Proceedings of German Society for Stem Cell Research - 2010 (PGSSCR)
(5th Annual Meeting)
Invited speakers – IS1

Cardiovascular derivatives of pluripotent stem cells in cardiac repair, drug discovery and disease
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Heart and vascular endothelial cells from human pluripotent stem cells are of interest for applications in cell therapy and cardiovascular disease. Differentiation protocols are now sufficiently refined that production of cardiomyocytes, endothelial- and smooth muscle cells is fairly efficient and reproducible. Genetically marked hESCs have been produced in which expression of eGFP is ubiquitous or under lineage specific control. We have used various tagged hESC-lines to trace cardiomyocytes following transplantation into a mouse heart after myocardial infarction and to select cardiovascular progenitors as a source of the different cardiovascular cell types. Although cardiomyocytes survive for months in the mouse heart and cause early improvements in function, these are not sustained. Cardiovascular progenitors from hESC and hiPSC however, present more immediate applications in drug discovery, toxicity and cardiovascular disease modelling. Results of these studies, in particular drug responses of hESC-derived cardiomyocytes and a hiPSC model for vascular disease, will be shown.
Proceedings of German Society for Stem Cell Research - 2010 (PGSSCR)
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Invited speakers – IS2

Prominin-1/CD133 and the cell biology of (cancer) stem cells
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Our group investigates the cell biological basis underlying the proliferation versus differentiation of neural stem and progenitors cells. In this context, we have been studying the symmetric versus asymmetric division of neuroepithelial (NE) and radial glial (RG) cells during embryonic development of the mouse brain. We have focused on the fate of apical constituents, notably prominin-1/CD133, during cytokinesis of these highly polarized cells. Prominin-1 is a pentaspan membrane protein that is specifically retained in plasma membrane protrusions. This specific subcellular localization reflects prominin's association with a novel, cholesterol-based membrane microdomain. We recently found that prominin-1-bearing extracellular membrane particles are present in the ventricular fluid of the embryonic mouse brain. These particles are derived from microvilli and the primary cilium of interphase NE/RG cells, as well as from the midbody of symmetrically dividing NE/RG cells. The release of these membrane particles appears to be associated with the switch of these neural stem cells from proliferation to differentiation, and to differ between cancer and physiological stem cells.

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Proceedings of German Society for Stem Cell Research - 2010 (PGSSCR)
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Invited speakers – IS2

Transcription factor - induced pluripotency
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Reprogramming of mouse and human somatic cells to induced pluripotent stem (iPS) cells has been possible with retroviral expression of the pluripotency-associated transcription factors Oct4, Sox2, Nanog and Lin28 as well as Klf4 and c-Myc. Considering that ectopic expression of some of these factors can cause tumors and expression vectors themselves are potentially mutagenic due to insertion into the host genome, reducing the number of transgenes during the generation of iPS cells is a crucial step towards their clinical applicability as an alternative pluripotent stem cell source.

The initial studies were undertaken inducing pluripotency in mouse and human fibroblasts. Based on the hypothesis, that in inducing pluripotency the number of reprogramming factors can be reduced when using somatic cells that endogenously express appropriate levels of the reprogramming transcription factors, we were able to demonstrate, that mouse and human neural stem cells (NSC), that express Sox2, Klf4 and c-Myc, can be reprogrammed to iPS cells by retroviral expression of Oct4 alone. Human Oct4 iPS cells resemble human embryonic stem cells morphologically, at the molecular level by gene expression microarrays, global miRNA and epigenetic profiling, as well as in their in vitro and in vivo differentiation potential.

We will describe the current status of our strategies to induce pluripotency from mouse and human somatic cells with a minimal number of defined transcription factors and small molecules for patient-specific disease modeling and cell therapeutic applications.
Factors such as regulatory proteins, mRNA and miRNA are transported by stem cell specific microparticles that are released into the cerebrospinal fluid following traumatic brain injury (TBI)

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Intercellular exchange of protein and RNA-containing microparticles is an increasingly important mode of cell-cell communication. Microparticles, which include exosomes, micro-vesicles, apoptotic bodies and apoptotic microparticles, are small (50 - 400 nm in diameter), membranous vesicles that can contain DNA, RNA, miRNA, intracellular proteins and express extracellular surface markers from the parental cells.

Our primary aim is to elucidate the role of microparticles in damage induced neurogenesis following traumatic brain injury in vivo.

Methods:

Cerebrospinal fluid was serially centrifuged. Pelleted microparticles were analysed by FACS, electron microscopy, RT-PCR and mass spectroscopy. Quantification of miRNA was performed by Small RNA Chip (Agilent Tech.). Specific miRNAs were identified by miRNA microarrays (Affymetrix).

Results:

We verified the presence of cerebral cell derived microparticles in cerebrospinal fluid of healthy volunteers and patients of TBI by FACS analysis and electron microscopy. Microparticles contained RNA, miRNA and protein. RNA was not susceptible to RNase digestion underlining RNA to be contained in protective vesicles. Approximately 50\% of the RNA content was demonstrated to be pre- and miRNA. A variety of specific miRNA species indicated in the regulation of regenerative processes were identified. Furthermore, RT-PCR analysis revealed cerebral microparticles to carry transcripts for MAP2, \( \beta \)-actin and CaMKII. MAP2 mRNA was not detected in cerebral microparticles of healthy controls. The presence of \( \beta \)-actin and CaMKII in microparticles might indicate neuronal RNA granules to be also packed into microparticles. Proteomic analysis indicated microparticles to carry proteins involved in motility (ankyrin-3), membrane regulation (stabilin-2, synaptotagmin), neuronal development (growth factor independence-1), as well as typical neuronal receptors (glutamate receptor). Within the first three days following traumatic brain injury 14\% \pm 3\% of microparticles in...
cerebrospinal fluid was CD133+, indicating a substantial fraction of microparticles to be derived from neuronal precursor cells.

**Summary:**

We were able to demonstrate for the first time the release of stem cell derived microparticles into cerebrospinal fluid. The detection of specific RNA transcripts and pre/miRNA underlines their predicted role for microparticles in cell-cell communication. To our knowledge there are no reports to date on the role of microparticles in the cerebral environment. We are presently studying the role of microparticle in endogenous neurogenesis following cerebral injury.
In a previous study we have identified a novel murine gene (GenBank X83587) which was found to be the mouse homologue of human Co-REST, a co-repressor to the neuronal silencer REST (repressor element-1 silencing transcription factor). Co-REST mediates the transcriptional repression of REST-responsive genes by recruiting histone deacetylases and other chromatin modifying enzymes to the repression site. In recent studies a REST-independent role of Co-REST in gene repression has been documented. A major role of the REST/Co-REST repressor complex is to restrict neuron-specific gene expression in extra-neural tissue. Accordingly, the occurrence of REST and Co-REST in neural tissue at early developmental stages has raised much interest in a potential role of this repressor complex in the timing of neural stem cell maturation.

Although expression of Co-REST has been related to long-term gene silencing mechanisms, its role in stem cell biology is still debatable. In a previous study we have shown that, in contrast to REST, Co-REST expression persists in the developing mouse CNS beyond newborn stage and - at low levels - throughout adulthood.

In this project we have been focussing on the stage-specific expression of Co-REST in the mammalian olfactory epithelium (OE), which is known to harbour a pool of highly dynamic neurogenic cells (neural stem/precursor cells, NSCs/NPCs). Using immunohistochemistry and in situ hybridization we have determined the spatio-temporal expression pattern of Co-REST in the developing and adult mouse OE. Co-expression studies have been performed using antibodies against stem cell-related transcription factors, NPC-associated cytoplasmic proteins, and neuron-specific terminal differentiation markers.

Here we have shown that the stem cell-associated proteins Sox2 and Nestin are co-localized in the young OE, showing prominent staining in basal and apical regions. Interestingly, expression of Co-REST is detectable exclusively in Sox2/Nestin-free areas. On the other hand, a considerable overlap of Co-REST and Doublecortin expression is visible in the OE from E10.5 on, which provides evidence to suggest that Co-REST may be considered a reliable marker for early neurons or neural precursor cells but does not indicate stem cell quality of OE cells.
Isolation and functional characterization of alpha-smooth muscle actin expressing cardiomyocytes from embryonic stem cells

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Early mammalian heart development is characterized by transient expression of alpha-smooth muscle actin (Acta2). To date, cardiomyocytes expressing Acta2 in the early stages of in vivo development have not been characterized. To functionally characterize Acta2-expressing cardiomyocytes, we used a transgenic ES cell line expressing both the puromycin acetyl transferase (Pac) and enhanced green fluorescent protein (EGFP) cassettes under the control of the Acta2 promoter. The onset of Acta2 expression occurred in parallel with the appearance of beating areas, indicating the formation of cardiomyocytes. Antibiotic selection resulted in a high yield of cardiomyocytes and smooth muscle cells. The green fluorescent beating areas stained positively for multiple cardiomyocyte markers. Quantitative real-time PCR and semiquantitative RT-PCR analyses of beating cell clusters indicated a maximal expression level of the cardiac-specific genes. Comparative electrophysiological analysis including fetal and alpha-MHC-expressing ES cell-derived cardiomyocyte controls showed that Acta2-positive cardiomyocytes contained pacemaker-, atrial- and ventricular-like phenotypes. Interestingly, the proportion of ventricular-like cells was much higher in the Acta2-positive cardiomyocytes population than in control alpha-MHC-expressing cardiomyocytes (75 % and 12 %, respectively). The findings of the present study provide a novel approach for the identification and enrichment of Acta2-positive cardiomyocytes, especially of the ventricular phenotype under in vitro conditions.
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Differentiation – O21

Transcriptom analysis of cardiovascular progenitors
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Aims:

Generation of cardiomyocytes from pluripotent stem cells provides a future perspective to overcome the complications of heart transplantation including in particular the shortage of donor organs. Thereby, stem cell therapy will require the availability of various cardiac cell types. Whereas early cardiovascular precursors appear to be important for novel approaches such as reseeding decellularized hearts, direct cell transplantation may require specified terminally differentiated cells. Our previous work demonstrated that MesP1 represents a master regulator sufficient to induce cardiovasculogenesis in pluripotent cells. Yet, a profound comprehension of the developmental processes as well as the molecular background is fundamental in order to specifically generate these cell types.

Methods and Results:

To gain further insights into MesP1 driven cardiovasculogenesis we initially compared global mRNA expression patterns of MesP1 overexpressing cells with control cells relying on the Affymetrix platform. The resulting data yielded a number of factors known to be involved in early mesodermal development confirming the importance of MesP1 during the earliest cardiovasculogenic events. In an extended experimental setting we generated cell clones bearing the cDNA encoding a deleted CD4 surface marker under control of the native mesp1 promoter. We subsequently followed the time course of endogenous MesP1 and mesp1 promoter driven CD4 expression. In western blotting and FACS analyses we compiled eight-hour-time curves over 4 days during differentiation. The results showed a high correlation between the appearance of endogenous MesP1 and CD4.

Based on CD4 expression magnetic cell sorting (MACS) of MesP1 positive cells will be utilized for deep sequencing (RNA-Seq) and transplantation approaches.
Sex-Chromosomal verified differentiation from human glandular stem cells to cardiomyocyte-like Cells in co-culture with human myocardial biopsies

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Background:

Human adult pancreatic (parotic) stem cells were brought in a co-culture with human myocardium. The origin of the resulting troponin-I-positive cardiomyocyte-like cells was considered as unclear. Were they derived from adult stem cells or the added myocardium? To clarify their origin, sex-chromosomal analyses were to be performed.

Material and Methods:

Male adult stem cells were harvested from pancreatic (parotic) tissue of patients undergoing operative procedures due to pancreatic (parotic) but not malignant diseases of female patients (n=6). The cells were selected, cultured, and passaged. Simultaneously with a troponin-I-staining, a Fluorescence In Situ Hybridization (FISH) is performed to evaluate the X and Y chromosome signals present in each cell. Human myocardial biopsies for co-cultures were taken from female patients.

Results:

We could show by simultaneously applied immunocytochemistry for troponin-I and FISH that human adult stem cells from pancreas and parotis with a positive immunocytochemistry for troponin-I differentiated into cardiomyocyte-like cells which were male (XY) likewise the applied glandular stem cells.

Conclusion:

The differentiation of human adult pancreatic and parotic stem cells enhanced by a cardiomyocyte coculture is reliably proven now. These glandular stem cells might become a clinically relevant autologous source of regenerative tissue for the repair of irreversible damaged myocardium.
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Differentiation – O28

Bone morphogenetic protein 4 role on in vitro differentiation of primordial germ cells from mouse embryonic stem cells.
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Background:
The purpose of this study was to determined optimal dose of BMP4 and its time period on differentiation Promordial Germ Cells (PGCs) from mouse Embryonic Stem Cells (mESCs) in vitro.

Methods:
To differentiation of PGCs, EBs from mouse ES cells were cultured in concentrations 0, 5, 10 and 50 ng/ml BMP4 at the different time interval. Viability of PGCs was assayed by MTT. Also, germ cell markers Oct-4, Stella and Mvh were analyzed by flow cytometry, Immunocytochemistry, electrophoresis gel and Real Time PCR.

Results:
BMP4 at 10 ng/ml concentrations for day 4 had the best on the viability. The data of flowcytometry demonstrated most of Mvh+ cells was observed in D4B10. Immunocytochemistry of EBs in D2B5, D2B10, D4B5 and D4B10 groups identified cells that are positive to Mvh. The result of Real time PCR was illustrated that expression of gene Oct-4 in the control groups was high and it gradually decreased with adding concentrations 5 ng/ml and 10 ng/ml of BMP4 on days 2nd, 4th and 6th. The maximum expression of Stella was observed in D4B10 group. However, the expression of Stella with BMP4 concentration of 50 ng/ml on the day 2nd, 4th and 6th considerably were decreased. The expression of Mvh with BMP4 concentrations of 0 ng/ml and 50 ng/ml was none. However, the expression of Mvh were increased in D2B5, D2B10, D4B5 and D4B10 groups (not significantly).

Conclusions:
The results propose that concentrations of 10 ng/ml BMP4 at days 4th had the optimal effects on differentiation of PGCs to mESCs.

Key words: BMP4, Mouse Embryonic Stem Cells, Primordial Germ Cells, Mvh+ Cells
Efficient generation of hepatic-like cells from multipotent adult mouse germline stem cells using OP9 co-culturing
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Previously, we reported the establishment of multipotent adult germline stem cells (maGSCs) from mouse testis. Similar to mouse embryonic stem cells (ESCs), these cells are able to self-renew and differentiate into derivatives of all three germ layers. These properties make maGSCs a potential cell source for the treatment of disease such as diabetes or liver cirrhosis. In this study, we describe the efficient generation of maGSC-derived hepatic-like cells by two differentiation systems using maGSCs and ESCs. Induction into the hepatic lineage through embryoid body formation resulted in a gradually decrease of undifferentiated Oct3/4- and Nanog-positiv cells during differentiation in contrast to an increase of endoderm-and hepatocyte-specific markers. To receive a higher amount of hepatic progenitors and mature hepatocytes we established a co-culture differentiation protocol using OP9 stromal cells with activin A. By reverse transcription polymerase chain reaction and immunofluorescence we show that OP9 co-culture, and furthermore activin A treatment increased and accelerated the expression of endodermal and liver-specific genes and proteins such as SOX17, HNF4, AFP, AAT, TTR, and ALB. Flow cytometry analysis resulted in 51% AFP-, 61% DLK-1-, and 26% ALB-positive maGSCs-derived hepatic like cells at late differentiation stages. Furthermore, these maGSC-derived hepatic-like cells successfully demonstrated in vitro functions associated with mature hepatocytes including albumin-and urea-secretion, glycogen storage, uptake of acetylated low density lipoprotein (LDL), and uptake and release of indocyanine green (ICG). Taken together, these data show that maGSCs show similar endodermal differentiation capabilities as ESCs and make them to a potential autologous and alternative source for pluripotent stem cells in regenerative medicine.
Generation of Flk1+ cells from Oct4-reprogrammed spermatogonial stem cells
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Organ regeneration with stem cells requires a delicate balance between the loss of donor cell pluripotency (so that teratoma formation is avoided) and retention of donor cell proliferative capacity prior to terminal differentiation (so that a sufficient number of target cells are generate to effect a therapeutic benefit). The aim of this study is to generate proliferating cardiovascular progenitors (Flk1+ cells) from pluripotent stem cells derived from spermatogonial stem cells (SSCs) of double transgenic mice (MHC-neo/MHC-EGFP) and to investigate their heart regeneration potential. We have generated four SSC lines with MHC-Neo-EGFP+ background from adult male mice. The SSC culture can be expanded over one year in vitro in the presence of glial cell line-derived neutrotrophic factor (GDNF) required for the maintenance of SSC self-renewal. The established SSC culture show typical SSC morphology and express SSC-specific markers like DAZL, VASA and GFR1a at both mRNA and protein levels. Furthermore, these SSCs from the MHC-Neo-EGFP+ mice were reprogrammed into pluripotent stem cells by overexpression of Oct4 alone. The reprogrammed cells showed similar characteristics as multipotent adult germline stem cells and are positive for pluripotency markers such as Oct4, Nanog, Sox2 and SSEA-1. They were able to spontaneously differentiate into cells of all embryonic germ layers in vitro by using the hanging drop method. The GFP+ beating cardiomyocytes can be selected by G418 treatment. After transplantation of these cells in SCID-beige mice, teratomas were detected 6 weeks later. In addition, we established a protocol to induce these cells to differentiate into Flk1+ cardiovascular progenitors. After coculture of these cells (n = 30000 per 10 cm dish) with OP9 cells for 6 days, about 25-35% of the cells were positive for Flk1. This efficiency is comparable to those derived from multipotent adult germline stem cells. For investigating the regenerative potential of these cells we used a myocardial infarction mouse model. We transplanted 50000 Flk1+ cells per mouse in an infarcted heart. At different time points after cell transplantation (2 days, 2 weeks, 4 weeks and 8 weeks) we took the hearts out and analysed them to identify the Flk1+ cells and their derivatives. Our first preliminary data showed that the transplanted cells survived in the infarcted heart. Investigations on the integration of the cells into the host myocardium and on the functional improvement by echocardiography are in progress.
Establishment of an extended in vitro assay to detect the most primitive human hematopoietic cells at a clonal level

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Hematopoietic stem cells (HSCs) can either self renew or give rise to multipotent hematopoietic progenitor cells (HPCs). As the most primitive hematopoietic cells both cell types obtain the ability to reconstitute all mature blood cell types. According to the classical model of hematopoiesis, these HPCs next become either restricted to the lymphoid or to the myeloid lineage, the common lymphoid (CLPs) or common myeloid progenitor (CMPs) cells. Via more restricted HPCs, CLPs then finally give rise to T, B and natural killer (NK) cells as well as to a subset of dendritic cells (DCs), while CMPs create macrophages, granulocytes, megakaryocytes and erythrocytes as well as a second subtype of DCs.

Due to the recent characterization of HPCs containing partial myeloid and partial lymphoid developmental potentials the classical model of hematopoiesis has been challenged. A bundle of new data suggests the existence of additional or alternative developmental pathways.

Aiming to set up a functional in vitro read out system for the most primitive human hematopoietic cells and having the classical model of hematopoiesis in mind, we originally developed a so called myeloid-lymphoid initiating-cell (ML-IC) assay. Within this assay individual candidates for very primitive human hematopoietic cells are initially expanded on a murine stromal feeder cell layer. To test for their lymphoid or myeloid lineage developmental capacity, offspring are then either transferred into an assay allowing NK cell or granulocyte and macrophage development, respectively. Before discovering the novel HPC types, deposited primitive hematopoietic cells whose offspring gave rise to NK cells as well as granulocytes and macrophages were retrospectively claimed as primitive cells containing the potential to develop into all hematopoietic cell types. However taking the new findings into account, this conclusion can not be justified anymore. Since we are still interested in an in vitro read out system allowing the functional detection of the most primitive human hematopoietic cells and also to define novel hematopoietic routes, we decided to extend the ML-IC assay for additional lineage read outs including T cell, B cell, megakaryocyte and erythrocyte development. Our experimental strategy to extend this assay and our ongoing results will be presented.
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Differentiation – P15

Enhancement of cardiac differentiation of human pluripotent stem cells by overexpression of multiple transcription factors
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Cardiomyocytes derived from human pluripotent stem cells possess a high potential for regenerative treatment of cardiovascular diseases as well as for drug screening and drug safety tests in pharmaceutical industry. However, one of the main obstacles to overcome towards in vitro-derived cardiomyocytes is the low efficiency of cardiac differentiation in human induced pluripotent stem (hiPS) or human embryonic stem (hES) cells. Several approaches have been taken to improve this situation including the overexpression of multiple transcription factors. Recent publications provided evidence that transcription factors known to be essential for cardiac development could indeed increase cardiomyogenesis upon constitutive (over)expression in mouse ES cells[1] or preformed mouse mesoderm[2]. Our aim is to systematically test and assess combinations of transgenic transcription factors for their cardiogenic potential in human pluripotent stem cells.

