the treatment of diseases like age-related macular degeneration (AMD). Current reprogramming techniques require retroviral transduction of four transcription factors which in part possess oncogenic potential. Given the associated risk of tumor formation, the use of iPS cells reprogrammed with a reduced number of these factors would be advantageous. We therefore evaluated human iPS cells that were generated using only two exogenous transcription factors for their capacity to differentiate into RPE cells. iPS cells were generated from primary human epidermal keratinocytes by lentiviral two factors transduction (Oct4, Klf4) and additional treatment with small molecules or, alternatively, from human fibroblasts using standard four factor transduction (Oct4, Sox2, Nanog, Lin28). RPE cells could be differentiated from both two factor- and four factor-derived iPS cells. These cells exhibited homogenous polygonal morphology and pronounced pigmentation, formed epithelial monolayers with intracellular tight junctions, and expressed RPE-specific markers (bestrophin, CRALBP, RPE65). Moreover, the cells phagocytosed photoreceptor outer segments and exhibited barrier function with apical-to-basolateral fluid transport. Thus, we demonstrate the capability of two factor-derived human iPS cells to differentiate into cells with RPE-specific morphology and function. Further optimization of reprogramming and differentiation efficiency is crucial for future therapeutic application of iPS cell-derived RPE cells as autologous grafts in diseases like AMD.

Keywords: Retina; retinal pigment epithelial cells; small molecules; differentiation; age-related macular degeneration
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Adipogenic lineage differentiation and its influence on primary aortic andothelial cells as a model for the metabolic syndrom

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Adipositas is a well known risk factor for diseases such as diabetes or atherosclerosis. In this, mainly the differentiating adipocytes and not the fully differentiated adipocytes are metabolically active. To investigate the influence of differentiating adipocytes on primary endothelial cells, human mesenchymal stem cells have been differentiated towards the adipogenic lineage to collect the secreted adipokines within the medium. Primary endothelial cells isolated from bovine aorta have been exposed to the conditioned medium. The migration was investigated in a wound assay model. To access the underlying mechanisms of the cell migration on a molecular level, ROBO1 and ROBO4 were investigated, since the expression of these transmembrane receptors, has been found to be involved in the promotion of vascular stability. Thus their expression was investigated on a molecular level. The cell migration was increased under the influence of secreted adipokines in a proliferation independent manner. Simultaneously the expression of ROBO1 and ROBO4 was down regulated. These data give new preliminary insights into the underlying mechanisms of endothelial dysfunction under the influence of a developing adipositas, by using mesenchymal stem cells in a wound assay model.

Keywords: mesenchymal stem cell; adipogenesis; endothelial cells; migration; ROBO 1 / 4
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Trim32 regulates skeletal muscle stem cell differentiation and is necessary for normal adult muscle regeneration

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Limb girdle muscular dystrophy type 2H (LGMD2H) is an inherited autosomal recessive disease of skeletal muscle caused by a mutation in the TRIM32 gene. Currently its pathogenesis remains unclear. TRIM32 regulates the fate of mammalian neural progenitor cells through controlling their differentiation. Given that skeletal muscle harbours a tissue specific stem cell population termed satellite cells that controls the regeneration process of adult skeletal muscle during growth or following injury, we asked whether TRIM32 could also be essential for the regulation of these myogenic stem cells. Here we demonstrate for the first time that TRIM32 is expressed in the skeletal muscle stem cell lineage of the adult and plays a key role in controlling muscle stem cell differentiation. Moreover, we show that the ubiquitin ligase TRIM32 controls this process through the regulation of c-Myc, a similar mechanism to that previously observed in neural progenitors. Importantly we show that in the absence of TRIM32, skeletal muscle develops a LGMD2H like phenotype and is greatly perturbed in its rate of *in vivo* regeneration. Our studies provide evidence that the loss of TRIM32 results in dysfunctional muscle stem cells which could contribute to the development of LGMD2H.

Keywords: Trim32, satellite cell, LGMD2H, muscle regeneration
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Cell-mediated transgenesis in rabbits: Chimeric and nuclear transfer animals

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Rabbits are important laboratory animals and used in a wide range of biomedical research areas. These would benefit considerably if cell mediated transgenic techniques such as gene targeting could be established in rabbit. However, rabbits have so far proved unusually refractory to the key enabling technologies: embryonic stem cells (ESC) and somatic cell nuclear transfer (SCNT). Therefore, we established and examined both pluripotent and multipotent stem cells for their ability to produce viable animals by nuclear transfer. Rabbit putative ESCs were derived and shown capable of *in vitro* and *in vivo* pluripotent differentiation. We obtained the first reported live born ESC-derived rabbit chimera. Rabbit mesenchymal stem cells (MSC) were derived from bone marrow and their multipotency was demonstrated by *in vitro* differentiation. Nuclear transfer was performed with both ESCs and MSCs. After embryo transfer, the development of resulting embryos was assessed *in vitro* and *in vivo*. As nuclear donors, MSCs were markedly more successful than ESCs. Moreover, MSCs were transfected with fluorescent reporter gene vectors and assessed for nuclear transfer competence. Transfected MSCs supported the development with similar efficiency as normal MSCs and resulted in the first live cloned rabbits from genetically manipulated MSCs. The expression of reporter genes in reconstructed embryos was investigated as a means of identifying viable embryos *in vitro*, but was not found to be a reliable indicator. Additionally, we examined serial nuclear transfers as a means of rescuing dead animals.

Keywords: rabbit; embryonic stem cell; mesenchymal stem cell; somatic cell nuclear transfer
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Ethical, Legal & Social Issues

The challenging search for new stem cell research rules between the poles of the recent preimplantation genetic diagnosis sentence of the Federal Supreme Court and the impact of reprogrammed stem cells in Germany

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A surprising decision of Germany's Federal Supreme Court in a preimplantation genetic diagnosis (PGD) case and groundbreaking discoveries in reprogramming pluripotent stem cells (iPSC) may facilitate a revision of Germany's stem cell law. On the one hand, the recent ruling of the Federal Supreme Court, which now allows discarding certain PGD embryos, sheds new light on Germany's stem cell law and may help to ease existing restrictions in stem cell research. On the other hand, the discovery of iPSC coincided with the latest amendment of Germany's stem cell law and could have been almost a reason to legally cancel further research with embryonic stem cells in Germany, because iPSC were seen as a scientific alternative for the use of human embryonic stem cells. On July 6, 2010, the German Federal Supreme Court decided that certain PGD methods are permissible under the German Embryo Protection Act. That decision is surprising because one consequence of PGD is the necessity of discarding those embryos in which genetic disorders have been detected. This finding seems contrary to the central provision in the German Embryo Protection Act, which states that every usage of the embryo that does not guarantee the maintenance of the embryo *in vitro* is prohibited. Therefore, this ruling may pave the way for a new legal understanding of *in vitro* embryos and. If it is now permissible to discard certain PGD embryos, it must be clarified whether those embryos must be discarded or if they could be used for research purposes. If it was permissible to use PGD embryos for research purposes, should it be also permissible to use surplus *in vitro* fertilisation embryos for research purposes? After political debates and pressure by the scientific community, German lawmakers amended the German Stem Cell Act in 2007. The German Stem Cell Act states the provisions for the import and work with imported human embryonic stem cells in Germany. The discovery of less ethically charged iPSC during the amendment process of the German Stem Cell Act could have put a total stop to German human embryonic stem cell research. It was argued, that the discovery of the iPSC as an alternative for embryonic stem cells would be the right time to exit completely from research with human embryonic stem cells in Germany. Instead of accepting this opinion the lawmakers only changed the import provisions. However, since its first announcement, the German Stem Cell Act has contained a subsidiary provision, which states that the import of human embryonic stem cells to Germany is not permitted if there is a scientific alternative for the use of human embryonic stem cells. The scientific and medical success of reprogramming research could therefore legally inhibit the further import of and research with human embryonic stem cells in Germany, if iPSC were an alternative for the use of human embryonic stem cells. The subsidiary provision could therefore also be a possibility to stop or to hinder the further embryonic stem cell research in Germany without any changes in the law.

Keywords: stem cell law; induced pluripotent stem cells; preimplantation genetic diagnosis
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Cell-based medicinal products require a unique approach for the development of GMP-compliant processes and manufacturing of safe and high-quality products

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When performing the GMP process development and scale up of cellular therapies, a critical review of the manufacturing process and all the materials and reagents involved in the production steps is the mandatory starting point to avoid potential issues related to the quality and safety of the product. The choice of the raw materials and all components such as plastics and other equipment that comes into direct contact with the product must be performed always keeping in mind that the cells as drug products cannot be terminally sterilized. The quality of the materials and reagents utilized is therefore directly related to the quality and degree of purity of the final product. Information about the available certification must be gathered for every component and, for critical materials, audits must be performed to the manufacturing sites to qualify the supplier. The protocol used for the cell expansion and processing must be designed trying to reduce at a minimum the dependance on growth factors and medium supplements. Each additional component that is added to the culture medium must be justified and its absence from the final product must be validated. Supplements such as FBS are still allowed for the manufacturing of cellular therapies, as long as the serum is sourced from a TSE-free area. Anyway, the choice of a medium with FBS must be done only in the absence of efficient alternatives. In this case, continuos research and development is strongly advised at the laboratory level in order to keep up to date with the latest developments in medium formulations, being ready to switch to an animal-free medium as soon as it is feasible. The reduction of growth factors and supplements is also important in order to control the manufacturing costs of a cell therapy. An evaluation of the economical aspects and market sustainability should be performed at an early stage if an industrial development of the cellular product is desired. The manipulation steps performed during manufacturing stage should be kept to a minimum, in order to reduce the human intervention and the possibility of contaminations. Media fill simulations must be performed in purposely stressed conditions to ensure that the process and the facility are able to support the production of a sterile product. When manufacturing patient-specific therapies, extensive efforts should be directed to reduce the variability of starting material, usually a tissue sample from the patient. Working with well-defined starting material allows for the set-up of a robust process with comparable characteristics between batches.The specifications of the final product for parameters such as cell number, purity and potency must be wide enough to tolerate the normal biological variability of living organisms, but sufficiently narrow to generate comparable batches of drug. This uniformity is mandatory for the set up of clinical trials aiming at gathering a reliable analysis of the safety, tolerability and efficacy data obtained from treated patients, in order to speed up the clinical development of innovative medicinal products such as cellular therapies.

Keywords mesenchymal; GMP; regulatory; quality; manufacturing
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Homing & Migration

Intracellular labeling of multiple cell types for MRI-based *in vivo* cell tracking

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Cell transplantation is an area of intense investigation especially with respect to stem cell therapy and transplantation studies of embryonic stem cell derived cell-types for treatment of degenerative diseases like Parkinson or heart failure. There is a need to serially image cells after intravenous administration or direct injection into tissue, in order to track migration into the target tissue. Monitoring the location and migration of grafted (stem) cells is essential for understanding their interaction within the host and their therapeutic effects. Therefore, cell tracking is of significant importance for basic research, preclinical evaluation as well as monitoring of early clinical trials applying cell transplantation. Magnetic resonance imaging (MRI) is the most frequently used technique for *in vivo* cell tracking applications due to strong high resolution of soft tissues, which makes it especially useful for imaging of the brain, muscles or the heart. Detectability of transplanted cells by MRI depends on their contrast characteristics. In order to produce a strong contrast against surrounding tissue, intracellular labeling of cells with iron oxide particles before transplantation has been described. We have established a two component protocol for *in vitro* intracellular labeling of multiple cell types with superparamagnetic iron oxide (SPIO) particles. Contrast particles and loading reagent were optimized for proper *in vitro* labeling of cell lines (NIH-3T3, Jurkat), primary cells (granulocytes, neural progenitors) and stem cells (hematopoietic, mesenchymal and embryonic stem cells) from different species (mouse, rat, human). Highly efficient intracellular labeling of various cell types with contrast particles was proven by prussian blue staining and anti-dextran immunofluorescence analysis. Intracellular labeling neither affected viability nor proliferation. Additionally, similar results with labeled and unlabeled hematopoietic stem cells, mesenchymal stem cells or embryonic stem cells were obtained in CFU assays and upon differentiation into osteoblasts, adipocytes and neurons, indicating biocompatibility of intracellular labeling with contrast particles. In order to evaluate feasibility of the newly developed SPIO particles for magnetic resonance imaging, we analyzed intracellular labeling of neural progenitors after transfection of contrast particles. MRI analyses revealed an *in vitro* detection limit of 250 labeled cells. After transplantation into mouse cortex, intracellular labeled cells were clearly identified with high field MRI scanners. In contrast, extracellular labeling of cells with MicroBead-conjugated antibodies failed to give similar signal intensities. We have developed a new method for *in vitro* intracellular labeling of multiple cell types with SPIO particles without affecting cellular characteristics or fates for high resolution MRI-based cell tracking.

Keywords: cell tracking; MRI; intracellular labeling; SPIO; stem cells
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Potential implications of vascular wall-resident progenitor cells

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New formation of blood vessels has undoubtedly been shown to be essential in physiologic as well as pathologic processes such as tumor growth and metastasis and cardio-vascular diseases. While until a decade ago it was generally accepted that new blood vessels in the adult are only provided by angiogenesis the discovery of endothelial progenitor cells (EPC) circulating in the peripheral blood and their contribution to new formation of blood vessels led to crucial revision of this concept. Today, it is widely accepted that new vessels in the adult are formed by both angiogenesis and postnatal vasculogenesis. Thus, it is essential to know where EPC and/or other progenitors contributing to the morphogenesis of the vascular wall are residing and how they are mobilized during new vessel formation. While the role of the circulating and bone marrow-derived EPC has intensively been studied despite some still controversial findings the potential contribution of the vascular wall itself to new vessel formation was neglected for a long time. Evidence provided during the last few years strongly suggests the existence of self-renewal potential in the vascular wall by the presence of not only EPC but also other progenitor cell types in distinct zones of the vascular wall supporting vascular morphogenesis. Here we show that not only embryonic and fetal aortas as well as adult human blood vessels harbour vascular wall-resident EPCs (VW-EPCs) and hematopoietic stem cells (HPCs) clearly indicating the presence of stem cell niches outside the bone marrow and the peripheral blood. Moreover, the vascular wall seems to harbour other types of progenitor cells which are capable to differentiate to smooth muscle cells, pericytes, fibroblast and macrophages. Additionally, the vascular adventitial layer which predominantly contains these progenitor cells produces factors such as SDF-1 which acts as a chemoattractant for recruited bone marrow-derived circulating cells and guides them to the vascular adventitia. These cells enhance angiogenic activities of endothelial cells via secretion of pro-angiogenic factors. It becomes clear that EPCs and other types of progenitors and cells accumulated in the vessel wall build a pro-angiogenic and pro-vasculogenic potential within the vascular wall which is of relevance not only for repair and self-renewal of vascular wall cells or vasa vasorum but also for local capacity of neovascularization in disease processes such as growth of tumor, metastatic lesions, atherosclerotic plaques and revascularization of ischemic tissue. The understanding of this potential in the vascular wall is also relevant for therapeutic manipulations.

Keywords: VW-EPC, MSC, tumor vascularization
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In vivo imaging of i.m. transplanted MSCs in rat

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INTRODUCTION Autologous mesenchymal stem cells (MSCs) have been shown to improve the functional outcome after skeletal muscle trauma. In previous work our group has shown a dose-response relationship between the amount of transplanted cells and the resulting muscle force. For our experimental work it was necessary to set up a reliable tracking system for the localisation of transplanted MSCs to evaluate distribution and migration of the cells *in vivo*. For this purpose a viral system was used which inserts copGFP alternatively dscopGFP-luciferase into the DNA of the MSCs. Cells expressing these proteins could be observed *in vitro* and *in vivo* by fluorescence microscopy and live-Imaging via IVIS® Lumina. To ensure that these proteins did not alter the function of the MSCs, a differentiation and proliferation assay was conducted. MATERIALS AND METHODS Bone marrow (BM)-derived MSCs were isolated from harvest of BM from tibial biopsies. The MSCs were transduced via a lentiviral system to insert copGFP or dscopGFP-luciferase (LV510A-1, TR011PA-1; Lentivector Expressing Systems; System Biosciences) into the DNA of the cells. The transduction was performed by adding the HEK cell produced virus to the adherent MSCs. After five days incubation at 37°C and 5% CO2 the expression of copGFP/luciferase was detectable. Female Sprague Dawley rats received an open crush trauma of the left soleus muscles. One week after trauma 1.5 x 10E6 of protein expressing MSCs were transplanted locally into the traumatized muscle. *In vivo* data of the transplanted cells were obtained by examination of the animals in an IVIS® Lumina Imaging System (VivoVision System). Measurements were carried out immediately after transplantation as well as 1, 2, 4 and 7 days post transplantation. The differentiation assays were performed over a period of 21 days. 2.4 x 10E4 cells per well were stimulated with osteogenic or adipogenic media and stained with Alizarin Red and Oil Red for photometric quantification. RESULTS After an effective transduction of MSCs with dscopGFP-luciferase we performed a successful transplantation of the cells. The locally transplanted MSCs could be detected for a period of 48 hours in the muscle with decreasing intensity (day 1 = 3.42 x 10E6 Photons, day 2 = 7.33 x 10E5 Photons). The copGFP- or luciferase expressing cells showed no significant differences in their differentiation and proliferation potential compared to non-transduced MSCs. CONCLUSION The described *in vivo* tracking methods are excellent for experimental set ups with the local transplantation of stem cells. They did not alter MSCs differentiation, survival and function in the investigated parameters and allowed the detection of the cells. This method may be used to quantify surviving cells in the target tissue compared to cells applied on day 0.

Keywords: MSC, Differentiation, ***in vivo*** **imaging, GFP, luciferase,**
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Hybrid cells derived from spontaneous fusion events between human mammary breast epithelial cells and breast cancer cells exhibit altered migratory properties

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Several data of the past 10 to 15 years provided evidence that tumor cell x tumor cell and tumor cell x normal cell hybrids exhibited novel properties, such as an increased drug resistance, an increased resistance to apoptosis, an increased proliferation as well as enhanced metastatic capability, which can be associated with cancer progression. Here we investigated the migratory activity of M13HS-X and M13MDA435-X (X marks the clone number) hybrid cells, which derived from spontaneous fusion events between the human M13SV1-EGFP-NEO mammary breast epithelial cell line exhibiting stem-like characteristics and the human breast cancer cell lines MDA-MB-435-Hyg and HS578T-Hyg, respectively. Analysis of cell migration by applying the 3D collagen matrix migration assay combined with computer-assisted cell tracking revealed that each hybrid cell clone exhibited a unique migratory behavior. For instance, M13MDA435 hybrid clones -1,-2, -4 responded to EGF stimulation with an increased migratory activity, whereas the locomotory activity of parental MDA-MB-435-Hyg breast cancer cells was rather blocked by EGF. Flow cytometry data indicated that MDA-MB-435-Hyg were positive for c-erbB-2 and erbB3, but negative for EGFR expression. By contrast, M13MDA435 hybrid clones -1,-2, -4 as well as M13SV1-EGFP-Neo mammary epithelial cells expressed all three members of the EGFR family, thus exhibiting a functional EGFR family member machinery. Analysis of signal transduction pathways initiated by EGFR/c-erbB-2 and c-erbB-2/erb3 heterodimer signaling showed that the PLC-γ1 pathway, the PI3K/AKT pathway and the Ras-Raf-MAPK pathway were activated in M13SV1-EGFP-Neo cells, whereas in M13MDA435 hybrid clones -1,-2, -4 EGF stimulation only resulted in the initiation of the PI3K/AKT pathway and the Ras-Raf-MAPK pathways. By contrast, non of these pathways were activated in MDA-MB-435-Hyg breast cancer cells. Similar findings have been observed for M13SV1-EGFP-Neo mammary epithelial cell, HS578T-Hyg breast cancer cells and the hybrid clones M13HS-2 and M13HS-8. Here, the parental tumor cell lines and the hybrids were positive for CCR7, whereas the mammary epithelial cells were negative for this chemokine receptor. Cell migration data revealed that M13SV1-EGFP-Neo cells did not respond to CCL21 treatment, whereas the migratory activity of HS578T-Hyg breast cancer cells was rather impaired by this chemokine. By contrast, both M13HS-2 and M13HS-8 hybrid clones responded to CCL21 treatment with an increased locomotory activity. Analysis of signal transduction cascades indicated treatment of M13SV1-EGFP-Neo breast epithelial cells with U73122 and Ly294002 did not alter the cells locomotory activity. Co-treatment of HS578T-Hyg breast cancer cells with U73122 and CCL21 resulted in a markedly reduced migratory activity, whereas the CCL21-mediated impaired migration of HS578T-Hyg cells was completely abrogated by the PI3K inhibitor Ly294002. These data suggest that the inhibitory effect of CCL21 on HS578T-Hyg cells is transmitted via the PI3K/AKT pathway. By contrast, both inhibition of PLC-γ1 activity and PI3K activity blocked the CCL21 induced migration of M13HS-2 and M13HS-8 hybrid cells. In summary, our data indicate that due

to cell fusion the non-migratory phenotype of a (parental) cancer cells can be switched towards a pro-migratory phenotype. Moreover, since both EGF and CCL21 have been associated with the (organ)-metastatic spreading of breast cancer cells our findings let assume that cell fusion not only could lead the origin of migratory active tumor cells, but also to organ-specific metastatic spreading cancer cells.

