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Epigenetic Regulation and Reprogramming

In vitro differentiation of reprogrammed murine somatic cells into hepatic precursor cells

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Background: Recently, a new approach to reprogram somatic cells to pluripotent stem cells was shown by fusion of somatic cells with embryonic stem cells (ESC), avoiding generation of totipotent embryos. These fusion hybrids morphologically resemble normal ESCs but have a tetraploid karyotype. Normal hepatocytes often are polyploid, so we preferentially investigate the differentiation potential of fusion hybrids into hepatic cells. Methods: Mononuclear bone marrow cells from C3H and Rosa26 mice were fused with HM-1 (hypoxanthine-phosphoribosyltransferase –deficient) or OG2 (Oct4-GFP transgenic) ESC, respectively. Unfused ESCs were eliminated by selection with hypoxanthine, aminopterine, thymidine (HAT) for C3H/HM-1 hybrids or G418 for Rosa26/OG2 hybrids and fusion-derived colonies could be subcloned. Results: Tetraploidy of fusion hybrids was confirmed by FACS analysis of DAPI stained cells and by the presence of the transgenes from both fusion partners in all colonies. The published hepatic precursor differentiation protocols needed slightly modifications. Briefly, pluripotent hybrid cells were cultivated in hanging drops for 5 days before plating onto gelatine-coated dishes for 7 days. Outgrows of these colonies were re-plated on collagen-coated dishes for additional 9 days in Hepatocyte Culture Medium (HCM) to induce hepatic differentiation. Gene expression analyses of these cells showed a hepatic precursor-like expression profile, but weak Oct4-expression still was detectable. In the first set of experiments 3 mice were analyzed after 6 weeks. Transplanted cells show engraftment distributed in all lobes of the liver and no teratoma formation was detectable. But in a second set of experiments 5 out of 8 mice show teratoma formation after 3 -5 weeks after transplantation in the spleen (injection site) and the liver. Conclusions: Murine bone marrow cells are reprogrammed after PEG-mediated fusion with mouse embryonic stem cells and resulting hybrids can be cultured like normal ESCs. A hepatic precursor cell type can be achieved and preliminary transplantation experiments proof engraftment, but functional integration has to be proven and teratoma formation needs to be excluded by introducing selection strategies.

Keywords: reprogramming; cell fusion; differentiation; liver cells

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Epigenetic Modification of Neurosphere Cells Induces Early Embryonic and Hematopoietic Stem Cell Genes

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Treatment with the cytokine granulocyte-colony stimulating factor (G-CSF), alone or in combination with stem cell factor (SCF), can improve hemodynamic cardiac function after myocardial infarction. Apart from impairing the pump function myocardial infarction causes an enhanced vulnerability to ventricular arrhythmias. Therefore, we investigated the electrophysiological effects of G-CSF/SCF treatment and the underlying cellular events in a murine infarction model. G-CSF/SCF treatment improved cardiac output after myocardial infarction. Although it led to a twofold increased, potentially proarrhythmic homing of bone marrow-derived cells to the area of infarction, less than 1% of these cells adopted a cardial phenotype. Inducibility of ventricular tachycardias during programmed stimulation was reduced five weeks after G-CSF/SCF treament. G-CSF/SCF increased cardiomyocyte diameter, arteriogenesis, and expression of connexin43 in the border zone of the infarction. An enhanced expression of the G-CSF receptor (G-CSFR) demonstrated in cardiomyocytes and other cell types of the infarcted myocardium indicates a sensitization of the heart to direct influences of this cytokine. In addition to paracrine effects, potentially caused by the increased homing of bone marrow-derived cells, these might contribute to the therapeutic effects of G-CSF.

Keywords: Epigenetic regulation; pluripotency; hematopoietic stem cells; ES cells; neurosphere cells

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Efficient differentiation of adult stem cells into glucose regulated insulin producing cells using histone deacetylating agents

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Histone modification is central for the genomic reprogramming. Histone acetylation is associated with open DNA conformation (euchromatin) that facilitates transcription of target genes and conversely, deacetylation is associated with a closed chromatin confirmation (heterochromatin) and facilitates repression of transcription. Therefore, inhibition of histone deacetylation (HDAC) typically leads to derepression of transcription. We evaluated the effects of different HDAC inhibitors on differentiation of bone marrow stem cells (BMSC) to insulin producing cells. We report here, that BMSC from mouse, when pretreated for 3 days with inhibitors for HDAC, differentiate under high glucose into insulin producing cells within 10 days. Trichostatin A (TSA) a potent HDAC inhibitor induced most efficiently formation of islet-like cell-clusters. The clustered cells showed endocrine gene expression, specific for pancreatic ß-cell development and function, such as Glut-2, pancreatic duodenal homeobox-1 (PDX-1), SUR-1, Pax-4, and insulin (I and II), glucagon, and somatostatin. Immuno-cytochemistry confirmed that the cells produced various pancreatic hormones such as insulin, and C-peptide, glucagon and somatostatin. Double immunofluorescence staining for insulin and C-peptide confirmed co-localization of both proteins and indicates de novo synthesis of insulin in these cells. Western blot analysis showed further the expression of insulin in the clustered cells. ELISA analysis demonstrated glucose regulated secretion of insulin from differentiated cells. Electron microscopic analysis of islet like cells revealed vesicles containing insulin granules similar to pancreatic ß cells. These findings suggest that HDAC inhibitors may induce differentiation of BMSC due to chromatin remodeling into functional insulin producing beta cells in vitro.

Keywords: adult stem cells; histone deacetylation; pancreatic ß cells; chromatin remodeling

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Human Fat-Derived Nuclei Code for Expression of Muscle Proteins after Cell Fusion with defect Myoblasts

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Recent evidence indicates that organotypic stem cells in fat tissue can in culture be treated to express several different lineage markers (Zuk et al., 2002, Lee et al., 2004; own observations) including myogenic ones (Mizuna et al., 2002). We co-cultured this stem cell fraction, defined by their capacity to adhere to plastic surface (Zuk et al., 2001; van den Bogaerdt et al., 2002), with desmin-deficient mouse myoblasts (from desmin-/- knock-out mice; see Li et al., 1996). Following PEG treatment a total of 102 desmin expressing cells were evaluated in detail. 90 of these cells were myotube-like as defined from their size, shape, presence of more than one nucleus and dystrophin (the latter not always tested). 85 of these 90 cells contained at least one human nucleus (detected from the presence of human specific lamin) in addition to one or several mouse derived nuclei; besides expressing desmin, all such cells tested for also expressed human specific NCAM. In contrast, only in 5 of the 90 myotubes we failed to detect a human nucleus and 12 desmin positive cells contained human nuclei only. We conclude that nuclei of fat tissue derived cells after fusion with myotubes are re-programmed to express myogenic proteins. Supported by BMBF grant 01GN 0122 provided to AW and HIFF to ET.

Keywords: stem cells; human fat tissue; fusion; desmin-deficient myotubes; re-programming

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DNA Methylation and Collagen Type X Induction in Mesenchymal Stem Cells and Chondrocytes

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Articular cartilage has only limited potential for the regeneration of defects. By using tissue engineering new cell therapy methods are in development, which try to treat cartilage damages by ex vivo culturing of adult mesenchymal stem cells (MSC's) and differentiating them towards chondrocytes. For the therapeutic use of tissue engineering products from mesenchymal stem cells it is very important that the regenerate is phenotypically stable and functional. The differentiation process of articular cartilage not only requires the cells to differentiate into the right direction but also the maturational arrest which locks the tissue in the state of hyaline articular cartilage preventing terminal hypertrophic differentiation. In current in vitro protocols of MSC chondrogenesis premature induction of hypertrophic molecules like collagen type X and MMP13 occurs and ectopic transplants undergo vascularisation, calcification and microossicle formation in SCID mice. In contrast, similarly treated expanded articular chondrocytes form stable ectopic cartilage with no evidence for hypertrophic differentiation. The aim of this study was to analyse whether the induction of collagen type X and MMP13 in chondrocytes is blocked on the DNA level by CpG methylation which potentially locks the corresponding DNA regions in an inactive state. Bisulfite treatment of DNA followed by CpG site specific restriction analysis and DNA sequencing of collagen type X promoter and coding region indicated no differences between MSC's and chondrocytes. Consistent with these results, the treatment of chondrocytes with 5-Aza-deoxycytidine only slightly enhanced the very low expression of collagen type X in chondrocytes. These results indicate that the inhibition of hypertrophic marker gene induction in expanded chondrocytes seems to be independent of the DNA methylation status of collagen type X.

Keywords: tissue engineering; mesenchymal stem cells; chondrocytes; DNA methylation; collagen X

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Genetic Control and Transcriptional Regulation of Stem Cell Potency

Pegylated G-CSF (Neulasta) mobilizes a higher proportion of HSC with increased cycling activity while in G-CSF-mobilized CD34+ cells a larger fraction undergoes differentiation to megakaryocyte-erythrocyte progenitors

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Current regimens for peripheral blood stem cell (PBSC) mobilization in patients with multiple myeloma are based on daily subcutaneous injections of G-CSF starting shortly after cytotoxic therapy. Recently, a polyethylenglycole (PEG)-conjugated G-CSF (pegfilgrastim) has been introduced which has a substantially longer half-life than the original formula and therefore provides the basis for continuous G-CSF serum-levels after a single injection. Here, we compared the stem and progenitor cells mobilized by pulsatile G-CSF stimulation phenotypically and functionally with those mobilized by continuous G-CSF stimulation with unconjugated G-CSF and pegfilgrastim, respectively. We examined immunomagnetically enriched CD34+ cells from leukapheresis products of 9 patients who received G-CSF and of 7 patients who were given pegfilgrastim using Affymetrix HG Focus GeneChips covering 8793 genes. The statistical scripting language 'R' was used for data analysis. Significantly differentially expressed genes were identified with the Significance Analysis of Microarrays (SAM) algorithm. Key functional genes identified by GeneChips were verified by RT-PCR. Subset analysis and fluorescence based cell sorting has been conducted to assess the effects of continuous vs. pulsatile G-CSF stimulation on CD34+ subset composition and to obtain HSCs. Cell cycle assays using BrdU and 7-AAD staining and clonogenic assays were performed for functional corroboration. Gene expression analysis revealed a higher expression of genes characteristic for erythroid differentiation in cells mobilized by pulsatile G-CSF stimulation. Utilizing clonogenic assays we were able to functionally corroborate this finding as G-CSF-mobilized cells gave rise to significantly higher numbers of burst-forming units erythroid compared to colony forming units granulocyte-macrophage. In cells mobilized by continuous stimulation with pegfilgrastim, early stem cell markers, i.e. HOXA9 and MEIS1 had a higher expression level. Concordantly, a higher proportion of megakaryocyte-erythrocyte progenitors in G-CSF-mobilized cells were seen while hematopoietic stem cells and common myeloid progenitors prevail in pegfilgrastim-mobilized cells applying cell subset analysis. Looking at cell cycle regulating genes, human HTm4, which causes cell cycle arrest in hematopoietic cells, was upregulated in the G-CSF group, as opposed to cell cycle-promoting genes including Cyclin D2 in pegfilgrastim-mobilized cells. This is emphasized by a significant higher proportion of actively cycling cells after pegfilgrastim-mobilization and a significant higher proportion of cells in G1 phase mobilized by G-CSF. Adressing the question, if the differrences observed are due to mobilisation of different subsets or differential induction of transcription factors in HSCs or both, we performed RT-PCR analysis of key functional genes in HSCs. We found a higher expression level of HOXA9, MEIS1 and PBX3 in cells mobilized by continuous G-CSF stimulation by pegfilgrastim, whereas erythropoiesis-related genes HBB, KLF1 and the myelopoiesis-related transcription factor C/EBP delta were not differentially expressed or could not be detected in HSCs. Conclusively, despite the similar active component G-CSF we found a miscellaneous effect on blood stem and progenitor cell biology.

This may be due to the different pharmacodynamics of continuous and pulsatile G-CSF stimulation by pegfilgrastim and unconjugated G-CSF injections, respectively.

Keywords: PBSC; mobilization; G-CSF, gene expression

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Use of transgenic UTF1 hES cell lines in differentiation studies and compound screening

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Undifferentiated transcription factor 1 (UTF1) was identified first in the mouse as a marker for pluripotent embryonic stem (ES) cells. Subsequent analyses demonstrated that the gene was also expressed in human ES (hES) cells where it seemed to be under the control of transcription factors OCT3/4 and SOX2. We generated transgenic hES cell lines which expressed a neomycin resistance marker placed under the control of the human UTF1 promoter/enhancer. Our analysis of these cell lines showed that the endogenous UTF1 gene as well as the neo transgene were co-expressed in undifferentiated hES cells and that both genes were quickly down-regulated upon induction of differentiation by DMSO. Treatment of transgenic cell lines with both DMSO and G418 leads to the induction of massive apoptosis. However, a treatment with another differentiation-inducing compound, retinoic acid (RA), in the presence of G418 did not result in massive cell death, and expression of both endogenous UTF1 and neo transgene was unaffected. Our results indicate that the regulation of UTF1 expression is tightly coupled to the commitment of hES cells to particular cell lineages and that our transgenic cell lines emerged as powerful tools in studying hES cell differentiation. These cell lines show also great potential in identifying new compounds which direct hES cell differentiation into particular cell lineages.

Keywords: stem cell differentiation; UTF1; pluripotency; transgenic cell lines; drug discovery

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

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We are interested in the regulation and function(s) of the extracellular matrix glycoprotein Tenascin C (Tnc) during central nervous system development. Tnc occurs in the adult neural stem cell niche and in the ventricular zone of the developing brain, where it is thought to control neural stem cell (NSC) development. Studies in Tnc knockout mice revealed its involvement in neural progenitor maintenance and maturation. Structurally, Tnc consists of several protein domains including 8 constitutive fibronectin type-III (FNIII) domains. By independent alternative splicing of six additional FNIII domains, theoretically, up to 64 different Tnc isoforms could be generated, and in cerebellum 27 different Tnc isoforms have been detected. The analysis of the complexity of Tnc isoform expression in NSCs, which were grown as free-floating neurospheres, revealed 20 different Tnc isoforms to be present. The isoform pattern was comparable to the one documented in P6 cerebellum that contains mostly Tnc expressing progenitor cells. One novel Tnc isoform could be identified that might be neural stem cell specific. Its expression could only be detected in neurospheres, but not in embryonic brain tissues. In order to study the regulation of Tnc and its isoforms in neurospheres grown from the embryonic brain cell suspensions we transfected different plasmids coding for the transcriptional regulators Pax6, Otx2 and Tlx, which resulted in the overexpression of these factors. When Pax6 was overexpressed the large Tnc isoforms containing four, five and six additional alternatively spliced FNIII domains were upregulated whereas the small isoforms without any or with one additional domain were downregulated. We also analysed the Tnc isoform complexity after Pax6 overexpression, but to our surprise we did not observe any significant change in the combinatorial code of Tnc isoform expression after the analysis of several hundred clones. Neurospheres were cultured in the presence of different growth factors, which were thought to have different impact on the expression level of Tnc. Epidermal growth factor and basic fibroblast growth factor upregulated the expression of Tnc and had a differential influence on the transcription of Tnc splice variants. Transforming growth factor beta showed no regulatory effect on Tnc in neurosphere cultures, which differs form the situation found in primary cortical rat astrocytes. These findings show that the expression of the extracellular matrix molecule Tnc and its isoforms is regulated by defined intrinsic and extrinsic factors. We conclude that the differential expression of Tnc isoforms is controlled by transcriptional regulators and growth factors, both in the developing central nervous system and in neural stem cells.

Keywords: neural stem cells; tenascin C; transcription factors; growth factors; gene regulation

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Regulation of human and mouse blastocyst inner cell mass and embryonic stem cell pluripotency and differentiation

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Galanin (GA) is a secreted neuropeptide found abundantly in the CNS and highly conserved between species. SAGE libraries in the mouse embryonic stem (ES) cells and embryonic carcinoma (EC) cells revealed that high expression of GAL is characteristic of pluripotent stem cell lines (Anisimov et al., 2002). This project addresses the role of GAL within human and mouse embryos and human EC and ES cells. The expression of GAL was examined in relation to other pluripotency related genes thought to have a function in murine ES cells such as Nanog and FoxD3 in human and mouse embryos as well as ES cells. GAL, Nanog, and FoxD3 are expressed from 8-cell stage of preimplantation human embryo and there was no change in their expression with the addition of Growth factors such as Leukaemia Inhibitory Factor (LIF), Insulin like Growth Factor-1 (IGF-1), Heparin binding-Epidermal Growth Factor (HB-EGF). In the mouse embryo, GAL is expressed throughout the preimplantation developmental stages; from oocyte to blastocyst. In Ntera2/D1 EC cell line, GAL, Nanog, and FoxD3 are expressed in undifferentiated cells and their expression is down regulated upon Retinoic Acid (RA) induced differentiation. GAL, Nanog and FoxD3 are also expressed in undifferentiated mouse and human ES cells. These date suggest a role for GAL in maintenance of the ES cell phenotype. We are currently examining the effect of GAL knockdown on cell phenotype in mouse and human ES cells.