To this end, an optimized electroporation protocol was used to simultaneously transfec up to three different non-viral expression plasmids into hiPS or hES cells. Combining this technique with antibiotic selection allowed the establishment of transgenic cell clones. Various clones with different transgene expression levels, as tested by qPCR, were then differentiated and assessed for cardiac markers. In a second approach, hiPS cells were transiently transfected by electroporation, seeded as a monolayer and directly subjected to differentiation in a fully defined serum-free medium.

[1] David et al., Cardiovascular Research 2009, 84: 263-72
[2] Takeuchi & Bruneau, Nature 2009, 459: 708-71
Proceedings of German Society for Stem Cell Research - 2010 (PGSSCR)
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Differentiation – P22

Cell-context specific target genes of Notch signaling: regulation by chromatin marks?
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Notch signaling is a pivotal mechanism throughout development, controlling cell lineage decisions in a cell-context dependent manner. Notch ligand binding, located on a neighboring cell, results in the consecutive cleavage of the Notch receptor by an ADAM protease and $\gamma$-secretase, followed by the release of the intracellular domain of Notch (NIC), which translocates then into the nucleus and converts the RBP-J repressor into an activator of Notch target genes. Although the cell-context dependency has been proposed for a long time, little is known about which target genes are affected in which cell type and how the cell-context dependency is mediated mechanistically. Here, we employed a Tamoxifen-inducible Notch1 system in embryonic stem cells at different stages of mesodermal differentiation combined with genome-wide transcriptome analysis and qPCR verification. Furthermore, using this system we were able to screen for potential direct targets using the protein synthesis inhibitor cycloheximide. Our results revealed that a large number of the identified target genes are unique for the cell type and vary highly in dependence of other signals. Among the potential direct Notch1 targets are, in addition to the known direct Notch1 target genes of the Hes and Hey family, key regulatory transcription factors such as Sox9, Pax6, Runx1, Myf5 and Id proteins that are critically involved in lineage decisions [1]. Activation of target genes by Notch1 is associated with the presence of activating histone modifications and strongly correlates with bivalent domains at RBP-J sites of promoters of these genes. Furthermore, increasing H3K4 methylation by the LSD1 inhibitor Tranylcypromine (PCPA) positively influences Notch1 induction, and alters the chromatin status of the promoters of Notch1 target genes. Thus, we propose that the cell-context dependency of Notch target genes is determined by a chromatin configuration poised for activation and that Notch signaling determines lineage decisions and expansion of stem cells by directly activating both, key lineage specific transcription factors and their repressors (Ids and Hes/Hey proteins).

Literature:
1. Meier-Stiegen, F., Schwanbeck, R., Bernoth, K. Martini, S. et al., PLoS One, 2010, accepted
Generation and selection of hepatocyte-like cells from transgenic murine embryonic stem cells at different differentiation stages

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Potential therapeutic applications of embryonic stem cell (ESC)-derived hepatocytes as an alternative to the transplantation of the whole liver or of primary hepatocytes is currently confirmed to be a highly topic issue. Crucial points thereby are high-yield generation and efficient selection of cells displaying hepatocyte-specific features from differentiating ESC cultures. In our study, we establish culture conditions for a large-scale production of hepatocyte-like cells from ESCs in a murine in-vitro model, using stable transgenic ESC clones which contain the live eGFP reporter gene and a puromycin resistance cassette, both driven by a common alpha-fetoprotein (AFP) gene promoter. From that clones, suspension- and adherent cultures with the activated AFP promoter were generated, which derived from the endoderm-like cell population. These cultures are supposed to express eGFP fluorescence as well as to acquire puromycin resistance, thus allowing for live monitoring of both differentiation and antibiotic selection.

We found out that a 95%-yield of eGFP-positive cells and of their localization pattern in the outer rim of EBs. Features of cells selected from the EB culture by puromycin application were shown to be dependent on the time point of the initial drug application. Thus, the puromycin treatment of EBs on their relatively early developmental stage yielded highly eGFP-expressing cell clusters that, after their plating onto adhesive substrates, developed to a culture comprising both highly proliferative hepatocyte-precursor-like- and advanced differentiated hepatocyte-like cells. Exposure of a long-term adherent culture to the drug resulted rather in selection of a significantly more mature cell population displaying a low proliferation capacity. We also investigate the effect of different adhesive substrates on the developmental pattern of adherent cultures.

Hence, alteration of culture conditions allows for differentiation of murine ESCs toward cells of the hepatic lineage and their selection on defined developmental stages, which is of interest in regard to toxicology screening- and transplantation models.
Cytarabine stimulates neuronal markers in hESC derived embryoid bodies
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Cytosine arabinoside (cytarabine) is an antimetabolic agent, which damages DNA when the cells cycle holds in S phase. Cytarabine is mainly used in the treatment of AML and lymphomas. It has been reported to cause severe neuropsychiatric side effects in human patients under going therapy for AML. Cytarabine is also reported to be used in the study of nervous system. In the present study, we have investigated the effect of cytarabine on human embryonic stem cells (hESCs) derived randomly differentiated embryoid bodies. Cytarabine, being a developmental toxicant, exhibited cytotoxicity at high concentrations, while sub-lethal concentration of cytarabine was found to stimulate neuronal markers such as PAX6 and MAP2. In addition, low concentration of cytarabine also caused inhibition of developmental markers. To find the significant toxicity markers microarray analysis was performed with Illumina HumanHT-12 v3 Expression BeadChip, revealing cytarabine promoted axon guidance and other pathways known to be involved in the regulation of neurogenesis in hESC embryoid bodies. In addition 124 upregulated and 416 down regulated significant genes (p ≤ 0.05) were observed in cytarabine treated EBs compared with untreated. The significantly (p ≤ 0.01) upregulated annotations for cytarabine treatment in the GO biological process relate to neuronal differentiation like neural morphogenesis, axonogenesis, nervous system development etc. In our work, it is shown that cytarabine can stimulate pathways and markers for neuronal differentiation and inhibit developmental markers.
Differentiation of human embryonic stem cells into multiple lineages- a toxicogenomic platform for developmental toxicity.

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The differentiation of human embryonic stem cells (hESC) into multiple organotypic cells has great potential in developmental biology and regenerative medicine. In recent years mouse embryonic stem cells were used for in vitro toxicity studies, in particular developmental toxicity studies. We combined hESCs with toxicogenomics for the construction of a developmental toxicity platform. hESCs were randomly differentiated into multiple lineages such as endoderm, ectoderm and mesoderm and challenged with thalidomide at sublethal doses. To identify significant toxicity markers, microarray analysis was performed with Illumina HumanHT-12 v3 Expression BeadChip. We could identify 40 significantly upregulated and 407 significantly downregulated genes (P<0.05) in day 14 Thalidomide treated samples compared to day 14 untreated samples. Among these transcripts, we found that germ line markers expressed on day 14 were down regulated when challenged with thalidomide. Gene ontology enrichment analysis reveals thalidomide treatment downregulated organ development, anatomical structure development, multicellular organismal development and circulatory system development in biological process. Shortlisted developmental markers were further validated with real time quantitative PCR (RT-qPCR) in independent experiments. To validate these markers a similar experiment was carried out with penicillin as a control compound where no changes were observed in the expression level. Further, to study the interspecies differences, a similar experiment was carried out with CGR8 mouse embryonic stem cells. Interestingly we found a battery (Markers shortlisted in hESC study) of markers was significantly repressed with thalidomide at sublethal concentration in RT-qPCR studies. Thus, the multilineage differentiation of pluripotent stem cells combined with transcriptional profiling of developmental markers may be a strong tool for the developmental toxicity platform.
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Differentiation – P34

Implications of chemopreventive agents in differentiation of mouse embryonic stem cell derived embryoid bodies

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Embryonic stem cells (ES) are precursor cells with the ability of pluripotency and self-renewal and bear the capability to differentiate into tissue specialized cells found in three primary germ layers of an embryo (endoderm, mesoderm and ectoderm) in response to appropriate signals. The coax ES cells fate is controlled by both intrinsic regulator and the extracellular environment (niche), which under appropriate conditions in cell culture are apparently spontaneous, which generally are inefficient and leads to heterogeneous population of differentiated and undifferentiated cells that are not useful for cell-based therapy and moreover complicate the biological studies of particular differentiation program. A lot of work has been poured in recent years to improve the differentiation process leading to purified or semi purified cell lineages, most of them generally are controlled by a cocktail of growth factors, signaling molecules and/or genetic manipulation. However these protocols restrict derived cells for basic research as they utilize either xeno confined substitutes or are transgenic, limiting their therapeutic usage. Employment of small molecules gives an advantage for such application for the best reasons as they not only are cell permeable but have been wisely studied tools for modulating complex cellular pathways. In the present study, differentiation potential of murine ES cells under the pressure of chemopreventive agents was studied. Curcumin, EGCG and quercetin are phytocompounds and part of our daily intake in form of many foods or food supplements. The differentiation of ES cell was performed in sub toxic levels. It was found via elaborative gene expression and protein expression data that EGCG cause significant stimulatory effect on cardiomyogenesis and mesoderm in general. Curcumin had a stimulatory role on ectoderm in particular neuroectoderm formation seen via numerous ectoderm gene markers expression studies. Quercetin was cytotoxic and yielded to heterogeneous cell after differentiation. In summary we show the use of small molecules that enhances the induction of differentiation of cardiomyocytes and/or neuroectoderm. Our study helps to explore the effects of chemopreventive drugs in ES cell differentiation to form germ layers.
The role of AMP Kinase on the beta-cell differentiation of mouse embryonic stem cells.
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Background:
Beta-cell differentiation from embryonic stem cells involves complex signaling mechanisms. Nutritional status of the mother was shown to have a profound effect on offspring’s beta-cell numbers, islet cell size, proliferation capacity and islet-insulin content. This prompted us to investigate the role of whole-body energy sensor AMP Kinase on the expression of pancreas specific transcription factors and on beta-cell differentiation of mouse embryonic stem cells.

Materials and methods:
CGR8 mouse embryonic stem cells were used for this study. Chemical activators and inhibitors were used for modulating the AMP Kinase signaling in differentiating embryonic stem cells. RT-PCR experiments were used for monitoring the mRNA expression of several pancreas specific transcription factors (Hnf-6, Mac A, Neutrogena 3, Neuron D1, Foxa2, Pdx-1) during the course of embryonic stem cell differentiation, in the presence and absence of AMP Kinase activators and inhibitors. The relationship between TGF-beta signaling pathway and AMP Kinase signaling was studied using RT-PCR and western blot.

Results:
The results showed that modulating AMP Kinase signaling pathway of mouse embryonic stem cells has a profound influence on the expression of pancreas specific transcription factors. Inhibition of AMP Kinase during the earlier stages of embryonic stem cell differentiation increases the fold expression of pancreas specific transcription factors while activation of AMP Kinase has an opposite effect. Experiments with AMP Kinase activators show that TGF-beta mRNA expression was down-regulated by metformin, an AMP Kinase activator.

Conclusion:
Initial experiments showed that AMP Kinase signaling pathway has a strong effect on the expression of pancreas specific transcription factors in mouse embryonic stem cells. Studying the relationship between AMP Kinase and TGF-beta pathway will help in understanding the interplay of these signaling molecules in the beta-cell differentiation of embryonic stem cells.
Characterization of endothelial cells derived from adult mouse germline-derived pluripotent stem cells

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Present cell-based therapies of ischemic diseases are predominantly relying on autologous endothelial progenitor cells (EPCs). However, their purification from peripheral blood or bone marrow is laborious. Furthermore, patient-derived EPCs are numerically reduced and mostly functionally impaired. Consistently, the efficacy of current EPC-based therapies is low. Therefore, the evaluation of alternative sources of autologous cells suitable to promote adult vascular growth is required. Recently, we have obtained germline-derived pluripotent stem (gPS) cells from adult mouse unipotent germline stem cells. The objective of our study is to reveal whether functional endothelial-like cells (ECs) capable to support angiogenesis and arteriogenesis in ischemic diseases can be derived from this new type of pluripotent stem cells.

Briefly, PECAM-1 (CD31)-positive cells were isolated from single cell suspensions of gPS-derived embryoid bodies (EBs) by fluorescence-activated cell sorting (FACS) and subcultivated on OP9 stromal cells. Subsequently, EC-like colonies were mechanically isolated and expanded on collagen IV-coated cell culture dishes. Using FACS analysis it was demonstrated that the cells expressed the endothelial cell-specific markers PECAM-1, von Willebrand Factor, Tie2, Flk1, and vascular endothelial-cadherin. These results were confirmed by immunofluorescence staining and RT-PCR. The cells were successfully maintained in in-vitro culture for many passages without a significant loss in expression of the endothelial cell markers. Furthermore, the cells were capable of forming capillary-like structures when cultured on Matrigel. Dil-conjugated acetylated low-density lipoprotein uptake confirmed the functionality of the gPS-derived ECs. Their functionality in vivo, i.e. the integration of transplanted gPS cell-derived ECs into growing collateral arteries and the potential augmentation of blood flow recovery by these cells is currently being investigated in a mouse hindlimb ischemia model to reveal the potential of gPS cells to serve as a new source of endothelial cells for therapy of ischemic diseases without being limited by the ethical concerns associated with the use of embryonic stem cells.
Somatic cell reprogramming by transfection with liposomal agents

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The induction of pluripotent stem cells from differentiated, somatic cells by genetic reprogramming with the transcription factors, Oct3, Sox2, c-Myc and Klf4 has given rise to new aspects in stem cell research and gene therapy by enabling the circumvention of legal and ethical issues involved in the use of embryonic stem cells. However, the use of retroviruses as transfection agents also raises questions concerning the application of these reprogrammed cells in gene therapy for patients due to the possible increase in tumor occurrence through viral agents. In the search for an effective, alternative method for transfecting cells, we first attempted to transfect these plasmids with the usage of liposomal transfection agents into the Human Embryonic Kidney cell line, HEK 293. In our experiment, we have incubated a liposomal transfection agent with three commercially available plasmid constructs that contained the transcription factors Oct3, Sox2 and Essrb respectively, whereas Essrb has been reported to up regulate the expression of the genes c-Myc and Klf4. Each of the plasmids transfected is marked with genes coding different fluorescent proteins. After incubation with the liposome-DNA complex, cells that have incorporated the plasmids are detected by fluorescence microscopy as they express the fluorescent marker proteins coded by the plasmids. Our preliminary results show that it is possible to obtain single cells with multicolor fluorescent signals and that this method may prove to be a possible alternative to the use of retroviruses as vectors in reprogramming mature somatic cells to create induced pluripotent stem cells.

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Differentiation – P40

Chondrogenic differentiation of murine and human induced pluripotent stem cells in vitro: challenges and differences in comparison to ES cells
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Induced pluripotent stem (iPS) cells hold great promise for research and potential therapeutic applications but the question whether iPS cells actually represent an equivalent alternative to embryonic stem (ES) cells regarding its use as an in vitro model system of cell differentiation remains to be answered.

Our data shows that there are remarkable differences with respect to the chondrogenic differentiation capacity as well as the expression of pluripotency markers between undifferentiated murine and human iPS and ES cells.

Murine iPS and ES cells were cultured as cell aggregates, the so called embryoid bodies (EBs), by the hanging drop method. Both cell lines could be differentiated into chondrocytes but iPS cells were found to be significantly less efficient. Further, iPS cell aggregations were smaller and less stable compared to ES cell EBs.

The differentiation of human iPS cells is posing a challenge as established methods failed to produce EBs. EB formation by fragmentation of cell colonies was inefficient and aggregates from single cell suspension disaggregated.
Differentiation – P49

Defective chondrogenic differentiation of murine embryonic stem cells treated with RGD-containing peptides

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The interplay between cells and their extracellular matrix (ECM) is of utmost importance in tissues like cartilage where cells are completely surrounded by ECM and cell-cell contacts thus take a minor role. A large number of these interactions are mediated by members of the integrin family. Chondrogenic development has been characterized in detail by using numerous model systems. Of these, murine embryonic stem cells (ESC) are of special importance because they are widely available without ethical concerns and because they render a large portion of animal experiments unnecessary. It has been proven that all physiologic stages of chondrogenic development are adequately mimicked by ESC in vitro. Since we found high expressions of the ECM molecule fibronectin (FN) and its major cellular receptor in cartilage, \( \alpha_5\beta_1 \)-integrin, during early chondrogenic development, namely during the formation of mesenchymal condensations that require active cell migration, we treated murine ESC either with RGD-containing blocking peptides mimicking the cell attachment domain of FN or RGE-containing control peptides to test the importance of FN-\( \alpha_5\beta_1 \)-integrin-interactions. RT-PCR analysis of \( \alpha_5 \) and FN-expression showed significant alterations demonstrating activity of the RGD-containing peptides. As shown by Alcian blue staining, the formation of chondrogenic nodules was significantly reduced although the number of PNA-positive cellular condensations and Collagen II-positive nodules remained unchanged. Confocal laser scanning microscopy did not show morphological differences between cells treated with blocking peptides and cells treated with control peptides. In addition we performed a phosphorylation-sensitive western blot to analyze Focal Adhesion Kinase (FAK)-activity as an integrin downstream signaling target. Taken together our results suggest that interactions between FN and \( \alpha_5\beta_1 \)-integrin play an important role during early chondrogenic differentiation stages of murine ESC that is independent from the formation of mesenchymal condensations.
Induction of chondrogenic differentiation after Notch1 activation is regulated by Sox9

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The Notch pathway is an evolutionary highly conserved intercellular cell communication mechanism, involved in various cell lineage determination processes during embryonic development. Here we show the influence of Notch1 signalling on chondrogenic differentiation via Sox9 as a regulatory protein. We have previously demonstrated, that stages of early chondrogenesis can be recapitulated during ES cell differentiation in vitro. Condensed mesenchymal cells developed into mature chondrocytes. After prolonged cultivation cells showed the phenotype of hypertrophic chondrocytes and at later stages signs of ossification. For studying the influence of Notch signalling on chondrogenic differentiation in vitro, we used murine ES cells carrying a tamoxifen inducible form of the Notch intracellular domain (NICD) of the Notch1 receptor. The cells were differentiated in vitro as embryoid bodies (EBs). Activation of Notch1 at an early stage of EB differentiation results in an initial upregulation of Sox9 expression and induction of chondrogenic differentiation at later stages of EB cultivation. Using siRNA targeting Sox9 we were able to knock down and adjust this early induced Sox9 expression peak to non-induced levels accompanied by reversion of late chondrogenic differentiation induction. Our data indicates that Notch1 signalling plays an important role during early stages of chondrogenic lineage determination by regulation of Sox9 expression.
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Differentiation – P56

**Differentiation of adipose-derived stem cells towards the epithelial lineage**
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**Question:**
Mesenchymal-epithelial interactions play a pivotal role in tubular morphogenesis and in the integrity of the kidney. During renal repair similar mechanisms may regulate cellular re-organisation and differentiation. Kidney regeneration of the renal tubular epithelium has been proven to be enhanced by treatment with mesenchymal stem cells. The mechanisms underlying this effect, however, remain unclear. Some in vivo studies suggest differentiation of MSC into tubular epithelial cells. Adipose-derived mesenchymal stem cells (ASC) can easily be isolated from lipoaspirates and possess a high plasticity towards different lineages. Previous studies of our group showed that retinoic acid (ATRA) induced initiation of epithelial differentiation of ASC.

**Methods:**
We tested the influences of nephrogenic factors on epithelial differentiation of ASC. Therefore, ASC were cultured with media containing different concentrations of Activin A (ActA), bone morphogenic protein –7 (BMP-7), and ATRA. ASC proliferation was analyzed by a fluorimetric assay, while differentiation into epithelial lineage was assessed by morphologic changes and by the induction of characteristic markers like cytokeratin-18 (CK) and zona occludens protein 1 (ZO-1). Expression of the markers was shown by qPCR, Western blotting and immunofluorescence stainings.

**Results:**
Addition of a mixture of ActA, BMP-7, and ATRA induced epithelial differentiation of ASC, whereas cell proliferation was not enhanced. Expression of the epithelial markers CK-18 and ZO-1 was significantly induced after 14 days of incubation.

**Conclusion:**
Our study highlighted that ASC are able to differentiate towards the epithelial lineage by cultivation with a combination of ActA, BMP-7, and ATRA. Such predifferentiated stem cells might be advantageous in regenerative medicine by improving stem cell based therapeutic options.
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Differentiation – P59

The effect of BMP4 on SSEA-1 expression during mouse embryonic stem cell differentiation to PGC
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Background:

The ability to generate germ cells from embryonic stem cells (ESCs) provides a powerful in vitro model to study germ cell development.

Aim:

The purpose of this study was to determine 10 ng/ml of BMP4 on SSEA-1 expression in process of mouse Embryonic Stem Cells (mESCs) differentiation to PGCs in vitro.