Keywords: cancer stem cells; migration; EGF; CCL21; SDF
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TBF-treated adipose tissue-derived stem cells increase the migratory activity of endothelial cells *in vitro*

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Introduction: Adipose tissue-derived stem cells (ASC) express the mesenchymal stem cell (MSC) markers CD44, CD68, CD105 and CD166 and can differentiate along different lineages. Since MSC are known to have immunomodulatory effects and since freshly isolated ASC express the perivascular marker CD34, we investigated whether inflammatory stimulation of ASC influences migration of human dermal microvascular endothelial cells (HDMEC). To this end, we treated ASC with tumor necrosis factor (TNF), transferred the cell culture supernatant to a culture of HDMEC and observed the migratory activity of the endothelial cells in Scratch and Boyden Chamber Assays. ELISA-based techniques were used to find factors that are secreted by ASC. Results: We found that ASC-conditioned medium significantly increased the migratory activity of HDMEC both in Scratch and Boyden Chamber Assays. Under TNF treatment, ASC-mediated migratory activation of HDMEC was further increased. Out of 31 factors that were analyzed by ELISA-based techniques, ASC were found to secrete 18 to the supernatant, and 13 of those factors were more strongly secreted following TNF treatment. Conclusions: Our findings indicate that there is an indirect interaction between ASC and HDMEC via diverse soluble factors. Although we can so far not decipher the individual contributions of the large variety of factors involved, we can nevertheless assume that ASC *in vivo* modify HDMEC-mediated processes such as e. g. wound healing, tissue infiltration by leukocytes or the development of new blood vessels. Therefore, ASC are a promising source for cell-based regenerative therapies.

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Keywords: ASC, HDMEC, inflammation, migration
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Induction & Maintenance of Pluripotency

Can proteomics techniques discover stemness proteins?

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Although multiple efforts have been made to understand the pluripotent networks using gene expression microarrays, there is an apparent paradox in the fact that different cell lineages with stem cells characteristics do not share a meaningful set of genes. The focus on transcriptomics measurements in previous studies shows that there is no consensus on the stemness genes, suggesting that perhaps there is no "universal stemness" property governed by a unique mechanism across different stem cell lineages. Bearing in mind that the proteins and not the genes are the cellular machinery and the structural building blocks, we have revisited the stemnnes problem using a label free proteomics approach. To improve the quality of our results, in difference to the previous transcriptomics studies we substituted the heterogeneous hematopoietic stems cells (HSCs) by a homogeneous germ stem cells (GSCs) line, and the neurospheres by neural stem cells (NSCs), we also used embryonic stem cells (ESCs). And to enhance the contrast of the results we used a negative non-stemness control, the mouse embryo fibroblasts (MEFs). In this sense it is noteworthy that although adaptation of established stem cell lines to *in vitro*–culture conditions may lead to alterations in the measured profiles, compared with the *in vivo* counterparts, *in vitro* ESCs, NSCs, and GSCs not only retain all stemness properties, but also comprise a homogeneous population of cells, thus producing more accurate results. We selected as potential stemness proteins those that were simultaneously detected in the three stem populations (ESC, NSC and GSC) but not in the MEF. Our analysis do not find exactly the same stemness markers as in the transcriptomics approaches but the overlap between the detected stemness proteomics and the previous stemness genes is in the same order as the overlap of stemness genes among several transcriptomics studies. But more interestingly our stemness proteomics highlights similar gene ontology biological processes, cellular components, and molecular functions such as transcriptional regulation, DNA repair, cell cycle regulation, translation to the stemness genes found by the former transcriptomics studies. Even more intriguingly, after performing a synteny analysis we discovered stemness hot spots loci across the genome, observing a persistent significance of the enrichment among other loci the t-complex locus of chromosome 17. It is noteworthy that such locus has already been identified by the previous transcriptomics studies. Thus, in some sense we can see our stemness proteins as a bridge connecting the stemness genes found by the previous transcriptomics studies. The stemness protein interaction reveals four main connected clusters Namely, the cluster formed by the transcription factor TFIID complex (Taf1, Taf2, Taf5, Taf6, Tbp); the cluster formed by cilium related proteins (Alms1, Pcnt); the cluster formed by the mitochondrion proteins (Lias, Dbt); and the cluster formed by the ataxia telangiectasia (Atm, Atr), Fanconi anemia (Fanca, Fancd2, Fancg) and other DNA repair proteins (Chek2/Rad53, Mdc1, Xrcc6/Ku70, Blm). Thus, our approach sheds a new light over the stemness searching problem, where the proteomics measurements provide a synergetic view to the transcriptomics approaches.

Keywords: stemness; proteomics; gene ontology; syntheny; bioinformatics
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Functional comparison reveals significant differences among different epiblast stem cell lines

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Epiblast stem cells (EpiSC) are pluripotent stem cells derived from the mouse postimplantation embryo. These cells – although derived from mouse embryos –share fundamental characteristics with human embryonic stem cells (hESC) and are therefore considered to be the murine counterpart of hESC. The basic characteristics of EpiSC (e.g. expression of pluripotency markers Oct4, Sox2 and Nanog, the ability to differentiate into cells of all three germ layers *in vivo* and *in vitro*, etc.) are widely accepted. However, the complete potential of these cells have been described differentially. Some reports demonstrate that EpiSC can be reprogrammed to a naïve pluripotent state upon addition of exogenous factors like Klf4 or Nanog. Others, conversely, show the reversion/conversion of EpiSC to a mESC-like state just by applying stringent mouse ESC conditions. These reverted EpiSC show characteristics of mESCs like chimera formation as well as germ line contribution. In order to understand discrepancies between different lines we carried out different analyses. Six different epiblast stem cell lines were analyzed in regard to gene expression profile, neural induction capacity as well as reversion/conversion ability. Whereas differences in neural induction seemed not to be significant, the ability to revert/convert to a state similar to mESC varies strongly among the lines. We conclude that different EpiSC lines resemble distinct developmental stages, some of which still have the ability to undergo reversion, some of which are already determined to only undergo somatic differentiation.

Keywords: Epiblast stem cells; reversion; pluripotency
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Proteomic analysis of mouse oocytes reveals 28 candidate factors of the 'reprogrammome'

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The oocyte is the only cell of the body that can reprogram transplanted somatic nuclei, and sets the gold standard for all reprogramming methods. Therefore, an in-depth characterization of its proteome holds promise to advance our understanding of reprogramming and germ cell biology. To date, limitations on oocyte numbers and proteomic technology have impeded this task, and the search for reprogramming factors has been conducted in embryonic stem (ES) cells instead. Here, we present the proteome of metaphase II mouse oocytes to a depth of 3699 proteins, which substantially extends the number of proteins identified until now in mouse oocytes and is comparable by size to the proteome of undifferentiated mouse ES cells. Twenty-eight oocyte proteins, also detected in ES cells, match the criteria of our multi-level approach to screen for active reprogramming factors, namely: nuclear localization, chromatin modification and catalytic activity. Of these 28 proteins, 17 show upregulated mRNA levels during the transcription factor-induced reprogramming of mouse fibroblast to induced pluripotent stem (iPS) cells. We propose that at least some of the identified 28 factors could confer speed, effectiveness and specificity on the processes that take place during transcription factor-induced reprogramming, which otherwise relies on the window of opportunity offered by the cell cycle and on endogenous components that may be present in insufficient amounts or not at all in a given cell type. To test our proposal we will have to see if oocytes can still support cloned embryo development when candidate maternal proteins have been depleted prior to SCNT (loss of function), or if iPS cells can be induced at higher rates when the oocyte proteins are coexpressed in precursor somatic cells along with the four factors Oct4, Sox2, c-Myc and Klf4 (gain of function). Our oocyte proteome catalog advances the definition of the 'reprogrammome', the set of molecules – proteins, RNAs, lipids and small molecules – that enable reprogramming. Moreover, our catalog provides a basis to further explore and understand the mechanisms of active reprogramming achieved by the oocyte for the benefit of all reprogramming platforms.

Keywords: oocyte; pluripotency; proteome; reprogramming; SCNT
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Somatic memory in pluripotent hybrids of mouse hematopoietic stem cells and ES cells

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Reprogramming of somatic cells has emerged as a particularly appealing approach for inducing pluripotency. Hematopoietic stem cells (HSC) give rise to all mature blood cells and blood-born cells in peripheral organs. Embryonic stem cells (ESC) are pluripotent cells that contain reprogramming activity and induce pluripotency in somatic cells upon fusion. HSC are an attractive cell type for reprogramming by ESC fusion, because our studies suggested the presence of shared signalling pathways and a significant overlap of the gene networks between HSC and ESC. Reprogramming by transduction of specific sets of transcription factors represents a frequently applied strategy for generating pluripotent cells, referred to as induced pluripotent stem cells (iPS cells). Here we compared reprogrammed pluripotent cells derived from Flt3 HSC by adding the four transcription factors Oct4, Klf4, c-Myc and Sox2 (Flt3 iPS cells) to those reprogrammed by HSC/ES fusion (Flt3 ESC hybrids). Reprogrammed Flt3 ESC hybrids were generated by isolating Flt3 HSC from bone marrow of Oct4-eGFP transgenic mice and fusion with ESC *in vitro*. These hybrids were stable 4n and acquired various features of ESC, including (i) ESC morphology, (ii) prolonged self-renewal ability and (iii) pluripotency, as demonstrated *in vitro* by embryoid body (EB) assay and *in vivo* by teratoma formation. Genome-wide gene expression profiling demonstrated that Flt3 ESC hybrids cluster with ESC and iPS cells, but also expressed gene clusters from both parental populations (in collaboration with Fügemann, C., Breitbach, M., Fleischmann, B. K., University of Bonn, Bonn, Germany). To compare the rate of differentiation of Flt3 ESC hybrids with Flt3 iPS cells, we studied spontaneous differentiation in EB assay *in vitro* (in collaboration with Kuzmenkin, A., Xu, G., Hescheler, J., University of Cologne, Cologne, Germany). Interestingly, Flt3 ESC hybrids showed an accelerated differentiation in comparison to Flt3 iPS cells. Additionally, such EB exhibited a well-structured tissue organization similar to a developing embryo, which was not observed for Flt3 iPS cells. In conclusion, Flt3 ESC hybrids acquired various features of pluripotency from the ESC fusion partner and gave rise to cells of all three germ layers upon differentiation. So, Flt3 ESC hybrids are very similar to ESC and Flt3 iPS cells. Interestingly, Flt3 ESC hybrids showed an enhanced and accelerated differentiation potential towards the mesodermal lineage, indicating memory of the somatic origin.

Keywords: reprogramming; somatic memory; iPS cells; ES cell fusion; hematopoietic stem cells
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Identification of NANOG2, a novel transcript in pluripotent human stem cell populations

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The transcription factor NANOG is one of the core components within the transcriptional network of pluripotent stem cells such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Recent studies revealed the existence of various NANOG pseudogenes (NANOGP2-P11) and, more importantly, of a gene duplicate termed as NANOG2. The latter was initially described as NANOG pseudogene 1 (NANOGP1), before it became evident that NANOG2 is a real gene with an own promoter and regulatory elements. When we investigated several NANOG transcripts in human carcinoma cells, hematopoietic stem cells and leukemia cells, the detected NANOG transcripts were identified as NANOG2. Moreover a 5´RACE analysis revealed novel 5´-exons and novel promoter regions for both the NANOG and NANOG2 gene. Thus, the alternative promoter activation results in complex splice variant formation leading to the expression of two putative NANOG and three putative NANOG2 protein variants. In our study, we addressed the question, which NANOG variants are expressed in ESC or iPSC and are relevant for induction and maintenance of pluripotency. RT-PCR experiments showed transcript variants of NANOG in human ES cells, which contain the novel 5'-exons. The same transcripts were also investigated in mesenchymal stem cells (MSCs) derived- and fibroblast derived-iPSCs. In comparison to MSCs, where no NANOG expression but strong NANOG2 expression is detectable, we could detect weak, but significant levels of NANOG2 in ESC and iPS cells. Our results revealed that other NANOG or NANOG2 splice variants are also transcribed in these stem cell populations and might support or even overtake the function of NANOG. The latter aspect is currently being investigated in iPSC generation experiments. In conclusion, we demonstrate the existence of mRNA transcripts from different NANOG variants in human pluripotent stem cells and provide preliminary evidence that the recently described variant NANOG2 might contribute to the pluripotency-related transcriptional network.

Keywords: pseudogene; gene duplicate; stem cells; splice variant; NANOG
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A unique linker interface is crucial for the biological activity of Oct4 in reprogramming

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Reprogramming terminally differentiated fibroblasts into pluripotent stem cells is achieved by forced overexpression of Oct4, Sox2, Klf4 and c-Myc. While first disease models are established and scientists are eager to challenge the potential of iPS cells in medical applications, surprisingly little is known about the molecular mechanism of the reprogramming process. It was shown that Sox2, Klf4 and c-Myc can be replaced by other factors, while Oct4 remains to be indispensible for successful reprogramming. We solved the crystal structure of Oct4:PORE which indicates a unique linker helix that distinguishes Oct4 from its paralogs. Here, we demonstrate the importance of the linker domain by mutating single amino acids and by generating linker chimeras that lead to a complete loss-of-function phenotype in reprogramming. However, protein degradation, protein localization, DNA binding and transactivation activity were not altered in the mutants and can not explain the lost reprogramming potential. Interestingly, point mutations that showed a partial or complete loss-of-function phenotype in an alanin scan reassemble a potential interface in the unique helix 5 in our crystal structure. Thus, we determined the interaction partners of WT Oct4 and mutated Oct4 proteins by label-free mass spectrometry and speculate that the linker region plays an important role in recruiting important factors to Oct4 target genes which is bona fide a prerequisite for successful reprogramming.

Keywords: reprogramming, iPS, ES, Oct4, epigenetics
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In vivo analysis of pluripotency related transcription factors

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The transcription factors Oct4, Sox2 and Nanog represent the core transcriptional network in mammalian embryonic stem cells. This network is also present in early germ-cells and some individual factors are expressed in somatic cells. Recently it has been shown that Oct4/Sox2 in combination and Oct4 alone can reprogram somatic cells into induced pluripotent stem (iPS) cells. As these factors are commonly introduced into cells by retroviral or lentiviral transduction, a reactivation in the adult organism is possible. Since "iPS cell-derived" mice show a higher rate of tumour formation during adulthood, a systematic approach to study the effects of these transcription factors *in vivo* is needed. Here we describe the development of an *in vivo* system to investigate the effects of ectopic Oct4, Sox2 and Nanog over-expression on somatic tissues of adult mice. For this purpose, a derivative of the Tet-On system was utilised. Depending on the organ analysed, induction rates of $1x10^3$ to $1x10^5$ fold could be observed using quantitative Real-Time PCR analysis mimicking endogenous expression levels of mouse embryonic stem cells. It could be shown that over-expression of Nanog imposed moderate proliferative effects on some selected organs (e.g. intestine and colon). These findings were further evaluated by global gene expression analysis and immunostainings of relevant markers. The tools presented here, will give a better insight into the oncogenic properties of these pluripotency related genes and will provide a risk-assessment for the use of genetically altered iPS cells for future therapies. Moreover, these mice will provide a defined system for studying the mechanism of reprogramming and for setting up screens to replace factors or enhance the efficiency of reprogramming.

Keywords: Oct4; Sox2; Nanog; inducible; trangenic
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Expression of neural markers by human mesenchymal stem cells after mesengenic differentiation

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AIMS Mesesenchymal Stem Cells (MSCs) are multipotent cells able to differentiate in mesengenic and non mesengenic lineages under specific stimuli. The knowledge of the human MSCs (hMSCs) biological properties is very important to optimize their promising clinical application. Literature data have demonstrated the expression of neural markers by undifferentiated MSCs, but there are no studies that evaluate the neural marker expression after mesengenic differentiation.Our preliminary results have demonstrated that undifferentiated hMSCs express high levels of β tubulin III and NeuN and that this expression is independent from both serum presence and number of culture passages. So the purpose of this work was to evaluate the expression of these neural markers after adipogenic, osteogenic and chondrogenic differentiation. METHODS Bone marrow hMSCs were plastic-adherent, expressed specific surface antigens and differentiated in mesengenic lineages under specific conditions. By immunofluorescence experiments, we evaluated the expression of the neuronal markers β tubulin III and NeuN by hMSCs cultured in differentiation medium at different time points depending on the specific protocol used. Cells grown in culture medium without any differentiative factor represented the control. RESULTS In our cell cultures, we observed that most of hMSCs express β tubulin III and NeuN after adipogenic and osteogenic differentiation, while cells differentiated toward the chondrogenic lineage didn't express these neuronal markers. CONCLUSIONS The finding that hMSCs differentiated into adipogenic and osteogenic lineages express neuronal markers such as β tubulin III and NeuN raises doubts about the reliability of these markers as indicators of neuronal differentiation. Moreover, further studies are necessary to understand the specific biological role of these proteins, known to be neuronal ones, in hMSCs.

Keywords: mesenchymal stem cells; mesengenic differentiation; neural markers
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Direct reprogramming of fibroblasts into epiblast stem cells

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Epiblast stem cells (EpiSCs) derived from epiblast tissue of post-implantation embryos are pluripotent and can give rise to all three germ layers in teratoma assays. Introduction of the 4 transcription factors Oct4/Sox2/Klf4/c-Myc into somatic cells has been shown to generate induced pluripotent stem cells (iPSCs), which are very similar to embryonic stem cells (ESCs) in a number of characteristics. However, generation of EpiSCs by the direct reprogramming of somatic cells using these transcription factors has not been shown to date. Here we show that Yamanaka's 4 transcription factors can be used to directly generate induced EpiSCs (iEpiSCs) under EpiSC culture conditions. iEpiSCs resemble EpiSCs in morphology, gene expression pattern, epigenetic status, and chimera forming capability, demonstrating that the culture environment in transcription factor–mediated reprogramming determines the cell fate of the reprogrammed cell. To our knowledge, this is the first report describing the direct conversion of fibroblasts, by transcription factors, to a stem cell other than ESCs.