Keywords: human embryos, human embryonic stem cells, Galanin, Growth factors

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Gene expression trees for modeling differentiation during hematopoiesis

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The regulatory processes that govern cell proliferation and differentiation are central to developmental biology. Particularly well studied in this respect is the hematopoietic system. Gene expression data of cells of various distinguishable developmental stages fosters the elucidation of the underlying molecular processes, which change gradually over time and lock cells in certain lineages. Large-scale analysis of this data requires a computational framework for tasks ranging from visualization, querying, and finding clusters of similar genes, to answering detailed question about the functional roles of individual genes and their similarities and differences. We present a statistical framework designed to analyze gene expression and further heterogeneous data such as microRNA binding as it is collected during the course of development. We extend conditional trees to continuous variates. These trees model differentiation with their inherent dependencies naturally, and enable data visualization and querying. Several trees are combined in a mixture model to allow inference of potentially overlapping groups of co-expressed genes. Computational results for a wide range of data from the hematopoietic system demonstrates the large biological relevance of our framework. We recover well-known biological facts and also identify putative but convincing regulatory elements, genes and functional assignments. Additionally,we include microRNA target prediction in our framework.

Keywords: hematopoiesis; differentiation tree; gene expression clustering; microRNA

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Planarian neoblasts as a model system to study toti- and pluripotency

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Freshwater planarians have prodigious regenerative abilities that enable them to form a complete organism from tiny body fragments. Planarian regeneration in the adult body relies on the presence of specific cells, named neoblasts, which are capable of differentiating into all cell types of the adult body, including germ cells. This makes Planaria a unique model for studying the complex events involved in both maintenance of toti- and/or pluripotency as well as cell differentiation. The principles underlying regeneration in planarians have been explored for over 100 years through surgical manipulations and morphological observations. However, the molecular pathways responsible of neoblasts' proliferation and differentiation still need to be determined. MicroRNAs (miRs) are untranslated RNAs about 22 nucleotides long and were first identified in the nematode Caenorhabditis elegans. In C. elegans, they regulate the activity of specific genes by binding to messenger RNAs (mRNAs), preventing their translation into proteins. MiRs are highly conserved throughout evolution, suggesting an essential role in the biochemical pathways directing cell homeostasis, while the expression of unique sets of miRs in undifferentiated and differentiating Embryonic Stem Cells (ESCs) insinuates a role in plastic regulation of cell differentiative events. Here we present the first study on miR expression in the planaria Schmidtea mediterranea intended to characterize putative regulatory pathways for neoblast proliferation, differentiation and cell homeostasis. Down-regulation of SmPiwi2, encoding for a protein that belongs to the Argonaute/PIWI family, was shown to block Planaria regeneration by inhibiting the neoblast capacity to proliferate and differentiate. We used this approach to derive a miR expression profile in animals with impaired neoblast trim. Several miRs confirmed high homology with mammalian ones and, among those, some showed up- or down-regulation in SmPiwi2 RNAi animals. Interestingly, almost all of them have been found to be specifically expressed in undifferentiated ESCs or to be involved in Central Nervous System (CNS) development and/or differentiation. These findings suggest that miRs are potentially implicated in governing the potency of neoblasts, as they play important roles in maintaining the plasticity that permits these cells to correctly regenerate a whole animal. Our results represent a first attempt to elucidate the molecular mechanisms at the basis of "stemness" and cellular differentiation during regeneration in Planaria. Given the high homology of S.mediterranea genome with those of vertebrates (~70% of coding sequences), this study might provide us with basic knowledge of post-transcriptional mechanisms involved in ES cells proliferation, differentiation and cell commitment during early mammalian embryogenesis.

Keywords: Schmidtea mediterranea; Neoblast; microRNA; Regeneration; Stemness

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Establishment and analysis of a neural crest stem cell line (JoMa1) using conditional transgenesis

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Murine neural crest stem cells (NCSCs) are a multipotent transient population of stem cells. After being formed during early embryogenesis as a consequence of neurulation at the apical neural fold, the cells rapidly disperse throughout the embryo, migrating along specific pathways and differentiating into a wide variety of cell types. In vitro the multipotency is lost rapidly, the cells differentiate and undergo apoptosis, making it difficult to study differentiation potential as well as cell fate decisions. We initiated a neural tube outgrowth culture system and could show in vitro that cells migrating from the neural tube show expression of Sox-10 and p75 (LNGFr) indicative for the presence of Neural Crest Stem Cells. Using a transgenic mouse line (Jäger et al., 2004), allowing for spatio-temporal control of the transforming c-myc oncogene, we derived a cell line (JoMa1) which expressed NCSC markers in a transgene-activity (c-MycER) dependant manner. We observed, that when proliferative (Myc active) and differentiation (BMP2) stimuli were given, enhanced cell death could be detected, suggesting that the two signals are incompatible in the cellular context. However, the cells regain their differentiation potential after inactivation of MycER. When induced with BMP2, neuronal differentiation was observed. Using TGFbeta leads to differentiation into smooth muscle. Using Glial-growth factor the culture is driven into a glial fate. JoMa1 cells can also differentiate into chondrocytes and melanocyte precursors. The fact, that chondrocytic differentiation could be achieved challenges the dogma, that trunk neural crest (where JoMa1 is derived from) is unable to generate cartilagenous cells. This indicates an enhanced plasticity/spectrum of differentiation of trunk neural crest in vitro. Hence, JoMa1 represents a neural crest stem cell line, which is able to differentiate into the major neural crest derivatives. In summary we have established a system which allows for the biochemical analysis of the molecular pathways governing neural crest stem cell biology. Also we should be able to obtain neural crest stem cell lines from crossing the c-MycER mice with mice harboring mutations affecting neural crest development enabling further insight into genetic pathways controlling neural crest differentiation.

Keywords: Neural crest stem cell line; cMycER; Multipotency

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Analysis of Connexin31 gap junction channel and protein properties in mouse placental development using trophoblast stem cell lines

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As the first organ to form, the placenta is essential for the survival of the embryo. To initiate the development of the placenta, the blastocyst cells need to communicate. The expression of gap junction channels, which consist of connexin (Cx) proteins, enables the cells to exchange molecules of a molecular weight of up to 1 kD, such as signal molecules, metabolites or ions. In mice, the expression of gap junction channels starts with the blastocyst stage, where predominantly connexins 31, 31.1 and 43 are expressed. Cx31 is of particular interest, since it is expressed throughout the early trophoblast cell lineage and later on in the spongiotrophoblast of the placenta. Inactivation of Cx31 leads to a placental phenotype with an enhanced differentiation of trophoblast stem cells along the giant cell pathway. This lead to the hypothesis that Cx31 is involved in maintaining the proliferative diploid trophoblast cell population in early placental development. Recently, we could show that trophoblast stem cell (TSC) lines generated from mouse blastocysts provide a powerful in-vitro model to investigate the specific role of connexins for placental development. Undifferentiated TSC lines express Cx31 which is involved in regulating TSC proliferation and differentiation (Kibschull et al., 2004). After the establishment of Cx31-deficient TSC lines from blastocysts of the knockout mouse we were able to investigate Cx31 mediated signalling pathways and further discriminate between Cx31 channel and protein properties. For this purpose the Cx31-deficient TSC lines are in process of stable transfection with several Cx31 mutants, which will be analysed concerning proliferation and differentiation properties along the trophoblast cell lineage. In wildtype TSC, a peak of Mash2 expression, which is an early marker gene of trophoblast differentiation, is reached at day three. The expression of placental lactogen (Pl-1), a marker of trophoblast giant cells, and Tpbpa, which serves as a marker of spongiotrophoblast cells, is increased from day five onwards throughout differentiation in wildtype TSC. In Cx31 ko TSC, the peaks of these three marker genes are anticipated for two days, which points to the above stated role for Cx31 in differentiation control. Transfection of Cx31 ko TSC with a Cx31 full length expression construct resulted in clones with a restored (wildtype) differentiation pattern. Because of the constitutive expression of Cx31, proliferation was not only restored but strongly enhanced. To further test the proliferation capacity of Cx31 transfected TSC, we performed a tumor assay using nude mice. The tumors formed by Cx31 rescue clones are larger than those from wildtype cells. Taken together, these data provide further evidence that Cx31 is involved in proliferation control in the mouse trophoblast cell lineage. Citation: Kibschull M, Nassiry M, Dunk C, Gellhaus A, Quinn JA, Rossant J, Lye SJ and Winterhager E (2004); Dev Biol 273: 63-75

Keywords: trophoblast stem cells, connexin31, mouse placenta

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Differentiation Potential of Somatic versus Embryonic Stem Cells Analysed by Microarray Analysis

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Accumulating evidence suggests that differentiation of somatic stem cells tends to depend strongly on the microenvironment (plasticity) whereas embryonic stem cells create a multitude of niches more independently and behave more autonomously. In order to investigate in more detail how this may translate into differences in developmental potential we performed a comparative study of somatic stem/progenitor cells versus embryonic stem cells. We set up an in vitro system that allows autonomous self-differentiation to occur in multicellular spheroids and traced gene expression profiles using microarrays. As a source of somatic stem cells we used the apical pad-like tissue of extracted immature third molar teeth obtained from young human adults. The tissue contains neural crest-derived mesenchymal progenitor cells which were used for analysis. As embryonic stem cells the rhesus monkey cell line 366.4 (WiCell) was used. All cells were cultured in vitro under conditions that provide a homogeneous environment avoiding instructive external impact. Cells were first cultured for four days in hanging drops to trigger aggregation in a three-dimensional space. The multicellular spheroids thus formed were subsequently put in a rotating (gyratory) culture system that conserved aggregation but also allowed growth and differentiation. RNA obtained at different time points of culture was collected and analysed on whole genome microarrays (Agilent Technologies). Gene expression profiles obtained from these experiments demonstrate on one hand as expected the ectomesenchymal character of our somatic stem/progenitor cells as well as the embryonic character of the embryonic stem cells. On the other hand our data clearly depicted a distinct difference in the molecular signature developing during autonomous three-dimensional growth of embryonic stem cells versus ectomesenchymal progenitor cells. The results suggest that the gene marker sets defined in our study may be useful for characterization of the developmental potential from various types of pluripotent or multipotent stem/progenitor cells. This will help to estimate their value as a possible tool for therapy of human diseases or tissue regeneration.

Keywords: stem cells, ectomesenchyme, embryo, microarray, potency

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Molecular phenotyping of a pad like tissue (PLT) of human immature wisdom teeth: a source for ectomesenchymal progenitor cells

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Besides the well known sources of mesenchymal stem cells from bone marrow, umbilical cord blood and fat tissue, neural crest-derived ectomesenchymal progenitor cells may be obtained from human dental tissues. In particular, the dental pulp, filling the center of the tooth, and the periodontal ligament, attaching the tooth to the alveolar bone, are reported to hold multipotent progenitor cells. In recognition of the developing tooth as an easy source of stem cells, we characterize a pad like tissue (PLT) at the apical side of immature wisdom teeth as a novel niche of ectomesenchymal progenitor cells. Surgically removed teeth of adolescent patients served for total RNA isolation with subsequent cDNA-synthesis. Thereafter, we analyzed the dental PLT by a set of transcripts using semi-quantitative RT-PCR. This includes various markers reflecting the developmental position in tooth formation e.g. Msx2 (msh homeobox homolog 2), Barx1 (BarH-like homeobox 1) and Dlx6 (distal-less homeobox 6). In addition, we tested the samples for signature markers of pluripotency such as Nanog or Oct4 (octamer binding protein 4). In order to elucidate an osteogenic or neurogenic fate we investigated the expression level of osteocalcin and beta-3 tubulin, respectively. We compared all analyzed genes of dental PLT with i) dental pulp tissue from mature human wisdom teeth and ii) progenitor cells grown from processed dental PLT in vitro as ectomesenchymal derived cells. Resembling a set of stem cells from mesodermal tissues we enclosed iii) human bone marrow derived stem cells and iv) stem cells from human umbilical cord blood. As a control, we included v) neonatal human dermal fibroblasts into our trials. By this comparison of different types of tissues and cultured cells it will be possible to evaluate for the first time the molecular characteristics of the ectomesenchymal dental PLT niche.

Keywords: wisdom tooth; gene expression;ectomesenchyme; progenitor cells

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MicroRNA profiling of induced Osteo-differentiation in Unrestricted Somatic Stem Cells and Renal Carcinoma Cells

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Micro-RNAs represent a relatively new class of small (average 22nts) RNA molecules capable to either facilitate degradation or translational repression of target mRNAs. As global translational regulators, certain microRNAs putatively play important roles in initation and maintenance of cell differentiation although for only a few microRNAs the precise biological function has yet been described. To evaluate the potential influence of microRNA expression on osteogenesis, a tightly controlled developmental process, microRNA expression profiles were generated from the recently isolated CD45-negative pluripotent cell population from cord blood (Unrestricted Somatic Stem Cell, USSC) and the renal cancer derived cell line DH-1, both capable of in-vitro osteogenic differentiation upon induction with DAG (dexametasone, acsorbic acid, glycerolphosphate). MicroRNA expression profiles of undifferentiated cells as well as of osteo-induced cells 3 and 6 days after induction were analysed using two independent approaches: Construction and subsequent sequencing of microRNA libraries (about 1900 clones each library) as well as filter-based microRNA arrays comprised of 240 known human microRNAs hybridized with 32P 5'-end-labeled low molecular weight RNA fractions. Regarding the renal carcinoma cell line DH-1, both, library- and array data for microRNA expression were found in very good agreement to each other whereas USSC gave weaker array-hybridization signals in particular from undifferentiated cells, possibly pointing to lower microRNA abundance in USSC. However also in this latter stem cell model, the comparison of library and array data for microRNAs was concurrent with each other. Common to both cell types, a significant increase in expression of microRNAs let-7a and let-7b and, to a lesser extend, of let-7f and mir-368 was observed already 3 days after induction. Let-7 a and b were found among the most strongly expressed miRNAs in both cell types. Interestingly, let-7 has already been described as a heterochronic microRNA functionally linked to development and differentiation and as a negative regulator of RAS. Mir-125, involved in cell proliferation, was found moderately expressed in both cell types. In USSC, a significant increase of expression of microRNAs mir-16, mir-24, mir-27a, and mir-143, the latter already assigned to adipocyte differentiation, was seen 3 days after osteo-induction. In DH-1 cells, mir-16, mir-27a, mir-29b, showed clearly decreased expression 3 days after osteo-induction. To a lesser extend, mir-21, mir-22, mir-99b, and mir-143 were found downregulated. In summary these data demonstrate a differential expression pattern of certain microRNAs during osteogenic differentiation of USSC and DH-1 with the let-7a/b/f expression pattern being common to both cell types analysed. Nevertheless, the USSC data in particular require further validation by a functionally independent method. To this end, the real-time PCR TaqMan microRNA assay (human panel) already established in our lab and presented separately as well as northern-blot verifications will help to select "final" candidate microRNAs for further experiments that may reveal functional cell fate specificity of distinct microRNAs. These include target-mRNA identifications, conditional overex-

pression and inhibition of miRNAs and target mRNAs together with comparisons to the global mRNA expression profiles of USSC.

Keywords: microRNA Expression Profiling; Unrestricted Somatic Stem Cells; Renal Cancer Cells; Differentiation

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Transcriptional Control of Hematopoietic Stem Cell Populations in Zebrafish

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The differentiation of hematopoietic stem cells (HSCs) into distinct lineages is orchestrated by timely expression of critical regulatory genes. To describe the transcriptional control of the early steps in blood and blood vessel formation in greater detail, we analyzed the expression profiles of putative hemangioblast and HSC populations by cell-sorting transgenic fli1-GFP+/+, lmo2-GFP+/+, flk1-GFP+/+, and gata1-GFP+/+ zebrafish embryos at the 10 somite stage (14 hours post fertilization (hpf)). 520 genes were found to be strongly expressed in these progenitor populations. Consistent with hematopoietic and vascular lineage potential, the established hemangioblast markers scl, hhex, lmo2, flk1, flt4, tie2, and the HSC markers gata1, drl, znfl2, and cmyb were strongly expressed in all populations. These populations also transcribed genes with previously unknown hematopoietic or vascular expression during early development, for instance bmp3, nodal modulator 2, yes-related kinase, interferon regulatory factor 1, and nuclear receptor subfamily 4. Despite their similar expression profiles, fli1-GFP+/+, lmo2-GFP+/+, flk1-GFP+/+ and gata1-GFP+/+ populations were distinct in their differential regulation of a subset of genes that included for instance dual specific phosphatase 2. When the transcriptional regulation of these early progenitors was compared to the expression profiles of lmo2-GFP+/+ and of cd41-GFP+/+ populations at 36 hpf, genes such as cxcr4, lmo1, klf13 showed predominant expression in early progenitors, whereas for instance prostaglandin-endoperoxide synthase, T-box gene 6, or adiponectin receptors showed increased expression primarily in precursors at later developmental stages. In summary, our results describe the transcriptional control of primitive as well as definitive HSCs in zebrafish. In conjunction with the evolutionarily highly conserved genetic program of vertebrate hematopoiesis, our study demonstrates a powerful approach to unravel the genetic regulation of HSC differentiation.