Materials and Methods:

Mouse ES cells were generated as embryoid bodies (EBs) in vitro by hanging drop method. In order to differentiation of PGCs, EBs was cultured in concentration 10 ng/ml BMP4 until 4 day. Germ cell markers SSEA-1 and Mvh were analyzed by flow cytometry, Immunocytochemistry and electrophoresis gel.

Results:

By flow cytometry detection, the differentiation SSEA-1 positive cells in control and treated groups were 67% and 31% respectively. Immunohistochemical analysis of the EBs demonstrated that SSEA-1 positive cells after 4 days culture localized only in the edge of EBs. We found that addition of 10 ng/ml BMP4 increased expression of the germ cell-specific marker Mvh during differentiation of mESCs to PGCs.

Conclusions:

We conclude that concentrations of 10ng/ml after 4 days had the effects on differentiation PGCs of mESCs.

Key words: BMP4, mouse Embryonic Stem Cells, Primordial Germ Cells, SSEA-1 positive cells
Visualization, tracking and quantification of bone-marrow mesenchymal cells homing in rabbit heart
(nanoparticles labeling and imaging by magnetic resonance and SPECT)

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Methods:

Rabbit BMMCs were isolated and cultured for 1-3 weeks. (A) Labeling was based on incubation of cells with complex 111 Indium-tropolone for 5-15 min. Labeling efficiency was determined. Surviving of BMMCs during 1 week was monitored. Different amounts of labeled cells were placed in phantom of rabbit chest and underwent basic gamma camera imaging. (B) Two types of iron oxide particles (Resovist or supermagnetic maghemite) were added to the BMMCs culture up to final concentration 100µg/ml. After several days particles were washed out and labeled BMMCs were placed in phantom of rabbit chest.

Results NMR imaging was evaluated. BMMCs displaying about 3 Bq/cell. Viability was not significantly decreased by this procedure. BMMCs numbers as low as 50 x 10^3 could be easily localized and imaged using gamma camera. Resovist and deadherence process resulted in surviving of 80% of cells. Cluster as small as 50 x 10^3 cells could be detected by NMR imaging.

Background and objectives:

Monitoring of stem cell homing plays a crucial role in preclinical evaluation of evolving stem cells therapies. Stem cells are wandering of desired organs and tissues very often. In case of heart is essential to visualize the right position of the delivered cells and ideal to quantify (recognize) the amount of remaining cells in the infarcted area. Most common were histological methods of recognizing remaining cells. Noninvasive methods would be more efficient and less time consuming. For tracking is very important to label the cells with particle which does not effect viability and would be specific enough to mark even a small number of cells. Bone-marrow mesenchymal cells (BMMCs) labeling methods were already described for canine, pig and rat models. Evaluation and comparison of labeling methods for rabbit BMMC is still lacking. Nuclear magnetic resonance is common cardiovascular diagnostic method with ideal recognition of heart geometry and motion. Radioactive indium can be tracked for four weeks and amount of radioactivity in the tissue is measurable. Combination of these two methods seems to be ideal.
Conclusion:

Resovist labeling of rabbit BMMCs were proved working and optimized. These methods can be used as base for in vivo studies of tracking of cells delivered into the infarcted rabbit heart. Washout of radioactive/contrast substances does not affect our methods and viability of the cells is not impaired.
Factors regulating stem cell behaviour – O2

Migratory progenitor cells in osteoarthritis and rheumatoid arthritis are driven by inflammatory mediators and sex hormones

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The regeneration of diseased hyaline cartilage continues to be a great challenge, mainly because degeneration—caused either by major injury or by age-related processes—can overextend the tissue’s self-renewal capacity. Adult osteoarthritic cartilage and cartilage from rheumatoid arthritis were obtained from the knee joints of patients (ages: 65 - 75 years) suffering from late-stage osteoarthritis (OA) or rheumatoid arthritis (RA) after total knee replacement. Light microscopy, ultrastructural investigations, cell isolation, cloning and immortalization, as well as multipotent differentiation experiments were performed. Furthermore, quantitative real-time RT-PCR, Western blotting, SILAC proteomics as well as RNA interference were applied. We have recently shown, that repair tissue from late stages of osteoarthritis in humans harbors a unique progenitor cell population, termed chondrogenic progenitor cells (CPCs). These exhibit stem cell characteristics such as clonogenicity, multipotency, and migratory activity. CPCs are governed by the osteogenic transcription factor runx-2 and the chondrogenic transcription factor sox-9. They show gender differences and exhibit estrogen and progesterone receptors. Treatment, especially with estrogen, at least in vitro, can enhance their chondrogenic potential. We have now isolated a similar CPC population from RA cartilage tissue, RA-CPC are regulated via IL-17 and novel TCR receptors. Our results offer new insights into the biology of progenitor cells in the context of diseased cartilage tissue and are relevant in the development of novel therapeutics for osteoarthritis and rheumatoid arthritis. DFG IMMUNOBONE Bl 1122/1-1 Koelling S, Kruegel J, Irmer M, Path J, Sadowski B, Miró X, Miosge N (2009) Migratory chondrogeniprogenitor cells from repair tissue during the late stages of human osteoarthritis Cell Stem Cell 4:324-335. Koelling S, Miosge N (2010) Sex differences of chondrogenic progenitor cells in late stages of osteoarthritis. Arthritis and Rheumatism 64:1077-1087. Koelling S, Miosge N (2009) Stem cell therapy for cartilage regeneration in osteoarthritis. Expert Opinion on Biological Therapy 9:1-7.
Release of soluble CD40L by matrix metalloprotease-2 (MMP-2)-dependent shedding of platelets and its subsequent accumulation in stem cell products of autologous donors

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Introduction:
Conditional on apheresis techniques, stem cell products contain a considerable amount of thrombocytes. Platelets are the major source of soluble CD40 ligand (sCD40L) (1) in the blood. It has been demonstrated that CD40L is cleaved from the surface of activated platelets: sCD40L is well known to show immunomodulatory functions and high concentrations in blood products (2). Therefore we examined sCD40L concentrations in stem cell apheresis.

Material and Methods:
In four patients suffering from multiple myeloma and undergoing autologous stem cell apheresis, sCD40L concentrations were measured in peripheral blood samples before, during and after apheresis procedure and in the respective stem cell product. sCD40L concentrations were determined by a commercially available ELISA Kit (R&D Systems). In an additional approach, platelet-rich plasma (PRP) from healthy volunteers (n=6) was incubated with different pharmacological inhibitors (MMP-2/MMP-9 Inhibitor I, MMP-9 Inhibitor I, MMP-2 Inhibitor I, recombinant ADAM 10, and recombinant ADAM-17) during platelet activation.

Results:
During stem cell apheresis, a decrease in platelet count could be observed from 94,822/µL ± 56,734/µL at the beginning to 55,007/µL ± 26,567/µL at the end of the procedure. The thrombocyte loss was accompanied by a significant lowering of sCD40L concentrations in peripheral blood samples from 241 pg/mL ± 137 pg/mL to 124 pg/mL ± 73 pg/mL (dependent on platelet count, linearly correlated, r = 0.95). In stem cell products, sCD40L concentrations were manifold elevated (range from 2189 to 3641 pg/mL) in comparison to concentrations of peripheral blood samples. Using the MMP-9 inhibitor (100 nM) and the MMP-2/9 inhibitor (3 µM) sCD40L release by platelets could be inhibited by >60%. Interestingly, the MMP-2
inhibitor (17 µM) completely prevented the shedding of sCD40L from activated platelets.

**Conclusions:**

During stem cell apheresis, sCD40L concentrations in peripheral blood were mainly influenced by alterations of platelet count. As known from platelet concentrates, an accumulation of sCD40L could also be observed in stem cell products pointing out the importance of sCD40L release by platelets. Additionally, these data support the hypothesis that MMP-2 might be the protease, primarily responsible for sCD40L cleavage from platelet surface.

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Role of LIF/STAT3 signaling in the regulation of major histocompatibility complex class I molecules in murine embryonic stem cells

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Teratoma formation from embryonic stem (ES) cells and immune rejection of their derivatives represent a major impediment to their therapeutic use. The primary effector cells mediating immune rejection of transplanted cells are cytotoxic T cells, which recognize major histocompatibility (MHC) class I/peptide complexes on target cells. In this study we demonstrate that MHC class I molecules are expressed only at very low levels on murine ES cells and they are not induced by the interferon gamma (IFNg) despite the presence of IFNg-receptors on their cell surface. Thus, we aimed at dissecting the molecular mechanism responsible for the low level of MHC class I expression and unresponsiveness to IFNg in murine ES cells. First, we postulated that leukemia inhibitory factor (LIF), a standard component of ES cell culture media, may be a factor responsible for suppression of MHC class I expression, because of the induction of STAT3 by LIF. Indeed, removal of LIF resulted in upregulated levels of MHC class I molecules on ES cells and its addition to differentiated cells in day 4 embryoid bodies suppressed their expression. STAT3-knockdown (STAT3-KD) in ES cells significantly increased the MHC class I expression even in the absence of IFNg and this increase was further enhanced by IFNg treatment. Using flow cytometry and STAT3-KD we demonstrated that STAT3 negatively affects IFNg-signaling by blocking STAT1 phosphorylation in ES cells. Luciferase reporter assay also indicated that GAS promoter responded strongly to IFNg in STAT3-depleted ES cells and only weakly in intact cells. Upregulation of MHC class I levels by STAT3-KD resulted in reduced lysis of ES cells by activated syngeneic natural killer (NK) cells. However, lysis of ES cells, which are normally not efficiently killed by cytotoxic T lymphocytes (CTLs), was increased by CTLs after STAT3-KD. These data indicate that STAT3 pathway plays an important role in regulating the MHC class I expression in murine ES cells and the susceptibility of these cells to lysis by NK cells and CTLs. Interfering with the inhibitory pathways that suppresses MHC class I expression may help control teratoma formation from contaminating ES cells in therapeutic cell transplants and may be used to eradicate cancer cells known to evade immune recognition by downregulating the MHC class I expression.
Hormonal regulation of human hair follicle epithelial stem cell functions \textit{in situ} and \textit{in vitro}

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The hormonal controls of adult epithelial stem cells \textit{in situ} are largely unknown. Here, we investigated (neuro-)endocrine controls of normal adult human hair follicle epithelial stem cells (eHFSCs) as a model to characterize these controls \textit{in situ} and \textit{in vitro}. Non-retroviral transfection of a human \textit{K15}-promotor-GFP/geneticin-resistance expression system in microdissected, organ-cultured adult human scalp skin HFs generates specific \textit{K15}-promoter-driven GFP expression in bulge eHFSCs \textit{in situ} or in isolated \textit{K15}-GFP+progenitors \textit{in vitro}. This novel tool was used to investigate the influence of thyroid hormones (THs), thyrotropin (TSH), thyrotropin-releasing-hormone (TRH), calcitriol, and prolactin (PRL) on selected eHFSCs functions \textit{in situ} and \textit{in vitro}. THs up-regulate \textit{K15}-promoter activity, \textit{K15} transcription and protein expression in eHFSCs \textit{in situ} and \textit{in vitro}. Moreover, THs reduce the colony forming efficiency (CFE), proliferation, viability and induce apoptosis in cultured \textit{K15}-GFP+ progenitors. THs may also induce epithelial-mesenchymal-transition, indicated by fibroblastoid morphology and vimentin expression, and up-regulates expression of the immuno-inhibitory eHFSCs marker CD200 \textit{in situ} and \textit{in vitro}. THs accelerate TSH-b-receptor expression, and become sensitive to TSH stimulation, which induces K15-GFP+progenitors to differentiate along the keratinocyte lineage and to express e.g. keratin-6. While TRH demonstrated no effects on eHFSCs \textit{in situ}, influences, TRH induced proliferation and differentiation in isolated K15-GFP+progenitors. Calcitriol induced morphological changes, up-regulated vitamin-D-receptor and CD200 expression and impaired both CFE and proliferation. Finally, PRL up-regulated \textit{K15}-promoter-driven GFP expression in the HF bulge region and extended the area \textit{K15}-promoter activity beyond the bulge region, and stimulated \textit{K15} protein expression. We document that human eHFSCs underlie prominent (neuro-)endocrine controls, not only by classical steroid hormones but also by peptide neurohomones. This encourages one to systematically explore the impact of these hormonal controls on human epithelial stem cells \textit{in situ} and \textit{in vitro} and to explore how these may be clinically exploited.
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Factors regulating stem cell behaviour – 14

Cathepsin X is activated by cathepsin L, inactivates the chemokine SDF-1 and reduces adhesion of hematopoietic stem and progenitor cells to osteoblasts

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Hematopoietic stem and progenitor cells (HSPC) are known to reside in specialized niches at the endosteum in the trabecular bone. It is well established that proteases can take part in the cytokine-induced mobilization process. However, migratory processes such as the regular trafficking and induced mobilization of HSPC are not fully understood.

In the present study we showed that the osteoblast-secreted activated cathepsin X is able to reduce the direct interaction of HSPC with human bone-forming osteoblasts. Immature cathepsin X is also bound to the cell surface of human osteoblasts. Knocking-down endogenous cathepsin X in osteoblasts with siRNA and subsequent HSPC adhesion studies led to a significant increase of HSPC binding to the adherent cells confirming its proteolytic influence on HSPC adhesion. In this context we studied the activation of cathepsin X and elucidated with different biochemical methods that cathepsin X can be activated by cathepsin L, a protease that is known to get secreted by activated osteoclasts.

Applying MALDI-TOF analysis we showed that the chemokine SDF-1, which is secreted by bone marrow stromal cells, can be readily digested with the carboxymonopeptidase cathepsin X. SDF-1 is a highly potent chemoattractant and a mediator of cell adhesion for HSPC. Migration assays with cathepsin X-digested SDF-1 showed a significant decrease in migration of HSPC compared to the non-truncated chemokine indicating that the protease is capable to inactivate SDF-1alpha. Furthermore, cathepsin X can convert the other isoform SDF-1beta to SDF-1alpha.

Current studies focus on the interaction between HSPC and their niche, especially the involvement of secreted proteases of the cathepsin family, their regulation and their extracellular substrate specificity to investigate the trafficking of HSPC in more detail.
Improving cell yield and differentiation potential of PCMOs: Effect of cell source and growth conditions in culture

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We have previously shown that peripheral blood monocytes can be differentiated \textit{in vitro} into hepatocyte-like and insulin-secreting cells following intermittent dedifferentiation into a more plastic stem cell-like intermediate designated PCMO (Programmable Cell of Monocytic Origin). The present study aimed at further optimizing the process of PCMO generation with respect to increasing both total cell yield and the cells' differentiation potential through studying the effects of different culturing conditions. Human monocytes isolated from buffy coats or by elutriation were cultured for 6 days in RPMI-based medium containing AB serum, M-CSF, and IL-3, under either adherent conditions or in suspension. The results revealed a gradual increase in cellular proliferation, as examined by immunofluorescence of Ki67 until the fourth day of culture and decreased thereafter. Proliferation indices were stronger i) in cells isolated from buffy coats than in those derived by elutriation, and ii) in monocytes from adherent compared to suspended cultures. The proliferative effect was paralleled by the expression of pluripotency markers such as Nanog and Oct3/4 and a decrease of monocytic markers such as p47phox. These results suggest that some as yet unidentified components in buffy coats may enhance both monocyte proliferation and dedifferentiation and that adherent growth may be superior to suspended growth conditions in the process of PCMO generation.
Factors regulating stem cell behaviour – P3

Effect of different culture condition on development of stem cell like cells from IVF derived early developing embryos in buffalo
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So far it has been difficult to generate a proven stem cell line in animal which has been attributed to the lack of proper culture condition. The present experiment was carried out to study the role of different culture conditions on multiplication of early stage IVF derived embryonic cells of buffalo. The IVF embryos of 32-cell stage were made zona free. The clumped blastomere were cultured on inactivated murine embryonic fibroblast (MEF) with culture condition viz. (C-I) DMEM+ITS +FBS +LIF + SCF, (C-II) DMEM+ITS +FBS +LIF + SCF + IGF1, (C-III) DMEM+ITS +FBS +LIF + SCF +IGF1+ bFGF4. The blastomere were cultured at 37oC, 5% CO2 and 90% relative humidity in CO2 incubator. Once the isolated blastomere clumped made stem cell clone, they were passaged mechanically. In the first culture condition, blastomere did not maintain for long time in culture. In culture condition-II, the multiplication was better as compared to C-I but no cell line could be derived in this culture condition also. In culture condition C-III, the cell clones could be propagated upto 3rd passage. The stem cell like clone were positive for Oct, AP and Nanog expression. The results indicated that multiplication of buffalo IVF derived embryonic cells from early stage embryos were better when they were cultured in presence of LIF, SCF, IGF1 and bFGF4 than culture condition excluding these cytokines.
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Factors regulating stem cell behaviour – P4

Effect of feeder free culture system on development of stem cell like cells from IVF derived early developing embryos in caprine
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So far it has been difficult to generate a proven stem cell line in animal which has been attributed to the lack of proper culture condition. The present experiment was carried out to study the role of feeder free culture conditions on multiplication of early stage IVF derived embryonic cells of goat. The blastomeres isolated from IVF embryos were cultured directly or after giving activation with 10μl of 7% ethanol/ml medium for 5 min on three types of feeder free coated plates viz. gelatin, matrigel and poly-l-lysine. Four different media were used viz. (Media-1) CR11aa supplemented with BSA, FBS, LIF, ITS (0.1%), IGF-1, bFGF, EAA (1%) and NEAA (0.5%), in Media-2; FBS supplementation was reduced to 10%; in Media-3, EAA was not incorporated, rather supplementation of NEAA was increased to 1% and in Media-4, 1mg/ml glucose was supplemented additionally. The blastomere were cultured at 37°C, 5% CO2 and 90% relative humidity in CO2 incubator. The results indicated that when blastomere were cultured on gelatin coated dish, the formation of ES cell clone were significantly higher (P<0.05) in Media-1 & 3 than Media 2 & 4. Again there was no significant difference was observed between Media 1 & 3 and Media 2 & 4. It was observed that there was no significant difference between gelatin and matrigel on formation of ES cell clones irrespective of media used. Further when all the media condition was considered together, there was no significant difference of ES cell clone formation when blastomere were cultured either on gelatin or matrigel. None of the embryos cultured on polylysine coated plate were found to be attached and developed to stem cell clones in any media and all of them died within few days. The result indicated that gelatin or matrigel coating could be used as a feeder free culture system for making ES cell clone of caprine.
Factors regulating stem cell behaviour – P13

Functional analyses of cell polarity organization in human hematopoietic stem and progenitor cells
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Freshly isolated hematopoietic stem and progenitor cells (HSPCs) are small, round cells which adopt a polarized cell shape upon cultivation. Depending on the activity of the phosphoinositol-3-kinase (PI3K) they form a leading edge at the front and a uropod at the rear pole. We have recently shown that in addition to different lipid raft associated proteins, the lipid raft organizing molecules Flotillin-1 and -2 get highly concentrated at the tip of the uropod. Performing pharmaceutical inhibitor studies we dissected mechanisms controlling HSPC polarization and were able to discriminate two levels of cellular polarization. According to our observation the vast majority of freshly isolated human HSPCs, i.e. umbilical cord blood derived CD34⁺ cells, show a random distribution of the Flotillins and other lipid raft associated molecules like ICAM3. Upon cultivation they redistribute these molecules to form a crescent and thus become intrinsically polarized, before they adopt their characteristic morphological polarized cell shape. Using this discrimination, we obtained evidence that PI3K and atypical protein kinase C (aPKC) activities are required to organize the intrinsic polarity while the morphological polarization process also depends on protein synthesis, actin polymerization and rho-GTPases activities.

Since aPKCs form an evolutionary conserved complex with the partitioning defect proteins Par3 and Par6 as well as with the rho-GTPase Cdc42 and this complex has been found to organize cell polarity in many organisms and tissues, we decided to investigate the function of the individual components on the cell polarization process of human HSPCs next. Due to the fact that the Par/aPKC complex also coordinates asymmetric cell divisions in a number of systems and as we showed that human HSPCs can divide asymmetrically, we have started to study the impact of these proteins on the cell fate of human CD34⁺ cells in parallel. Our pharmaceutical studies as well as our experimental strategy within the Par/aPKC project together with some preliminary results will be presented.
Effects on differentiation of early murine embryonic and hematopoietic stem cells under stimulation with Granulocyte Colony Stimulating Factor

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Question: Granulocyte colony-stimulating factor (G-CSF) is widely used in clinical practice for human stem cell mobilization and transplantation, and to reduce chemotherapy-induced febrile neutropenia. While the effect of G-CSF to stimulate neutrophil-progenitor cell development is well established, accumulating evidence implies additional functions of G-CSF as well as an impact on other cell-types, it is effects on stem cells are widely unknown. Our group now addressed the question about a possible effect of G-CSF on embryonic stem cells and early hematopoietic stem cells.