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Evaluation of basal membrane-derived and biomaterial matrices for endothelial cell expansion and maintenance of their primitive potential

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The discovery of so-called circulating endothelial progenitor cells (EPCs) has highly stimulated the field of vascular biology. During recent years different protocols have been applied to raise and expand such cells. However, a recent comprehensive comparison of obtained EPCs revealed that many of them are of hematopoietic origin and just mimic an endothelial cell surface phenotype. According to the published studies only the endothelial colony forming cells (ECFCs) seem to represent true circulating endothelial progenitor cells. Of note, ECFCs raised from human umbilical cord blood (UCB) are phenotypically almost identical to human umbilical cord vein endothelial cells (HUVECs). Furthermore, similar to HUVECs their proliferation capacity is limited to a few passages. In this context it has been suggested that both cell types become senescent and stop to proliferate. Provided that both endothelial cell types are organized in a hierarchical manner similar to primitive hematopoietic cells, such a limited expansion would also be expected if the culture conditions would not allow self-renewal of the more primitive cells. Favoring the latter hypothesis we aim to optimize the culture conditions for these endothelial cells. For a number of different cell systems cell fate modulating features of matrices and biomaterials have been reported. To this end we decided to compare the impact of basal membrane-derived matrices as well as distinct biomaterials on the adherence capacity, the metabolic activity, the necrosis and apoptosis rate and the expansion rate of primary human endothelial cells. So far, we compared the impact of collagen IV, fibrin, gelatin, heparin, hyaluronic acid, laminin, platelet lysate coated plastic dishes with conventional plastic ware and a novel commercially available Advanced plastic dish type (Greiner Bio-One GmbH). In addition, we studied biological features of ECFCs expanded on different biomaterials used in tissue engineering including PVDF-gf, PTFE, PET, Texin 950, PDMS, L209S (PLLA), R203S (PDLLA), LR704, RG503, LT706, PCL, BAK1095, PEA-C and Alginate. Furthermore, we improved these new conditions by changing the components of the conventional media.

Keywords: endothelial progenitor cells; ECFC, matrices, biomaterials, primitive potential
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Oct4-reprogramming of spermatogonial stem cells to generate cardiovascular progenitor cells

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Organ regeneration with stem cells requires a delicate balance between the loss of donor cell pluripotency (so that teratoma formation is avoided) and retention of donor cell proliferative capacity prior to terminal differentiation (so that a sufficient number of target cells are generate to effect a therapeutic benefit). The aim of this study is to reprogram spermatogonial stem cells (SSCs) derived from double transgenic mice (MHC-neo/MHC-EGFP) by overexpression of Oct4 alone and to differentiate these cells into proliferating cardiovascular progenitors (Flk1 cells). We have generated four SSC lines from adult male mice. The SSC culture can be expanded over one year *in vitro* and show typical SSC morphology and express SSC-specific markers like DAZL, VASA and GFR1α at both mRNA and protein levels. Furthermore, these SSCs from the MHC-Neo-EGFP mice were reprogrammed into pluripotent stem cells by overexpression of Oct4 alone via a lentivirus system. The reprogrammed cells are positive for pluripotency markers such as Oct4, Nanog, Sox2 and SSEA-1 and show similar characteristics as pluripotent stem cells. They were able to differentiate spontaneously into cells of all three embryonic germ layers *in vitro* by using the hanging drop method. GFP beating cardiomyocytes can be selected by the G418 treatment. After transplantation of these cells in SCID-beige mice, teratomas were detected 6 weeks later. Moreover we established a protocol to induce these cells to differentiate into Flk1 cardiovascular progenitors via coculture of these cells (n = 30.000 per 10 cm dish) with OP9 cells. After differentiation for 6 days 25-35% of the cells were positive for Flk1. The Flk1 cells were then sorted using the fluorescence-activated cell sorting technique. For investigating the regenerative potential of these cells we used a myocardial infarction mouse model and transplanted 500.000 Flk1 cells per mouse in an infarcted heart. At different time points after cell transplantation (2 days, 2 weeks, 4 weeks and 8 weeks) we took the hearts out and analysed them to identify the Flk1 cells and their derivatives. Investigations on the integration of the cells into the host myocardium and on the functional improvement of the heart by echocardiography are in progress.

Keywords: SSCs; reprogramming; iPSCs; pluripotency; Flk1-cardiovascular progenitors
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Generation of patient-customized iPSC from umbilical cord and adult peripheral blood

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Generation of human induced pluripotent stem cells (iPSC) from peripheral blood or umbilical cord blood offers several advantages over other cell sources. Peripheral blood mononuclear cells (PBMCs) are readily available, collection is less invasive than dermal fibroblast or keratinocyte sampling, and there is no requirement to establish primary cell cultures from skin biopsies which may take several weeks. Umbilical cord blood cells (UCBCs) are highly proliferating cells and represent a juvenescent cell source that can be expected to carry relatively few fixed mutations, which upon reprogramming may be transferred to iPSCs lines. In the present study we aimed to transduce either Macs-isolated CD34 UCBCs or unfractionated adult PBMCs. Cells were prestimulated for 48 hours with a cytokine cocktail consisting of SCF, TPO FLT3L (UCBCs) or SCF, TPO, FLT3L, GM-CSF, IL-3 and G-CSF (PBMCs) to activate in particular myeloid progenitor cells. For transduction we used polycistronic LV vectors encoding reprogramming factors (Oct3/4, Sox2, and Klf4 with or without c-Myc). Factors were expressed from a Spleen-Focus-Forming-Virus (SFFV) derived promoter/enhancer element ensuring high transgene expression in hematopoietic target cells and factor-cDNAs was separated by P2A motifs. To easily identify transduced cells the fluorescence reporter gene dTomato coupled to an Internal-Ribosomal-Entry-Site (IRES) was used (Warlich et al, Mol Ther, 2011). Although reprogramming efficiency was rather low, after 20 - 30 days approx. 50 iPSC-like colonies were obtained from $5x10^5$ CD34 UCBCs (0,01%) with the three factors LV vector. Approx. 25 iPSC-like colonies were obtained from $5x10^5$ PBMCs (0,005%). Additional use of c-Myc improved reprogramming efficiency 1-1,5 folds. This construct only was used in initial experiments, however to avoid the risk of malignant transformation of iPSCs due to c-Myc (re) activation. Efficiency of reprogramming in PBMCs was improved 2-3 fold by using thiazovivin in combination with inhibitors of the TGFβ receptor and the MEK pathway. Of note, reprogramming also was achieved from frozen PBMCs samples. So far, 8 – 10 UCBCs-derived and 10 – 12 PBMCs –derived iPSC lines were established. All iPSC lines displayed typical ES-like morphology, immunocytochemistry and FACS analysis confirmed that lines were positive for NANOG, OCT4, Tra-1-81, Tra-1-60, SSEA3, SSEA4, and alkaline phosphatase staining. All iPSC lines were cultured for 8 – 10 passages before they were frozen down. Further characterization with regard to their differentiation and teratoma forming potential are ongoing. Using state-of-the-art LV gene transfer technology we here demonstrate efficient generation of human cord blood or peripheral blood derived iPSC. As hematopoietic cells clearly represent one of the most interesting starting material to generate iPSC for patient-specific cell therapies, these findings should be of considerable clinical relevance.

Keywords: human iPSC, peripheral blood, cord blood, polycistronic lentiviral vectors
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Non-viral reprogramming strategies in somatic cells: Approach for the study of diseases

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Patient-specific induced pluripotent stem (iPS) cells are an ideal tool to explore *in vitro* the pathogenesis of diseases such as diabetes mellitus allowing the improvement of current therapeutic strategies. Generally, most iPS cells are generated using viral strategies, hindering their clinical applicability.Therefore, we established a non-viral, non-integrative protocol for the generation of iPS cells. Stepwise induction of pluripotency was achieved by: (i) a strong initial impulse, (ii) enhancement, and (iii) maintenance and preservation of pluripotency. (i) A strong initial impulse was given by the STEMcircleTM vector, which contains the four reprogramming factors Oct4, Nanog, Sox2 and Lin28 (ONSL) as well as a GFP. (ii) To enhance reprogramming, we used hypoxic culture conditions, small molecules as well as micro RNAs (miRs). (iii) The maintenance and preservation of the established potential iPS cells required sophisticated culture conditions. Therefore, also small molecules were tested for their ability to support viability during and after the reprogramming procedure. Cell type adapted transfection protocols allowed sorting (FACS) and propagation of about 30 40 % GFP-positive potential iPS cells. Expression of the endogenous pluripotency factors (ONSL) and pluripotency-supporting factors (such as Klf4) was up-regulated. (ii) Culture under hypoxic conditions also induced expression of pluripotency-associated factors (e.g. Nanog, Oct4). To further accelerate the reprogramming, small molecules such as RG 108, SB-431542 or valproic acid were used to suppress signalling-pathways of apoptosis and early differentiation as well as epigenetic modelling (methylation and acetylation) of DNA and histones. A cocktail of hESC-specific mature miRs was used to induce endogenous pluripotency-associated miRs. In addition to the increase of mature miRs, the induction of pre-miRs as well as pri-miRs confirmed the permanent change of the miR-ome. Moreover, the expression pattern of miRs was comparable to that of hESC. Direct (Wdr61, Klf13, Dnmt1, Myst3, CyclinA1) and indirect (Thy1, Fsp1) miR target genes were down-regulated. We conclude that induced endogenous miRNA-expression strengthens the reprogramming process by shifting the transcriptome to a pattern similar to that of human embryonic stem cells (hESCs). (iii) The small molecules BIO, (±)-Bay K 8644, and Pifithrin alpha improved preservation of potential iPS cells. We provide new strategies for the non-viral generation of iPS cells suitable for *in vitro* test systems and potentially clinical applications.

Keywords: miRNA; reprogramming; pluripotency; iPS; hypoxia
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Lineage conversion of murine extraembryonic trophoblast stem cells to pluripotent stem cells

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In mammals, the first cell fate decision is initialized by cell polarization at the 8-16-cell stage of the pre-implantation embryo. At this stage outside cells adopt a trophectoderm (TE) fate, whereas the inside cell population gives rise to the inner cell mass (ICM). Prior to implantation, transcriptional interaction networks and epigenetic modifications divide the extraembryonic and embryonic fate irrevocably. Here, we report that extraembryonic trophoblast stem cell (TSC) lines are converted to induced pluripotent stem cells (TSC-iPSCs) by overexpressing Oct4, Sox2, Klf4 and cMyc. Methylation studies and gene array analyses indicated that TSC-iPSCs had adopted a pluripotent potential. The rate of conversion was lower compared to somatic reprogramming experiments, probably due to the unique genetic network controlling extraembryonic lineage fixation. Both *in vitro* and *in vivo*, TSC-iPSCs differentiated into tissues representing all three embryonic germ layers, indicating that somatic cell fate could be induced. Finally, TSC-iPSCs chimerized the embryo proper and contributed to the germ line of mice, indicating that these cells had acquired full somatic differentiation potential. These results lead to a better understanding of the molecular processes that govern the first lineage decision in mammals.

Keywords: trophectoderm, pluripotency, trophoblast stem cells, lineage conversion
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Transposon-derived porcine induced pluripotent stem (piPS) cells and live imaging of pluripotency

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Reprogramming of porcine somatic cells to pluripotent stem cells is promising for the assessment of functionality and safety of stem cell applications. The pig is a well established large animal model for preclinical testing, including potential iPS-based cell therapies. The first attempts to produce porcine induced pluripotent stem cells were made using retro- and lentiviral vectors, which are associated with an increased risk of insertional mutagenesis and which are not easily removable after reprogramming. Here, we describe a non-viral transposon-based method for the derivation of porcine induced pluripotent stem cells, employing transgenic fibroblasts carrying a pluripotency reporter construct (Oct4-EGFP). The Oct4-EGFP reporter allows live cell imaging of reprogrammed cells [1]. The transposon-reprogrammed porcine iPS expressed typical markers of embryonic stem cells, showed long term proliferation under feeder-free culture conditions, differentiated into cell types of the three germ layers *in vitro*, and formed teratomas after subcutaneous injection into immunodeficient nude mice. The polycistronic transposon construct can be removed from the porcine genome, and cells free of the reprogramming construct can be selected. These results are a major step towards preclinical testing of iPS-derived cells in the pig model.

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Keywords: large animal model, germ line, embryonic stem cell, Sleeping Beauty
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Functional analyses and chromatin studies of ES cell pluripotency by reversible knockdown of Bmi1 paralog Pcgf6

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Polycomb group (PcG) proteins are chromatin modifiers involved in heritable gene repression. Two main PcG complexes have been characterized: Polycomb repressive complex 2 (PRC2) is involved in the initiation of gene silencing, whereas Polycomb repressive complex 1 (PRC1) participates in the stable maintenance of gene repression. Bmi1(Pcgf4) is one of the most studied PRC1 members with essential functions for embryonic development and adult stem cell self renewal. In embryonic stem cells (ESCs) Bmi is poorly expressed while its paralogs (Pcgf1, 2, 3, 5, 6) are expresse at higher levels. The relevance of Bmi1 paralogs for the establishment and maintenance of ESC pluripotency and ESC chromatin biology has so far not been addressed. To investigate the function of the Bmi1 paralog Pcgf6 in ESCs, we established a doxycycline (Dox) inducible shRNA-targeted knockdown system according to Seibler et al. [NAR, Vol.33, Pp e67, 2005]. We observed that Pcgf6 is highly expressed in ESCs. However, upon ESC differentiation, gene expression of Pcgf6 strongly declines. Following Dox-induced knockdown (KD) of Pcgf6, we observed decreased ESC proliferation and colony formation. In parallel, gene expression of pluripotency markers such as Oct4, Nanog and Sox2 was reduced in Pcgf6-KD-ESCs, while mesodermal lineage markers became up regulated. Upon *in vitro* differentiation of Pcgf6-KD-ESCs into embryoid bodies (EBs), FACS analyses showed increased frequencies of Flk1 cells in day 4 EBs, indicating increased hemangioblast formation. To further analyse the role of Pcgf6 during hematopoiesis we performed directed hematopoietic differentiation of Pcgf6-KD-ESCs via EB formation and hematopoietic methylcellulose cultures. We found increased frequencies of CD45 and Mac-1 cells, reflectiv of increased hematopoietic differentiation. These analyses give first indications that the Bmi1- paralog Pcgf6 is non-redundantly involved in regulating ESC differentiation properties.

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Analyses of the function of Lrrc34 in pluripotent stem cells and in the pluripotency network

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Embryonic stem cells (ESCs) derived from the inner cell mass of blastocysts are pluripotent cells (Evans and Kaufmann, 1981; Martin, 1981). They can give rise to all types of differentiated cells (Suda et al., 1987), including germ cells (Geijsen et al., 2004; Nayernia et al., 2006). Also they possess the capacity to self-renew and to differentiate into any of the three germ layers. Pluripotent stem cell lines can also be generated from Spermatogonial Stem Cells (SSCs) derived from adult mouse testis (multipotent adult germline stem cells, maGSCs) (Guan et al., 2006) which show ESCs properties. We have performed DNA microarray analysis with undifferentiated and differentiated ESCs and maGSCs from different genetic backgrounds. The results have shown an 97-99% identity in their transcriptomes (Meyer et al., 2010). Moreover the maGSCs and ESCs express the same set of known pluripotency-related genes, such as Oct4, Nanog and Sox2. These transcription factors are known to be indispensable for maintaining pluripotency in ESCs. Inactivation of one of these genes leads to loss of pluripotency and to differentiation of the cells. Therefore we were searching for unknown pluripotency-related genes. The gene Lrrc34, leucine rich repeat containing 34, was found to be expressed in undifferentiated ESCs, maGSCs and other pluripotent cell lines and in the adult testis. After differentiation with RA, the expression was strongly downregulated in ESCs and maGSCs. Methylation studies of the promoter of Lrrc34 have shown that the promoter is hypomethylated in undifferentiated cells and becomes hypermethylated upon differentiation. Furthermore ChIP- qPCR analyses of histone modifications at gene-specific level were done. The modification patterns of Oct4, Sox2 and Nanog in maGSCs are nearly identical to those of male ESCs (Khromov et al., 2010). These genes and Lrrc34 were enriched for activating histone modifications and depleted of repressive histone modifications. To further analyse the function of Lrrc34, siRNA experiments were performed in ESCs to have a closer look on the effect of the downregulation of Lrrc34 to other known pluripotency-related genes. We found that i.e. Oct4, Zfp206 and Klf4 are downregulated while differentiation markers like Vimentin and Hnf4 are strongly upregulated. To confirm these data, stable downregulation of Lrrc34 in ESCs is in progress. In another approach overexpression of Lrrc34 in ESCs was performed. First results showed an increased expression of Nanog, Stra8 and Lin28 and a decreased expression of Oct4, Zfp206 and Vimentin. To further characterize the function of Lrrc34 SILAC experiments will be carried out to find putative interaction partners and a conditional Knock-out mouse model will be generated. Our results indicate that Lrrc34 has an effect on other pluripotency marker genes and might play a role in maintenance of pluripotency in stem cells.

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Keywords: pluripotency; stem cells; gene expression; differentiation
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A full miRNA library screen reveals a new miRNA family enhancing reprogramming through direct regulation of the homeobox transcription factor Meox2

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Introduction: Induced pluripotent stem cells (iPSCs) can be generated by ectopic overexpression of the four transcription factors Oct4, Sox2, Klf4 and c-Myc. Even though re-activation of the pluripotency-related transcriptional network during reprogramming is well studied, only limited information is available on the role of microRNAs (miRNAs), which support induction of pluripotency in somatic cells. Here, conducting a full miRNA library screen during the early phase of iPSC generation, we investigated if particular miRNAs can improve reprogramming and sought to elucidate further molecular pathways involved in the induction of pluripotency. Materials/Methods: We transduced murine embryonic fibroblasts (MEFs) from OG2 mice (Oct4 promoter-driven GFP expression) with a polycistronic lentiviral construct expressing Oct4, Klf4 and Sox2 from a spleen focus forming virus (SFFV)-derived promoter/enhancer. Twenty-four hours after transduction, we individually transfected murine miRNAs from a Pre-miR™ miRNA Precursor Library–Mouse V3 containing 379 miRNAs (Ambion) into these MEFs. At day 7 to 10 after transduction, GFP-positive colonies were counted. Results: Applying a threshold of 4-fold induction of reprogramming events over the controls, we could identify miRNAs previously demonstrated to improve reprogramming, such as members of the mir-290 and mir-302 clusters. In addition, we were able to find several hitherto unpublished miRNAs profoundly enhancing iPSC generation. Interestingly, we identified a miRNA family targeting the same downstream mRNAs, consisting of the miRNAs 130b, 301b and 721. Based on in-silico predictions (Targetscan and miRBase), we investigated various putative miRNA targets by western blot and luciferase assays. Indeed, we were able to identify the homeobox transcription factor Meox2 to be directly downregulated by this miRNA family. Further validation revealed that ectopic overexpression of the protein from a lentiviral construct significantly decreased reprogramming events, however, specific siRNA-mediated knockdown of this factor enhanced reprogramming efficiencies comparable to the miRNA-mediated effects. Conclusion: Applying a full library miRNA screen, we were able to identify a novel miRNA family significantly increasing the induction of pluripotency in MEFs. This newly characterized miRNA family specifically targets the transcription factor Meox2, which upon knockdown facilitates and upon lentiviral overexpression significantly blocked reprogramming. Collectively, these data show that a decrease of Meox2 expression is greatly supporting efficient iPSC generation from murine fibroblasts. Further studies aim at elucidating downstream effectors of the transcription factor identified.