Keywords: hematopoiesis, zebrafish, microarray, hemangioblast

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Bulk and single cell quantitative analysis of microRNA expression of cell fate definition of unrestricted somatic stem cells (USSC)

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Unrestricted somatic stem cells (USSC) from human cord blood were identified from this laboratory possesses a pluripotent differentiation potential. In-vitro cultured USSC showed differentiation into osteoblasts, chondroblasts, adipocytes, and neural cells. MicroRNAs (miRNA) are a class of 17-25 nt non-coding RNAs that have been shown to have critical functions in a wide variety of biological processes, like cell cycle regulation, cell differentiation as well as maintanance of imprinting, during development. To correlate the changes in miRNA expression from the USSC to the different cellular stages of its osteo-induced differentitation (day 0 to day 14) the newly developed human panel miRNA assay from ABI was employed. For this approach, small fraction RNA was isolated and the expression of 150 different microRNAs was determined using stem-loop reverse transcription primers followed by miRNA-specific TaqMan probes. Comparable to other profiling technologies, this novel real-time quantification method detects specific mature miRNAs in a sensitive manner. 14 miRNAs were upregulated, whereas expression of 38 miRNAs during osteogenic differentiation were decreased. The most promising canditates for regulation of the osteogenic cell fate are mir-143, mir-146, mir-181a, and mir-181b. In further experiments this miRNA transcriptom is analyzed on the single cell level. In this way, it is possible to assess the true clonal frequency of cells with unrestricted stem cell potential within this somatic stemm cell differentiation model.

Keywords: Stem cell, expression profiles, microRNA

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New Technologies

Embryonic Stem Cell Differentiation using Combinatorial Cell Culture

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Embryonic stem (ES) cell research would be greatly advanced by convenient and reliable in vitro methods to direct ES cell differentiation to specific lineages. Here we present a general method wherein ES cells are grown on microscopic beads which are then systematically shuffled through manifold combinations of growth media, such that cells which differentiate to a desired phenotype by passage through the correct series of conditions can be isolated and their culture history traced using fluorescent labels. This new technique is capable of multiplexing up to hundreds of thousands of ES cell differentiation experiments. Combinatorial Cell Culture was used to screen an experimental matrix comprising almost 14,000 different cell culture protocols for conditions leading to differentiation of pluripotent mouse ES cells to the monocyte-macrophage lineage. By screening such a large number of potential pathways it was possible to identify multiple protocols, and to overcome the use of embryoid bodies, semi-solid media and animal serum, combinations of which are normally required for monocyte-macrophage development in vitro.

Keywords: Stem Cell; Combinatorial; Macrophage; Differentiation

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Sequential application of cell permeable Cre and Flp recombinases allows tightly controlled reversible overexpression in embryonic stem cells

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Reversible and tightly controlled overexpression of transgenes in embryonic stem (ES) cells becomes increasingly important for the analysis of gene functions involved in stem cell maintenance and targeted differentiation. In this study we present a novel gene switch enabling reversible gene induction by the combination of two site specific recombinase (SSR) activities. SSRs such as Cre from bacteriophage P1 or FLP from S. cerevisiae can be used to recombine DNA in vitro and in vivo under spatial and temporal control. Recently, we reported a cell-permeable version of Cre that is able to translocate into mammalian cells and induce recombination with high efficiency. Here we present the adaptation of this technique to another commonly used SSR, i.e. FLP. We constructed a fusion protein consisting of the FLP recombinase, the protein transduction domain TAT, as well as a nuclear localization signal (NLS). The His-tagged TAT-NLS-FLP (HTNF) can be readily expressed in and purified from E. coli using Ni-(II)-affinity chromatography. The recombinant HTNF is approx. 54 kDa in size and soluble in physiological buffers at concentrations up to 400 Ìg/ml. The biological activity of HTNF was analyzed employing a fibroblast and a murine ES cell line both carrying a FLP-inducible LacZ reporter gene. We observed that HTNF induces FLP-mediated recombination in up to 80 % of the NIH 3T3 cells simply by adding recombinant HTNF to the medium. HTNF-mediated recombination is strictly time-dependent between 20 and 180 minutes reaching a plateau after three hours as observed in time course experiments. Almost 100% of recombination efficiency was achieved by an additional gancyclovir selection. This study demonstrates that engineered recombinant, biologically active FLP recombinase can be directly delivered into mammalian cells with high efficiency. Transducible FLP in combination with our previously published cell permeable Cre will greatly expand our capabilities to manipulate mammalian genomes by dual recombination strategies. Supported by the Ministry of Science and Research of North Rhine Westphalia and the Volkswagen Foundation.

Keywords: Overexpression, conditional mutagenesis, site specific recombination, reporter gene, transgene

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Analysis of Embryonic Stem Cell Pluripotency using a dual-color Fluorescence-based protocol

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Pluripotent stem cells, including Embryonic Stem (ES) cells, Embryonal Carcinoma (EC) cells and Embryonic Germ (EG) cells exhibit unique properties that have been exploited for stem cell derivation, purification, and analysis 1,2,3. The transcription factor protein Oct-3/4, and enzyme markers, such as phosphatases, have commonly been investigated to allow researchers to identify and distinguish in vitro differentiation in embryonic stem cell populations. Immunocytochemical methods including the Gomori technique, azo dye methods and BCIP/NBT have been employed to detect endogenous alkaline phosphatase activity in cells 4,5. Using the parental mouse embryonic stem cell line, R1, and a subclone, R1/E, we demonstrate a novel, dual-color fluorescence-based protocol for detecting and comparing Oct-3/4 expression and phosphatase activity simultaneously in embryonic stem cells. The results were then compared to our germline transmission data for these ES lines, assessing the feasibility of complete in vitro qualification of ES pluripotency.

Keywords: stem cells; embryonic; fluorescence; cell line

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A sensitive and specific method for detecting G-protein coupled receptor mRNAs reveals the receptor repertoire of human bone marrow stromal cells

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Signal transduction across the plasma membrane is a fundamental aspect of cellular regulation. This progress relies on a large number of extracellular ligands and specific receptors that respond to them. Among these receptors are the families of proteins that interact with intracellular signal cascades via trimeric G-proteins. These G-protein coupled receptors (GPCRs) are essential for mammilian cell biology. In addition, approximately 30% of all drugs in use today modulate GPCR activity. However, these drugs target only about 10% of all GPCRs; it is likely therefore that agonists or antagonists of additional receptors will prove to be beneficial in the future. GPCRs are encoded by low-abundant mRNAs and are fully functional at levels $<1x10^4$ protein copies per cell. Therefore, determining the expression profiles of GPCRs with conventional cDNA- or oligonucleotide arrays has been very difficult. We have developed a sensitive and specific method to profile GPCR transcripts in cell/tissue extracts using as little as 1µg of total RNA. We combine multiplex (50 primer pairs) one-step reverse transcription PCR (RT-PCR) to amplify and dye-label GPCR transcripts, and hybridization of the labeled amplicons to an array of 55 base-long oligonucleotides with sequences flanked by, but not including, those of the primers. To look for spurious signals and to validate the primer pairs chosen we profiled 6 human tissues (bone marrow stromal cells, liver, kidney, cerebral cortex, ovary, and hippocampus). 84% of the GPCR transcripts were expressed in at least one of these six tissue samples. The sensitivity of the method seemed excellent. On average, $3x10^3$ receptor transcripts could be detected. This is equivalent to ~1 molecule in 10^6. Furthermore, the false positive rate seemed reasonably low. Out of 96 GPCR detected with this system, 92 gave the correct signals on the array after individual amplification and hybridization. Thus, four of the original signals (4.2%) appeared to be false positives. To distinguish abundant and rare GPCR mRNAs, profiling was done using 100ng, 10ng and 1ng of total RNA. Quantitative RT-PCR (Q-PCR) was then performed on GPCR transcripts, which have been detected with 100ng, 10ng and 1ng of RNA versus transcripts, which could only be detected at the 100ng RNA-level. Q-PCR results with two independent sets of primers confirmed the difference in abundance between these two groups. To show the power of our method, we studied human bone marrow stromal cells (BMSC) from two donors. We detected a list of 162 GPCR mRNAs expressed in both samples. Among these, 50 were detected when 10ng of RNA were used for amplification and 24 were detected when only 1ng was used. Among the receptor mRNAs detected in these cells, some were known, while many had not been recognized previously. Based on these results we have developed a sensitive method to identify low abundant mRNAs of GPCRs in cells or tissues that may play significant roles in cell physiology.

Keywords: G protein coupled receptor, bone marrow stromal cell, microarray

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Hematopoietic and Embryonic Stem Cells from Nonhuman Primates (Callithrix jaccus): Efficient Transduction by Foamyvirus Vectors

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Preclinical animal models are important for evaluating the safety and therapeutic efficacy of new therapeutic modalities such as gene therapy. From the different large animal models, nonhuman primate models have emerged over the last decades as highly desirable experimental systems from both a pathophysiologic and pharmacokinetic viewpoint and the study of nonhuman primates has provided important information on the efficacy and safety of gene therapy systems in vivo prior to human trials. The common marmoset (Callithrix jacchus) has the advantage that it is a small, and thus relatively inexpensive nonhuman primate model. Currently, very little data on the transduction efficiency of foamyviral vectors for the transduction of marmoset stem cells exists. We therefore performed a direct comparison using identically designed gammaretroviral, lentiviral and foamyviral vector constructs expressing the enhanced green fluorescent protein (EGFP) from the spleen focus forming virus (SFFV) promoter pseudotyped with either a modified prototype foamy virus (PFV) envelope or the G-protein of vesicular stomatitis virus (VSV-G) for the transduction of marmoset CD34+ hematopoietic progenitor cells as well as common marmoset embryonic stem cells (CMES). Virus stocks of these vectors were prepared by polyethyleneimine-mediated transfection of 293T cells and concentrated approximately 10-fold by centrifugation for 4 hours at 10.000 g at 4oC. Three different target cell populations were transduced: Previously cryopreserved CD34-enriched cells from bone marrow of a common marmoset either (1) after a two-day prestimulation in the presence of IL-6, FLT3L, cSCF and TPO at a concentration of 100 ng/mL each, (2) after overnight incubation with 100 ng/mL SCF only and (3) common marmoset embryonic stem cells (ESC). Equal numbers of cells were exposed to the four different vector preparations for 14 hours in 12-well dishes coated with CH-296. The read-out was based on fluorescence microscopy of colonies plated in methyl cellulose as well as flow cytometry (FACS). Foamyviral vectors with the foamyviral envelope were the most efficient gene transfer tool for marmoset hematopoietic CD34-positive cells with stable transduction rates of over 80% as assessed by flow cytometry at both 2 or 7 days after the end of transduction and on average 88% transduction efficiency into colony forming cells (CFU-C). Transduction of CFU-C with the other vectors was always below 60%. In CMES, initial gene transfer rates of over 80% were achieved with the VSV-G pseudotype lentiviral vector, however, expression decreased to 13% after 7 days. In contrast, the foamyviral vector pseudotyped with the foamyviral envelope decreased only from 49% to 24% after 7 days. In conclusion, we achieved stable viral gene transfer and expression in CMES cells as well as highly efficient gene transfer into common marmoset hematopoietic CD34 positive cells using foamyviral vectors. In conclusion, these results suggest that foamyviral vec-

tors may be highly feasible vectors for stem cell gene transfer and thus set the stage for a more detailed analysis of this vector system in transplantation studies in this nonhuman primate model.

Keywords: embryonic stem cell; hematopoietic stem cell; transduction; nonhuman primate; foamyvirus vector

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Gene knock down of maternal and embryonic expressed GFP in murine embryos by the injection of a short interfering RNA (siRNA)

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RNA interference (RNAi) is becoming a widely used technology for gene silencing in various biological systems. Injection of long double stranded RNAs has been shown to specifically knock down gene expression in mammalian embryos, however, the utilization of short interfering RNAs (siRNA) to target specific embryonic genes would make this approach much more flexible and efficient. As a demonstration that a single class of siRNA molecules has long lasting effects after injection into mammalian zygotes, we have used siRNAs to knock down expression of the green fluorescent protein (GFP) in transgenic murine embryos of the OG2 transgenic line. Homozygous OG2-animals, carrying the Oct4-GFP transgene, were mated with NMRI animals to produce OG2 hemizygous zygotes, which show an inheritance-dependent expression pattern of the marker gene. Hemizygous 1-cell embryos with a maternally inherited Oct4-GFP gene express the GFP marker at the zygote stage, hemizygous embryos with a paternally inherited Oct4-GFP start transcription of the GFP gene at the 4-8 cell stage. Thus efficacy and duration of gene silencing could be tested under conditions where (i) GFP mRNA was already present at the time point of injection, and (ii) where GFP transcription started 2-3 cell cycles after siRNA injection. Fertilized embryos were microinjected with a 21 base GFP-siRNA or a control siRNA and then cultured in vitro. The siRNAs were conjugated with the fluorochrome rhodamine, so that injection and subsequent degradation of siRNAs could be followed. GFP fluorescence was recorded, and at the end of the in vitro culture period, the developmental stage, the number of nuclei and the GFP mRNA levels were determined. The results proved that injection of a synthetic siRNA is sufficient to knock down a target gene transcript in mammalian embryos for at least 4.5 days. The advantages of this approach are that (i) off target effects of long double stranded RNAs can be avoided, (ii) siRNAs against any known transcript can be rapidly designed and synthesized, (iii) developmentally important genes can be silenced in embryos for at least 4.5 days following injection.

Keywords: RNA interference; siRNA; Oct4-GFP marker gene; hemizygous

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Radiation Retinopathy in the GFP-Chimeric Mouse Model Compromises Stem Cell Research in Long-term Neovascular Ophthalmologic Pathologies

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Purpose: Construction of chimeric GFP+-mice has become a powerful tool in the field of stem cell-participation and -homing in various models of ocular pathology. This study evaluates the course of retinal pathology following lethal irradiation for bone marrow depletion and substitution. Methods: Lethal irradiation in C57/Bl6 mice was conducted with a low voltage radiation unit (U=120kV, I=25mA). Applied radiation was varied between 8 and 13 Gy. Subsequently, mice were transplanted with 1.5 million cells of whole bone marrow derived from mice expressing GFP under a b-actin promoter. GFP-transformation rates were investigated by FACS analysis. Mice were sacrificed at various timepoints between one and ten months after irradiation. In order to localize and quantify bone marrow derived cells within the retina in relationship to the retinal vasculature, transcardial perfusion with rhodamine-coupled concanavalin A lectin was performed. Extravasal cells were counted via an image analysis software. For colocalization studies flatmounts were stained with F4/80 and CD11c antibodies (for labelling of macrophages and dendritic cells, respectively) and evaluated using confocal and fluorescence microscopy. Results: Lethal irradiation was achieved at a total dose of 11 Gy, higher doses were lethal notwithstanding successful transplantation procedure. FACS-analysis revealed average transfomation rates of 75% with a trend towards higher rates with increasing age of recipient mice, ranging as high as 90%. Perfused flatmounts 4 weeks after transplantation were virtually free of extravasal GFP+-cells, whereas in the 4-months group cluster cell infiltrations, preferentially in the peripheral retina, became apparent. Cell morphology appeared from oval to cells with few extensions to dendritiform cells. In the 10-month group a variable cell number was found, and cells with a typical macrophage-like morphology were noted. Colocalization staining showed GFP+- as well as GFP–macrophages and dendritic cells. Conclusions: Following lethal irradiation, bone marrow-derived leukocytes seem to infiltrate the retina, commencing at about 4 months after irradiation. With regard to the observed radiation-related inflammatory reaction after long-term transplantation, this model may be unsuitable for investigation of prolonged retinal diseases.

Keywords: chimera; GFP; retinopathy; radiation; neovascularization

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Aspects of auto- and paracrine regulation and stem cell activation by reorganization in the bioreactor liver cell cocultures.

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Extracorporeal liver support using in vitro liver cell cultures offers an option for assisting or replacing the failing organ until regeneration occurs or until transplantation can be performed. For these purposes the multicompartment bioreactor was developed and applied therapeutically with success. The system provides cells with decentralized plasma or medium supply and oxygen. Primary human liver cells obtained from explanted organs discarded for transplantation because of different reasons were used as a cell source. Successful long-term maintenance of differentiated hepatocytes in vitro requires an appropriate microenvironment including an adequate supply of hormones, mediators and growth factors. Cocultivation with other liver cell types may support the organization of the extracellular matrix and provide hepatocytes with hormones and mediators in an auto- and paracrine way. In this study we investigated the bioreactor cocultures with RT-PCR and immunohistochemical techniques to detect and localize HGF (hepatocyte growth factor), HGFA (HGF-activator), and c-Met (HGF-receptor). We also studied the 3-D arrangement of cultured cells using serial sections and specific antibodies. To further characterize the regeneration processes stem (c-kit) and progenitor cell (AFP) markers were applied. It was demonstrated, that liver cells are organized in tissue-like structures inside the bioreactor system and support each other with the growth factors in an auto- and paracrine way. CK19-positive cells formed bile duct-like structures which exhibited inordinate branching in part with blind ending. The positive signal for HGFA was localized mainly to the CK19 positive cells, HGF was detected in random scattered cells resembling non-parenchymal liver sinusoidal cells, and c-Met receptor was abundantly expressed in parenchymal cells. The findings were supported by RT-PCR data. There was also evidence of stem cells activation during the regeneration and reorganization processes in the bioreactor cocultures. This study provides an overview of the steady state auto- and paracrine regulative processes in bioreactor cocultures and gives some starting points for optimizing the culture medium.