Methods: We used murine embryonic E14 stem cells which provide a homogeneous cell system to study cellular and molecular events that occur during early hematopoietic development. The embryonic stem cells were cultured in standard medium or medium continuously supplemented with G-CSF (50 ng/ml) in hanging drops for 3 days and the resulting embryoid bodies were kept in culture for a further period of 8 days. On days 3 (d3), 6 (d6) and 11 (d6+5), cells were harvested and separated with the Anti Sca-1- microbeads for analysis with Realtime-PCR, FACS and protein-arrays.

Results: Our data show that G-CSF influences the early embryonic and hematopoietic cell development. Most hematopoietic genes (e.g. Gata-2, Pu1, Lmo2) are upregulated as early as day 3 in the cell pool. When the cells were separated with MACS-microbeads (sca1-antibody), we see that the stem cells show a delayed expression of hematopoietic stem cell surface-markers (e.g. CD34, CD117, CD133) and a downregulation in hematopoietic genes when stimulated with G-CSF.

Discussion: Our results suggest that G-CSF is influencing hematopoietic stem cell development, and thus not only neutrophil-cell development. The protein and gene analysis of the complete cell cultures imply that G-CSF acts on these immature cells to promote hematopoietic development, and that this effect is not limited to hematopoietic cells. The downregulation of genes and stem cell markers of immature stem cells bearing the sca1-protein interestingly implies that G-CSF has a direct or indirect effect which preserves stem cell properties of the immature stem cells from the stem cell pool.
Hypoxia enhances proliferation and attenuates differentiation capacity of human mesenchymal stromal cells - and prolongs their lifespan

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Low oxygen tension is thought to be an integral component of the human mesenchymal stem cell (MSC) native bone marrow microenvironment. MSCs (n=9) were maintained under hypoxic atmospheres (1%O₂ and 5%O₂) for up to ten in vitro passages. This resulted in approximately 3,000 (1%O₂) and 1,800 (5%O₂) fold higher cumulative cell numbers after five passages compared to MSCs cultured at normoxic (20%O₂) conditions. When compared with MSCs expanded at normoxic atmospheres, sixfold (1%O₂) or fourfold (5%O₂) higher number of colony-forming units were found; MSCs also expressed higher levels of stem cell markers (STRO-1, Oct-4, SSEA-1, SSEA-4 and NANOG). However, under 1%O₂ adipogenic and osteogenic differentiation was suppressed, while chondrogenic differentiation was inducible but diminished compared to standard in vitro conditions. Using 5%O₂ tension attenuated differentiation capacity was detected for osteogenic and adipogenic pathway while chondrogenesis was enhanced. In turn, with the exception of chondrogenesis (5%O₂), MSCs obtained from hypoxic cultures also expressed higher levels of osteogenic and adipogenic differentiation markers when subsequently were differentiated under normoxic conditions. Additionally, in vitro proliferation lifespan was significantly increased with about 7 additional passages (1%O₂) before reaching terminal growth arrest. Thus, the low oxygen tension is a key parameter that influences in vitro characteristics of MSCs by providing a micromilieu for extension of lifespan and enhanced proliferation capacity. Simultaneously the maintenance of stemness and differentiation capacity was improved.
Reduced oxygen tension has related effects of toll-like receptor mediated immunmodulatory properties of human mesenchymal stromal cells

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Mesenchymal stromal cells are (MSCs) are widespread in adult organisms and are involved in tissue repair as well as in the regulation of immunoregulatory responses. Up to now it is not clear how MSCs respond to unfavorable conditions like ischemia. Toll like receptors (TLRs) play a major role in immune system, participating in the recognition of microbial pathogens and pathogen-associated components. We investigated in seven experiments the induction of TLR expression of MSCs which were exposed to low oxygen tension (1%O₂ and 5%O₂) without the additional substitution of classical TLR ligands like heat shock proteins or lipopolysaccharides. In flow cytometry analyses MSCs showed at 1%O₂ respectively 5%O₂ significantly upregulated expression of TLR2, TLR3, TLR4, TLR7, TLR8, TLR9 and TLR10. In 1%O₂ treated cultures TLR3 and TLR4 expression was significantly more represented compared to 5%O₂ exposed MSCs. Furthermore, the toll-like receptor associated protein MyD88 was also upregulated. Additionally, in ELISA based analyses the cytokine expression of CCL2, CXCL10, interferon-1β, interleukin-1α, interleukin-1β, interleukin-6, interleukin-8, interleukin-10 and tumor-nekrosis-factor-α was significantly enhanced, e.g. in the case of CXCL10 126.099±29.28pg/mL (1%O₂) respectively 102.461±23.486pg/mL compared to normoxia (37.854±11.329pg/mL). In the supernatant of low oxygen cultures, endogeneous danger proteins heat shock protein 60 and fibronectin were found, indicating that MSCs at low oxygen tension produces these proteins. Thus, low oxygen tension may have direct effects on immunmodulatory responses of MSCs, as well as on proliferation capacity or stemness. In hypoxic cell niche of MSCs, immunmodulatory effects may suppressed by other factors.
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Factors regulating stem cell behaviour – P31

Heparansulfate proteoglycan suppresses in vitro proliferation and differentiation of human mesenchymal stromal cells

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The basement membrane extracellular matrix (ECM) is composed of proteins like collagen IV and laminin-5 and also of mobilized proteoglycans like heparansulfate proteoglycan (HP). In recent studies, the in vitro application of ECM-proteins improved proliferation and differentiation capacity of mesenchymal stromal cells (MSCs). In six co-culture experiments we investigated the influences of HP on the proliferation and differentiation capacity of in vitro cultured MSCs. Interestingly the proliferation capacity was downregulated when compared to plastic standard conditions. The cumulative cell numbers after five passages as determined by flow cytometry approaches was about 634±154fold lower than on plastic cultured MSCs. Furthermore, the in vitro proliferation life span was significantly shortened (38±5,60d; on plastic:78±13,39d). On the other hand, when status of terminal growth arrest was reached, the viability of HP treated MSCs was about 95±3,67% for a period of about 45d in culture. In contrast, viability of on plastic expanded and then proliferation inactive MSCs was only for 15±7,71d over 90%. The differentiation of MSCs cultured with HP was diminished, e.g. in the osteogenic lineage the alkaline phosphatase activity (APase) was 3,45±1,29 fold lower for HP treated MSCs. In turn, after the exclusion of HP, differentiation capacity was enhanced; e.g. APase was 2,81±0,89 fold higher compared to standard conditions. Additionally, immunmodulatory responses maybe suppressed by HP. After induction of toll-like receptor (TLR) expression by low oxygen tension, the supplementation of HP diminished the expression of TLR-receptors 2,3,4,and 9. Similar effects of HP were previously described for pericytes. Thus, parallelism of HP influences on MSCs and pericytes maybe another advice for the in vivo identity of MSCs.
Factors regulating stem cell behaviour – P38

New microfluidics-based technologies enable the high throughput screening for optimal cultivation and differentiation conditions: First adaptation to stem cell culture

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Stem cells have the potential to significantly improve future therapies [1]. But new approaches can only be realised safely with reproducible, xeno-free, and efficient culture and differentiation protocols. Until now, these goals have not been completely mastered for clinical purposes. None of the available state-of-the-art methods do allow fast and affordable screenings for optimal conditions, because of high consumption of expensive growth factors and media additives. Therefore, the HYPERLAB consortium aims at improving protocols by adapting microfluidics-based cell cultivation technologies to the specific needs of stem cells [2]. Mainly miniaturisation and parallelisation will enable a rapid, cost efficient, and precise screening by using a pipette robot, a modified hanging droplet approach, and a medium throughput system based on segmented flow, the pipe based bioreactors-system. These techniques enable the analysis of a multitude of test conditions with a parallel reduction of the necessary cell material [3]. After analysing the physicochemical changes in miniaturised compartments, we show that an automated medium exchange using a pipette robot has no effects on H1 and MSCs by observing the cells with a live cell imaging system. As cells showed very good viability and proliferation without increased differentiation, this setup can be used for screenings of growth and differentiation factors.

We also demonstrate in this work, that with the pipe based bioreactors, cells can be cultivated, transported and observed in small medium volumes, so the screening of many conditions in parallel is possible. For this, culture of various stem cell types on microcarrier was established and cultivation in separated medium compartments was analysed in terms of viability, proliferation, and pluripotency.

The modified hanging droplet technology, based on electrowetting or in combination with a pipette robot, will enable manipulation of cells, concentrated in droplets of medium. For this innovative approach, we established the hardware and adapted protocols for the use of hanging droplets.
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Factors regulating stem cell behaviour – P58

Repair of spinal cord injury by co-transplantation of embryonic stem cell-derived motor neuron and olfactory ensheathing cell
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Background:

The failure of regeneration after spinal cord injury (SCI) has been attributed to axonal demyelination and neuronal death. Cellular replacement and white matter regeneration are both necessary for SCI repair. In this study, we evaluated the co-transplantation of olfactory ensheathing cells (OEC) and embryonic stem (ES) cell-derived motor neurons (ESMN) on contused SCI.

Methods:

OEC cultured from olfactory nerve rootlets and olfactory bulbs. ESMN was generated by exposing mouse ES cells to retinoic acid and sonic hedgehog. Thirty female rats were used to prepare SCI models in five groups. Control and medium-injected groups were subjected to induce lesion without cell transplantation. OEC or ESMN or both were transplanted into the site of the lesion in other groups.

Results:

The purity of OEC culture was 95%. Motor neuron progenitor markers (Olig2, Nkx6.1 and Pax6) and motor neuron markers (Isl1, Isl2 and Hb9) were expressed. Histological analysis showed that significantly more (P<0.001) spinal tissue was spared in OEC, ESMN and OEC+ ESMN groups but the OEC+ ESMN group had a significantly greater percentage of spared tissue and myelination than other groups (P< 0.05). The numbers of ESMN in co-transplanted group were significantly higher than ESMN group (P<0.05). A significant (P<0.05) recovery of hindlimb function was observed in rats in the transplanted groups.

Conclusion:

We found that the co-transplantation of ESMN and OEC into an injured spinal cord has a synergistic effect, promoting neural regeneration, ESMN survival and partial functional recovery.
Oct4/Pou5f1 controls tissue-specific repressors in early zebrafish embryo
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The transcription factor Pou5f1/Oct4 controls pluripotency in mammalian ES cells, but little is known about its role in early development. pou5f1 homologues are ubiquitously expressed during the gastrulation stages in vertebrates, and loss of its function leads to early embryonic lethality. Mouse Oct4 rescues severe gastrulation and axial defects of zebrafish pou5f1 null mutant MZspg, suggesting the phylogenetic conservation of Pou5f1 functions. Using time-resolved transcriptome analysis of zebrafish MZspg mutant embryos we found, that multiple differentiation and patterning genes are prematurely expressed in MZspg embryos, while expression of soxB1 class genes and some transcriptional repressors is reduced or absent. Our findings demonstrate that Pou5f1 controls differentiation timing by direct activation of transcriptional repressor sets specifically expressed in each main embryonic tissues: mesendoderm (foxD3), klf5 in non-neural ectoderm (klf2b), neuroectoderm (her3 and hesx1). Significant overlap between zebrafish and mammalian Pou5f1 targets together with the ability of mouse Oct4 to replace for zebrafish Pou5f1 function suggests that the mammalian stem cell network may have evolved from a basal situation similar to what is observed in teleosts. In support of this idea, tissue specificity of klf5 and foxD3 expression in fish, correctly predicts the tissue-specificity of differentiation upon the knockout of respective mammalian homologues in ES cells (ectoderm and mesendoderm). The analysis of Pou5f1 downstream tissue-specific subnetwork will be presented. We envision that the knowledge of molecular interactions downstream of Pou5f1 in experimentally amenable models will contribute to the effort of directing differentiation of pluripotent stem cells to defined cell fates.
Investigation of zebrafish homologues to specifically upregulated TUFs in cardiomyocytes derived from murine ES cells

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Heart failure due to loss of functional cardiomyocytes is one of the most frequent cardiovascular diseases. Understanding the genetic network that leads to functional cardiomyocytes is the first step to develop future therapies. A global transcriptome analysis yielded up-regulated genes in cardiomyocytes, which were derived from murine embryonic stem (ES) cells (Doss et al., 2007). In the present study we were on the one hand interested in a fast screen for the functional role of transcripts with unknown function (TUFs) for an intact activity of the heart. Therefore we searched for homologues in the zebrafish genome and performed a morpholino-based knockdown approach. We tested several TUFs of the zebrafish and found most of them to be expressed in the cardiovascular system. Morpholino-oligonucleotide injections caused highly specific cardiovascular defects in the majority of them such as altering of heart morphology, vascular defects or accumulation of blood cells to a different extent and penetrance. This pilot approach thus shows the potential of the zebrafish to identify TUFs in the cardiovascular system. Based on that, on the other hand we want to investigate promising candidate genes from the zebrafish approach in ES cells. ES cells are pluripotent cells which were isolated from early mouse embryos and because of their pluripotency able to differentiate into derivatives of the three primitive layers: ectoderm, endoderm and mesoderm. Using ES cells which were differentiated into three-dimensional structures, so-called embryoid bodies (EBs), we want to study early developmental stages. For this we have generated stable knockdown clones of mouse ES cells expressing a shRNA construct against the desired genes. These clones were also labeled with a green fluorescent protein (GFP) reporter to track the silencing effect. Clones, expressing GFP, were initiated to generate EBs and will be reviewed via PCR, western blotting and beating activity.
B cell to myeloid reprogramming by the transcription factor C/EBPβ

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Background:
CCAAT enhancer binding protein (C/EBP) transcription factors may reprogram cells from the lymphoid lineage into the myeloid lineage. However, little is known about the molecular mechanism of this cell plasticity and its potential role in leukemogenesis.

Material and Methods:
B cell progenitors were substituted with the wild type or functional mutants of the transcription factor C/EBPβ to elucidate the mechanisms of reprogramming. After in vitro culturing in a stromal cell co-culture system that supports both, B cell and myeloid development, cells were analyzed for surface marker expression, cell morphology, protein expression, functionality and rearrangements in the immunoglobulin locus.

Results:
Our data show that 1) the long C/EBPβ isoforms LAP and LAP*, but not the short isoform LIP may reprogram B cell progenitors to mature myeloid cells; 2) distinct amino acid substitutions, and even some deletion mutants of the transactivation domain, maintain reprogramming functions; 3) the deletion mutant ΔCR3,4 is incapable of reprogramming, while the individual deletion mutants ΔCR3 and ΔCR4 may retain reprogramming functions. Reprogrammed B- to myeloid cells display common macrophage characteristics, including phagocytic activity, and exhibit rearranged IgH locus, thus confirming their B cell origin.

Conclusion:
Our results show that the C/EBPβ regulatory domain and conserved regions (CR) 1 and 2 from the transactivation domain are dispensable for the lineage switch. CR 3 and 4 display redundant functions, as the presence of either one of them comprises reprogramming function, whereas the lack of both abrogates reprogramming. Our data suggest that the molecular mechanisms of B cell to macrophage reprogramming resides in CR3,4. It appears as important to determine the interaction partners of CR3,4 in B cells to work out the mechanism of transdifferentiation.
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Molecular Mechanisms-P12

Modulation of epigenetic marks at cell-context dependent Notch target genes
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Notch proteins are transmembrane receptors which influence cell fate decisions, differentiation, proliferation and apoptosis in many developmental systems including neurogenesis and myogenesis. After ligand binding and activation, the Notch intracellular domain (NIC) is cleaved from the cytoplasmic membrane and translocates into the nucleus to act as a transcription factor. NIC binds to DNA via the adapter protein RBP-J (also termed CBF-1) and converts the transcriptional repressor RBP-J into a transcriptional activator. Recently we have shown that Notch signaling regulates expression of genes playing key roles in cell differentiation, cell cycle control and apoptosis in a highly context dependent manner. Epigenetic events like histone modification, DNA methylation and chromatin remodeling are tightly involved in the control of gene expression. To investigate the role of chromatin modifications for cell-context dependent activation of direct Notch target genes, we analysed the chromatin modifications present at the regulatory regions of genes that we identified to be regulated in embryonic stem cells (ESC) by activated Notch using public databases. We found that the promoter regions of Notch target genes are marked by an enrichment of H3K4me3 and bivalent domains (H3K4me3 and H3K27me3). We further screened the regulatory regions of the Notch1 target genes Hes5, Sox9 and Myf5 for potential RBP-J binding sites and analysed the modifications and their changes upon Notch activation in ESC and mesodermal cells. After activation of Notch signaling for 4 h, H3K4me3 and H3K4me2 marks at the regulatory regions of Notch target genes, Hes5 and Sox9 in ESC and Myf5 in mesodermal cells, were increased, suggesting that Notch signaling may lead to remodeling at the regulatory regions of its target genes. In addition, we tested the effect of LSD-1 (Lysine specific demethylase 1), a histone modifying enzyme that controls gene repression, on the expression of Notch target genes. After inhibition of LSD-1 by PCPA, Notch induced gene expression of the target genes Hes5, Hey1, Id4 and Pax6 was increased in ESC, further indicating an important role for histone modifications in the regulation of Notch target gene activation.
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Molecular Mechanisms-P16

Characterisation of the endosomal machinery in human hematopoietic stem and progenitor cells
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Somatic stem cells give rise to self-renewing and differentiating daughter cells. To maintain the regenerative capacity of given tissues over each organism’s life time, it is required that somatic stem cell pools are kept relatively constant. There is good evidence that decision processes controlling whether somatic stem cell progeny self-renew or get committed to differentiate are tightly controlled. At the example of hematopoietic stem and progenitor cells (HSPCs), the best investigated somatic stem cell system so far, it has been shown that extrinsic factors provided by the surrounding environment, the stem cell niches, are required to maintain primitive hematopoietic cell fates. In addition, at the example of human umbilical cord blood derived CD34⁺ cells we recently identified four different proteins that segregate asymmetrically in a proportion of dividing HSPCs, demonstrating that HSPCs can - as it has long been suggested - indeed divide asymmetrically. Remarkably, three of the four proteins identified, the tetraspanins CD53 and CD63 as well as the transferrin receptor (CD71), are associated with the endosomal compartment. Together with recent findings in model organisms it becomes tempting to speculate that the endosomal machinery essentially participates in cell fate decision processes. To gain more insight into the underlying mechanisms, we decided to analyze the functional impact of distinct endosomal proteins on the biology of HSPCs. Since the endosomal trafficking largely depends on the activity of Rab-GTPases, we aim to analyze the functional impact of certain Rab-GTPases on processes controlling self-renewal versus differentiation of HSPCs as well as on their cell polarity organizing machinery. In this context we have cloned constitutive active, dominant negative and wild-type gene variants of selected Rab-GTPases into lentiviral vectors. Furthermore, we established staining protocols for several endosomal antigens, allowing us to discriminate different endosomal compartments in HSPCs as well as in other somatic stem cell entities like mesenchymal stem and endothelial progenitor cells. Preliminary results as well as our experimental strategy to analyze the impact of the endosomal machinery will be presented.
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**Molecular Mechanisms-P21**

**BioMark dynamic arrays for single-cell gene expression analysis**

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Variation in gene expression at the single-cell level may be a critical parameter in understanding the differentiation of stem cells; however, single-cell samples have been difficult and expensive to analyze. BioMark\(^{\text{TM}}\) dynamic arrays provide a convenient and cost-effective method for performing multiple RNA expression assays on multiple single-cell samples. The BioMark 96.96 Dynamic Array enables running up to 96 real time PCR assays on up to 96 samples, i.e. up to 9,216 different reactions.

