5th International Meeting
Stem Cell Network
North Rhine-Westphalia

_March 24th – 25th, 2009

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Tuesday, March 24th

8:00 - 9:00 am _Registration

SPP 1356 'Pluripotency & Reprogramming':
Reprogramming I, Chair: A. Müller

9:00 - 9:30 am _Austin Cooney, Houston
Alternative pathways to maintain pluripotency

9:30 - 10:00 am _Theodore Rasmussen, Storrs, Connecticut
Direct reprogramming of somatic cells: From ES cell fusion to iPS

10:00 - 10:30 am _Paul Robson, Singapore
Insights into blastocyst formation revealed by single cell analysis

10:30 - 11:00 am _Miodrag Stojkovic, Valencia
Potential of embryonic and adult stem cells

11:00 - 11:15 am _Coffee Break, Poster Session

11:15 - 11:45 am **Opening of the NRW-Meeting**
_Thomas Rachel (Parliamentary State Secretary of Education and Research)

Keynote Lectures, Chair: H. Schöler

11:45 - 12:30 am _John Gurdon, Cambridge, UK
Nuclear reprogramming by eggs and oocytes

12:30 - 1:15 am _Bartha Knoppers, Montreal
Title to be announced

1:15 - 2:30 pm _Lunch Break, Poster Session

Reprogramming II, Chair: M. Zenke

2:30 - 3:00 pm _Huck-Hui Ng, Singapore
Deciphering and reconstruction of embryonic stem cell transcriptional regulatory network

3:00 - 3:30 pm _Alexander Meissner, Cambridge, USA
Dissecting the mechanism of reprogramming

3:30 - 4:00 pm _Coffee Break, Poster Session

Mechanisms Regulating the Stem Cell State, Chair: A. Faissner

4:00 - 4:30 pm _Ian Chambers, Edinburgh
Transcription factor control of ES cell self-renewal

4:30 - 4:45 pm _Jens Schwamborn, Münster

4:45 - 5:15 pm _Niall Dillon, London
Combinatorial histone modifications and the epigenetic regulation of stem cell commitment and differentiation

5:15 - 6:45 pm **Poster Session**

7:00 - 10:30 pm _Networking Event at the historic Aula Carolina (bus transport provided)

Wednesday, March 25th

9:00 - 10:00 am _Ethical Issues regarding Therapeutic Experiments (Panel Discussion)

Stem Cell Differentiation, Chair: S. Schlatt

10:00 - 10:30 am _Renee Reijo-Pera, Stanford
Human germ cell differentiation from hESCs and iPSCs

10:30 - 10:45 am Kinarm Ko, Münster

10:45 - 11:15 am _Timm Schroeder, Munich
Tracking of stem cell behavior at the single cell level: New tools for old questions

11:15 - 11:45 am _Coffee Break, Poster Session

Cancer Stem Cells, Chair: T. Dittmar

11:45 - 12:15 am _Maarten van Lohuizen, Amsterdam
Polycomb repressors controlling stem cell fate: Implications for cancer and development

12:15 - 12:45 am _Andeas Trumpp, Heidelberg
Dormancy in stem cells

12:45 - 2:15 pm _Lunch Break, Poster Session

Therapeutic Devices I, Chair: E. Tobiasch

2:15 - 2:45 pm _Benjamin E. Reubinoff, Jerusalem
Human embryonic stem cell – towards future therapy of neurological disorder

2:45 -3:00 pm _Sabine Neuss, Aachen

3:00 - 3:30 pm _Mathis Riehle, Glasgow
Engineering cell function and differentiation in 2 and 3 dimensions
with mirco- and nanotopographies

3:30 - 4:00 pm _Coffee Break

Therapeutic Devices II, Chair: O. Brüstle

4:00 - 4:30 pm _Christine Mummery, Utrecht
Cardiomyocytes from human embryonic stem cells

4:30 - 4:45 pm _Ruth Olmer, Hannover

4:45 - 5:15 pm _Markus Grompe, Portland
Hepatic stem cells and their medical use

following **Poster Awarding and Conclusion**

_Register

Bioengineering

Cancer & Stem Cells

Chromatin and Epigenetics

Development and Regeneration

ELSI (ethical, legal, social issues)

Embryonic Stem Cells

Genomics and Proteomics

Induction of Pluripotency

Somatic Stem Cells/Stem Cell Niche

Company Profiles

Bioengineering

Automated selection and harvesting of pluripotent stem cell colonies using the CellCelector

[1]Simone Haupt, [1]Jan Grützner, [2]Marc-Christian Thier, [1]Barbara Helen Rath, [2]Frank Edenhofer, [3]Heike Möhlig, [1,2]Oliver Brüstle

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The ability of human embryonic stem cells (hESC) to differentiate into specialized cells of all three germ layers (pluripotency), their capability for unlimited cell division (self-renewal) and their amenability to genetic modification provide fascinating prospects for the generation of genetically modified human cell lines for biomedical and pharmaceutical research. Recently, induced pluripotent stem (iPS) cells have emerged as an additional source of pluripotent cells, which can be derived from adult somatic tissues (Takahashi K et al., 2007). Both, the selection of successfully engineered hESC and the derivation of iPS cells depend on the harvesting of individual stem cell colonies, which are subsequently further expanded to obtain homogenous cell lines. In this study we implemented the CellCelector technology to automatically detect, isolate and propagate human ES cells as well as murine iPS cells. The feasibility of this approach was assessed by comparing the biological properties of automatically harvested hESC with those, of manually transferred cells. Our data show that hESC colonies harvested with the CellCelector maintain their viability, pluripotency, proliferation and differentiation properties even after repetitive (up to 3x) passaging. The CellCelectorTM technology was also used to select and harvest primary murine iPS cells derived from Oct4-GFP mouse embryonic fibroblasts (MEF) 14 days after retroviral transduction with oct4, sox2, klf4 and c-myc. Primary iPS cell colonies were selected by virtue of their morphology or Oct4-GFP fluorescence. From a total of 42 selected and harvested colonies, 33 (78,6%) could be further propagated to stable cell lines exhibiting pluripotency marker expression and multi-germlayer differentiation. Thus, the CellCelector technology provides a useful tool for identifying and isolating pluripotent stem cell colonies in a highly selective manner at the phase contrast, bright field or immunofluorescence level.

Keywords: stem cell technology

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The Impact of Adhesion Molecules on Stem Cell/ Biomaterial Interactions

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Stem cells represent an ideal source for tissue engineering, because they are readily isolated, expanded, differentiated and transplanted. In many instances stem cell transplantation will require their application on biomaterial scaffolds. Our studies on the interaction of a large panel of stem cell types with an extensive array of artificial biomaterials demonstrated that at the current state of knowledge a rational prediction of the behavior of a particular stem cell type on a specific biomaterial is difficult (Neuss et al., Biomaterials 29, 302-313, 2008). Surprisingly, even ontogenetically related stem cell types, such as mesenchymal stem cells (MSC), preadipocytes and dental pulp stem cells (DPSC), exhibit distinct adhesion properties on the very same biomaterial surface. To this end, we started to investigate integrin and extracellular matrix (ECM) protein expression of stem cells in order to relate gene expression to adhesion behavior. MSC, preadipocytes and DPSC were cultivated on selected synthetic polymers like Texin, Poly(dimethyl siloxane), Poly-D,L-lactic acid and L-lactic acid-trimehylene carbonate. Integrins and ECM proteins were analysed by RT-PCR, Real Time PCR and immunohistochemistry. The results demonstrated constitutive gene expression of fibronectin and collagen I. Thus, these ECM proteins are most unlikely responsible for the differential adhesion behavior of stem cells on diverse polymers. In contrast, integrin alphaV and integrin alpha4 and the ECM molecules collagen III and collagen IV contribute to and/or are responsible for the differential stem cell adhesion. Thus, our studies on the molecular interactions between stem cells and polymers are expected to lead to a more profound understanding of the stem cell/biomaterial interactions to eventually allow for a rational biopolymer design.

Keywords: biomaterials, scaffolds, tissue engineering

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Influence of engineered magnetic nanoparticles on hematopoietic stem cells

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Hematopoietic stem cells (HSC) reside in the bone marrow and are capable of differentiating into all cell types of the blood. They have been successfully established in the field of regenerative medicine as a therapeutic tool for the treatment of various diseases of the hematopoietic system. However, a better understanding of the migratory and/or homing properties of hematopoietic stem- and precursor cells might help to improve emerging cellular therapeutic approaches. Magnetic nanoparticles (MNP) have been implicated as a means to non-invasively monitor transplanted cells in vivo using molecular imaging approaches such as magnetic resonance imaging (MRI). MRI is well suited for obtaining high-resolution three-dimensional images of anatomical structures and the detection of cell deposits and their migration after stable labeling of cells with contrast agents including MNP. However, little is known about the molecular mechanisms of HSC/MNP interaction. Questions still remain as to whether MNP shell composition might influence crucial HSC properties. In order to address these questions, we investigated the uptake properties of iron-oxide based MNP with various surface characteristics into bone marrow derived HSC in vitro. To achieve this goal, we selectively engineered such MNP as well as employing bacterial magnetosomes to cover a broad range of nanoparticle characteristics (charge, size, origin, surface biomolecule composition) and introduced them into bone marrow derived HSC. Assays related to MNP uptake, intracellular MNP localization and iron concentration gave insights into possible trafficking routes, degradation kinetics and MNP clustering effects. Phenotypical analysis displayed alterations due to MNP incorporation and physico-chemical MRI relaxometry studies provided valuable information into the contrast agent potential of the employed MNP. Taken together, our results provide new insights for further optimization and development of engineered MNP with improved biocompatibility and labeling specificities for use in clinical therapies employing HSC.

Keywords: Hematopoietic stem cells (HSC), magnetic nanoparticles (MNP), magnetic resonance imaging (MRI)

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Cancer & Stem Cells

Identification of Cancer Stem Cell-Like Cells and Stromal Cells in Renal Cell Carcinoma

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Cancer stem cells are crucial to the development and progression of tumors. However, other tissue specific stem cells like bone marrow-derived mesenchymal stem cell-like cells constitute components of tumor stroma and also contribute to these processes. We have established and characterized three renal cell carcinoma (RCC)-derived cell lines with stem cell characteristics. One of these cell lines showed epithelial morphology whereas the two other cell lines had a mesenchymal appearance. All three cell lines had a CD13+, CD29+, CD44+, CD45-, CD71+, CD73+ and CD105+ immunophenotype in common. The mesenchymal cell lines were CD90+, CD133-, CD326- and cytokeratin 8/18-, whereas the epithelial cell line was CD90-, CD133+, CD326+ and cytokeratin 8/18+. Chromosomal aberrations typical for RCC were only observed in the epithelial cell line. All three cell lines had differentiation capacity. They were able to differentiate along the mesodermal osteogenic differentiation pathway in the presence of dexamethasone, ascorbic acid and glycerol phosphate and formed bone nodules positive for Alizarin-red. Upon xenotransplantation, the epithelial cell line formed tumors and revealed self-renewal capacity. The mesenchymal cell lines were non-tumorigenic but one of the cell lines promoted tumor formation from a normally non-tumorigenic RCC cell line. Thus, the newly established epithelial-type human RCC-derived cell line shares typical features with cancer stem cells, including immunophenotype, tumorigenicity, self-renewal capacity and differentiation potential. In contrast, the mesenchymal-type human RCC-derived cell lines show characteristics of mesenchymal stem cells and apparently are derived from RCC stroma.

Keywords: Cancer stem cells, tumor stroma, MSC, CD133, renal cell carcinoma

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Apoptosis resistance of glioblastoma stem cells – a question of Notch?

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The purpose of this study was to investigate the regulation of apoptosis in stem cell-like glioblastoma cells (GSC). These tumor initiating cells exhibit an increased resistance to apoptotic cell death compared to non-stem cell-like glioma cells (N-GSC) and might therefore contribute to the resistance to treatment in glioblastomas. To investigate the mechanisms of apoptosis resistance in glioma stem cell-like cells, we characterized the apoptotic signaling cascades in glioblastoma stem cell-like cells and their non-stem like counterparts. First, we analysed the expression levels of several apoptosis-related proteins. The anti-apoptotic proteins Mcl-1, Bcl-2, phospho-Bad, and Survivin showed elevated expression levels in GSC. For another apoptosis-related protein, the transmembrane receptor Notch1, we could also observe a prominent elevation in GSC compared to N-GSC. The up-regulation of Notch1 is mediated at the transcriptional level since RT-PCR analysis indicated a 7- to 10-fold increase of Notch1 mRNA in GSC compared to N-GSC. To investigate the effects of Notch1 on apoptosis we overexpressed the Notch1 intracellular domain (NICD1) in U251MG glioma cells. This resulted in an increased expression of the anti-apoptotic proteins Mcl-1 and Bcl-2. In contrast, downregulation of Notch1 in U251MG glioma cells via siRNA led to decreased expression levels of Mcl-1 and Bcl-2. Given the striking difference in Mcl-1 expression in GSC and N-GSC, we focused our following studies on the mechanisms of Notch1-dependent regulation of Mcl-1 expression. To determine whether Mcl-1 is regulated at the transcriptional or the protein level, we performed RT-PCR analyses and studied the effects of the translational inhibitor cycloheximide on Mcl-1 expression after NICD1 transfection. Both methods indicated a Notch1-dependent regulation of Mcl-1 expression at the protein level. This finding was consistent with RT-PCR analyses of GSC versus N-GSC demonstrating comparable Mcl-1 mRNA levels. In conclusion, our results suggest that glioblastoma stem cell-like cells are characterized by an increased expression of anti-apoptotic proteins. The strongly up-regulated expression of Notch1 in GSC might act in an anti-apoptotic manner by increasing the levels of anti-apoptotic proteins via post-transcriptional mechanisms. Further studies will elucidate the molecular mechanisms of the Notch1-dependent regulation of apoptosis-related proteins.

Keywords: glioblastoma stem cell-like cells, Notch1, apoptosis

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siRNA-mediated targeting of the adult progenitor cell marker Musashi-1 in endometrial carcinoma leads to a modulation of p21WAF-dependent cell cycle progression and apoptosis via downregulation of the notch-1/Hes1-pathway

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Adult stem cells are thought to be responsible for the high regenerative capacity of the human endometrium, and have been implicated in the pathology of endometrial carcinoma (1-3). The RNA-binding protein Musashi-1 is associated with maintenance and asymmetric cell division of neural and epithelial progenitor cells (4). Musashi-1 is a repressor of numb-1, a negative regulator of notch-1 expression (4), thus acting as a promoter of notch-1 signalling. We could recently demonstrate significantly increased Musashi-1 expression in clinical endometrial carcinoma specimen compared to healthy controls, indicating an involvement of Musashi-1 in endometrial carcinoma pathogenesis (5). In order to elucidate the role of Musashi-1 in vitro, we performed siRNA-mediated knockdown of Musashi-1 expression in the endometrial carcinoma cell line Ishikawa. Musashi-1-silencing resulted in significantly decreased expression of notch-1 and its downstream target, the HES1 transcription factor, while expression of the cell cycle regulatory protein p21/WAF was upregulated at the protein level. Of note, siRNA-mediated knockdown of Musashi-1 expression significantly increased the apoptosis rate of endometrial carcinoma cells, and reduced cell proliferation, as indicated by a relative increase of cells in the G1-phase of the cell cycle, and a relative decrease of cell numbers in the S- and G2M-phase in Musashi-1 silenced vs control cells. Our results support the concept of a stem cell origin of endometrial carcinoma, and identify Musashi-1 as a potential target for induced differentiation of endometrial cancer stem cells (6).

(1) Moreno-Bueno G et al. Oncogene 2002; 21:7981–7990.
(2) Rossi DJ & Weissman IL. Cell 2006; 125:229–231.
(3) Lehner R et al. Gynecol Oncol 2002; 84:120–125.
(4) Okano H et al.; Exp Cell Res. 2005; 306:349-356
(5) Götte M et al. J Pathol. 2008; 215:317-329.
(6) Yip GW et al.; Mol Cancer Ther. 2006; 5:2139-2148

Keywords: endometrial carcinoma, Musashi, notch, cancer stem cells, adult stem cells

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The influence of in vitro conditions for the enrichment of stem-like cell population in primary human brain tumor cultures: hypoxia-mediated upregulation of Sonic Hedgehoc (SHH)-pathway

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Objective: Recently demonstrated slowly proliferating stem-like cell population (BTSC) in malignant brain tumors, responsible for tumor initiation and propagation, allowed the identification of the molecular mechanisms underlying their resistance to both chemo- and radiotherapy. Reduction of BTSC population in glioblastoma cell lines by inhibition of Notch, WNT/ β-catenin and SHH pathways diminished in vitro proliferation and ability to form tumors in vivo. Our project is focused on defining cell culture conditions for enrichment of patient-derived glial tumor cultures in stem-like cell population for further ex vivo manipulations.
Methods Freshly resected malignant human glial tumors and fetal-derived neural tissue were propagated as free-floating culture in serum-free media with mitogens. Two media-compositions (DMEM/F12 and Neurocult® based) and oxygen concentrations (21% and 3%) were tested. Culture proliferation kinetic was investigated with MTS-Assay and BTSC population was analysed using FACS against stem cell marker CD133 and aldehyde dehydrogenase activity (Aldefluor®). Additionally, semi-quantitative PCR allowed the expression analysis of oncogenic and stem cell specific genes.
Results The growth curve of all investigated tumor- and fetal-derived cell lines showed significantly higher proliferation in DMEM based medium and under atmospheric oxygen concentration. In contrast, exposure to 3% oxygen in Neurocult® based media resulted in significant decrease of proliferation rate. FACS analysis revealed that slower proliferating cultures contained significantly more CD133+ stem-like cells (up to 15% in case of tumor-derived and 61% in fetal-derived cultures) compared to its fast proliferating counterparts. Aldefluor-Assay showed increase of BTSC in Neurocult® media under lowered oxygen concentration. Quantitative gene expression analysis confirmed higher expression of stem cell specific genes in the latter culture condition (in the case of tumor derived culture: CD133 up to 16-fold; SOX2 15-fold; NANOG 1,8 fold; NESTIN 6,8-fold). Interestingly, the effect of relative hypoxia (3% O2) on BTSC enrichment was stronger then any changes of the culture media. Furthermore, preliminary evaluations of quatitative gene expression levels revealed an upregualtion of SHH- pathway (SMO, NOTCH, PTCH, SHH) in BTSC-enriched cultures. Conclusions Neurocult® based media combined with lowered oxygen concentration increases the stem-like cell population in malignant glial tumor cultures

and in vitro propagated fetal-derived neural tissue. Hypoxia seems to play a key role in BTSC enrichment. This protocol allows the generation of significant numbers of BTSC for further genetic manipulations and ex vivo testing of novel therapeutic protocols. If the stemcell status in these cultures is direct proportional with overexpression of particualr signaling pathway, treatments with small inhibiting molecules could possible result in a diminishment of BTSC-fraction.

Keywords: cancer, stem cell, hypoxia, Sonic Hedgehoc

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Characterization of breast cancer cell and BMDC derived hybrid cells

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Cell fusion is perhaps one of the most important events in mammalian development. The beginning of mammalian life starts with the fusion of an egg with a sperm. But fertilization is not the only process where cell fusion plays a crucial role. It also participates in placentation, in maturation of skeletal muscle and bone, and in tissue repair. But what happens if cell fusion occurs not as an event of normal development? It has been shown that tumour cells are highly fusogenic and that fusion of tumour cells can give rise to 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 investigate the fusion of murine breast cancer cells to murine bone marrow-derived stem cells (BMDCs) in vitro. 67NR cells transfected with a hygromycin resistance gene and puromycin resistant BMDCs from Tg(GFPU)5Nagy/J mice were co-cultured for 24 hours. After this time hygromycin and puromycin were added to select fusion derived double resistant hybrid cells. Hygromycin-puromycin resistant hybrid cell clones were selected for further characterization on chromosome number, proliferation, migration and differentiation capacity. Determination of the chromosomal number revealed that each tested clone of hygromycin-puromycin resistant hybrid cells showed a chromosome set from nearly 50 up to 60 chromosomes per cell. The murine karyotype constitutes of 40 chromosomes for the diploid set, while 67NR cells harbor more than 70 chromosomes. The proliferation rate of hybrid cell clones was in between their parental cells. 3D-collagen-matrix migration assay was used for examining the migratory potential under the influence of epidermal growth factor (EGF), norepinephrine and phorbol myristate acetate (PMA). EGF, norepinephrine and PMA had no effect on the migration of 67NR cells, whereas BMDCs as well as the tested hybrid cell clones revealed a different responsiveness towards these three factors. Norepinephrine had a stimulatory effect on the average migration rate of BMDCs (control: 100%, norepinephrine: 119%) and hybrid clone 1 (control: 100%, norepinephrine: 129%), while there was no effect on clone 2, but a rather inhibitory effect on clone 3 (control: 100%, norepinephrine: 85%). EGF enhanced the average migration rate of hybrid clones 1 (control: 100%, EGF: 123%) and 3 (control: 100%, EGF: 123%), whereby there was no effect on clone 2 or BMDCs. A high stimulating influence on the average migration rate of hybrid clone 2 (control: 100%, PMA: 122%) and 3 (control: 100%, PMA: 150%) was observed with PMA, while there was an inhibiting effect on BMDCs (control: 100%, PMA: 60%) and hybrid clone 1 (control: 100%, PMA: 85%). Further studies suggested that some hygromycin-puromycin resistant hybrid cell

clones possess differentiation capacity. When cultured in osteogenic differentiation medium hybrid cells showed the ability to express bone specific alkaline phosphatase, indicating the differentiation potential into osteogenic direction. No alkaline phosphatase expression was detectable in 67NR cells after culturing in osteogenic differentiation medium. Our conclusion is that cell fusion occurs between breast cancer cell line 67NR and BMDCs in vitro. The fusion derived hybrid cells are showing a mix of parental characteristics like immortality, migratory competence and a certain kind of differentiation potential.

Keywords: breast cancer cells, bone marrow-derived stem cells, cell fusion, hybrid cells

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The seminoma-like cell line TCam-2 gains differentiation potential upon FGF4 treatment independent of a seminoma to embryonal carcinoma transition

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While embryonal carcinomas (EC), which belong to the class of pluripotent germ cell tumors are able to differentiate into cells of all three germ layers, not much is known about the differentiation potential of seminomas (S), another germ cell tumor entity. It is postulated that seminomas are able to undergo a transition to EC's and thus gain the ability to differentiate similarly. To verify this hypothesis and to shed light on the differentiation processes of seminomas we treated the seminoma-like cell line TCam-2 with various agents known to induce differentiation. Finally activation of FGF signalling by cultivation in mouse-fibroblast conditioned medium, supplemented with FGF4 and heparin led to the differentiation of TCam-2 cells. After a few days of treatment the cells change their morphology, growth characteristics and marker gene expression profile. We could show for the first time that seminomas are able to differentiate directly into yolk-sac tumor lineage and skip a transition step to EC's. The differentiated cells were analysed and characterised under molecular genetic standpoints and the results were used to develop a model of a putative seminoma differentiation pathway.

Keywords: germ cell tumors, seminoma, FGF4, differentiation

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Influence of cytokines and chemokines on breast cancer cells, breast stem cells and their hybrid cells

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The biological phenomenon of cell fusion is discussed controversially in cancer research. Cell fusion among tumor cells as well among tumor cells and magrophages can give rise to hybrid cells exhibiting new properties such as an enhanced metastatic potential, drug resistance, high proliferative rate and resistance to apoptosis. Whether the fusion of cancer cells and stem cells give rise to so-called cancer stem cells (CSCs) is not clear. Nonetheless, recent findings of our group have shown that breast stem cells and breast cancer cells fuse spontaneously, thereby giving rise to stable breast stem cell / breast cancer cell hybrids. Here we worked with M13SV1-EGFP positive breast stem cells, invasive HS578T breast cancer cells and the hybrid cell line M13HS-8, derived from a spontaneous fusion of the parental cell lines. Flow cytometry data revealed that all cell lines were positive for EGFR, c-erbB-2, CCR7 and negative for CXCR4, FGF-R1, CXCR1 and CXCR2. Calcium measurements performed by flow cytometry revealed that M13SV1-EGFP breast stem cells responded to EGF, FGF, a combination of both, and IL8, but not CK6, IL4, SDF and TNF-alpha, with increased cytosolic calcium concentrations. By contrast, HS578T breast cancer cells and the M13HS-8 hybrid cell line solely showed moderately increased intracellular calcium levels only after stimulation with EGF and a combination of EGF and FGF, whereas the other tested factors had no effect. The calcium measurements correlated well with analysis of the PI3K-Akt pathway. Increased pAkt levels were observed in M13SV1-EGFP cells after EGF stimulation, but not after FGF and CK6 stimulation. In accordance with the moderately increased calcium levels after EGF stimulation, HS578T and M13HS-8 cells revealed only weak increased pAkt levels after EGF stimulation. Both FGF and CK6 had no effect on Akt phosphorylation in HS578T and M13HS-8 cells. Because of the discussed influence of CK6 on guiding metastasizing breast cancer cells into lymph nodes, we additionally explored the influence of CK6 on the migration of all three cell lines by applying the 3D-collagen matrix migration assay combined with computer-assisted cell tracking. Both M13SV1-EGFP breast stem cells and HS578T breast cancer cells did not respond to CK6 with an increased migratory activity, which is in accordance with calcium measurements. By contrast, the migratory activity of M13HS-8 hybrid cells was increased by up to 20% in the presence of CK6. Whether this might point to an enhanced metastatogenic capacity of the breast stem cell/breast cancer cell hybrid is not yet clear. Ultimately, we tried to figure out whether EGF, FGF, and CK6, as well as other factors, including IL4, IL8, SDF, TNF-alpha, and factor combinations might have an influence on the fusion of M13SV1-EGFP breast stem cells and HS578T breast cancer cells. Therefore,

CellTracker-Green labeled M13SV1-EGFP cells were co-cultured with CellTracker-Red stained HS578T breast cancer cells for 24h in the presence of different combinations of the above mentioned factors. Subsequently, the amount of CellTracker-Green/CellTracker-Red double positive cells were determined by flow cytometry. Interestingly, both EGF and FGF, as well as a combination of both, decreased the amount of CellTracker-Green/Cell-Tracker-Red double positive cells (control 100%; EGF down to 57%), FGF (control 100%; FGF down to 75%) and a combination of both factors (control 100%; EGF+FGF up to 75%) suggesting and influence of these factors on M13SV1-EGFP breast stem cell/HS578T breast cancer cell contacts. CK6 and the other factors tested had no influence on the amount of double positive cells. In summary, our data show that breast stem cells, breast cancer cells, and its hybrid clone responded differentially to cytokines and chemokines.

Keywords: EGF, FGF, CK6, Breast cancer cell, breast stem cell, hybrid cells

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Genetically modified mammary stem cells define distinct roles for Stat5 in mammary gland development and breast cancer

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The mammary gland represents a unique organ with mainly postnatal development, which is suited to study gene function in adult stem cells. Mammary stem cells (MSCs) are required for the generation and maintenance of the mammary epithelium in successive rounds of pregnancy and lactation. The current notion is that breast cancer originates from transformed MSCs. The characterization and purification of MSCs has proven elusive due to the lack of specific markers. However, the stem cell activity can be measured by its ability to reconstitute an entire functional gland upon transplantation of mammary epithelial cells (MECs) into the cleared fat pad. We optimized the transduction of adherent primary MECs with lentiviral vectors and were able to genetically modify MSCs in an unfractionated cell population. Transplantation of a mixture of MECs expressing different color fluorescent proteins resulted in the formation of separate unicolored ductal trees probably originating from individual MSCs. We then used genetically modified MSCs to investigate the effects of the loss or gain of function of the transcription factor signal transducer and activator of transcription 5 (Stat5) in mammary gland development and breast cancer. Freshly isolated mouse MECs were genetically modified by transduction with lentiviral gene transfer vectors encoding a Stat5 specific small hairpin RNA (shStat5a/b) or a constitutively active variant of Stat5a (cS5-F). The cS5-F mutant carries a point mutation (S711F) and is constitutively tyrosine phosphorylated in the absence of inducing cytokine signals. The downregulation of Stat5a/b in MSCs did not affect the outgrowth of primary ducts, but resulted in the formation of thinner ducts, reduced side branching and impaired alveologenesis. Conversely, constitutive activation of Stat5a in MSCs and their progenitors caused hyperproliferation of the epithelial cells, thickening of the ducts and precocious development of alveoli in virgin mice. These results indicate that Stat5 activity regulates the emergence of mature alveolar cells from luminal progenitors. The persistent activation of Stat5 during the involution stage prevents apoptosis of the epithelial cells and caused formation of adenocarcinomas with short latencies. These tumors were highly proliferative and expressed activated Stat3. Microarray analysis of the tumors revealed novel Stat5 target genes associated with malignant transformation. Our approach allows the genetic manipulations of stem cells ex vivo and the study of the consequences in progenitor and differentiated cells upon transplantation in vivo. This represents an alternative to conventional transgenic mouse models to decipher the function of genes during mammary gland development and tumorigenesis.