Keywords: bioreactor, cocultures, paracrine regulation, stem cells, acute liver failure

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Cre protein transduction: A tool for site-specific recombination in human embryonic stem cells and their neural progeny

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Human embryonic stem (hES) cells have become a major focus of scientific interest both as a potential donor source for regenerative medicine and as a model system for tissue development and pathobiology. Stringent and efficient methods for genetic engineering are required to exploit hES cells as disease models and to instructively guide their differentiation towards specific somatic phenotypes. During the last decade the application of site-specific recombinases such as Cre has revolutionized mammalian genetics by providing a reliable and efficient tool to manipulate chromosomal DNA in a conditional manner. Despite these significant advances, the conventional technology is hampered by unwanted side effects caused by the random integration of Cre expression vectors and leak activity of inducible or presumptive cell type-specific Cre expression systems. These limitations can be overcome by the recently developed technique of Cre protein transduction, which is based on the direct delivery of biologically active cell-permeable Cre protein into mammalian cells. Here we report Cre-mediated site-specific recombination of a chromosomally integrated allele in hES cells for the first time. Moreover we demonstrate that hES cells and hES cell-derived neural precursors are amenable to Cre protein transduction with an exceptionally high efficiency. Using a hES double reporter cell line we achieve recombination efficiencies of virtually 100% as determined by flow cytometry and Southern blot analysis. The Cre-transduced hES cells are not impaired in growth, maintain a normal karyotype and their ability to differentiate into derivatives of all three germ layers in vitro and in vivo. Furthermore we demonstrate that Cre protein transduction can be extended to hES cell-derived neural precursors with similar efficiencies. We expect this method to represent an important progress for the genetic engineering of hES cells as well as their differentiated progeny.

Keywords: Protein transduction; Site specific recombination; Cre

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A novel method for isolation and separation of whole brain derived PSA-NCAM positive adult neuronal progenitor cells

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Neural stem cells are defined as early precursors of neuronal and glial cells. During mammalian brain development these cells undergo mitosis, migrate and differentiate into specific lineages. Recently, neural stem cells within the adult central nervous system, especially in the subventricular zone (SVZ) were identified. It has been described that immature neuronal progenitors migrating out of the SVZ via the rostral migratory stream express high levels of polysialylated neural cell adhesion molecule (PSA-NCAM). Demonstrably, the expression of PSA-NCAM in the adult brain is largely declined compared to embryonic and early postnatal stages of development. Therefore, investigation of adult PSA-NCAM positive (PSA+ cells) is very limited. Existing isolation protocols like fluorescence activated cell sorting (FACS) provide relatively pure PSA+ cell population - however the yield of cells obtained by this method remains usually very low. Here we describe a novel method for isolation and enrichment of rat and mouse derived PSA+ cells using a monoclonal antibody against PSA coupled to superparamagnetic particles. In addition we established a novel whole-brain dissociation method. Using these novel tools we were able to obtain high purity and yield. The isolated cells possess all crucial properties of neuronal progenitor cells – they are able to form 3D neurospheres, to migrate, differentiate and proliferate.

Keywords: PSA-NCAM, separation, MACS, cell sorting, cell enrichment

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Establishment of multilineage hematopoietic readout systems in a nonhuman primate model (Callithrix jaccus) for comparative in vivo/in vitro analysis

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To investigate the properties of human hematopoietic stem and progenitor cells for therapeutic safety and efficacy after in vitro manipulation, reliable readout systems are required for preclinical evaluation. Although stroma dependent long-term in vitro systems are capable of measuring human progenitor properties up to a single cell level, they fail to predict stem cell engraftment. Similarly, the low engraftment frequency as well as the non-species specific microenvironment limits the use of xenogeneic transplantation models. Since the commonly used murine models differ greatly in pathophysiology and pharmacology to the human system, nonhuman primate models have emerged as highly desirable experimental systems to assess the properties of modified hematopoietic stem cells closely related to the clinical setting in humans. However, these animals are very expensive and in addition require expensive labor efforts as well as large facilities. A small new world monkey, the common marmoset (Callithrix jacchus) could overcome these disadvantages. It is small, easy to bread, inexpensive and does not require special facilities. Here we report the establishment of long-term hematopoietic in vitro readout systems to functionally characterize and enumerate primitive and stem cell equivalent progenitors of numerous hematopoietic lineages from the common marmoset. The animals (n=3) have been put into short term general anaesthesia and approximately 1 ml of bone marrow was aspirated from the femur. After density centrifugation CD34+ cells were enriched by magnetic separation using a recently described monoclonal mouse anti-marmoset CD34-antibody (MA24). To further characterize the CD34 marmoset-population several human-specific antibodies, such as AC133 have been evaluated. After an initial short-term expansion culture (using flt-3L, SCF and IL-7) frequency assessment and functional characterization of CD34+ progenitors were performed by transferring equal aliquots of cells from the primary cultures into human specific myeloid as well as lymphoid readout systems. Myelo-erythroid progenitors were assessed as Long-Term Culture Initiating Cells (LTC-IC), more committed cells were enumerated as Colony Forming Units (CFU) that can be generated within 2 weeks in clonogeneic methylcellulose cultures. Alternatively, the lymphoid differentiation potential was assessed by measuring Natural-Killer-Cell Initiating Cells (NK-IC) under human specific in vitro conditions. Mature NK-cells were further characterized by flow cytometry to assess the reactivity of NK-cell specific anti-human antibodies in the marmoset system. We demonstrate that similarly to the human system LTC-IC and NK-IC can be enumerated after 8 weeks of in vitro culture with an initial inoculation of only 500-1000 total CD34+ cells. After approximately 4fold initial expansion the LTC-IC frequency in marmoset CD34+ cells was assessed between 10 to 59% (35±14%), the NK-IC-frequency was enumerated between 10 and 43% (25±9%). Further characterization of NK-cell maturation revealed that marmoset NK-cells can be evaluated using specific clones of anti-human CD56, NKp30 and NKp46. The frequency of colony forming cells (CFC) was measured between 23 and 61% (42±11%) after primary expansion. Thus, this marmoset readout system can easily be adapted to our previously described human single cell setup. This will allow

comparative in vivo/in vitro analysis as well as clonal studies i.e. for the preclinical evaluation of novel stem cell-directed gene therapy approaches.

Keywords: marmoset; hematopoietic stem cells ; in vitro assay; primate

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Gen transfer into neural stem cells of the rodent brain via in utero electroporation – Investigating Rap1B function in vivo

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Most studies investigating the development of neuronal differentiation use cultures of dissociated hippocampal neurons as a model system. This system, has many advantages, including the well described development of neurons under in vitro conditions. Nevertheless, as an in vitro system these cultures do not completely reflect the situation in a developing brain. To overcome this limitation, an in vivo system is necessary. A Prerequisite for such a system is the possibility to transfect neural stem cells of the ventricular zone in the intact brain of a living mouse embryo. Furthermore, after this transfection the normal development of the embryo must continue in the abdominal cavity of the mother. A technique combining these features is the in utero elctroporation. In cultures of dissociated hippocampal neurons, it was shown that the GTPase Rap1B is essential for the development of neuronal polarity. Here we present our results concerning the in vivo function of Rap1B in neural stem cells during their division, migration and differentiation. We transfected neural stem cells of the ventricular zone during embryonic development by in utero electroporation. Expression vectors for constitutively active Rap1BV12 (gain-of-function) or a shRNA against Rap1B, that mediates the knock-down of Rap1B (loss-of-function) were used in these experiments. Our results not only highlight the function of Rap1B, but also serve as an example for the applicability of in utero electroporation to study the differentiation of neural stem cells during embryonic development in vivo.

Keywords: Neural Stem Cells; In Utero; GTPases

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Niches and Biomaterials

Gene expression of chondroitin/dermatan sulfotransferases in neural stem cell niche

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Chondroitin/dermatan sulfotransferases (C/D-STs) are responsible for the generation of diverse sulfated structures in chondroitin/dermatan sulfate (CS/DS) chains. It has been suggested that the biological roles of chondroitin/dermatan sulfate proteoglycans (CS/DS-PGs) are closely linked to the sulfation profiles of the corresponding CS/DS polymers. As an initial step to elucidate the functional roles of CS/DS-PGs in the neural stem cell niche, we have examined the gene expression profile of C/D-STs in the neurogenic regions of embryonic and adult mouse CNS. Using RT-PCR analysis, all presently known C/D-STs were detected in the dorsal and ventral telencephalon of the embryonic day 13 (E13) mouse embryo, with the exception of C4ST-3. In situ hybridization for C4ST-1, D4ST-1, C6ST-1, -2 and UA2OST revealed a cellular expression of these sulfotransferase genes in the embryonic germinal and adult subventricular zones. Expression of these enzymes was also recorded in neurosphere cultures obtained from E13 mouse telencephalon that represents a model system for the study of neural stem cells. We have recently reported that the unique sulfate CS/DS structure called 473HD-epitope is expressed in the periventricular germinal zones of the developing and in the subventricular zone of the adult mouse CNS. Expression of this epitope is also detected on the neurosphere-forming cells. In the present study, we document that neurosphere-forming cells express alternatively spliced isoforms of RPTP-β/ζ, which are modified by 473HD-epitope. Altogether, these results suggest that in the neural stem cell niche proteoglycans are modified by differential sulfation of CS/DS chains. This observation implies that these CS/DS-PGs may play important roles in the extracellular microenvironment surrounding neural stem/progenitor cells.

Keywords: Chondroitin/Dermatan sulfate proteoglycan; Sulfotransferase; Sulfation; Neural stem cell niche; Neurosphere

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Outgrowth endothelial cells from human peripheral blood in co-cultures with osteoblasts, bone- and adipose stroma cells in vitro

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Introduction One aspect of particular interest in tissue engineering is an improved vascularization of larger size defects, which would lead to a sufficient supply with oxygen and nutrients in the central regions of a larger tissue engineered construct. The process of vascularization is mainly mediated by endothelial cells, nevertheless other cell types are affecting the vascularization by a close interaction with endothelial cells, based on several factors such as production of extracellular matrix or secretion of growth factors. In this study we investigated the interaction of outgrowth endothelial cell (OEC) from human peripheral blood with other cell types relevant for tissue engineering applications. OEC are supposed to derive from bone marrow stem cells and develop in culture a fully differentiated phenotype. Furthermore, they reveal a high phenotypic and functional stability during their expansion. In addition, we have shown that these cells can be used in combination with different kinds of scaffolds. Here, we investigated the interaction of OEC in co-cultures with various cell types, as well as growth factors such as VEGF and endothelin involved in the cross talk of different cell types during the vascularization process. Materials and methods Endothelial cells (HUVEC and OEC) and osteoblasts or bone marrow or adipose stromal cells were first co-cultured on coverslips. To differentiate the two populations, the MG63 or stromal cells, respectively, were labelled with Cell Tracker green using standard protocols. Co-cultures were stopped and further processed by staining for endothelial markers such as van Willebrand factor or others. These co-cultures were then analysed by confocal laser scanning microscopy. Endothelin produced by OEC and VEGF produced by osteoblast or stromal cells were investigated by ELISA and RT-PCR. Furthermore the reaction of OEC by VEGF stimulation was tested by RT-PCR for caveolin-1. Results and Discussion In the co-culture experiments endothelial cells and osteoblasts or stromal cells were clearly distinguishable using a pre-labelling of MG63 or stromal cells with cell tracker green and staining for highly specific markers for endothelial cells such as CD31 and vWF. In these culture systems outgrowth endothelial cells formed a network-like structure resembling the structure of a microcirculation. Such networks were not observed when OEC were cultured in monoculture on the same substrate. The mechanisms controlling how OEC form these networks when co-cultured with stromal cells are currently under investigation. Using RT-PCR we observed a strong upregulation of collagen-type 1 when the bone marrow cells were cultured in a culture medium supplemented with growth factors for osteoblast differentiation. Furthermore in immunofluorescence studies endothelial cells seem to use the collagen fiber-matrix produced by bone marrow cells or osteoblasts as a substratum for angiogenesis. Fat stroma cells and MG63 produced VEGF under hypoxic conditions, whereas OEC produced increased levels of endothelin. Furthermore OEC responded on VEGF stimulation with downregulation of caveolin-1. In conclusion, outgrowth endothelial cells in co-culture with osteoblasts, bone marrow cells and adipose stroma cells form network-like structures. Furthemore, we found in OEC the expression of growth factors involved cross talk of different cell types during the vascularization process. This findings can serve as a promising basis to use OEC for pro angiogenic applications.

Keywords: EPC, Vascularization, co-culture, osteoblasts, adipose tissue

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Searching for a Stem Cell Home: Primary Feeder Cells for Co-Culture Expansion of Hematopoietic Stem Cells

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During the last years, umbilical cord blood has been established as a source of hematopoietic stem cells (HSC) for transplantation. But due to low absolute stem cell counts, this source still cannot be fully exploited. Thus, the ex vivo-expansion of HSC from cord blood is needed. Earlier experiments showed that best results are obtained when HSC are co-cultivated with feeder cells, but murine cell lines used for this purpose cannot be used in a clinical setup. To overcome this problem, we investigated several human primary cell types, namely mesenchymal stem cells (MSC), umbilical cord vein endothelial cells (HUVEC) and cells from the connective tissue of the umbilical cord called the Wharton's jelly (WJC) for their potential to support the expansion of cord blood HSC. In contrast to MSC, HUVEC and WJC could be obtained from the same donors as cord blood, but HUVEC could not be isolated from every umbilical cord due to vein ruptures or thrombus formation. Cryoconservation was possible for all three cell types with 86 - 93 % viability after thawing. All potential feeder cells were shown to be CD45-, thus enabling a sorting procedure before transplantation. Allogeneic T-cells were activated by HUVEC and WJC at all concentrations tested, but also by low concentrations of MSC as shown by up-regulation of T-cell activation markers CD25 and CD71. Feeder cells survived a short-term culture in serum-free HSC culture medium, albeit in low numbers. After a one-week culture period, an excess of glucose was still found in the supernatant, but no glutamine was left demonstrating the need of higher concentration. The experimental design for co-culture procedures was kept as short and easy as possible to fit clinical demands. Both feeder cells and CD34+ cells from cord blood were thawed just before use with only one day pre-culture of feeder cells and seven days of co-culture. During cultivation, no additional feeding was needed. Harvesting was done by simply rinsing the HSC-derived cells from the plates leaving the adherent feeder cells behind. Supernatant analysis showed that even at the time of harvest the medium contained an excess of glucose and glutamine. The concentrations of possibly growth inhibiting metabolites were low with 1.2 - 3.4 mM lactate and 0.7 - 0.9 mM glutamate. Expansion of mononuclear cells (MNC), colony-forming cells (CFC) and cobblestone-area forming cells (CAFC) from CD34+ cord blood cells co-cultured with MSC, HUVEC or WJC were significantly higher than in isolated suspension cultures used as controls. Expansion factors were 30- to 60fold (MNC), 20- to 40fold (CFC) and 10- to 50fold (CAFC). These results indicate that all three primary cell types might be used for ex vivo-expansion of HSC from umbilical cord blood in a clinical setting with WJC probably being the best candidate due to the possibility of an autologous co-culture. For enabling the use of the co-culture system in clinical application, next steps should include completely serum-free culture of feeder cells as well as automation of co-culture procedures.

Keywords: umbilical cord blood; MSC; HUVEC; Wharton's jelly cells; co-culture

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Characterization of murine skin derived neural precursors

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Recently, stem cells derived from skin have attracted much attention. Further characterization and isolation of these cells is hampered by the absence of specific cell surface markers. Here we describe the characterization, isolation and localization of several possible stem cell populations in newborn murine skin. Dermal and epidermal cells were characterized via FACS regarding their expression of prominin (CD133), stem cell antigen-1 (Sca-1), c-kit (CD117), Thy1.2 (CD90), CD45 and lineage markers. After separation, cells were cultured in vitro and different cell fractions were examined concerning their sphere forming ability. Differentiated spheres were examined via immunohistochemistry. Putative stem cell populations were localized in the skin by means of immunohistochemical stainings of cryosections.

Keywords: murine; skin; presursor

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Towards developing biomaterials for stem cell-based tissue engineering

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A consortium of six groups at RWTH works on different stem cell types: hematopoietic stem cells (HSC), mesenchymal stem cells (hMSC), endothelial progenitor cells (EPC), dental pulp stem cells (DPSC) and preadipocytes from man, and mouse embryonic stem cells (mES) and mesenchymal stem cells (mMSC). Each group aims at developing a three-dimensional biohybrid, consisting of biomaterials and stem cells, to substitute for specific tissues, such as skin, soft tissue, bone, teeth, blood vessels, etc. Natural and synthetic biomaterials have been used for decades in medicine to reconstruct tissue and organs. Yet the biocompatibility of biomaterials is defined for a specific function and a specific environment, and hence clinically tested biomaterials can vary e. g. in adhesion, vitality, and proliferation of different cell types. Therefore, a biomaterial platform was established for high throughput screening of different biomaterials in contact with different stem cell types. With our cooperation partners at the Department of Textile and Macromolecular Chemistry at RWTH, we have established a biomaterial bank consisting of both natural and synthetic polymers with different properties (biodegradable or stable, rigid or flexible) to allow an easy and fast access for each biomaterial. 20 different polymers are subjected to systematical testing in contact with all stem cell types monitoring cytotoxicity, adhesion, morphology, vitality, proliferation, differentiation, apoptosis, and cytokine secretion. Cellular responses triggered by contact with biomaterials will be investigated and compared between stem cell types and materials using standardized morphological and biochemical methods. Furthermore, the gene expression pattern will be investigated by DNA chip analysis before and after contact with biomaterials. Biomaterials supporting stem cell properties (self-renewal capacitiy and maintenance of the undifferentiated state) or inducing specific differentiation pathways will be further optimized e. g. by surface coating with peptides, proteins or glycostructures to enhance cell/biomaterial interactions. The optimal biomaterials will be constructed as three-dimensional porous scaffolds and seeded with stem cells to develop a biohybrid for tissue replacement. Supported by a grant from the Interdisciplinary Centre for Clinical Research „BIOMAT." within the faculty of Medicine at the RWTH Aachen University (VVB110).