Here we demonstrate the use of gene expression assays for Stem Cell biologists and other users. These assays use the detection dye EvaGreen - a second generation dye less inhibitory than SYBR Green. Assay design involves a preamplification step (STA) followed by nested priming within the preamp product. To date, these approaches provide robust qPCR assays for a variety of eukaryotic and prokaryotic systems. Here we use that approach and EvaGreen to examine single cell gene expression.
Notch1 signaling promotes neuro-ectodermal differentiation of embryonic stem cells via the Notch target gene Sox9
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The Notch pathway plays a pivotal role in the control of cell fate decisions during differentiation of various tissues. In early embryogenesis, activated Notch signaling favours the formation of ectoderm at the expense of mesendoderm. In the development of the nervous system, Notch promotes the maintenance of neural stem cells, inhibits differentiation into neurons and commits neural progenitors to a glial fate. Recently we have shown that Notch signaling regulates expression of genes playing key roles in cell differentiation, cell cycle control and apoptosis in a highly context dependent manner. In embryonic stem cells under neuro-ectodermal differentiation conditions, Notch1 activation results in the upregulation of Pax6 and Sox9 RNA and protein. Sox9 and Pax6 expression is induced by activated Notch1 also in the absence of protein synthesis, suggesting that Pax6 and Sox9 may be direct Notch1 target genes. Pax6 promotes neural stem cell maintenance and is required for the specification of a neuronal fate. Sox9 specifies glial cells, promotes glial differentiation and inhibits neuronal differentiation. To understand the molecular mechanisms by which Notch mediates the neuro-ectodermal lineage choice, we combined a tamoxifen-inducible system to activate Notch signaling in embryonic stem cells differentiating in vitro towards the neural lineage with an siRNA-based strategy to simultaneously knock down Notch target genes. Using a neural monolayer differentiation protocol that mimicks neural differentiation in vivo, we activated Notch1 signaling and simultaneously knocked down Sox9 or Pax6 and monitored differentiation along the neuro-ectodermal lineage by analyses of morphology, RNA expression and FACS analysis of neuronal and glial cell specific markers. Activation of Notch signaling in ESC inhibited neuronal differentiation and promoted gliogenesis. These effects were reversed by adjusting Sox9 expression levels back to uninduced levels during Notch1 activation. Knock down of Notch1-induced Pax6 expression in ESC did not alter the outcome of Notch signaling on neural differentiation. We thus conclude that Sox9 but not Pax6 is involved in mediating Notch1-induced neuro-ectodermal lineage determination.
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Pluripotency and stem cell plasticity-O4

The transcription factor TCFAP2C/AP-2gamma cooperates with CDX2 to maintain trophectoderm formation
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In mammals, cell lineage specification is established at the blastocyst stage. At this stage, transcription factor Cdx2 represses pluripotency genes, thus promoting extraembryonic trophoblast fate. Recently, transcription factor Gata3 was shown to act in a parallel pathway in promoting trophoblast cell fate, suggesting that there are more factors working in the trophoblast lineage. Here, we report that the transcription factor Tcfap2c is expressed at a high level in the trophectoderm and is able to induce trophoblast fate in embryonic stem cells. Trophoblast fate induced by Tcfap2c does not require Cdx2 and vice versa, suggesting that the molecules act in alternative pathways. However, both Tcfap2c and Cdx2 are required for the upregulation of Elf5, a marker of trophoblast stem cell maintenance, suggesting that both factors are required for stable trophoblast induction. Tcfap2c-induced trophoblast-like cells are stable in long-term culture, indicating that they are capable of self-renewal. Tcfap2c-controlled trophoblast maintenance involves the induction of Cdx2 and the repression of the pluripotency factor Nanog. Tcfap2c-induced trophoblast-like cells differentiate to trophoblast derivatives in vitro and contribute to the trophectoderm in blastocysts in vivo. Taken together, these observations suggest that Tcfap2c and Cdx2 cooperate to override the pluripotency program and establish the extraembryonic trophoblast maintenance program in murine embryos.
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Pluripotency and stem cell plasticity-O17

Sprouty4 is a critical negative regulator of the pluripotent state in embryonic stem cells
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A hallmark feature of embryonic stem (ES) cells is their ability for self-renewal to maintain pluripotent. ES cells can be derived and maintained independently of serum and cytokines using compounds that negatively regulate signalling pathways. Here, we report that innate Sprouty4 (Spry4) action deploys a similar strategy by inhibiting specific signalling cascades. We find that Spry4 is highly enriched in ES cells and is likewise confined to the inner cell mass of mouse blastocysts. Chromatin immunoprecipitation and overexpression assays validate Spry4 as a direct target of the pluripotency factor Nanog. Constitutive Spry4 expression increases expression of important stem cell regulators, thus lodging ES cells in an uncommitted state. Importantly, sustained Spry4 expression voids the need for chemical inhibition under 3i culture condition. Conversely, expressing a dominant negative Spry4 mutant sensitizes ES cells to differentiation inducing endoreduplication and adopting a preferential trophectoderm differentiation. Altogether, our results show that in ES cells Spry4 is critically suppresses differentiation. In order to remain pluripotent an ES cell uses the Spry4 activity to curtail ES cell signalling pathways.
Analysis of the lymphoid system in NOD/LtSz-scid IL2Rγnull mice engrafted with purified cord blood derived stem cells

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Background:

The NOD/LtSz-scid IL2Rγnull mice present an in vivo model for human HSC engraftment, differentiation and analysis of human immune system responses. This study aimed to investigate the time course of HSC engraftment in NOD/LtSz-scid IL2Rγnull mice using purified cord blood derived stem cells.

Methods:

Purified human CD34+ cells derived from frozen cord blood were transplanted into non-lethally irradiated newborn mice. Peripheral blood was collected for flow cytometric analysis using two novel eleven colour flow cytometric panel for T-/NK-cells and B-/DC-cells to analyze subtypes and differentiation status of human cells in the peripheral blood and lymphoid organs. Spleen, thymus, lymph nodes and bone marrow were analyzed by flow cytometry and plasma by ELISA measurements of human IgG, IgM, IgA.

Results:

In transplanted animals human IgM-plasma level became detectable eight weeks after transplantation and increased over time to up to 50 µg/mL. IgG and IgA-plasma level remained undetectable between week 8 and 26. At week eight after transplantation human lymphocytes became detectable, with low frequency of human T-cells and high frequency of human B-cells in peripheral blood. Between week 16 and 22 huCD45+ cells reached >40 %. Over time, the rate of the human T cells increased whereas the rate of the B-cells decreased. High frequencies of B-cells in the spleen and low frequencies in the lymph nodes were detected, whereas the T-cells showed an inversed ratio in spleen and lymph nodes. Both cell types remain stable over the time. At week 22 after transplantation 40 % of thymic cells are human T cells. This rate decreased to 25 % at week 29. Additionally, the proportion of the human CD4 and CD8 double positive T cells decreases from 50 % to 10 % at week 22 and 29, respectively. Immune histology of bone marrow, thymus, lymph nodes and spleen
demonstrated presence of huCD45⁺, huCD4⁺, huCD8⁺, huCD11⁺, huCD20⁺ and huCD23⁺ cells. Especially spleen and lymph nodes showed distinctive human lymphoid structures.

**Conclusion:**

NOD/LtSz-scid IL2Rγnull mice present a robust model for in vivo studies of human HSC engraftment and differentiation. Starting 12 weeks post transplantation stable multilineage chimerism could be detected by 11-colour FACS. This model allows studies of human biological processes in vivo.
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Pluripotency and stem cell plasticity-P24

Efficient multi-genetic modification of human ESC and iPS cells for cardiomyocytes enrichment and tracking
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Purpose: To date, the generation of stable transgenic hESC lines remains inefficient and transgenic hiPS lines allowing lineage-specific enrichment of progenies have not been reported. Herein, we describe a non-viral strategy for the efficient, parallel introduction of multiple-plasmid constructs into hESC / hiPS by means of a single drug resistance. This enables, for example, the antibiotic-based purification of cardiomyocytes (CMs) that also express several reporter genes facilitating donor cell monitoring, quantification, and eventually functional assessment in vivo.

Methods: Our method allows single cell dissociation of common hESC / hiPS cultures without limiting cell vitality. Co-introduction of multiple plasmids was established by optimized electroporation. Cells were seeded in feeder-free cultures to facilitate antibiotic-based clonal selection; colonies were individually expanded and tested for the presence of multiple transgenes.

Results: Two factors were critical for the generation of stable lines in only 12 days: high transduction efficiency (of up to 60%) and high cell vitality post transduction thereby supporting high colony recovery. On average, about 20 transgenic clones were reproducibly achieved from 1.5 million treated hESC or hiPS; about 25% of these clones carried multiple transgenes as demonstrated by PCR and functional assessment. Clones were also analysed for the expression of pluripotency markers and the capacity to differentiate into all germ-layers in vitro and in vivo. Successful enrichment of essentially pure CMs was established following cardiomyogenic differentiation of transgenic clones. We also report the current application of these CMs for the in vivo optimization of donor-cell survival in rodent hearts and the electrophysiological assessment of drug toxicity in vitro.

Conclusion: This is the first report of stable hiPS clones resulting in the purification of functional CMs and the parallel expression of additional marker genes. These clones were generated using a novel, fast, and highly efficient method of transgene introduction, which is of broad interest for numerous gain and loss of function applications in pluripotent stem cell research. Notably, stable multi-transgene expression was observed for at least 25 passages, the latest time point assessed. Thus, the method overcomes the common issue of vector silencing in pluripotent stem cells often associated with viral transduction methods.
Clara cells are non-mucous and non-ciliated secretory cells of the conducting airways. One of their major functions is to protect the bronchiolar epithelium of mammals and the upper airways of some species such as mice by producing several products like the Clara cell secretory protein (CCSP). Furthermore, they may serve as progenitor/stem cells. Currently, allogeneic lung transplantation seems to be the only alternative to treat patients with terminal pulmonary failure. However, the limiting factor is the shortage of donor organs. Therefore, the ability to produce Clara cells in vitro would offer new therapeutic options to treat pulmonary injuries and diseases, including genetic disorders like cystic fibrosis. Pluripotent stem cells might be a suitable source to produce Clara cells in vitro. In contrast to ESCs the new technique of producing iPSCs offers perspective to produce patient-specific Clara cells in the future. The aim of this work is to increase the yield of Clara cells derived from mESCs and miPSCs via enhanced definitive endoderm formation. In this context, we are testing the influence of different substrates and growth factors on the monolayer-based differentiation. To determine the percentage of mesendoderm and definitive endoderm in the early differentiation cultures, we are using a transgenic mESC line, kindly provided by the group of G. Keller, that expresses eGFP from the brachyury locus and a truncated version of the human CD4 from the foxa2 locus. Moreover, in collaboration with M. Mall of the University of Heidelberg, a transgenic mice was established that expresses eGFP and lacZ from the CCSP locus. A miPSC clone from these transgenic mice was established and successfully differentiated into lacZ positive Clara cells.
Spermatogonial stem cells gene expression in different media condition
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Introduction: Spermatogonial stem cells (SSCs) are unique cells in testis that can proliferate, differentiate and transmit genetic information to next generation. However the effect of different media and feeders on gene expression of these cells is not well known. So in this study, we compare the in vitro effect of adult sertoli cells, fetal sertoli cells, mouse embryonic fibroblast (MEF) and SIM mouse embryo-derived thioguanine and ouabain resistant (STO) as different feeder layers with or without GDNF, GFR- α1, bFGF on gene expression of SSCs.

Materials and Methods: For this purpose we isolated SSCs from 3-6 day mouse by enzymatic digestion, and then SSCs were enriched by magnetic activated cell sorting (MACS) against Thy-1 antibody. The purity of the isolated cells was assayed by flow cytometry with α6-integrin and β1-integrin antibodies. Adult and fetal sertoli cells were isolated by DSA lectin. For isolation adult sertoli cells we used 8-12 week male mouse and for fetal sertoli cells we used 14.5 day male embryos. For MEF we used 13-16 day mouse embryos. Identities of cells were confirmed by fluorescent immunostaining against vimentin for sertoli cells and alkaline phosphatase activity for SSCs. After enrichment, SSCs were cultured for 7 days in different feeders and media. The expression of specific genes for SSCs (α6-integrin, β1-integrin, DAZL and stra-8) was studied by RT-PCR.

Results: The percentage of SSCs purification was 85-90%. Expression of these genes were higher significantly (p<0.05) in sertoli cells group compared to other.

Conclusion: Our findings suggested that co-culture with sertoli cells had a positive effect on in vitro culture of SSCs and had best effect on SSCs gene expression than others.

Key Words: Spermatogonial stem cell, Proliferation, sertoli cell, feeder.
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Pluripotency and stem cell plasticity-P55

Spermatogonial stem cell: isolation, purification and assessment
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Introduction: Spermatogonial stem cells have self renewal and differentiation that produce mature sperm in development and differentiation. This cells, so uses in regenerative medicine and gene therapy. This cells is necessary because 1/250 persons have cancers in 2010 and so suggested %50 couples will have infertility until 2050. Because infertility increased gradually so maintenance SSCs are necessary. SSCs are few in testis (%0/03). So isolation and culture them is important, also it is more importance in some cases (cancer of testis, Obstructive asospermia)

Materials and methods: tunica albuginea removed from adult mouse testes and cells isolated, then mechanical digestion of somniferous tubules, Enzymatic digestion did and cell suspension transferred to Percoll (Discontinuous percoll centrifugation) and after cell suspansion transferred to coated dishes with Laminin, then supernatant removed and MACS with thy-1 immunobids was done. Before and after MACS isolation, cells purification percent measured with α6, β1 integrins. After purification, cells cultured in MEMα medium and after one week colony formation assayed by Alkaline phosphatase staining, Immunocytochemistry, RT-PCR and Flow cytometry analysis. Viability of cells was done in all of steps.

Results: purification of cells about 80% after MACS, colony assessment showed Alkaline phosphatase positive and α6, β1 integrins in SSCs by Immunocytochemistry, Flow cytometry and Stra8 and DAZL by RT-PCR was detected.

Conclusion: MACS is the best method for isolation and purification cells, α6, β1 integrins are the best markers for detection of Spermatogonial stem cells.

Key words: Spermatogonial stem cells, Isolation, MACS, α6 integrin, β1 integrin, Flowcytometry.
Derivation of induced pluripotent stem (iPS) cells from a patient with an arrhythmogenic right ventricular cardiomyopathy (ARVC)

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Human iPS cells are very similar to human embryonic stem (ES) cells but do not require an embryo for their derivation. They can be derived from patients with complex genetic defects to create in vitro disease models and thus represent an opportunity to study disease pathophysiology, develop new drugs and test methods for delaying disease progression or reversing its phenotype. We have generated iPS cells from a patient suffering from arrhythmogenic right ventricular cardiomyopathy (ARVC) carrying a novel spontaneous heterozygous autosomal dominant mutation in the gene desmin (N116S). The mutation affects filament formation leading to protein aggresomes in ventricular myocardium in vivo. Lentiviral overexpression of combination of four transcription factors Oct4, Sox2, cMyc and Klf4 from a single vector (gift of Gustavo Mostoslavsky, Boston University, USA) was used to induce pluripotency in the patient-derived dermal fibroblasts. These iPS cells show a human ES cell-like colony morphology, express pluripotency markers at the protein (alkaline phosphatase, Tra-1-81, Tra-1-60, OCT4, NANO, and SSEA4) and transcript level (OCT4, SOX2, NANO, REX1), and exhibit the methylation pattern in promoter regions of OCT4 and NANO genes, which is indistinguishable from that of conventional ES cells. In addition, these iPS cells carry the same genotype and disease-specific mutation as parental somatic cells, form teratomas in immunodeficient animals and differentiate to spontaneously beating cardiomyocytes in vitro. Further analyses are being carried out to assess the functional properties of ARVC-specific cardiomyocytes so as to determine whether they recapitulate the patient's disease phenotype in vitro. Thus, the ARVC-specific iPS cells generated in this study may serve as a replenishable source of cardiomyocytes for disease modelling and drug discovery.
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Proliferation and stemness – O1

A stem cell niche dominance theorem
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Tissue homeostasis and cancerous transformations are a consequence of reciprocal interactions between the epithelial tissue and lineages arising from cells with stem cell properties. This view has important consequences for the way we investigate complex multilevel systems. Motivated by experimental evidence for the dominance of individual stem cells in tissue organization, we developed a conceptual framework to analyze cross-level principles. Our analysis of cell-tissue relationships then led us to the formulation and proof of a dominance theorem, which states that the only logically feasible relationship between the stemness of cell lineages and the emergent fate of their tissue is one of dominance from a particular lineage. Our analysis is important to the discussion of stem cells as the cells-of-origin for cancer and provides a new perspective on the notion of stemness. We develop and discuss our mathematical analysis within the context of colorectal cancer. Our analysis provides support for the concepts of niche succession and monoclonal conversion in intestinal crypts as bottom-up relations, while crypt fission is postulated to be a top-down principle. The dominance theorem is an adaptation of Arrow’s (im) possibility theorem from collective choice theory. The present work is the first formulation and proof of a theorem related to cross-level principles tissue organization and possible cancerous transformations.
Hematopoietic stem cell (HSC) transplantation is a routinely used therapeutic method. Most of all currently registered stem cell-related clinical trials focus on HSCs. Selecting the most potent HSC population might improve the beneficial stem cell related therapeutic effect.

To analyze c-kit⁺ subpopulations for their proliferation and differentiation capacity, murine bone marrow c-kit⁺ cells were sorted for lin⁻, CD34, Sca1 and CD45. Proliferation potential was analyzed using a Colony-Forming-Unit (CFU) assay. Surface markers and gene expression were characterized by flow cytometry and real-time PCR.

Prior to CFU assay entire cell population was viable, lin⁻ and c-kit⁺. Their purity exceeded 92%. The major subpopulation was CD45⁺CD34⁻Sca1⁻ (21.25 ± 4.25%), followed by CD45⁺CD34⁺Sca1⁻ (15.00 ± 4.23%). Cells positive for CD45, CD34 and Sca1 and cells negative for all three markers formed smaller populations (0.74 ± 0.40% and 1.03 ± 1.25%). After CFU assay we found significant differences in the CFU count of analyzed subpopulations (P≤0.0026).

CD45⁺CD34⁺Sca1⁺ cells displayed the highest CFU count per utilized cell (0.470 ± 0.055; n=3), followed by CD45⁺CD34⁺Sca1⁻ cells (0.162 ± 0.019; n=5). CD45⁺CD34⁻Sca1⁻ and CD45⁻CD34⁺Sca1⁻ cells showed low CFU frequency (0.0032 ± 0.0009 and 0.0035 ± 0.0027, n=5, difference n.s.; Figure 1).

Interestingly, original CD45⁺CD34⁺Sca1⁻ cells after CFU displayed a larger Sca1⁺ population than CD45⁻CD34⁺Sca1⁺ cells (14.51 ± 5.21% versus 3.84 ± 0.72% CD34⁺Sca1⁺-kit⁻lin⁻ CD45⁺ cells). In CD45⁺CD34⁺Sca1⁺-derived cells, SPP1 and GATA2 genes, which are present in HSC niche, were upregulated whereas Wnt3a and VEGFA genes, which are mainly expressed in active and differentiating HSC, were strongly downregulated compared to cells derived from the CD45⁺CD34⁺Sca1⁺ subpopulation.

In conclusion, we present viable lin⁻c-kit⁺ cell subpopulations, specified by differential CD45, CD34 and Sca1 protein surface expression, are highly heterogeneous in proliferation and differentiation capacity. CD45⁺CD34⁺Sca1⁺ cells proliferate more readily and may differentiate more distinctively than CD45⁻CD34⁺Sca1⁻, while CD34 Sca1⁻ cells display a low proliferation potential. These findings might help deepen
the knowledge in c-kit+ HSC plasticity and therapeutic potential.

Figure 1
In the past years it has been shown that bone marrow-derived mesenchymal stem or stromal cells (BM-MSCs) have a high potential for cell-based therapies and tissue engineering applications because of their multilineage differentiation potential and their immunomodulatory properties. However, bone marrow presents several disadvantages, namely low frequency of MSCs, high donor-dependent variations in quality and the isolation procedure is painful and implies the risk of infection. In search of alternative sources of MSCs, the umbilical cord (UC) tissue gained more and more attention. Since the UC is discarded after birth, the cells are easily accessible without ethical concerns. We isolated a population of plastic-adherent mesenchymal stem cell-like cells from human UC-tissue, which exhibit a high proliferative potential and express several MSC markers, including CD44, CD73, CD90 and CD105 (negative for CD31, CD34 and CD45). Furthermore, the cells display multilineage differentiation potential.

The aim of this study was to shape the microenvironment of the cells with regard to different oxygen concentrations and cell-cell interactions with immune cells. Therefore, the oxygen consumption, as well as the metabolic activity and HIF-1α target gene expression were determined. In addition, immunomodulatory properties of MSC-like cells were analyzed by direct and indirect co-culture experiments using peripheral blood mononuclear cells (PBMC) in CFSE-based proliferation assays.

Our study revealed that UC-derived MSC-like cells consume 2-3 times less oxygen under hypoxic conditions (1.5% O₂, 2.5% O₂ and 5% O₂) as compared to 21% O₂ control. Hypoxic culture conditions caused stabilization of HIF-1α protein and subsequent regulation of its target genes, involved in glucose metabolism. Moreover, UC-derived MSC-like cells showed increased proliferation at 2.5% O₂.

Furthermore, our results demonstrated that MSC-like cells do not induce proliferation of allogeneic PBMCs in vitro. Additionally, co-culturing of MSC-like cells and PHA-stimulated PBMCs in direct or in transwell co-culture experiments led to a decrease of PBMC proliferation compared to PHA-stimulated control PBMCs, indicating invitro immunomodulatory properties.
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.
The discovery of so-called circulating endothelial progenitor cells (EPCs) has highly stimulated the field of vascular biology. During recent years different protocols have been applied to raise and expand such cells. However, a recent comprehensive comparison of obtained EPCs revealed that many of them are of hematopoietic origin and just mimic an endothelial cell surface phenotype. According to the published studies only the endothelial colony forming cells (ECFCs) seem to represent true circulating endothelial progenitor cells. Of note, ECFCs raised from human umbilical cord blood are phenotypically almost identical to human umbilical cord vein endothelial cells (HUVECs). Furthermore, similar to HUVECs their proliferation capacity is limited to a few passages. In this context it is often suggested that both cell types become senescent and stop to proliferate. Provided that both endothelial cell types are organized in a hierarchical manner similar to primitive hematopoietic cells, such a limited expansion would also be expected, if the culture conditions would not allow self-renewal of the more primitive cells. Favoring the latter hypothesis we aim to optimize the culture conditions for these endothelial cells.