Keywords: reprogramming, micro RNAs, screen, iPSCs
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Doxycycline - inducible transposon vector system for generation of induced pluripotent cells based on the porcine transcription factor sequences

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Efficient delivery of reprogramming transcription factors to the target cells is essential for generation of induced pluripotent stem cells (iPSC) from non-pluripotent cells. Transposon vectors, such as Sleeping Beauty (SB) or PiggyBac (PB), offer the advantages of relatively high efficiency of transfection combined with improved safety for use. For these reasons, we have developed a SB vector system for the expression of the porcine cDNA sequences coding for OCT4, SOX2, C-MYC, KLF4, and NANOG, under the control of Doxycycline (DOX) -inducible tetO- promoters. The system consists of the following vectors: SB-tetO-rtTA-SV40pA-tetO-OCT4-IRES-Tomato-bGHpA (SB-rtTA-O4-T), SB-tetO-SOX2-IRES-Neor-bGHpA (SB-SOX2-N), SB-tetO-C-MYC-IRES-Puror-bGHpA (SB-MYC-P), SB-tetO-KLF4-IRES-Puror-bGHpA (SB-KLF4-P), SB-tetO-rtTA-SV40pA-tetO-NANOG-IRES-Puror-bGHpA (SB-NAN-P), and the SBx100 transposase. Testing of the SB-rtTA-O4-T vector in transfected mouse embryo fibroblasts showed that there was no detectable Tomato fluorescence in absence of DOX, but it was up-regulated within 8 hours upon addition of 1 µg/ml DOX. Antibody staining showed that OCT4 was expressed in all cells that had red fluorescence, confirming that Tomato expression is a reliable indicator for the expression of the main gene. Combined transfection of porcine embryo fibroblasts with all six plasmids described above resulted in increased proliferation and formation of ESC-like, alkaline phosphatase-positive colonies by day 9 post transfection. RT-PCR analysis showed that these cells expressed all 5 transcription factors. While SB-NAN-P was dispensable for the formation of these colonies, omission of either SB-OCT4-T, SB-SOX2-N, SB-MYC-P, or SB-KLF4-P did not result in colony formation. The cells generated with the described vector system are being expanded and analysed to evaluate the extent of reprogramming.

Keywords: iPSC, transposons, porcine, doxycycline
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Lentiviral vector design and imaging approaches to visualize the earlystages of cellular reprogramming

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Gene transfer of 4 reprogramming transcription factors (RF) (Oct4, Sox2, Klf4 and c-Myc) can convert somatic cells to induced pluripotent stem cells (iPSC)[1]. However, the underlying mechanism is poorly understood and it is to be elucidated why only a fraction of cells expressing the RF fully converts into iPSC and where this fraction derives from. The "stochastic" model, in which most of the differentiated cells have the potential to become iPSC presupposing epigenetic remodelling, has only recently been substantiated using a conditional transgenic model2. This and other studies recently investigated morphological and molecular changes associated with reprogrammig monitoring these processes every few hours/days albeit without directly marking RF expression [2,3]. In the present study, we extended these findings employing fluorescence-coded lentiviral reprogramming vectors in combination with single-cell tracking[4,5] in short intervals (minutes) and filmed the "birth" of iPSC in cell clusters expressing and silencing the RF[6]. For this purpose, we constructed either 1-factor vectors (expressing one RF) or combinatorial (3-in-1 or 4-in-1) constructs co-expressing Oct4, Klf4, Sox2 and optionally c Myc via different 2A-proteinase sites and further linked them to fluorescent proteins. We improved RF and fluorochrome expression by a set of modifications including (1) the incorporation of a Kozak consensus sequence, (2) codon-optimization of the RF, and (3) the addition of a woodchuck post-transcriptional regulatory element (wPRE). Importantly, we chose a retroviral promoter (spleen focus-forming virus, SFFV), which mediates efficient expression in many somatic cell types, but is rapidly silenced in cells undergoing epigenetic remodelling such as reprogramming cells. Transducing murine embryonic fibroblasts (MEFs) of a well characterized Oct4-EGFP reporter mouse strain (OG2)[7] these vectors triggered reprogramming with high efficiency (>10% Oct4-EGFP+ cells 11 days post transduction (pt)). Resulting iPSC lines demonstrated ES cell-like morphology and marker expression and were competent to form teratoma with differentiation to the three germ layers. Flow cytometric analysis of reprogramming colonies revealed a nearly complete downregulation of SFFV-driven dTomato expression (co-expressed with the 4 RF) in emerging EGFP+ cells (11 days pt). Apoptome 3D-microscopy based reconstruction (13 days pt) showed that colonies were composed of both, EGFP+ potential iPSC and dTomato+ cells still expressing the RF, whereas cells co-expressing both markers were rare. Combining live cell imaging with single cell tracking based on the software tool TTT we monitored the early phases of reprogramming and were able to document that most colonies did not arise from single transduced cells, but incorporated neighboring transduced cells

while proliferating, resulting in genetic patchwork colonies. Furthermore, we found that only a fraction of the genetically identical progeny from a single transduced cell switched on Oct4-EGFP, supporting the notion of stochastic epigenetic gate-keeping events. In summary, improved vector design with continuous live single cell observation at high temporal resolution creates a powerful system to assess the subtle kinetics and morphology during biological processes such as the early stages of reprogramming. The experimental system described herein could be useful to further explore reprogramming events as well as to screen pluripotency markers, reprogramming factor candidates, roadblock inhibitors, and novel tools for reprogramming.

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Keywords: induced pluripotent stem cells; lentiviral vector; fluorescent reporters; live cell imaging; single cell tracking
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Generation of induced pluripotent stem cells from cynomolgus monkey fibroblasts and the differentiation towards cardiomyocytes

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Cynomolgus monkeys are frequently used as an animal model especially in safety pharmacology, but may also become important for preclinical evaluation of stem cell based therapies. As mouse and human induced pluripotent stem (iPS) cells do not allow for establishment of preclinical allogeneic transplantation models, it was aim of our study to generate cynomolgus monkey iPS cells (cyiPS), and to differentiate these iPS cells towards CMs. In this study we could show the reprogramming of fibroblasts isolated from skin biopsies from a cynomolgus monkey. We transduced these fibroblasts with a lentiviral vector containing the four reprogramming factors Oct4, Sox2, c-myc and Klf4. First cynomolgus iPSC (cyiPSC) colonies became visible after 26 days. Isolated cyiPSC clones were characterized for expression of pluripotency markers and for their potential to differentiate into derivatives of all three germ layers. Quantitative real time PCR analysis and immunofluorescence staining of undifferentiated cyiPS cells revealed expression of Oct 3/4, Nanog, SSEA-4, TRA 1-60 and Sox2. Differentiation of cyiPSCs resulted in expression of marker genes typical for endoderm, mesoderm, cardiac mesoderm and CMs as demonstrated through semi-quantitative RT-PCR. Moreover, an average of 17 % of spontaneously contracting embryoid bodies (EBs) from cyiPS cells could be observed. Immunohistological studies showed expression of CM-typical proteins including sarcomeric actinin and troponin T. Electrophysiological studies by means of multi electrode arrays (MEA) to show the functionality, electrical coupling and β-adrenergic signaling of the generated cardiomyocytes are ongoing. In conclusion, cynomolgus monkey iPS cells were generated that may provide an important cell source for establishment of preclinical allogeneic cell transplantation models and the development of high-throughput assays for cardiac safety pharmacology according to the 3R concept.

Keywords: cynomolgus monkey; induced pluripotent stem cells; cardiac differentiation; pharmacological screening
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Reprogramming cells of the nonhuman primate common marmoset towards pluripotency

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Within the context of somatic gene therapy of the hematopoietic system, treatment of monogenetic diseases by targeted gene correction within, autologous hematopoietic stem cells (HSCs) and subsequent expansion of selected clones prior to transplantation would be highly desirable. However, purification of HSCs to homogeneity, homologous recombination and their selective expansion, *in vitro*, have remained unsolved tasks until now. In contrast, pluripotent stem cells may represent an attractive alternative source for HSC-based therapies as they offer all required aforementioned prerequisites and, in addition, can in principle be differentiated towards somatic hematopoietic stem and progenitor cells (HSPCs), *in vitro*. To test the medical relevance of this notion in the long run we sought to employing an animal model system more closely related to humans than commonly used mouse model. Thus, we wished to generate induced pluripotent stem (iPS-) cells from the non-human primate common marmoset (Callithrix jacchus) as a starting point for the generation of transplantable HSPCs, *in vitro*. Marmosets are increasingly being used as valuable models for human diseases. Because the conditions for reprogramming marmoset cells are ill-defined, we initiated comparative studies with marmoset skin fibroblasts and human umbilical endothelial cells (HUVECs) using different vector systems for ectopic gene expression. In particular, we transduced these cells with a lentiviral vector coexpressing the cDNAs for the human transcription factors OCT3/4, SOX-2, KLF4 and c-MYC (in the order 5´-OSKM-3´) and a fluorescent reporter protein. In addition, we used a PiggyBac-transposon-based approach for coexpression of a reprogramming cassette containing the cDNAs of either four (OSKM) or five (OSKM Lin28) mouse transcription factors. Formation of colonies with ES-cell like morphology was observed approximately 15 days after gene transfer using either approach, both with human and marmoset cells. Whereas human colonies could be propagated and expanded for extended periods of time and showed hallmarks of pluripotency, marmoset colonies ceased further proliferation under the employed culture conditions as well as in medium supporting proliferation of a marmoset ES-cell line, suggesting only partial reprogramming of the primate cells so far. Such partial reprogrammed marmoset cells may already represent an attractive source for transdifferentiation of autologous cells towards HSPCs, *in vitro*. Nevertheless, to support the identification and isolation of fully reprogrammed iPS cells, a recently described pluripotency reporter system driven by the Oct-4 core enhancer from the conserved region 4 (CR4) is currently being employed.

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Keywords: common marmoset; pluripotency, induced pluripotent stem cells, hematopoietic stem cells, HUVEC
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Metabolism

Cytoplasm-nucleus interaction in oocyte-induced somatic nuclear reprogramming

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The mammalian oocyte has the remarkable ability to reprogram nuclei of somatic cells after somatic cell nuclear transfer (SCNT). Defining aspects of this process are qualitative (gene silencing/ activation) and quantitative (levels of transcripts) changes in gene expression, resulting in an embryo-like pattern that supports pluripotency and occasionally even totipotency. Since after NT into the ooplasm the somatic nucleus faces a cytoplasm-to-nucleus ratio one to two orders of magnitude higher than before, feedback signals are expected to tune the level of nuclear activity on the new cellular features, including (although not limited to) biomass and energy metabolism. In this context, we aim to determine if cytoplasm-to-nucleus signaling takes place correctly after SCNT and how it contributes to gene expression pattern reshaping during reprogramming. Our results show that compared to ICSI-fertilized counterparts, SCNT mouse embryos have lower ATP levels and higher hydrogen peroxide content at the 4-cell stage, indicating metabolic disturbances during early stage reprogramming. To directly investigate how gene transcription/ translation is tuned in this energetically demanding environment, we performed SCNT with cumulus cells that harbor an actin promoter-driven Cox8-GFP transgene. Cox8-GFP is transcribed in the nucleus, and the product is specifically translocated to the mitochondrial membrane, allowing tracing/ visualization of a nucleus-encoded product (mRNA/protein) specifically resulting from embryonic genome activation. Interestingly, while the onset of COX8-GFP expression is synchronous in SCNT and ICSI embryos (early morula), lower protein levels are found in the SCNT blastocysts. As mitochondria numbers start increasing at peri-implantation, our observation is unlikely due to differential mitochondria numbers in SCNT and ICSI blastocysts, but rather indicates that SCNT mouse embryos exhibit a generally lower gene expression, potentially fostered by the metabolic disturbances in the early embryonic stages. Ongoing work includes clarification of cause-effect relations between the observed metabolic and gene expression dysfunctions in the SCNT embryo.

Keywords: somatic cell nuclear transfer; reprogramming; metabolism
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Niche & Microenvironment

Isolation of novel multipotent neural crest-derived stem cells from adult human inferior turbinate

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Adult human neural crest-derived stem cells are of extraordinary high plasticity and promising candidates for the use in regenerative medicine. The ideal source of such adult stem cells has to satisfy at least the criteria of easy accessibility, sufficient amount of stem cells within the tissue, a high yield and purity of the isolated population and finally a possible cultivation without complementation with animal serum. Here we describe for the first time a novel neural crest-derived stem cell population within the respiratory epithelium of adult human inferior turbinate. In contrast to superior and middle turbinates, high amounts of source material can be isolated from human inferior turbinates even in older patients efficiently and safe using minimally-invasive surgery methods. Within their niche in the lamina propria of respiratory epithelium, inferior turbinate stem cells (ITSCs) expressed high levels of nestin, p75NTR and S100. Cultivated ITSCs expressed nestin and S100 as well as the neural crest markers Slug and Sox10. Furthermore, the majority of samples investigated expressed p75NTR and the transcription factors associated with pluripotency: Sox2, Klf4, c-Myc and Oct4. ITSCs were able to differentiate into ectodermal, mesodermal and endodermal cell types. Finally, we developed a separation strategy based on magnetic cell sorting of p75NTR positive ITSCs, which formed larger neurospheres and proliferated faster than p75NTR negative ITSCs. Moreover, the expression of p75NTR correlated with the expression of Oct4 at RNA and protein level. Taken together our study describes a novel, readily accessible and safe source of multipotent human neural crest-derived stem cells.

Keywords: human stem cells, neural crest-derived stem cells, inferior turbinate, p75NTR, reprogramming factors
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VSEL cells and CFU-f activity in murine bone marrow diminish with age

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Introduction: Murine bone marrow (mBM) is a rich source of multipotent adult stem cells of hematopoietic and mesenchymal origin. However, also putatively pluripotent stem cells like very small embryonic-like (VSEL) stem cells have been isolated from mBM. Recently, a new subpopulation of platelet-derived growth factor receptor alpha (PDGF-Ra) positive cells has been identified within the VSEL population. This PaS-population is highly enriched in primitive mesenchymal progenitors, and gives rise to mesenchymal progeny both *in vitro* and *in vivo*. Here we report an optimised isolation protocol for VSEL / PaS cells and the herein contained primary mesenchymal progenitors based on enzymatic digestion of long bone fragments. We have used this method to monitor the abundance of VSEL / PaS cells and functional mesenchymal progenitors in young and aged mice. Results: The frequency of VSEL cells in conventional crude bone marrow preparations is very low (4.0•10(3) /- 3.5•10(3) VSEL cells/mice). However, by applying enzymatic treatment to the bone tissue, the number of accessible VSEL cells increases by 4-fold (1.6•10(4) /- 1.0•10(4) VSEL cells/mice). A time-course experiment revealed that the enzymatic liberation of CFU-f (colony forming units-fibroblast) activity correlates well with VSEL / PaS cells, but not with e.g. KSL HSCs. A minimal digestion time of 90 minutes is necessary to liberate ~ 90% of all VSEL cells. It was previously reported, that the number of VSEL cells decreases with age. However, no enzymatic treatment was used in these studies, such that probably not all VSEL cells were subjected to that analysis. Given the strong adherence of VSEL cells to bone tissue, we asked if the observed age dependant decrease might be due to a technical difficulty to liberate the cells from the bone. Thus, we re-examined the abundance of VSEL cells and CFU-f in mice at three different age groups between 2 and 12 month, using our established digestion protocol. Our data show that VSEL cells and CFU-f are both firmly attached to bone tissue in young and old mice, and require the same amount of enzymatic digestion. We could confirm a significant drop of CFU-f activity from young (2.1•10(3) /- 0.4•10(3) CFU-f/mice) to old mice (1.2•10(2) /- 1.4•10(2) CFU-f/mice), which was paralleled by a decrease of the VSEL population (4.7•10(3) /- 1.3•10(3) VSEL/mice in 2 month old mice to 1.9•10(3) /- 1.4•10(3) VSEL/mice in 12 month old mice). Conclusion: Both, VSEL cells and CFU-f are firmly attached to bone tissue in young and old mice and require enzymatic treatment for their liberation. Older mice carry a significantly lower total number of VSEL cells paralleled by a lower number of functional mesenchymal progenitors compared to young animals.

Keywords: bone marrow; primary stem cell isolation; mesenchymal stem cells; VSEL cells; aging
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LewisX glycans modulate growth factor signaling in neural stem cells

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Glycosylation is a common modification of proteins and lipids. Elimination of the major classes of glycans has proven lethal during embryogenesis. Therefore, it is accepted that glycosylation is critical for proper protein function. Genetic disruption of various glycosyltransferases that determine distal parts of glycan chains gives rise to viable animals. However, these suffer from significant functional deficits. These findings indicate that glycans serve manifold biological roles, most of which remain to be uncovered. Additionally, glycans serve as excellent biomarkers at different stages of cellular differentiation. LewisX, also known as CD15 or SSEA-1, is a carbohydrate moiety expressed by a subpopulation of neural stem/progenitor cells. LewisX expression is highly regulated in the forebrain during embryonic development. Throughout neurogenesis, LewisX is present on radial glia which represent the neural stem cell population in the developing embryonic cortex. The expression of the LewisX glycan can be used to isolate neural stem/progenitor cells from embryonic as well as adult brains. *In vitro* assays have shown that LewisX influences cell migration and adhesion. Moreover, it is involved in growth factor signalling, such as FGF and wnt. Surprisingly, the function of the LewisX glycan seems to differ depending on the cell type investigated. Albeit its prominent expression on cortical neural stem/progenitor cells its functional relevance during cortical development is not known. Hence, we are interested in the role of the LewisX glycan for cortical neural stem cells. Especially since neural stem/progenitor cells of the ganglionic eminence, the future striatum, show a modified glycosylation pattern. Using neurospheres derived from embryonic cortical cells as a model system, we investigated the function of LewisX for neural stem/progenitor cells. Firstly, we confirmed that Fucosyltransferase 9 (Fut9) is the enzyme responsible for LewisX synthesis in neural stem/progenitor cells. Secondly, we showed that LewisX glycosylation on neural stem/progenitor cells increases their responsiveness to FGF2. We further investigated which molecules in the embryonic nervous system carry the LewisX glycan. We identified Tenascin-C and LDL-receptor related protein 1 (Lrp1) as LewisX carrying proteins. Tenascin-C is a protein of the extracellular matrix expressed by radial glia. It regulates growth factor responsiveness in the neural stem cell niche. Lrp1 is widely expressed by many neural cell types. Interestingly, not all glycoforms of these proteins carry the LewisX epitope. Further experiments are planned to investigate how LewisX alters the function of its carrier proteins within the neural stem cell compartment. In summary, our results demonstrate that the LewisX glycosylation pattern is responsible for the fine tuning of neural stem cell behaviour.

Keywords: LewisX; glycoprotein; neural stem cell; FGF; cortical development
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Increased substrate elasticity favors hematopoietic stem and progenitor cell migration

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In the bone marrow, hematopoietic stem cells (HSCs) reside in endosteal and vascular niches. The interactions with the niches are essential for the maintenance of HSC number and properties. Although the molecular nature of these interactions is quite well understood, little is known about the role of physical parameters such as matrix elasticity. Osteoblasts, the major cellular component of the endosteal HSC niche, flatten during HSC mobilization. We show that this process is accompanied by osteoblast stiffening, demonstrating that during mobilization not only biochemical signals but also mechanical properties of the niche are modulated. HSCs react to stiffer substrates with increased cell adhesion and migration, which could facilitate the exit of HSCs from the niche. These results indicate that matrix elasticity is an important factor in regulating the retention of HSCs in the endosteal niche and should be considered in attempts to propagate HSCs *in vitro* for clinical applications.