Keywords: Mammary stem cells, lentiviral gene transduction, organ reconstitution, Stat5 gene function analysis

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Dehydroepiandrosterone (DHEA) and its sulphate ester (DHEAS) exert a prominent effect on chromaffin PC12 cell differentiation

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In the adrenal gland, the coalescence of steroid-producing cells with catecholamine-producing cells reflects a striking example of differential stem cell commitment of various cell-types in one organ. Our in vivo observations in humans revealed that congenital adrenal hyperplasia due to 21-hydroxylase (OH) deficiency, resulting in androgen excess, is accompanied by severe adrenomedullary dysplasia and chromaffin cell dysfunction. Recent studies have shown a beneficial effect of DHEA and DHEAS on neuronal stem cell survival and proliferation. Furthermore, DHEA is the only steroid produced by the fetal adrenal when sympathoadrenal progenitor cells invade the adrenal anlagen. Based on this evidence, we hypothesize that DHEA and DHEAS influence adrenomedullary differentiation and/or proliferation. Recently, we could show an effect of DHEA and DHEAS on bovine adrenomedullary chromaffin cells proliferation. In the present study, chromaffin pheochromocytoma PC12 cells were used since they harbour features of early chromaffin progenitor cells. Cell survival and differentiation processes were studied in this model. DHEA and DHEAS significantly reduced nerve growth factor (NGF)-induced cell survival as well as markers of neuronal differentiation, such as neurite outgrowth and expression of neuronal marker proteins, SNAP-25 and VAMP-2. Accordingly, DHEA was found to stimulate NGF-stimulated cells towards a more neuroendocrine phenotype. Thus, DHEA largely increased catecholamine release from NGF-induced PC12 cells and enhanced expression of the neuroendocrine marker chromogranin A. In a next step, we explored the molecular mechanisms of DHEA and NGF interaction in more detail. DHEA and DHEAS significantly reduced NGF-mediated ERK1/2 MAPK activation. Differentiation as well as proliferation processes in PC12 cells are accompanied by ERK 1/2 activation. In summary, our data demonstrate that DHEA and DHEAS influence differentiation processes in PC12 cells. DHEA drives the cells in the presence, but not in the absence, of NGF towards a more neuroendocrine phenotype. Our studies further suggest that this effect might be due to interference of DHEA with NGF-induced ERK1/2 activation by a rapid, non-genomic signalling mechanism. This data provides further evidence for an impact of DHEA and DHEAS on chromaffin cells during adrenal tissue development.

Keywords: chromaffin PC12 tumor cells, progenitor characteristics, neuroendocrine differentiation

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Chromatin and Epigenetics

Characterization of the early embryo upon loss of histone methyltransferase Setd1a

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Epigenetics highly determine chromatin structure and enable inheritance of genes in a temporal and spatial depended manner. During embryonic development, epigenetic mechanisms are essential to establish and further maintain gene expression patterns. Activation or silencing of specific gene loci correlates with posttranscriptional modifications at histone tails of the eukaryotic chromatin. Among those modifications, histone tail methylation originating from trithorax group (trxG) protein function has been shown to be crucial to the developing embryo. These trxG proteins specifically methylate nucleosomes at their histone tail 3 at lysine residue 4 (H3K4) that is associated with active gene expression. However, how functional trxG methylation complexes accomplish precise gene activation ultimately determining cell fate is still unclear. Complexity increases with the fact that there are six functional H3K4 histone methyltransferases, namely Mll1 - Mll4 and Setd1a and Setd1b. Our laboratory focuses on conditional mutagenesis of all six methyltransferases to ultimately understand the individual role of each histone methyltransferase in the process of self-renewal and differentiation of mouse ES cells as well as their impact on mouse embryonic development. Here we report results from conditional mutagenesis of Setd1a. We were able to target one allele of Setd1a using homologous recombination in ES cells. Several attempts to target the second allele of Setd1a failed. Two independent targeted ES cell clones were used to generate chimeras that gave germline transmission of the targeted allele. In mice, we found that null embryos die shortly after implantation. Further, Set1a -/- blastocysts can produce outgrowth albeit with a smaller inner cell mass (ICM). Attempts to establish a Set1a -/- ES cell line have equally failed. So far all above results indicate that Setd1a is crucial for the derivation of ES cell lines. Since we implemented a conditional targeting strategy our plan is to generate ES cell lines in which a Setd1a knock out can be induced using tamoxifen.

Keywords: embryonic stem cells, histone methylation

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HMG proteins: the new guardians of embryonic stem cell genomes

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It is estimated that between 10.000 and 100.000 abasic (AP) sites are generated per day and genome in human cells as a result of chemical base modifications or due to spontaneous hydrolysis of the N-glycosilic bond. It is also well established that AP sites are highly mutagenic if left unrepaired. Because pluripotent embryonic stem cells eventually produce all somatic cell types and give rise to germ stem cells, it is of utmost importance for ES cells to be able to efficiently repair this type of DNA lesion in order to minimize the risk of accumulating mutations during evolution. High mobility group (HMG) proteins are divided into three subfamilies: HMGA, HMGB, and HMGN. They are the most abundant non-histone chromatin factors in a eukaryotic nucleus where they contribute to the formation of special chromatin structures and serve as positive and negative transcriptional regulators. Here we show that HMG proteins are actively involved in base excision repair (BER); the main pathway in human cells that repairs base damage. We demonstrate that HMGA and HMGN proteins are highly active as DNA lyases, which are key components in BER and cleave AP sites during the initiation of the repair process. We demonstrate that the lyase activity of HMGA2 promotes cellular resistance against DNA damage that is targeted by BER, and that this protective effect directly correlates with the level of HMGA2 expression in cancer cells. HMGA2 is highly expressed in pluripotent ES cells and using siRNA knock down and Comet assays, we demonstrate that HMGA2 also protects hES cells from DNA damage induced by the methylating agent methyl methanesulphonate (MMS). Together with the previous finding that HMGB1 has dRP lyase activity, we propose a model in which HMG proteins, as key components of ES cell chromatin, constantly patrol the genome for the presence of AP sites and initiate BER through their associated lyase activities.

Keywords: DNA repair, AP/dRP lyase, HMG proteins, hESCs, genome stability

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Mammalian embryos respond to 5-cytosine methylation at CpG or GpC dinucleotides

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Mammalian genomes carry DNA methylation marks specifically at CpG dinucleotides. The aim of this study was to investigate the effects of different 5-cytosine methylations on gene expression. A reporter construct was selectively methylated at cytosines either in 5´-CpG or in 5´-GpC dinucleotides. Naturally, cytosine methylation did not occur at 5´-GpC dinucleotids in mammals and thus these positions represent artificial methylations sites. The differentially methylated plasmids were then injected in bovine and murine embryos or were electroporated into primary fibroblasts and cell lines. The reporter DNA (pEGFP) was treated with CpG- or GpC-methyltransferases, and completeness of DNA methylation was verified. The onset of GFP expression in both bovine and murine plasmid-injected embryos was directly correlated to the methylation pattern. The GpC methylated plasmid gave GFP expression from 4 cell stages onwards, whereas the CpG methylated plasmid caused a delayed GFP expression beginning from 8 cell stages. The unmethylated plasmid gave an expression which was indistinguishable from the GpC methylated plasmid. Electroporation of the differentially methylated plasmids into cultured primary fibroblasts and immortalized cell lines did not reveal any differences in terms of onset of GFP expression or expression levels. Bisulfite sequencing of recovered plasmids indicated that the methylation patters were unchanged over the observation period. Thus unlike the cultured cells, the mammalian embryos seem to be able to discriminate between 5-cytosine methylations in either CpG or GpC dinucleotides.

Keywords: CpG methylation, embryos, GFP, Bisulfite sequencing

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Fetal and adult hematopoiesis requires continuous Mll1 function

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Mll1 (Mixed lineage leukemia) belongs to the SET1 super family catalyzing the methylation of H3K4 leading to transcriptional activation. Translocations resulting in fusion proteins of Mll1 with over 50 different partner genes are known to cause acute lymphocytic leukemia and acute myeloid leukemia. Understanding the role of Mll1 in the hematopoietic system is therefore of critical importance. In order to explore the function of Mll1 we are using a conditional knockout mouse line in which the gene is ablated according to the knock-out-first strategy. A stop cassette inserted into the first intron truncates the transcript before the second exon. Removal of this cassette restores wildtype function. Removal of exon 2 by Cre-mediated recombination in this case with the tamoxifen inducible ROSACreERT2 line results in a frameshift. Mll1-/- embryos die before E13.5 and show a characteristic hemorrhage in the abdomen suggesting a fetal hematopoietic defect, which is currently under investigation. Acute loss of Mll1 in 12-week-old mice after tamoxifen gavage led to rapid death after approximately 20 days. The heterozygous control mice that were also tamoxifen treated were healthy beyond 6 months. Analysis of peripheral blood revealed a decreased hematocrit along with reduced erythrocyte counts in Mll1-/- mice. Thrombocyte and leukocyte numbers were also decreased. Blood cell morphology was unchanged determined by measurements of mean cell volume. Inspection of internal organs revealed a reduction in the size of thymus and spleen. However, the architecture of thymus and spleen was generally maintained. Histological analysis of paraffin embedded decalcified humerus sections revealed a decreased cellularity in the bone marrow. Flush outs of the humerus followed by red blood cell lysis and subsequent cell counts confirmed this drop in cell number. One mechanism which can account for this bone marrow failure is the reduced expression of several Mll1 target genes namely hox a7, hox a9 and hox b4. We assume a cell-intrinsic defect, which will be further investigated by bone marrow transplantation experiments.

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Analyzing epigenetic modifications in pluripotent stem cells

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Pluripotent embryonic stem (ES) cells are in the focus of current epigenetic research as chromatin configurations mirror the cellular developmental potential. Understanding the epigenetic code of pluripotency is of fundamental importance both for basic and applied research. Here we aimed to develop flow cytometric protocols that display the levels of different histone modifications. Since the cells remain intact through the fixation procedure diverse flow cytometric markers and applications can be combined to analyze large quantities of cells at the single cell level. Applying the intranuclear flow cytometric method to ES cells, we detected increased global histone H3 lysine 9 acetylation (H3K9ac) levels in cells that were treated with the HDAC inhibitor TSA. Further, we observed a reduction of global histone H3 lysine 9 di-methylation (H3K9me2) levels in ES cells that were treated with the selective G9a-HMTase inhibitor BIX-01294. Interestingly, we detected an increase of H3K9ac levels in BIX-01294-treated and a decrease of H3K9me2 levels in TSA-treated ES cells. This indicates a crosstalk between different histone modifications, in this case probably due to spatial expulsion of one modification by another one. Additionally, we found elevated H3K9ac levels in an SSEA-1 positive subpopulation as compared to SSEA-1 negative cells following all trans retinoic acid-induced ES cell differentiation, suggesting that less differentiated cells within heterogeneous differentiation cultures are characterized by hyper-acetylated histones, i.e. a more open chromatin structure. Together, we developed a new method for the analysis of global histone modifications by intranuclear flow cytometry. Chromatin flow cytometry offers several advantages over chromatin analysis by Western blot or ELISA, since it allows a quantitative, high-throughput and simultaneous analysis of multiple intranuclear and cell surface markers on a single cell level. Although this method cannot display gene locus-specific chromatin stages, it has the potential to uncover subpopulations of cells with distinct chromatin-modification-specific phenotypes which can be separated by sorting for further analysis. This method represents a promising tool to simultaneously study cellular properties, such as cell proliferation, apoptosis, surface marker expression and intranuclear chromatin-phenotype, on the single cell level of large quantities of cells.

Keywords: histone, epigenetic, flow cytometry

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Incomplete epigenetic repression of embryonic stem cell pluripotency genes in unrestricted somatic stem cells (USSC) from human umbilical cord blood

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The transcription factors OCT4 (POU5F1) and SOX2 are involved in the control of self-renewal and pluripotency of embryonic stem cells. In the course of embryonic stem cell differentiation, these stem cell factors are silenced by epigenetic mechanisms, namely DNA methylation and chromatin remodelling. We have previously shown, that unrestricted somatic stem cells (USSC) from human umbilical cord blood display a broad differentiation potential for ectodermal, mesodermal and endodermal cell types in vitro as well as in animal models. Here, we report the epigenetic status of the pluripotency genes OCT4 and SOX2 for this stem cell type in its undifferentiated and its osteo-induced state. The OCT4 promoter shows incomplete methylation in all undifferentiated USSC lines examined. This methylation level remains unaltered within 14 days after osteogenic induction. Furthermore, although the SOX2 gene is inactive in all USSC lines analysed so far the corresponding distal SRR1 enhancer and the promoter region are completely free of DNA methylation. In addition, both the OCT4 and SOX2 promoter region show absence of the histone modification dimeH3K9, which is characteristic for constitutively inactivated promoter regions. Instead both promoters are marked by the „activating“ histone modification dimeH3K4 and in various undifferentiated USSC lines the SOX2 promoter region exhibits a bivalent histone modification signature consisting of dimeH3K4 and trimeH3K27, which is a marker for facultatively inactivated gene promoters. This „bivalency“ has been documented to be characteristic for silenced but developmentally regulated gene promoters which are “poised” for transcription. Our data collectively suggest that the undifferentiated state of the USSCs is reflected by an incomplete epigenetic repression of pluripotency genes. Furthermore, we hypothesize that „poised“ epigenetic status of pluripotency genes could preserve the USSC potential to be able to react adequately to distinct differentiation or reprogramming cues.

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Keywords: epigenetics, stem cell

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Comparative methylation profiles and telomerase biology of mouse multipotent adult germline stem cells and embryonic stem cells

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Multipotent adult germline stem cells (maGSCs) have been derived from adult mouse testis and, like embryonic stem cells (ESCs), have the potential to differentiate into various types of somatic cells. We comparatively analyzed gene-specific and global DNA methylation profiles as well as the telomerase biology of several maGSC and ESC lines. We show that undifferentiated maGSCs are very similar to undifferentiated ESCs with regard to global DNA methylation, methylation of pluripotency marker gene loci, telomerase activity, and telomere length. Imprinted gene methylation levels were generally lower in undifferentiated maGSCs than in undifferentiated ESCs, but, compared to other undifferentiated multipotent germline stem cells, more similar to those of ESCs. Differentiation of maGSCs increased the methylation of three of the four analyzed imprinted genes to almost somatic methylation patterns, but dramatically decreased global DNA methylation. Chromatin immunoprecipitation (ChIP) PCR experiments to also comparatively determine gene-specific active and repressive histone modifications are still in progress. Our findings further substantiate the pluripotency of maGSCs and their potential for regenerative medicine.

Keywords: multipotent adult germline stem cells, pluripotency, genome-wide methylation, imprinted genes, pluripotency marker genes, telomerase biology

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Development and Regeneration

Inhibition of Notch signaling in human ES cell-derived neural stem cells accelerates their differentiation into functional neurons

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During development, Notch acts as a key regulator of neural stem cell maintenance by contributing to self-renewal while preventing neurogenesis. We have recently derived stably proliferating neural stem cells from human embryonic stem cells (hES-NSCs), which stably maintain multipotency and the capacity for self-renewal in the presence of bFGF and EGF. Here we assess the expression of known Notch pathway components in hES-NSCs and show that Notch signaling is active under self-renewing culture conditions. Inhibition of Notch activity by the gamma-secretase inhibitor DAPT diminishes the expression of the human homologues of Hes5 and Hey1 bHLH repressor genes, which are known to be targets of Notch in other vertebrates. Furthermore, treatment of hES-NSCs with DAPT reduces proliferation and promotes their neuronal differentiation. Notch and bFGF/EGF signaling synergize in preventing the differentiation of human neural precursors. Conversely, inhibition of Notch signaling in addition to withdrawal of growth factors permits a significant reduction of the in vitro differentiation time required for the generation of electrophysiologically mature hES-NSC-derived neurons.

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Keywords: Neural stem cell, neuron, Notch

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Growth/differentiation factor (GDF) 15 regulates cell cycle exit of secondary progenitors in the developing mouse ganglionic eminence

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Although several extrinsic signals regulate the proliferation of telencephalic precursors, few selective regulators of the proliferation and differentiation of secondary progenitors are known. Members of the transforming growth factor (TGF) superfamily appear to play an important role in the regional and temporal regulation of neural precursors proliferation and differentiation. Growth/differentiation factor 15 (GDF15) is a member of the TGF-β superfamily which is widely expressed in several organs and tissues of rodents. Within the CNS, GDF15 mRNA and protein have been detected in the periventricular germinal epithelium of embryonic rat brain, suggesting a possible function of this protein in NSC. In this study we analyse the role of GDF15 in embryonic neurogenesis in mouse ganglionic eminence (GE). We show that expression of GDF15 in the germinal epithelium of the ganglionic eminence increases from mid embryonic development onwards. Although GDF15 is highly expressed in purified neural stem cells, their proliferation and maintenance are not directly affected by GDF15 both in vitro and in vivo. Instead, we found that in vitro GDF15 directly regulates the timing of cell cycle exit of neural stem cell derived progenitors. In addition, analysis of proliferation in vivo shows that absence of GDF15 results in increased proliferation of precursors dividing distally from the apical border, whereas proliferation of apically dividing neural precursors is not affected. Finally, cell tracing of the dividing cells by BrdU incorporation indicates that the extra dividing cells after division migrate towards the site of differentiation. Taken together our data indicate that although GDF15 is secreted mainly by multipotent precursors it regulates both in vivo and in vitro the timing of cell cycle-exit of secondary progenitors.

Keywords: neural stem cells, GDF15, ganglionic emminence, secondary progenitors, EGFR

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Characterization of Novel Nurr1 Splicing Variant During Developent of Dopaminergic Neurons

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The Nurr1 gene belongs to the nuclear receptors superfamily of transcription factors which play a diverse role during tissue development, homeostasis and their maintenance. Nurr1 in particular has been implicated in the generation, maintenance and survival of midbrain dopamineric neurons which degenerate in Parkinson's disease. Given the role of Nurr1 in early specification of the dopaminergic phenotype of midbrain dopaminergic neurons we investigated and identified a novel splice variant of Nurr1 generated through a partial deletion of the ligand binding domain, leading to in-frame deletion of 37 amino acids. We found that the splice isoform although co-expressed along with the wildtype Nurr1 during early developmental stage of dopaminergic neurons and it differs significantly from the wildtype in its functional characteristics. Furthermore, our results suggest its alternative role in the differentiation of midbrain dopaminergic neurons.

Keywords: Nurr1, splicing variant, dopaminergic neuron, development

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BMI1 Transcriptional Repressor Promotes Hematopoietic Cell Development from ES Cells

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Hematopoietic stem cells (HSC) are multipotent stem cells that give raise to all mature blood cells throughout life. Many protocols for in vitro HSC expansion have been described, yet expansion with preserving the multilineage differentiation potential of HSC has remained a major challenge. Pluripotent stem cells, such as ES cells, represent another source of HSC. Here we have expressed the BMI1 transcriptional repressor in mouse ES cells and found that BMI1 promotes hematopoietic cell development from ES cells. BMI1 is one of the key components of PcG (Polycomb group) complexes and acts as transcriptional repressor. In BMI1-/- mice adult HSC are profoundly defective in their self-renewal capacity. Conversely, forced BMI1 expression promotes HSC self-renewal. By RT-PCR analysis we found that BMI1 was not expressed in ES cells. Here we studied the impact of lentivirus mediated BMI1 expression in ES cells. BMI1 overexpression did not affect morphology and proliferation of ES cell colonies. Upon differentiation in embryoid body (EB) assays, BMI1 over-expressing ES cells yielded Flk1+ mesodermal precursors with the same frequencies as control. However, BMI1 strongly enhanced hematopoietic cell generation from Flk1+ cells, as determined by colony forming assay in methylcellulose. Such BMI1 hematopoietic progenitors showed a growth advantage under serum free liquid culture conditions and gave more cobble-stone forming cells in OP9 co-cultures. Thus, forced BMI1 expression ES cells enhanced hematopoietic cell development and caused a growth advantage and sustained growth of ES cell-derived HSC. Thus, regulated BMI1 expression might be an appealing strategy for enhancing hematopoietic cell development from ES cells and for studying hematopoietic cell related disease.

Keywords: PcG, BMI1, embryonic stem cells, hematopoietic stem cells, differentiation

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Molecular and functional characterization of macrophage subtypes involved in cell-material interactions and wound healing

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Macrophages are professional phagocytes critically involved in pathogen defense, particle and debris clearing, as well as removal of apoptotic cells. The inflammatory reaction of macrophages is critically involved in biomaterial implant performance and wound healing. In the laboratory, macrophages are routinely differentiated from bone marrow precursors cells, yielding bone marrow derived macrophages (BM-MΦ). In embryonic life, macrophages are derived from hemangioblasts. Later on in fetal development, macrophages differentiate from the erythromyeloid and/or hepatic hematopoietic lineage. We studied embryonic stem cell derived macrophages (ES-MΦ) as an alternative source of naïve macrophages resembling the embryonic macrophage subtype. We hypothesized that BM-MΦ may be more mature and may predominantly mediate inflammation related immune responses while ES-MΦ may be more embryonic and may predominantly mediate non-inflammatory clearing of apoptotic cells, and possibly scar-free wound healing. Genome-wide expression analysis using Affymetrix® gene chips and immunohistochemistry were employed to reveal differences in their specific expression profiles. Both BM-MΦ and ES-MΦ macrophage subtypes were analyzed with respect to cytokine/chemokine expression, particle endocytosis, and apoptotic cell clearing. We analyzed the influence of both macrophage subtypes in vivo using a mouse tail wounding model. Preliminary results of the expression studies and the wounding model will be presented.

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Role of NKG2D-ligands and ICAM-1 in NK cell-mediated lysis of murine embryonic stem cells and embryonic stem cell-derived cardiomyocytes

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The transplantation of cardiomyocytes derived from embryonic stem (ES) cells into infarcted heart has been shown to improve heart function in animal models. However, immune rejection of transplanted cells may hamper the clinical application of this approach. Natural killer (NK) cells could play an important role in this process both in autologous and allogeneic settings by eliminating cells expressing low levels of MHC class I molecules. Here we characterize ES cell-derived cardiomyocytes (ESCM) in terms of their sensitivity to NK cells. We show that despite expression of very low levels of MHC class I molecules murine ESCM were neither recognized nor lysed by activated syngeneic NK cells in vitro. In contrast, undifferentiated ES cells expressing similarly low levels of MHC class I molecules as ESCM were recognized and lysed by NK cells. This differential susceptibility results from the differential expression of ligands for the major activating NK cell receptor NKG2D and the adhesion molecule ICAM-1 on ES cells versus ESCM. NKG2D ligands and ICAM-1 were expressed on ES cells but were absent from ESCM. Undifferentiated ES cells were lysed by NK cells in a perforin-dependent manner. However, simultaneous blockade of NKG2D and ICAM-1 by antibodies inhibited this killing. These data suggest that in the course of differentiation ESCM acquire resistance to NK cell-mediated lysis by down-regulating the expression of ligands required for activation of NK cell cytotoxicity.

Keywords: murine ES cells, NKG-2D ligands, cardiomyocytes, ICAM-1

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An in vitro investigation of the effects of multipotent progenitors and their astroglial derivates on DRG axonal growth

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Neural and non-neural progenitor cells have been suggested to be promising candidates for promoting tissue repair following traumatic injury to the spinal cord. We are investigating the in vitro axon growth promoting properties of highly enriched populations of adult human mesenchymal stromal cells (hMSC), human foetus-derived neural progenitors (hNP), hNP–derived immature type I astrocytes (hNP-AC) and early postnatal rat-derived astrocytes (pr-AC). A random, non-oriented outgrowth of neurites was observed from dissociated DRG neurons seeded onto a PLL/laminin positive control substrate. Neurite growth over hNP, hNP-AC and pr-AC was similarly non-orientated. Of all cell types tested, the pr-AC performed the poorest, supporting the lowest number of regenerating primary neurites as well as the lowest extent of neuritic growth (both values being significantly lower than those of DRG neurons plated onto the control substrate). Confluent cultures of hMSCs tended to form arrays of similarly orientated cell bodies and processes which supported the regrowth of significantly more primary neurites than any other substrate, but with a tendency for a slightly reduced overall neuritic length than that observed on the control substrate. The hMSC did, however, exert a strong influence on the orientation of neuritic outgrowth; many of the regenerating processes following the overall orientation of underlying cells and processes. The most extensive neuritic outgrowth was supported by the hNP-AC. The production of extracellular matrix and release of growth factors appear to contribute to this beneficial effect of hNP-AC on DRG neuritic outgrowth. Therefore, the present in vitro investigation suggests that further investigations into the properties of hNP-AC may be of particular interest in the development of a transplant-mediated intervention strategy for promoting functional tissue repair in experimental models of spinal cord injury.

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Keywords: regeneration, neural stem cells, mesenchymal stromal cells, astrocytes, dorsal root ganglia

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Experimental modulation of telomerase activity in bovine embryos

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Telomeres are repetitive, noncoding sequences at the end of the linear chromosomes that are shortened with each cell division. They play an important role in ageing and affect the regeneration capacity of cells. The holoenzyme telomerase rebuilds telomeres and is composed of two components, i.e. the catalytic protein component telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC). TERC acts as template for the synthesis of telomeres. In mammals, telomerase is active during embryogenesis, in germ cells and a subset of stem and progenitor cells. In the present study we attempted first to express only TERC component and second TERT and TERC components of human telomerase in bovine embryos. DNA expression constructs encoding TERC, TERT and a GFP reporter construct were co-injected into bovine zygotes. GFP fluorescence was used to identify successfully injected embryos. Injected and control embryos were cultured in vitro up to the blastocyst stage and the impact on early embryonic development and the physiological consequences of an ectopic over-expression of telomerase in early bovine embryos were assayed. Embryos with GFP-fluorescence were frozen for PCR analysis, or the blastocysts were spread on glass slides for quantitative fluoresence in situ hybridization (qFISH) to monitor telomere length. Control groups were analysed for the endogenous levels of TERC and TERT. Results indicate that endogenous TERC and TERT are expressed at low levels from the 2-cell to 16-cell stage and are upregulated in morulae and blastocyts. We show that human TERC and TERT can be expressed after cytoplasmic injection of plasmids into bovine zygotes. The expression of the hTERC component resulted in a significant extension of telomere length in early embryonic stages. Results from the expression analysis of both components hTERC and hTERT are underway. Results from this study will allow a comprehensive analysis of the function of TERT and TERC in early embryogenesis. The ectopic expression of telomerase components in bovine embryos could pave new avenues for generating stem cells and for the development of novel regenerative therapies.

Keywords: hTERC, hTERT, telomerase, cytoplasmatic injection

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Essential role for Smed-LSm protein for planarian stem cell proliferation

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A novel member of the (L)Sm family is essential for regeneration in planarians. Planarians (Phylum Plathelmyntes, Class Turbellaria) are free-living flatworms best known for their striking ability to regenerate lost body parts. The extreme plasticity is endowed by neoblasts, namely somatic totipotent stem cells that are distributed throughout the entire body of the animal. Experimentally accessible and easily targetable by RNA interference, neoblasts have been considered to be a homogeneous population for over a century. Here we report the first planarian member of the (L)Sm protein superfamily, Smed-LSm, whose expression is restricted to the stem compartment. Intact planarians injected with Smed-LSm dsRNA showed a degenerative phenotype progressing from anterior to posterior, and died within 3-4 weeks. Regenerating animals manifested a broad inhibition to blastema formation due to massive neoblast proliferation failure, and died within 2 weeks. Interestingly, a small number of cells presenting the morphological and molecular traits of neoblasts and organized in precise spatial clusters could still be found immediately before the animals' death, suggesting that some stem cells, though incapable of proliferation, can outlive the effects of Smed-LSm knockdown. Our functional studies show that Smed-LSm plays an essential role in maintaining neoblast' ability to self-renew, and strengthen the recent theory postulating the heterogeneity of the neoblasts population.

Keywords: (L)Sm proteins, Planaria, Proliferation, Stem cell, Regeneration

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Is Bone Marrow Concentrate Suitable to Treat Local Bone Defects?

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Background: During the last years controversy has arisen regarding the role and relevance of mesenchymal stem cell (MSC) in orthopaedic surgery. Besides cartilage regeneration, bone regeneration is one major focus for potential clinical application of MSC. Although autologous bone grafting is still the "gold standard" to heal critical size bony defects, this procedure is associated with significant donor site morbidity. Therefore some investigators suggested that the application of bone marrow aspiration concentrate (BMAC) is a valuable tool to stimulate local bone formation and reduce donor site morbidity. Recent data support the concept that MSCs are not the exclusive source of osteoblast but also hematopoietic cells promote osteoblastic differentiation. However, it is questionable if the relatively small number of living spongious MSC is sufficient to induce relevant bone regeneration in vivo. We report about our clinical and experimental data of BMAC in patients with local bone defects. Materials and Methods: Clinical trial: 40 patients with pseudarthrosis or local bone defects (bone cysts, benign bone tumors, bone defects related to revision endoprosthetic surgery) were treated with autologous BMAC. Therefore, 60 ml bone marrow aspirate (BMA) from the iliac crest was harvested by vacuum aspiration at the beginning of surgery. BMAC was prepared by density gradient centrifugation (Smart prep2 Harvest system centrifuge) to a volume of 7-10 ml while cancellous bone from the iliac crest was obtained to fill up the bone defect with a maximum volume of 50% of the defect size. The BMAC was incubated on bovine hydroxyapatite (HA) carrier (Orthoss) or a collagen membrane (Gelaspon) for at least 15 minutes and transplanted in the osseous defect afterwards. Bone regeneration was determinated by clinical and radiological (standard x-rays in 2 planes) examinations after 2, 6 and 12 weeks and 6 months. Experimental data: The number of BMA and BMAC mononuclear cells was controlled by cell counter. To determine colony forming (CFU-F/-ALP) units BMA and BMAC cells were cultivated. In addition, cellular adherence and proliferation on both scaffolds (HA, collagen) were analyzed (LDH-assay, HE/DAPI staining). To evaluate osteogenic potential BMAC samples were cultivated for 28 d either with an osteogenic mixture (DAG) or without any osteogenic supplement. Both groups were stained for CD105, ALP and von Kossa. Results: All of the 40 patients showed new bone formation or bone healing during follow up. Except for one persisting hematoma and 3 prolonged wound secretions no other perioperative complications were observed. 1 patient underwent revision surgery due to incomplete bony bridging of a pseudarthrosis. The average concentration factor for BMAC was 5.7 (SD: 1.01). CFU appeared earlier and were larger suggesting a higher regenerative potential in BMAC. It was also shown that BMAC were able to adhere on the scaffolds in significant

numbers, proliferate in vitro and follow for an osteogenic in vitro-differentiation with and without DAG supplementation. Also significant amounts of RNA were extracted from the HA scaffold corresponding to cellular proliferation. Conclusion: Our interim data showed that the application of BMAC is easy to handle, a safe procedure and successful in treatment of local bone defects. However, it is unclear if additional supplements such as thrombin, fibrin or growth factors (e.g. BMPs) are able to improve the clinical and radiological outcome of BMAC.