Keywords: polymers, biohybrids, tissue engineering

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Expression pattern of human mesenchymal stem cells cultivated on textile scaffolds

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Bone marrow derived mesenchymal stem cells (MSC) can form bone, cartilage, and fat. Therefore human adult stem cells are a promising cell source for tissue engineering. Scaffolds are a powerful tool for applying such cells to the body for reconstruction or replacement of damaged tissues or organ. Practical considerations in tissue engineering include i) how to deliver and keep the cells to their target tissue and ii) whether the undifferentiated status is modified by growing stems cells in a three-dimensional environment. A variety of different materials are being used as cell carriers, but little is known about the influence of three-dimensional cultivation of stem cells on their differentiation capacity. We therefore cultivated human adult mesenchymal stem cells on textile scaffolds (nonwovens/warp-knitted meshes) manufactured from medical-grade poly(vinyliden flouride) and compared their genome-wide expression profiles to those of cells cultured on cell culture plastic. MSC (eight human donors) were grown individually until P3. 3x106 cells were pooled and amplified to 60x106 over two passages. MSC were seeded at 1.25x105 cells/cm2 on textile scaffolds. After cultivation for 21 days the scaffolds were analysed by electron-microscopy and histology. Genome-wide expression-profiling (22000 genes) was performed using DNA-chip-technology (Affymetrix). About 9800 genes were expressed independently from growth conditions of MSC. Around 3000 genes were changed specifically when MSC where grown in three-dimensional PVDF-scaffolds. Comparing the two manufacturing techniques we found 400 genes expressed only on woven-meshes, whereas 200 genes are specifically expressed after cultivation on textile-fleeces. No altered expression of markers for adipogenic, osteogenic and chondrogenic differentiation could be detected. Despite changes in gene expression MSCs in contact with PVDF-scaffolds did not spontaneously differentiate into one of the three classical tissue types bone, cartilage or fat. Therefore textile PVDF-scaffolds seem well suited for tissue engineering purposes employing MSC specifically to deliver cells to target tissues.

Keywords: mesenchymal stem cells; Biomaterials; DNA-Array; Tissue Engineering

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Signaling, Self Renewal and Differentiation

RNAi-mediated Suppression of OCT4 function in Human ES cells Recapitulates Primary Differentiation at the Blastocyst stage of Development

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The POU domain transcription factor OCT4, a key regulator of pluripotency in the early mammalian embryo, is expressed in primordial germ cells, unfertilised oocytes, totipotent preimplantation embryos and the inner cell mass of the blastocyst. Consistent with its essential role in maintaining pluripotency, its expression is down-regulated during formation of the trophoblast lineage. To enhance our meagre understanding of the molecular basis of this first differentiation event in the human embryo, we adopted an in vitro and functional genomics paradigm involving RNAi-mediated ablation of OCT4 function in the human ES cell line - H1 and analysed transcriptional changes at 24 and 72 hours after initiating its knockdown. Approximately 1,104 differentially expressed genes identified included positively (NANOG, SOX2, ZFP42, LEFTY1, LEFTY2, DPPA4, FGF2, THY1, FLJ10884, FOXD3 and TDGF1) and negatively (CDX2, EOMES, BMP4, FGF8, GSC, DKK1, HLX1, GATA2, GATA6, ID2 and DLX5) direct and indirectly regulated targets of OCT4 as well as a significant number of novel genes. Based on Gene Ontologies, we could assign these genes to diverse functions and processes, such as transcriptional regulation, epigenetics, chromatin remodelling, apoptosis, cell cycle, extracellular matrix, the cytoskeleton and metabolism. These findings point to the underlying molecular mechanisms that regulate the maintenance of pluripotency and trophoblast differentiation in human. We noted expression changes in components of several annotated metabolic and signalling pathways. These include known pathways described to be pertinent for self-renewal and pluripotency, namely, WNT, MAPK, TGF-ß/ACTIVIN/NODAL, FGF, and NOTCH. Conversely, active Hedgehog and BMP signalling induces trophoblast differentiation. Significant concordance between this data set and previously identified differences between ICM and trophectoderm in humans confirms that the analysis of human ES cell differentiation in vitro can serve as a useful tool for studying the early differentiation events in human embryos.

Keywords: human embryonic stem cells; pluripotency; OCT4-RNAi; trophoblast; signalling pathways

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Horizontal gene transfer from HUVEC to rat cardiomyocytes via apoptotic bodies

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Background: In recent years, several studies have examined the transdifferentiation potential of human umbilical vein endothelial cells (HUVECs). The observation that green-fluorescent-protein-(GFP)-positive HUVECs transdifferentiate into cardiomyocytes when co-cultured with neonatal rat cardiomyocytes in vitro, implicated the therapeutical use of these cells. Whether this holds true under in vivo conditions has not been examined. Methods and Results: To study the fate of HUVECs in vivo, we injected a bolus of 250.000 111Indium- or enhanced GFP (EGFP)-positive cells into the coronary arteries of wistar-rats utilising a catheter-based technique, whereby 18% of the administered HUVECs stably attached to the coronary endothelium as determined by SPECT measurement. One day after transplantation EGFP-positive HUVECs had passed the endothelial barrier. Two days later all EGFP-embodying cells displayed rat cardiomyocytic morphology and, surprisingly, immunostaining using species-specific antibodies in combination with confocal microscopy excluded the phenomena of transdifferentiation and cell fusion. However, we measured increased levels of caspase-3-activity in the HUVEC-receiving rat heart as an indication of apoptotic processes. Twenty-one days after cell transplantation no EGFP-positive cells were found in the rat heart. Additionally, heart function was unaltered throughout the study period. To verify these results we conducted co-culture experiments using neonatal rat cardiomyocytes and EGFP-positive HUVECs and again did not observe evidence for transdifferentiation but otherwise confirmed that rat cardiomyocytes contain the green fluorescent protein after a short time of co-cultivation. Apoptotic bodies generated in vitro from EGFP–expressing HUVECs were shown – by means of polymerase chain reaction – to carry the genetic information for the green cell marker. In vitro co-incubation of apoptotic bodies with neonatal rat cardiomyocytes resulted in cardiac myocytes expressing EGFP. Conclusions: Our data suggest that HUVECs, effectively engrafted into the coronary arteries, do not transdifferentiate into or fuse with cardiomyocytes. In fact, we provide first evidence for a horizontal transfer of the EGFP-gene from HUVECs into cardiomyocytes most likely via apoptotic bodies. This mechanism has to be considered when EGFP is used for following the fate of cells.

Keywords: HUVEC; transdifferentiation; cell fusion; catheter based cell transplantation; apoptotic bodies

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Asymmetric cell division of primitive human hematopoietic cells

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Somatic stem cells are defined as undifferentiated cells which can self-renew over a long period of time and give rise to progenitor cells which differentiate upon their further development. Since both uncontrolled expansion as well as loss of stem cells would be fatal for multi-cellular organisms the decision of self-renewal versus differentiation needs to be tightly controlled. Therefore one important aspect in the field of stem cell biology is to elucidate the mechanisms that govern this decision. Studies on model organisms like Drosophila melanogaster and Caenorhabditis elegans revealed two different strategies to control the cell fate of stem cell progenies: i) the asymmetric cell division, in which intrinsic cell fate determinants segregate differently, resulting in the maintenance of the stem cell fate in only one of the daughter cells, and ii) the stem cell niche, a microenvironment which provides a special combination of extrinsic factors, sustaining stem cells in their primitive state. Although there is good evidence for the existence of hematopoietic stem cell niches, it is often predicted that primitive hematopoietic cells divide asymmetrically to give rise to one daughter cell maintaining the primitive state and one being more specified. This assumption is mainly supported by the finding that primitive human hematopoietic cells often realize different proliferation kinetics and give rise to daughter cells adopting different cell fates. Although these data generally fit into the model of asymmetric cell division, it cannot be ruled out that the two daughter cells are initially identical and mutually influence each others cell fates post-mitotically; maybe in a way similar to the well known process of lateral inhibition mediated by the Notch signaling pathway. To unequivocally demonstrate that primitive hematopoietic cells can divide asymmetrically, we searched for molecules, which – similar to cell fate determinants in model organisms – obviously segregate differentially during mitosis. As it will be presented we could identify proteins helping to define a more primitive sub-fraction of short-term cultivated umbilical cord blood derived CD34+ cells, which indeed segregate differently in a portion of these cells.

Keywords: asymmetric stem cell division; cell fate; hematopoietic stem cells; HSC

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Long-term dark treatment induces neurogenesis in the mouse hippocampus

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Adult neurogenesis is a complex phenomenon known to be modulated by a large number of genetic and environmental factors. Here we investigate if dark treatment of mice affects Ras activity and adult neurogenesis in the hippocampus. Endogenous Ras-activity of defined brain regions were measured by a sensitive Ras pull-down assay. There was a dramatic decrease in Ras activity in the visual cortex and hippocampus after 7 days persisting for 3 weeks of dark treatment, the longest time period measured. We have shown previously that down-regulation of neuronal Ras activity by hyperoxia is associated with a disseminated apoptosis in various brain regions (Felderhoff-Mueser et al., 2004, Neurobiol. Dis. 17, 273). This insult-induced apoptosis can be greatly prevented by constitutive enhancement of Ras in neurons suggesting that activated Ras maintains low levels of neuronal apoptosis during pathological conditions. Next we tested if the observed down-regulation of Ras in the hippocampus during dark treatment would lead to a reactive neurogenesis, as previously shown for numerous toxic insults. Adult mice were put into a dark room for 1 to 3 weeks, while controls were kept under normal light/dark cycles (12h/12h). The numbers of neuronal precursor cells were assessed in the dentate gyrus of the hippocampus by immunohistochemical staining of doublecortin (DCX). While the number of DCX positive cells remained unchanged after one week of dark treatment, a significant 30% increase of these cells could be observed after three weeks in the dark. Our results support the working hypothesis that down-regulation of Ras activity by light deprivation triggers reactive hippocampal neurogenesis.

Keywords: Ras/neurogenesis/hippocampus/dark treatment/mice

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Regulation of adult hippocampal neurogenesis by transgenic activation of Ras in neurons: impact on working memory

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Adult neurogenesis is known to be modulated by a number of factors but its mechanism of regulation is still poorly understood. Previous studies have shown that the intracellular signaling protein Ras promotes survival of differentiated neurons in the central nervous system and protects against neurodegeneration. Indeed, degeneration of motorneurons was completely prevented after a lesion of the facial nerve of the synRas transgenic mice, a model which express constitutively activated V12-Ha-Ras in neurons (Heumann et al., 2000, J. Cell. Biol. 151:153). Furthermore, neurotoxin-induced degeneration of dopaminergic substantia nigra neurons and their striatal projections was greatly attenuated in these mice. Another study showed that postnatal treatment with the NMDA open channel antagonist MK801 led to disseminated apoptosis in the brain that is strongly reduced by the enhancement of Ras activity in neurons of synRas animals. Here we ask if the activation of Ras in differentiated neurons could modulate the production of their progenitors in the adult hippocampus. Proliferation and survival rate of newborn cells were measured in the dentate gyrus of synRas mice at day 1 or day 28 after BrdU injections, respectively. Double labelling experiments as well as the number of doublecortin positive cells revealed a dramatic decrease of the neuronal cell population displayed by synRas mice compared to wild type siblings. Interestingly, while the proliferation rate is reduced, the survival rate is increased in the synRas mice compared to the controls. Furthermore, the number of caspase 3 positive cells was strongly reduced in the granule cell layer of the synRas mice. Analysis of spatial memory abilities revealed a specific impairment of the working memory in the radial maze task. In order to investigate if neurogenesis can be rescued in synRas mice we injected recombinant erythropoietin intraperitoneally. The results showed that erythropoietin partially reversed the blocked neurogenesis in the hippocampus of synRas mice. Moreover, the adverse effect on the working memory abilities is attenuated in the radial maze test, improving the scores of the EPO-injected synRas mice. These results support the hypothesis of a reduced adult neurogenesis by Ras that is activated in the neuronal granule cell layer of the hippocampus regulating specific working memory processes.

Keywords: neurogenesis; ras; hippocampus; EPO; behavior

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In vitro clonogenic capacity of ectomesenchymal progenitor cells derived from human dental tissue

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The immature tooth is a potential source for progenitor cells. In particular, neural crest-derived dental cells raise the hope for successful cell therapy for curing tooth-related defects. However, a basic characterization of these ectomesenchymal progenitor cells beforehand is essential. To evaluate the clonogenic capacity, single cell cultures were established. As one parameter closely linked to their desired functionality, we analyzed the clones with regard to their osteogenic differentiation potential. Cells were enzymatically isolated from apical tissue of immature human wisdom teeth surgically removed from a 14 year old patient. Cultures were maintained in DMEM containing FCS. In passage eight, single cells were sorted by means of FACS (FACS ARIA, Fa. BD Biosciences, USA) into 96 well plates. Automated microscope images of the whole well (Cellscreen, Fa. Innovatis, Bielefeld, FRG) allowed non-invasive cell counting while culturing. Counting the recorded cells indicated the proliferation capacity of the clones. After 45 days some clones proliferated sufficiently to perform further experiments and they were subsequently cultured in osteogenic differentiation medium containing dexamethason, ß-glycerolphosphate and ascorbic acid. After 28 days, the calcification of these cultures was verified by Alizarin Red staining. From 50 single cells, 14 cells were able to adhere (28%). Their specific growth rate varied from μ = 0.00 d -1 to μ = 0.43 d -1. Those clones displaying a high growth rate (μA = 0.23 d -1; μB = 0.29 d ¬1; μC = 0.31 d -1; μD = 0.43 d -1) were further cultured in an osteogenic differentiation medium. Alizarin Red staining shows a clear calcification of these clones compared to controls. However, the intensity of mineralisation varied among the clones. Here we demonstrate that ectomesenchymal tissue from immature wisdom teeth contains progenitor cells with clonogenic capacity, i.e. a single cell is able to proliferate and to differentiate in case of osteogenic stimulation. Future experiments will demonstrate whether these clones exhibit multipotency, thereby showing the capability of undergoing expansion and variant differentiation in vitro.

Keywords: tooth, progenitor cells, ectomesenchyme, clone, ossification

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Osmolyte profile of human hematopoietic CD34+ stem and progenitor cells (HSPC) and analysis of murine HSPC of taurine transporter TAUT knockout mice

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Introduction: Intracellular accumulation of compatible organic osmolytes is not only important for regulation of cell volume homeostasis but also for cell protection. Betaine, myoinositol and taurine are compatible organic osmolytes which are accumulated by a lot of cells exposed to hyperosmotic medium. In this study, we assessed an osmolyte profile of human CD34+ hematopoietic stem and progenitor cells (HSPC). Taurine is one of the major osmolytes. This prompted us to examine if there are changes in hematopoiesis and clonogenic function of HSPC, in taurine transporter TAUT knockout mice compared to wildtype mice. Methods: CD34+ cells were isolated immunomagnetically from peripheral blood of patients following G-CSF mobilization. After hyperosmotic exposure (405 mosmol/l) the mRNA levels for the betaine/Á-amino-n-butyric acid (GABA) transporter BGT-1, the sodium-dependent myoinositol transporter SMIT and the taurine transporter TAUT as well as the osmolyte uptake of betaine, myoinositol and taurine was measured in comparison to normoosmotic (305 mosmol/l) exposure. Murine surface expression of lineage and stemcell markers on isolated cells of spleen, bone marrow and blood was examined by flow cytometry. Clonogenic growth of murine HSPC was analysed by applying isolated bone marrow cells in complete and Pre-B-Cell methylcellulose colony-forming assays. Results: BGT1-mRNA levels were significantly increased 1.5-fold after 6h exposure to 405 mosmol/l. SMIT mRNA and TAUT mRNA levels were increased 2.5-fold and 2-fold, respectively, compared to normoosmotic conditions. After hyperosmotic exposure for 2h betaine uptake was decreased, whereas uptake of myoinositol was 1.5-fold enhanced. Taurine was uptaken significantly up to 2.5-fold higher after hyperosmotic exposure. At that, uptake of taurine at 405 mosmol/l with 18 nmol/mg proteine is about 9-fold higher then uptake of betaine and myoinositol. Murine surface expression of lineage and stemcell markers showed no significant difference in any analysed organ. Additionaly, clonogenic growth of knockout and wildtyp murine HSPC in complete colony-forming assays showed no change in numbers of CFUs, neither in CFU-E nor in CFU-GM. However, in Pre-B-cell colony-forming assays the total number of CFUs, and thus CFU-GM and CFU-Pre-B, from knockout HSPC was significantly lower. Conclusions: These data show that taurine plays the leading part in regulation of cell volume homeostasis in human CD34+ cells. Myoinositol also plays a major role, whereas betaine is only partly involved. The finding of a significant decreased number of CFU-GM in Pre-B-cell assay, with a similar quantity of CFU-GM in the complete colony-forming assay, leads us to the hypothesis that the HSPC of the knockout mice are more prone to stress. Lacking of taurine in vivo and complete medium seems to be compansated, whereas minimal conditions like the medium of the Pre-B-cell colony-forming assay which is lacking SCF, IL-3 and IL-6 brings forward this sensitivity.