Keywords: hematopoietic stem cells; substrate elasticity, mechanosensitivity / mechanotransduction, endosteal stem cell niche, osteoblasts
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Mesenchymal stem cells of the umbilical cord provide stromal support for cord blood hematopoietic stem cells in a three-dimensional, tissue-engineered stem cell niche

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Mesenchymal stem cells (MSC) control hematopoietic stem cell (HSC) differentiation and proliferation by production of growth factors/cytokines, extracellular matrix (ECM) remodelling as well as cell-cell-interactions. It was shown that the co-culture of bone marrow-derived MSC (BM-MSC) with umbilical cord HSC (UC-HSC) can be utilized for cord blood expansion purposes as they preserve the necessary hematopoietic microenvironment. For clinical transplantation, it may be preferred to obtain HSC and MSC from the same donor, thereby eliminating complications resulting from a HSC and MSC genetic mismatch. In a previous study, we established a three-dimensional bone microenvironment by culturing MSC from the Wharton's jelly of the umbilical cord (UC-MSC) in a collagen gel while subjecting the cells to osteogenic differentiation (Schneider et al., Biomaterials 2010). In this artificial bone niche, UC-MSC extensively remodelled the collagenous matrix and efficiently differentiated into osteoblasts. In the current study, we analyzed the potential of UC-MSC to act as a stromal support for UC-HSC in our established three-dimensional bone niche compared to monolayer culture. As previously described, we generated 3D-collagen gels with and without embedded UC- and BM-MSC. HSC were isolated by MACS separation from umbilical cord blood. Enrichment for CD34 cells was performed using immunomagnetic beads. We simultaneously analyzed cell divisions and immunophenotypic differentiation of HPC by using cell division tracking with CFSE. Further, we analyze HSC migration as well as remodelling of the extracellular matrix by MSC. We demonstrated a positive influence of the cell-free, three-dimensional collagen gel on HSC proliferation in comparison to monolayer culture. The proliferation rate was even enhanced when HSC were co-cultured on collagen gels containing UC-MSC. Nevertheless, the co-culture of HSC and UC-MSC also accelerated the differentiation of HSC. CD34 expression decreased after a cultivation period of up to 13 days, whereas CD38 expression was up-regulated as well as the expression of the differentiation markers CD13, CD45 and CD56. In contrast, without co-culture the more primitive CD34( ) CD38(-) fraction of HSC was maintained. Histological analysis demonstrated that UC-MSC might have a chemotactic influence on HSC as migration into the collagen gel was accelerated by embedded MSC. Migrated HSC showed the typical cobblestone-like pattern of proliferating hematopoietic progenitor cells in the collagenous matrix. Besides, HSC maintained their differentiation potential in the collagenous matrix and showed signs of maturation. Interestingly, UC-MSC caused intensive extracellular matrix remodelling as shown by immunohistochemistry and produced a dense matrix of fibronectin, osteopontin and collagen I, typical for the bone marrow niche. Concluding, UC-MSC can act as a stromal support for HSC and build an optimal microenvironment for stem cell differentiation as well as proliferation. Our three-dimensional culture system even accelerates the effect of UC-MSC on HSC maintenance and allows for studying matrix remodelling processes in physiological and pathophysiological processes.

Keywords: umbilical cord; mesenchymal stem cells; hematopoietic stem cells; bone marrow niche; tissue engineering
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Functional role of Vav3 for the regulation and differentiation of neural stem/progenitor cells in the developing visual system

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Chondroitinsulfate proteoglycans (CSPGs) are highly enriched extracellular matrix (ECM) components in the developing ventricular zone and in the neurogenic niches of the adult CNS. The degradation of their covalently attached gylcosaminoglycan chains induces a switch from neurogenic to gliogenic differentiation, diminishing neuronal progenitors while simultaneously promoting astrocytic development. But the mechanisms, by which CSPGs regulate the proliferation and differentiation of neural stem cells, are still unknown. Previous experiments showed that digestion of glycosaminoglycans with the enzyme chondroitinase ABC leads to the upregulation of the guanine nucleotide exchange factor Vav3, an activator of RhoGTPases (Lehmann et al., submitted). In order to investigate the potential role of Vav3 for mediation of CSPG-dependent signaling in neural stem cells, we focused on the embryonic retina and cortex of Vav3 -/- mice. By analysing the cellular composition of the Vav3 deficient retina *in vitro* and *in vivo*, we demonstrate an essential role for Vav3 during mouse retinal development. In early developmental stages, we found significantly more BLBP+-progenitor cells, while there was an overproduction of retinal ganglion cells in later developmental stages. Furthermore, the analysis revealed a role of Vav3 in fate decisions of neural stem/progenitor cells in the ventricular zone. Acutely dissociated cells from the embryonic cortex of Vav3 deficient mice displayed a higher proportion of nestin positive progenitor cells and significantly less GLAST and 473HD positive gliogenic radial glia at E18, a stage when gliogenesis occurs. Consistently, Vav3 deficient neurospheres from the cortex and ganglionic eminence formed significantly less astrocytes and more stem/progenitor cells. This is supported by previous studies of the vice versa situation, where disruption of CS-GAGs and the accompanying upregulation of Vav3 signaling affected cell lineage decisions favoring gliogenesis at the expense of neurogenesis (Sirko et al., 2007). Together, we could show two potential functions of Vav3 in cell fate decisions of the embryonic retina and brain. In the retina, Vav3 seems to be involved in ganglion cell differentiation, while it influences radial glia maturation in the cortical ventricular zone. This study will probably lead to a better understanding of the role of CSPGs on stem cell behaviour and neurogenesis/gliogenesis during development of the retina and brain.

Keywords: extracellular matrix; stem cells; proteoglycans; guanine nucleotide exchange factors, cell fate
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Engineering an artificial, hematopoietic stem cell niche: Mimicking signals of the extra cellular matrix

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Adult, hematopoietic stem cells (HSC) have been used for many years for the treatment of hematopoietic malignancies and exhibit a high potential for further therapeutic applications. The HSC niche in the bone marrow consists of specialized populations of cells, extracellular matrix (ECM) and soluble factors that all play an essential role in regulating stem cell fate. It has been proposed that the ECM provides specific adhesion signals to retain stem cells in their niche. Furthermore, ECM molecules of the bone marrow are capable of inducing cell proliferation, survival, migration and differentiation. In contrast to past opinion, that only the nature of the offered ligand influences cultured cells, it is now widely accepted that the nanometer-scale geometry of the presented ligands and the elasticity of the environment play an important role, too. However, successful expansion of HSCs *in vitro* has not yet been fully accomplished possibly due to the fact that the properties of the niche, especially the composition and biophysical characteristics of the ECM have not been elucidated in detail. The aim of our research is to develop a strategy to expand human umbilical cord blood-derived HSCs by simulating the natural signals of the niche. For this purpose, we are optimizing a system based on a nanopattern, embedded in a hydrogel, presenting, in a defined geometry, bioactive molecules to cells. This allows us to study the influence of spatial, chemical and mechanical parameters on cells. Hydrogel-supported gold nanopatterns with varying interparticle distances were produced using the transfer diblock copolymer micelle nanolithography technique. After biofunctionalization, cellular ligands were presented in a defined nano geometry on elastic substrates. We have shown that the critical adhesive interparticle distance for HSCs on RGD (integrin recognition sequence) functionalized hydrogels is 40 nm. HSCs remained spherical on the nanostructured surface and contacted the ligand via filapodia. Quantification of cell attachment to different adhesive ligands of the HSC niche revealed the highest HSC adhesion to fibronectin and osteopontin derived ligands as well as for bio-active peptides derived from the laminin alpha5 chain on nanostructured surfaces. Further investigations concerning the proliferation and differentiation of HSCs in contact with niche signals are currently in progress. A systematic *in vitro* expansion would have far-reaching consequences for the clinical application of HSCs and could provide a basis for the development of a new generation of biomaterials for stem cell bioengineering.

Keywords: hematopoietic stem cell niche; extracellular matrix; nanopattern; hydrogel
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The microenvironment affects differentiation of the seminoma cell line TCam-2 and infusion into the mouse testis provides a novel model for the study of CIS

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All type II germ cell tumors (GCTs) are thought to originate from a common precursor lesion, called carcinoma-in-situ (CIS). Seminomas are highly similar to CIS cells and are considered to be their default developmental pathway. While embryonal carcinomas (ECs) are prone to differentiate into all three germ layers, seminomatous differentiation processes are observed very rarely and the molecular mechanisms involved in seminoma formation are only poorly understood. In this study, we like to shed light on seminomatous differentiation abilities in dependence to the cellular microenvironment utilizing a seminoma-like cell line called TCam-2. Therefore, TCam-2 cells were injected into the seminiferous tubules and into the flank of nude mice. Additionally, TCam-2 cells were cultivated *in vitro* in murine embryonic fibroblast conditioned medium plus FGF4 and Heparin. We could demonstrate that TCam-2 cells mimic a CIS-like state in the testis and develop into EC-like tumors in the murine flank. Furthermore, TCam-2 cells could be differentiated directly into mixed non-seminomatous lineage *in vitro*. We identified BMP-signalling, the Hippo-pathway and the BLIMP1/PRMT5-mechanism as crucial mediators of this *in vitro* differentiation process. Our findings indicate that the cellular microenvironment strongly influences seminomatous differentiation abilities and that no additionally acquired genetic aberrations, like mutations are necessary to cause a change in the tumor class. Furthermore, we were able to reliably and quickly generate a CIS mouse model that offers new opportunities to study the molecular pathways involved in seminoma development, progression from CIS as well as drug response.

Keywords: seminoma; differentiation; microenvironment; TCam-2, CIS mouse model
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Generation of novel cell lines from bone marrow stroma for the investigation of normal and leukemic haematopoiesis

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A well-regulated haematopoiesis is dependent on the direct interaction of a haematopoietic stem (HSC) or progenitor cell with stromal cells in the bone marrow (BM) microenvironmental niche and the exchange of soluble mediators. Among other cell types mesenchymal stem cells (MSC) are found in the BM in a considerable frequency. These adult stem cells are able to differentiate into various cell types such as osteoblasts, adipocytes, and chondrocytes and can self-renew. In murine systems MSC have been shown to represent a most important component of the stem cell niche for appropriate HSC differentiation. Cumulative evidence indicates that in haematopoietic malignancies the BM stroma is altered in a way to support neoplastic cells at the expense of normal haematopoietic differentiation. Thus, comparative study of normal vs. leukemic stroma with more standardized tools may contribute to the understanding of mechanisms of malignant transformation and define novel therapeutic targets in the stroma compartment. To this end, we established novel cell lines from BM stroma of two patients suffering from acute myeloid leukemia, two lymphoma patients without malignant infiltration of the BM, and, in addition, from the BM of a healthy donor. Plastic-adherent primary BM cells were cultured under conditions favouring outgrowth of fibroblasts. After transduction with different immortalizing genes we established five independent polyclonal cell lines. These cell lines showed a robust proliferation and expressed surface markers reminiscent of MSC. Two of the cell lines were also able to differentiate into osteoblasts, adipocytes, and chondrocytes. With regard to cell lines derived from leukemic and non-leukemic stroma a differential expression pattern was observed for membrane-anchored and soluble molecules (HLA-G, MICA, MICB, ULBP2, ILT-4) known to be implicated in the suppression of the innate and adaptive immune system. These novel bone marrow stroma cell lines are considered as a robust and reliable *in vitro* test system to investigate the microenvironment required for the comparative investigation of leukemic vs. normal haematopoiesis.

Keywords: Bone marrow stroma; HSC; leukemia; cell lines
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Effects of nanostructures and mouse embryonic stem cells on cord formation of rat Sertoli cells *in vitro*

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Male sexual differentiation of the indifferent gonad begins with the SRY-dependent appearance of Sertoli cells in the embryonic gonad. Their aggregation is the crucial starting point for generation of plaques which leads to a morphogenetic cascade terminating in the establishment and subsequent longitudinal outgrowth of testicular cords. It is not known whether Sertoli cells use topographical cues to direct or stimulate morphogenetic events and whether germ cells have an impact on this process. Here we analyze the morphogenetic patterns of primary Sertoli cells from day 7 rats after seeding on variable nanostructures with or without coating of laminin. We also tested if the addition of mouse embryonic stem cells to Sertoli cells cultures affects cord formation in a dose dependent manner. Immature rat testicular cells were obtained from day 7 rat pups by two-step enzymatic digestion using collagenase and hyaluronidase. In experiment 1, Sertoli cells were seeded into either nanostructured (ridge/groove width: 400, 700, 104 nm, height: 350nm) or flat PDMS in 24 well culture plates which were eventually pre-coated with laminin. In experiment 2, Sertoli cells (5×10^5) were seeded into standard uncoated wells (24 well culture plates) and 10^1, 10^2...10^5 OG2-cells (embryonic stem cells carrying a GFP-reporter in the OCT3/4 promoter) were added to each well either immediately or 5 days later. Cultures were maintained for several weeks without media changes and the cells were monitored with time-laps (fluorescence)-microscopy. Cell growth and aggregation as well as orientation of cord-like structures were evaluated. While 400nm and 104nm had less effect, the 700nm nanostructure induced an alignment of peritubular and Sertoli cells in the direction of the grooves not visible in the flat PDMS. The tendency to align in Sertoli cells, but not in peritubular cells, was clearly associated with focal cell densities and was most intense in areas with low cell densities. Laminin coating provided a more random spread of cells but had no effect on alignment of cells. Plaque and cord formation occurred inconsistently and the formation and orientation of cords did not depend on the presence and direction of nanostructures. In experiment 2, addition of OG2-cells induced a cell-number dependent stimulation in terms of speed and intensity of cord formation irrespective of adding the cells to freshly isolated cells or after establishment of monolayers. OG2-cells expanded in contact with Sertoli cells and colonized the newly established cord-like structures. We conclude that nanostructures exert strong cues on testicular somatic cells irrespective of laminin coating. The effect on Sertoli cells vanishes at higher cell density and aggregation and cord formation occurs independently of cues from nanostructures indicating that crosstalk of Sertoli cells at higher densities overcomes the effects of cues from the matrix. The presence of ES cells stimulates aggregation and cord formation in a dose dependent manner indicating that they provide factors promoting testicular differentiation. These studies show that an orchestration of external stimuli from the underlying matrix and from germ cells as well as crosstalk between densely growing cells control the Sertoli cell-intrinsic mechanism leading to aggregation and cord formation during testicular morphogenesis.

Keywords: nanostructures, embryonic stem cells, Sertoli cells, cord formation
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Assessment of cornerstones during HOXB4-assisted hematopoietic development of pluripotent stem cells, *in vitro*

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In hematopoietic stem cells (HSCs), many mutations can lead to diseases associated with blood formation and effector cell function. Thus, the ability to correct the underlying genetic defect within these cells is of prime medical importance. However, isolation, gene targeting and selective expansion of gene-corrected HSC clones is currently not feasible. An alternative solution could be the utilization of pluripotent embryonic stem cells (ESCs) because they allow the efficient application of the aforementioned procedures and can, in principle, be differentiated towards HSCs, *in vitro*, prior to transplantation. An attractive candidate to support their hematopoietic differentiation is the homeodomain transcription factor HOXB4. Our group and others have repeatedly shown that ectopic expression of this protein enhances formation of hematopoietic stem and progenitor cells (HSPCs) from mouse ES-cells and mediates expansion of HSCs, *in vitro* and *in vivo*. To explore the influence of HOXB4 during the development of HSPCs in more detail, we differentiated ESCs as embryoid bodies (EBs) for 6 days and subsequently cultured the dissociated cells in suspension and on OP9 stroma with appropriate cytokine support. The ESC-derived hematopoietic cells (ES-HCs) cultured in suspension were periodically characterized by FACS analyses using the cell surface markers c-kit (CD117), CD41 (gpIIb, integrin alpha IIb chain) and CD34. Surface expression of CD41 marks the earliest known hematopoietic cells during embryonic development. Cells with the phenotype c-kit /CD41 /CD34- have been shown to be capable of engraftment after transplantation into lethally irradiated recipient mice. 9 days after initiation of differentiation, first CD41 cells were detectable in ES-HC cultures. Noteworthy, increased HOXB4 expression tightly correlated with the presence of a CD41hi subpopulation in suspension cultures. On OP9 cells, clusters of round, semiattached cells first appeared on day 9 after EB dissociation with dramatically increasing cell numbers during the following days. These putative hematopoietic cells emerged from islands presumably containing hemogenic endothel cells. To this end, our observations in the ES-EB differentiation system suggest that HOXB4 influences hematopoietic development around the stage of hemogenic endothelium generation. Tracking the appearance of Runx1/AML1 expressing cells (via a Runx1 reporter) in combination with analysis of Tie2, c-Kit, and CD41 expression will enable us to define the stage of earliest HOXB4 activity during hematopoiesis more precisely.

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Keywords: HOXB4; embryonic stem cells (ESCs); embryoid bodies (EBs); hematopoietic stem cells (HSCs); hemogenic endothel

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Somatic & Cancer Stem Cells

Identification of cancer stem cell-like cells in a papillary renal cell carcinoma-derived cell line

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Tumor stem cells play an important role in the development und progression of tumors. They can be defined as a subpopulation of cells within a tumor which shows self–renewal capacity, pseudo/differentiation activity and most importantly, they are able to initiate tumor growth when xenotransplanted in immunocompromized mice. We have established and characterized a tumor cell line, MG-1, from a papillary renal cell carcinoma. MG-1 showed an epithelial morphology, expressed epithelial markers like Ep-Cam (CD326) and cytokeratins 8 and 18 as well as markers on at least subpopulations which have been identified on tumor stem cells of other tumor entities, including CD24, CD44 and CD133. They differentiated along the mesodermal osteogenic pathway and formed tumorspheres when single cells were plated in growth-factor supplemented serum-free medium. Moreover, MG-1 cells were tumorigenic in nude, NOD/SCID and NOD/SCID IL2rcÐnull mice when $1x10^6$ cells were xenotransplanted, and from tumors tumorigenic cells could be re-grown (evidence for self-renewal activity). After cell sorting based on CD133 expression, MG-1 subclones have been generated. All tumorigenic clones were derived from CD133- cells, indicating that the tumor stem cells reside within the CD133- population. One CD133- subclone, M-G1/F7, was highly tumorigenic. Tumor development was observed when as few as 1000 tumor cells were transplanted and several rounds of serial transplantations were possible. MG-1/F7 cells showed a phenotype similar to the parental line and revealed osteogenic differentiation but no sphere-forming activity. Interestingly, although expression of the stem cell marker Oct-4 was observed in about half of the clones, it was not associated with tumorigenicity and MG-1/F7 cells were Oct-4 negative. In conclusion, the newly established epithelial renal cell carcinoma cell line MG-1 and particularly its CD133- subclones share typical features with cancer stem cells, including immunophenotype, tumorigenicity, self-renewal and differentiation potential.

Keywords: Tumor stem cells
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Analysis of the differentiation potential of murine fetal somatic stem cells

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Pluripotent fetal somatic stem cells (FSSCs) have been isolated from murine and porcine primary cell cultures and could be maintained in medium supplemented with 30% fetal calf serum, i.e. T3 medium consisting of 67% DMEM, 30% FCS, 1% glutamine, 1% non essential amino acids and 1% antibiotics (Kues et al., 2005). However, the differentiation potential of FSSCs has not yet been assessed. Here, the differentiation potential of murine FSSCs was characterized either by determining spontaneous differentiation, or by applying protocols for directed differentiation *in vitro*, or by injection of FSSCs into immunodeficient nude mice. For directed differentiation *in vitro*, existing protocols for differentiation into osteogenic and adipogenic lineages were applied. FSSCs were chemically induced to initiate osteogenesis by supplementing T3 medium with 0,1 µM dexamethasone, 50 µM ascorbate and 10 mM β-glycerophosphate (Berg et al., 2007) for 28 days prior to staining with alizarin red S (Guo et al., 2008). Adipogenesis was induced by supplementing T3 medium with 0.1 µM dexamethasone, 2.07 µM insulin, 0.45 mM isobutyl-methylxanthine and 15% new born calf serum for 4 days followed by 0.5% serum supplementation for another 4 days prior to staining with Oil Red O (Berg et al., 2007). Tissue specific stainings with alizarin red S and Oil Red O, respectively, yielded positive results in both cases. Staining of FSSCs with antibodies indicative for cell types of the three germ layers (ectoderm: Rat-401-s, RT97-s, 5A6-s; mesoderm: T14-s and CIICI-s; endoderm: PAX6-s and GS-9A8-s) brought positive results for neuronal cells which stained positive by Rat-401-s, RT97-s and 5A6-s and for cartilage-tissue stained by CIICI-s, but not for cells indicative for endoderm. The tumorigenic potential of FSSCs was investigated by injecting transgenic FSSCs into immunodeficient nude mice. FSSCS from reporter-transgenic mice lines (Oct4-EGFP and CAGGS-Venus) allowed to determining the origin of the resulting tumors. Tumor growth could be induced by injecting FSSCs into immunodeficient mice, and the investigation of these tumors showed that FSSCs differentiated predominantly into cells of mesodermal origin. In conclusion, a limited differentiation potential into meso- and ectodermal cell lineages was found for murine FSSCs. Accordingly these are multipotent stem cells.