Keywords: osteoblast, mesenchymal stem cell, bone regeneration, bone marrow concentrate

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Differentiation of Human Embryonic Stem Cells into Hepatocytes-like cells

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In a mouse model hepatocytes derived from mouse embryonic stem cells have been shown to integrate into liver tissue and produce albumin. Human embryonic stem cells possess obvious therapeutic potential and capacity to increase the number of functional hepatocytes in a diseased liver. The main aim of this project is to examine how specific cytokines, growth factors and transcription factors support differentiation of human embryonic stem cells to hepatocytes in an efficient and reproducible manner. As an initial step in this differentiation protocol, Activin A has been used to promote definitive endoderm differentiation. This was then validated by analysing the expression of the definitive endoderm markers SOX17, FOXA2, HNF4A at the mRNA and protein level. In the next steps the differentiation protocol is mimicking embryonic liver development. Molecules important for this step are, FGF4, HGF, on-costatin M and dexamethasone. We employed previously published protocols which led to differentiation into cells possessing morphologic and molecular features typical for hepatocytes. To understand the developmental biology of the liver, whole genome expression profiling of hepatocytes derived from hESCs has been done. In comparison with fetal and adult human hepatocytes reveal an overlap of genes crucial for functional hepatocytes.

Keywords: endoderm, hepatocytes, differentiation

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Regulation of neural stem cell behaviour in the developing spinal cord by extracellular matrix molecules

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During development of the central nervous system neural stem/progenitor cells give rise to three major cells types, namely neurons, astrocytes and oligodendrocytes. The processes that lead to the generation of distinct cell lineages are highly regulated by environmental cues such as growth factors or cytokines. Recent studies in the developing forebrain have shown that extracellular matrix (ECM) molecules including glycoproteins such as Tenascin-C (Tnc) are involved in neural stem cell proliferation and differentiation. However, little is known about ECM effects on neural stem cell behaviour in the developing spinal cord. Therefore, we initially undertook a systematic description of the expression of Tnc in the developing spinal cord both on mRNA and protein level. In addition to the expression analysis a correlation of the expression of Tnc with growth factor responsiveness was attempted using the neurosphere culture system. We found that during neurogenesis at E11.5 Tnc is absent from the spinal cord. At that age the isolated neural stem/progenitor cells were primarily FGF-responsive, as revealed by neurosphere formation assays. When neurogenesis has ceased and oligodendrocyte precursor cells appear in the spinal cord (E13.5-E15.5) Tnc becomes strongly up-regulated, in particular in the ventral horns, but also at the ventricular zone. This up-regulation is accompanied by the emergence of a highly proliferative EGF-responsive neural stem/progenitor cell population. Furthermore many Tnc positive cells were also positive for the radial glia cell markers Nestin or the glutamate/aspartate transporter (GLAST). Interestingly the ventral spinal cord harbours significantly more neurosphere forming cells than the dorsal part. These results prompted us to investigate BrdU incorporation in vivo and neurosphere formation of Tnc deficient mice in comparison to their wildtype littermates. Taken together this study will lead to further insights into the role of extracellular matrix molecules on neural stem/progenitor cell behaviour in the developing spinal cord. Our approach may also lead to a better understanding of the elusive neurosphere-forming cell in the spinal cord.

Keywords: spinal cord, neural stem cells, extracellular matrix

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Development of earliest hematopoietic cells and HOXB4

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Somatic cells which have been reprogrammed back to an induced pluripotent state (iPS) will likely become a key source for the in vitro generation of patient-tailored hematopoietic stem cells (HSCs) in future gene therapy. This opens avenues for efficient selection of molecularly characterized, "safe", gene-corrected clones at the pluripotent, undifferentiated level. However, protocols for directed differentiation of pluripotent cells towards hematopoietic stem cells (HSCs) have to be improved and subsequent expansion of the artificially generated stem cells must be achieved up to levels useful for transplantation. Ectopic expression of a member of the homeodomain containing family of transcription factors, HOXB4, has been shown to mediate HSC expansion, both in vitro and in vivo, and also enhances the in vivo repopulation ability of in vitro differentiated mouse ES-cells in a dosage dependent manner (1-3). Although its constitutive expression perturbs hematopoiesis (1,2), HOXB4 is still considered one the most attractive candidates for therapeutic use, provided that its activity can be tightly regulated. How HOXB4 promotes the conversion of ES-cell derivatives to HSCs is not yet known.Here we demonstrate that HOXB4 expression enforces the development of the earliest known hematopoietic cell (4) during pluripotent stem cell differentiation and promotes its subsequent expansion in stroma-cell free suspension culture. A subpopulation expressing the surface marker CD41 at high levels was selectively observed in the HOXB4 expressing cultures. These cultures lead to long-term engraftment when transplanted into immunodeficient, Rag2(-/-)γC(-/-) recipient mice, suggesting that the ability to engraft may correlate with the presence of CD41hi cells in these cultures. To be able to test whether the appearance and expansion of these early hematopoietic cells depends on the expression levels of HOXB4, we designed new regulated cassettes based on the tetracycline-inducible system, in which all necessary components were embedded in a single gammaretroviral SIN-vector. For optimization of the so-called TetOn-System we modified the transcriptional regulatory (response) unit to minimize activity in the uninduced state (i.e. without doxycycline) while conserving high transgene expression after induction. These novel vectors allow for a more graduated analysis of HOXB4 dosage effects by using different concentrations of the inducing drug. Using this "all-in-one" vector, we show that the presence of a CD41hi / c-Kit+/- / CD45- subpopulation correlates with HOXB4 expression levels in the clonal ES-HC cultures.

This subpopulation is capable of reconstituting the entire heterogeneity of the primary suspension culture, in vitro, after flowcytometrical isolation and can differentiate towards all lineages measurable in colony assays. In summary, we show that induced HOXB4 expression using a novel, tightly regulated expression cassette enhances the in vitro generation and expansion of the earliest known HSCs from mouse pluripotent stem cells. Thus, tight and fine-tuned regulation of HOXB4 may also support the generation and safe expansion of patient-specific pluripotent stem cell derived HSCs.

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Mid-term cell loss of mouse embryonic stem cell derived cardiomyocytes after intramyocardial injection into cryo-injured hearts is similarly high, but occurs earlier than in sham-operated mouse hearts

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Purpose: Cardiac cell replacement therapy is a promising strategy to restore impaired cardiac function. Embryonic stem cell derived cardiomyocytes (ES-CM) integrate into host myocardium and improve cardiac function after transplantation into injured hearts. Here, we quantified engraftment, persistence and survival of transplanted ES-CM as these are crucial factors for this therapy's effectiveness.
Methods: Male murine ES-CM were generated from a transgenic clone of D3 embryonic stem cells (αPIG44) and were highly purified (>99%) using an antibiotic selection strategy and a genetic resistance under cardiac specific promoters. In female adult mice (129/S2; syngeneic to ES-CM), 300.000 ES-CM were transplanted with 2 direct intramyocardial injections (10µl each) into healthy regions at the border of previous cryo-injury (CRYO) or into sham-operated (SHAM) hearts. In control samples (to assess the 100% signal), the cell suspension was added to explanted hearts ex vivo. After surgery and randomization, hearts were explanted immediately (0h) or after 6h, 24h, 48h, 5 days or 3 weeks, and genomic DNA was isolated. The number of transplanted cells in each sample was determined by quantitative real-time PCR with Y-chromosome specific primers.
Results: Engraftment efficiency was similar in both groups with detection of 15.1±6.7% (SHAM) and 16.6±5.8% (CRYO) of the transplanted ES-CM at 0h, which was significantly less than in controls (both P<0.001). At 6h, numbers remained unchanged in SHAM (11.4±3.6%) but tended to decrease in CRYO (2.8±0.9%, P<0.08 vs 0h, P=0.1 vs SHAM). At 24h, numbers declined significantly in SHAM (0.9±0.3%, P<0.05 vs 6h) to similar levels as observed in CRYO (1.6±1.4%). At later time points, numbers remained without changes in both groups with 1.9±0.9% (SHAM) and 3.4±3.3% (CRYO) at 48h, 0.9±0.3% (SHAM) and 5.7±5.6% (CRYO) at 5 days and 1.1±0.6% (SHAM) and 2.3±1.9% (CRYO) at 3 weeks.
Conclusions: Direct intramyocardial injection of ES-CM into mouse hearts leads to low mid-term persistence and survival of only 1-2% of the transplanted cells. Cell loss occurs in 2 phases: immediately during/after injection (>80% of transplanted cells lost) and within 24h after cell injection (>90% of successfully engrafted cells lost). The second phase occurs earlier in injured than in healthy hearts but leads to similarly poor mid-term persistence and survival of transplanted cells. Strategies to improve engraftment,

persistence and survival of transplanted cells must be identified in order to optimize the effectiveness of cardiac cell replacement therapy.

Keywords: embryonic stem cells, cardiomyocytes, cardiac cell therapy, myocardium, heart, myocyrdial infarction, cryo-injury, transplantation, engraftment, survival, persistence

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Overcoming restricted neuronal migration and integration of human ES cell-derived neural transplants

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Controlled differentiation of human embryonic stem cells (hESC) towards different neural populations provides a perspective to generate donor cells for neural repair in high purity. Presently, transplantation of hESC-derived neural precursors (hESNP) is hampered by limited neuronal integration into the host tissue. Typically, neural precursors grafted into an adult host brain form dense clusters, which might continue to proliferate, a phenomenon also known as neural overgrowth. We hypothesized that post-grafting cluster formation is initiated by chemoattractive interactions between neural precursors and their differentiated neuronal progeny. To test this hypothesis, we generated purified hESC-derived neurons via a recently established doublecortin (DCX)-EGFP-based lineage selection approach (Ladewig et al., Stem Cells 26:1705-12, 2008) and grafted them either alone or as a mixture with undifferentiated hESNP. We found that pure neuronal grafts show significantly enhanced migration and integration both in hippocampal slice cultures and upon transplantation into the adult rat brain. In contrast, control populations containing hESNP only or a mixed population of hESNP and neurons formed prominent clusters at the transplantation site with only few neurons integrating into the surrounding host tissue. Enhancement of donor cell integration correlated with an acceleration of functional maturation. Whereas neurons derived from purified neuronal grafts exhibited spontaneous postsynaptic currents already four weeks after deposition on hippocampal slice cultures, control populations showed no evidence of synaptic integration at this time point. Results from Boyden chamber assays suggest that FGF and VEGF signaling might play an important role in mediating auto-attraction between hESNP and their neuronal progeny. In line with this, endostatin, an inhibitor of FGF2- and VEGF-mediated chemotaxis, enhanced the emigration of neurons from mixed precursor/neuron clusters. These data provide a perspective for the development of pharmacological strategies to enhance tissue integration of neural transplants.

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Endodermal differentiation capabilities of germ-line derived pluripotent stem cells

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Background and Aims: Recent reports described the generation of pluripotent stem cells from the mammalian germ-line. In females parthenogenetic activation of oocytes can give rise to blastocysts, whose inner cell mass can be isolated and propagated as parthenogenetic embryonic stem cells (pES). In males, testis-derived pluripotent germline stem cells (gPS) can be derived from spermatogonial stem cells. In our present study we investigated, whether pES and gPS harbor similar hepatic differentiation characteristics as normal embryonic stem cells (ES).
Methods and Results: Both germ-line-derived pluripotent cell lines (pES and gPS) gave rise to hepatic progenitor cells (HPC) using an embryoid body formation-based protocol. However, the generation of these EB-HPC is rather inefficient and we failed to develop a strategy for further expansion of EB-HPC. Therefore we evaluated a cytokine based differentiation protocol on adherent monolayers of ES and gPS. Activin A treatment of these cells resulted in characteristic morphological changes accompanied with upregulation of mesodermal and definitive endodermal markers in qRT-PCR analyses. Further cultivation of these cells in serum-free differentiation medium supplemented with Activin A, BMBP4, and basic FGF improved endodermal specification and further cultivation in hepatocyte culture medium induced expression of hepatic genes such as alpha-fetoprotein and albumin. In conclusion, pluripotent stem cells derived from either female or male germ-line can give rise to hepatic cells after exposure to suitable in vitro differentiation protocols. However, further refinements are needed to obtain more mature cell phenotypes and to get expandable hepatic precursor cells from these resources.

Keywords: pluripotent stem cells, germ-line, endodermal differentiation

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Human spongiosa mesenchymal stem cells fail to generate cardiomyocytes in vitro

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Introduction: Human mesenchymal stem cells (hMSCs) are broadly discussed as a promising cell population for regenerative therapy of ischemic disease and its consequences. Although the cardiac-specific differentiation of hMSCs in vitro was shown in several studies, the types and characteristics of these stem cells remain poorly defined, and the efficiency of transdifferentiation greatly varies between publications. We report the results of our complex study on directed cardiac differentiation of hMSCs in vitro, in which we attempted to cover all principal trends discussed in literature, such as use of growth factors, chemical inductors, biomaterial scaffolds, and co-culture techniques to find the most promising one.
Materials and methods: Differentiation of hMSCs towards cardiomyocytes was induced by use of different published medium compositions. To examine the influence of biomaterials on the efficacy of cardiac differentiation protocols, five biodegradable matrices were selected on the basis of their compatibility with hMSCs culture judged by cytotoxicity, cell vitality, morphology, apoptosis and proliferation studies: Resomer RG503, Collagen, PCL, Texin 950, PEA C. Direct co-culture of hMSCs with murine cardiomyocytes was established by seeding passage 2 hMSCs and mouse atrial-like cardiomyocytes (Cor.AT cells) together at ratios 5000:3000 cells in 48-well plates. Differentiation towards cardiomyocyte-like cells was estimated through examination of expression of cardiomyocyte-specific markers in comparison to human heart cells and untreated hMSCs in immunofluorescence and RT-PCR assays. The results of cardiac specific genes (MZH7B, Mef2A, Mef2D, Nkx2.5) expression analysis were ranked from 0 to 4 in accordance with the intensity of fluorescence stain of RCR products in agarose gel in relation to housekeeping gene (GAPDH). Average linkage cluster analysis based on Euclidian distances was then applied to the obtained data matrix using PRIMER6 software (PRIMER-E Ltd., 2006). The dissimilarity between tested cell cultures was tested with ANOSIM routine of PRIMER.
Results: The main findings of our work are as follows: i) Three-dimensional culture in the presence of 1 mg/ml bovine insulin, 0,1 mM ascorbate phosphate and 1 nM dexamethasone appeared to be the most promising method of cardiomyocyte-like cells generation from hMSCs in vitro relied on the use of simple chemical inducers of cardiac differentiation pathways (P = 0,036, ANOSIM). ii) The increase in expression of cardiac-specific genes by differentiated hMSCs has a transient character and does not prove the true cardiac differentiation since even untreated hMSCs demonstrate some level of cardiac expression. The expression of MYH7B and Nkx2.5 in differentiated cells was high by the day 15

and then leveled off both in long-term culture and passaged cells (P = 0,01, ANOSIM). iii) The biomaterials Resomer RG 503 and Texin 950 could be the most appropriate for use as scaffolds in techniques of cardiac-like cells generation from hMSCs in vitro (P = 0,05, ANOSIM). iv) Co-culturing of hMSCs with cardiomyocytes does not result in a "real" transdifferentiation of hMSCs. However, the expression of some heart specific markers by hMSCs in co-culture is achievable. Conclusion Human MSCs fail to generate functionally active ardiomyocytes in vitro, although part of the cells demonstrated increased levels of cardiac-specific genes expression when treated with differentiation factors and chemical substances or co-cultured with native cardiomyocytes. Therefore, MSCs should be considered as an ideal tool for gene therapy of ischemic heart disease or as antiapoptotic, immunotherapeutic agents in myocardial regeneration after infarction rather than crude for mechanical substitution of dead cardiomyocytes.

Keywords: MSCs cardiac differentiation

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Therapeutic angiogenesis for patients with chronic lower limb ischaemia by autologous transplantation of bone-marrow cells with and without granulocyte colony-stimulating factor

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Background: In a substantial number of patients suffering from lower limb ischemia (LLI), the distribution and extent of the disease leads to limited therapy options and therefore amputation of the affected limb is usually the only solution to unbearable symptoms. However, application of stem cells by implantation of autologous mononuclear cells into ischemic limbs has emerged as a new alternative for treatment of severe LLI. This strategy could be performed either by isolating stem cells directly from bone marrow (BM) aspiration or through mobilization into the peripheral blood. The latter route requires granulocyte colony-stimulating factor (G-CSF) to be administered a few days before transplantation in order to mobilize progenitors from the BM compartment to the peripheral blood. It has also been proposed that G-CSF may have a direct effect on collateral growth and perfusion recovery. However, it is still unclear at present whether G-CSF administration could be useful clinically for enhancing neovascularization following BM derived mononuclear cell transplantation. Therefore, the purpose of this study was to investigate the efficacy and safety of autologous transplantation of mononuclear cells with and without G-CSF in patients with chronic lower limb ischemia.
Methods: Fifteen patients with chronic lower limb ischemia were enrolled and were injected with bone marrow-mononuclear cells into the gastrocnemius of the ischaemic limb. The patients divided into two groups by randomization and received autologous bone marrow mononuclear cells with or without G-CSF adjuvant. Primary outcomes were safety and efficacy of treatment, based on ankle-brachial index (ABI), visual analog pain scale (VAS) and pain-free walking distance (PFWD).
Findings: On initial assessment at 4 weeks' follow-up, all clinical parameters showed a mean significant improvement from baseline. In 12 patients (80%), improvement of ischemic condition was maintained during 24 weeks follow-up. In these patients, no significant differences were found in monitored parameters (ABI, VAS and PFWD) evaluated at weeks 4 and 24. A comparison between group 1 (BM-MNCs without G-CSF) and group 2 (BM-MNCs with G-CSF) was made which revealed no significant differences between the groups in clinical outcomes including ABI, VAS and PFWD at both weeks 4 and 24. An overall therapeutic improvement of ischemic ulcers defined as a regression in the size of the ulcers and necrotic regions occurred in 9 of 13 patients (69%). During the 24 week follow up, complete healing of the ulcer was seen in 6 (46%) patients.

Keywords: angiogenesis, lower limb ischemia , granulocyte colony-stimulating factor

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New polyester fleeces for the spatial regeneration of renal tubules

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Sound knowledge concerning the cell biological mechanisms controlling the regeneration of renal tubules in acute and chronic renal failure after application of stem/progenitor cells is lacking. The integration of stem/progenitor cells in a diseased environment, the development into nephron specific cell types and the spatial formation of tubules are unresolved issues. For that reason a new technique for the generation of renal tubules was developed. Following this strategy new biomaterials have to be found promoting an improved spatial development of tubules. To gain novel information about the growth of tubules, tissue containing stem/progenitor cells was isolated out of neonatal rabbit kidney and mounted in a tissue carrier between Positech® (Posi) polyester fleeces. This method creates an artificial interstitium and replaces coating by extracellular matrix. The introduced technique supports spatial development of tubules within 13 days of perfusion culture in chemically defined Isocove`s modified Dulbecco`s medium (IMDM) containing aldosterone (1 x 10-7 M) as tubulogenic factor. Features of polyester fleeces were investigated by scanning electron microscopy. The spatial development of tubules was registered on whole mount specimens and on cryo-sections labeled with soybean agglutinin (SBA) and tissue-specific antibodies indicating tubule differentiation. It is found that polyester fleeces such as Posi-4, Posi-5, Posi-6 and Posi-7 are promising new candidates for the generation of renal tubules in future biomedicine. In the presently used Posi polyester fleeces tubules develop in the space between the polyester fibers. Surprisingly, each of the tested Posi polyester fleeces promotes the spatial development of tubules, but each of them produces its individual growth pattern.

Keywords: Tissue engineering, perfusion culture, kidney, tubule, polyester fleece, artificial interstitium, stem/progenitor cells

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The role of the transcription factor GCNF in germ cell differentiation and reproduction

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The germ cell nuclear factor (GCNF) is a member of the nuclear receptor super family of transcription factors. GCNF expression during gastrulation and neurulation is critical for normal embryogenesis in mice. GCNF represses expression of the POU domain transcription factor Oct4 during mouse post-implantation development in vivo. Oct4 is thus down-regulated during female gonadal development, when the germ cells enter meiosis, which is a process important for reproduction, but one that is rare in germ cells derived from embryonic stem cells in vitro. One aim of our work is to better define the role of GCNF during mouse germ cell development in vivo. We observed a steady decrease in pluripotency-associated gene activity with a concomitant up-regulation of GCNF expression in germ cells derived from developing fetal gonads one day prior to the onset of meiosis. Meiosis-associated genes were then up-regulated at onset of meiosis. These findings suggests that GCNF may repress Oct4 expression in female germ cells and that it plays a role in initiation of meiosis or in activation of meiosis-associated genes in female germ cells. Examination of gene expression profiles in whole gonad in vitro culture will provide insight into the molecular mechanisms of meiosis and germ cell differentiation, and may help optimize procedures in germ cell differentiation and gamete derivation in vitro.

Keywords: Germ Cell, Meiosis

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Gene Modified Mesenchymal Stem Cells for Therapy of Liver Copper Disease

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In the rare disorder of Wilson's disease (WD) excess accumulation of copper leads to fatal hepatocellular injury. The causative mutation of the hereditary disease has been assigned to the liver copper transporter gene ATP7B. Transplantation of whole liver or hepatocytes with intact ATP7B have previously been shown to cure liver disease. Mesenchymal stem cells (MSC) are important sources for cell therapy of liver disease, however, in vitro gene transfer may further enhance the therapeutic effect. MSCs were derived from bone marrow of LEC (Long Evans Cinnamon) rats that lack functional ATP7B and are a valuable model of WD. ATP7B was transferred to MSCs by retroviral vector. As controls, different cell lines that either lack (chinese hamster ovary cells CHO) or express ATP7B (human embryonal kidney cells HEK 293; human hepatoma cells HepG2) were also investigated. In cell lines high gene transfer rates of up to 95±7% were achieved by empty vector and 69±17% with vector carrying ATP7B (n=6). For MSCs 42±10% and 19±17% of cells could be transduced, respectively (n=5). ATP7B specific RNA was detected by real-time RT-PCR analysis and demonstrated high rates of ATP7B overexpression that exceeded levels found in human hepatoma cell line HepG2 by factor of 7-60 depending on the cell line analyzed. ATP7B protein expression was correspondingly analyzed by Western-blot and immunofluorescence. In order to determine the level of copper resistance due to ATP7B overexpression cells were cultivated in different concentrations of copper, and viability was determined by MTT assay. Whereas no (0%) or 13±3% of viable cells could be detected after 4 days of cultivation in untransduced MSCs, more than 65±17% of MSCs survived after ATP7B transduction at copper concentration ranging between 0.75-1.0 mM (n=3). The proliferative resistance to high copper was further studied in MSC populations consisting of a few ATP7B transduced cells (< 5%) and untransduced cells (> 95%) modelling a clinical situation after hepatocyte transplantation. In this scenario, high concentrations of copper (> 750 microM) led to a specific selection of transduced MSCs that overgrew (88±7%; n=3) the majority of untransduced cells within a few days. Selection of MSCs was stable thereafter (up to day 70) even in the absence of copper. Our observations are significant for further exploration of cell-based therapeutic effects after transplantation of gene modified cells into animal models of liver disease.

Keywords: Mesenchymal stem cells, hepatocytes, liver, Wilson's disease

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The asymmetrically segregating determinant TRIM32 prevents self-renewal in mouse neural progenitors

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In the mouse neocortex, neural progenitor cells generate both differentiating neurons and daughter cells that maintain progenitor fate. Here, we show that the TRIM-NHL protein TRIM32 regulates protein degradation and micro-RNA activity to control the balance between those two daughter cell types. In both horizontally and vertically dividing progenitors, TRIM32 becomes polarized in mitosis and is concentrated in one of the two daughter cells. TRIM32 overexpression induces neuronal differentiation while inhibition of TRIM32 causes both daughter cells to retain progenitor cell fate. TRIM32 ubiquitinates and degrades the transcription factor c-Myc but also binds Argonaute-1 and thereby increases the activity of specific micro-RNAs. We show that Let-7 is one of the TRIM32 targets and is required and sufficient for neuronal differentiation. TRIM32 is the mouse ortholog of Drosophila Brat and Mei-P26 and might be part of a protein family that regulates the balance between differentiation and proliferation in stem cell lineages.

Keywords: neurogenesis, asymmetric cell division

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Gene Regulation of Tenascin C and its Isoforms in the Developing Mouse Central Nervous System and Neural Stem Cells

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During neurogenesis the temporal and spatial generation and organisation of neural progeny emerging from neural stem cells is tightly controled. Radial glia cells represent a distinct type of neural stem/progenitor cells in the brain that serve as source of neurons and glia. Newborn neurons migrate along these cells to their final position. Radial glia cells in the developing brain express the extracellular matrix glycoprotein tenascin C (Tnc). In these progenitor cells, the expression of Tnc is modulated by several developmentally relevant extrinsic and intrinsic factors. We show here that the growth factors EGF and bFGF that stimulate proliferation of neural stem cells strongly induce the expression of Tnc in neurosphere cultures. The EGF- and bFGF-responsive populations react differentially to growth factor stimulation depending on their spatial and temporal appearance during brain development. Structurally, Tnc consists of several protein domains including 8 constitutive fibronectin type-III (FNIII) domains. By independent alternative splicing of six additional FNIII domains, theoretically up to 64 different Tnc isoforms can be generated, and 27 different Tnc isoforms have been detected in the cerebellum. The analysis of Tnc complexity in neural precursor cells grown as free-floating neurospheres revealed the presence of 20 different Tnc isoforms. During brain development, we detected a differential expression of the alternatively spliced FNIII domains in regions of active cell proliferation and neuronal migration. The expression of Tnc is intrinsically regulated by transcription factors that control the relative abundance of different isoforms. We show here that in the pax6-deficient small eye (sey) mutant the expression of Tnc is impaired, which mainly affects the large isoforms. The transcription factors pax6 and otx2 selectively regulate differently sized isoforms of Tnc. Upon transfection of neural progenitor cells with expression plasmids for various transcription factors, we found that pax6 and otx2 preferentially support the large Tnc isoforms that are important for cell migration. We could prove a direct binding of pax6 to different positions in the Tnc upstream regulatory sequence. Neurosphere cultures from pax6-mutants show a higher proliferation capacity and impaired neurogenesis, which is due to the loss of the neurogenic signal provided to neural precursor cells by pax6. Under certain conditions neural precursors in culture extend radial cell processes which are reminiscent of radial glia and serve as migration substrate for neurons. Cells in sey cultures also generated these functional radial glia cells, although the morphology of this cell type in vivo is strongly disturbed. Time-lapse imaging of these cultures revealed that the migration of neurons along radial glia cells in pax6-defective

cultures is slowed down, which may result from the missing stimulatory influence of large Tnc isoforms. Our results suggest that Tnc partakes in the regulatory and functionally relevant mechanisms of radial glia cells during embryonic CNS development and that distinct Tnc isoforms mediate different functions.

Keywords: Neural stem cells, Radial glia, neuron migration, small eye mutant, Pax6

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ELSI
(ethical, legal, social issues)

The Impact of iPS on Stem Cell Legislation and Administration in Germany

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In 2008 the German lawmaker has reformed the national Stem Cell Act (Stammzellengesetz – StZG). This reform applies to the provisions for the import of human embryonic stem cells to Germany and to the provisions for the infringement of the Stem Cell Act. This revision of the German Stem Cell Act coincided with unexpected results of stem cell research in the field of the creation of ethically unloaded stem cells by techniques of reprogramming. These techniques lead to so-called induced pluripotent stem cells (iPS). However, the German Stem Cell Act contains a subsidiary provision which states that the import of human embryonic stem cells to Germany is not allowed if there is a scientific alternative for the use of human embryonic stem cells. But, if the further import of embryonic stem cells is legally cut off there is the probability that further stem cell research in Germany flags. Scientists argue that there is still an urgent and constant need to do research with newly derived human embryonic stem cells. For Germany-based researchers the import of human embryonic stem cells is the only possibility of getting access to these cells because due to the provisions of the German Embryo Protection Act (Embryonenschutzgesetz – ESchG) the derivation of human embryonic stem cells out of human embryos is banned. Therefore, the scientific and medical success of reprogramming research could legally inhibit the further import of and research with human embryonic stem cells in Germany if iPS were an alternative for the use of human embryonic stem cells. But, this subsidiary provision is only applicable if the "scientific alternative" could fully replace the scientific use of human embryonic stem cells. For this reason, the legal (and scientific) status of iPS must be clarified. This scientific project gives a broad overview for these topics and gives answers regarding the legal impact of reprogrammed stem cells in legislation and administration.

Keywords: iPS, Stem Cell Legislation, Reprogramming, Stammzellgesetz, Embryonenschutzgesetz

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Embryonic Stem Cells

Exploring non-genetic activation of transcription factors Oct4 and Sox2

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Embryonic stem cells are unique in their properties of unlimited self-renewal and the ability to differentiate into any cell type of the adult body. Research activities during the last five years enabled comprehensive insight into the molecular mechanism controlling stem cell identity. In this respect the transcription factors Oct4 and Sox2 turned out to be molecular key players. By cooperatively binding regulatory regions of promoters they are able to regulate the expression of several pluripotency and differentiation factors. Moreover these factors were found to be not only essential, but sufficient to induce pluripotency in somatic cells, a phenomenon designated as reprogramming, enabling the derivation of embryonic stem cell-like cells from any somatic source. In this study we aim at using Oct4 and Sox2 for the modulation of stemness properties. Since the importance of non-genetic modification of cells is rising constantly we employed protein transduction as a means of delivering functional factors into the cell. For this, proteins were fused to a so-called protein transduction domain and directly applied in cell culture. We present the pSESAME-Vector system, which facilitates the generation of transducible proteins. After identifying optimal constructs for expression and purification of transducible Oct4 and Sox2 fusion proteins we show that both recombinant factors are able to specifically bind to a target sequence in a manner similar to the endogenous counterparts. Data will be presented demonstrating that directly delivered recombinant transcription factors Oct4 and Sox2 do exhibit biological activity.