Keywords: osmolyte; taurine; CFU

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AP-2g in trophectoderm stem cell determination, maintenance and differentiation

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Transcription factor AP-2g belongs to a family of five closely related and developmentally regulated genes. AP-2g can be detected in all cells of the morula and becomes restricted to the trophectoderm lineage at embryonic day 3.5. Subsequently, extraembryonic derivatives like the ectoplacental cone and the extraembryonic ectoderm are positive for AP-2g suggesting a role of this gene in regulating trophoblast gene expression programs. Knockout mice established by us and others die shortly after implantation around embryonic day 7.5 due to a defect in the extraembryonic derivatives highlighting the essential role for AP-2g in early embryogenesis. To investigate the regulatory function of AP-2g for placental development, we established murine trophoblast stem (TS) cell cultures. Expression analysis of all five AP-2 genes during differentiation of TS cells revealed that AP-2g is expressed abundantly suggesting a function in the trophectoderm lineage. Notably, efforts to generate AP-2g deficient TS cells failed indicating a requirement of AP-2g in establishing the TS lineage. Consequently TS cells harboring conditional alleles for AP-2g were established. Here, Cre mediated deletion of AP-2g resulted in rapid death of the TS cell population indicating the absolute requirement of AP-2g for TS cell maintenance. Thus, Cre activity using a fluorescent Cre-transducing protein is being employed in order to elucidate the molecular mechanisms of AP-2g induced cell death in more detail. Recently, it has been shown that the markers of pluripotency Oct4, Nanog and Sox2 bind to the promotor of AP-2g eventually repressing its transcription in ES cells. Hence, AP-2g might serve, besides Cdx2 as master regulator, for the TS lineage specification. To pursue this hypothesis a gain of function approach in ES cells is being tested currently.

Keywords: trophoblast stem cells; AP-2g

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Enhanced neuronal activation of Ras promotes survival and stabilization of dopaminergic properties in differentiating neurospheres

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Parkinson's disease is a progressive neurodegenerative disorder which affects 2% of the aged population. Its primary pathology involves degeneration and loss of dopaminergic neurons in the substantia nigra. In order to replace the lost dopaminergic neurons, the concept of neural transplantation / replacement has been developed. However therapeutic transplantation of dopaminergic neurons generated from neural/precursor cells suffers from a dramatic loss in number after implantation by as yet unknown mechanisms. Various neurotrophic factors and cytokines are being investigated to reduce dopaminergic cell death and further enhance its survival. Most of the neurotrophic factors and cytokines mediate their action by pathways which are also activated by Ras, an intracellular plasma membrane bound protein. We utilized the synRas mouse model (Heumann et al, 2000), with constitutively activated Val 12-Ha-ras expression under the control of the neuronal synapsin-1 promoter.
We generated ventral mesencephalic neurospheres from single genotyped litter mate embryos, and upon differentiation the number of tyrosine hydroxylase cells was found to be significantly increased in synRas derived cultures compared to the wildtype. We found a small but significant reduction in the number of GAD65 immunoreactive GABAergic neurons and GFAP positive astrocytes in synRas derived cultures compared with wildtype cultures. Cultures from both genotypes exhibited other midbrain dopaminergic markers. No change in the mRNA expression pattern of En1, Lmx1b, Pitx3 and Pax2 genes was observed between differentiated wildtype and synRas derived neurospheres.
Protein expression determined by western blot revealed significantly enhanced expression of active GTP-bound Ras along with enhanced expression of its downstream effectors consisting of phosphorylated MAPK 1/2, Akt, Bad and CREB in differentiated synRas derived cultures compared to the wildtype. Interestingly, we also observed an increase in Nurr1 expression in synRas derived cultures. Tyrosine hydroxylase promoter assay revealed enhanced transcriptional activation of the promoter in the synRas derived cultures. We found that transient transfection with Nurr1 leads to significant increase in the number of tyrosine hydroxylase positive neurons in both wildtype and synRas derived cultures. Cell death studies involving serum deprivation and 6-OHDA toxicity assays revealed significant reduction in cell death mediated by both apoptotic dependent and apoptotic independent pathways in synRas derived cultures compared to wildtype cultures. Functional neurochemical analysis using HPLC-ECD revealed that cultures generated from both genotypes produces and release dopamine. Electrophysiological analysis of identified dopaminergic neurons in wildtype and synRas derived cultures exhibited action potential and spontaneous electrical activity as characteristic of functional neurons.

Keywords: synRas, Dopaminergic neurons, Neurospheres, Parkinson's disease

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Analysis of Extracellular Matrix Derived Signalling Cues on the Lineage Progression of Oligodendrocyte Precursor Cells

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Oligodendrocyte precursor cells (OPCs) are specified to become the myelin-forming cells of the central nervous system (CNS) by default. However, they seem to have a greater developmental potential. At least in vitro it is possible to dedifferentiate OPCs into neural stem-like cells which are in consequence able to generate all three major CNS cell types. This feature could be of particular interest due the fact that in the adult CNS the availability of OPCs is not limited. Clearly, we need to understand the mechanisms that drive OPCs along their lineage. Hence our research interest is to analyse the influence of microenvironmental factors on CNS precursor cells, i.e. the extracellular matrix (ECM) which consists of a network of macromolecules that are synthesized and secreted into the extracellular space by the cells of their tissue of origin. Among the large variety of ECM-components we focus on the ECM-glycoproteins Tenascin C (Tnc) and Tenascin R (Tnr) which have both been shown to be expressed dynamically during CNS development. In order to gain insight into the cellular and molecular events that trigger OPC behaviour the in vitro culture of purified OPCs represents a useful system to assay the role of defined extrinsic factors for its lineage progression. Our results show that the transition from an early A2B5-positive OPC to an O4-positive immature oligodendrocyte occurs more rapidly in Tnc -/- in comparison to the Tnc +/+ OPCs when the cells are kept in proliferation medium. This effect could not be overcome by exposing Tnc -/- OPCs to astroglial-derived Tnc. The investigation of Tnc-isoforms expressed by OPCs and astrocytes revealed that, different from astrocytes, OPCs also express the large alternatively spliced Tnc isoforms. In addition, we observed that Tnc is a major component of the astroglial ECM that exerts strong inhibitory effects on myelin-gene expression in differentiating oligodendrocytes. In contrast to Tnc, Tnr has opposite effects on myelin-gene expression. OPCs derived from Tnr -/- mice display decreased expression-rates of the myelin basic protein, as compared to Tnr +/+ mice. Taken together, we show in the present study that the ECM components Tnc and Tnr influence oligodendrocyte development in an opposite manner at different stages of lineage progression. Our results provide insights towards a better understanding of OPC behaviour in a complex environment, which could be useful also for understanding pathological situations such as the loss of oligodendrocytes in multiple sclerosis.

Keywords: Precursor; Tenascin; Development; Extracellular Matrix; Forebrain

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Regional specification of long-term propagated murine and human ES cell-derived neural precursors

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Homogenous, long-term in vitro expandable ES cell-derived neural stem cells could provide a useful donor source for the treatment of neurological diseases and the study of nervous system development and disease. We used quantitative RT-PCR and immunocytochemistry to explore whether the expression of region- and cell type-specific markers in stably proliferating neural precursors changes upon prolonged expansion. Proliferation in FGF/EGF was used to enable short-term (<10 passages) and long-term (>28 passages) propagation of ES cell-derived neural precursors. Human ES cell-derived neural precursors showed highest levels of the telencephalic marker FoxG1 at early passages, followed by expression peaks of ventral forebrain (Dlx2, Mash1) and midbrain (En1, TH) markers in intermediate passages; highest levels of the hindbrain marker HoxB6 and the rhombomeric marker Krox20 were found at late passages. Increased passaging resulted in a significant loss of FoxG1-expression, which could be confirmed by immunocytochemical analysis. Similar results were obtained with murine ES cell-derived neural precursors: Telencephalic markers peaked in early passages, whereas mid- and hindbrain markers were found at highest levels in ES cell-derived NS cells1 that had been propagated for 30 passages. These results indicate a posterior shift in ES-cell derived neural precursors upon prolonged FGF/EGF based cultivation. Current work focuses on the question whether transplantation of ES cell-derived neural precursors from early and late passages into different brain regions can influence and overcome this phenotypic restriction. To that end, we set up co-culture and organotypic slice culture systems2 which enable transient exposure of fluorophore-labelled neural precursors to different brain regions. 1. Conti, L. et al. Niche-Independent Symmetrical Self-Renewal of a Mammalian Tissue Stem Cell. PLoS Biol 3, e283 (2005). 2. Scheffler, B. et al. Functional network integration of embryonic stem cell-derived astrocytes in hippocampal slice cultures. Development 130, 5533-41 (2003).

Keywords: Regionalisation; human and murine ES cell-derived neural precursors

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Conditional ablation of BMPRIA indicates essential roles for BMP in ES cell self renewal and early neural commitment

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Embryonic stem (ES) cells differ from somatic cells by their capacity to renew themselves and the ability to form all cell types of the body. The molecular and developmental mechanisms controlling pluripotency and differentiation of ES cells are largely unknown. Both the derivation and the maintenance of ES cells in vitro depend on mostly undefined feeder cell-derived growth factors. Beside the leukemia inhibitory factor (LIF) two recent publications report on bone morphogenic protein 4 (BMP4) as another essential cytokine for sustaining pluripotency of ES cells. BMPs act through heterodimers of type I and type II serine/threonine kinase receptors. BMPRIA seems to play a major role in controlling pluripotency of ES cells. Using ES cells with a conditional BMPRIA allele we analyzed its function by a direct genetic approach. We employed a cell-permeable form of Cre recombinase to inactivate the loxP-modified BMPRIA allele in proliferating ES cells. Data will be presented indicating impaired proliferation after BMPRIA inactivation. BMPRIA activation is known to induce expression of BMPRIB in neural precursor cells and to promote their differentiation. Therefore we examined the effect of BMPRIA deletion during neural development as well. We demonstrate that loxP-modified BMPRIA ES cells together with Cre protein transduction serves as a reliable model to study BMP signaling during early neural development.
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Keywords: bmp signaling, self renewal, pluripotency, neural differentiation, conditional mutagenesis

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Stem cells under remote control - Protein transduction of stemness factors modulates self renewal of ES cells and induces transformation of NIH 3T3 cells

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Embryonic stem (ES) cells differ from somatic cells by their unique capacity to renew themselves and to give rise to all differentiated cell types. The transcription factors Oct4, Nanog and Sox2 are thought to play essential roles in the maintenance of ES cell properties. These three factors co-occupy the promoters of a large population of genes encoding developmentally important homeodomain transcription factors. However, the molecular mechanism of pluripotency maintenance, particularly the functional interactions of the intracellular molecular players and signalling pathways triggered by extrinsic factors remain elusive. We present a novel experimental strategy to induce Nanog activity in cells by nonviral, non-DNA based means in a reversible manner thereby modulating stem cell properties without genetic modification. Based on our previously reported Cre protein transduction system we engineered cell permeable versions of the stem cell factors Nanog and Oct4. Cell permeable versions of both Nanog and Oct4 support self renewal of ES cells as determined by growth morphology and staining for pluripotency markers such as alkaline phosphatase and SSEA-1. We observed that TAT-Nanog treatment induced self renewal properties even in the absence of leukemia inhibitory factor (LIF) in a reversible manner. TAT-Nanog-treated ES cells maintain a typical undifferentiated morphology, self-renewal and markers for pluripotency such as alkaline phosphatase, SSEA-1 and Oct4 for at least 20 passages in the absence of LIF. Moreover, we observed that TAT-Nanog together with LIF induces a characteristically altered, compact morphology of colonies and marked increased activation of an Oct-4 promoter driven GFP reporter gene indicative for a synergistic interaction of both stemness factors. Finally, we assessed the potential of TAT-Nanog to modulate growth properties of NIH 3T3 cells as a model to study pluripotency mechanism in differentiated cells. TAT-Nanog induced the formation of three-dimensional cell clusters similar to cell foci of transformed fibroblast cells. Foci formation was not observed in cells treated with control proteins indicating that the transforming activity is specific to TAT-Nanog. In conclusion our system of cellular manipulation provides a powerful model to study molecular and functional interactions of stemness factors. Supported by funds from the Ministry of Science and Research of NRW and the Volkswagen Foundation.

Keywords: Self renewal, Reprogramming, Transcription factor, Protein transduction

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Integrin-linked kinase is involved in vascular development and signalling

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Integrin-linked kinase (ILK) is a key molecule of the cell- extra cellular matrix (ECM) adhesion and interacts with the beta1-integrin cytoplasmic domain. Recent studies have shown the importance of beta1-integrins integrity and of the cytoskeleton organization in receptors signalling and clustering as well as in vasculogenesis. ILK deficient (-/-) embryoid bodies (EBs) showed a disrupted morphology of endothelial tubes as well as a decreased number of vessel-like structures when compared to the wild type. The analysis of ECM by immunocytochemistry experiments revealed major defects in collagen IV, laminin and fibronectin deposition. We further analysed the role of ILK on different receptor signalling pathways involved in angiogenesis. For this purpose we performed [Ca2+]i imaging experiments on magnet-associated cell sorting (MACS)-sorted endothelial cells from wild type and ILK (-/-) EBs loaded with the dye fura-2. After perfusion with VEGF (20ng/ml), wild type cells displayed a classical [Ca2+]i transient which was absent in most of the ILK (-/-) cells. On the contrary Bradykinin (100nM) effect was similar in both clones. Moreover, we examined the expression of the VEGF receptor 2 (VEGFR 2) in MACS-sorted endothelial cells. Western blot analysis indicated an equal expression pattern of the VEGFR 2 in wild type as well as ILK (-/-) cells. So far, these experiments point out the important role of ILK in embryonic vascular development by affecting ECM stability, regulating morphogenesis as well as the VEGF-signalling pathway.

Keywords: vasculogenesis; VEGF-signalling; ECM

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Genomics of developing cardiomyocytes from recombinant murine embryonic stem cells

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Introduction Embryonic stem cells (ESC) have the potential to develop differentiated tissues. However, little is known on the regulation of the transcriptome during differentiation of ESC. We analysed a recombinant ESC line with a neomycine-resistance gene under the control of the alpha myosin heavy chain promotor, which allows selection of pure ESC-derived murine cardiomyocytes. We analysed by microarrays the transcriptome of undifferentiated ESC and samples during differentiation to cardiac muscle cells and compared the profiles to adult murine myocardium. Methods ESC-line CM7/1 was cultivated with LIF-containing medium until confluency. Withdrawal of LIF induced ESC differentiation and embroid body (EB) formation in spinner cultures. Selection with geniticine leads to formation of beating cardiobodies (CB), from which isolated cardiomyocytes can be derived by trypsinization. Total RNA of 7 timepoints was isolated by standard methods and hybridized on Affymetrix MOE430A arrays. Analysis of expression data was performed using Genedata Analyst software. Expression of selected genes was confirmed by Real-time PCR (TaqMan). Results One dimensional clustering of transcription factors (TF) revealed 3 characteristic groups of TF, which appear transiently in the early and late phase of the cell culture and are different from isolated cardiomyocytes. The TF-pattern of the ESC-derived cardiomyocytes showed only few differences to ventricular myocardium. Dissociation of cardiobodies leads to downregulation of TF regulating heart development (hand2) or from cardiac precursor cells (Isl1), respectively. Early transcription factors, which regulate pluripotency like Oct4 and Nanog are downregulated early after induction of differentiation. During formation of EBs hand2 and GATA6 are expressed throughout differentiation to cardiomyocytes, whereas SNAI2 and HMGA2 are transiently expressed. Discussion The differentiation of pluripotent embryonic stem cells to cardiomyocytes is well established. However, little is known on the regulation of transcription factors needed for differentiation from ESC to cardiomyocytes. From our data we conclude, that in the early undifferentiated state of the ESC a set of transcription factors is transiently transcribed, which is distinct from those needed for pattern formation in embroid bodies and differentiated cardiomyocytes. The expression pattern of isolated cardiomyocytes and the ventricular myocardium was found to be extremely similar.

Keywords: stem cells, genomics, transcription factor, cardiomyocyte

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The cyclin dependent kinase inhibitor p16INK4a modulates hematopoetic stem cell ageing

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Hematopoiesis is maintained throughout an animals lifetime, but clinical practice as well as experimental evidence show that ageing hematopoiesis has an altered ability to react to stress and that ageing stem cells have a changed capability to differentiate. Up to date, the precise phenotype and the underlying mechanisms of hematopoietic ageing remain unknown. The cell cycle inhibitor p16, a member of the INK cyclin dependent kinase family, has been shown to accumulate in ageing cells in vitro and is an important element of the in vitro process of replicative senescence. Recent research ties p16 to ageing in vivo (Krishnamurthy et al, JCI 2004; Melk et al., Kidney Int 2003). Surprisingly though, p16 deletion on its own does not cause abortion of replicative senescence in vitro, but it facilitates development of immortal cell lines in cultured cells (Sharpless et al., Nature 2001; Cancer Res 2002). As we have recently shown, CDKIs are important regulators of hematopoietic stem cell fate (Cheng et al., Nature Med 2001; Stier et al., Blood 2003). We therefore hypothesize that p16INK4a is a regulator of stem cell fate with a role in hematopoietic ageing. We demonstrated that p16INK4a expression, but not p19ARF expression increases in ageing bone marrow, and more specifically in the lineage negative, c-Kit negative, Sca1-positive progenitor population (L-K-L+). Using a p16 KO mouse model, we have shown that in homeostasis p16 KO bone marrow has slightly more colony forming units (CFC-U) than the wild type controls, but less early progenitors (LTC-IC) and a lower repopulating cell frequency in comptetitive repopulation assays. In serial transplantation assays testing for stem cell function / self-renewal capability, p16 KO bone marrow performance was inferior to wild type. In ageing mice (> 12 months), the base line differences were lost. But in the serial transplantation experiment, we observed a superior self-renewal capability of aged p16 KO bone marrow. Cell cycling, apoptosis and differentiation were comparable in both KO and WT progenitor populations. To further assess this surprising result, we performed RT/PCR analysis of several genes connected to stem cell function and self-renewal. We observed an up-regualtion of hes-1 and gfi-1, but not Bmi-1 in old p16 KO bone marrow pointing towards a shift towards self-renewal function in old p16 KO bone marrow as the underlying mechanism. Whereas p16 deletion does not alter the major characteristics of ageing hematopoiesis in FVB/N and Bl/6 mice, and minor differences of young hematopoiesis are lost with age, we observed a major advantage of p16 KO bone marrow under conditions of stress. The advantage in serial BMT points towards an improved stem cell function. This hypothesis is supported by detected up-regulation of hes-1 and gfi-1 which have been shown to be important for stem cell function. Therefore, for the very first time we can demonstrate that deletion of a single gene has a benficial effect in the ageing organism.