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Keywords: fetal somatic stem cells; differentiation potential; tissue specific staining; induced tumors; immunodeficient mice
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Expression profiles of cancer stem cell markers in breast cell lines

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Introduction: Cancer initiating cells or cancer stem cells (CSCs), respectively, are thought to be responsible for tumor progression and therapy resistance. They have been identified in a variety of human solid tumors as well as in cancer cell lines. Cell lines might therefore serve as an attractive source for CSC *in vitro* research. We investigated to which extent breast cell lines of benign and malign origin contain CSC-like cell clones and if their expression profiles correlate with clinical parameters. Material & Methods: Altogether, eleven breast cell lines of normal mammary tissue, breast carcinomas and metastases were analyzed by flow cytometry using a panel of six cell surface markers of CSC-like phenotype. Markers were chosen based on literature search and comprised CD326, CD133, CD44, CD24, CD29 and CD49f. At least four replicates of each cell line were analyzed. Expression frequency of CSC-like markers was divided into four categories with high (> 70% of cells), moderate (< 70% and > 30%), low (< 30% and > 1%), and absent (< 1%) expression. Results: All cell lines showed a stable expression pattern throughout all four replicates. CD326, CD44 and CD133 distinguished benign from malignant origin with absent to low expression in normal cell lines and high expression in carcinoma and metastasis cell lines. Based on CD133 expression we were able to separate not only benign from malignant, but also early from late carcinoma and carcinoma from metastasis. Conclusion: Breast cell lines do harbour to a substantial amount cells with CSC-like properties. The frequency of CD326, CD44 and CD133 shows distinct separation ability between benign and malignant cell lines and even different tumor stages. The eleven breast cell lines seem to represent stable *in vitro* models of distinct CSC-like expression profiles for different clinical phenotypes. They could thus serve for functional, molecular characterization of marker specific expression profiles.

Keywords: cancer stem cells, breast cancer, cell line model, clinical phenotypes
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Putative cancer stem cells in conjunctival neoplasia

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Introduction: Conjunctival intraepithelial neoplasias (CIN) and invasive smooth cell carcinomas (SCC) belong to the most common ocular tumors. They often involve the limbal or the fonical area which are the locations of the corneal epithelial and conjunctival stem cells (SCs) respectively. The aim of the study was to analyze the expression of SC markers in CINs and SCCs. Material and Methods: Invasive and preinvasive conjunctival lesons were excised and exactly classified by experts from the Department of Pathology. Samples, which involved the limbus were compared to samples of histological unaltered limbus. Lesions from conjunctiva were compared to healthy bulbar and fornical conjunctiva. The expression of epithelial differentiation markers (K3, K15, K19), putative limbal SC markers (ABCG2, p63), and molecules expressed in pluripotent/multipotent SCs (NANOG, OCT4, SOX2, MUSASHI 1, KLF4, KIT, NESTIN, PAX6) was examined using real-time PCR. Paraffin sections were stained with antibodies against K3/K12, K15, SOX2 and Oct4. Results: In limbal CIN III SOX2 is remarkable up regulated in comparison to the normal limbus. The analyzed conjunctival samples did not express K3 and CIN samples showed an extremely low expression of K3. From CIN II to CIN III a tendency toward further dedifferentiation is obvious. The expression of SOX2 was in neoplasia samples significantly higher than in both conjunctival regions. All other analyzed markers showed a lower or similar expression in neoplasia samples in comparison to conjunctiva samples. In high grade CIN at the fornical region some cells are stained with K15 in a pattern like putative stem cells at the limbal region. Antibodies against SOX2 and Oct4 are non-uniform disseminated within the epithelium in high grade CIN and SCC. Conclusions: The activation of a transcriptional program in somatic epithelial stem cells of the ocular surface may induce pathologic self-renewal characteristic for cancer stem cells. The genes coding stem cell markers Oct4 and SOX2 are over expressed in high grade CIN. It remains to be clarified if the expression of these genes is associated with tumor progression or bad prognosis.

Keywords: cancer, stem cell, CIN, conjunctiva, limbus
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‚En route' to novel drugs for glioblastoma therapy: Validation of primary hits from a compound library screening

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Glioblastoma (GBM) accounts for one of the most aggressive types of cancer. Despite a multimodal therapeutic approach involving surgical removal of the tumor mass in combination with radiation and chemotherapy the median overall survival time after diagnosis is 14.6 months. Our work aims to improve the very poor prognosis of GBM by developing new antitumor agents that inhibit the growth of every cancer cell, including the population of cancer-initiating stem cells (CSCs). In a previous study, we had applied the commercially available Killer Collection® compound library in a high-throughput screening on CSC-enriched human primary GBM cultures, and we identified 31 candidate compounds that had a significant cytotoxic or cytostatic effect. In this study, we conducted validation experiments on 6 compounds that promised most for future clinical application. Hit validation included thorough analysis of pharmacodynamics based on an alamarBlue® assay of metabolic activity and AnnexinV-analysis of apoptosis. Subsequent *in vitro* examination of growth kinetics, cell cycle analysis, quantification of self-renewing multipotent CSCs, including stem cell marker expression profiling as well as *in vivo* studies on tumorigenicity are conducted to further promote individual candidate compounds in the process of drug development. Preliminary data of these studies will be presented indicating that some of these compounds indeed could become suitable pipeline drugs, while others failed to demonstrate efficiency.

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Keywords: glioblastoma; cancer stem cells; drug validation
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Functional analyses of cell polarity organization in human hematopoietic stem and progenitor cells

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Freshly isolated hematopoietic stem and progenitor cells (HSPCs) are small, round cells which adopt a polarized cell shape upon cultivation. Depending on the activity of the phosphoinositol-3-kinase (PI3K) they form a leading edge at the front and a uropod at the rear pole. We have recently shown that in addition to different lipid raft associated proteins, the lipid raft organizing molecules Flotillin-1 and -2 get highly concentrated at the tip of the uropod. Performing pharmaceutical inhibitor studies we dissected mechanisms controlling HSPC polarization and were able to discriminate two levels of cellular polarization. According to our observation the vast majority of freshly isolated human HSPCs, i.e. umbilical cord blood derived CD34 cells, show a random distribution of the Flotillins and other lipid raft associated molecules like ICAM3. Upon cultivation they redistribute these molecules to form a crescent and thus become intrinsically polarized, before they adopt their characteristic morphological polarized cell shape. Using this discrimination, we obtained evidence that PI3K and atypical protein kinase C (aPKC) activities are required to organize the intrinsic polarity while the morphological polarization process also depends on protein synthesis, actin polymerization and rho-GTPases activities. Since aPKCs form an evolutionary conserved complex with the partitioning defect proteins Par3 and Par6 as well as with the rho-GTPase Cdc42 and this complex has been found to organize cell polarity in many organisms and tissues, we decided to investigate the function of the individual components on the cell polarization process of human HSPCs next. Due to the fact that the Par/aPKC complex also coordinates asymmetric cell divisions in a number of systems and as we showed that human HSPCs can divide asymmetrically, we have started to study the impact of these proteins on the cell fate of human CD34 cells in parallel. Our pharmaceutical studies as well as our experimental strategy within the Par/aPKC project together with preliminary results will be presented.

Keywords: CD34; cell polarity; HSPC
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Numb, a cell fate determinant known from Drosophila, inhibits maintenance of human primitive haematopoietic cell fates

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Hematopoietic stem cells (HSCs) are the most intensively investigated mammalian stem cells. Like other stem cells they are undifferentiated cells that can self-renew over a long period of time and give rise to progenitor cells containing the capability to reconstitute the whole blood system. It has been found that HSCs reside in special stem cell niches that provide a certain combination of extrinsic signals being required to maintain their primitive cell fates. Furthermore, we could recently show that primitive hematopoietic cells can divide asymmetrically. Therefore, both, extrinsic as well as intrinsic factors control whether HSCs self-renew or become committed to differentiate. This, together with the findings that the Notch signaling pathway plays important roles in controlling presumptive HSC fates suggests mechanistic parallels to the development of the peripheral sensory organs of Drosophila. In flies, the Notch signaling pathway, its extrinsic ligands and its intrinsic modulators specify the cell fates of the four cells of the developing external sensory organs. It has been demonstrated that the cell fate determinant Numb segregates asymmetrically mainly into one of the arising daughter cells during cell divisions of the developing sensory organs. Since Numb acts as an intrinsic inhibitor of the Notch signaling pathway, the Notch signal, which is normally activated by its extrinsic ligands Delta and Serrate (the homolog of mammalian Jagged), can only be transduced into one of the two arising daughter cells. Since Numb is conserved during evolution, we investigated whether Numb also controls cell fate specification processes in primitive hematopoietic cells. After showing that Numb is expressed in primitive human hematopoietic CD34 cells, we performed functional analyzes. Over-expression and siRNA-mediated knock down studies on primary human CD34 suggest that Numb acts as a cell fate determinant during early hematopoiesis, similar to the process of the peripheral sensory organ development in Drosophila. Thus, our results suggest that Numb activity inhibits maintenance of primitive human hematopoietic cell fates.

Keywords: CD34 ; HSC; Numb; Notch
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Vav-iCre mediated deletion of the von Hippel Lindau gene does not alter the cell cycle status of hematopoietic stem cells *in vivo*

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Hematopoietic stem cells (HSCs) are located in a highly specialized cellular micro-environment within the bone marrow known as the stem cell niche. The bone marrow HSC niche is positioned at the lowest end of an oxygen gradient, which implies that hypoxia might play a role in regulating HSCs. Consequently, the oxygen sensing pathway with its central negative regulator von Hippel Lindau protein (pVHL) might play a central role in controlling HSC function. Previous studies using conditional Mx1-Cre;VHLloxP/loxP mice were hampered by immediate lethality (Takubo et al., Cell Stem Cell 2010). Furthermore, phenotypic studies of HSCs in Mx1-Cre;VHLloxP/loxP bone marrow transplant models led to conflicting data. Therefore, we here crossed VHL conditional mice with vav-iCre mice to induce constitutive hematopoiesis specific VHL deletion (vav-iCre;VHLloxP/loxP). Complete VHL gene deletion within bone marrow, thymus and spleen was confirmed using a three primer multiplex PCR protocol. Vav-iCre;VHLloxP/loxP mice were viable, however died prematurely within the first six month of life (mean survival 13 weeks). Pathology analysis revealed the formation of cardiac hemangiomas as the most likely cause of death. Detailed flow cytometric analysis of six-week-old vav-iCre;VHLloxP/loxP mice showed that Flk2-CD34- LSK (lin-Sca1 ckit) as well as CD48-CD150 LSK long-term HSC bone marrow proportions and absolute numbers did not differ from control mice. Furthermore, analyzing the vav-iCre;VHLloxP/loxP HSC cell cycle status by combining Hoechst dye and an intracellular Ki67 staining we did not observe any alterations induced by hematopoietic VHL deletion. The most obvious difference between vav-iCre;VHLloxP/loxP and control HSCs was the increased proportion of CD150 expressing cells within the LSK population of VHL null bone marrow. This was mainly due to the increased proportion of CD150 high LSK cells and a simultaneously decrease of CD48-CD150low LSK cells. This is of particular importance as it has been demonstrated that especially CD48-CD150low LSK cells are lymphoid biased HSCs (Morita et al., J Exp Med 2010; Beerman et al., PNAS 2010; Challen et al., Cell Stem Cell 2010). In contrast to the unchanged bone marrow long-term HSC numbers we observed increased LSK and CD48-CD150 LSK cells in the spleens of vav-iCre;VHLloxP/loxP mice. In parallel we also analyzed mice with vav-iCre driven ablation of the positive oxygen sensing pathway regulator HIF-1alpha. In contrast to the vav-iCre;VHLloxP/loxP model, 6-week-old mice with hematopoietic HIF-1alpha ablation did not show any HSC alterations consistent with data reported for the Mx1-Cre;HIF-1alpha model. These data demonstrate that hematopoietic VHL deficiency does not directly lead to an altered cell cycle status of HSCs. However, pVHL deficient HSC trafficking is altered in terms of spleen colonization.

Keywords: hematopoietic stem cells, hypoxia, von Hippel Lindau protein
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Analysis of transcription factors differentially expressed in the primitive human hematopoietic compartment

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Somatic stem cells are required to maintain homeostasis in different tissues. In this context stem cells give rise to differentiating cells which replace cells getting lost in the lifetime of a multicellular organism. To fulfil this function over a long period of time, it is essential that the pool of stem cells remains a constant size. Since both the abnormal loss as well as the uncontrolled expansion of stem cells is fatal for organisms, the decision of self-renewal versus differentiation needs to be tightly regulated. The understanding of such mechanisms will not only be essential for the clinical use of these cells in regenerative medicine but will also increase our understanding of certain aspects of tumor formation and degenerative diseases. A number of transcription factors have been identified to take part in the decision process self-renewal versus differentiation of primitive hematopoietic stem cells, including HoxB4, AML1/Runx1, SCL/Tal1, Meis1. While loss of function of these transcription factors is generally associated with defects in the development of the hematopoietic system, the aberrant expression often results in an expansion of primitive hematopoietic cells and seems to be connected to different forms of leukemia. With the aim to identify additional transcription factors required for the self-renewal process of primitive human hematopoietic cells, we have performed genome wide GeneChipTM analyses of different cell fractions, containing either primitive or more mature hematopoietic cells. We identified a number of transcription factors encoding genes which are specifically expressed in the most primitive hematopoietic cell fractions, whose function has not yet been associated with hematopoiesis. In order to characterize the early hematopoietic function of some of these candidate genes, we decided to perform over expression as well as RNAi mediated knock down experiments. We are using a lentiviral transduction to genetically manipulate primary human umbilical cord blood derived CD34 cells and analyze effects on the cell fates of transduced cells in different functional read out systems.

Keywords: hematopoiesis; somatic stem cells; transcription factors; cell fate; CD34
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Doxycyclin inducible expression of cytidine deaminase (CDD) for myeloprotection

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Myelosuppression is a major side effect of cytotoxic chemotherapy and one way to facilitate hematopoietic regeneration is the usage of stem cells genetically modified to express chemotherapy resistance (CTX-R) genes. One CTX-R gene investigated in this context is the cytidine deaminase (CDD). Recently, we could show that overexpression of CDD from a constitutive spleen focus forming virus (SFFV)-derived promoter profoundly protects hematopoiesis from Ara-C. However, this study failed to show transduced B- and T cells *in vivo*, arguing for a potential CDD induced lymphotoxicity. To circumvent the potential CDD induced lymphotoxicity, we here have generated a conditional expression system allowing for externally controllable transgene expression (TET-ON). Doxycyclin (DOX) controllable expression of CDD was investigated in murine protection assays *in vitro* and *in vivo*, respectively. CDD-mediated cytostatic drug resistance was first evaluated in 32D cells. Therefore, cells were transduced with a CDD expressing lentiviral construct (TET-CDD) and a lentiviral vector expressing the reverse transactivator protein (rtTA3). Two days post-transduction (p.t.), transduced cells were treated with different concentrations of Ara-C for 48h. Subsequently, cell viability was determined with Propidium iodide. In addition, primary murine (lineage negative) cells were transduced using the same lentiviral vectors. Two days p.t. transduced cells were plated in clonogenic assays for 10 days in the presence of different concentrations of Ara-C. Thereafter, numbers of hematopoietic colony were determined. For *in vivo* experiments, two cohorts of C57Bl/6 mice were transplanted with Lin-cells from R26-M2rtTA mice previously transduced with TET.CDD or control SIN lentiviral vectors. Transgene expression was determined through consecutive DOX administration four weeks post transplantation. *In vivo* protection analysis was performed 7 weeks post transplantation by administering 500 mg/kg Ara-C for four consecutive days. Inducible CDD expression protects 32D cells within 24h against Ara-C up to 5000 nM, whereas control- or untransduced cells die at a 25-fold lower dose. After DOX withdrawal, CDD expression remains detectable for three days in 32D cells. Primary murine cells were protected within 24h against Ara-C up to at least 300 or 600 nM. In contrast, control- or untransduced cells die at 50 nM Ara-C. DOX administration *in vivo* revealed stable transgene expression in peripheral B-, T- and myeloid cells 15 days post DOX administration and transgene expression was detectable for 21 days after DOX withdrawal. Moreover, the TET.CDD vector conveys significant protection against Ara-C to peripheral blood cells of transplanted R26-M2rtTA mice. Thus, in summary, we demonstrate inducible hCDD expression in 32D cells, primary murine HSCs and in peripheral blood cells of transplanted animals. In all models DOX-induced CDD expression mediates protection from Ara-C toxicity. In contrast to constitutive CDD expression, no lymphotoxic effects were observed arguing for DOX induced CDD expression and a reliable gene transfer system to reduce transgene toxicity.

Keywords: gene therapy, hematopoiesis, stem cells
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Long-term follow-up of cellular in-vitro immunity after allogeneic peripheral blood stem cell versus bone marrow transplantation

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Immune reconstitution appeared to be superior in allogeneic peripheral blood stem transplantation (PBSCT) versus bone marrow transplantation (BMT) within the first year post transplantation (Ottinger et al. Blood 1996; 7: 2775). However, long-term follow up data on cellular immune function are lacking. In the present study we extended the previous data and analyzed 408 patients after PBSCT and 324 after BMT (1231 and 787 samples, respectively) by lymphocyte transformation test (LTT) for up to 20 years post transplantation. Here, mononuclear cells were stimulated *in vitro* by 4 mitogens and 12 recall antigens of viral, bacterial and fungal origin. Data within the first year could be confirmed. Thereafter, within the second and third year, cellular in-vitro responses were comparable in both groups. Starting from year 4, patients after BMT exhibited significantly higher reactions towards mitogens ($P < 0.05$ in the 4th year) and recall antigens ($P < 0.01$ in the 4th and 6th year). The later differences were mainly caused by an increased LTT reaction towards herpes viruses (HSV-type 1 and VZV) and influenza virus A and B in the BMT group. Finally, responses towards recall antigens reached levels of healthy controls (n = 374) approximately 2 1/2 years after transplantation. Recipients benefit from PBSCT within the critical first year post transplantation when they are at high risk for infections but not thereafter.

Keywords: Immune reconstitution; peripheral blood stem transplantation; bone marrow transplantation; cellular immune function; lymphocyte transformation test
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Hematopoietic stem cell transplantation from donors immunized against hepatitis B results in clearance of infection in recipients

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After previously having shown that immune transfer can occur via hematopoietic stem cell transplantation and also after liver transplantation we implemented this knowledge in clinical patient care. We here describe two patients who suffered from non-Hodgkin lymphoma or acute myeloid leukemia plus either acute or chronic hepatitis B (HBV) infection, respectively, prior to peripheral blood stem cell transplantation (PBSCT). In the patient with acute HBV infection occurring at month 6 prior to PBSCT, we actively immunized the HLA-identical sister 4 times (day 0, week 2, week 4, and month 5) using Bio-Hep-B, an HBV vaccine containing PreS1, PreS2 and S antigens and thereby obtained humoral and cellular HBV immunity prior to PBSCT (anti-HBs titer: 347 IU/L, HBV-specific stimulation index (SI): 8.5, and HBV-specific interferon-gamma ELISpot: 12 spots increment). The corresponding recipient was transplanted after HBV DNA became undetectable. He was followed up for 19 months and remained HBV DNA negative. At month 19 anti-HBs and HBV-specific cellular immunity was still measurable (1,483 IU/L, SI of 4.8, and 8.5 spots increment) indicating that HBV infection was controlled by donor immunity. The patient with chronic HBV infection received a graft from an HLA-identical unrelated donor who had been immunized using a German standard HBV vaccine containing the S antigen (anti-HBs titer 265,647 IU/L). She was furthermore treated by antiviral drugs prior to and post transplantation. After PBSCT the patient cleared her chronic HBV infection and became HBV DNA negative. Furthermore, she displayed humoral and cellular HBV immunity at month 1 after transplantation (anti-HBs titer 588,497 IU/L, SI of 5.4, and 8 spots increment). At month 3 she was tested again and humoral HBV immunity further increased (anti-HBs 1,301,100 IU/L). Cellular HBV immunity, however, was undetectable at that time (SI of 1.0 and 2 spots increment). Most likely, after PBSCT HBs antigen had persisted outside the peripheral blood and "boostered" anti-HBs production. The data demonstrate that in PBSCT recipients the reactivation of acute HBV infection could be prevented and chronic HBV infection could be cleared if donors were immunized against HBV.