Keywords: protein transduction, stem cells, pluripotency, reprogramming

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Analyzing the neural potential of androgenetic murine ESCs

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Uniparental (androgenetic, parthenogenetic and gynogenetic) ESCs are interesting cell lines both for basic research and regenerative medicine. Apart from any therapeutic application, however, is the question of whether uniparental ESCs are compromised in their ability to develop into tissue stem cells that are functional and safe after transplantation. To address this issue, we analyzed the neural potential of AG ESCs following blastocyst injection and by stereotactic transplantation into a mouse model of traumatic brain injury. The results show a widespread and balanced distribution of AG donor cells in E12.5 and E16.5 chimeric brains and neural differentiation of AG donor cells in recipient brains after transplantation. In addition, we investigated the imprinting status of AG cells using real-time RT-PCR. The AG ESC-derived neural progenitor cells reveal parent-of-origin-specific expression of imprinted brain genes.

Keywords: androgenetic, ESCs, neural differentiation

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The early steps of germ cell development in a novel non-human primate embryonic stem cell line

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Embryonic stem cells (ESC) are a very useful tool to investigate aspects of early embryonic development and differentiation. Previously, it has been shown that ESC can also give rise to germ cells in vitro. Here, we employed a novel non-human primate (common marmoset monkey, Callithrix jacchus) ESC line (Müller et al., Human Reproduction, in press) to study early germ cell marker expression likely representing the earliest stages of germ cell development occuring in this ESC culture. Details on the general characterization of this novel cell line are presented on the poster by Müller et al. ESC were analysed by RT-PCR and immunofluorescence after two weeks of standard culture on mouse embryonic feeder cells without passaging. The first clear morphological evidence of differentiation of the colonies was a crater-like structure in the center of the colonies. The craters' bottoms consisted in the central part of flat, enlarged cells, which constitute a single-layered tissue. This central part of the crater was surrounded by smaller cells forming a multi-layered fringe. The cells in the multi-layered area maintained expression of stem cell markers, e.g. OCT4, NANOG, and SOX2, while the single-layered cells lost expression of some pluripotency markers. Interestingly, individual flat cells on the bottom of the crater started expressing germ cell markers characteristic for testicular stem cells. We first observed coexpression of the germ cell marker VASA together with CD9 and CD49f, respectively. Both markers were recently successfully used to select testicular stem cells. Furthermore, we colocalized VASA and SSEA-4 in individual cells. Even tough SSEA-4 is also expressed in undifferentiated ESC, coexpression with VASA could only be detected in a small subpopulation. Interestingly, we have recently shown that adult spermatogonial stem cells in the non-human primate also express SSEA-4. Later on, these VASA/SSEA-4-positive cells detach from the crater bottom as small roundish cells maintaining VASA/SSEA-4 coexpression. Moreover, these detaching cells also coexpress VASA and PGP 9.5. PGP9.5 was originally described as a neuronal marker, but is also strongly expressed in human and non-human primate spermatogonia. These data on early germ cell development in ESC cultures were corroborated by RT-PCR for the germ cell markers VASA, BOULE, DAZL, GCNF, and the meiotic marker SCP3. In summary, we provide evidence for spontaneous early germ cell development in this novel common marmoset ESC line. These findings underline the close relation between ESC and primordial germ cells / testicular stem cells and may, at least in part, explain the ability of spermatogonia to spontaneously dedifferentiate into pluripotent cells in vitro.

Keywords: germ cells, embryonic stem cells, Primates

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Manipulation of MHC expression in non human primate cells

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Embryonic stem cells (ESC) hold tremendous potential for therapeutic applications, including regenerative medicine, as well as for understanding basic mechanisms in stem cell biology. Many experiments cannot be conducted in human ESC because of ethical or practical limitations and thus nonhuman primate ESC serve as invaluable clinically relevant models. In regenerative medicine expression of the major histocompatibility complex (MHC) is a major problem for transplantation. To avoid rejection of transplant and overcome the immune barrier, recipients are typically treated with immunosuppressants. Disadvantages for recipients are different adverse effects including tumor formation. ESC display a relatively low expression of MHC class I and lack expression of MHC class II. During differentiation of ESC to other cell types MHC expression increases remarkably. An approach to avoid rejection of graft is silencing MHC expression in ESC with shRNA by RNA interference with lentiviral vector constructs. We expressed shRNA sequences targeting different regions of the marmoset β2-microglobulin (β2m) in marmoset fibroblasts, B lymphoblastoid cell lines (B-LCLs) as well as in marmoset ESC. The down regulation of the MHC class I expression was followed on mRNA level by real time RT-PCR and on protein level by flow cytometry. The transduction of RNAi cassettes containing the sequences for shRNAs targeting β2m suppressed MHC class I protein expression by up to 85%. These data demonstrate the feasibility of controlling MHC expression by genetically modifying non-human primate cells, setting the stage for a clinically relevant evaluation of this innovative approach in a large-animal model.

Keywords: Embryonic stem cells, MHC expression silencing

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Establishment of the novel gene-trap screen to understand the molecular basis of pluripotency in vivo

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Despite its direct relevance to the reproductive medicine and stem cell research, molecular mechanism of the lineage specification in early mammalian embryos remains elusive. Full understanding of the program leading to formation of the pluripotent inner cell mass should provide the molecular basis of pluripotency in embryonic stem cells. My laboratory has established a conceptual model for the early embryonic patterning (Hiiragi and Solter 2004; Motosugi et al. 2005; Motosugi et al. 2006; Dietrich and Hiiragi 2007), yet the underlying molecular mechanism remains to be clarified. Very few players are known, since a systemic search has been hampered by technical difficulties, and it is very likely that essential components are thus far unidentified. Thus, we have recently established two complementary screens to identify genes involved in this process: i) a fluorescence (Venus)-based promoter gene trap screen, and ii) single blastomere-gene expression profiling. Here I report that we have successfully developed the Venus-trap screen by lentiviral transgenesis. This method combines generation of knock-in fluorescent reporters to investigate early embryonic patterning with the advantage of potentially identifying novel key players. It is, to our knowledge, the first promoter gene-trap successfully applied to early mouse development. 4D live-imaging of the trapped lines enables us to precisely track lineage segregation during mouse preimplantation development. Of 97 mouse lines established in the pilot screen, 21% have indeed gene expression detectable in preimplantation embryos. In particular, 38% of the positive lines have an expression specific to one of the lineages, including a few indeed marking the ICM population. Recent analyses will be presented.

Keywords: Pluripotency, mouse embryo, gene-trap, live-imaging, patterning

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Characterization of early cardiac-specific transcripts in embryonic stem cell-derived multilineage progenitor cells

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Embryonic stem (ES) cells are undifferentiated cells with the capacity to develop into cells ofall three primary germ layers. During in vitro differentiation, ES cells recapitulate cellular developmental processes and gene expression patterns of early embryogenesis. Here, we characterize early stages of cardiac differentiation of mouse ES cells. ES cells (line R1) were cultured as embryoid bodies (EBs) for 5 days (d). After plating EBs were spontaneously differentiated for 9d into a multilineage progenitor population representing cells of all three primary germ layers including early cardiomyocytes. To investigate this stage of multilineage progenitor cells and of early cardiac cell types at the transcript level, microarray analysis was performed using Affymetrix chips comparing undifferentiated ES cells and 5+9d progenitors followed by RT-PCR analysis. Transcript levels of transcriptional regulators (e.g. GATA4, Mef2c, Pitx2), extra-cellular matrix components (e.g. procollagens I, III, IV, MMPs), and cytoskeletal proteins (troponin T2 and C, cardiac alpha actin) involved in cardiac differentiation and function were found to be up-regulated. Specifically, we detected a subset of up-regulated transcripts of genes known to be specifically expressed in the cardiac neural crest or neural crest cell derivatives. One of these up-regulated transcripts, Lbx1, is know to play a role in the migration of muscle progenitor cells in limb buds and also in neural determination processes. In addition, involvement of Lbx1 in cardiac neural crest related cardiogenesis was postulated. To verify the presence of Lbx1 in cardiac cells double immunocytochemistry of ES cell-derived cardiomyocytes and a quantification assay were performed at different developmental stages. Co-labeling of Lbx1 and cardiac specific markers troponin T or alpha actinin suggested a potential role in early myocardial development.

Keywords: Embryonic stem cells, microarray analysis, cardiac differentiation, Lbx1

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Simvastatin modulates cartilage nodule formation in murine ES cell-derived embryoid bodies

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It has been studied in detail that cellular differentiation steps during chondrogenesis can be recapitulated in vitro using the embryonic stem (ES) cell model system. First, mesenchymal cells form condensations within ES cell-derived embryoid bodies. During further differentiation expression of collagen type II and aggrecan can be found within the developing Alcianblue-positive cartilage nodules. Finally, loss of Alcianblue staining during later cultivation stages indicates dedifferentiation and the development of hypertrophic chondrogenic cells into the osteogenic direction. Previously, we have described that ES cell-derived cartilage nodule formation is induced by bone morphogenetic protein (BMP)-2. Because HMG-CoA reductase inhibitors enhance BMP-2 expression, we now asked the question how the application of Simvastatin influences the chondrogenic differentiation of ES cells and the maintenance of chondrocytes in vitro. The clinical link of the study was to support the understanding of the basic mode of possible action of statins in bone and joint diseases. Moreover, the study underlines that the ES cell system is an established in vitro-model to screen for principles of teratogenicity. We found that Simvastatin enhanced cartilage nodule formation in vitro. Prolonged application of the statin during cultivation resulted in a continued expression of cartilage marker molecules and cartilage nodules were also stained Alcianblue-positive in late stages. Expression of collagen type II and aggrecan were significantly upregulated during Simvastatin-induced chondrogenic differentiation as demonstrated by quantitative real time PCR. The mediating stimulus might be the enhanced BMP-2 gene expression, which we confirmed in EBs under the influence of simvastatin. Immunostaining for cartilage marker molecules revealed that the main cellular differentiation steps of chondrogenesis take place after statin application. However, cartilage nodules within Simvastatin-treated EBs were defective as demonstrated by confocal laser scanning microscopy, and showed cell cavities, which may be due to an anti-proliferative effect of statins demonstrated in cultures of primary chondrocytes. In addition, in comparison to untreated controls Simvastatin-treated EBs were significant smaller in size, although apoptosis-indicating Caspase activity was reduced. Anti-proliferative effects point out the teratogenic potency of statins.

Keywords: embryonic stem cells, HMG-CoA reductase inhibitors, statin, cartilage, teratogenicity

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Efficient generation of oligodendrocytes from gliogenic human embryonic stem cell-derived neural stem cells

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In many diseases including multiple sclerosis, stroke and spinal cord injury, demyelination is considered a major pathogenetic component. Failure of adequate remyelination has been associated with the limited availability and myelination potential of endogenous oligodendrocyte precursor cells. Thus, one potential therapeutic strategy is transplantation of myelin-forming cells. In recent years, human embryonic stem cells (hESC) and other pluripotent stem cell populations have emerged as particularly versatile and unlimited source of neural donor cells. While the generation of neural precursors and differentiated neurons from hESC is well established, oligodendroglial differentiation, so far, requires complex and lengthy in vitro differentiation protocols. Here we present a retinoic acid-based approach to generate an adherent population of hESC-derived neural stem cells (NSC) with efficient oligodendroglial differentiation potential. These cells express markers typically associated with neural stem/radial glia cells, including nestin, sox2, BLBP, GLAST, 3CB2 and vimentin. Upon growth factor withdrawal they differentiate into neurons, astrocytes and oligodendrocytes. Importantly, using defined differentiation conditions, these NSC populations can be efficiently differentiated into oligodendrocytes and their precursors. Following terminal differentiation by growth factor withdrawal for 6 weeks, >70% of the surviving cells expressed the oligodendrocyte progenitor marker NG2; >50% were positive for the oligodendrocyte-specific O4 antigen. This efficient differentiation paradigm might be particularly useful for studying human oligodendrocyte differentiation, screening of compounds for the therapy of myelin disorders and the development of transplant-based neural repair strategies.

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ILK deficiency affects vascular development and Tyrosine Kinase Receptors function

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Integrin linked kinase (ILK) is a key molecule of the cell-extracellular matrix (ECM) adhesion complex, linking the cytoskeleton with the cytoplasmic domain of beta1 integrin. The integrity of beta1 integrin and consequently of the cytoskeletal organization is relevant for cellular signalling and for vasculo-and angiogenesis. We aimed to further determined the role of ILK for vasculo- and angiogenesis using embryoid bodies (EBs) and MACS sorted endothelial cells (MEC) derived from ILK deficient (-/-) and wild type (wt) embryonic stem (ES) cell lines. ILK (-/-) EBs showed delayed development and differentiation of vessel-like structures which correlated with major defects in BM formation as revealed by the analysis of collagen IV, laminin and fibronectin distribution. The proliferation and apoptosis data revealed an increase rate in ILK (-/-) compared to the wt, but the ratio was moved toward proliferation in ILK (-/-) at later stages. Combined with the higher migration rate, these data could explain the delayed development and differentiation of vessel-like structures which are increased in later stages of development. VEGF signalling plays an important role in EC development regulating cell survival and migration; analysis of the VEGF receptor distribution by immunohistochemistry revealed that it was similarly distributed in both wt and ILK (-/-) MECs, but the higher phosphorylation level of both the receptor and of one of the most important intracellular target, ERK1/2, suggests an alteration of its modulation. We therefore investigated the function of this receptor in MEC analysing [Ca2+]i transients. 67.1% of wt cells responded to VEGF (20ng/ml) with a [Ca2+]i transient whereas this was absent in 99.1% of the ILK (-/-) cells. In contrast [Ca2+]i transients were evoked in both wt and ILK (-/-) endothelial cells when applying the G protein coupled agonists carbachol (1µM) and bradykinin (100nM). Interestingly, failed [Ca2+]i stimulation with EGF, another RTK agonist, in ILK (-/-) cells suggests that ILK deficiency prevented specifically the function of tyrosine kinase receptors, whereas G-Protein-coupled receptors are not affected. Furthermore, ILK deficiency caused perturbation of microtubules contact with the cortical actin filaments at the plasma membrane. These data suggest that ILK is essential to organize cytoskeleton networking and ECM and affecting VEGFR2 signaling alters cell survival, inhibits the progression in the differentiation, the stabilization and development of vessels.

Keywords: VEGFR2, integrin, cytoskeleton, integrin-linked kinase

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A novel embryonic stem cell line derived from the common marmoset monkey (Callithrix jacchus)

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Embryonic stem cells (ESC) hold great promise for the treatment of degenerative diseases. However, before clinical application of ESC in cell replacement therapy can be achieved, the safety and feasibility must be extensively tested in animal models. The common marmoset monkey (Callithrix jacchus) is a useful preclinical non-human primate model due to its physiological similarities to human. Yet, few marmoset ESC lines exist and differences in their developmental potential remain unclear. Blastocysts were collected and immunosurgery was performed. cjes001 cells were tested for euploidy by karyotyping. The presence of markers for pluripotency was confirmed by immunofluorescence staining and RT–PCR. Histology of teratoma, in vitro differentiation and embryoid body formation revealed the differentiation potential. cjes001 cells displayed a normal 46,XX karyotype. Alkaline phosphatase activity, expression of telomerase and the transcription factors OCT4, NANOG and SOX2 as well as the presence of stage-specific embryonic antigen SSEA-3, SSEA-4, tumor rejection antigens TRA-1-60, and TRA-1-81 indicated pluripotency. All pluripotency transcription factors tested were down-regulated upon ESC differentiation as revealed by RT–PCR; other differentiation-specific genes were switched on, such as CD34 for hematopoietic progenitors, NESTIN for neuronal progenitors, as well as FOXD3. Interestingly, this forkhead transcription factor, is not expressed in both undifferentiated human and marmoset ESC, but appears later in differentiation with its antagonistic effect on OCT4. Subcutaneous injection of cjes001 cells into immunodeficient mice resulted in tumors expressing marker mRNAs representing all three embryonic germ layers, bIII tubulin for differentiated neural cells (ectoderm), Brachyury for mesoderm and AFP for endoderm. Also, histological evaluation of the teratoma revealed tissues indicative of a tumor derived from pluripotent cells, such as chondrocytes, bone tissue, bone marrow, mesenchyme, muscle, nerves and epithelia. Altogether, the data show that this novel marmoset ESC line can form teratoma and, thus, is pluripotent. The cjes001 cells represent a new pluripotent ESC line and will be very valuable for comparative studies on primate ESC biology.

Keywords: embryonic stem cell, common marmoset, non-human primate, pluripotency

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Stemness factor Nanog suppresses replicative senescence in somatic cells

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The transcription factor Nanog plays a key role in the maintenance of stem cell properties. Moreover, several studies assign Nanog a role in the induction of pluripotency in somatic cells employing cell fusion and induced pluripotent stem cells (iPS cells) as model systems. Increased levels of Nanog are able to promote pluripotency transfer to the somatic cell shown in cell fusion experiments. iPS studies revealed that in contrast to Oct4 and Sox2, Nanog appears to be dispensable but seems to have a promoting effect though. A speculated role of Nanog in stemness maintenance and reprogramming could involve cell cycle regulation, a mechanism that is fine-tuned through small non-coding RNAs as well. A further understanding might come from assessing the activity of Nanog in somatic cells. In this study, we addressed this topic employing genetic overexpression of Nanog on the one hand and direct delivery of Nanog protein on the other hand as a non-genetic alternative to intracellularly activate Nanog. For that we used NIH 3T3 cells and murine embryonic fibroblasts (MEFs) as model systems. NIH 3T3 cells transfected with a vector carrying a conditional Nanog gene show expression of the transgene after the application of cell-permeable Cre. The Nanog activity can be detected for 2 days via RT-PCR. After this period of time the cells undergo apoptosis. To circumvent this limitation we engineered a cell-permeable version of the stem cell factor Nanog (TAT-Nanog fusion protein) based on our previously reported Cre protein transduction system. Treatment with TAT-Nanog induces self-renewal properties in embryonic stem cells even in the absence of leukemia inhibitory factor (LIF) in a reversible manner. With the TAT-Nanog fusion protein we are now able to induce Nanog activity in somatic cells in a titratable manner. We show the potential of TAT-Nanog to modulate growth properties of mature cells. Introduction of Nanog activity into NIH 3T3 cells results in an increased growth rate and a transformed phenotype as demonstrated by foci formation and colony growth in soft agar. TAT-Nanog transduction into primary fibroblasts induces an increased proliferation and bypasses replicative senescence. In conclusion our system of cellular manipulation provides a useful model to introduce the stemness factor Nanog into somatic cells without altering the genome and overcoming gene silencing. We expect this approach to further elucidate the potential role of Nanog in the regulation of the cell cycle and thereby its speculated function during the process of reprogramming.

Keywords: Nanog, pluripotency, senescence

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Expansion of undifferentiated (non) human primate iPS and ES cells in suspension culture using a largely defined medium

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Therapeutic application of pluripotent stem cell derivatives requires large quantities of cells produced in xeno-free defined media. This cannot be achieved by conventional culture techniques, and expansion of pluripotent stem cells under scalable suspension culture conditions is mandatory. Aiming at the development and scale-up of ES / iPS cell suspension culture, we have generated reporter lines of (non) human primate ES and iPS cells expressing GFP under control of the human Oct4 promoter. Stable transgenic cell clones were isolated by means of flow cytometry after lentiviral transduction. Immunocytology and qPCR demonstrated robust GFP expression in undifferentiated colonies of resulting cell clones. Moreover, a gradual decrease in GFP expression during differentiation was observed using qPCR. Anti-Oct3/4 staining revealed a strong correlation between endogenous Oct3/4 and GFP expression at the single cell level, indicating the usefulness of these clones for direct monitoring of the differentiation status. Together with these Oct4P-eGFP transgenic clones, we were able to establish the expansion of primate ESCs and human iPS cells in suspension culture using a largely defined medium. ES / iPS cells were expanded for up to 15 passages in suspension while maintaining their potential for differentiation into derivatives of the three germ layers, including beating cardiomyocytes. The ability to expand (non) human primate pluripotent stem cells in suspension culture represents a first step towards a controlled and scalable production of large cell numbers in stirred bioreactors. Furthermore, Oct4-dependent transgene expression can be applied for automated monitoring of the differentiation status in stirred bioreactor systems.

Keywords: (non)human primate iPS and ES cells, Suspension culture, Oct4P-eGFP transgenic clones

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Dissecting RNA Mediated Gene Silencing Pathways in Murine Embryonic Stem Cells

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In this study, Dicer-deficient mouse embryonic stem (ES) cells (A3 cells) were rescued with the two Dicer proteins from Drosophila melanogaster, dcr-1 and -2. This resulted in a structure and function analysis of Dicer with the potential of specifically rescuing either the miRNA or the siRNA pathway. To dissect these two RNA mediated gene silencing pathways, dcr-1 and dcr-2 from Drosophila were separately targeted into dcrΔ/Δ (A3) cells. The phenotypical rescue of these cells through gene targeting experiments with hDicer (mammals have only one dicer gene) served as a control and was accomplished. The successful gene targeting of the rosa locus was confirmed by Southern analysis; protein expression of DCR-1 and -2 could be shown by Western blot analysis using DCR-1 or DCR-2 specific antibodies, respectively. Since dcr-1 is believed to play an important role in the processing of microRNAs, the processing of microRNAs precursors into mature miRNAs was observed after successful gene targeting; the processing of microRNAs was shown by Northern analysis. In contrast, cells that had been targeted with Drosophila dcr-2 did not show any processing of microRNAs but siRNA processing. DCR-2 is believed to mediate the processing of siRNA in cells. Since such important processes like V(D)J recombination in B cells are regulated by (antisense) siRNA, it is of high value to have cells on hand that either express the miRNA or siRNA pathway to study these regulatory processes and especially their malfunctions in tumor genesis.

Keywords: Stemcells, RNAi, Gene targeting, microRNA, siRNA

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Human embryonic stem cells as model system for early neural crest development

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Neural crest cells (NCCs) are an intermediate embryonic cell population with remarkable characteristics. They delaminate during early development from the dorsal part of the closing neural tube and exhibit extensive migration throughout the body. NCCs give rise to a wide variety of different cell types, including neurons and glia from the peripheral nervous system, pigment cells, smooth muscle, as well as bone and cartilage of the head region. The delamination process has been studied in animal models by isolating the neural tube and allowing NCCs to emigrate. Up to now, little is known about the delamination process of NCCs in primates, due to the difficulties to access primary tissues. Human embryonic stem cells (hESCs) offer a valuable source for studying NC development. Focusing on the early steps of NC induction in spontaneously differentiating hESC cultures, we have developed a cell culture model recapitulating NC development in vitro. Specifically, we demonstrate that hESC-derived neural rosettes exhibit a regional organization, indicated by expression of the dorsal markers Pax3 and Pax7 at the outer edges. NCCs delaminate from these structures, migrate excessively, and accumulate in the periphery of neural rosettes. NCCs were identified by their expression of p75, Sox10, HNK-1, and AP2. During delamination and migration typical transient expression of specific cadherins was observed. Isolation of NCCs can either be achieved by manual picking of the NC aggregates or by fluorescence activated cell sorting (FACS). Following isolation, NCCs can be replated at low densities and induced to differentiate into mesenchymal NC phenotypes, but also to peripheral glia and different types of peripheral neurons. This ESC-based NCC culture system represents an attractive tool to study molecular and cellular mechanisms involved in the development of human NCCs and could provide important insights into NC-related diseases. The opportunity to derive multipotent human NCCs in vitro also opens interesting perspectives for the generation of various NC-derived cell types for tissue regeneration.

Keywords: Human embryonic stem cells, neural crest

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Preparation of microvascular endothelial cells for co-cultivation with embryonic stem cell-derived cardiomyocytes

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Differentiation experiments using embryonic stem (ES) cells are traditionally performed in conventional tissue culture plates by adding soluble growth factors and extra-cellular matrix (ECM) components to the medium. However, this approach does not consider the three-dimensional (3D) organization of in vivo differentiating cells and the influence of a cell specific ECM composition. For engineering mature and long-term functional tissues in vitro, sophisticated cultivation systems are required that mimic the spatiotemporal regulation of tissues developing in vivo. It is well known that endothelial cells in the heart exhibit the property of close anatomical and functional interaction with cardiomyocytes. They express and release a variety of auto- and paracrine agents (e.g. endothelin, prostaglandin I2, angiotensin II) as well as extracellular matrix proteins (fibronectin, collagen IV, laminin) which directly influence cardiac metabolism, development and function. Therefore, we want to establish a co-cultivation system of mouse ES cell-derived cardiomyocytes and freshly isolated microvascular endothelial cells. Our studies will focus on the influence of primary microvascular endothelial cells on the differentiation and maturation of ES cell-derived cardiomyocytes. For the generation of ES cell-derived cardiomyocytes we used a transgenic ES cell line exhibiting puromycin resistance and expressing eGFP under the control of the alpha-myosin heavy chain (MHC) promoter. ES cells were cultured as embryoid bodies (EBs) using the conventional hanging drop system. During EB formation eGFP fluorescence increased and was microscopically detectable after 8 to 10 days. During following puromycin treatment puromycin-resistant eGFP-expressing alpha-MHC positive cells within beating clusters were progressively enriched. Microvascular endothelial cells were isolated from murine heart tissue by using an endothelial cell specific antibody and immunomagnetic purification via magnetic dynabeads®. In order to optimize the co-cultivation conditions different media compositions were tested. Therefore both cell populations were analysed by RT-PCR and immunocytochemistry at various time points. Further studies will focus on the influence of the ECM secreted by organ specific microvascular endothelial cells on the differentiation of ES cells and their maturation into functional cardiac phenotypes. By using this cultivation system fundamental aspects of cell-cell interactions during stem cell differentiation can be investigated, which will help to overcome the limitations of conventional tissue culture.

Keywords: microvascular endothelial cells, ES cell-derived cardiomyocytes, co-cultivation system

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A new gene transfer tool: SEVI

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Today, gene transfer systems are widely in use not only for the genetic modification of Stem Cells (SC) including Embryonic Stem Cells (ESC), but also to generate induced Pluripotent Stem Cells (iPS cells) with retroviral gene transfer systems. The gene transfer rate is the most limiting factor of stable genetic modification. Recently, an enhancement of retroviral infection has been postulated using a naturally occurring fragment of the abundant semen marker prostatic acidic phosphatase (PAP). This peptide forms amyloid fibrils and is able to capture HIV virions and to promote their attachment to the target cells (Münch et al., Semen-derived Amyloid Fibril Drastically Enhance HIV Infection, Cell, 2007). The fibrils were termed Semen-derived Enhancer of Virus Infection (SEVI) and it was proposed that their function also applies to other retroviral vector systems. Our preliminary results show that not only HIV virions can bind to SEVI, but also other retroviruses, which are pseudotyped with different envelopes. The increase of the gene transfer rate was tested on cell lines like 293T, HeLa and K562 cells and also on murine and human hematopoietic stem cells (HSC). Lentiviral vectors were pseudotyped with four different envelopes: VSV-G, a foamyviral envelope, GALV and RD114 were selected because they attractive for use in gene therapy trials. Virus and SEVI (concentration 50 µg/ml) were simultaneously added to the cells. The gene transfer rate with GALV and RD114 in the presence of SEVI was typically increased 6 - 7-fold on cell lines compared to controls without SEVI. For VSV-G and the foamyviral envelope such a high increase was not detected and the effect of SEVI was strongly dependent on the cell line used. The gene transfer rate of the foamyviral pseudotyped vector was increased up to 6-fold on murine HSC. Furthermore, on human HSC the gene transfer rate was increased 8-fold with RD114 and 6-fold with FV. No toxic effect of the SEVI treatment was observed on any tested cell type and also the colony assays of the HSC show a normal growth and differentiation rate. This first data suggest that SEVI has no toxic effect on murine ESC and the cells do not lose their pluripotent status, if SEVI is present in the culture system. In conclusion, the application of SEVI protein is a reliable and feasible tool to increase the gene transfer rate. This effect is not only limited to fibroblasts, but can also be used in HSC, which are more difficult to transduce. Further research is needed to investigate whether SEVI can increase the gene transfer rate into ESCs and/or iPS cells.

Keywords: Embryonic stem cells, Retroviral gene transfer, SEVI

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miR-290 cluster prevents differentiation of embryonic stem cells towards mesoderm

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Changes in expression levels of miR-290 cluster have been associated with pluripotency and differentiation in mouse by a number of studies and homologues of this family have been identified also in human with similar expression profiles. However, role of miR-290 cluster during differentiation of embryonic stem cells (ESCs) remains unclear. We present the biological effects of miR-290 cluster via gain-of-function or loss-of-function experiments in mouse embryonic stem cells (ESCs) cultured under differentiation conditions. Previous studies on this miRNA cluster were based on cell systems depleted by all miRNAs. Thus, it was not possible to separate the effects observed due to loss of miR-290 cluster with those effects due to loss of other miRNAs that share the same miRNA seed with miR-290 cluster and may have similar functions. This study overcomes these problems by applying specific inhibition or overexpression of miR-290 cluster in a well defined system like that of ESCs deprived of LIF, without affecting any other miRNAs. We show that miR-290 cluster inhibition under differentiation conditions results in earlier downregulation of Oct-4 compared with the negative control. We found that miR-290 cluster regulates negatively differentiation of ESCs towards mesodermal lineage by affecting expression of key mesodermal genes like Brachyury, Fgf-8 and Eomesodermin. These results suggest that although incapable to maintain pluripotent state alone, miR-290 cluster inhibits ESC differentiation and it is involved in the pathways controlling mesoderm differentiation. Finally, we show that miR-290 cluster members regulate positively Wnt pathway, which can partially explain the reason why miR-290 cluster favours pluripotency against differentiation.