Keywords: hematopoetic stem cell, ageing, self-renewal, p16, senescence

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BMP-4 induces a Smad-dependent apoptotic cell death of mouse embryonic stem cell derived neural precursors

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Embryonic ectoderm is fated to become either neural or epidermal, depending on patterning processes that occur before and during gastrulation. It has been stated that the differentiation of the epidermis requires inductive signals and proceeds from the inhibition of the dorsal ectoderm neuralisation. BMP-4, a member of the Transforming Growth Factor-beta (TGF-ß superfamily, is a key regulator of this commitment since it can directly induce the epidermal cell fate and inhibit the formation of the neural tissue. Using embryonic stem (ES) cell technology to study the initial molecular mechanisms underlying this lineage differentiation, we recently demonstrated that murine ES cells treated by physiological concentrations of BMP-4 underwent effective keratinocyte commitment. Focusing on the precise role of BMP-4 in the early choice between neural and epidermal commitment, we show that BMP-4 induces a severe inhibition of the neural differentiation process leading to epidermal commitment. We show that a high proportion of ES cells, engaged in differentiation towards neural cell types, undergo a dose and time dependent apoptotic cell death when treated early with BMP-4. This apoptotic cell death involves the characteristic mitochondrial damages (DiOC6/IP) leading to the cleavage of caspase-3 (western blot and IF) and targets early neural Sox-1 positive precursor cells. Specific inhibition of the p38 signalling pathway (which has been involved in BMPs effector functions) did not prevent this cell death. However, the overexpression of Smad6 (specific endogenous inhibitor of the Smad pathway), with adenoviral infection of differentiating ES cells, prevented as expected the BMP-4 inhibition of the neural differentiation. Altogether, our results indicate that part of the inhibition of neuralization by BMP-4, in mammals, involves the apoptotic cell death of neural precursor cells, resulting in enrichment of epithelial/epidermal lineages. This cellular model of early development can be useful to discriminate efficiently between neural and ectodermal precursors, while the established dogme of a common precursor for these two cell lineages is currently called into question.

Keywords: BMP-4, mouse ES cells, apoptosis, neural precursors, Sox-1

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Human umbilical cord blood progenitor cells as an alternative neuronal cell source

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There is an increasing interest in stem cell research both from basic science and clinical perspective. Data from in vitro and in vivo experiments demonstrates the wide potentials of these cells. Human Cord Blood (HCB) is ethically less controversial stem cell source and is now considered a new valuable pool in the stem cells research. HCB contains in its mononuclear cell fraction a certain number of mesenchymal stem cells able to proliferate and differentiate in vitro in many cell types. Our objective is to proliferate and differentiate these cells into functional neuronal cells in vitro. HCB is the blood remaining in the placenta and umbilical cord after the baby is born. HCB are collected after informed consent of mothers according to the German guidelines for blood donation. Mesenchymal stem cells derived from HCB have the same potential as stem cells derived from bone marrow. Mononuclear cells were isolated from HCB by Ficoll density-gradient centrifugation and cultured in MSCB medium (Cambrex). The mesenchymal stem cells are growing as adherent cell fraction and they are cultured until their morphology becomes spindle-shaped. In this stage, the cells are pre-differentiated with a bFGF containing medium for one week. Pre-differentiated cells are plated on fibronectin coated cover slips and cultured in cAMP containing differentiation medium. After 24 hours the cells are fixed and processed for immunocytochemistry against Nestin, ,-tubulin, MAP2, NeuN, GFAP, Neuro D, Neurofilament 200, and Neurofilament M. We see a wide range in number and survival pattern of mononuclear cells between the different donors. After three weeks in MSCB culture medium the cells reach the stage of morphology of spindle-shaped and start proliferating faster. At that stage, bFGF exposure changes their morphology into neuronal like cells. Our results show that it is possible to isolate mesenchymal stem cells out of umbilical blood, proliferate and differentiate them to neuronal like precursors that expressing Neuro D, MAP2, Nestin and ,-tubulin. The detailed analysis of the expression pattern of these cells is an ongoing study and the results will be presented at the meeting.

Keywords: neurodegeneration, neuroregeneration, transplantation

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PSA-NCAM overexpression modulates the sensitivity of ES cell-derived glial precursors to migration guidance cues

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The generation of neural precursors from ES cells opens new perspectives for the generation of donor cells for neural repair. The success of transplant-based therapies will critically depend on the ability of the donor cells to migrate across long distances within the host CNS. Previous studies have suggested a crucial role of PSA-NCAM in mediating precursor cell migration in the adult brain. Based on these observations, we explored overexpression of PSA-NCAM as a strategy to enhance the migratory properties of ES cell-derived glial precursors. Transduction with a retrovirus encoding the polysialyltransferase STX resulted in a six-fold increase in PSA-NCAM expression. Overexpression of PSA-NCAM had no effect on the bipotential differentiation of ESGPs into astrocytes and myelinating oligodendrocytes. PSA-NCAM-overexpressing ESGPs showed enhanced migration in monolayer cultures and an increased penetration of organotypic slice cultures. Upon transplantation into the adult striatum PSA-overexpressing cells, unlike control-transduced cells, displayed a targeted migration towards the ventricular wall. In vitro transfilter migration assays confirmed an enhanced response of PSA-overexpressing cells to putative chemotactic factors in different brain regions, but also to known chemoattractants including FGF2, PDGF and BDNF. Based on these data we propose that PSA plays a crucial role in modulating the ability of migrating precursor cells to respond to regional guidance cues within the brain tissue.

Keywords: ES cell-derived glial precursors; PSA-NCAM; cell migration; chemotaxis; guidance cues

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Neural stem cells and neurogenesis in the adult zebrafish brain: origin, proliferation dynamics, migration and cell fate

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Lifelong neurogenesis in vertebrates relies on stem cells producing proliferation zones that contain neuronal precursors with distinct fates. Proliferation zones in the adult zebrafish brain are located in distinct regions along its entire anterior-posterior axis. We show a previously unappreciated degree of conservation of brain proliferation patterns among teleosts, suggestive of a teleost ground plan. Pulse chase labeling of proliferating populations reveals a centrifugal movement of cells away from their places of birth into the surrounding mantle zone. We observe tangential migration of cells born in the ventral telencephalon, but only a minor rostral migratory stream to the olfactory bulb. In contrast, the lateral telencephalic area, a domain considered homologous to the mammalian dentate gyrus, shows production of interneurons and migration as in mammals. After a 46d chase newborn highly mobile cells have moved into nuclear areas surrounding the proliferation zones. They often show HuC/D immunoreactivity but importantly also more specific neuronal identities as indicated by immunoreactivity for tyrosine hydroxylase, serotonin and parvalbumin. Application of a second proliferation marker allows us to recognize label-retaining, actively cycling cells that remain in the proliferation zones. The latter population meets two key criteria of neural stem cells: label retention and self renewal.

Keywords: adult neurogenesis, proliferation zones, neural stem cells, Danio rerio

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Maintenance of pluripotency in human ES and EC cells

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Embryonic stem cells are unique in that they are pluripotent and may be propagated indefinetely in culture (self-renewal). Differentiated derivatives of human embryonic stem cells may serve as donor material for cell replacement therapies in the future. Therefore, it is crucial to unravel the mechanisms of self-renewal control in these cells in order to enable more precise manipulation in differentiation protocols. Despite the identification of several important transcription factors the mechanisms of self-renewal in ES cells remain poorly understood. We are investigating the regulatory network controling pluripotency by making use of microarrays. Data gained so far from transcription factor knockdowns in embryonic carcinoma cells, growth factor stimulation experiments, and expression profiles of hES cells reveal the activity of defined pathways and regulatory relationships between a range of specific TFs as being crucial for maintaining the undifferentiated state.

Keywords: human ES cells, EC cells, self-renewal

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Neuronal differentiation of unrestricted somatic stem cells from human umbilical cord blood

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It has recently been shown that human unrestricted somatic stem cells from umbilical cord blood (USSC) represent pluripotent, neonatal, non-hematopoetic stem cells with the potential to differentiate into the neural lineage. However, functional characterization of the neural phenotype and evaluation of the degree of maturity of the resulting cells is still lacking. In this study we address the question of neuronal differentiation and maturation induced by a defined composition of growth and differentiation factors (XXL-medium). We used immunocytochemistry and quantitative real-time PCR to demonstrate the expression of different neuronal marker proteins and their enrichment in USSC derived cultures during XXL-medium incubation. Further, enrichment of tyrosine hydroxylase (TH) expressing USSC, the key enzyme of dopaminergic neurons, could be shown being expressed in theses stem cells. Functional neuronal properties of USSC have been analyzed by means of patch-clamp-analysis and high performance liquid chromatography (HPLC). Although a rare event, voltage-gated sodium channels could clearly be identified by patch-clamp recordings in laminin pre-differentiated USSC. In addition, HPLC-analysis revealed synthesis and release of the neurotransmitter dopamine by USSC, thus correlating well with the immunocytochemical and Q-PCR detection of TH.

Keywords: neural differentiation, neurofilament, tyrosine hydroxylase, USSC, voltage gated sodium-channels

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No evidence of transdifferntiation of human endotherial progenitor cells into cardiomyocytes after co-culture with neonatral rat cardiomyocytes

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Background - Transplantation of endothelial progenitor cells (EPCs) has been shown to improve cardiac function after myocardial infarction. Whether this is due to in vivo differentiation of EPCs into cardiomyocytes is unclear. Recently, in vitro differentiation of human EPCs (huEPCs) after co-culture with neonatal rat cardiomyocytes (NRCMs) has been reported. We investigated whether underlying observations are based on differentiation, or alternatively on cell fusion or on the inability of conventional 3-colour-2D immunofluorscence microscopy to discriminate between double positive cells and CMs lying on top of labeled EPCs. Methods and Results - Co-culture experiments with huEPCs and NRCMs have been performed. EPCs have been labeled with DiIAcLDL, or with the membrane dye CM-DiI. Co-cultures have been analyzed by RT-PCRs, specific for early human cardiac transcription factors, by confocal laser microscopy after co-staining for different cardiac markers and by electron microscopy. Suitable software allowed for measurement of EPC morphology and three-dimensional analysis of apparently double-stained CMs. No evidence for cardiac differentiation of huEPCs has been obtained. No mRNA-expression of human cardiac transcription factors was detected. Contractions of labeled huEPCs were found to depend on mechanical coupling to CMs. No human EPC-derived CMs could be detected after DiIAcLDL or CM-DiI labeling. Cells, apparently double positive for cardiac markers and DiI were found to represent labeled EPCs lying over or under NRCMs or decaying NRCMs with high autofluorescence as confirmed by electron microscopy. Conclusion - We were not able to confirm reports on cardiac differentiation of adult human blood-derived EPCs. Although we cannot entirely exclude that recent contrary data are due to slightly different protocols, our studies revealed that 2D-immunofluorescence microscopy is unsuitable to analyze differentiation events in co-culture experiments, resulting data should be judged with caution.

Keywords: Cardiomyocytes, differentiation, endothelial progenitor cells

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The role of the survival promoting peptide, SPP/Y-P30 in differentiation of stem cell-derived neurons

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SPP (Y-P30) was purified from the medium of organotypic rat cortex cultures (Landgraf et al. FASEB J 2004). SPP promotes the survival of thalamic explants, growth of thalamocortical axons and neurite outgrowth of cerebellar neurons. SPP is expressed by peripheral blood mononuclear cells of pregnant female rats and human during corticogenesis to about birth. It becomes imported via the umbilical cord to the brain and enriches in cortical neurons of early postnatal rats and prenatal humans suggesting multiple roles during development. Here, we investigate if SPP affects stem cell-derived neural cells. Murine neurospheres derived from E13 cortex and ganglionic eminence (GE) (grown in presence of bFGF and EGF), were devoid of SPP immunoreactivity suggesting that precursors at the origin of neurospheres do not incorporate SPP. Differentiation assays revealed that the ratios of beta-III-tubulin-positive neurons, GFAP-positive astrocytes and Nestin-positive precursors were not altered by SPP. Neurospheres derived from GE showed significantly higher proliferation rates under normal conditions than those of cortical origin, and the SPP treatment selectively decreased proliferation in GE-derived neurospheres. SPP upregulated the mRNA expression of GAD-65, NT3, and NGF in undifferentiated cortical, but not GE derived neurospheres. On other hand there was an upregulation in NT4 expression levels in GE neurospheres, and downregulation of BDNF mRNA in both cortical and GE derived neurospheres. Murine ES cell-derived neurons were obtained from embryoid bodies (EB) produced by hanging-drop cultures treated with retinoic acid to induce neural differentiation. EB were plated to allow nuerite out growth, SPP treatment resulted in significantly longer axons. The growth of dendrites and number of primary dendrites was unaffected. The results suggest that SPP promotes axonal elongation and neurochemical differentiation in a regionally specific manner. Supported by Stem Cell Network NRW, LSA NP2TP5 and GRK-736.

Keywords: Survival promoting peptide, Neurospheres, aon growth

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Mesenchymal multipotence of ovine cord blood progenitor cells (sUSSC)

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Introduction: It was shown before that stem cells derived from umbilical cord blood display mesenchymal multipotency and can differentiate into osteoblasts, chondroblasts and adipoblasts in vitro under defined stimuli. Although sheep have been used as experimental models for investigations on xenoreactivity after transplantation of stem cells isolated from human umbilical cord blood, the potential of ovine cord blood stem cells to differentiate has so far not been examined. Materials and Methods: Mononuclear cells from the placentoms of 3 lambs were isolated via density gradient centrifugation and cultivated. After expansion up to 3 passages, the cells were stimulated to differentiate toward osteogenic (dexamethasone, ascorbic-acid-2-phosphate, ,-glycerolphosphate), chondrogenic (TGF-beta3, insulin, transferrin, selenium, dexamethasone, ascorbic-acid-2-phosphate) and adipogenic (indomethacine, insulin, 3-isobutyl-1-methylxanthine, dexamethasone) lines for 20 days. The cells were characterized morphologically by transmission and phase contrast light microscopy during lineage-specific stimulation.
Immunocytochemistry and conventional stains were used to detect lineage-typical markers: fat vacuoles and peroxisome proliferation-acitivated receptor A2 (PPAR) served to detect adipoblasts, whereas osteopontin (OP) was used to charaterize osteoblasts. A positive antibody reaction to collagen II and chondrogenic oligomeric protein (COMP) revealed the presence of chondroblasts. Results: The osteogenic line formed bone nodules, adipogenic cells developed lipid droplets and the cells of the chondrogenic line showed typical chondroblast-like morphology. Moreover the cells expressed typical marker proteins after lineage specific stimulation. As a sign of biomineralization conventional stainings showed the presence of CaP within the osteogenic stimulated line. Conclusion: It was demonstrated that ovine mesenchymal stem cells, derived from umbilical cord blood (sheep unrestricted somatic stem cells, S-USSCs), can be isolated via gradient density centrifugation and expanded in vitro. Under lineage-specific stimulation, S-USSCs differentiated into osteo-, chondro- and adipoblasts with typical morphological characteristics. Significant quantitative differences between the stimulated and control groups in lineage-typical immunocytochemical markers verified these findings.