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Keywords: Hematopoietic stem cell transplantation; immune transfer; clearance of hepatitis B
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Endosomal machinery in hematopoietic stem and progenitor cells (HSPCs)

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One important aspect in the field of stem cell biology is to elucidate the mechanisms which control the decision whether progenies of somatic stem cells are maintained as stem cells or whether they become committed to differentiate. In principal two different settings have been identified that can control such decisions, the process of asymmetric cell division and the stem cell niche. For the most investigated mammalian stem cells, the hematopoietic stem and progenitor cells (HSPCs), it has been elaborated that they reside in special niches, which are required for their maintenance. In addition, due to the identification of asymmetrically segregating proteins, we have previously proven for the first time that HSPCs also have the ability to divide asymmetrically. Remarkably, three of the four identified, asymmetrically segregating proteins are associated with the endosomal machinery. According to recent findings that endosomes also can segregate asymmetrically in model organisms and that they participate in the regulation of cell fate decisions, we have started to elvaluate impacts of the endosomal machinery on cell fate decisions within the human primitive hematopoietic cell compartment. Since small GTPases play key roles in regulating fission, fusion and trafficking of endosomal vesicles, we decided to set up a technical platform to genetically manipulate human HSPCs with constructs expressing normal as well as constitutive active and dominant negative isoforms of a variety of different Rab-GTPases. After cloning corresponding coding regions into lentiviral vectors allowing to stably transduce human somatic stem cells, we are now exploring their functional impacts on the decision self-renewal versus differentiation of human HSPCs in different *in vitro* assays.

Keywords: hematopoietic stem cells; GTPases; endosomes
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Cell fusion: A step towards cancer progression?

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Data of the past 10 to 15 years provided evidence that cell fusion is not only a process in normal development, but also takes place in the course of cancer disease. It has been shown that tumour cells are highly fusogenic and that fusion of tumour cells can give rise to hybrid cells exhibiting new properties, such as an enhanced metastatic potential, an enhanced drug resistance, a higher proliferation rate as well as an increased resistance to apoptosis. In the present study we investigated hybrid cells derived from spontaneous fusion of murine 67NR-Hyg mammary carcinoma cells with bone marrow derived cells (BMDCs) from Tg(GFPU)5/Nagy mice *in vitro*. First evidence of cell fusion was investigated by detection of EGFP and determination of chromosomal number in hybrid cells compared to parental cells. Furthermore, Real Time PCR Arrays for analysing Mouse Cancer Drug Resistance and Metabolism, Western Blot, FACS analysis, proliferation and migration studies were accomplished. Like in parental BMDCs the gene encoding for EGFP could be detected in hybrid cells by PCR analysis. Determination of chromosomal number revealed that each tested hybrid clone exhibited a mean chromosome set of nearly 50 to 60 chromosomes per cell, which, interestingly, was in between the mean chromosomal number of parental cells with 40 chromosomes for murine BMDCs and more than 70 chromosomes in 67NR-Hyg cells. PCR results as well as Western Blot analysis indicated a strong up-regulation of ABC-transporters, especially Abcb1a and Abcb1b, the murine homologue of human P-glycoprotein, in hybrids cells. Protein expression analysis could be verified by measuring ABC-transporter mediated Rhodamine123 efflux by FACS analysis. Both, 67NR-Hyg cells and BMDCs, showed only a small side-population of ABC-transporter expressing cells, whereas in hybrid cells this small side-population seemed to be the main population. Rhodamine efflux could be inhibited by blocking ABC-transporters with Verapamil. We investigated cell proliferation and viability among different well known cytostatic drugs like Doxorubicin, 17-DMAG, Etoposide, Paclitaxel and 5-Fluoruracil. For instance, hybrid cells exhibited an enhanced drug resistance to concentrations up to 10µM Doxorubicin and 17-DMAG, whereas parental cells showed a strong reduced viability after three days of incubation. This enhanced drug resistance of hybrid cells could be reversed by addition of 50µM Verapamil and hybrid cells died at low concentrations of 1µM Doxorubicin or 17-DMAG. In case of high concentrations of Etoposide and 5-Fluoruracil 67NR-Hyg as well as hybrid cells, revealed a distinct reduced viability compared to parental BMDCs, which only could be further reduced by addition of Verapamil to Etoposide. Investigating the migratory behaviour within a 3-dimensional collagen matrix revealed an altered migratory activity of hybrid cells caused by influence of EGF, IL-8 and SDF as compared to parental cells. For instance, clone 3 hybrid cells showed an enhanced migratory activity under influence of Il-8 and SDF, whereas clone 1 hybrid cells only seemed to respond to EGF. Both parental cells showed no enhanced migratory activity under influence of examined factors. Our results clearly reveal that each hybrid clone showed a unique pattern of alterations related to proliferation under cytostatic drugs, gene and protein expression as well as to cell migration capacity. We thus conclude that cell fusion between breast cancer cells and BMDCs can give rise to hybrids with novel properties including an enhanced ABC-transporter mediated drug resistance as well as an altered migratory behaviour, which may promote cancer (hybrid) cell survival during chemotherapy and which may enable such hybrid cells to initiate the metastatic cascade.

Keywords: cell fusion; cancer; bone marrow derived cells; drug resistance; migration
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Establishment of an extended *in vitro* assay to detect the most primitive human hematopoietic cells at a clonal level

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Hematopoietic stem cells (HSCs) can either self renew or give rise to multipotent hematopoietic progenitor cells (HPCs). As the most primitive hematopoietic cells both cell types obtain the ability to reconstitute all mature blood cell types. According to the classical model of hematopoiesis, these HPCs become either restricted to the lymphoid or to the myeloid lineage, e.g. to the common lymphoid (CLPs) or common myeloid progenitor (CMPs) cells. Via more restricted HPCs, CLPs then give rise to T, B and natural killer (NK) cells as well as to a subset of dendritic cells (DCs), while CMPs differentiate to macrophages, granulocytes, megakaryocytes and erythrocytes as well as a second subtype of DCs. Due to the recent characterization of HPCs containing partial myeloid and partial lymphoid developmental potentials the classical model of hematopoiesis has been challenged. A bundle of new data suggests the existence of additional or alternative developmental pathways. Aiming to set up a functional *in vitro* read out system for the most primitive human hematopoietic cells, we originally developed a so called myeloid-lymphoid initiating-cell (ML-IC) assay. Within this assay individual candidates for very primitive human hematopoietic cells are initially expanded on a murine stromal feeder cell layer. To test for their lymphoid or myeloid lineage developmental capacity, the daughter cells are then either transferred into an assay allowing NK cell or granulocyte and macrophage development, respectively. Before discovering the novel HPC types, deposited primitive hematopoietic cells whose offspring gave rise to NK cells as well as granulocytes and macrophages were retrospectively claimed as primitive cells containing the potential to develop into all hematopoietic cell types. However taking the new findings into account, this conclusion can not be justified anymore. Since we are still interested in an *in vitro* read out system allowing the functional detection of the most primitive human hematopoietic cells and also to define novel hematopoietic routes, we decided to extend the ML-IC assay for additional lineage read outs including T cell, B cell, megakaryocyte and erythrocyte development. Our experimental strategy to extend this assay and our ongoing results will be presented.

Keywords: HSC; in vitro assays; hematopoiesis; classical model; ML-IC
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DNA damage leads to senescence and astrocytic differentiation of neural stem cells

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Tissue homeostasis failure and ageing are thought to be the manifestations of the loss of somatic stem cell activity. Stem cell failure in turn may likely happen due to accumulation of DNA damage in stem cells, yet the present knowledge of DNA damage impact on somatic stem cells is sparse. - DNA damage response (DDR) pathways have been extensively studied in various cellular models, predominantly tumour cell lines and also in untransformed systems like primary fibroblasts. In those systems it was shown that damage to genomic DNA evokes prompt cellular responses, such as cell cycle arrest, apoptosis or senescence. We addressed the role of DDR in the recently established model of embryonic stem cell-derived murine neural stem cell (NSC) lines, which grow in homogenously undifferentiated NSC monolayers. We have discovered that DNA damage by X-rays leads in these cells to a swift cell cycle arrest and senescence, but also to the loss of their stem cell markers such as Nestin, Sox2 and Pax6. Moreover, irradiated NSC acquire the expression of typical astrocyte markers such as GFAP and S100b, while still being cultured in NSC proliferation medium, without addition of any known astrocyte differentiation stimuli. We also studied the mechanisms behind this phenomenon. Strikingly, inhibition of key DDR factors such as ATM, Chk2 and p53 in fact strongly promotes the astrocytic differentiation of X-ray irradiated NSC. Instead, the onset of astrocytic differentiation is acutely dependent on the activation of JAK/STAT and BMP/SMAD signaling pathways. Their inhibition prevents upregulation of GFAP, yet does not allow bypass of senescence or cell cycle re-entry of irradiated NSC. Hence, we propose a two-stage model of DNA damage effect on NSC: 1. rapid cell cycle arrest and senescence, associated with the loss of stem cell features 2. subsequent acquisition of astrocyte-similar characteristics and gene expression. This mechanism may account for depletion of stem cells and tissue homeostasis failure in pathological conditions of genotoxic insult to somatic stem cells.

Keywords: neural stem cells; DNA damage; differentiation; astrocytes
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Human spermatogonial stem cells as a source for pluripotent stem cells?

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The use of patient-specific adult stem cells for stem cell based therapies forms a good alternative to human embryonic stem cells. Recent studies showed an amazing plasticity of murine spermatogonial stem cells *in vitro*. These unipotent stem cells can be converted into pluripotent stem cells without direct intervention into the genome. The access to healthy human testis tissue for further research is difficult. Aim of this study was to find an alternative source to common organ donation. Tissues from organ donors, tumor tissues and testis tissues next to the tumor, so called "tumor-free" tissues from tumor patients, were analysed regarding spermatogonial and pluripotential markers on both RNA- and protein levels. RT-PCR experiments showed that the spermatogonial marker genes VASA, DAZL and Stra8 are significantly down-regulated in both tumor and "tumor-free" tissues compared to tissues from organ donors. Pluripotential marker genes like NANOG, OCT4 and GDF3 are significantly up-regulated in both tumor and "tumor-free" tissues compared to tissues from organ donors whereas FOXD3 is suppressed. There are no significant changes regarding genes encoding SOX2, LIN28, KLF4 and c-MYC among different tissues. However, clear expression of OCT4, NANOG, SOX2, FOXD3, c-MYC and KLF4 were found in all organ donor samples. Western blot analyses demonstrated the down-regulation of spermatogonia-specific marker VASA and up-regulation of pluripotency markers OCT4, NANOG and LIN28 in both tumor and "tumor-free" tissues compared to tissues from organ donors. Immunohistochemical staining showed that spermatogonia in testis tissues of organ donors were positive for Ki-67 and VASA but negative for NANOG, OCT4 and LIN28 whereas NANOG, OCT4 and LIN28 positive cells were found in tumor and "tumor-free" tissues. These findings indicate that cells expressing pluripotency markers exist in healthy human testis tissue, but testis tissues from tumor patients are not suited for the isolation of spermatogonial stem cells. Thereupon we isolated cells from adult testis tissues of organ donors and cultivated them on mouse embryonic feeder layer. Within the first week of isolation we saw putative SSCs. These cells grow in colonies and could be further cultivated until passage 8. RT-PCR analyses showed the expression of the transcription factors OCT4, NANOG, SOX2, c-MYC and KLF4 with a slide decrease in higher passages. Immunocytochemical staining showed that these cultured putative SSCs were positive for spermatogonia-specific markers VASA and DAZL as well as pluripotency marker SSEA4. It is of paramount importance to establish the culture condition for human SSCs to maintain their pluripotency properties *in vitro*. Successful conversion of these cells into pluripotent stem cells may provide a new source for basic research and potentially therapeutic application.

Keywords: human spermatogonial stem cells, pluripotency, human testis, cancer
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Limbal epithelial stem cells of the cornea: characteristics and clinical relevance

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Background: Human limbal epithelial stem cells (HLECs) are tissue specific stem cells which are located in the transitional region between the cornea and conjunctiva known as the limbus. They are capable of differentiation into corneal epithelial cells and form the reservoir for corneal epithelial cell renewal. HLEC display several characteristics attributed to stem cells including slow cell cycles, expression of stem cell- (ABCG2, p63, K15) and pluripotency markers (OCT4, SOX2, c-MYC, NANOG) and lack of corneal epithelial differentiation markers (K3, CX43) as well as absence of gap-junctional communication. Tissue engineering: Transplantation of HLECs cultivated on amniotic membrane (AM) is a novel method in regenerative medicine for the treatment of damaged corneal epithelium leading to limbal stem cell deficiency. Samples form limbal tissue are partly digested with dispase and cultivated *ex vivo* with AM as a growth substrate. Clinical use in regenerative medicine: Cultivated HLECs from autologous or allogenic tissue samples are used in regenerative medicine in order to restore the damages of the corneal surface caused by injuries (e.g. chemical burn) or diseases (e.g. Pterygium, congenital aniridia) which result in limbal stem cell deficiency. They are capable of restoring the stem cell reservoir and provide regeneration and maintenance of the corneal epithelium. Conclusions: HLECs are adult stem cells that can be cultivated *ex vivo* from autologous or allogenic donor tissue and transplanted in regenerative medicine in order to reconstruct a regular corneal epithelium thereby maintaining and restoring vision.

Keywords: tissue specific stem cells, regenerative medicine, tissue engineering
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Influence of the secreted fraction from bone marrow-derived stroma cell lines on NK-mediated lysis

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The impairment of the immunologic response against tumors is a known issue in cancer biology. Besides alteration of the tumor cells themselves components of the tumor microenvironment may play a role in this decreased immune response. Tumor-derived stromal cells (TStrC) were previously shown to impair NK cell proliferation and function. However, the mechanisms underlying the influence of stromal cells in tumor development are poorly understood. In this work we sought to investigate the potential influence of the secreted fraction from different TStrC lines as well as stromal cell lines derived from healthy subjects in NK cell function. Six cell lines were derived from bone marrow stroma by immortalization with different transgenes. Primary plastic-adherent stroma cells prior to immortalization were cultured from patients suffering from acute myeloid leukemia, lymphoma patients without malignant infiltration in the bone marrow, and a healthy donor. These cell lines displayed robust proliferation and expressed surface markers reminiscent of MSC. Two of these lines were also able to differentiate into osteoblasts, adipocytes and chondrocytes. All stromal cell lines expressed the ligands for the activating NKG2D receptor i.e. MICA, MICB, ULBP2 which have been described to have activating properties when in membrane form and, conversely, inhibitory features when in the soluble form, as well as cytokines. These molecules were also present in the ultracentrifuge (UC) pellet fraction, composed of protein aggregates, microvesicles, and exosomes. Exosomes derived from the TSrC also displayed significant amounts of cytokines, such as IL-8, which was high in all lines, but also variable levels of IL-1b and IL-6 and lower amounts of other cytokines. When analyzing NK92, a NK lymphoma cell line, cell-mediated lysis against K562 and K562-derived lines we observed that NK92 mediated lysis was strongly inhibited by stromal cell supernatants. However, the UC pellet fraction did not seem to inhibit NK cell lysis, which could be due to the high amount of pro-inflammatory cytokines. Taken together, our results support the use of cell lines derived from bone marrow stroma as an interesting model for analyzing the interaction between tumor-derived stromal cells and the immune system in patients with malignancies in order to evaluate their potential role in the protection of tumor cells from immune system aggression.

Keywords: Tumor-derived stroma cells; microvesikel; exosomes;MCS; NKG2D ligands
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HLA-G expression in undifferentiated human embryonic stem cells

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Human leukocyte antigen G (HLA-G) is a tolerogenic molecule that acts on cells of both innate and adaptive immunity. This molecule has been associated with tumor progression, transplantation, placentation as well as the protection of the allogeneic fetus from the maternal immune system. We investigated HLA-G mRNA and protein expression in undifferentiated human embryonic stem cells (hESC) derived from the inner cell mass (ICM) of blastocysts. HESC self-renew indefinitely in culture while maintaining the potential to differentiate into all cell types of the body, providing an unlimited source of cells for therapy. HLA-G mRNA was detected in three early and late passage hESC, as assessed by real time RT-PCR. Qualitative RT-PCR showed a different isoform expression pattern, although HLA-G1 mRNA was found in all three lines. Protein expression was demonstrated by immunocytochemistry and confirmed by flow cytometry and ELISA on a hESC extract. Binding of HLA-G with its immunoglobulin-like transcript 2 (ILT2) receptor demonstrated the functional active status. In order to verify this finding in a physiologically relevant setting, HLA-G protein expression was investigated during preimplantation development. We demonstrated HLA-G protein expression in blastocysts in which we found it expressed in trophectoderm (TE) as well as in ICM cells. Co-expression of HLA-G and NANOG, known to be expressed in some nuclei of blastocyst ICM cells, confirmed these cells to be ICM cells. During blastocyst development, a down-regulation of HLA-G in the ICM cells was present. This data may also be of relevance for the development and transplantation of ESC-derived cellular therapeutics since undifferentiated hESC can contaminate the transplant of differentiated stem cells and develop into malignant tumors.

Keywords: human embryonic stem cells; HLA-G; preimplantation development; blastocyst development; NANOG
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The adult stem cell marker Musashi-1 modulates endometrial carcinoma cell cycle progression, apoptosis and tumor growth *in vivo* via Notch-1 and p21WAF1/CIP1

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The RNA-binding protein Musashi-1 has been proposed to maintain stem cell function during development and regenerative processes as a modulator of the Notch-1 signaling pathway. Musashi-1 expression is up regulated in endometrial carcinoma, however, its pathogenetic role in this tumor entity is unknown. Here we investigate the functional impact and mode of action of Musashi-1 on endometrial carcinoma cell behavior *in vitro* and in a nude mouse xenograft model. Aldehyde dehydrogenase-1 activity and side population (SP) measurement by Hoechst dye exclusion revealed that the Ishikawa endometrial carcinoma cell line contains a pool of putative cancer stem cells. Musashi-1 expression is up regulated in SP+ subpopulations compared to SP- cell pools. siRNA-mediated knockdown of Musashi-1 mRNA expression lead to an altered expression of the signaling receptor Notch-1 and its downstream targets, the transcription factor Hes-1 and the cell cycle regulators p21WAF1/CIP1 and cyclin B1, as determined by Western blotting and quantitative real-time PCR. Flow cytometric and ELISA analyses revealed that Musashi-1-mediated modulation of these factors exerted an antiproliferative effect on the cell cycle, and increased apoptosis in endometrial carcinoma cells. In a nude mouse xenograft model, siRNA-mediated depletion of Musashi-1 significantly inhibited tumor growth *in vivo* by 43% ($p<0.05$, n=15 mice/group). We conclude that Ishikawa cells contain a subpopulation of cells with stem cell-like properties. Musashi-1 modulates endometrial carcinoma cell cycle progression and apoptosis via the stemness-related factors Notch-1, Hes-1 and p21WAF1/CIP1, thus emerging as a novel future target for endometrial carcinoma therapy.