Keywords: microRNAs, embryonic stem cells, differentiation, mesoderm

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Genomics and Proteomics

A data integration approach to mapping OCT4-regulated transcriptional networks required for sustaining self-renewal and pluripotency in embryonic stem cells

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Background: Deciphering the transcriptional networks operative in human embryonic stem cells (hES) and human embryonal cancinoma cells (hEC), is essential for enhancing our understanding of self-renewal and pluripotency. The transcription factor OCT4, is a master regulator of the transcriptional networks required for inducing and maintaining pluripotency. Therefore, employing a systems biology approach whereby correlating gene expression resulting from the ablation of OCT4 function with potential OCT4-binding sites within the promoters of target genes allows a higher predictability of motif-specific driven expression modules important for maintaining self-renewal and pluripotency.
Methology/Principal Findings: We have conducted ChIP-on-Chip experiments using OCT4 antibodies to obtain a defined dataset related to OCT4-bound regions close to the transcription start sites of target genes. To achieve this, we compared several peak finding analysis programs to arrive at a refined list of OCT4 targets in hEC cells and compared this data to hES specific OCT4-binding and expression. We identified and verified a highly enriched POU/OCT4 -motif by employing a de novo approach, this enabled us to uncover six distinct OCT4-binding modules which are evolutionary conserved. Of these are for instance, the classic OCT4-SOX2 motif present within the NANOG proximal promoter. Other target genes such as USP44 and GADD45G have the POU-motif but not the classical HMG/SOX2 motif. Additionally, we observed preferred distances for the HMG and the POU motif, thus further evidence for additional binding modules other than the classical HMG-POU consensus sequence. In undifferentiated hEC and hES cells, USP44 and GADD45G are positively and negatively regulated by OCT4 respectively. Furthermore, over-expression of GADD45G in hEC cells resulted in an enrichment of up-regulated genes related to differentiation pathways. Due to the large nature of available datasets pertinent to embryonic stem cell biology, we have integrated our and already published datasets and developed an interactive embryonic stem cell database.
Conclusion/Significance: Employing a systems biology approach, we have uncovered new OCT4-binding modules and regulated targets, and highlighted their importance in the hEC/hES self-renewal circuitry. In this era of high-throughput functional genomics, which results in large datasets, our database allows rapid and convenient assess and comparisons between published datasets related to embryonic stem cell biology.

Keywords: OCT4, Transcriptional regulation, ChIP-on-chip, systems biology, embryonic stem cells, embryonal carcinoma cells, data integration, self-renewal, pluripotency

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Adipogenic Differentiation of Human Mesenchymal Stromal Cells is Affected by MicroRNAs HSA-MIR- 371 and HSA-MIR-369

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Mesenchymal stromal cells (MSC) display a limited lifespan in vitro and enter senescence after a certain number of cell divisions. Recent studies have indicated that this process of replicative senescence impairs the differentiation potential of MSC and might affect their therapeutic applications. Although the molecular mechanism of this process is still unknown, it might be regulated by micro RNAs (miRNA), a group of endogenous small, non coding RNAs. In this study we analyzed the role of specific miRNAs on the differentiation potential and replicative senescence of MSC. Upon 43 to 77 days of cultivation (7 to 12 passages) MSC demonstrated a proliferation arrest. Within this process adipogenic differentiation decreased whereas the osteogenic differentiation potential increased. MiRNA expression profiles of MSC upon in vitro expansion were analyzed by miCHIP technology and revealed an up-regulation of hsa-mir-371, hsa-mir-369-5P, hsa-mir-29c, hsa-mir-499 and hsa-let-7f. To gain insight into the functional role of these miRNAs MSC were transfected with the different miRNA molecules and effects on differentitation capacity, morphology and proliferation were subsequently analyzed. Upon transfection with hsa-mir-371 the adipogenic differentiation potential of MSC was significantly increased. In correlation with this gene expression of the adipogenic associated markers adiponectin (ADIPOQ), C/EBP alpha (CEBPA) and fatty acid binding protein 4 (FABP4) was upregulated. In contrast transfection with hsa-mir-369-5P resulted in a significant decrease of adipogenic differentiation and downregulation of adiponectin (ADIPOQ). As changes in methylation pattern have also been shown to play role in senescence and aging we analyzed the effects of miRNA transfection on gene expression of DNA methyltransferases. In this context hsa-mir-371 induced gene expression of DNA methyltransferases 3A (DNMT3A) and 3B (DNMT3B) whereas hsa-mir-29c down-regulated DNMT3A. This study demonstrates that specific miRNAs are up-regulated upon cellular aging of MSC. These include hsa-mir-371 and hsa-mir-369-5P that increase and decrease adipogenic differentiation respectively and hsa-mir-371 and hsa-mir-29c that influence gene expression of DNA methyltransferases. Thus, these microRNAs might indirectly affect replicative senescence and differentiation potential of MSC by epigenetic modifications.

Keywords: mesenchymal stromal cells, microRNA, adipogenic differentiation, replicatice senescence

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Tagging methods for proteomics and regulomics in mouse embryonic stem cells

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Proteomic approaches in mammalians require simple and specific protein purification methodologies that are amenable to high-throughput approaches for the isolation of protein complexes. The most prominent technique used to pull down protein complexes is the tandem affinity purification (TAP) tag method, so far successfully applied in yeast but still inefficient in mammalians. Here, we describe an approach for a single-step purification of protein complexes based on Green Fluorescent Protein (GFP). The GFP tag was fused to the C-terminus of Ash2l, a component of histone H3K4 methyltransferase complexes. The fusion protein was expressed in the E14tg2A mouse embryonic stem cell line. From our results we can conclude that the GFP tag altered neither the factor's protein interactions or DNA binding properties in vivo nor its sub-nuclear distribution. Therefore, GFP tag provides a promising basis for the analysis of the mammalian proteome.

Keywords: TAP, tag, GFP

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Transcriptomics and proteomics of mouse embryonic and multipotent adult germline stem cells

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Stem cells have the capacity to self-renew and the ability to generate differentiated cells. Until recently embryonic stem cells (ECSs) which are derived from the inner cell mass of blastocysts, embryonic germ cells (EGCs) which can be obtained from primordial germ cells and germline stem cells (GSCs) from neonatal mouse testis were the only known pluripotent cells. In 2006 we reported that also spermatogonial stem cells (SSCs) from adult mouse testis acquire embryonic stem cell properties and give rise to pluripotent stem cell lines in culture. These multipotent adult germline stem cells (maGSCs) retain their pluripotency in culture, are able to differentiate into derivatives of the three germ layers in vitro, generate teratomas in immunodeficient mice and when injected into blastocysts show germline transmission in chimaeras. To further define maGSCs as pluripotent cells, we compared ESCs and maGSCs of different genetic backgrounds cultured under standard ESC-culture conditions and in differentiation-promoting conditions at the transcriptional level by performing DNA-microarray experiments. Therefore we isolated RNA from ESC- and maGSC-lines, amplified it by in vitro reverse transcription and hybridized the samples to microarrays containing 44,000 genes. Scanned arrays were analyzed to identify fold changes in gene expression between the cell lines. The results of the microarray experiments concerning the expression of several randomly chosen genes were validated by quantitative Real Time PCR. Pluripotent cell lines are very similar based on their global gene expression pattern. Their transcriptomes reveal 98% identity. Only 306 genes are more than fourfold stronger expressed in ESC-lines, and 553 genes are higher expressed in maGSC-lines. Both cell types express the same genes involved in the regulation of pluripotency and apoptosis, and even show no difference in the expression level of these genes. Also after differentiation of maGSCs and ESCs the transcriptomes of the cell lines showed 95% identity what suggests that both cell types differentiate spontaneously in a very similar way. To show the similarities and differences of ESCs and maGSCs on protein level we performed 2D-Gelelectrophoresis and DIGE-analysis of undifferentiated cell lines. With this approach we could show a 96% identity of the proteomes between ESCs and maGSCs. Only 18 proteins show differences in expression between the two cell types, whereas proteins known to be specific for ESCs could also be detected in maGSCs. We now promote differentiation of both cell types and compare their proteomes.

Keywords: ESCs, maGSCs, transcriptomics, proteomics

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Cell-permeant FLP and ligand-inducible Dre fusion proteins as two novel recombinase tools for genetic engineering of stem cells

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Site-specific recombinases (SSRs) such as Cre and FLP have become increasingly important for conditional mutagenesis to study gene functions involved in stem cell maintenance or in committing cell differentiation. Combined application of two recombinases offer sophisticated strategies to address complex biological questions. However, in laboratory practice such dual recombinase strategies are hampered by the comparably low efficiency of FLP recombinase and the outage of efficient alternative recombinase systems. In this study we designed a recombinant cell-permeant FLP protein and a ligand-inducible version of a newly identified SSR, Dre. Our modified FLP protein induces recombination in more than 75 % of fibroblasts and embryonic stem (ES) cells. The FLP transduction system ideally complements the application of cell-permeant Cre recombinase previously developed in our laboratory for genetic engineering. We exemplify this strategy by reversible expression of a lacZ transgene in mouse ES cells and by a proof-of-principle study in human ES cells enabling reversible GFP transgene expression. To further expand the genetic toolbox we exploited Dre recombinase, a recently identified Cre-like SSR that holds the promise to have the remarkable properties of Cre. We generated an expression construct encoding for the Dre*PR fusion protein (progesterone ligand binding domain) and assessed its recombination potential in the presence of the synthetic progesterone agonist RU486. We found RU486-dependent recombination under exceptionally tight control in both fibroblast and ES reporter cells. Combined application of both systems, FLP-transduction and Dre-induction, together with our previously reported Cre transduction system will greatly enhance our capabilities to address intricate biological questions by complex genetic engineering.

Keywords: Recombination, Genetic engineering, Conditional mutagenesis, Cre

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Aging and Replicative Senescence Have Related Effects on Human Stem and Progenitor Cells

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The regenerative potential diminishes with age and this has been ascribed to functional impairments of adult stem cells. Cells in culture undergo senescence after a certain number of cell divisions whereby the cells enlarge and finally stop proliferation. This observation of replicative senescence has been extrapolated to somatic stem cells in vivo and might reflect the aging process of the whole organism. We have addressed the impact of replicative senescence on mesenchymal stromal cells (MSC) from human bone marrow. Within 43 to 77 days of cultivation (7 to 12 passages), MSC demonstrated morphological abnormalities, enlargement, attenuated expression of specific surface markers, and ultimately proliferation arrest. Adipogenic differentiation potential decreased whereas the propensity for osteogenic differentiation increased. Gene expression profiles were analyzed by Affymetrix GeneChip technology and this revealed a consistent pattern of alterations in the signature of MSC at different passages. These changes are not restricted to later passages, but are continuously acquired with increasing passages. In continuation of this work, we have analyzed effects of aging on gene expression profiles of MSC or of human hematopoietic progenitor cells (HPC). MSC were isolated from bone marrow of 12 donors that were between 21 and 92 years old. 67 genes were age-induced and 60 were age-repressed. CD34+ HPC were isolated from cord blood of 4 donors and from mobilized peripheral blood of 15 healthy donors between 27 and 73 years. 432 genes were age-induced and 495 were age-repressed. The overlap of age-associated differential gene expression in HPC and MSC was moderate. However, it was striking that several age-related gene expression changes in both HPC and MSC were also differentially expressed upon replicative senescence of MSC in vitro. Especially genes involved in genomic integrity and regulation of transcription were age-repressed. These studies have demonstrated that aging causes gene expression changes in human MSC and HPC that vary between the two different cell types. Changes upon aging of MSC and HPC are related to those of replicative senescence of MSC in vitro and this supports the notion that our stem and progenitor cells undergo replicative senescence also in vivo.

Keywords: Aging, replicative senescence, HSC, MSC, gene expression

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Induction of Pluripotency

Characterization and culture of spermatogonial stem cells from infantile marmoset testes

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Background: Spermatogonial stem cells (SSCs) are capable of self-renewal and giving rise to differentiating daughter cells that maintain gamete production. As the histological appearance of spermatogenesis in the non-human primate Callithrix jacchus is similar to that in man, we have used the marmoset monkey as a primate model for studies addressing both reproductive medicine and stem cell physiology. But markers for characterizing cells in culture have not been determined. In the present study, our aims were (a) to identify specific spermatogonial and pluripotency markers in native testicular tissue, and (b) to establish culture conditions for the maintenance and expansion of spermatogonia and putative stem cells.
Materials and methods: Four testes of newborn and four testes of 8-week-old monkeys were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned and immunohistochemically stained for the presence of Vasa, Oct4 and AP-2g. In parallel marmoset testicular cells from two 8-week-old monkeys were isolated by two-step enzymatic digestion and cultured initially in medium according to Guan et al. (2006) on gelatin-coated coverslips. After 7 days cells were transferred to StemPro medium containing the growth factors bFGF, EGF and GDNF and a cytokine (LIF) according to Kanatsu-Shinohara et al. (2004). We tested the effects of different concentration of FBS and frequent media changes. The obtained cell aggregates were embedded in paraffin in order to analyze the stem cell and germ cell markers Oct4, AP-2g and Vasa immunohistochemically.
Results: Immunohistochemical localization revealed that the number of positive cells in seminiferous tubules was similar for Vasa in newborn and 8 week old monkeys but lower for AP-2g and Oct4 at 8 weeks. Further morphometric analysis to reveal total number of germ cell populations is in progress. The cultured cells showed the same growth patterns irrespective of the frequency of medium changes. The appearance of the cultured cells changed drastically in the presence of FBS. At low amounts of FBS (1%) fewer fibroblasts were observed and after two weeks cells formed alkaline phosphatase-positive aggregates. At high amounts of FBS (15%) an overgrowth of fibroblasts occurred and no cell aggregates were obtained.
Discussion: These results show that a subpopulation of immature marmoset germ cells expresses the germ/pluripotency markers Vasa, Oct4 and Ap-2g Differences in the number of immunopositive cells for each marker indicate a dynamic growth of spermatogonial subpopulations between birth and 8 weeks of age. The outgrowth of colonies containing potentially pluripotent cells was not affected by frequent media changes but was highly dependent on the amount of FBS in the medium since fibroblasts out-competed the

slowly growing germ cells. Further characterisation of the cell aggregates and optimisation of culture conditions will provide an opportunity to establish long-term cultures of immature marmoset pluripotent germ cells.

Keywords: spermatogonial stem cells, germ cell, marmoset

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Induced Pluripotent Stem Cells (iPS cells): Implementation of the Reprogramming Technology for Human Disease Modeling

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The study of neurological disorders at the cellular level is hampered by poor access to patient-specific neurons and glia. This limitation may be overcome by the generation of human induced pluripotent stem cells (hiPSCs) derived from patient-specific fibroblasts. Shinya Yamanaka and colleagues have recently demonstrated that transgenic overexpression of the pluripotency-associated transcription factors Oct4, Sox2, Klf4 and c-Myc in somatic cells yields hiPSCs resembling human embryonic stem cells (hESCs) with respect to morphology, gene expression profile and differentiation potential. Subsequent in vitro differentiation of hiPSCs may provide an avenue to generate unlimited numbers of disease-specific neurons and glia. We have implemented the reprogramming technology and co-transduced adult dermal fibroblasts from skin biopsies with retroviruses coding for the four reprogramming factors. After three weeks underhESC culture conditions, monolayer colonies with distinct borders emerged and were picked for clonal expansion and further analysis. Established hiPSC lines displayed a hESC-like morphology cells and expressed the pluripotency-associated markers SSEA-3, SSEA-4, Nanog, Tra 1-60 and Tra 1-81. Silencing of transgene expression, which is essential for the differentiation potential of hiPSCs, could be demonstrated by RT-qPCR analysis. Furthermore, pluripotency of the cells was confirmed by multi-germ layer differentiation in vitro as well as by teratoma formation in vivo in SCID beige mice. SNP analyses revealed occasional microduplications, which would have gone unnoticed using classic metaphase G-banding, thus stressing the need for high-resolution genotyping in iPS cell validation. Controlled differentiation of proper validated hiPSCs into neural cells may permit the establishment of cellular disease models for neurodegenerative disorders and thus provide a tool for disease-related basic research and compound development.

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Lineage selection and characterization of murine iPS cell-derived cardiomyocytes

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Conversion of adult somatic cells into a pluripotent state by transient overexpression of reprogramming factors opens new possibilities for autologous cell replacement therapy, establishment of human in vitro disease models, drug discovery and toxicology. However, before induced pluripotent stem (iPS) cells can be used for any of these purposes it must be determined whether differentiated cells derived from iPS cells have the same characteristics as their embryonic stem (ES) cell counterparts. In this study we have compared structural, functional and molecular properties of highly purified murine iPS and ES cell-derived cardiomyocytes generated from corresponding transgenic lines expressing puromycin N-acetyltransferase and green fluorescent protein under the control of a cardiospecific α-myosin heavy chain promoter. We demonstrate that murine iPS and ES cells differentiate into spontaneously beating cardiomyocytes at comparable efficiencies. Both iPS and ES cell-derived cardiomyocytes express typical cardiac transcripts and structural proteins and possess similar ultrastructural organization. Action potential recordings revealed that iPS- and ES-cardiomyocytes respond to β-adrenergic and muscarinic receptor modulation, express functional voltage-gated sodium, calcium and potassium channels and possess comparable current densities. Comparison of global gene expression profiles of iPS and ES cardiomyocytes revealed that these cells cluster close to each other but are highly distant to undifferentiated ES or iPS cells as well as unpurified iPS and ES cell-derived embryoid bodies. Transplantation of purified iPS cardiomyocytes did not result in teratoma formation demonstrating the lack of contamination with tumorigenic pluripotent cells. These data suggest that iPS cardiomyocytes obtained by lineage selection are highly similar in their structural, functional and molecular properties to ES cell-derived cardiomyocytes and represent a valuable and safe source of cells for a variety of in vitro and in vivo applications.

Keywords: induced pluripotent cells, cardiomyocytes, Es cells, transplantation

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Generation of induced pluripotent stem cells from mouse neural stem cells by transient plasmid nucleofection reprogramming in chemical inhibitor (iSTEM®) culture medium

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Induced pluripotent stem (iPS) cells hold great potential for cell therapy and drug discovery. The increased risk of tumorigenicity by viral integration into the host genome is however of serious concern. Therefore, it is desirable to develop protocols that enable iPS generation with transient transfection methods. To this end we engineered the cDNAs for Oct-4, Klf4, c-myc and Sox2 linked via an IRES (internal ribosome entry site) to puromycin resistance into plasmid vectors under the control of the constitutively active CAG promoter. The plasmids were transfected a single time (Amaxa nucleofection system) into mouse neural stem (NS) cells derived from the foetal brain of OCT4-GFP reporter mice. Before transfection the NS cells were negative for expression of the OCT4-GFP gene by fluorescence as expected. Transfected cells were plated feeder-free onto gelatine coated plates in NS expansion medium for 24 hours to allow recovery. They were then cultured in serum-containing medium and leukaemia inhibitory factor (LIF) under puromycin selection until colonies with ES-like morphology emerged. These were initially negative for Oct-4, but when subsequently plated into N2B27 supplemented with the two differentiation signal inhibitors PD0325901 plus CHIR99021 and LIF (2i/LIF; Silva, 2008: PLoS Biol 6, e253) serum-free conditions for two weeks they became OCT4-GFP fluorescence positive, indicative of true iPS cells. The cells expanded rapidly and were maintained with a stable ES-like morphology and OCT4-GFP expression in serum- and feeder-free culture for >15 passages. Immunofluorescence analysis confirmed that iPS cell colonies were also positive for SSEA-1 and Nanog. To determine the differentiation capacity of the presumed iPS cells, monolayer (Ying, 2003: Meth Enzymol 365, 327-41) and embryoid body differentiation protocols were performed. In the monolayer condition within seven days differentiated cells of mixed morphology were noted and the majority of cells had become OCT4-GFP negative. Over the course of two weeks multiple beating areas of cardiomyocytes appeared, some of which had endothelial-like structures entering them. At this stage there was no evidence of residual iPS cells in the cultures. The pluripotent differentiation potential of the presumed iPS cells into progeny from all three germ layers was further confirmed by RT-PCR for Pax6, GATA4 and PDGFRalpha. In summary these results suggest that full reprogramming by transient transfection followed by culture in 2i/LIF might be feasible.

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Generation of functional cardiac myocytes from human induced pluripotent stem cells

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Background: Recent establishment of induced pluripotent stem (iPS) cells opened new avenues for the generation of human patient-specific stem cell derivatives that can be used for in vitro modeling of human disease, drug development or cell replacement therapies. The major objective of this study was to characterize the molecular and functional properties of cardiomyocytes differentiated from human iPS cells.
Methods and Results: Human iPS cells were differentiated into cardiomyocytes on a layer of the murine endodermal cell line END-2. Clusters of synchronously beating cells were first observed at day 11 of iPS cell differentiation. Beating areas that were microdissected at day 18 of differentiation expressed high levels of cardio specific transcripts NKx2.5, alpha-MHC, MLC2v, alpha-actinin and troponin T. Immunocytochemical stainings for alpha-actinin and troponin T revealed that these structural proteins form cross-striations, typical for cardiomyocytes. Functional assessment of iPS cell-derived cardiomyocytes showed that these cells possess intact calcium transients and respond to stimulation of beta-adrenergic and muscarinic signaling pathways. Molecular, structural and electrophysiological properties of iPS cell-derived cardiomyocytes were highly comparable to those of human ES cell-derived cardiomyocytes at the same differentiation stage.
Conclusion: Human iPS cells can differentiate into functional cardiomyocytes in vitro and thus fulfill the basic requirement for their use in disease modeling, drug screening and therapeutic applications.

Keywords: human iPS cells, cardiomyocytes, electrophysiology, differentiation

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Differentiation of human cord blood derived induced pluripotent stem (iPS) cells into functional cardiomyocytes

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Reprogramming of somatic cells into a pluripotent state can be achieved by the overexpression of several transcription factors. Here we report, that human induced pluripotent stem (iPS) cells can be generated from cord blood (CB) derived endothelial cells using lentiviral vectors expressing Oct3/4, Sox-2, Nanog and lin28. These iPS cells show typical DNA methylation characteristics of ESCs, express endogenous pluripotency factors and form derivatives of all three germ layers in vitro. Spontaneously beating iPS-derived cardiomyocytes were characterized on a molecular and functional level. Our results suggest that therapeutically useful cardiac myocytes can be produced from blood via the induction of pluripotent stem cells. Furthermore, CB may represent a useful cell source for the production of patient-specific and allogeneic iPS derivatives as i) it is routinely harvested, without risk to the donor, for public and commercial CB banks, and ii) much lower frequencies of accumulated mutations one expected as compared to cells from aged individuals.

Keywords: Induced pluripotent stem cells, IPS, Cardiomyocytes, Reprogramming, Cord blood

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Sox2 Fully Unfolds Pluripotency of Mouse Epiblast Stem Cells

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Pluripotent EpiSCs, one type of epiblast-derived pluripotent stem cell, have been newly established, but their pluripotential capabilities have not been adequately defined. Thus, in the current study, we elucidated the pluripotential capacities of EpiSCs by investigating their reprogramming potential using a cell fusion protocol. While the fusion of ES cells and F9 EC cells resulted in the efficient production of fusion hybrid colonies, epiblast-derived pluripotent stem cells (EpiSCs) and P19 EC cells showed extremely low and delayed reprogramming patterns. The low reprogramming potential of these cells could be rescued by overexpressing Sox2, which is underexpressed in both EpiSCs and P19 EC cells. Sox2 overexpression also resulted in a reduction in the time required for reprogramming as well as in the enhancement of epigenetic modifications of the hybrid cells, exemplified by DNA demethylation of Oct4 regulatory regions and reactivation of the X chromosome. Sox2-overexpressing F9 cells, P19 EC cells, and EpiSCs all showed compact ES-like morphology and the capability to proliferate under ES culture conditions for many passages. Moreover, EpiSCs, which very rarely form chimeras, were capable of efficiently forming chimeras and contributing to germ cell formation following Sox2 overexpression. Therefore, Sox2 overexpression alone is sufficient to dramatically rescue the restricted pluripotential capacities of mouse epiblast-derived pluripotent stem cells.

Keywords: Epiblast, pluripotency, reprogramming, cell fusion, Sox2

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Oct4-Induced Pluripotency in Adult Neural Stem Cells

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The four transcription factors Oct4, Sox2, Klf4 and c-Myc can induce pluripotency in mouse and human fibroblasts. We previously described direct reprogramming of adult mouse neural stem cells (NSCs) by Oct4 and either Klf4 or c-Myc. NSCs endogenously express Sox2, c-Myc, and Klf4 as well as several intermediate reprogramming markers. Here we report that exogenous expression of the germline-specific transcription factor Oct4 is sufficient to generate pluripotent stem cells from adult mouse NSCs. These one-factor induced pluripotent stem (1F iPS) cells are similar to embryonic stem cells in vitro and in vivo. 1F iPS cells can be efficiently differentiated into NSCs, cardiomyocytes and germ cells in vitro, and they are capable of teratoma formation and germline transmission in vivo. Our results demonstrate that Oct4 is required and sufficient to directly reprogram NSCs to pluripotency.

Keywords: 1F iPS, Oct4, Neural Stem cells, Reprogramming

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Conversion of Unipotent Germline Stem Cells into Pluripotent Stem Cells

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Reprogramming of mouse and human somatic cells into pluripotent stem cells, designated as induced pluripotent stem (iPS) cells, was first described for fibroblasts and required the introduction of the virally expressed transcription factor quartet Oct4, Sox2, c-Myc, and Klf4, as well as Nanog and LIN28. Recently, we have shown that Oct4 is required and sufficient to directly reprogram neural stem cells to pluripotency. Strikingly, the Oct4 gene is already active in adult germline stem cells (GSCs) despite these cells being unipotent. Here we demonstrate that mouse pluripotent stem cells can be derived from established adult unipotent GSCs by using a defined culturing procedure without virally expressed transcription factors. Germline stem cells (GSCs) are unipotent cells of the testis capable of self-renewing and of giving rise to sperm. Genome-wide gene expression profiling demonstrates that GSCs are more closely related to ESCs than other cell types that had been previously reprogrammed, and shows that germline pluripotent stem (gPS) cells converted from GSCs are more similar to ESCs than other reprogrammed pluripotent stem cells. gPS cells were also derived after clonal expansion from single GSCs. Pluripotency of gPS cells was confirmed by in vitro and in vivo differentiation analyses, including germ cell contribution and germ cell transmission. We also show that functional somatic cells could be derived by in vitro differentiation of gPS cells. The establishment of pluripotent stem cells without the introduction of any virally expressed transcription factors is an important step forward in generating patient-specific pluripotent stem cells for medical purposes.

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Induced pluripotent stem cells from a murine model of tyrosinemia

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Background: The direct reprogramming of somatic cells into pluripotent cells by retrovirus-mediated expression of pluripotency-associated genes in mice and in humans is a promising approach to derive disease-specific induced pluripotent stem (iPS) cells. In this study we focused on a mouse model for tyrosinemia type 1 (fumarylacetoacetate-hydrolase-deficiency, FAH-/- mice).
Methods and Results: Fetal fibroblasts (day 13.5 p.c.) were prepared and used at passage 4 for retroviral expression of Oct4, Sox2, c-Myc and Klf4. Embryonic stem cell–like colonies that emerged 3 weeks after transduction were subcloned based on morphological selection and maintained as individual iPS cell lines. RT-PCR and immunofluorescence analyses show the expression of pluripotency markers (Oct4, Nanog and Sox2) in iPS cells with similar levels as in wild-type ES cells and teratoma formation demonstrates pluripotency of the iPS cell lines. Furthermore, we demonstrate contribution to various tissues of all three germ layers and the germ-line after morula aggregation. In addition, we obtained fetal mice after tetraploid embryo aggregation, that were delivered by caesarian section on day 18.5 dpc. However, due to a premature pulmonary function these pups died from respiratory insufficiency in the first hour after birth. Applying an in-vitro differentiation protocol hepatic precursor cells could be derived from these disease-specific iPS cells and could be visualized after transduction of a lentiviral albumin-GFP reporter construct. Further characterization of these cells demonstrate a hepatic phenotype of the iPS-derived cells. In conclusion, we provide evidence that metabolic liver disease-specific iPS cells can be generated from murine somatic cells and can be re-differentiated into the diseased cell phenotype. If this technology is transferred to humans, it might be a versatile tool to study the underlying molecular mechanisms of the respective disease.

Keywords: induced pluripotent stem cells, disease models, endodermal differentiation

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Oct4-EGFP Transgenic Pigs – Large Animal Model for Reprogramming Studies

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The domestic pig is an important biomedical model mainly due to its similarity in size and physiology to humans. However, the lack of true pluripotent embryonic stem cells severely limits the value of the pig model for testing novel stem cells therapies. A major hurdle for the maintenance and proliferation of porcine pluripotent cells is that suitable culture conditions for in vitro culture are largely unknown. The Oct4 gene is an essential factor for the maintenance of pluripotency. The Oct4-EGFP reporter facilitates the detection of porcine germline and pluripotent cells, Here, we report the production and stem-cell-specific expression pattern of transgenic pigs carrying a murine Oct4 promoter – enhanced green fluorescent protein (Oct4-EGFP) reporter construct. Fetal porcine fibroblasts were transfected with the Oct4-EGFP construct (18kb) and transgenic cell clones were used for somatic cell nuclear transfer to produce reconstructed embryos, these cloned embryos were surgically transferred to the oviducts of recipient animals and 11 live piglets were delivered. As expected, the Oct4-EGFP construct is active in cloned pig blastocysts (day 7) and exclusively in the primordial germ cells of day 25 fetuses, whereas somatic tissues do not express the transgene. Preliminary results from sacrificed piglets confirmed stable integration of the Oct4-EGFP construct, and suggested that the Oct4-EGFP construct is expressed in a small subpopulation of testis cells, presumably the precursor cells of spermatogonia. Transgenic porcine fibroblasts were fused with murine embryonic stem cells to demonstrate reactivation of the Oct4 gene. The fused hybrids displayed stem cell morphology, a high proliferation rate and expressed EGFP fluorescence for a period of at least 72 hours after fusion. In summary, we report here the production of viable Oct4-EGFP transgenic piglets, which express EGFP exclusively in germline and pluripotent cells. This transgenic pig line should simplify the derivation and maintenance of porcine embryonic stem cells and will be of utmost interest for reprogramming studies based on porcine cells and for the preclinical testing of stem cell therapy.