For a number of different cell systems cell fate modulating features of biomaterials and matrices have been reported. To this end we decided to compare the impact of distinct biomaterials as well as basal membrane-derived matrices on the adherence capacity, the vitality, the cytotoxicity, the apoptosis rate and the expansion rate of primary human endothelial cells.

So far, we compared the impact of collagen V, fibrin, gelatin, heparin, hyaluron acid, laminin platelet lysate coated plastic dishes with conventional plastic dishes and a novel commercially available advanced plastic dish type from Greiner Bio-One GmbH. Additionally, we studied biological features of ECFCs expanded on different biomaterials used in tissue engineering including PVDF-gf, PTFE, PET, Texin 950, PDMS, L209S (PLLA), R203S (PDLLA), LR704, RG503, LT706, PCL, BAK1095, PEA-C and Alginate. Our current results will be presented.
CD133+ cells from bone marrow have a greater stem cell potential than CD133+ cells from stem cell apheresates

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Question: Endothelial progenitor cells (EPCs) play an important role in angiogenesis, which is essential for physiological processes as well as tumor growth. The primitive hemangioblast make up only 0.1-0.5% to total CD34+ cells and have the surface phenotype CD34+, CD133+, VEGF-R2+, CD31+. Cells exhibiting these markers are predominantly found in bone marrow (BM) and in stem cell apheresates (SA). However, the exact molecular mechanisms regulating the proliferation, differentiation and migration of EPCs as well as the interaction with niche cells still remain elusive. Our study focus on the biological distinctions between CD133+ cells from the BM and SA, using to analyse expression profile for EPC surface marker and genes as well as micro RNA (miRNA).

Methods: We characterised the differences between EPCs cells from the BM and SA by using CD133+ cells separating with Anti-CD133- microbeads. These cells were cultured for 21 days and samples were obtained at day 0, 2, 4, 8, 14 and 21. Surface marker e.g. CD133, CD31, CD34 and VEGF-R2 were determined by FACS. Genexpression had been analysed by realtime PCR and analysis for stem cell miRNAs.

Results: Results of FACS showed that CD133+ cells from BM as well as SA expressed the different surface markers in a similar level at day 0 and in all probes of the expansion culture (day 2-21). We could show that there are no differences at day 0 in the expression of different genes which are associated with endothelial differentiation like Prominin or VEGF-R2. Only the CD133+ cells from the BM showed up regulation for e.g. Prominin and VEGF-R2 in expansion culture from day 2 to 21. Interestingly the miRNA302 is up regulated in the purify EPCs from BM at day 0 as well as at day 8 and 21 of the expansion culture.

Conclusion: In summary we suggest that there is no difference between the phenotype of CD133+ cells whether they from BM or SA. Also there is no difference in gene expression in the purify EPCs. Only in the expansion culture the CD133+ cells from BM showed up regulation of some genes. We suppose that CD133+ cells from BM have more stem cell potential then cells from SA. This hypothesis will be supported by the observation that only
in CD133+ cells from the BM miRNA302 is up regulated. MiRNA302 is also highly expressed in embryonic cells. We postulate that CD133+ cells from BM have more stem cell potential than from SA although they have the same phenotypes.
Aging and replicative senescence of human mesenchymal stromal cells is accompanied by increased loss of RS-cell-subpopulation

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The role of adult mesenchymal stromal cells (MSC) in tissue maintenance and regeneration has received significant attention of late. MSCs in culture undergo senescence after a certain number of cell doublings whereby the cells enlarge and finally reach terminal growth arrest. In this study we have analyzed the behavior of subpopulation of rapidly self-renewing cells (RS-) cells. 22 samples of MSCs were isolated from bone marrow of donors between 22-87 years old. In general, RS-cell fraction was significant lower after 10 days cultivation in samples obtained of donors >50 years (end passage 2: 13,74±4,45% to 22,51±6,21%; p<0.01). With the increase of passage number RS-cell fraction decreased, independent of age of donors. In samples of younger donors (<50 years), the approval of RS-cells was slighter (passage 5: 7,45±3,45% to 16,38±6,29%). The seven samples of donors under 50 years old could always been cultivated until passage 10 (100 days), while MSCs from older donors stopped proliferation before. Interestingly, after RS-cell fraction was dropped down under about 6,65%, the terminal growth arrest was reached reproducibly in all samples, indicating a essential effect of RS-cells on proliferation behavior of the whole population. In flow cytometry approaches only 0,9±0,15% of RS-cells were positive for senescence indicating staining with β-Galactosidase, while the rest of population offered passage-dependent larger numbers of positive cells. Although telomere length varied particularly in donors, an telomere-dependent correlation to the decline of RS-cell subpopulation could not be statistically verified. Thus, the study demonstrated that in vivo and in vitro aging is accompanied by a loss of RS-subpopulation until its dropped under a certain niveau, which is characterized by terminal growth arrest.
Chemoresistance and apoptosis induction in head and neck squamous cell carcinoma
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**Background:** Resistance to apoptosis plays an important role in cancer therapies such as chemotherapy and radiation; whereas molecular processes for avoiding apoptosis mechanism are mostly unknown. A conventional therapy by HNSCC (head and neck squamous cell carcinoma) is a cyclic application of the cytostatic drug Paclitaxel. Usually a tumor recurs after chemotherapy.

**Method and Materials:** Human HNSCC cell lines were treated with Paclitaxel to induce apoptosis. Apoptosis induction was quantified by flow cytometry and western blot analysis. The processing and activation of caspase 3 was used as a read out system for Paclitaxel induced apoptosis.

**Results:** Paclitaxel induced programmed cell death in each treated HNSCC cell line. Nevertheless in every experiment at least some cells avoided cell death and finally survived.

**Conclusion:** These Paclitaxel resistance subpopulation could be cancer stem cells and should be further analyzed related to stem cell characteristic and their tumor inducing potential.
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Proliferation and stemness – P46

Stem cell characteristics in Polyposis nasi
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Background: Chronic Rhinosinusitis with Nasal Polyps (Polyposis nasi) is a chronic inflammatory disease of the upper airway with polyps extending from the middle meatus or ethmoid into the nasal cavity. Symptoms include nasal congestion, chronic rhinorrhea, hyposmia and facial headache. Histologically, the polyps are characterized by a highly edematous stroma and infiltration of inflammatory cells. Several hypotheses have been put forward regarding the underlying mechanisms including chronic infection, aspirin intolerance, epithelial disruptions, allergies and alteration in aerodynamics with trapping of pollutants. The underlying molecular mechanisms of the benign cell progression and the chronic recurrent nature of nasal polyps are still unknown. In parallel to the development and progression of malignant tumors, we postulate that the chronic growth of nasal polyps could be promoted by the activation and recruitment of a pool of tissue stem cells.

Methods: Total RNA from nasal polyps was isolated and a cDNA library was synthesized. Polymerase chain reaction (PCR) was performed in order to detect stem cell markers like CD133, Nestin, ABCG2 and Sox2. Cryostat sections of nasal polyps were prepared and stained against various stem cell marker proteins, such as Oct4, using immunohistochemistry (IHC).

Results: Using PCR we were able to detect the expression of the specific stem cell markers CD133, Nestin, ABCG2, Sox2. Furthermore, immunostaining revealed the existence Oct4-positive cells in nasal polyp tissue.

Conclusions: Our data strongly suggest the existence of cells with stem cell characteristics. We postulate that these cells are involved in the development and progression of Polyposis nasi. Further investigation of the role of stem cells in nasal polyps could allow for innovative rudiments for future treatment strategies.
Introduction: Culture medium for mesenchymal stromal cells (MSC) is frequently supplemented with fetal calf serum (FCS). FCS can induce xenogeneic immune reactions, transmit bovine pathogens and has a high lot-to-lot variability that hampers reproducibility of results. Human supplements have been used as substitutes for FCS before and several other groups demonstrated that pooled human platelet lysate (HPL) provides an attractive alternative. However, the composition of heterogeneous subpopulations might be affected by serum supplements and lot-to-lot variation of individual platelet lysates has not yet been addressed in detail.

Methods: In this study we compared the impact of serum supplements on initial fibroblastoid colony forming units (CFU-F). Subsequently, we addressed the activity of lysates from platelet units of individual donors on proliferation, in vitro differentiation and long-term culture of MSC. These data were correlated with chemokine profiles of HPLs.

Results: Isolation of MSC with either HPL or FCS resulted in similar CFU-F frequency, colony morphology, immunophenotype, and adipogenic differentiation potential. Osteogenic differentiation was more pronounced in HPL than in FCS. There was some variation in MSC proliferation with individual lysates but it was always higher in comparison to FCS. Proliferation of MSC correlated with the concentration of platelet-derived growth factor (PDGF) and there was a moderate association with platelet counts. All HPLs facilitated expansion for more than 20 population doublings.

Conclusions: Reliable long-term expansion was possible with each lysate of individual platelet units and this supports the notion that donor recipient matched or autologous HPL can be used for therapeutic MSC products. However, there was some variation in growth supportive potential and this correlated with PDGF concentration.
Identification and manipulation of endogenous adult stem cells
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Stem cells (SC) are characterized by the ability to renew themselves through symmetric and asymmetric cell division and to differentiate into a diverse range of specialized cell types. This implicates their potential for therapy in regenerative medicine. However, our understanding of how SCs are maintained or replenished during life-time until senescence is poor. In this project signaling cascades involved in the self-renewal process of SCs are examined. Furthermore, since SCs display an undifferentiated phenotype, novel genetic approaches are needed to specifically address SCs. Modern genetic techniques not only enable researchers to visualize individual cells in order to identify morphological characteristics but also place them in a position to simultaneously manipulate cellular responses. In order to monitor and trace stem cells we have generated and tested novel reporters to monitor Notch as well as Oct4 positive stem cells in vitro and in vivo. In combination with conditional gene inactivation and cell ablation experiments these reporters provide new tools to characterize endogenous adult SCs and will give valuable information not only for researchers but also for clinicians in search for novel therapeutic approaches.
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Proliferation and stemness – P57

Isolation and characterization of mesenchymal stromal cells from adult mouse kidney
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After an acute renal failure the kidney is able to regenerate. A role in this process is awarded to renal mesenchymal stromal cells. These cells may support the self-healing process via interaction with injured tubule cells by producing erythropoietin (Epo) or via stem/progenitor cell attributes with direct replacement of injured cells.

The aim of this study was to isolate and characterize renal mesenchymal stromal cells in vitro.

A population of mesenchymal stromal cells from adult mouse kidney was isolated by plastic adherence. The cells were characterized by different attributes: proliferation capacity (CFU-F), cell proliferation in NOX or HOX (2%O₂), telomerase activity, FACS analysis, gene and protein expression, as well as differentiation capacity. To explore whether isolated renal cells (k) have similarity to mesenchymal stromal cells from other organs, they were compared to mesenchymal lung cells (l), colon cells (c), bone marrow cells (bm) and fibroblasts (fb, isolated from murine skin).

No significant difference between renal cells and cells of other mesenchymal origin were shown in CFU-F-, cell proliferation and telomerase analysis. Cell proliferation ascertained showed a 2-fold better proliferation in NOX than HOX.

In FACS analysis, cells from kidney and colon could not be distinguished. Significant difference was shown by comparing kidney and lung (CD44 [k>l]), kidney and bone marrow (CD117 [k<bm], CD292 [k<bm], OCT ¾ [k<bm]) and fibroblasts and all other cell cultures (CD105 [f<k/l/c/bm], CD117 [f>k/l/c/bm], Sca-1[f<k/l/c/bm]).

Analysis of differences of renal cells to fibroblasts by immunoflourescence and RT-PCR showed differences in aSMA (k+/f-), cytokeratin (k+/f-), lim1 (k+/f+), pax8 (k+/f-), ksp (k+/f-) and erythropoietin (k+/f-). The gene expression of nephrin (k+/f+) and podocin (k+/f+) was different to the protein expression of nephrin (k+/f-) and podocin (k-/f-).

Analysis of differentiation capacity showed that only single cells are able to obtain adipogenic, chondrogenic or osteogenic characteristics.

In conclusion, renal mesenchymal stromal cells can be significantly distinguished to
different mesenchymal cell cultures. Only rare renal cells express Epo or are able to undergo an adipogenic, osteogenic or chondrogenic differentiation.
Proceedings of German Society for Stem Cell Research (PGSSCR)
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Regenerative medicine – O3

Unrestricted somatic stem cells support HSC proliferation in vitro and engraftment in vivo and possess no tumorigenic potential
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Hematopoietic stem cell (HSC) transplantation is a therapeutic option in the treatment of inherited diseases and leukemias. In many cases HSC transplantation is associated with complications including engraftment failure or long-term pancytopenia [1]. Mesenchymal stem cells have been identified as playing an important role in the support of HSC engraftment in animal models and patients due to their cytokine production [2, 3]. Recently, a new multipotent population of umbilical cord blood cells, unrestricted somatic stem cells (USSC), was identified which possesses intrinsic potential to develop into mesodermal, endodermal, and ectodermal tissue. It was shown that USSC produce various cytokines. [4]. Co-cultivation of HSC and USSC in feeder layer assays resulted in an amplification of the amount of CD34⁺ HSC demonstrating that USSC support proliferation of HSC in vitro [5].

Here, we investigated the effect of USSC on the engraftment of cotransplanted human HSC in the NOD/SCID mouse model. After 4 weeks, homing and engraftment of human cells to the bone marrow was significantly increased in mice cotransplanted with HSC and USSC (30.9%), as compared to the control group (HSC only) (5.9%, p=0.004). After 8 weeks, the median proportions of human cells detected in bone marrow were 24.2% in the cotransplanted group and 11.3% in the control group. The percentage of human cells maintaining their CD34 expression in the bone marrow was unaffected by cotransplantation of USSC. Apart from short-term entrapment in the lungs, USSC themselves could not be detected in the bone marrow or other organs. According to migration assay results and to expression of genes known to mediate HSC homing and migration, USSC showed no potential to migrate towards bone marrow. An in vivo tumorigenicity assay in nu/nu mice showed no tumorigenic potential of USSC which is a very important finding in regard to a possible use of these cells for regenerative medicine.

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Regenerative medicine – 07

Lineage of conversion of skeletal muscle derived precursor cells into cardiomyocytes – a promising autologous cell source for cardiac cell therapy

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Question: Cardiac cell transplantation is a promising approach for cardiac regeneration in heart failure patients. However, the ideal cell source has not been found yet. In the presented work we induced a lineage conversion of skeletal muscle derived precursors into cardiomyocytes avoiding gene manipulation, which can be easily translated into a clinical application.

Methods: Skeletal precursor cells were isolated from adult C57/BL6 mice. Following a primary expansion and purification according to a skeletal myoblast isolation protocol, the cell product was further cultured under hanging drop culture conditions. The forming cell clusters were characterized by immunohistochemistry and single cell patch-clamping.

Results: Under hanging drop culture conditions the purified cells showed a high lineage conversion rate towards cardiomyocyte-like phenotype. Besides synchronous beating of the clusters, these cells were highly positive for cardiac troponin, connexin43, cardiac myosin heavy-chain. Electrophysiological assessment under 8 Hz stimulation showed cardiomyocyte like shape of the action-potentials.

Conclusions: Despite an ongoing controversial discussion about skeletal precursor cells a cell source for cardiac cell therapy, we confirmed successful lineage conversion of those cells into a cardiomyocyte-like phenotype. This provides an outstanding alternative cell source for cardiac cell therapy which can be easily translated to clinical application.
Proceedings of German Society for Stem Cell Research (PGSSCR)  
(5th Annual Meeting)  
Regenerative medicine – O8

Noninvasive in vivo tracking of mesenchymal stem cells by MRI and evaluation of cell therapeutic effects

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**Question:** Stem cell transplantation is emerging as a promising approach for the regeneration of infarcted myocardium. However, there is little known about the fate of the transplanted cells since the means for tracking transplanted cells and measuring their therapeutic effects in vivo are limited. The goal of this study is the in vivo analysis of stem cell transplantation into cryo-infarcted mice hearts by magnetic resonance imaging (MRI).

**Methods:** Murine mesenchymal stem cells (mMSC) were isolated from bone marrow. After expansion lineage specificity was confirmed in vitro by adipo-, chondro- and osteo-differentiation and FACS-analysis for CD44 and Sca-1. mMSC were labeled with paramagnetic microspheres (Ø 1µm) and transplanted into the border zone of the infarcted myocardium subsequently after cryo-infarction. In vivo cell tracking and measurements of functional cardiac parameters were performed using a clinical 3T MRI-scanner (Philips Achieva) applying ECG-gated T2*-weighted imaging using a dedicated magnification coil for mice.

**Results:** Microspheres were phagocytosed by mMSC efficiently, without interfering with their proliferation and differentiation potential. A minimum of 50,000 transplanted mMSC could be clearly detected and co-localized by MRI up to five days after transplantation. Furthermore, compared to sham-controls, cell transplanted animals showed a significant improvement left ventricular function and reduction of the infarct scar three weeks postoperatively.

**Conclusion:** Transplantation of mesenchymal stem cells results in reduction of infarct size and improvement of left ventricular function. In addition, we could show that in vivo tracking of transplanted cells is feasible by MRI and opens new options to elucidate the mechanisms of cardiac cell therapy.
Cord blood-derived stem cells administered intravenously to newborn rats can engraft in brain and skin lesions and produce neuronal or epithelial progenitor cells

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CD133(+) cells isolated from umbilical cord blood (UCB) represent an established source of transplantable hematopoietic progenitors, and subpopulations of such cells have been shown to be capable of differentiating into mesenchymal lineages. Here we investigated whether CD133(+) UCB cells may contribute to the regeneration of injured brain and skin regions. A purified population of CD133(+) cells from human UCB was expanded in a stem cell medium, labeled with a fluorescent cell tracker and administered intravenously to 9-day-old rats subjected to unilateral carotid artery ligation and subsequent hypoxia (8% O₂) for 120 minutes on day 7 after birth. The capability of these cells to differentiate into the neurogenic lineage was confirmed in vitro by RT-PCR and immunostaining of differentiation markers including NeuN and MAP2B. Their biodistribution in vivo was investigated by fluorescence microscopy and immunostaining of human nuclear antigen. Cell differentiation in vivo was assessed histologically and immunohistochemically using various markers. Animals which received a cell-free buffer and sham-operated animals treated with the stem cells served as controls. In caspase-3-positive brain lesions of all rats subjected to hypoxic-ischemic injury, numerous labeled cells were detected that expressed human nuclear antigen and CXCR4. Overlapping staining of NeuN and MAP2B revealed neurogenic differentiation of the transplanted cells. Furthermore, in the wounded jugular skin of all rats subjected to hypoxic-ischemic brain damage or sham-operation abundant human cells were detectable at the site of injury. Human cells located around the epidermal basement membrane and hair follicle bulges were positive for CD133 and epidermal differentiation markers, whereas CD133(-) human cells were found in the dermal connective tissue. Thus, UCB-derived stem cells can be mobilized from blood vessels into brain and skin lesions, where they may contribute to tissue regeneration.
Efficient ZFN-based gene inactivation in transgenic human iPS cells as a model for gene editing in patient-specific cells
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Gene targeting by homologous recombination via customized zinc-finger nucleases (ZFN) is a powerful method to manipulate the genome and correct genetic defects. Although efficiency of ZFN based homologous recombination has been shown to be significantly higher than by means of conventional gene targeting, the selection of suitable clones still requires cells that proliferate in culture. Clinically applicable ZFN-based gene correction in patient-specific cells was hardly possible so far, due to the inability to sufficiently expand most adult (stem and progenitor) cells in vitro. However, the availability of human induced pluripotent stem (hiPS) cells with their almost unlimited potential for proliferation and differentiation now offers novel opportunities for the development of patient-specific regenerative therapies.

As a first step towards ZFN-based gene targeting, a non-viral gene-transfer approach with transfection rates of up to 60% and high cell vitality was established for hiPS cells. Aiming at the development of a general ZFN-based recombination approach in hiPS cells, we investigated the functionality of an eGFP specific ZFN in an eGFP transgenic hiPS cell clone. Targeting of the eGFP via non-homologous end joining resulted in up to 3% eGFP<sup>neg</sup> cells, and mRNA expression of genetically modified eGFP was shown in sorted eGFP<sup>neg</sup> hiPS cell clones.