Keywords: Musashi-1; Notch-1; cancer stem cells; apoptosis
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Myelinating Schwann Cells can be reprogrammed to multipotency by culture

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Adult neural crest related-stem cells are persisting in the adulthood making them an ideal and easily accessible source of multipotent cells for potential clinical use. Recently, we reported the presence of neural crest-related stem cells within adult palatal ridges raising the question of their localization in their endogenous niche. Using immunocytochemistry, RT-PCR and correlative fluorescence and transmission electron microscopy, we identified myelinating Schwann cells within palatal ridges as a putative neural crest stem cell source. Palatal Schwann cells expressed nestin, p75NTR and S100. Correlative fluorescence and transmission electron microscopy revealed the exclusive nestin expression within myelinating Schwann cells. Palatal neural crest stem cells and nestin-positive Schwann cells isolated from adult sciatic nerves were able to grow under serum-free conditions as neurospheres in presence of FGF-2 and EGF. Spheres of palatal and sciatic origin showed overlapping expression pattern of neural crest stem cell and Schwann cell markers. Expression of the pluripotency factors Sox2, Klf4, c-Myc, Oct4, the NF-κB subunits p65, p50 and the NF-κB-inhibitor IκB-β were up-regulated in conventionally cultivated sciatic nerve Schwann cells and in neurosphere cultures. Finally, neurospheres of sciatic origin were able to differentiate into ectodermal, mesodermal and endodermal cell types emphasizing their multipotency. Taken together we show that nestin-positive myelinating Schwann cells can be reprogrammed into multipotent adult neural crest stem cells under appropriate culture conditions.

Keywords: neural crest stem cells, stem cell niche, Schwann Cells, cellular reprogramming, correlative fluorescence and transmission electron microscopy
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Aberrant expression of the pluripotency marker SOX-2 in endometriosis

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The endometrium is constantly regenerated during the menstrual cycle, and it has been proposed that the enourmous regenreative capacity of this tissue is due to the activity of stem cell activity. In the present study, we addressed the question if pluripotency-associated putative stem cell markers are present, and differentially expressed between normal and pathologically altered endometrial tissue. We focussed on the transcription factor SOX-2, which is essential for maintaining the high self-renewal potential of undifferentiated embryonic and somatic stem cells, and on asssociated pluripotency factors. Dysregulated SOX-2 expression has been described in at least 14 different tumor entities, and is associated with poor prognosis, however, little is known about a potential contribution of SOX-2 dysregulation to the pathogenesis of endometriosis. Expression of SOX-2, OCT-4, KLF-4, and NANOG was analyzed by quantitative real-time polymerase chain reaction, immunohistochemistry, and immunofluorescence microscopy in the endometrium, myometrium, and endometriotic tissue of 36 patients. We demonstrate that SOX-2 expression is significantly more frequent in proliferative vs secretory endometrium, and in endometriotic tissue compared to healthy secretory endometrium. Aberrant expression of SOX-2 may indicate a stem cell origin of endometriosis, whereas the presence of all progenitor markers in endometrial tissue marks the endometrium as a potential source for induced pluripotent stem cell generation.

Keywords: endometriosis; pluripotency; disease; SOX-2; OCT-4; NANOG
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Transcriptional Regulation & Micro-RNAs

Prox1 determines oligodendrocyte differentiation in the adult mouse subventricular zone

Eva Christine Bunk, Sandra Stelzer, Jens C. Schwamborn

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Prox1, a homeobox transcription factor has previously been shown to play an important role during embryonic development of many different organs such as the spinal cord. Moreover, Prospero, the Drosophila homologue of Prox1 in vertebrates, suppresses the genetic program of self-renewal of NSCs and cell cycle progression, while it drives terminal neuronal differentiation. The transient expression pattern of Prox1 in the developing mouse CNS seems to be restricted to the subventricular region where progenitor cells proliferate and give rise to neurons. So far, remaining Prox1 expression within the adult brain was only shown for the hippocampal granule neurons in the dentate gyrus. Here, we provide evidence that Prox1 is also present in the adult SVZ and is associated with neural stem cells and their progeny. However, Prox1-positive cells from the SVZ do not seem to migrate along the RMS into the OB to generate neurons. Moreover, newly generated Prox1-positive cells where found within in the corpus callosum where they express the oligodendrocyte marker GST℗. Further *in vitro* experiments showed that Prox1 gain-of-function drives oligodendrocyte differentiation in NSCs. Recently, evidence was provided that Prox1 directly interacts with the Notch1 promoter region within the developing spinal cord of mice and chick in order to promote neuronal differentiation. Notch1 is known to be one of the key players in the maintenance of NSCs, but also seems to be important for oligodendrocyte differentiation. Using an oligodendrocyte-like cell line (OLN 93) we could show that Prox1 up-regulates Notch1 mRNA levels. In conclusion, we here for the first time report a function of Prox1 as fate a determinant for oligodendrocyte differentiation in the adult mammalian brain.

Keywords: Prox1; neural stem cells; oligodendrocytes; differentiation
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Computational approached to understand the regulation of transcription by POU factors

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The transcription factor Oct4 plays key roles in the maintenance of embryonic stem cell pluripotency and cellular reprogramming. Conserved in vertebrates, Oct4 belongs to the family of POU transcription factors that interact with DNA through two DNA binding domains, the POU specific (POUS) domain and the POU homeodomain (POUHD) which are connected via a flexible, non-conserved linker. POU factors regulate transcription in a combinatorial fashion using different monomer, homo-, and hetero-dimmer configurations on the DNA. Despite the strong similarities among these factors, Oct4 is unique in its ability to regulate the transcription of embryonic stem cell marker genes. On several promoters, Oct4 interacts directly with other transcription factors such as Sox2 to initiate transcription. Although several crystal and NMR structures provided insights into the DNA recognition by POU factors, the mechanisms underlying the Oct4 specificity are not understood. I present here the application of state-of-the-art molecular modeling methods to investigate the dynamics of Oct4 and other POU factors on different DNA binding sites, in the presence and the absence of co-regulators such as Sox2. These methods will enable the characterization of the structural fingerprints of different POU factors, thus providing an explanation for their functional diversity.

Keywords: molecular dynamics, structural biology, transcription regulation, pou factors
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miRNA and mRNA expression in cardiomyocyte differentiation in murine ESCs

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Embryonic stem cells (ESCs) have the ability of differentiating to specialized cells. ESCs containing the EGFP gene and the PuromycinR -MHC promoter control provide us a modelα(Pac) cassette under cardiac to observe cardiomyocyte specific differentiation of ESCs therefore to obtain cues of stimulation of cardiac and muscle formation. After induction of cardiomyocyte specific differentiation under puromycin selection, total RNAs including miRNA were isolated from samples collected at day0 (undifferentiated ESCs) as well as day12, day19 and day26 during differentiation and maturation. miRNA expressions were investigated with Affymetrix miRNA1.0 GeneChip as well as Febit Geniom mouse miRNA array. The results from both platforms were analyzed and compared in order to distinguish regulated miRNAs plausibly. miRNAs ,whose regulation were platform and analysis methods insensitive, were identified as valid candidate miRNAs. To correlate miRNA expression to the expression of potential target gene, mRNA from the total RNA from the same time points were profiled with Affymetrix mouse exon arrays. After normalization and statistically analysis, significantly regulated genes were identified. The effect of miRNA regulation on cardiomyocyte specific gene expression was studied. These results will help to elucidate the complete regulation pathway during cardiomyocyte specific differentiation.

Keywords: miRNA; murine ESCs; differentiation; regulation; gene expression
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Inhibition of NF-kappa B translation by miRNA-cluster 290 maintains embryonic stem cell pluripotency

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Nuclear factor kappa B (NF-κB) is in the center of active research since its discovery twenty years ago, when it was identified as a regulator of the κB light chain expression in B cells. Besides its important role in the immune system, a large amount of studies established a more general role for NF-κB as a central regulator of different cellular processes like cell survival, differentiation, proliferation and neuroprotection. Only recently a function for NF-κB signaling in embryonic stem cells (ES-cells) was discovered. Our study reports a posttranscriptional mechanism for inhibition of canonical NF-κB signaling in ES-cells. During retinoic acid (RA) induced differentiation of ES-cells protein levels of NF-κB subunits c-Rel and p65 and its inhibitor IκB-alpha were upregulated, whereas mRNA levels remained constant. Ectopic expression of bioinformatically predicted micro-RNAs and flag-tagged p65 in HEK 293 cells revealed a downregulation of p65 by the ES-cell specific micro-RNAs 293 and 291b. We also observed a decrease of micro-RNA 293 and 291b expression after addition of retinoic acid. Furthermore inhibition of these micro-RNAs in ES-cells increased p65 protein levels in a dose-dependent manner. Overexpression of p65, among other components of the NF-κB pathway, causes loss of pluripotency in ES-cells, but overexpression of IκB (Inhibitor of κB) had no effect. Moreover, we detected an upregulation of neural crest markers after p65 overexpression during ES-cell differentiation. These markers also play an important role in the epithelial to mesenchymal transition (EMT), a crucial process in the formation of the body plan. During delamination of the neural tube neural crest cells also undergo epithelial to mesenchymal transition. Differentiation of ES-cells with retinoic acid and further cultivation in the presence of 10% FCS results in a predominant mesenchymal phenotype. To further investigate the role of NF-κB during this differentiation process, we established a lentiviral knockdown of both NF-κB kinases (IKK1 and IKK2) leading to a decreased NF-κB activation. This inhibition of NF-κB signaling resulted in an increased number of neuroectodermal cells and a reduced number of mesodermal cells. Our results identify a novel intermediate neural crest state during ES-cell differentiation, induced by retinoic acid. We suggest a crucial role for NF-κB in the formation of this neural crest competence by inducing EMT *in vitro*.

Keywords: NF-kappa B; microRNAs; epithelial to mesenchymal transition; embryonic stem cells
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P2 receptor signalling in human mesenchymal and ectomesenchymal stem cells

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Human adult mesenchymal stem cells (MSCs) are of major interest for Regenerative Medicine, in particular due to their ability to differentiate into cells forming muscle, cartilage or bone. In contrast to this, the ectomesenchymal stem cells derived from dental follicle are further committed towards hard tissues. Purinergic receptors are well known to participate in important cellular processes, such as proliferation and migration, but the role of these receptors during mesenchymal differentiation has been deciphered only fragmentarily so far. To evaluate the potential influence of P2 receptors in stem cell differentiation, these two stem cell types with their differences in lineage potential have been compared for the receptor expression. The role of metabotropic P2Y and ionotropic P2X receptor subtypes has been examined in mesenchymal and ectomesenchymal stem cells during differentiation. Calcium imaging following agonist stimulation was used to demonstrate the functional activity of P2 receptors. Both cell types expressed several functionally active P2X and P2Y receptor subtypes, but with differences in the expression level. Interestingly, some particular P2 receptor subtypes were found to be differently regulated during adipogenic and osteogenic differentiation. Moreover, the administration of agonists and antagonists of P2 receptors had a direct influence on those differentiations. Taken together, purinergic receptors play an important role during the differentiation towards the adipogenic and osteogenic lineage. Here we present for the first time a model for purinergic signalling in human MSCs. In the future, artificial P2 receptor ligands might be used to control mesenchymal stem cell fate.

Keywords: Human mesenchymal stem cells, dental follicle, purinergic receptors, adipogenic differentiation, osteogenic differentiation
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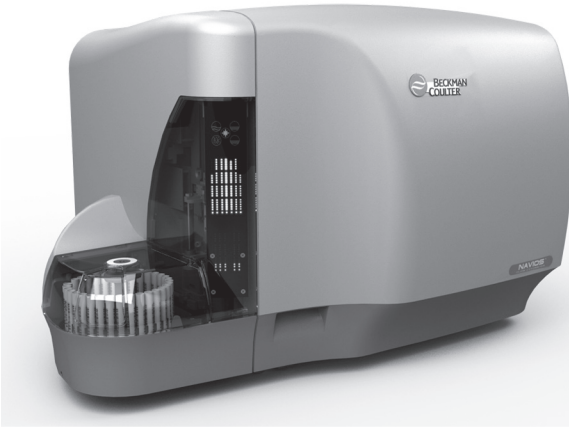

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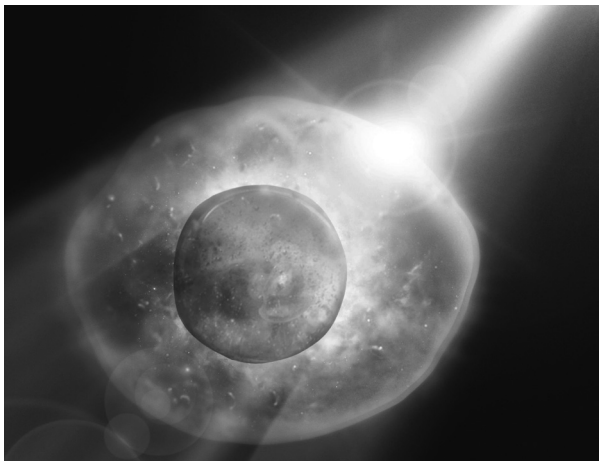

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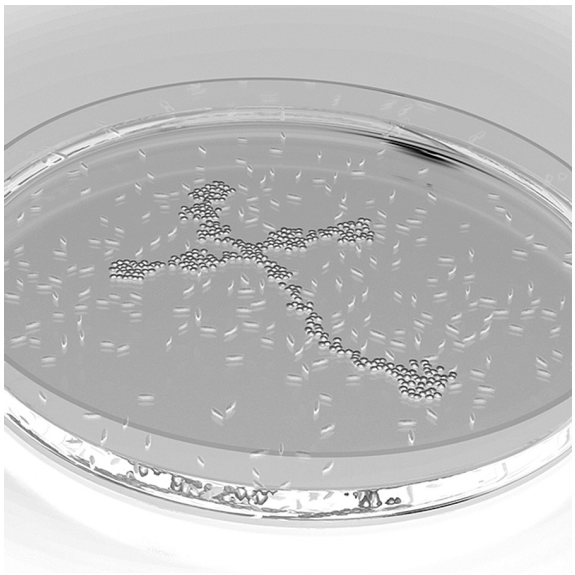

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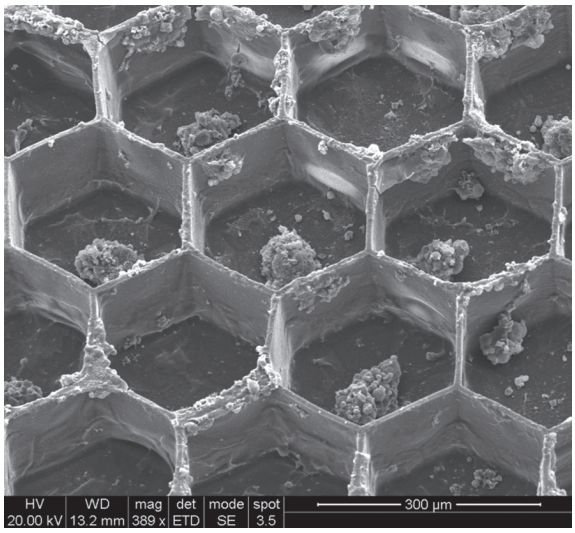

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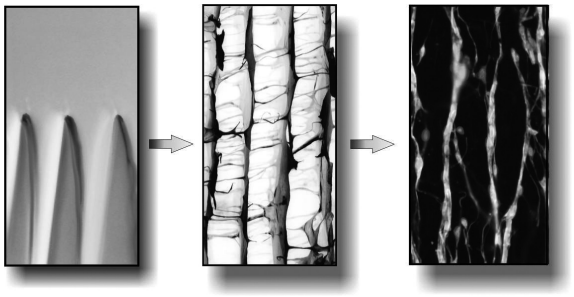

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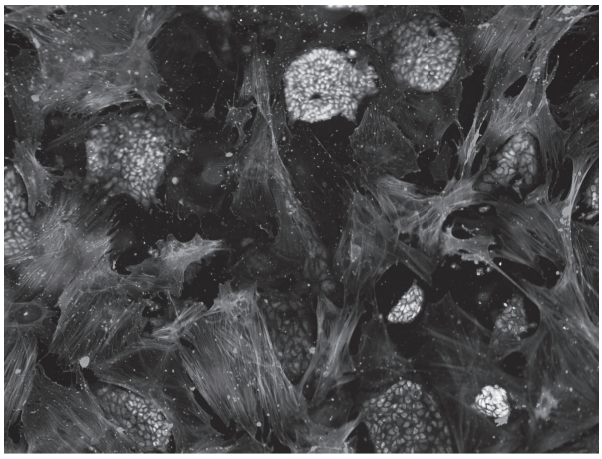

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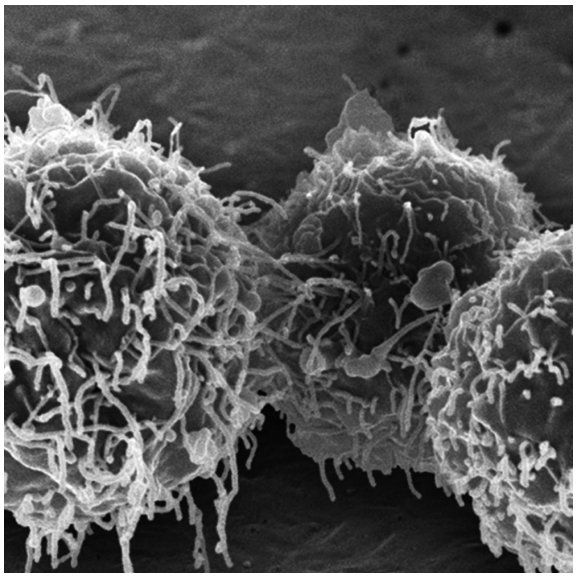

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SERVA Electrophoresis is a German life science company founded in 1953. Its unique product portfolio includes electrophoresis and laboratory devices, accessories, fine biochemicals and enzymes for research and clinical applications.

SERVA Collagenase NB qualities are the first choice for researchers aiming for isolation of high-yield viable cells including stem cells. The application of pharmaceutical manufacturing standards guarantees stringent quality control, reliable lot-to-lot consistency and excellent performance. SERVA was the first supplier of GMP Grade collagenases designed for cell isolation which are today an established tool world wide.

SERVA's superior Collagenase NB 6 GMP Grade is particularly suitable for isolation and passaging of stem cells destined for clinical applications. TSE safety is has been certified by EDQM and low endotoxin level assures high yield of viable cells.

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STEMCELL Technologies

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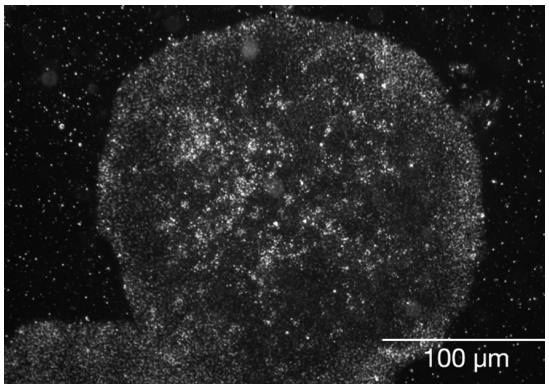

STEMCELL Technologies is a leading provider of specialized cell culture media for human and mouse embryonic and induced pluripotent stem cells as well as hematopoietic, mesenchymal, neural, endothelial, mammary epithelial cells.

We offer a wide range of highly specialized cell culture media optimized for maintenance, reprogramming, differentiation, cryopreservation, characterization and isolation of iPSC and ESC. mTeSR™1 is a fully defined media for the maintenance of human embryonic and induced pluripotent stem cells without the use of feeders. AggreWell™ is a unique tool that allows researchers to generate uniformly-shaped embryoid bodies, the size of which can be controlled by the researcher. A full line of cell separation products are available for the isolation of virtually any cell type from virtually any species. Other products and services include primary human cells, sera, cytokines, antibodies, training courses, Proficiency Testing, and Contract Services. For more information please visit www.stemcell.com or join our "Friends Ð mTeSR™1, too" at www.stemcell.com/forms/friends-love-mtesr1.aspx.

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