Keywords: pig, cell fusion, reprogramming

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Generation of induced pluripotent stem cells (iPS) from murine bone marrow

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Introduction: Induced pluripotent stem (iPS) cells have emerged as an alternative to ES cells as a source of pluripotent stem cells for use in regenerative medicine. These iPS cells originally have been generated from murine and human fibroblastoid cells by retroviral expression of the transcription factors Oct3/4, Sox2, Klf4 and c-Myc. More recently additional reprogramming factors and other gene transfer systems have been used to produce iPS from different sources such as fetal and adult fibroblasts, keratinocytes, hepatocytes, and neural or mesenchymal stem cells. Given their high abundance and easy accessibility, hematopoietic cells appear as another promising source for the generation of iPS cells. However, reprogramming of blooad cells has proven difficult and so far has been only successful for differentiated B-lymphocytes using a rather complex experimental system.
Methods: In order to assess the feasibility of producing hematopoiesis-derived iPS cells, we transduced bone marrow (BM) cells from OG2 mice, which express GFP under control of the Oct3/4 promoter as an indicator of pluripotency. OG2 bone marrow cells were sorted immunomagnetically into an immature precursor (lineage negative, Lin-) and a more differentiated (lineage positive, Lin+) population and were subsequently transduced with lentiviral vector constructs expressing human Oct3/4, Sox2, Klf-4 and c-Myc from spleen focus forming virus (SFFV)-derived promoter/enhancer sequences. These vectors are known to promote high transgene expression levels particularly in the hematopoietic system. Two days prior to and up to 7 days after lentiviral infection, Lin- and Lin+ cells were cultured in the presence of suitable hematopoietic growth factors, thereafter cells were transferred to standard ES cell culture conditions.
Results: Five (Lin-) and seven (Lin+) days after transduction, single GFP-positive cells were detected, which during the next 2 weeks gave rise to iPS cell colonies, characterized by GFP-expression, typical ES-cell morphology, positive alkaline phosphatase activity and SSEA-1 expression. Expression of important pluripotency markers, such as Oct3/4 and Nanog, was demonstrated by qRT-PCR and Western blot analyses at levels similar to OG2 ES cells. Reprogramming efficiencies were 0.013% for Lin- and 0.005% for Lin+ cells, respectively. Currently, additional experiments are performed to further characterize our BM-derived iPS cell lines as well as to define the contribution of individual subpopulations of Lin- and Lin+ BM cells to iPS generation. In conclusion, our data suggest that both immature as well as more differentiated murine bone marrow cells can be reprogrammed

to a pluripotent state and therefore may serve as a convenient source for the generation of iPS cells.

Keywords: Reprogramming, iPS cells, Hematopoiesis, Lentiviral Vectors

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Colony morphology based selection is an ambiguous indicator for stable pluripotent murine induced pluripotent stem (iPS) cells whereas UTF1-Neo selection generates stable pluripotent iPS cell lines

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Somatic cells can be reprogrammed by viral transduction with defined factors to form induced pluripotent stem (iPS) cells. It has been reported that selection of fully reprogrammed cells can be done by screening for colonies that exhibit a morphology typical for embryonic stem (ES) cells. We analysed the clonal iPS cell line TiB7-4 and subclones of this cell line for maintainance of pluripotency during culture and found them to be diverse with respect to maintaining the typical ES cell-like colony morphology and expression of SSEA1. Undifferentiated transcription factor 1 (UTF1) promoter driven G418 resistance has recently been shown to significantly improve the quality of ES cells in culture. Transfection of TiB7-4 cells with this selection cassette resulted in the formation of subclones after an initial G418 selection for 14 days. Even without further selection pressure these subclones could be maintained stable for at least 40 passages in culture and were able to differentiate into all three germ layers in vitro. Control transfections with a construct expressing G418 resistance under the control of the ubiquitously active SV40 early promoter formed a variety of subclones with different colony morphology, some of them showing a perfect ES cell like shape. Two of these subclones could be also propagated for more than 8 passages without loosing their pluripotency as determined by monitoring the SSEA1 expression and colony morphology. Loss of pluripotency occurred in other colonies in an unpredictable manner, independently of the subclone's initial morphology and SSEA1 expression. The data presented here indicate that colony morphology is an ambiguous indication of stable pluripotent cells and that temporary selection with UTF1-Neo can form subclones that maintain pluripotency for a prolonged period of time.

Keywords: pluripotency, stem cells

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Ooplasm reprograms two nuclei at once and yields stable NT-ES cell lines

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Nuclear cloning entails the transplantation of a diploid nucleus into an oocyte to generate a diploid cloned embryo capable of full development. In contrast to diploidy, pure tetraploidy is incompatible with full development in mammals, although tetraploid cells exist in mammals and participate in normal (and pathological) organ physiology. We put forward the question whether one ooplasm can reprogram two diploid nuclei to a pluripotent state, and if so, whether the resultant tetraploid embryos may serve as a source of stable cell lines able to participate in normal organ physiology. To address these questions, we modified the classical nuclear cloning approach by injecting two somatic nuclei (Oct4-GFP transgenic donor) into the mouse ooplasm, whose amount of the pluripotency factor Oct4 is 16 times higher than that of an ES cell as estimated by Western blot. Despite the multiple somatic centrioles transferred along with the two nuclei, single bipolar spindles invariably formed in oocytes. These constructs developed to blastocyst at comparable rates to diploid clones. Metabolic profiles of diploid and tetraploid clones overlapped as measured by aminoacid net uptake and ATP content. Comparison of gene expression by Q-PCR revealed lower levels of Oct4, Nanog and Cdx2 transcripts in the tetraploid morulae, however the corresponding proteins, as determined by immunofluorescence, were at diploid levels. Since Oct4 is essential for pluripotency of mouse and human ES cells, and it is also present in the ooplasm as a maternal factor, we next asked if an ooplasm challenged with two nuclei would support development beyond blastocyst i.e. to an ES cell state. Bi-nuclear transplantation supported the derivation of Oct4-GFP-expressing tetraploid cell lines under LIF and feeder cell culture conditions. These cell lines maintained a stable karyotype for more than six passages and populated the inner cell mass of normal blastocysts. Therefore we likened these cell lines to ES cells. Chimeric blastocysts gave rise to postimplantation embryos with tetraploid contribution however subcutaneous injection of tetraploid NT-ES cells into SCID mice did not give rise to teratomas. In conclusion, we have for the first time generated tetraploid NT-ES cells by bi-nuclear cloning and we have shown that the mouse ooplasm is not limited to a single nucleus in its reprogramming capacity. Future experiments will clarify whether 1) subvolumes of an ooplasm can be used to reprogram single somatic nuclei; 2) tetraploid NT-ES cells stay free of teratoma concerns also when transplanted in niches other than the subcutaneous; and whether 3) these cells can be differentiated into different lineages in vitro.

Keywords: somatic cell nuclear transfer, reprogramming, pluripotency, tetraploidy

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Reprogramming of human somatic cells to pluripotency: establishment of new disease models and generation of alternative reprogramming strategies

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Reprogramming human somatic cells to a pluripotent state has been shown achievable by ectopically forcing the stable expression of a combination of genes normally expressed in human Embryonic Stem Cells (hESCs). This technique, called direct reprogramming, allows the generation of induced Pluripotent Stem (iPS) cells from primary cells derived from skin biopsy (1, 2). iPS cells appear indistinguishable from hESCs in terms of developmental potential, cell growth and chromatin state. Thus, these cells hold the potentiality to generate patient or disease-specific pluripotent cells without the need for human embryonic stem cells (3, 4). Our first aim is the generation of stable iPS cell lines from healthy and patient-derived somatic cells using a retroviral cocktail containing the four transcriptional factors OCT4, KLF4, SOX2 and C-MYC (2). Skin-derived fibroblasts obtained from a healthy neonate (HFF1, ATCC) and from an 84 year-old woman affected by type II diabetes have been infected with the retroviral cocktail. Several hESC-like clones have been picked and propagated in an hESC-like fashion. Comparative (hESCs and iPS cells) analysis of transcriptional profiles, differentiation potential and teratoma formation are currently ongoing. Our second aim is to formulate efficient alternative reprogramming strategies that could overcome the use of viruses for iPS cell induction. These strategies include mRNA electroporation (5) and the use of epigenetic-modifier compounds which have already been shown to improve iPS cell generation (6). Overall, our long term goal is to generate in vitro cellular model systems of complex genetic diseases, e.g. neurodegenerative disorders, and to establish non-viral reprogramming approaches that could bring the concept of iPS cells one step further towards their use in cell-based therapies, drug screening and toxicology studies.

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Keywords: reprogramming, ips, disease modeling

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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. Frequently, reprogramming by defined factors and/or culture conditions requires 2-3 weeks. Reprogramming by cell fusion occurs within 1-2 days and readily allows the analysis of the molecular events ongoing during reprogramming. 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. Flt3+ HSC from bone marrow of Oct4-eGFP+ transgenic mice were fused with ESC in vitro and reprogrammed Oct4-eGFP+Flt3+ HCS/ESC hybrids were isolated. These hybrids acquired various features of the ES cells like ESC morphology and prolonged self-renewal ability. The Flt3+ HCS/ESC hybrids were stable 4n in the undifferentiated state as determined by flow cytometry and karyotype analysis. Pluripotency was demonstrated in vitro by embryoid body (EB) assay and in vivo by teratoma formation. Interestingly, microarray and real-time RT-PCR analyses revealed that the reprogrammed Flt3+ESC hybrids express gene clusters from both parental population but also clusters that were not expressed in either parental cell population. Gene array data were analysed on a global scale by principle component analysis. Here, we demonstrate that Flt3+ HCS/ESC hybrids cluster with ESC and induced pluripotent stem cells (iPS cells). The demethylated status of CpG sites of Oct4 and Nanog promotor/enhancer regions demonstrated efficient reprogramming of the Flt3+ HCS/ESC hybrids. To determine whether Flt3+ESC hybrids differentiate preferentially towards a specific lineage, we studied spontaneous and directed differentiation. After spontaneous differentiation in EB assay, Flt3+ESC hybrids showed an enhanced differentiation into mesodermal lineages as evidenced by real-time RT-PCR and analysis of beating structures. Directed differentiation in OP9 cocultures revealed an increased and accelerated propensity towards hematopoietic cell differentiation. 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. Interestingly, Flt3+ESC showed an enhanced and accelerated differentiation potential towards the

mesodermal lineage, indicating memory of the somatic origin.

Keywords: Reprogramming, Cell Fusion, Flt3+ESC hybrids; Somatic Memory, Pluripotency

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Large-scale-production of cell-permeable proteins for pluripotency induction in somatic cells

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The homeodomain transcription factors Oct4 and NANOG are of great importance in recent experimental research on pluripotency. For the complete understanding of the self-renewal processes many experiments have to be done. To assure the performance of the necessary experiments, great quantities of the relevant proteins are needed. The production of Oct4 and NANOG in E. coli is a way to solve the problem of great quantities of proteins for several experiments. A producing method for Oct4 and NANOG in Erlenmeyer flasks does already exist. But this method is not appropriate to satisfy the demand of proteins for all necessary experiments. Therefore an upscale of this method is required to make these proteins available in sufficient quantities. The upscale in bioreactors to cell high densities is the aim of this work. Many problems like toxicity for the E. coli host, must be solved until it is possible to produce the proteins with good quality and quantity. The development of a specific analysis is also required to determine and study all essential parameters of the upscaled process. Higher Volumes also require new kinds of reconditioning of the proteins to assure a high rate of yield with minimal work and costs for the reconditioning process.

Keywords: Oct4, NANOG, Large-scale-production

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A modular lentiviral vector system for reprogramming of somatic cells into induced pluripotent stem cells

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As originally described by Takahashi and Yamanaka, genetic modification of fibroblasts or other somatic cells with 4 transcription factors (Oct3/4, Sox2, Klf4, c-Myc) facilitates the induction of pluripotent stem (iPS) cells. The ultimate goal is the generation of patient- or disease-specific iPS cells to create new avenues for disease models, drug discovery and regenerative medicine. Yet the low reprogramming efficiency remains a major drawback of the procedure. Here, we describe a modular lentiviral expression system for enhanced expression of these four reprogramming factors and simple exchange of all relevant components. For optimized expression levels murine as well as human cDNAs with confirmed sequence information were equipped with Kozak consensus sequences. Optionally, murine factors were also codon-optimized as previous work has demonstrated that codon-optimization can lead to a significantly improved titer and expression of retrovirally delivered transgenes (Moreno-Carranza et al., 2008). The vector system allows for constitutive expression from the murine spleen focus-forming virus promoter, which mediates strong transgene expression and is frequently silenced in embryonic stem cells thus facilitating subsequent differentiation of iPS cells. In addition, regulated expression from a tetracycline-inducible promoter is possible. Correct processing of the reprogramming factors was confirmed by northern and western blot analysis, respectively. Furthermore, we linked each factor to a fluorescent marker of choice to follow expression kinetics. Based on the coexpressed fluorescent marking titration is facilitated, demonstrating titers in the range of 10 mio transducing units/ml. Titer determination and usage of defined multiplicities of infection enables higher standardization of the reprogramming protocol. Proof of concept for lentiviral reprogramming was obtained when transducing murine embryonic fibroblasts containing an EGFP-tagged Oct4 allele. iPS clones exhibiting embryonic stem cell-like morphology were derived from the different vector constructs at considerable high frequency (up to 14% of OG2 cells were induced to express GFP by day 9 with first detectable GFP expression as early as day 5). Clonal iPS lines showed expression of the ES cell markers (1) alkaline phosphatase, (2) stage-specific embryonic antigen 1 and (3) reactivation of endogenous alleles highly active in embryonic stem cells, such as Oct3/4 and Nanog. Teratoma formation further demonstrated the pluripotent potential of derived iPS lines. In conclusion, we have generated a flexible and modular vector system which

may ease the generation of iPS cells and represents a valuable tool to dissect the underlying mechanism.

Keywords: induced pluripotent stem cells, gene therapy, vectorology

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Construction of bovine Oct4 recombinant adenovirus and functional analysis of Oct4 over-expression in bovine embryonic fibroblasts

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Derivation of embryonic stem (ES) cells has not yet been truly achieved in farm animals like bovine and pig. Recent advances in the field of induced pluripotent stem (iPS) cells provide a circumvent path to generation of pluripotency. As the first step to iPS cells, pluripotency associated factors like Oct4 and Nanog need to be explored. Here, we explored the expression pattern of Oct4 and Nanog in bovine pre-implantation stage. Then we cloned the Oct4 cDNA from bovine blastocysts for the first time, and inserted it into the AdEasy recombinant adenoviral system, which is a safer gene delivery system widely used for gene therapy. Bovine Oct4 was transduced into bovine embryonic fibroblasts (BEF) with the adenovirus vector. It suggested that bovine Oct4 expressed in cytoplasm and translocated to the nucleus. No obvious changes were observed morphologically after 12 days overexpression of Oct4 in BEF. Transfection efficiency was evaluated in BEF and MEF with recombinant adenovirus, and as a result 80% BEF cells was positively transfected, evidenced by immunostaining and PCR analysis, which demonstrated the feasibility of generation of iPSCs with adenoviral vectors. Thus recombinant adenovirus is a viable gene delivery system for transfection in BEF, and this may facilitate generation of bovine iPSCs and other gene function analysis. Our study lays the foundation for further assessing reprogramming ability of Oct4 in bovine cells and for reprogramming of fibroblasts to iPSCs.

Keywords: Bovine, Oct4, Pluripotency, AdEasy adenoviral system

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Somatic Stem Cells/ Stem Cell Niche

Generation of Functional Endothelial Progenitor Cells from Adult Mouse Germline-Derived Pluripotent Stem Cells

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Generation of functional somatic cells from pluripotent cells is critical step for cell-based regeneration therapy. Recently, we have obtained germline-derived pluripotent stem (gPS) cells from adult mouse unipotent germline stem cells. Objective of our study is to test whether functional endothelial cell suitable for the promotion of angiogenesis and arteriogenesis in ischemic diseases, can be derived from the new source of pluripotent stem cells. gPS-derived EPCs were isolated from 6-days old embryoid bodies via magnetic associated cell sorting technique using anti Phycoerythrin (PE) antibody-coated microbeads, PE-conjugated anti PECAM (CD31) antibody and LS-MACS columns, according to the protocol provided by Miltenyi Biotec Inc (Bergisch Gladbach, Germany). The sorted cells were plated on gelatine-coated culture dish , expanded and maintained at 37°C in a humidified atmosphere of 5% CO2/95% air in Iscove's modified Eagle's medium (PAN-Biotech) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 1 x non-essential amino acids (all reagents from Biochrom), and 10 µM 2-b-Mercaptoethanol (Gibco). We could show using indirect immunofluorescence staining that approximately 90% of the cells expressed endothelial cell specific genes, PECAM-1 (CD 31), von Willebrand Factor and vascular endothelial-cadherin, these results were confirmed by fluorescence-activated cell sorting and RT-PCR. The cells were successfully passaged and maintained in in vitro culture for long without a significant loss in expression of endothelial cell markers. Furthermore, the cells were able to form capillary-like networks when cultured on Matrigel. Dil-conjugated acetylated low-density lipoprotein uptake confirmed functionality of gPS-derived EPCs. Our results provide evidence that EPCs can be obtained from gPS cells, which have typical endothelial characteristics. Therefore, gPS cells can serve as a new source of stem cells for endothelial cell based cell therapy without the ethical limitations affecting the use of embryonic stem cells.

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The influence of hypoxia on generation, expansion and differentiation of unrestricted somatic stem cells (USSC) from cord blood (CB) and bone marrow stromal cells (BM-MSC)

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Adult stem/progenitor cells such as BM-MSC and USSC from umbilical CB (Kögler et al. 2004) have multipotent differentiation potential. USSC and MSC reside in cell niches creating a unique tissue microenvironment. Previous data (Cipolleschi et al. 1993) suggested that BM exhibits hypoxic conditions (1%-2% O2) in which MSC are localized. Oxygen (O2) conditions in the blood of the umbilical cord vein vary from 3 to 4% (Klinke 2005). Thus lower O2- concentrations better simulate the physiological situation compared to atmospheric O2-concentrations (21%, normoxia). Based on this observation it was shown that hypoxia maintains the undifferentiated state, prolongs life span and enhances proliferation of MSC (Grayson et al. 2007, Fehrer et al. 2007). Here the influence of hypoxia (1.5%, 3%, 5% O2) on generation, growth-kinetics, differentiation potential and gene expression profile of USSC in comparison to normoxia and to BM-MSC was analyzed. Generation of USSC under normoxic or hypoxic (5% O2) conditions revealed a more efficient generation rate in hypoxic (46%, n=35 CB) versus normoxic conditions (29%, n=35 CB). Tested O2 conditions had different influences on the growth of the USSC (n=16). 1.5% as well as 3% O2 resulted in a restricted growth of the USSC correlating with lower cumulative population doublings (CPD) compared to normoxic conditions whereas 5% O2 resulted in enhanced expansion and increased proliferative lifespan of the USSC. In cultures of BM-MSC (n=5) only 1.5% O2 resulted in decreased growth rates, while both 3% and 5% O2 caused increased growth associated with higher CPD. Differentiation capacity towards the adipogenic lineage was diminished for BM-MSC (n=4), CB MSC (n=2) and USSC (n=6) in all hypoxic conditions tested as documented by Oil Red O staining, immunohistochemistry for PLIN, ADIPOQ and Real Time PCR for PPARgamma2. Immunohistochemistry (n=16) and Real Time PCR of human neurofilament and beta-3-tubulin revealed a decreased neural differentiation capacity applying 3% and 5% O2. In contrast the osteogenic potential was enhanced under 5% O2 but reduced under 1.5% and 3% O2 (n=14) as visualized by Alizarin red staining and quantified by a calcium release assay (BioVision). Gene expression was analyzed for the ES-markers OCT4A, NANOG, SOX2, KLF4 and telomerase activity. USSC as well as BM-MSC do not upregulate OCT4A, NANOG, SOX2 and telomerase under hypoxic conditions, as suggested previously for OCT4 (Grayson et al. 2006). Hypoxia-inducible factors (HIF 1- and HIF 2-alpha) are known to play an important role in the response of stem cells exposed to hypoxia (Keith et al. 2007). USSC and BM-MSC revealed unaltered transcription levels as compared for normoxia and hypoxia.

We document here that low oxygen levels (5% O2) mimic the natural conditions of USSC in CB, thus providing a milieu that extends cellular lifespan and rises CPD, while suppressing adipogenic and neural differentiation.

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Keywords: hypoxia, human cord blood

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c-kit expression identifies cardiovascular precursors in the neonatal mouse heart

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Directed differentiation of embryonic stem cells indicates that mesodermal lineages in the mammalian heart (cardiac, endothelial, and smooth muscle cells) develop from a common, multipotent cardiovascular precursor. To isolate and characterize the lineage potential of a resident pool of cardiovascular progenitor cells (CPc), we developed BAC transgenic mice in which the expression of enhanced green fluorescent protein (EGFP) is placed under control of the full c-kit locus (c-kitBAC-EGFP mice). Discrete populations of c-kit-EGFP+ at different stages of differentiation were observed within the late embryonic heart, increasing in number to a maximum at about postnatal day (PN) 2; thereafter EGFP+ cells declined and were rarely observed in the adult heart. EGFP+ cells purified from PN 0-5 hearts were nestin+ and expanded in culture; 67% of cells were fluorescent after 9 days. Purified cells differentiated into endothelial, cardiac, and smooth muscle cells, and differentiation could be directed by specific growth factors. CPc–derived cardiac myocytes displayed rhythmic beating and action potentials characteristic of multiple cardiac cell types, similar to ES cell–derived cardiomyocytes. Single cell dilution studies confirmed the potential of individual CPc to form all three cardiovascular lineages. In the adult heart, cryoablation resulted in prominent c-kit-EGFP+ expression, peaking 7 days post cryolesion. Expression occurred in endothelial and smooth muscle cells in the revascularizing infarct, and in terminally differentiated cardiomyocytes in the border zone surrounding the infarct. Thus c-kit expression marks CPc in the neonatal heart that are capable of directed differentiation in vitro; however, c-kit expression in cardiomyocytes in the adult heart following injury does not identify cardiac myogenesis.

Keywords: progenitor cells, cardiac stem cells

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FGF9 secreted by prostate carcinoma favors mesenchymal stem cells differentiation toward osteoblasts at least through the transcriptional induction of alpha5 integrin

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Prostate cancer metastasis to bone results in mixed osteolytic and osteoblastic lesions associated with high morbidity. Factors secreted by Prostate cancer and molecular events that lead to mesenchymal stem cells (MSc) differentiation toward osteoblast during disease progression just start to be understood. By using conditionned media, here we first demonstrated that prostate carcinoma have an ex vivo pro-osteoblast potential on Msc differentiation . We next found by Q-PCR based screening, that one of the proficient osteoblastic effect of prostate cancer cells acts at least through the transcriptionnal upregulation of the integrin alpha 5 in MSc. We finally demonstrated that this upregulation is dependent on FGF9, one of the pro-osteoblatic factors secreted by the Prostate cancer cells. Indeed Neutralizing antibodies against FGF9 added on conditionned media or addition of recombinant FGF9 on MSc, are sufficient to modulate positively or negatively the level of the integrin alpha 5 mRNA. These results will help us to better understand molecular mechanisms controling osteoblastogenesis during bone metastasis.

Keywords: stem cell, osteoblast, prostate cancer, integrin

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In Vitro Proliferation and Differentiation of Ovine and Caprine Spermatogonia Stem Cells

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Proliferation and differentiation of spermatogonia in vitro provide a convenient approach for study of spermatogenesis, and an opportunity for manipulation of spermatogonial stem cells (SSC). This technique has many potential applications in animal production and medicine. For instance, it has been suggested as an alternative to the currently inefficient methods to generate transgenic domestic animals that can produce therapeutic proteins in their milk, or organs suitable for transplantation to humans. Although culture of spermatogonia has been reported, relatively fewer reports are available regarding in vitro maintenance of spermatogonia. Sheep and goats are not only important farm animals in that they produce wool, milk and meat, but also used as animal models in medical research. However, no method is available for in vitro proliferation and differentiation of spermatogonia in these two small ruminant species. The objective of this study was to develop a culture system that could sustain the proliferation of ovine and caprine spermatogonia and to test if higher concentration of FBS expedites their differentiation. Methods: Spermatogonia were enzymatically isolated from the testes of Small-tail Han rams and male dairy goats at 3 months old, respectively, purified by discontinuous Percoll gradient centrifugation, and cultured on the monolayer of testis somatic cells in DMEM/F-12 supplemented with 10 ng/ml of EGF and 0.01% 2-mercaptoethanol, with or without 2.5%, 5% or 10% FBS in an incubator with 5% CO2 at 37 °C. Medium was changed every 2-3 days and the monolayer changed monthly. Cells and their morphological changes in culture were studied and photographed with a Nikon inverted microscope (TS-100). The cells were also stained with PGP 9.5, BrdU and C-kit to help identify the types, mitosis and meiosis of the germ cells. Spermatogonia were collected after culture and cryopreserved in liquid nitrogen. Results and conclusion: A number of clones of spermatogonia were formed in a couple of weeks of culture in DMEM/F-12 containing 2.5% FBS, 10 ng/ml of EGF and 0.01% 2-mercaptoethanol, and the spermatogonia in the culture maintained over an experimental period of 3 months, while increasing FBS to 10% shortened it to 4-5 weeks as spermatogonia differentiated into spermatids rapidly. Complete removal of FBS resulted in poor proliferation and differentiation of spermatogonia in both species. The following cells and structures were observed and recorded: Type A and type B spermatogonia, paired and aligned spermatogonia, bridges, clones and colonies of spermatogonia, spermatocytes, Sertoli cells and their junctions, spermatids, and premature spermatozoa. In conclusion, DMEM/F-12, when supplemented with 2.5% FBS, 10ng/ml of EGF, and 0.01% 2-mercaptoethano significantly ($P<0.05$) enhances the proliferation of ovine and caprine

spermatogonia on the monolayer of testis somatic cells in 5% CO2 at 37 °C, while 10% FBS in the medium promotes rapid differentiation.

Keywords: Spermatogonia, stem cells, proliferation

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The switch from germline stem cell proliferation to differentiation is accompanied by dynamic changes in the expression of BMP signalling components and target genes

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Bone Morphogenetic Proteins (BMPs) are essential for normal specification, expansion and maintenance of the early primordial germ cell pool, but potential roles during post-migratory germ cell proliferation, meiotic differentiation and the formation of primordial follicles are less well defined. To investigate the role of these growth factors during germ cell development in the human ovary, we analysed the expression of BMP signalling components and downstream target genes in the fetal ovary from germ cell proliferation (8-9 weeks gestation) through the onset of differentiation and meiotic entry (14-16 weeks) to the assembly of the first primordial follicles and meiotic arrest (18-20 weeks) using specimens obtained following elective termination of pregnancy. BMP4 expression (by qRT-PCR) fell 60% between 9 and 14 weeks, whilst BMP2 expression increased more than 5-fold. Parallel changes in the expression of SMAD5 and SMAD1 (3-fold decrease and 1.3-fold increase respectively) were detected over the same period, suggesting a possible switch of BMP ligand during germ cell differentiation, accompanied by a change in the preferred downstream intracellular mediator. To identify the sites of BMP action in the developing ovary we performed immunohistochemistry to detect the BMP receptors BMPR1a and -1b and the active phosphorylated form of SMAD1/5/8. At 9 weeks BMP receptors BMPR1a and -1b were expressed by both germ and somatic cells, but became restricted to germ cells only by 14 weeks and continued through the period of development examined, up to 20 weeks gestation. Similarly, phosphoSMAD1/5/8 was exclusively localised to primordial germ cell nuclei at 9 weeks, identifying these cells as the target of BMP signalling in the first trimester fetal ovary. At later gestations, pSMAD1/5/8 staining remained germ cell-specific, but unexpectedly localised predominantly to the cytoplasm with few germ cells showing nuclear expression. This suggests that nuclear translocation of pSMAD1/5/8 may be impeded in oocytes, despite their continued stimulation by BMPs. The Inhibitor of Differentiation (ID) genes are well-characterised transcriptional targets of BMP action in many tissues, and repress differentiation by inhibiting the action of bHLH transcription factors. As a number of bHLH transcription factors are essential for oocyte differentiation, we hypothesised that the ID genes may be good candidates for regulation by BMP signalling in the human fetal ovary. Expression of ID1 and ID4 increased markedly with gestation (6-fold and 17-fold respectively between 9 and 19 weeks), coincident with increasing expression of key ovarian bHLH proteins. Culture of 9 week fetal gonads with BMP4 for 24h induced 3- and 1.8-fold increases in ID1 and ID4

respectively compared to untreated controls. Greater effects on ID1and ID4 expression (10- and 4-fold respectively) were seen when 14-15 week fetal ovaries were disaggregated and cultured for 24h with BMP4. BMP4 had no effect on the expression of key germ cell-specific genes OCT4, DAZL, and VASA, demonstrating that the effect on ID gene expression is specific. Immunohistochemistry confirmed that ID1 protein expression is germ cell-specific, and localised to the nucleus at all gestations examined. Together these data demonstrate the presence of a functional and developmentally-regulated BMP signalling system in the human fetal ovarian germ cell niche that may act to regulate germ cell differentiation by promoting ID gene expression.