Ultimately, the development of a generally applicable protocol for ZFN based site-specific recombination and gene correction in patient-specific hiPS cells may enable the development of cellular therapies for various genetic diseases.
Therapeutic application of stem cell derivatives requires large quantities of cells produced in defined media and cannot be produced via adherent culture. We have applied human induced pluripotent stem (hiPS) cells expressing eGFP under control of the OCT4 promoter to establish the expansion of undifferentiated human embryonic stem (hES) and hiPS cells in static suspension culture. A defined culture medium has been identified that results in up to six-fold increase in cell numbers within four days. Our culture system is based on initial single cell dissociation which is critical for standardized process inoculation. hES/hiPS cells were expanded for up to 17 passages. The cells maintained a stable karyotype, their expression of pluripotency markers and their potential to differentiate into derivatives of all three germ layers. The ability to expand hES/hiPS cells in a scalable suspension culture represents a critical step towards production in stirred bioreactors.

Standardized fully controlled stirred tank reactors are already widely used in biopharmaceutical industry for culture volumes of up to 10,000 L. However, standard cell lines used for the production of recombinant factors are usually homogenous and robust and therefore relative easy to maintain under standardized conditions. In contrast, cultures of hiPS/hES are relatively heterogeneous, particularly when combined with differentiation. A direct transfer of these complex cultures to established bioreactor systems is therefore challenging. Applying our experience in static suspension culture we have systematically performed translation to stirred bioreactors. This includes optimization of inoculation, control of hydrodynamics and feeding strategies to establish stepwise adaptation to scalable mass expansion of hiPS/hES cells. In addition we are exploring direct combination of expansion and differentiation processes in stirred bioreactors aiming at the translation of stem cell research to clinical practice.
Modulation of pathological phenotype of red blood cells generated ex vivo from hematopoietic stem cells in patients with sickle cell disease
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Background: In sickle cell anemia (SA), synthesis of pathological hemoglobin S (HbS) induces sickling of red blood cells (RBC), their reduced deformation capacity and increased adherence to the endothelial wall. This results in vaso-occlusive events, responsible for much of the morbidity and mortality. Fetal hemoglobin (HbF) is known as the most potent modifier in disease severity, since it protects RBC from pathological HbS polymerization. The aim of the study was the modification of RBC pathology by the ex vivo generation of RBC, containing a high level of HbF, derived from hematopoietic stem cells (HSC) of SA patients.

Material and Methods: Peripheral blood (PB) CD34+ HSC from SA patients were cultured over 25 days in an in-vitro erythropoiesis assay. Generated RBC were compared with native RBC from the same patient for their level of HbF (HPLC, flow cytometry), the expression of adhesion molecules CD36, Integrin a4ß1, CD239, ICAM-4, CD47, CD147 (flow cytometry), their deformation capacity (ektacytometry) and adhesion to human laminin (flow adhesion assay). Results: CD34+ HSC were found to be increased in PB of SA patients (~10fold) and could be isolated in a sufficient way. The applied in-vitro erythropoiesis assay was able to generate 100% enucleated RBC with an at least 16,500fold amplification. Compared to native SA-RBC, ex vivo generated RBC showed an increased HbF level (~ 5-fold). Whereas native SA-RBC showed strong adhesion to laminin, this was reduced or absent in ex vivo generated RBC. In line with this, cultured RBC showed a modified expression of adhesion molecules. In contrast to the reduced deformation of native SA-RBC, the deformation capacity of ex vivo generated RBC was also normalized.

Conclusion: RBC generation under ex vivo conditions allows for the modulation of the pathological phenotype in SA. Besides an increased level of HbF, ex vivo generated cells show a normalized deformation capacity and reduced adhesion to elements of the endothelial wall. Further work will be carried out to identify underlying pathways. As the most interesting clinical application, such ex vivo generated RBC might be suitable as an
autologous transfusion product, able to reduce the frequency of vaso-occlusive events and to circumvent storages of compatible RBC units.
Evidence of neurogenesis in the adult enteric nervous system in the postnatal human ENS brought new perspective for cell therapy and neural regeneration. Although these cells do show a decreased plasticity in the aging gut, they are a potential source for autologous neural stem cells. The appendix might be the appropriate location with a sufficient amount enteric nervous tissue where these cells could be easily harvested. Appendices from adults and children (age ranged from 6 month to 69 years) were obtained from two surgical centers. From each appendix tissue samples were collected and processed for immunohistochemistry. The remaining tissue was used for the isolation of myenteric and submucous plexus. Muscle and submucous layer were separated and enzymatically digested, so that pure myenteric plexus could be harvested, dissociated and seeded in 25 cm² flasks. Every 2 days, half of the medium was replaced. After 6 day in culture free floating neurospheres as well as neurons and glia cells were easily to discriminate. The supernatant with free floating neurospheres was cultivated for another ten days. Than neurospheres were dissociated and resuspended in an extracellular matrix gel with to mimick the tissue in which the stem cells will have to be transplanted. Immunostaining for neuronal and glial markers were performed and cells analyzed. Sections from the paraffin blocks were stained for nestin, Musashi 1 and Integrin-B1. After 6 days in cell culture first neurospheres were seen which could be cultivated up to 40 days. After dissociation of neurospheres, the neuronal spheres and isolated neuronal cells developed a intricated network with glia, neurons and interconnecting fibers, as seen in primary enteric cultures before. Immunostaining with PGP, β-tubulin III, GFAP and S-100 showed positive stainings in different cell types. The Immunohistochemistry of the histological preparations showed an increase of nestin positive cells in the inflamed appendices (Fig.1). The enteric nervous system is a autologous neural stem cell source. Using the appendix as a potential targeted opens up a new perspective which might lead to a relatively unproblematic harvest of neural stem cells without endangering the patient.
The IFN-γ-inducible enzyme indoleamine 2,3-dioxygenase (IDO) catalyzes the conversion of tryptophan to kynurenine. This enzyme is able to mediate antimicrobial functions which result in an inhibition of e.g. *Toxoplasma gondii* or *Staphylococcus aureus*. Furthermore, it is known that IDO-activity also leads to immunoregulatory effects, which inhibit T cell proliferation. Here, we analysed the influence of the human cytomegalovirus (hCMV) on IDO-mediated effects in human mesenchymal stem cells (MSC). This pathogen is known to be able to inhibit the IFN-γ-signalling pathway. As all herpes viruses hCMV has the characteristic ability to remain latent within the body over long periods. The infection or reactivation can be life threatening for patients, who are immunocompromised for example: patients with HIV, organ transplant recipients or neonates.

We recently detected that IFN-γ-stimulated human MSC mediate antibacterial, antiparasitical, antiviral and immunoregulatory effects in an IDO-dependent manner. The IDO-dependency of these effects was shown by the abrogation of antimicrobial effects by the supplementation of tryptophan or by the addition of the IDO-specific inhibitor 1-methyltryptophan.

Here, it is shown that an infection with hCMV reduces the IFN-γ-induced IDO-activity in human MSC. This suppressive effect of hCMV can be explained by an inhibition of the IFN-γ signalling pathway. We observed that the inhibition of IDO-activity results from a dramatic reduction of IDO transcription and translation in infected cells. Consequently, these cells were no longer able to restrict bacterial and parasitic growth and, furthermore, these hCMV-infected cells lost their IDO-mediated immunosuppressive capacity. This coherence between virus infection and inhibition of IDO-induction still gains more importance in the case of organ or haematopoetic stem cell transplantation. From clinical observations it is known that after transplantations an active hCMV infection results in an increased risk of infection and in a higher risk of transplant rejection. Both effects could be explained by the observations.
of the hCMV-infection’s influence on IDO-activity in our *invitro* systems.
New marine collagen source induces high level of collagen type II synthesis in chondrocytes but not in mesenchymal stem cells
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The extracellular microenvironment plays a significant role in controlling cellular behaviour. Identification of appropriate biomaterials that support cellular attachment, proliferation and most importantly lineage-specific differentiation is critical for tissue engineering. Collagens exert important functions as cellular microenvironment and therefore make them ideally suited to use as biomaterial for tissue engineering (TE).

Here we designed collagen scaffolds from marine sources for cartilage TE. Since mesenchymal stem cells (MSCs) have multipotent capabilities including differentiation towards the chondrocytic lineage and MSCs have been used successfully in animal models to regenerate articular cartilage, we tested the MSCs concerning their cartilage-forming ability in marine collagen scaffolds. Using adult mesenchymal stem cells from human bone marrow biopsies analyses show low cell attachment, low new extracellular matrix deposition as well as low differentiation capacity towards the chondrocytic lineage implying this as an unsuitable way for cartilage TE. However, primary adult chondrocytes seems to be a suitable cell source for engineering hyaline-like cartilage. Qualitative analyses of these bioscaffolds show chondrocyte cell adhesion to the matrix, cell proliferation as well as new matrix deposition. Based on (immuno)histological analyses this newly synthesized matrix consists of proteoglycans and collagens - the major constituents of hyaline, articular cartilage. Quantitative analyses on protein level show a collagen ratio of the chondrocyte specific marker collagen type II to the dedifferentiation marker of chondrocytes, collagen type I, of up to 50:1. From this, the in vitro engineered marine bioscaffold resembles to a hyaline-like cartilage with a mechanically functional collagen type II network.

Since in vivo cartilage damage often ends in a fibrocartilagenous, collagen type I containing matrix which is unable to withstand the demands of the mechanical environment of articular joints, the marine scaffolds may show promising results for the functional repair of articular defects using chondrocytes but not MSCs. Moreover, the new collagen source is a good alternative to the widely used bovine collagen which is associated with higher risks of BSE and TSE.
Background: Neuropathic pain is a very complex disease, involving several molecular pathways. Current available drugs have a generalized nature and act only on the temporal pain symptoms rather than being targeted towards the several mechanisms underlying the generation and propagation of pain. Nowadays, pain research is directing towards new molecular and cellular methods, such as stem cell therapy. Aim of this study was to verify whether human mesenchymal stem cell (hMSC) transplantation could be an useful cell-based therapy for neuropathic pain treatment.

Material and Methods: We used spared nerve injury (SNI) mouse model of neuropathic pain to assess the possible use of hMSCs as anti-neuropathic tool. Biomolecular, immunocytochemical and immunohistochemical analysis were carried out in order to verify stem cell-mediated changes in molecular mechanisms underlying pain development and maintenance.

Key Results: Human MSCs were transplanted in the mouse lateral cerebral ventricle. Stem cells injection was performed 4 days after sciatic nerve surgery. Neuropathic mice were monitored 7, 10, 14, 17, and 21 days after surgery. Human MSCs were able to reduce pain like behaviours, once transplanted in cerebral ventricle. Anti-nociceptive effect was detectable from day 10 after surgery (6 days post cell injection). Transplanted MSCs reduced the mRNA levels of the pro-inflammatory interleukin IL-1ß mouse gene, astrocytic, microglial cell activation and premature senescence-associated neuronal suffering. Indeed, hMSCs were able to decrease the β-galactosidase over-activation positive profiles in the cortex of SNI/hMSC-treated mice compared to SNI/vehicle mice.

Conclusions: Despite over fifty years of research there are no valid treatments over time and neuropathic pain can be classified as an incurable disease without treatment. Mesenchymal stem cell therapy represents the new promising potential treatment for neuropathic pain relief.
**C-kit positive cells of the heart consists of mast cells and cardiac progenitor cells populations**

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**Background:** Stem cell therapy is actively being explored as a novel method to regenerate damaged myocardium. It has been established that the heart contains a reservoir of stem cells (c-kit+, sca-1+, isl-1+) having the capability for ex vivo and in vivo differentiation toward the vascular and cardiac lineages and showing cardiac regeneration potential. The aim of the present study is to characterize cardiac stem cells in the human heart tissue (appendix of the right atrium) harvested during coronary artery bypass grafting.

**Material and Methods:** We analyzed samples of heart appendix by fluorescence-activated cell sorting and immunohistochemical method for markers of stemness (C-kit), hematopoietic cells (CD34, CD45), blood lineage markers (Lin) and tryptase. We isolated c-kit cells using explant culture and anti-c-kit antibody. Coculture with neonatal rat cardiomyocytes was used to analyze cardiomyogenic potential of c-kit cells.

**Results:** C-kit positive cells consist about 0.79±0.32% of total cell population of appendix of the right atrium and were largely negative for CD34 and cocktail of blood lineage markers Lin. We identified two populations of C-kit positive cells: about 60% of cells were C-kit(+)CD45(+), which might populate the heart via circulation and another one were CD45 negative (40%). Immunohistochemical research of autopsy samples of left ventricular tissue showed that majority of C-kit are distinctly positive for CD45 and tryptase, suggested that they are mast cells and only a small population of C-kit(+)CD45(-)triptase(-) cells represent human cardiac stem cells.

Using magnetic cell sorting c-kit positive cells could be successfully isolated from human heart tissue and expanded in vitro. C-kit cells undergo cardiomyogenic potential when cocultured with neonatal rat cardiomyocytes.

**Conclusion:** Thus appendix of the right atrium could be an alternative source of autologous cardiac stem cells.
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Regenerative medicine – P17

Tendon progenitor cells - their appearance and distribution in degenerated and ageing tendon
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Tendons are specialized tissues that connect muscle to bone and transmit the forces generated by muscle to bone, resulting in joint movements. They are characterized by a sparse vascular bed, low cell density and a decreased healing capacity. Only little is known about the molecular composition of tendon cells, their extracellular matrix and their progenitor cells. We analysed biopsies of human supraspinatus tendon (ST) from patients of various ages for the existence and the distribution of progenitor cells using immunohistochemistry. The expression of various cellular markers: for tendon cells (Scleraxis, Tenomodulin), extracellular matrix (ECM) proteins (Col1A1 and 3A1, MMP-2, -9, Lox), progenitor cells (Nestin, CD 133, CD11b, VCAM-1) and for cell differentiation (Aggrecan, Sox-9, Osterix, REST, CoREST) was studied with RT-PCR and qRT-PCR. In tendon biopsies of patients >50 years the yield of total RNA was significantly lower than in biopsies of younger patients (<50 years) and the expression frequencies of progenitor cell markers was decreasing with age. To further study tendon ageing we isolated RNA from Achilles tendons of young adult (6 weeks) and old NMRI mice (>24 months) and performed expression analysis of Nestin, Aggrecan, Scleraxis, Col1A1, Col3A1, MMP-2, MMP-9 and Lox. The expression of Scleraxis and Aggrecan was significantly down-regulated and Nestin mRNA was not detectable in tendons of old mice. The ECM proteins MMP-2, Col1A1, Col3A1 and Lox showed significant down-regulation in old mouse tendons, whereas MMP-9 was up-regulated in old mice. We also analysed biopsies of ruptured human ST for the expression of the above mentioned markers, interestingly the age dependency was not detectable anymore. Regarding to tissue degenerations, like fatty infiltrations, hypervascularization or cartilagenous modifications as well as the increase of the dense connective tissue in the ST, the expression of cellular markers revealed significant differences. Between males and females we found the most striking and highly significant differences in the expression of ECM markers. In conclusion progenitor cells are still detectable in older individuals although to a lesser extent, depending on individual susceptibility and pathological status of the patients.
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Regenerative medicine – P47

Intra-myocardial homing of adult stem cells: glandular vs. mesenchymal stem cells

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Background: Applying stem cell therapy in a failing myocardium, the dimension of an intra-myocardial cell homing is significant. Thus a comparison of the homing potential between glandular (GSCs) and mesenchymal stem cells (MSCs) was performed within the myocardium of a big animal model.

Methods: In African Bore Goats the intramyocardial homing and the engraftment of glandular stem cells and MSCs (CD133+) were evaluated. Glandular stem cells were characterized by red PKH26 respectively green PKH67 (MSCs) markers. After a left lateral thoracotomy and exposure of the left heart ventricle a mix of one million of each cell type was injected into three locations of the goat’s myocardium of the left ventricle. Myocardial samples were taken after one resp. three hours, others were harvested 6 weeks after injection (n=5). Frozen tissue slices were generated and examined for the marked cells.

Results: Using a mix of an intra-myocardial injection of GSCs and MSCs, solely in MSCs (green) a significant cell migration into the surrounding myocardium (n=3) was observed, more expressed after 3 hours than after one hour. Additionally within 5 goats with three intra-myocardial injections in each after 6 weeks nearly all GSCs remained within the myocardium while the MSCs disappeared almost completely. Within the frozen myocardial slices 98% of the marked stem cells were identified as GSCs (red) but just 2% as green MSCs. (P≤0,05)

Conclusion: Due to a 98 % homing of GSCs combined with the ability developing cardiomyocyte like cells, glandular stem cells might become a very promising treatment option in the therapy of a failing myocardium.
Identification and characterization of the CD4+AT2R+ T cell subpopulation in rats and humans

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Introduction: Following acute myocardial infarction (MI), the heart suffers, beside ischemia-induced direct myocardial injury, from a subsequent indirect damage through improper inflammatory reaction. A wealth of information indicates that the renin-angiotensin system (RAS) can interfere with acute cardiac remodelling and inflammation processes during cardiovascular injury. Given the recent observations showing that AT2 receptors (AT2R) are abundantly expressed in immunocompetent cells and involved in cell-mediated inflammatory injury, it appears likely that AT2R may exert their actions through interfering with CD4+ T cell-involved inflammatory processes in response to ischemia-induced cardiac injury.

Methods: We isolated CD4+ T cells from peripheral blood of rats with acute myocardial infarction and healthy donors using Ficoll gradient centrifugation and MACS technology. The CD4+AT2R+ and CD4+AT2R- T cell populations were further purified by FACS sorting. The purity of isolated cells and the expression of AT2R and various cytokines were confirmed using FACS analysis, cytopsin staining and RT-PCR methods. To study the role of AT2R on cytokine production, the CD4+AT2R+ and CD4+AT2R- T cell were treated with AT2R agonist (compound 21), or angiotensin II in combination with AT2R antagonist PD123319 (PD).

Results and Discussion: Here, we defined the CD4+AT2R+ T cell subpopulation in the peripheral blood of rats and humans. These blood CD4+AT2R+ T cells were characterized by upregulated expression of transcription factor forkhead box protein FOXP3 [Figure Nr.1] and various cytokines (anti- and proinflammatory). In addition, AT2R activation enhanced production of anti-inflammatory cytokine IL-10 in the CD4+AT2R+ T cells, but not in the CD4+AT2R- T cells [Figure Nr.2]. This study suggested a novel AT2R-mediated cellular

Figure 1
mechanism via the CD4+AT2R+ T cell subpopulation in suppressing inflammatory injury in the heart.

figure 2
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Tumor stem cells – O13

Murine breast-cancer-cell/mesenchymal-stem-cell hybrids exhibit enhanced drug resistance to different cytostatic drugs
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Several data of the past 15 years suggest that cell fusion is not only a process of normal development and tissue homeostasis, it is also assumed to participate in cancer progression. Because of the high fusogenic capacity of cancer cells cell fusion may promote diversity in cancer cell populations. Therefore fusion of a cancer cell with another cancer cell, an immunocompetent cells or even an adult stem cell can give rise to hybrid cells with new properties.

Question: What is the role of cell fusion in cancer progression?

Methods: Characterisation of hybrid cells derived from spontaneous fusion of murine 67NR-Hyg breast cancer with puromycin resistant bone marrow derived MSCs, from Tg(GFPU)5Nagy/J mice in vitro, by Realtime PCR Arrays for analysing breast cancer and multi drug resistance associated genes, measurement of proliferation rate under influence of chemotherapeutic agents Doxorubicin, 17-DMAG, Etoposide, Paclitaxel and 5-FU over a period of 3 days, FACS analysis of Rhodamine 123 efflux and Western Blot analysis.

Results: Realtime PCR Arrays for analysing breast cancer and multi drug resistance associated genes revealed an increased expression of drug resistance proteins, in particular ABC-transporters. These findings correlated with high ABC-transporter mediated Rhodamine 123 efflux of hybrid cells in comparison to parental cell lines detected by FACS analysis. XTT-proliferation-assay after culturing cells over a period of 3 days among different concentrations of chemotherapeutic agents mentioned above showed an increased drug resistance of hybrid cells compared to parental cells for Doxorubicin, 17-DMAG, Etoposide and Paclitaxel. Hybrid cells exhibited an altered morphology under influence of chemotherapeutic agents, especially Doxorubicin and 17-DMAG, but even survived at concentrations of 10µM. Drug resistance of hybrid cells may be reversed by addition of 50µM Verapamil in some cases of tested chemotherapeutic agents.