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Specific lineage-priming of bone marrow mesenchymal stem cells provides the molecular framework for their plasticity

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Two models of stem cell (SC) differentiation have been proposed. In a first model, SCs are considered as a "blank slate" since they do not express differentiation-associated genes; specific differentiation markers are acquired step by step during differentiation. In a second model, SCs express a subset of genes associated to the differentiation pathways to which they commit; differentiation in a given pathway is then characterized by the increased expression of marker genes associated to this pathway and the decreased expression of genes related to other lineages. The second model corresponds to lineage-priming, which has been first described for a hematopoietic multipotential cell line maintained as self-renewing undifferentiated cells. BM MSCs are cells that actively proliferate in vitro and give rise in the presence of appropriate inducers to adipocytes (A), osteoblasts (O) and chondrocytes (C). However, certain report indicate that the multipotentiality of these cells may not be restricted to the 3 classical mesenchymal lineages, but would include endothelial (E), vascular smooth muscle (V), skeletal muscle (MSK), cardiac muscle (MCA), neural (N), hematopoietic (H) and hepatocytic (HEP) lineages. Because of the known plasticity of apparently differentiated mesenchymal cells to shift their differentiation pathway under modified external conditions, the self-renewal capacity may not be for MSCs a strict requirement, a daughter cell in the progeny being able to recover the multipotentiality of the mother SC. For these reasons, we have explored the theory of lineage-priming applied to these cells. In this work we have studied in human and murine clonal populations of BM MSCs 10 differentiation programs (from extracellular effectors to cytokine and adhesion receptors, major transcription factors and downstream structural or regulatory molecules). We show that proliferating primary layers and clones of BM MSCs have precise priming to the osteoblastic (O), chondrocytic (C), adipocytic (A) and the vascular smooth muscle (V) lineages, but not to skeletal muscle, cardiac muscle, hematopoietic, hepatocytic or neural lineages. Priming was shown both at the mRNA (300 transcripts were evaluated by a sensitive and reproducible method of quantitative RT-PCR) and the protein level. In particular, the master transactivator proteins PPARG, RUNX2 and SOX9 were co-expressed before differentiation induction in all cells from incipient clones. We further show that MSCs (non clonal and clonal populations) cultured in the presence of inducers differentiate into the lineages for which they are primed. Our data point out to a number of signaling pathways that might be activated in proliferating MSCs and would be

responsible for the differentiation and proliferation potential of these cells. Our results extend the notion of lineage-priming and provide the molecular framework for inter-A, -O, -C, -V plasticity of BM MSCs. Our data highlight the use of BM MSCs for the cell therapy of skeletal or vascular disorders, but provide a word of caution about their use in other clinical indications.

Keywords: Lineage-priming, mesenchymal stem cells, differentation, clinical indications

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Generation of clonal derived unrestricted somatic stem cells (USSC) from cord blood and analysis of their expression and differentiation potential

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Cord blood contains non-hematopoetic multipotent adherently growing cells: mesenchymal stromal cells (CB MSC) with high adipogenic differentiation potential and unrestricted somatic stem cells (USSC). USSC have the capacity to differentiate under defined conditions in vitro into osteoblasts and chondroblasts, neural cells as well as in cells of the endodermal lineage (Kögler et al., 2004, 2005, 2006, Sensken et al., 2005, Trapp et al., 2008). To analyze possible differences within a USSC cell population clonal USSC were generated. The AVISO CellCelector™, a high precision multifunctional robot system (Schneider et al., 2008), was used to harvest single cells. Clonal USSC were cultured in conditioned medium. Overall 10 different cell lines were analyzed and 577 single cells were generated with a median cloning efficiency of 27.1% (n=158). 26% (n=41) of clonal populations could be expanded for further 5 passages, 12% (n=19) for at least further 9 passages after single cell selection. USSC reached up to 50 CPD, clonal populations reached 58 CPD. At mean 6.2 x 106 ± 1.1 x 106 cells were generated from a single cell with a maximum of 3.7 x 107 cells. Clonal populations were compared to the initial cell line for their gene expression profile and differentiation potential. 4 USSC and 19 corresponding clonal USSC populations in passages 4 up to 9 were tested in RT-PCR for the embryonic stem cell markers NANOG, OCT4 and SOX2. None of the tested USSC or the clonal populations ever expressed NANOG, OCT4 or SOX2, confirming that USSC as well as CB MSC are multipotent but not embryonic like cells. 4 USSC, 9 clonal USSC populations, 1 CB MSC and 4 clonal derived CB-MSC populations were tested for their differentiation potential towards adipocytes by Oil Red O staining of lipid vacuoles. In contrast to CB MSC and the corresponding cell clones we never observed adipogenic differentiation in any of the USSC and their clonal derived cells. The preadipocyte marker DLK-1/ PREF1 is a key factor to suppress adipogenic differentiation (Smas, Sul, 1993). Expression of DLK-1/ PREF1 in USSC inhibits adipogenic differentiation and maintains the osteogenic as well as neural and endodermal differentiation potential of USSC. 3 USSC and 1 CB MSC and 24 clonal USSC populations in passages 4 up to 9 were tested for DLK-1 by RT-PCR. 3 USSC and 8 clonal populations were positive for DLK-1. We observed a reduction in the expression of DLK-1 in some USSC and clonal populations with increasing age (more than 50 CPD).

All of the USSC tested revealed a strong osteogenic differentiation whereas clonal populations (n=8) showed a restricted osteogenic differentiation (higher numbers of CPD at the same passage). In summary the results document that clonal derived USSC and CB MSC can be generated and expanded, but the clonal cells showed an even more heterogeneous developmental potential by aging as compared to the original cell line.

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Keywords: clonal, human cord blood

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Functional characterization of the impact of the Par/aPKC complex and Cdc42 on cell fate decisions, polarity and migration in human hematopoietic stem and progenitor cells

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Freshly isolated hematopoietic stem and progenitor cells (HSC/HPCs) are small round cells which acquire a polarized cell shape upon cultivation. They form a leading edge at the front and a uropod at the rear pole. We and others have shown that this polarization process depends on the activity of the phosphoinositol-3-kinase; evidence was provided that the GTPase Cdc42 is also involved in this process. As it has been shown in model organisms, polarity is an important prerequisite for asymmetric cell divisions and it is known that the Par/aPKC complex and Cdc42 are required for the establishment of this polarity. Since we showed that HSC/HPC cells can divide asymmetrically, we wondered whether these evolutionary conserved proteins are also involved in cell fate specification processes within the human hematopoietic compartment. First results of our group show that the main components of the Par/aPKC complex are expressed in cultivated HSC/HPCs. To analyse the impact of these proteins on primitive hematopoietic cells by means of gain or loss of function experiments, we have set up efficient methods to genetically manipulate primary human HSCs/HPCs first. Furthermore, we have established assays to analyse effects on the cell polarity, on the migration behaviour and the cell fate of the manipulated cells and show preliminary results here.

Keywords: hematopoietic stem cell, HSC/HPC, polarity, PAR/APKC complex, Cdc42, asymmetric cell division

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Adopting the endothelial colony forming cell system to functionally analyze mechanisms regulating the self renewal capacity of somatic stem and progenitor cells

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The discovery of circulating endothelial cells that are involved in neoangiogenesis and postnatal vasculogenesis has stimulated the field of vascular biology. During the recent years different protocols have been applied to raise cells termed circulating endothelial progenitor cells. Indeed, most protocols select for cells expressing cell surface antigens being claimed as endothelial specific (e.g. CD31, CD144, CD146, KDR). However, a recent comprehensive comparison of such cells revealed that some of the commonly applied protocols rather select for phagocytotic cells of hematopoietic origin that just mimic the endothelial cell surface phenotype (Yoder et al., Blood 2007; 109, 1801-1809). According to these studies only endothelial colony forming cells (ECFC) - also termed as late outgrowth colonies - represent true circulating endothelial progenitor cells. Similar to primitive hematopoietic cells, ECFC are organized in a hierarchical manner, i.e. more primitive ECFC contain higher proliferation capacities than more mature ones. Since we are interested in mechanisms that control whether progeny of primitive somatic progenitor cells remain primitive or become committed to differentiate, ECFC provide a novel cell system to study the underlying mechanisms. In our ongoing work we have started to raise ECFC from different cell sources and to characterize their cell surface phenotype in more detail. According to our aim to perform functional analyses of genetically manipulated ECFC, we have also set up techniques to stably introduce genes into ECFC. Our current results will be presented.

Keywords: endothelial colony forming cells (ECFC), HUVEC, hierachy, characterization cell surface phenotype, self renewal

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Consecutive development of multipotent c-kit+ Flt3+ hematopoietic progenitors in vitro

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All blood cells derive from a population of c-kit+ Flt3+ hematopoietic stem cells (HSC) in bone marrow (BM). Here we describe a culture system where we use the cognate ligands SCF and Flt3L to amplify c-kit+ Flt3+ cells from mouse BM to large cell numbers. The amplified cells grow synchronously and can be kept proliferating for several weeks. The cells represent HSC as judged by phenotype and surface antigens expression, and give rise to cells of all lineages of the hematopoietic system when adoptively transferred into lethally irradiated mice. We further investigated the developmental potential of the c-kit+ Flt3+ cells by employing in vitro assays. In co-cultures with mouse embryonic fibroblasts, the cells form cobblestone areas, indicating a primitive state. In the colony-forming cell assay the cells rapidly give rise to colonies of several lineages. In addition, the cells display multilineage potential giving rise to both lymphoid and myeloid cells in vitro. With appropriate cytokines the cells differentiate into dendritic cells (DC), monocytes/macrophages and B cells. A more comprehensive analysis showed that our cultures comprise two progenitor cell populations: Flt3-/lo c-kithi multipotent progenitors (MPP) and Flt3+ c-kitint M-CSFR+ cells that have recently been identified as common DC precursors (CDP). The MPP and CDP populations demonstrate distinct proliferation capacity and differentiation kinetics when sorted by flow cytometry. Only the MPP population is able to extensively proliferate and reproduce both MPP and CDP under amplification conditions. The CDP population shows a more limited proliferation capacity. CDP readily differentiate into DC whereas the differentiation kinetics of MPP is considerably slower. Taken all together, our data suggest a hierarchy from MPP to CDP and during the MPP to CDP transition the growth and differentiation potential becomes more restricted.

Keywords: hematopoietic stem cell, multipotent progenitor, c-kit, Flt3

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Isolation and ex-vivo expansion of tissue-resident immunologically responsive mesenchymal stromal cells (MSCs) from human nasal mucosa

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Multipotent mesenchymal stromal cells (MSCs) are present in bone marrow (BM) and other tissues such as adipose tissue, muscle, pancreas, liver, tendon etc. Recent evidence suggests that MSC interact with different immune cell subsets and thus may be important regulators of local tissue immunity. Until now, most immunological studies refer to bone-marrow-derived MSC. We report on the isolation and characterization of multipotent nasal mucosa-derived mesenchymal stromal cells (NM-MSCs). We show that nasal mucosa mesenchymal cells, isolated by enzymatic digestion, demonstrate plastic adherence and fibroblast-like morphology, are able to form colonies and can be expanded for at least 14 passages. Following an initial proliferation period and short-term culture for more than 4 passages these MSCs expressed the typical mesenchymal stem cell marker proteins CD29, CD44, CD90 and CD105. MSC could be differentiated along the adipogenic, chondrogenic and osteogenic pathway. Tissue-specific differentiation was confirmed by histochemical and immunofluorescense staining as well as by RT-PCR for defined marker genes. Nasal mucosa MSCs were immunologically active and responsive. They produced inflammatory cytokines and were responsive to IFNg, TNFa and SDF-1. In conclusion, our study is the first description of human tissue-resident MSC from nasal mucosa. These cells may be an alternative adult stromal cell resource for regenerative tissue repair and auto-transplantation in the ENT field and may represent important regulators of local mucosal immunity.

Keywords: MSC, mucosa, immunology

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Modulation of Mesenchymal Stromal Cell Biology by inflammatory signals

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Mesenchymal Stromal Cells (MSC) are non-hematopoietic adult stem cells with multilineage potential. MSC are defined by plastic adherence and differentiation potential into osteogenic, adipogenic and chondrogenic lineages under specific in vitro differentiating conditions. They lack expression of hematopoietic antigens but express markers such as CD71, CD90, CD117, CD120a, CD271, CD105, CD73 and CD90. However, the expression of specific combinations of markers appears to be microenvironment-dependent, suggesting a strong influence of tissue context on MSC phenotype. The regeneration capability of MSC, their ease to undergo gene modification, as well as their immunosuppressive capacity render them as popular candidates for tissue engineering, gene therapy, and immunotherapy. MSC reside mainly in bone marrow but have also been isolated from various sites other than the bone marrow including adipose tissue, connective tissues, umbilical cord blood and peripheral blood. MSC exhibit tropism for sites of tissue damage as well as the tumor microenvironment. Such extravasation of MSC from blood vessels into target tissues and tumors is controlled by chemokine receptors and adhesion molecules expressed on the cell surface of MSC and is also dependent on a multitude of signals ranging such as growth factors and chemokines secreted by target cells. In this study, we evaluated the effects of proinflammatory mediators on the immunological properties, cytokines/chemokines production, adhesion properties, migration potential and chemokine receptor gene expression using MSC cell line model. We demonstrated that MSC under basal unstimulated conditions have an immunosuppressive effect on T-cell proliferation, express immunomodulatory mediators and a wide range of chemokine receptors, and are able to migrate in response to different chemokines as well as tumor stroma. MSC secrete also several chemokines and cytokines, express particular adhesion molecules on their surface and are able to adhere to other cells or cell components. Moreover, we demonstrated that these functions and properties can be modulated by inflammatory mediators and thereby, extending current understanding on the possible effect of inflammatory signals on MSC functions and providing a firm basis for defining the migratory itinerary of MSC.

Keywords: mesenchymal stromal cells (MSCs), inflammatory signals

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Emerging Patterns of Local Progenitors in the Vascular Wall: Potential Implications in Health and Diseases

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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: Vascular wall, EPCs, HPCs, stem cells, pericytes

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Amniotic fluid derived stem cells contribute mesenchymal components to reconstituted mammary ducts, but fail to participate in epithelial differentiation

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The mammary gland is uniquely suited to study stem cell functions and communication with the microenvironment. Mammary gland regeneration can be achieved with the transfer of exogenously provided stem cells present in ductal fragments or dispersed epithelial cells into cleared fat pads. It is also possible to gauge exogenously added stem cells from other tissue compartments for their potential to contribute to mammary gland regeneration. We addressed the question whether amniotic fluid derived stem cells (AFS) can participate in the reconstitution of mammary tissue. AFS are multipotent foetal cells derived from embryonic and extra-embryonic tissues present in amniotic fluid which can differentiate into various cell lineages after stimulation with particular growth and differentiation factors. We derived murine AFS using a two-stage culture model and the expression of embryonic and mesenchymal stem cell markers was confirmed by RT-PCR, FACS analysis and immunocytochemistry. The majority of AFS appear fibroblastoid, are rapidly proliferating and co-express keratin and vimentin confirming their epithelial nature. The AFS were marked in order to distinguish them from recipient host cells. We performed cleared fat pad transplantation experiments in order to assess the capacity of AFS to adopt a mammary epithelial like phenotype in response to the intrinsic stimuli and growth conditions provided by the microenvironment. Transplantation of AFS in the absence of mammary epithelial cells caused the differentiation of AFS into connective tissue with increased matrix deposition and into adipocytes. When the AFS were co-transplanted with mammary epithelial cells ductal structures developed surrounded by a ring-like structure contributed by AFS. The AFS expressed myoepithelial markers but failed to integrate into the myoepithelial layer of the ductal structures. Furthermore, AFS contributed to wound healing and homed to other organs. In conclusion we could show that the signals provided by the mammary microenvironment override the epithelial nature of the transplanted AFS and triggered a mesenchymal differentiation program.

Keywords: Amniotic fluid stem cells, mammary gland regeneration

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Characterization of human testis biopsies and isolation of spermatogonial stem cells

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Objectives: It has recently been demonstrated that spermatogonial stem cell (SSC) lines, which display characteristics of multipotency, can be generated from human testis biopsies. These cells express embryonic stem cell markers and can differentiate to the three primary germ layers. However, their potential to differentiate into mature germ cells in vitro has not been shown. Here, we aim to a) generate and characterize the potential of human SSC lines for germ cell development and b) establish conditions in which this procedure can be translated into a clinical setting, allowing the infertility treatment of patients with Sertoli cell only (SCO) Syndrome and Klinefelter patients (47,XXY).
Methods: hMGSCs derived from a patient with normal spermatogenesis were analyzed using pluripotency assays. A specific gene expression panel was established allowing the quantification of pluripotency and germ cell markers in human testis. Testis biopsies from SCO and Klinefelter patient were obtained in the frame of fertility treatment procedures (TESE) after written informed consent and ethical approval.
Results: We report the generation of a putative hMGSC line from a patient with normal spermatogenesis. The cells express distinct markers of pluripotency and form embryoid bodies that contain derivatives of all three germ layers, demonstrating that human SSCs are multipotent. To investigate if testicular biopsies from SCO and Klinefelter patients can be used as a source for SSCs, their expression patterns were analyzed using quantitative real time PCR. The biopsies (n=8) showed expression of pluripotency and germ cell markers thereby indicating the presence of early germ cell populations.
Comments and Conclusion: Our data show that human SSCs are multipotent and that testicular biopsies of infertile men can be used as a potential source for SSCs. Future efforts focusing on the differentiation of human MGSCs into mature germ cells in vitro would offer new alternatives in male fertility preservation.

Keywords: Spermatogonial stem cells, hMGSCs, fertility preservation

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Enhanced Proliferation and Differentiation Capacity of Human Mesenchymal Stem Cells Cultured with Basement-Membrane Extracellular Matrix Proteins

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Purpose : In-vitro cultured mesenchymal stem cells are characterized by a short proliferative life span, an increasing loss of proliferation capacity and progressive reduction of differentiation potential. Laminin-1, laminin-5, collagen IV, and fibronectin are important constituents of the basement membrane extracellular matrix (bmECM), managing a variety of cellular activities including cell attachment and motility. We hypothesized improved proliferation and differentiation capacity of MSCs cultured in the presense of bmECM proteins.
Results : Cumulative cell numbers of MSCs, expanded in the presense of bmECM proteins, were 2500 fold higher after 50 days of cultivation compared to that of plastic expanded MSCs. Increase of proliferation capacity was correlated with enhanced content of rapidly self-renewing cells (RS-cells) in the MSC population (24±3% under standard conditions to 36±5%) and elevated expression of multipotentiality indicating markers (for example STRO-1: 34.8% to 77.4%). Furthermore, co-cultivated MSCs retained their multipotential differentiation capacity throughout many culture passages. For example, alkaline phosphatase activity as an indicator for osteblastic differentiation was 2.5 fold higher upregulated after 28 days of differentiation ($p<0.001$).
Conclusions : The results suggest that expansion of bone-marrow derived MSCs in the presense of bmECM-proteins is a powerful approach for the generation of huge numbers of MSCs, which can be induced into specific tissue lineages, and therefore be a valuable tool for tissue engineering and regenerative medicine in the near future.

Keywords: Mesenchymal Stem Cells, Extracellular Matrix, Expansion, Differentiation, Laminin-1, Laminin-5

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Cultured Early Passage Mesenchymal Stem Cells Could Exhibit Abnormalous Genotype, Karyotype and Metabolism

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Introduction: Accumulating clinical and preclinical evidence indicate mesenchymal stem cells (MSCs) are a promising cell source for regenerative medical therapies. However, unpredicted immortalization, spontaneous transformation and tumorigenic potential from long term cultured MSCs have been reported in human and mouse. As we have previously described, rat MSCs isolated from young donors could undergo transformation in early passage culture. In the current study, we aimed to investigate growth, senescence and tumorigenesis potential of abnormal MSCs (AMSCs) population. In addition, we characterized their metabolism, karyotype and level of proliferative and cancer related mRNA.
Materials and Methods: MSCs were isolated from bone marrow of Lewis rats according to standard protocols and cultured under standard conditions. Cell growth and senescence were evaluated among normal MSCs and AMSCs by sequential counting and senescence β-Galactosidase staining, respectively. For tumorigenesis assay 1×106 or 3×106 cells were injected intravenously or subcutaneously into BALB/c athymic (nude) male mice. After one and four months, organs including heart, lungs, liver, kidneys and brain were analyzed. Karyotype and genotype of growing cells was assessed by Giemsa staining and Quantitative Real Time PCR, respectively. Representative parameters for cell metabolism were measured by cell culture on chip and the innovative Bionas®2500 analyzing system.
Results: AMSCs revealed aberrant cell proliferation and show low β-galactosidase activity. They exhibited a very wide range of chromosome numbers, from 49 up to 221 with an average of 90. The mRNA level of c-myc, p53, cyclin D1, cdk1 and cdk4 was significantly increased. AMSCs showed distinctly higher metabolic activities. Their respiration and acidification rates were particularly elevated. Moreover, AMSCs showed a low impedance signal, a parameter that indicates cell adhesion or cell density. Within four months after cell injection to immunodeficient mouse there was no indication of tissue abnormalities or tumor formation.
Conclusion: The importance of this study is related to the clinical trial that administrates human MSCs to the patients with ischemic cardiovascular diseases for regenerating cardiac functions. Extensive investigations are needed to ensure the safe usage of MSCs in regenerative therapies. In our study, rat AMSCs showed aberrant karyotype and metabolism, up-regulation of oncogene c-myc and lost of cell cycle control. Although AMSCs did not induce sarcoma in nude mouse after four months the cells could hide undesirable effects after transplantation. Systematic characterization, standardized, rigorously tested protocols and quality control will be highly recommendable before MSCs in clinical application.

Keywords: Mesenchymal Stem Cells(MSCs), Metabolism, senescence, tumorigenesis

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Localized SDF-1alpha gene release mediated by collagen substrate induces CD117+ stem cell homing

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Objective: Stromal cell-derived factor-1α (SDF-1α) mediated mobilization and homing of stem cells showed promising potential in stem cell based tissue engineering and regenerative medicine. However local and sustained release of SDF-1α is indispensable for stem cell mediated regenerative process due to its short half-life under inflammatory conditions. In this study, a gene activated collagen substrate (GAC) was formed via assembly of plasmid encoding SDF-1α into a collagen substrate to create a microenvironment favoring stem cell homing. Local release of SDF-1α from the transfected cells on GAC and its effect on CD117+ stem cell homing were investigated in a mouse hindlimb model.
Methods: Non-viral poly-ethyleneimine (25kDa PEI)/DNA complexes were mixed with rat tail collagen solution to form the GAC. Optimization of GAC was carried out based on collagen effects on the PEI/DNA complexes, viability and luciferase expression of COS7 cells on GAC. CD117+ stem cells homing in response to SDF-1α local expression from transfected cells on GAC were investigated in a flow chamber in vitro and in a mouse hindlimb model in vivo. The gene expression, migration of CD117+ stem cells and the induced inflammation were investigated with immunostaining, reverse transcription polymerase chain reaction (RT-PCR) and H&E staining.
Results: The optimized parameters for GAC were DNA dosage 10μg/cm2, molar ratio of PEI nitrogen in primary amine to DNA phosphate (N/P ratio) 4 and mass ratio of collagen to DNA (C/D ratio) 1.0. It kept cell viability above 75% and transfection efficiency around 5.8×105 RLU/mg protein. GAC allowed the sustained gene release up to 60 days. GAC mediated SDF-1α gene release induced migration and homing of CD117+ stem cells in vitro and in vivo significantly, and the inflammation of GAC reduced significantly two weeks after transplantation. GAC is a promising stem cell based therapeutic strategy for regenerative medicine.

Keywords: CD117 cell, Collagen, gene delivery

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Heterogeneous growth factor expression by human mesenchymal stromal cells in an in vitro study of tissue damage

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The mechanism responsible for the beneficial outcome after transplantation of mesenchymal stromal cells (MSCs) into traumatic spinal cord lesions or into infarcted myocardial tissues still remains unclear. Three possible mechanisms have been proposed: (1) transplanted MSCs differentiate into cells of the injured tissue (i.e. neurons or cardiomyocytes); (2) grafted MSCs fuse with cells of the lesioned tissue; and (3) donor MSCs express and release growth factors which promote cell survival and proliferation of stem/progenitor cells residing in the damaged organ or tissue. The hypothesis that MSCs differentiate into either neurons or cardimyocytes has aroused great interest in research. However, these transdifferentiation results need to be judged carefully, since this appears to be a rare event. A similarly rare event seems to be cell fusion. In the present investigation, we have studied the expression of a number of growth factors by human MSCs exposed to different environments. Samples of human MSCs from 3 different donors were co-incubated with tissue homogenate from normal rat spinal cord or heart as well as homogenate obtained from spinal cord and heart that had been lesioned 7 days earlier. The expression profile of several growth factors was investigated using quantitative RT-PCR with human specific primers. The basal growth factor expression profiles in response to the co-incubation with tissue homogenates were widely heterogeneous. These data are of significant importance since cells intended for transplantation obtained from different patients may not have predictable repair promoting potential in spinal cord injuries as well as in myocardial infarction.

Keywords: mesenchymal stromal cells, growth factors

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Extracellular Matrix Molecules of the Neural Stem Cell Niche: A Gene Trap Approach to Elucidate their Signaling Role

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Neural stem cells (NSC) reside in a niche that is rich in extracellular matrix (ECM) molecules. The ECM glycoprotein Tenascin-C (Tnc) occurs in more than 25 isoforms and represents a major constituent of the privileged NSC milieu. To understand its role for NSCs, the induction gene trap technology was successfully applied to mouse embryonic NSCs and a library of more than 500 NSC-lines with independent gene trap vector integrations was established. Our pilot screen identified Sam68 as a target of Tnc-signaling in NSCs. The Tnc mediated down-regulation of Sam68, which we found expressed at low levels in the niche along with Tnc, was independently confirmed on the protein level. Sam68 is a multifunctional RNA binding protein and its potential significance for cultured NSCs was studied by overexpression. Increased Sam68 levels caused a marked reduction in NSC cell proliferation. In addition, Sam68 is a signal-dependent regulator of alternative splicing and its overexpression selectively increased the larger Tnc isoforms, while a mutated phosphorylation-deficient Sam68 variant did not. This emphasizes the importance of Sam68 for NSC biology and implicates an instructive rather than a purely permissive role for Tnc in the neural stem cell niche. Thus, our work has revealed novel pathways through which ECM molecules act that are potentially relevant for translational stem cell research.

Keywords: brain, differentiation, proliferation, splicing

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The Stem Cell Niche Connectome: Mapping Transcription Factors and Signalling Networks in Normal and Pathological Conditions

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Our realisation is that the stem-cell niche or microenvironment plays more than just a supporting role in tumour progression represented a radical shift in the study of stem-cell biology. Briefly, in the bone marrow, osteoblasts and endothelial cell constitutes the major cellular components contributing to the endosteal and vascular niches that serve as the microenvironment for maintaining haematopoietic stem cells (HSCs). Recent data suggest that mesenchymal stem cells (MSCs) themselves may reside in niches that are in close proximity to the HSC niche and co-regulate activities each other. Though number of niches has been identified up-to-date by using novel technologies, however, the niche is likely comprised of many different niche constituents including osteoblasts, endothelial cells, fibroblasts and or cancer-associated fibroblasts (CAFs), adipocytes, macrophages as well as vascular cells. Other cells known to participate in the generation of niche or its regulation are osteoclasts and the HSCs and progenitor cells themselves, which is only now becoming appreciated. In addition to the influence of stem-cell niche on HSCs, growing reports are also highlighting their extended role in development of leukaemia stem-cells (LSCs) as well as cancer stem-cells (CSCs). Although the profound influence of the stroma on tumorogenesis is now widely accepted, a full understanding of the cross talk between stem cells and the niche (which translates into changes in transcriptional networks and chromatin modifications), microenvironment role for self-renewal, stem-cell fate as well as role on heterogeneity of embryonic and adult stem cells etc., remains a nascent field. In this scenario, there is an urgency to bring together the scattered information and map the picture of transcriptional factors and cell signalling networks in different niches in one place. In order to accomplish this goal, we are trying to apply an interdisciplinary approach to address and documenting molecular networks that involves in normal and in disease conditions, which is including small nonprotein-coding RNAs (such as microRNA pathway that differentiate LSCs from CSCs, for an example), signalling by morphogens (which is known to be important for the self-renewal of many adult stem cell types), growth-factors (IGF1R is expressed exclusively in the hESCs, for an example) as well as functional assays (in order to distinguish normal HSCs from cells that have undergone some degree of neoplastic progression), novel methodologies (imaging technology), etc., in order to exploit stem-cell niche for potential therapeutic benefits. Hope our advanced 'connectome- review' initiative will eventually help us to increase survival rates and improve quality of life for survivors of breast cancer, leukaemia and brain cancers.

Keywords: Stem Cell Niche, Mapping, Transcription Factors, Signalling Networks, Chromatin modifications

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Conditional mutagenesis of Histone Methyltransferase Mll2 in Neural Stem Cells

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Post-translational modifications of histone tails act as epigenetic signals that maintain gene expression patterns during cellular development. Polycomb group (PcG) and trithorax group (trxG) methyltransferases counteract each other by repressing or preserving gene expression. Mll2 (Wbp7) is a mammalian member of the trxG involved in maintaining gene expression by methylating lysine 4 of histone 3. The Constitutive Knock-out of Mll2 in mice is embryonic lethal before E11.5 due to widespread developmental defects (Glaser et al., 2006) and Mll2 knock-out embryonic stem (ES) cells display defects in the differentiation towards all three germ layers (Lubitz et al., 2007). As differentiation to mature neurons (ectoderm) was most severely impaired, this study focuses on the role of Mll2 in mouse Neural Stem (NS) cells. NS cells were generated either from ES cells or mouse fetal forebrain. Both sources produced comparable results. Due to the embryonic lethality, a conditional knock-out of Mll2 was performed using the 4-OH-tamoxifen inducible Cre/loxP site-specific recombinase system. We found that self- renewal of NS cells was not affected by Mll2 knock-out, but Mll2 deficient cells exhibited a proliferative defect that was due to increased apoptosis. During in vitro differentiation most of the Mll2 deficient cells died. Surviving cells generated only a few astrocytes and no mature neurons. Thus, the differentiation deficiency of Mll2 knock-out cells seems to be a general effect that is not restricted to ES cells. These findings indicate a redundancy of Mll2 for maintaining gene expression patterns in NS cells. However, Mll2 seems to be essential for differentiation events when histone modification patterns have to be altered. This may be due to specific interactions with other chromatin modifications or the transcription machinery serving as signals for de novo methylation of histone tails.

Keywords: neural stem cells, differentiation, Mll2, Wbp7

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Reprogrammed Stem Cells and Tissue Engineering

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Reprogramming of somatic cells by transcription factors represents a particular appealing approach of generating pluripotent stem cells, referred to as induced pluripotent stem (iPS) cells (Takahashi & Yamanaka, Cell 2006; Kim et al., Nature 2008; Kim et al., Cell 2009). The somatic origin of iPS cells will allow for an autologous, patient-specific transplantation for tissue repair and/or regeneration. In tissue engineering, stem cell transplantation frequently requires their use in biohybrid systems, where cells are seeded in 3D biomaterial scaffolds with specific mechanical and chemical properties to ensure a spatial structure of the substituted tissue. Such biohybrids provide a microenvironment to allow for an efficient cell engraftment and survival in the recipient. Biomaterial scaffolds can influence stem cell survival, proliferation and differentiation both, in positive and negative ways. We have analyzed the interaction of a variety of stem cell types with a large panel of biomaterials (Neuss et al., Biomaterials 29, 302-313, 2008). To this end, we used a grid-based platform for the systematic assessment of stem cell-biomaterial interactions and (i) established a Biomaterial Bank of known and newly synthesized polymers and (ii) tested embryonic and adult stem cell types. Parameters such as cell morphology, adhesion, proliferation and differentiation, vitality, cytotoxicity and apoptosis were systematically analyzed. This has allowed to suggest and advise for or against specific stem cell-biomaterial combinations for tissue engineering. Recently, we included 2-Factor iPS cells in our biomaterial test platform. We have started to investigate whether biomaterials can (i) support a feeder-free growth of reprogrammed stem cells by maintaining pluripotency and (ii) direct the differentiation of reprogrammed stem cells towards cardiomyocytes and epithelial cells (smart biomaterials).

Keywords: biomaterials, scaffolds, tissue engineering

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Proliferation of EGFR+ neural precursor cells and lineage tracing in the postnatal SVZ

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In the adult mammalian brain neurogenesis continues after birth and proliferation of neural precursor cells (NPCs) is strictly regulated by regional and temporal cues. In the adult subventricular zone (SVZ) neurogenesis is regulated by the coordinated proliferation of two different stem cell types: astroglial stem cells expressing glial acidic fibrillary protein (GFAP), and rapidly dividing transit-amplifying precursors (TAPs). Previous studies have shown that fibroblast growth factor (FGF)-2 and epidermal growth factor (EGF) represent the main mitogenic signals for NPCs supporting their proliferation in vitro. At early stages of embryonic development NPCs respond only to FGF-2 whereas from late development onwards they acquire the ability to respond also to EGF. This transition in growth factor responsiveness coincides with an increase in expression of EGF receptor (EGFR). However, the impact of EGFR signaling in vivo is unknown. Here we have investigated the relevance of EGFR expression for NPC proliferation in the SVZ in vitro and in vivo during postnatal development. By using clonal assays to determine stem cell activity in purified populations of SVZ cells we found that cells expressing high levels of EGFR (EGFRhigh cells) displayed characteristics of TAPs and only a subset exhibited antigenic characteristics of astroglial stem cells. Confirming the hypothesis that most are TAPs, cell tracing by lentiviral transduction with a construct expressing eGFP under the control of the EGFR promoter in neonatal organotypic brain slices revealed that within two days the majority of eGFP/EGFR+ cells had exited the cell cycle and differentiated into a progenitor displaying intermediate antigenic and functional properties between TAPs and neuroblasts. Furthermore, after three days in culture the majority of the cells have underwent differentiation into neuroblasts. Additionally, analysis of EGFR expressing cells in brain slices from perfused adult mice revealed that all cells undergoing mitosis in the adult SVZ express EGFR including GFAP immunopositive cells. Thus, EGFR expression strictly correlated with stem cell activity and proliferation in vitro and in vivo and provides a useful tool for lineage tracing.

Keywords: neural stem cells, postnatal SVZ, EGFR, TAPs, lineage tracing

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Expression of Pluripotency/Multipotency Markers in Human Corneal, Limbal and Cultivated Limbal Epithelium

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Purpose: Corneal epithelial stem cells (SC) are located in the limbal zone. Recent studies have shown that several adult stem cells express pluripotency markers and can under certain conditions differentiate into a wide range of cell types. The objective of this study was to analyze the expression of key molecules needed for the maintenance of pluripotency in the human corneal and limbal epithelium, as well as in cultivated limbal epithelium.
Methods: Four samples of human corneal, limbal and on intact amniotic membrane cultivated limbal epithelium were analyzed. The expression of corneal epithelial differentiation markers (K3, K12, K15 and Cx43), putative limbal SC markers (ABCG2, p63), and molecules expressed in pluripotent/multipotent SCs (NANOG, OCT4 (POU5F1), SOX2, KLF4, KIT, NESTIN, PAX6) was examined using Real-Time PCR.
Results: The expression of all studied markers was detected in all samples of limbal and corneal epithelium. Limbal epithelium showed a significantly ($p<0.05$) higher expression of K15, ABCG2, OCT4, SOX2, and NESTIN, but a significantly lower expression of K3 in comparison to central corneal epithelium. Limbal epithelial cells did not express K3 after cultivation on amniotic membrane and showed a significantly lower expression of differentiation markers K12 and Cx43 in comparison to both corneal and limbal epithelium. The expression of all pluripotency markers was detectable in cultivated limbal epithelial cells, even though the expression was significantly lower than in native limbal epithelium.
Conclusion: The human limbal epithelial cells express genes that are associated with the maintenance of pluripotency/multipotency and preserve an expression of these genes even after cultivation on amniotic membrane. Limbal epithelial stem cells may have a higher differentiation potential than previously presumed.

Keywords: Limbal stem cells, Pluripotency/Multipotency Markers, adult stem cells, cornea, eye

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Cardiomyogenic potential of adipose tissue-derived stem cells

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Adipose tissue-derived stem cells (ADSCs) are autologous stem cells that are easily accessible in large numbers by uncomplicated surgery. Therefore they are an optimal source of stem cells for regenerative therapies. The aim of our study was to elucidate the potential of ADSCs to differentiate into functional cardiomyocytes. Methods: ADSCs were isolated from gonadal and inguinal white adipose tissue of six month old C57BL/6 mice. After three weeks of culture in DMEM 10% FCS (basic media) the expression of cardiac markers was analysed by reverse transcription (RT)-PCR and real-time PCR as well as antibody staining. Results: In order to test the stem cell character of the isolated ADSCs we successfully differentiated them into the adipogenic and osteogenic lineage. After three weeks of culture in basic medium the ADSCs expressed the cardiac transcription factors Nkx2.5 and Gata4, which were not present in freshly isolated ADSCs. Furthermore the cardiac marker Nppa, a downstream target of Nkx2.5 was expressed upon cultivation. The cardiac pacemaker ion channel HCN2 was also detected by RT-PCR. Cardiac contractile apparatus genes, like cTnI, Mlc2v and Mybpc3, were present in the ADSCs after 3 weeks of culture. In contrast the cardiac myosin heavy chain isoforms Myh6 or Myh7 were not expressed. The RT-PCR results were confirmed by positive antibody staining against Nkx2.5 and Gata4 in the nuclei of about 5% of the cultured ADSCs. Although specific genes of the contractile apparatus were expressed no striated structure was detected by antibody staining against sarcomeric myosin heavy chain. As Nkx2.5 is an early, highly specific cardiac transcription factor we further analysed its expression in the course of ADSCs culture. Expression was first detected after 3 days of culture and was 6 times up regulated till day 21. By implementing low density culture we could separate different cell clusters according to their morphology and identify a special subpopulation giving rise to the Nkx2.5 expression. Conclusion: From these results we conclude that ADSCs do not spontaneously differentiate into functional cardiomyocytes but can give rise to cardiac progenitors expressing a number of cardiac markers. However, differentiation appears to become blocked before development of a sarcomeric structure. Therefore in a future approach we want to overcome this differentiation blockade by lentiviral expression of a set of cardiac transcription factors in the ADSCs. We suggest that our cardiac primed ADSCs are a good cell source for a direct reprogramming strategy as it was already shown by others that the cell type – developmental origin of the cells used is important for this method to be successful.

Keywords: Adipose tissue-derived stem cells, cardiomyocyte, direct reprogramming

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Conditional immortalization to study lineage commitment of bone marrow mesenchymal stromal cells

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Bone marrow mesenchymal stromal cells (BM MSCs) can differentiate in vitro into cells of mesodermal origin (bone, fat, cartilage, muscle). The population of BM MSCs is heterogenic and probably contains progenitor cells committed to different lineages. However mouse BM MSCs have a low proliferative capacity that makes analysis at the single-cell level complicated. To overcome this problem, we developed a strategy for reversible, ligand regulated cellular immortalization using SV40 Large T-antigen. We have modified the tetracycline-regulated system so that its application is highly predictable. Conditional immortalization was achieved by introduction of the tetracycline-regulated system and tet-controlled SV40 large T-Antigen into mouse ES cells. Double stable ES clones expressed T-Antigen only upon treatment with Dexamethasone (Dex) and Doxycycline (Dox). Subsequently, transgenic mice were generated which can be used to isolate and easily expand the cells in culture upon induction of the T-Antigen. BM MSCs from adult transgenic mice multiplied rapidly upon the induction of T-antigen without signs of senescence for more than 30 passages. The cells expressed similar pattern of surface markers known for BM MSCs (positive for CD9, CD44, CD90 and negative for CD34, CD45, CD117). Moreover the cells were able to differentiate in vitro into adipocytic, chondrocytic and osteocytic lineages. Thus conditional immortalization did not affect those properties of BM MSCs. Two subsequent rounds of cellular cloning were performed by manual dilutions. The expanded single-cell derived clones were explored by in vitro differentiation assays into the osteocytic, adipocytic and chondrocytic lineages. Multipotential subpopulations with high ability for the three types of differentiation as well as clones restricted to two lineages (osteo- and chondrogenic, osteo- and adipo-, adipo- and chondrogenic clones) or one lineage (osteogenic, adipogenic clones) were found. Taken together, these results revealed that we established for the first time homogeneous subpopulations of progenitors and stem cells committed to different lineages. The expression profile of different clones is currently being analysed. Conditional immortalization provides a powerful tool to study mechanisms of differentiation and lineage commitment.

Keywords: MSC, bone marrow, conditional immortalization, differentiation

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TNF Exposure of Adipose Tissue-Derived Stem Cells Induces Cytokine Secretion and Incomplete Osteogenic Differentiation In Vitro

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Introduction: Throughout whole life, bone is continually renewed: It is degraded by osteoclasts and rebuilt by osteoblasts. In chronic inflammatory diseases, this fine-balanced system may be disturbed. In arthritis for example, increased osteoclast maturation rates promote osteoporosis. While osteoclasts derive from the hematopoietic lineage, osteoblasts develop from mesenchymal stem cells (MSC). Adipose tissue-derived stem cells (ASC) have MSC character, as they are able to differentiate e.g. along the adipogenic, chondrogenic and osteogenic lineage and express typical MSC surface markers. Thus, they are promising candidates for regenerative medicine and tissue engineering.
This work aims at elucidating the impact of chronic inflammatory conditions on osteogenic differentiation of ASC, simulated in vitro by long-term treatment with a low concentration of tumor necrosis factor alpha (TNF).
Results and Discussion: By flow cytometry, we confirmed that ASC express both TNF receptor 1 and 2 on their surface. Expression level of both receptors was not influenced by TNF treatment. To exclude the possibility that other TNF receptor bearing cells are present in culture and transmit TNF induced signals to the ASC, we proved absence of monocytes, macrophages and endothelial cells, according to CD markers 14, 68 and 31, respectively. As a response to pro-inflammatory stimulation with TNF, ASC secrete markedly increased levels of IL-6, IL-8, IL-15, MCP-1, G-CSF, RANTES and VEGF. These seven molecules represent a functionally consistent pattern in innate as wells as in adaptive immunity. Cell numbers of TNF treated ASC cultured for up to five weeks increased exponentially and considerably faster as in the unstimulated control cells, an effect that was found even more pronounced after osteogenic stimulation. As TNF is known to induce osteogenic differentiation of hematopoietic stem cells, we investigated expression of early osteogenic marker gene alkaline phosphatase (ALPL) by ASC. Within three weeks of analysis, ALPL expression increased continuously, but not as strong as in the osteogenically stimulated ASC. When looking for the activity of alkaline phosphatase protein, the increase of the gene‘s expression was not recovered. Only the osteogenically stimulated ASC exhibited significantly increased alkaline phosphatase activity. To go for a later marker of osteogenic differentiation, we investigated calcification of the ASC‘s extracellular matrix and found a similar result: Calcification occurred only following osteogenic stimulation for at least three weeks, but never after TNF treatment. Hence, TNF treatment of ASC strongly stimulates cytokine secretion, whereas osteogenic differentiation was induced

only at the transcript level, but not at the protein level.

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Keywords: Adipose Tissue-Derived Stem Cells, Inflammation, Osteogenic Differentiation

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Characterization of mesenchymal stem cells from umbilical cord and bone marrow under tissue-specific conditions of skin: Analysis of ectodermal and mesodermal differentiation

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Human mesenchymal stem cells (hMSC) are able to differentiate into mature cells of various mesodermal tissues. Recent studies even report that they might give rise to cells of ectodermal origin. Our study explores the ectodermal and mesodermal differentiation potential of hMSC from bone marrow (BM) and umbilical cord (UC) in a skin-specific microenvironment simulated in vitro. HMSC were cultured air-exposed on dermal equivalents (DEs) consisting of collagen types I and III with dermal fibroblasts and subjected to conditions similar to those used for tissue engineering of skin. To determine whether MSC maintain their multipotency when cultured in contact to the DE on one side and air-exposure on the other side, hMSC were also differentiated into adipocytes and osteoblasts. Under these conditions hMSC adapted to the epidermis-specific conditions without losing their multipotency. However, despite their viability and three-dimensional epidermis-like growth pattern, hMSC from BM showed a persistent expression of mesenchymal but not of epithelial markers. In contrast, hMSC from UC express cytokeratins after isolation and showed a persistent but faint cytokeratin expression and a distinct expression of the mesenchymal marker vimentin after cultivation under epidermis-like conditions. Interestingly, after osteogenic differentiation on dermal equivalents, hMSC from UC were negativ for epithelial markers. This lost of cytokeratin expression during cultivation on DEs under osteogenic differentiation indicates a shift towards mesenchymal phenotypes. Consistently, hMSC from both origins revealed the capacity of matrix remodelling under the varying differentiation conditions. Accordingly, electron microscopy and immunohistochemical analyses demonstrated that hMSC cultured in contact to the dermal matrix adopt myofibroblastic phenotype and function as well as express matrix-metalloproteinases and extracellular matrix proteins like laminin. These results indicate that hMSC in general might contribute to wound healing processes by matrix reorganization but not by differentiation into keratinocytes.

Keywords: mesenchymal stem cells, three-dimensional organotypic culture system, tissue-engineering of skin, matrix reorganization

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MicroRNA expression profiles point to networks of proteins involved in neuronal differentiation of unrestricted somatic stem cells from human cord blood

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Unrestricted somatic stem cells (USSC) from placental cord blood constitute fetal stem cells with the capability to differentiate into neuronal lineages. MicroRNAs (miRNAs) represent a population of small RNA molecules of approximately 22 nucleotides in size that inhibit protein synthesis by either mRNA degradation or inhibition of translation and have been shown to play important roles in development and differentiation. To investigate the impact of miRNAs on neuronal differentiation of USSC, three different USSC lines (SA5/03, SA5/73 and SA8/25) were neuronally differentiated in-vitro over a period of 14 days (SA5/03, SA5/73) and 28 days (SA8/25) using XXL-Medium and analyzed for miRNA expression profiles using the TaqMan qPCR Megaplex Assay (Applied Biosystems). Compared to native USSC a common set of 18 miRNAs was found downregulated in SA5/03 and SA5/73 at day 14 and in SA8/25 at day 28 of neuronal differentiation. Among the most prominently downregulated miRNAs were miR-138, miR-335, miR-18a, miR-20a, and miR-218. Interestingly, no miRNA was found significantly upregulated in SA5/03 and SA5/73, whereas in SA8/25, miR-483 was found strongly increased already after 14 days. To analyze the putative biological impact of this pattern of 18 downregulated miRNAs, we performed intensive target gene predictions using five different algorithms combined with in-silico pathway analyses. Upon downregulation of the corresponding miRNA, target genes might display increased expression. Target predictions for downregulated miRNAs revealed a large amount of proteins important for neuronal differentiation and neuronal signal transmission with many being predicted for up to 11 miRNAs and/or that even with different algorithms. Among the most prominently predicted targets, we found NEUROD1, NBEA, CRIM-1, NRP1, NEUROG1, all involved in neuronal differentiation. To further understand the underlying regulatory networks, predicted proteins were fed into the DAVID Pathway database and analyzed for allocation to biological pathways important in neuronal development. Most prominently, predictions within the Axon Guidance pathway were found, focusing on central ligand-receptor pairs. Furthermore, core components of TGF-ß signalling, Wnt-signalling and Long-term potentiation pathways were predicted. In addition, many other proteins involved in these pathways were predicted as putative targets. Overall, these target predictions and pathway analyses point to a network of potentially miRNA-influenced proteins involved in neuronal differentiation and signalling of USSC. Furthermore, we began to investigate changes within the transcriptome during USSC

neuronal differentiation and found downregulation of PTBP-1, an important nonneuronal splice-regulator and moderate upregulation of certain ß-chains of voltage gated sodium channels. Expression analysis of predicted core proteins will be performed to underline the participation of a miRNA network in neuronal USSC differentiation.

Keywords: Epigenetic microRNA, stem cells, neuronal differentiation

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Hepatocyte Growth Factor-Mediated Attraction of Mesenchymal Stem Cells for Apoptotic Tissues

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Human bone marrow-derived mesenchymal stem cells (MSC) hold promise in regenerative medicine due to their potential to form various mature cell types. Furthermore, they are applied as immunomodulating therapeutics because of their broad immunosuppressive activities. In this study, we have investigated the influence of apoptotic and necrotic cell death, thus, distinct types of tissue damage, on MSC migration. Concordant with an increased overall motility, MSC migrated towards apoptotic, but not necrotic neural and cardiac cells in an under-agarose chemotaxis assay. Total numbers of migrating cells as well as migration rates were higher for MSC from early passages. Upon induction of apoptosis, hepatocyte growth factor (HGF) was expressed in apoptotic but not in vital or necrotic neural and cardiac cells. MSC, in contrast, revealed expression of the HGF-receptor c-MET. Blocking HGF bioactivity in the chemotaxis assay resulted in significant reduction of migration rates. Moreover, recombinant HGF attracted MSC in a dose dependent manner. Thus, the HGF/c-Met axis plays an important role in attracting MSC to apoptotic but not to necrotic tissues.

Keywords: Mesenchymal Stem Cells, Migration, Regeneration

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Telomere length erosion and karyotype instability in long-term cultured mouse neurosphere cells

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Neural stem and progenitor cells (NSPCs) are isolated from forebrain of 14.5 days old mouse embryos. In selective conditions NSPCs form sphere-like structures – neurospheres cells. Extended cultures of progenitors and stem cells in vitro are considered as a source for transplantation therapies to replace CNS cells. Therefore, the possible impact of long-term culturing on overall cellular stability is of major importance in adjusting optimal culture conditions of NSPCs aimed for transplantation therapy. Therefore, distinct aspects of ageing in long-term NSPCs culture were tested by comprehensive analysis. Potential alterations that might occur due to ageing were monitored within 1-16 weeks of culturing. Long-term NSPCs displayed elevated self-renewal and proliferative capacity at the expense of a declined ability to differentiate into neurons. Little is known about molecular mechanisms underlying changes in self-renewal and differentiation in long-term culture. The high mobility group A (Hmga2) gene plays the key role in positive regulation of self-renewal. Hmga2 represses the Ink4-Arf locus and consequently p16Ink4a and p19ARF expression. We found a several fold upregulation of the Hmga2 gene, while p16Ink4a and p19ARF gene expression remained unchanged. Our results raise possibility that HMG2a promotes self renewal via negative regulation of p16Ink4a and p19ARF as described recently. Unlike our data, upregulated p53 was reported as overlapping factor with p16Ink4a and p19ARF in negative control of self renewal. Initially, tremendous structural and numerous chromosomal aberrations were observed upon 16 weeks of culturing such as gain of chromosome 1. Chromosomal gain in human ESCs was considered a 'culture adaptation' which gives selective advantage to some cell variants. In accordance with the prior studies on ESCs, we suggest that elevated self-renewal and higher proliferation rate give the aneuploid NSPCs subpopulation a selective advantage over other diploid NSPCs subpopulations in long-term culture. Nevertheless, culture adaptation may cause lack of stem cells' functions in transplantation therapies. Moreover, cellular transformation occurs due to tremendously impaired chromosomal integrity and may induce tumor lesions upon transplantation since genomic instability is the initiation of a transformed state and progression into tumorigenic stem cells in vivo. Telomere length measurements revealed a significant decrease after 4 weeks and stabilized up to 16 weeks of culturing. Generally, erosion of telomere length is assumed as an indication of potential replicative senescence that may lead to proliferation arrest and cells depletion. Oppositely, our findings strongly

suggest that telomere shortening may not reflect on self renewal or proliferation. Telomeres may reach a critical length that, nevertheless, allows cells to proliferate. Consequently, we found unaltered cell cycle kinetics over 20 weeks. Genetic instability and diminished differentiation capacity seem to be a consequence of long term culturing implying potential transformation. Therefore, analysis of self renewal, differentiation capacity, telomere length, tumor suppression genes and chromosomal instability should have high priority in the monitoring and quality control of neural stem cell cultures prior to transplantation. Our data could be instructive in future development of NSPCs culture conditions for transplantation therapies.

Keywords: long-term culture, telomere errosion, aberrations, ageing

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Hepatic differentiation of human cord blood-derived unrestricted somatic stem cells (USSC)

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Unrestricted somatic stem cells (USSC) are a defined CD45- human somatic stem cell population derived from cord blood with extensive expansion potential and the ability to differentiate into osteoblasts, chondroblasts, adipocytes, cardiac and neural cells as well as hepatic cells in vivo and in vitro. Due to their ability of in vitro hepatic differentiation diverse USSC lines (n=20) 1-5 fold could be differentiated into albumin secreting and glycogen storing cells with hepatic-like morphology. Representative expression of hepatic differentiation marker HNF4α and endodermal transcription factor SOX17 could be detected from d14 of diffenentiation in 18 of assayed celllines. Expression of AFP, FOXA2 and HNF1α could not be significantly detected. Since delta-like1 (DLK) seems to distiguish different USSC lines and indicates their broader expansion and differentiation potential (unpublished data) we analysed DLK expressing USSC lines for their endodermal differentiation potential. DLK has privotal roles in hepatoblast fate decision and is a useful marker for hepatoblasts. In order to differentiate DLK+ USSC a predifferetiation followed by a two step differentiation-protocol under serum free conditions was applied. Preinduction was carried out using ActivinA, FGF4, BMP2 and EGF for 6 days. During the following first period of 14 days the induction media contained hepatocyte growth factor (HGF), dexamethason, insulin, transferrin and sodium selenit (ITS). For the subsequent 14 days HGF was replaced by oncostatin M (OSM). 4 DLK+ USSC lines were differentiated in independent experiments. Differentiation was analysed by RT-PCR, IHC and individual assays for the functionality of differentiated cells. Preinduction led to an SOX17, GSC, FOXA2, GATA4 and HSA expressing cell population on day6 and expression of HNF4α could be observed on day8 by RT-PCR. AFP expression was detected between day2 and day14 of differentiation. Expression of SOX17, FOXA2, HSA and AFP on day 6 of differentiation was confirmed by IHC. After 5 weeks of culture we were able to show that assayed USSC differentiated into hepatic-like cells under influence of HGF and OSM. These differentiated cells expressed at day28 of differentiation hepatic markers like HSA, GYS2 and low content of CYP3A4 as well as hepatic transcription factors such as HNF1α, HNF4α and SOX17. Confirmation of nuclear presence of HNF1α and HNF4α could be demonstrated by IHC. To investigate the functional hepatic features of differentiated USSC, cells were analysed by individual assays: Glyconeogenesis and glycogen-storage correlating with the expression of glycogensynthase2 could be detected by periodic acid-shiff reaction (PAS) and albumin secretion by ELISA. Urea synthesis as the privileged function of hepatocytes could not be significantly detected.

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Keywords: cord blood, USSC, hepatic differentiation

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Cord Blood Expansion on Mesenchymal Stromal Cells: Proliferation and Self-Renewal of Primitive Immunophenotype are Regulated by Cellular Aging and Adhesion Proteins

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The use of umbilical cord blood for transplantation is limited by the available number of hematopoietic stem and progenitor cells (HPC). Co-culture with mesenchymal stromal cells (MSC) from bone marrow might provide a suitable cellular microenvironment for in vitro expansion of HPC. This necessitates that co-culture increases both, proliferation and self-renewal of primitive cells. For that reason, we have simultaneously analyzed the impact of co-culture with MSC on cell division history and differentiation of HPC during expansion using the fluorescent dye carboxyfluorescein diacetate N-succinimidyl ester (CFSE). Co-culture with MSC significantly enhanced the HPC proliferation rate, especially in the initial more primitive CD34+ / CD38- sorted cell fraction. With MSC co-culture, expression of the stem cell markers CD34 and CD133 lasted for more cell divisions than without. Furthermore, upregulation of the differentiation markers CD45 (common lymphocyte antigen), CD13 (myeloid marker) and CD56 (NCAM, expressed on NK-cells) was delayed to a higher number of cell divisions. CD38 expression was transiently upregulated and diminished in the fast dividing cell fraction. In addition, the total amount of primitive HPC after co-culture was higher than after culture expansion without MSC, especially in the slow dividing cell fraction. Thus, co-culture with MSC increased HPC proliferation and self-renewal of cells with a primitive CD34+ / CD133+ / CD38- immunophenotype. For further investigation of proteins that are possibly involved in cell-cell adhesion and signalling, we performed siRNA knockdown of CD44, integrin beta 1 (ITGB1), N-cadherin, cadherin-11, jagged-1, vascular cell adhesion molecule (VACM-1) and mitogen activated protein kinase 1 (MAPK1) in MSC. MAPK1 was also inhibited by use of PD098059. siRNA knockdown of N-cadherin or VCAM-1 increased the percentage of cells in the slow dividing fraction. Knockdown of ITGB1 or CD44 impaired their differentiation and hence, more HPC remain CD34+ / CD38-. This suggests that ITGB1 and CD44 take part in regulation of self-renewal of a primitive immunophenotype. siRNA treatment or inhibition of MAPK1 impaired proliferation and differentiation of HPC, but not maintenance of long term culture-initiating cells (LTC-IC). Thus, modulation of specific proteins in MSC feeder might increase their hematopoiesis supportive potential. Replicative senescence of MSC could also provide a relevant aspect for HPC expansion. To address this issue, we used MSC feeder of different passages of the same donor samples. MSC of early passages maintained CD34 expression in HPC for more cell divisions, whereas MSC of higher passages further enhanced the HPC proliferation rate. In conclusion, co-culture with MSC enhances the

HPC proliferation and also supports the self-renewal of HPC with a primitive immunophenotype. The use of early passages of MSC and modification of specific adhesion proteins might further enhance cord blood expansion on MSC.

Keywords: mesenchymal stromal cells, hematopoietic stem cells, stem cell niche, cord blood, co-culture, immunophenotype, proliferation, replicative senescence, adhesion proteins

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Adult palate as a novel source of neural-crest related stem cells

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Somatic neural and neural crest stem cells are promising sources for cellular therapy of several neurodegenerative diseases. However, due to practical considerations, e.g. inadequate accessibility (e.g. CNS) or severe bacterial (periodontal ligament) and fungal contamination (skin biopsies) of the source material, the application of neural crest stem cells from mammalian sources is strictly limited. As a potential novel source of adult stem cells, we investigated the secondary palate, a tissue which is highly regenerative and heavily innervated and develops embryonally under direct contribution of neural crest cells. Here we describe for the first time the presence of Nestin-positive Neural Crest related stem cells adjacent to Meissner Corpuscles and Merkel cell-neurite complexes within palatal rugae (rugae palatinae) of adult Wistar rats. After isolation the cells were cultivated in the presence of EGF and FGF-2 under serum-free conditions resulting in large amounts of Neurosphere-like cell clusters. These palatal Neural-Crest related Stem Cells (pNC-SCs) showed a population doubling time of ca. 65h. Complementarily, a limited dilution assay revealed a sphere-forming frequency of 1.8%. Using FACS analysis and chromosome counting, we confirmed chromosomal stability of the cultivated secondary neurospheres. We used immunocytochemical techniques and RT-PCR to assess the expression profile of pNC-SCs. In addition to Neural Stem Cell Markers as Nestin, Notch1 and Sox2 we detected Neural Crest specific gene products such as p75, Twist, Sox9 and Slug. Additionally, pNC-SCs isolated from palate of adult rats expressed three of the factors needed for reprogramming: c-Myc, Klf4 and Sox2. Adherence to poly-D-lysine and laminin, growth factor deprivation and retinoic acid treatment led to neuronal differentiation. Furthermore pNC-SCs were able to differentiate into glial lineage if cultivated in presence of 10% FCS without growth factors. Finally, we investigated the potential expression of stemness markers within human palate. We detected high levels of stem cell markers Nestin and CD133 and the transcription factors Sox2, Oct3/4, Klf4 and c-Myc. Taken together our study suggests that Neural Crest Stem Cells derived from mammalian palate could be an alternative, easily accessible source of multipotent or after reprogramming pluripotent adult stem cells for clinical and research use.

Keywords: Neural Crest, novel human stem cell source, tissue stem cells

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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 multi-cellular 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. At the example of the hematopoietic system, a few transcription factors, e.g. HoxB4, AML1/Runx1, SCL/Tal1, Meis1, have been identified, taking part in the decision process self-renewal versus differentiation of primitive hematopoietic stem cells. While loss of function of these transcription factors is generally associated with defects in the development of the hematopoietic system, the aberrant expression is 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 strategy 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: HSC, Transcription factor

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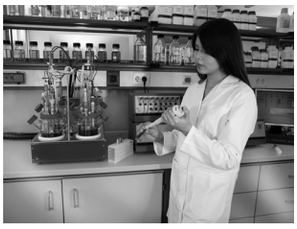

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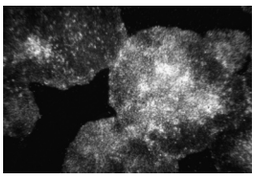

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