Conclusions: We conclude that cell fusion between breast cancer cells and MSCs can give rise to hybrid cells with altered properties that direct enhanced ABC-transporter mediated drug resistance and therefore may promote cancer cell survival during chemotherapy.
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Tumor stem cells – O18

Wnt/β-catenin activity is essential to turn the epigenetic state to “ON” in salivary gland stem cells to create cancer stem cells
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Little is known about the processes by which cancer stem cells arise in the different tissues. Our analysis of aggressive squamous cell carcinomas (SCCs) of the salivary gland in human patients suggested a specific link to the Wnt/b-catenin and Bmp signalling systems. Using a genetically modified strain of mouse in which Wnt signalling is up-regulated and Bmp is suppressed, we found that Wnt/b-catenin promotes the transformation of normal stem cells into cancer stem cells through an epigenetic mechanism. Mouse SCCs contained high numbers of CD24+CD29+ cancer stem cells. As few as 500 of these cells sufficed to cause tumours in NOD/SCID mice. This contrasts mice in which only one of the signalling systems was altered albeit high numbers of stem cells and efficient tissue regeneration, they had no apparent tumours. We discovered that the difference of normal compared to cancer stem cells in the salivary gland is an up-regulation of specific pluripotency genes, e.g. Dppa5, as well as a global changes in trimethylated Lysine 4 and 27 of histone 3. This indicates an increase of active chromatin and a decrease in the repressive form, which suggests a mechanistic explanation for the change of cell fate. Cancer stem cells of the salivary gland grow as non-adherent spheres and retain the capacity for differentiation if b-catenin is inhibited. This depends on repressive chromatin, as shown by the fact that 5-azacytidine or HDAC-inhibitors restore stemness. Our data opens new strategies for future cancer therapies in humans.
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Tumor stem cells – O27

Leukemia stem cell candidates in acute myeloid leukemia predict refractoriness to conventional chemotherapy and adverse clinical outcome
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We have shown that leukemia stem cells candidates (LSCC) can be prospectively identified by high activity of aldehyde dehydrogenase (ALDHbr) among the leukemia blasts from the marrow of patients with acute myeloid leukemia (AML). These LSCC demonstrated functional characteristics of stem cells in vitro and in xenogenic transplantation models.

Methods: In this report we have studied the relationship between the frequency of LSCC at diagnosis with persistence of leukemia blasts after induction chemotherapy as well as with long-term clinical outcome. We have identified subsets among the LSCC and correlated their individual functional properties with the corresponding marker profile using single cell sorting.

Results: The percentage of LSCC (ALDHbr) in 101 patients with AML ranged from 0.01% to 12.90% with a median of 0.51%. Frequencies of LSCC among the leukemia blasts at diagnosis correlated significantly with the persistence of leukemia after the first induction chemotherapy (n=79, Spearman R=0.7797, P<0.0001). During the observation period of 24 months, 21 of 60 patients with high levels of LSCC died as compared to 7 of 41 patients with low levels of LSCC (p=0.029). The overall survival (OS) probability for the patients with high levels of LSCC was significantly worse (p=0.05) than in those with low LSCC. Characterization of these LSCC at a single cell level showed that a varying proportion, i.e. 15% to 78% of their progeny cells demonstrated the same chromosomal aberrations as the original leukemia population, indicating the presence of normal HSC among our preparation of LSCC. The LSCC were more resistant to chemotherapy as compared to the other leukemia blasts and co-culture with MSC further increased the resistance of the ALDHbr cells against chemotherapy (n=3, p<0.001).

Conclusions: Thus high frequencies of LSCC at the time of diagnosis predict persistence of leukemia blasts, failure to achieve CR within the first cycle and poor overall clinical outcome and hence represent an independent poor prognostic factor.
Malignant brain tumors are amongst the most lethal solid tumors. The gold standard in therapy is concurrent radiochemotherapy, using the alkylating compound temozolomide (TMZ). It has been proposed that stem-like tumor cells mediate therapy resistance and regrowth.

We identified stem-like cells in human glioblastoma, gliosarcoma, oligodendroma and oligoastrocytoma. These cells exhibited a substantial heterogeneity with respect to proliferation rate and growth as spheres, adherent or semi-adherent cultures. Based on the expression of the intermediary filaments Nestin and GFAP, the transcription factors Sox2, Oct4, Nanog, as well as regulators and signalling molecules, such as p53, EGFR, PDGFRα, and PTEN we defined subtypes of stem-like brain tumor cells (SCIC). CD133 expression varied largely between different SCIC lines. We determined the responsiveness of SCIC subtypes to TMZ and chloroquine and related it to the expression of the repair enzyme MGMT and key players in apoptotic and autophagic processes.

Dose curves showed that responsiveness to TMZ was significantly different. Strong responsiveness to TMZ did not only depend on the methylation status of the MGMT promoter but on additional features, some of which appeared to be related to the SCIC stemness state. Co-application of chloroquine, a drug used in malaria prevention, which presumably affects autophagy, enhanced responsiveness of SCIC to TMZ in a dose-dependent manner. Both, TMZ and chloroquine induced cleavage of PARP, a key player in apoptosis and a measure for caspase 3 activity. The levels of cleaved PARP, however, differed largely between the various SCIC lines. Expression of Beclin and LC3B, proteins which are associated with different autophagy-associated processes were up-regulated in some but not all SCIC-lines. This indicates, that different SCIC subtypes respond differently to cell death inducing reagents and parallels our findings concerning the different proliferation and differentiation capacity of SCIC subtypes.
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Tumor stem cells – P8

Biomarkers associated to human esophageal stem cell-like
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The incidence of adenocarcinomas of the esophagus has increased during the last years. Many studies focused on the presence of cancer stem cells that have the capacity to regenerate tumors. Tumor initiating cells have the ability of self-renewal and proliferation, are resistant to drugs, and might express surface markers that were associated to stem cells. Moreover, recent studies demonstrated that radioresistance might be also caused by cancer stem cells (CSC). Isolation and identification of cancer stem cells in human tumors and in tumor cell lines are important steps for a further functional characterization of cancer stem cells, in order to find new ways to destroy them. The present study focused on characterization by flow-cytometry of the antigen expression of several biomarkers (CD24, CD44, CD71, CD105, CD117, CD133, CD166, CD200, EpCAM, E-cadherin, beta-catenin) associated to esophageal cells KYSE-150. Cells were cultivated in DMEM:F12 cell culture medium containing different amounts of fetal calf serum (FCS) (from 2% to 20%) or serum replacement (10% - 20%) and growth factors (bFGF, EGF). Moreover, CD44+ cells isolated after magnetic sorting using were further cultivated and antigenicity of subpopulations compared.

In addition, proliferation through cell cycle phases was also studied by using propidium iodide staining, followed by flow-citometry analysis. The results obtained demonstrated a differential expression of the biomarkers taken under study, depending on cell culture conditions used. Proliferation was also influenced since DNA analysis showed major changes in S-phase distribution. These results might add to identification of esophageal stem cells and facilitate the studies on carcinogenesis. Further studies will bring new data concerning evaluation of radio- and chemoresistance, in order to establish new protocols which might eliminate/diminish the tumours, significantly contributing to the immunotherapeutical management of esophageal tumors.
Cylindrospiradenomas are benign skin appendage tumors that can develop at multiple sites of hair follicle bearing skin as a result of Brooke-Spiegler Syndrome (BSS). The autosomal-dominant BSS is associated with mutations in the CYLD gene that encodes a deubiquitinase which inhibits NF-kB signalling resulting in an anti-inflammatory and anti-proliferative effect. Following-up our previous hypothesis that these tumors arise hair follicle stem cells (HFSC) or their progeny (Massoumi et al. JID 2005), we first immunostained cylindrospiradenoma sections from three BSS patients for the human HF epithelial stem cell (HFSC) markers keratin 15 (K15) and the immune privilege marker CD200. Interestingly, multiple K15 and CD200 positive, but β1-integrin-negative cells were found lining the tubular tumor structures, while most of the epithelial tumor nodules were K15-negative but brightly β1-integrin-positive. This suggests that cylindrospiradenoma nodules in BSS are derived from immunoprivileged HFSC-like cells and share some characteristics with highly proliferative, undifferentiated basal layer keratinocytes.

Interestingly, BSS patients also show extensive T cell infiltrates in tumors and tumor-free regions of their scalp and strong, ectopic expression of MHC class II molecules on the ORS of their hair follicles. This suggests that inflammatory processes precede or accompany tumor formation and growth.

To test whether anti-inflammatory agents inhibit tumor growth, we established a serum-free assay normally used for hair follicle organ culture that allows the maintenance of cylindro(spiradeno)ma fragments for up to 6 days in vitro. Addition of Na-salicylate resulted in increased cell death in treated compared to untreated cylindrospiradenoma fragments. Thus, administration of anti-inflammatory agents may offer a pharmacological alternative to surgical cylindrospiradenoma management.
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Tumor stem cells – P41

Heterogeneity of stem-like human glioma cells
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Glioblastoma multiforme (GBM) is one of the most lethal solid tumors. GBMs grow invasively, develop resistance to radiation and chemotherapy, and frequently recur. Recently, glioma were classified into four distinct molecular entities, the classical, mesenchymal, neural and proneural subtype based on genomic and transcriptomic data obtained with brain tumor tissue. It remains unclear whether stem-like glioma cells, which constitute a small subfraction of the brain tumor cells, could be grouped into similar categories. We analyzed the features of stem-like glioma cells from glioblastoma, gliosarcoma, astrocytoma, oligodendroma and oligoastrocytomas. Primary cultures of glioblastoma and gliosarcoma consisted of varying amounts of different cell types. Growth in serum-depleted medium containing the growth factors EGF and bFGF resulted in progressive enrichment of stem-like cells. Co-expression of the intermediary filaments Nestin and GFAP as well as prominin-1/CD133, factors typically found in adult neural stem cells/progenitors, was observed only in a subfraction of stem-like cells and expression of the transcriptional regulator Sox-2 varied largely. In keeping with their stemness features, coexpression of Nestin and the stemness factors Nanog or Oct4 was detected in all populations of stem-like cells. Expression of neurofilaments, doublecortin, DLX, Pax6, PSA-NCAM and other neural markers was additionally observed, suggesting that the majority of the populations of stem-like cells would belong to a neural or proneural group. mRNAs of the factors Myc, Gli, PTEN, Rest, Hif1α and p53, which affect stemness and tumorigenicity, respectively, were detected in all populations of stem-like cells. The major differences, however, concerned the protein expression levels. In particular, the level of p53 protein varied largely, even though p53 RNA was present in all primary cultures, suggesting malfunctioning of the p53-pathway in a subset of glioma cells. In addition, PTEN and AKT/PKB phosphorylation differed. Together our data reveals (i) a clear heterogeneity of stem-like glioma cells from different gliomas and gliosarcomas and (ii) differences between stem-like cells and their non stem-like counterparts from the same tumors.
Malignant brain tumors are amongst the most lethal solid tumors. The standard treatment consists of surgical resection followed by adjuvant radiochemotherapy. It has been proposed that glioma cells with stem cell features contribute to both, initiation and progression of primary and recurrent glioma. It remains elusive, how their growth is regulated and how the stem-like cells, which constitute only a subfraction of the brain tumor, could contribute to tumor progression and relapse. Here we investigated the responsiveness of stem-like tumor cells to mitogens and growth factor withdrawal. Moreover, we analyzed the expression of receptor tyrosine kinases and peptide growth factors in stem-like brain tumor cells and their non-stem-like counterparts from the same tumor.

We identified stem-like cells in all brain tumor biopsies analyzed so far. These cells exhibited a substantial heterogeneity with respect to morphology, proliferation rate, growth modus and the expression of neural markers and stemness factors. Therefore, we refer to these cells as subtypes of stem-like cells (SCIC-subtypes). Both, bulk tumor cells and SCIC-subtypes coexpressed several receptor tyrosine kinases (RTK), including members of the HER-family (HER: human epidermal growth factor (EGF) receptor), as well as receptors for PDGF (platelet-derived growth factor), FGF (fibroblast growth factor) and SCF (stem cell factor). Except for the SCF receptor c-kit, RTK expression appeared unrelated to the cell type, tumor type, and the WHO grade of the original tumor. SCIC-subtypes were maintained in serum-free medium containing the growth factors EGF and bFGF (basic fibroblast growth factor). Withdrawal of either one or both growth factors only moderately impaired growth in a cell line-specific way, indicating that stem-like glioma cells are largely independent of the exogenous growth factor supply. This is explained by the co-expression of EGF, heparin-bound EGF, bFGF, SCF and both, PDGF A and B in a large subset of the SCIC subtypes. The expression pattern showed some variation between the various SCIC subtypes and amongst SCIC and the corresponding bulk tumor. The responsiveness to EGF and bFGF differed, and PDGF AB mediated a cell line-specific growth reduction. Together our data suggest that the bulk tumor cells and SCIC within malignant gliomas exert autocrine growth control and that the various cell populations within the tumors may cross-talk via paracrine mechanisms.
Tumor stem cells – P43

Therapy responsiveness of stem-like human glioma cells
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Glioblastoma multiforme (GBM) is one of the most lethal solid tumors. GBMs grow invasively, develop resistance to radiation and chemotherapy, and frequently recur. The gold standard in GBM therapy is concurrent radiochemotherapy, using the alkylating compound temozolomide (TMZ). It has been proposed that stem-like tumor cells mediate therapy resistance and regrowth. Furthermore, resistance against TMZ seemed related to an altered apoptotic and autophagic death machinery. We determined the responsiveness of subtypes of stem-like glioma cells to TMZ and chloroquine, compared it to the behaviour of the established glioma cell line U87, related it to the expression of MGMT and ABC-transporters, and analyzed apoptotic and autophagic processes.

All of the primary cultures with stem-like cells exhibited self-renewal, although growth behaviour and proliferation rate differed largely. CD133 expression varied between different cell lines. Dose curves showed that responsiveness to TMZ was significantly different between the various stem-like cell lines, as BrdU incorporation was inhibited with different efficacy. Strong responsiveness to TMZ was related to the methylation of the MGMT promoter, but not to the expression levels of the ABC-transporters analyzed or p53. Co-application of chloroquine, a drug used in malaria prevention, which presumably affects autophagy, enhanced responsiveness of stem-like cells and U87 to TMZ in vitro. Western blot analysis showed that TMZ induced apoptotic cell death in stem-like cells, although less efficiently in the MGMT-positive cell lines. In addition, MGMT+ cells showed down-regulation of autophagy related proteins, which might contribute to processes that impair TMZ-induced cytotoxicity. The co-application of chloroquine and TMZ resulted in an increase of autophagic cell death in most cases. In addition, in several subtypes of stem-like cells, co-application of TMZ and chloroquin could additionally promote apoptotic cell death. Our results suggest that co-application of chloroquine might in part overcome the resistance to TMZ by promoting cell death.
Nanoparticel labelling of stem cell populations in head and neck cancer
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Head and neck squamous cell carcinoma (HNSCC) is one of the most common solid neoplasms worldwide. Unfortunately, the mortality rates are still high due to local tumor invasion and to a high predilection for the development of relapses and metastases. The cellular and molecular mechanisms responsible for tumor aggressiveness and its response to chemo- and radiation therapies remain mostly unknown. It is becoming more and more obvious that tumor progression and metastasis are stem cell driven processes and that these ‘tumor stem cells’ have to be in the focus of innovative therapeutic and diagnostic approaches.

Our aim is to identify and characterize tumor stem cell populations in HNSCC and to analyze and visualize their migration activity and their tumor inducing potential. Therefore, cells were labelled with superparamagnetic dextran coated iron oxide nanoparticles in order to make them detectable via ‘magnetic particle imaging’ (MPI), which is a new quantitative imaging technique capable of determining the spatial distribution of superparamagnetic nanoparticles at high temporal and spatial resolution. Tumor cells’ nanoparticel uptake was corroborated using flow cytometry analysis of FITC labelled particles as well as electron microscopy. We will show the progress of our investigations.
Monitoring adult stem cell response on superparamagnetic iron oxide nanoparticles for cancer therapy

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Superparamagnetic iron oxide nanoparticles (SPIO) are of increasing interest in biomedical applications such as targeted cancer therapy, magnetic field-triggered drug release or in vivo cell tracking. In early nanoparticle development stability, particle size and coating are optimized. For their use in clinical applications biodistribution and cytotoxicity of SPIOs are important parameters to be determined. Furthermore the function of human adult stem cells, as a pool of cells for self-renewal and repair, should not be reduced by the application of SPIOs. As a sensitive in vitro test system human adult stem cells can be used to determine uptake and cytotoxicity of SPIOs. In the current study we analyzed the uptake of newly developed dextran-coated SPIOs by confocal laser-scanning microscopy, transmission electron microscopy and prussian blue staining. To determine cell death, with regard to apoptosis and necrosis, cells were incubated with SPIOs, stained with Annexin V and propidium iodide and analyzed by flow cytometry. The viability of the human adult stem cells was monitored by MTT-Assay and FDA staining. Dextran-coated SPIOs were uptaken by human adult stem cells. The dextran-coated SPIOs had no influence on cell death or proliferation of the human adult stem cells. Our data further support dextran-coated magnetic nanoparticles as a well tolerated and promising tool for further surface modifications that and targeted cancer therapy.
Expression profiles of cancer stem cell markers in colorectal cancer cell lines

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Background: Cancer stem cells (CSCs) are thought to be responsible for tumor progression and therapy resistance. They have been identified in a variety of human tumors as well as in cancer cell lines. Cell lines might therefore serve as an attractive source for CSC in vitro research. We investigated to which extent colorectal cancer cell lines contain CSC-like cells and if their expression profiles correlate with clinical measures.

Methods: Altogether, 12 colorectal cancer cell lines of carcinomas and metastases were analyzed by flow cytometry using a panel of six CSC surface markers (CD326, CD133, CD44, CD166, Msi-1 and Gpr49). Expression frequency of CSC markers was divided into four categories with high (> 70% of cells), moderate (≤ 70% and ≥ 30%), low (< 30% and ≥ 1%), and absent (< 1%) expression.

Results: All cell lines but one (HT29) showed a stable expression pattern throughout all four replicates. HT29 showed an increased expression for CD133 and CD166 over time and was thus excluded from further analyses. The majority (91%) of cell lines showed high expression for CD326. About half to one third of the cell lines expressed at high frequency CD44 and CD166 (in 45%) and CD133 (in 36%).

In contrast, most cell lines expressed Msi1 and Gpr49 at low frequency. Since CD326, Msi1, and Gpr49 did not show any major expression differences in between the various cell lines, we analyzed for potential correlation of CD44, CD133 and CD166 expression differences with clinical parameters. However, we could not observe any significant correlation.

Conclusion: Colorectal cancer cell lines do harbour to a substantial amount CSCs. The frequency of such shows a distinct variability among different cell lines particularly for CD44, CD133, and CD166. This might be due to different clinical properties such as tumor progression and metastasizing as reported previously. In our study, case numbers were too small to validate such reports.

Interestingly, the frequency of CSC remained considerably stable over multiple passages within the individual cell line, except for HT29. We suggest excluding HT29 from in vitro analyses. In contrast, the remaining 11 cell lines seem to represent stable models of distinct CSC expression profiles and thus can serve for functional, molecular characterization of marker specific expression profiles.
Acquired resistance to cytostatics triggers cancer stem-cell-like phenotype in different tumor entities

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Background: Relapse of cancer occurs months or even years after apparently successful treatment. The principal cause of failure in second line chemotherapy is the acquired resistance to cytostatics. Probably, the reason for the appearance of chemotherapy refractory cancers is the survival of cancer stem cells (CSCs) since they are known to show MDR phenotypes and radio resistance. Our previous studies demonstrate that cancer cell lines of different tumor entities show characteristics of CSCs after induction of chemotherapy resistance to etoposide or doxorubicin. Here, we aim at characterizing the CSC properties in chemotherapy refractory cell lines.

Methods: Resistance and cross-resistance to etoposide and doxorubicin were monitored by MTT proliferation assay. The gene expression of CSC markers was performed using both conventional RT-PCR and qPCR. The protein expression of these markers was corroborated applying ICC and IHC. The resistance to ionizing radiation was analyzed by exposition of cells to gamma radiation.

Results: Our studies revealed that etoposide resistant entities displayed significant differences in the expression of stem cell markers compared to their parental cell lines. For neuroblastoma, prostate and glioblastoma cancer cell lines characteristic stem cell markers (CD34, CD44, CD117, CXCR4 and p75NTR) were found to be significantly and sustainably upregulated in etoposide-resistant sublines which also show cross-resistance to doxorubicin and high radioresistance. Furthermore, we corroborated that many of these pleiotropic effects were maintained when cells were xenographed into nude mice.

Conclusions: In view of the fact that etoposide and doxorubicin are commonly used clinical agents for treatment of many different types of cancer, the induction of CSCs by these cytostatics should be investigated in order to disclose selection of chemorefractory CSC during treatment, a phenomenon which might account for an eventual progression to intractable tumors. Moreover, such undesirable adverse events must be investigated for others MDR-phenotype inducing cytostatics. These novel findings could generate more knowledge about the pleiotropic effects of therapeutics on the cell biology particularly of clinically aggressive tumors. Consequently, improved cure rates
may be achieved via identification and therapeutic targeting of remanent chemotherapy resistant metastatic CSCs.