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Human dental pulp stem cells and their biological and antigenic properties

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Mesenchymal stem cells (MSCs) represent promising source of cells for replacement of damaged tissues. We isolated stem cells from human dental pulp (DPSCs) that shared the similar antigenic, morphological and differentiation potential characteristics with MSCs. This study provides extensive antigenic and biological characteristics of DPSCs in vitro and describes changes of their phenotype following differentiation in multiple cell types. DPSCs were isolated from extracted third molars using enzymatic dissociation and cultured in a serum-low-content medium supplemented with growth factors PDGF-BB and EGF. Dissociated single cells were analyzed by flow cytometry, plated on fibronectin-coated coverslips and examined immunocytochemically or reseeded into differentiation media and cultured adherently or as free-floating cell clusters. Isolated stem cells were expanded over the 50 population doublings (PD). Cell doubling time changed from 24-50 hours in the initial PD to 60-270 hours after 50 PD, during the whole culture DPSCs showed stable karyotype. DNA analysis demonstrated that 42% cells were in SG2 phase. DPSCs represent a cell population that is CD29+, CD44+, Thy-1+, HLA I+, CD49a,c,d,elow, CD11b,clow c-kitlow, CD34-, CD45- and HLA II-. Immunocytochemical and western blot analysis confirmed that DPSCs express markers typical for MSCs including STRO-1 and proved expression of stem cells associated markers including telomerase (hTERT), CXCR4, nucleostemin and nestin. Moreover, the cells were found to be immunopositive for ,1-integrins, NG2, vimentin and weakly for VEGFR2, Bcrp1 and MDR1. All DPSCs were negative for neural cell markers, vascular cells markers (CD31, CD34, VCAM-1, SMA, vWF) and hepatic marker ·-fetoprotein. When the cells were placed into medium commonly used for cultivation of neural stem cells supplemented with serum, they started to form spheroids. These cells exhibited positivity for markers of immature neural cells – nestin, A2B5, NG2, RC2, O4, SSEA-1 and increased immunoreactivity for Bcrp1. Exclusion of serum from media facilitated cell differentiation and expression of neuronal (Pan NF, ,-III tubulin) and glial (GFAP) markers. When using other defined differentiation media, DPSCs were found to differentiate in osteonectin a procollagen type I positive osteoblasts or chondroblasts generated in osteo- and chondrogenic pellets in which production of extracellular matrix typical for bone and cartilage tissue was detected. Endothelial differentiation was confirmed as well. Our results demonstrate that DPSCs are homogeneous cytogenetically stable population of multipotent, self-renewal stem cells that can be expanded indefinitely without any loss of viability, maintaining the phenotype of stem cells during the whole culture. Modification of media can induce differentiation of cells into neural, bone, cartilage and vascular cells which offers the possibility to use DPSCs as an alternative source of cells in tissue engineering. This work was supported by the grant MSM 0021620820.

Keywords: stem cells; dental pulp; immunocytochemistry; flow cytometry; differentiation

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Interleukin-6 (IL-6) as a culture supplement alters the migratory phenotype of CD34+ haematopoietic progenitor cells by influencing the role of Protein Kinase C α (PKC α)

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Flt3-ligand (FL) and the inflammatory cytokine IL-6 are common supplements used for haematopoietic stem cells (HSC) expansion. Recent studies of our group have shown that SDF-1α stimulated human CD133+ HSC co-cultured for 5 days with FL and IL-6 showed an altered migration pattern as compared to CD133+ HSCs solely cultivated with FL (Weidt et al., 2004, Stem Cells 22(6):890-896). Our current studies thus concentrate on these two supplements' effects on CD34+ human HSCs migration characteristics induced by SDF-1α stimulation and the role of protein kinase C α (PKCα) as part of the molecular machinery directing cell migration. After magnetic separation from human cord blood CD34+ HSC were incubated in two different culture conditions with FL or a combination of FL and IL-6. Expression levels of PKC isotype expression in foetal CD34+ HSCs were determined by Western-Blot analysis. Migration assays were carried out in a three-dimensional (3D) collagen matrix on the first and the fifth day of incubation after separation. Further analysis by the time-lapse video microscopy combined with computer-assisted cell tracking allows for detailed analysis of the migration pattern on a cell population and a single-cell level. Western Blot analysis showed no differential PKC-α expression between FL and FL/IL-6 cultivated CD34+ cells on day 1, but a significant decrease on day 5 in FL/IL-6 cultivated cells. Thus migration experiments were carried out using the highly specific PKC-α inhibitor Gö6976 (6nM). On day 1 of cultivation CD34+ HSCs, supplemented either with FL or with FL/ IL-6, showed a similar migration pattern. The spontaneous migration rate of the cells was around 30% (FL: 28%; FL/IL-6: 34%) and was increased by SDF-1α to 41% (FL) and 56% (FL/IL-6), respectively. Both the spontaneous and the SDF-1 induced migration of FL and FL/IL6 cultivated CD34+ HSCs was decreased by Gö6976 treatment (FL/Gö: 18%; FL/SDF/Gö: 31%; FL/IL-6/Gö: 31%; FL/IL-6/SDF/Gö: 36%). Interestingly, on day 5 of cultivation the migration pattern of FL and FL/IL-6 cultivated CD34+ HSCs was markedly different. Both the spontaneous and the SDF-1α induced migration of FL cultured cells was decreased by Gö6976 treatment (FL: 25%; FL/Gö: 21%; FL/SDF: 43%; FL/SDF/Gö: 37%). In contrast, the spontaneous migration of FL/IL-6 CD34+ HSCs on day 5 was not affected by Gö6976 treatment (FL/IL-6: 33%; FL/IL-6/Gö: 33%). Moreover we observed a slightly increased migration rate on CD34+ HSCs co-treated with SDF-1α and Gö6976 (FL/IL-6/SDF: 43%; FL/IL-6/SDF/Gö: 47%). These results indicate that IL-6 as a culture supplement has an influence on the PKC-α expression level, which in turn causes the observed differences in the migration rates of FL and FL/IL-6 cultivated CD34+ HSCs. We conclude from our results that on day 1 migration of FL and FL/IL-6 cultivated CD34+ HSCs depends on PKC-α activity, an effect that does not change if cells were solely cultivated with FL alone. In contrast, pro-longed IL-6 cultivation of CD34+ HSCs resulted in a transition from a PKC-α dependent to a PKC-α independent migration. Thus further investigations should focus on the mechanisms HSC migration is influenced by IL-6.

Keywords: CD34+ hematopoietic stem cells; IL6; Migration; Flt3-ligand; PKCalpha

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Pax6-Mediated Neuronal Induction and Regional Specification of Embryonic Stem Cells

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During CNS development, Pax6 plays a crucial role in neuronal induction and the specification of cortical projection neurons. Here we explore whether Pax6 can instructively promote distinct neuronal fates during the in vitro differentiation of embryonic stem (ES) cells. Under standard conditions, murine ES cell-derived neural precursors (ESNPs) are expandable in growth factors and exhibit pan-neural differentiation into neurons, astrocytes and oligodendrocytes. In contrast, Pax6-transfected ESNPs show impaired proliferation and differentiate almost exclusively into neurons (97,5 ± 0,6%). Remarkably, Pax6 also influenced the neurotransmitter phenotype of the newly generated neurons. Whereas ESNPs transduced with an EGFP control vector generated only few vGlut1-positive glutamatergic and mostly GAD67-positive GABAergic neurons, Pax6-transduced cells yielded 91.7 ± 1,7% glutamatergic neurons. A concomitant down-regulation of Dlx1, Mash1, Gsh2 and DARPP32 and an upregulation of Emx1 suggests that this transmitter shift coincides with a suppression of ventral and a promotion of dorsal telencephalic fates.

Keywords: Embryonic stem cells, neuronal differentiation

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Derivation and Regional Restriction of Neural Stem Cells from Human ES Cells

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An increasing number of recent studies demonstrate that the broad differentiation potential of human embryonic stem cells (hESC) permits the derivation of various cell types under controlled conditions in vitro. Following lineage commitment, isolation and stable expansion of tissue-specific somatic stem cells represents a further challenge. We have derived stably proliferating neuroepithelial stem cells from human ES cells. These cells can be expanded to >75 passages, display multipotentiality at a clonal level and maintain a constant neuro- and gliogenic differentiation pattern. When cells derived from passages 10, 25, 50 and 75 were differentiated by 4 weeks of growth factor withdrawal, they yielded stable neuronal and glial fractions of 55-65% and 25-35%, respectively. The ES cell-derived neurons are excitable, i.e., they express Na+ and K+ channels for action-potential generation and acquire predominantly GABA(+), GAD67(+) phenotypes. Their neurogenic potential is also conserved after transplantation into hippocampal slice cultures and newborn rat brains. Under standard in vitro culture conditions, hESC-derived neural stem cells express transcription factors compatible with a posterior regionalization. Remarkably, even after long-term culture, these cells remain responsive to instructive regionalization cues such as Shh/FGF8-mediated induction of midbrain fates. The derivation of stable neural stem cell populations from human ES cells represents a potent tool for the study of lineage specification and the generation of donor cells for biomedical applications. Supported by DFG, BMBF and the Hertie Foundation

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Retinoic acid and EGF-2 induced mouse embryonic stem cell-derived renal differentiation

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Objective: Embryonic stem (ES) cells are known to be pluripotent because of their capability to differentiate into cell types of all three germ layers including germ cells. Recently, we have demonstrated that ES cells differentiate into renal cell types in vitro. Here, we show that renal differentiation of ES cells can be modulated by growth factors and signalling molecules. Material and Methods: Differentiation of mouse ES cells via embryoid bodies (EB) is established as a suitable model to study cellular processes of development in vitro. Cellular differentiation within the EBs was analysed by RT-PCR, immunostaining and electron microscopy. Results: We found that genes were expressed during EB-cultivation, which have been previously described to be involved in renal development. Marker molecules characteristic for terminally differentiated renal cell types were found to be expressed predominantly during late stages of EB-cultivation, while marker molecules involved in the initiation of nephrogenesis were already expressed during early steps of EB-development. On the cellular level - using immunostaining - we detected cells expressing podocin, podocalyxin. nephrin and wt-1, characteristic for differentiated podocytes and other cells which expressed Tamm-Horsfall protein (THP), a marker for distal tubule epithelial cells of kidney tissue. Furthermore, the proximal tubule marker molecules renal-specific oxido reductase (RSOR), kidney androgen-related protein (KAP) and 25-hydroxyvitamin D3 1alpha-hydroxylase (D3H) were found to be expressed in EBs. In particular, we could demonstrate that cells expressing podocyte marker molecules assemble to distinct ring-like structures within the EBs. Ultrastructural analysis by electron microscopy demonstrated that cells resembling early podocytes formed these specific cellular entities. Because the differentiation efficiency into these cell types was relatively low, application of different nephrogenic growth factors and signalling molecules was tested for induction. We found that EGF-2 and retinoic acid (RA) enhanced the number of renal ring-like structures within the EBs. Conclusions: Renal differentiation can be analysed in vitro using the ES cell model system. Induction of renal ring-like structure formation by EGF-2 and RA indicates the modulation of the nephrogenic pathway by these exogenous factors.

Keywords: ES cells; kidney; renal differentiation; retinoic acid; EGF-2; growth factors

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Lineage selection of doublecortin-positive human ES cell-derived neurons

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A key prerequisite for the application of human embryonic stem cells (hESC) in neural repair is the generation of defined neural cell types at high purity. Neuronal replacement would require purified populations of immature migrating neurons capable of integrating into host tissue. To that end we have developed an efficient strategy combining controlled in vitro differentiation and genetic lineage selection of doublecortin-positive cells. Doublecortin (DCX) is expressed in neuronal precursors of the developing and adult CNS and plays an essential role in regulating neuronal migration during cortical development. Stably proliferating human embryonic stem cell-derived neural precursors (hESNPs) were transfected by nucleofection with a construct carrying EGFP under the control of the human DCX promoter. Out of 50 clones monitored, 2 showed faithful recapitulation of DCX expression by the EGFP reporter. Upon in vitro differentiation DCX/EGFP-positive cells expressed the neuronal markers ß-III-tubulin and MAP2ab but were negative for astrocytic and oligodendroglial antigens. FACSorting of immature DCX-EGFP-positive cells yielded neuronal populations at purities exceeding 95%. Sorted cells were amenable to replating and further in vitro differentiation. Upon transplantation, DCX-EGFP-positive cells showed efficient migration and integration into the rodent brain. DCX-EGFP-based lineage selection may thus provide a useful tool for deriving transplantable immature neurons from human ES cells. Supported by the DFG (1337-3/2).

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Identification of a Chondroitin Sulfate Proteoglycan Regulated GEF by Application of the Induction Gene Trap Technology in Neural Stem Cells

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The differentiation and proliferation potential of neural stem cells (NSCs) is controlled by intrinsic and environmental factors, including extracellular matrix (ECM) molecules. We are investigating the molecular basis of chondroitin sulfate proteoglycan (CSPG)–dependent signalling in NSCs. CSPGs are defined components of the ECM that are found in the germinal layers of the embryonic CNS and in the adult neural stem cell niche, where neural progenitors and/or NSCs reside. These molecules are also expressed in embryonic forebrain-derived NSCs cultivated as free-floating neurospheres. To obtain information about the molecular cascades that are triggered by CSPGs, we have adapted the induction gene trap technology for use in NSCs. For this purpose, the gene trap vector is electroporated into neurosphere-derived cells, which leads to a random integration of the gene trap vector into the NSC genome. In a 96-well-based screening procedure individual resistant gene trap clones are tested in parallel for changes beta-galactosidase activity in the presence or absence of chondroitinase ABC (ChABC). This enzyme removes chondroitinsulfate-glycosaminoglycan side (CS-GAG) chains from the CSPG core proteins and is used to identify regulated target genes by CSPGs. We have generated >650 independent NSC gene trap clones. More than 350 lines have been screened for regulation by ChABC treatment. So far, two induced gene trap vector clones 19.3 and N2.11 have been identified as a result of CS-GAG removal. The sequence analysis after 5'RACE of the regulated target genes identified them as a guanine nucleotide exchange factor (GEF) for the Rho-family of GTPases and a G Protein-coupled seven transmembrane receptor, respectively. The candidate 19.3 was independently confirmed as regulated target gene by RT-PCR and in situ hybridization, when ChABC-treated neurospheres were compared to control neurosphere cultures. The expression pattern of 19.3 during forebrain development was analysed by in situ hybridzation, and revealed the presence in the ventricular and subventricular zone of the telencephalon. To investigate the functional relevance of the identified target genes, gain and loss of function approaches are currently undertaken. For that purpose among others the RNAi technology is used and the constructs are introduced into NSCs by electroporation to analyse in vitro the influence of the identified GEF on proliferation and differentiation properties in the neurosphere model. We have shown that the induction gene trap technology works successfully in NSCs. This method gives rise to reasonable and interesting target gene candidates that are likely to contribute to the understanding the cell biology of neural stem cells. Acknowledgements: Supported by the IGSN (Bochum).

Keywords: neural stem cells; gene trap; ECM; proliferation; differentiation

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Hypoxic cultivation of human embryonic stem cells induces spontaneous differentiation

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During early development, the mammalian embryo encounters low oxygen concentrations, ranging from 1% to 10% oxygen in the oviduct and uterus. Cultivation of mammalian preimplantation embryos under these physiological conditions is known to promote embryo survival and blastocyst differentiation. On the other hand, there are reports that suggest that hypoxia supports proliferation and pluripotency of human embryonic stem cells (hESC). We found that hESC cultured in hypoxic conditions (ranging from 2% to 8% oxygen atmosphere) show a decrease in proliferation. In a 2% oxygen atmosphere, mean specific growth rates dropped to 0,31 ± 0,03 [1/d] as compared to 0,43 ± 0,01 [1/d] under normoxic conditions. BrdU incorporation assays revealed that the fraction of proliferating cells observed after a 6-hour-pulse dropped from 62,2 ± 1,9% under normoxic conditions to 46,9 ± 4,6% under 2% oxygen. In contrast, Annexin-V binding as indicator of apoptosis showed only a slight increase under hypoxic conditions (8,9 ± 3,0% versus 7,4 ± 1,6% of the cells). Effects of hypoxia on the pluripotency of hESCs were determined by flow cytometric analysis of the pluripotency-associated surface antigens Tra-1-60 and Tra-1-81. Under normoxic conditions, more than 90% of the cells expressed Tra-1-60 and Tra-1-81. After 30 days / 5 passages in hypoxia (2% oxygen), these values dropped to 18,5 ± 6,3% for Tra-1-60 and 17,8 ± 8,9% for Tra-1-81. Concomittantly, we observed an upregulation of the early differentiation marker SSEA-1. Increased differentiation under hypoxic conditions was also suggested by the downregulation of Oct-4 and Nanog and an increased expression of vimentin, nestin and alpha-fetoprotein in qRT-PCR studies. Thus, in contrast to data from previous studies, hypoxia appears to inhibit proliferation and support differentiation of hESC.

Keywords: low oxygen, pluripotency, hESC

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An Induction Gene Trap Screen for Tenascin-C Target Genes in Neural Stem Cells

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The self-renewal and differentiation of neural stem cells (NSCs) is regulated by intrinsic and extrinsic factors. Within the last years the stem cell niche concept emerged underlining the importance of environmental cues for NSC behaviour/responses including interactions of the NSCs with extracellular matrix (ECM) components. Tenascin-C (Tnc) is a modular ECM glycoprotein expressed in the ventricular and subventricular zone during embryonic forebrain development where NSCs reside. Postnatally, expression persists in the neural stem cell niche of the adult CNS. For Tnc several interaction partners (e.g. phosphacan, neurocan) including cell surface receptors have been described (e.g. integrin v 3, RPTP- ,F3 /Contactin). Moreover many of the known receptors have been mapped to specific Tnc domains but in most cases neither signalling pathways nor Tnc regulated target genes are known. Recently, it has been shown that Tnc regulates the NSC pool during embryonic development by modulating growth factor responses of NSCs. It increases the sensitivity of NSCs to FGF2 and interferes with the BMP4 signalling pathway, which results in the correct temporal EGFR expression during development. It is, however, incompletely understood which genes are regulated by Tnc signalling in NSCs and how the latter modulates responses of NSCs to intrinsic and/or extrinsic signals. To identifiy and characterize genes which are regulated by Tnc we applied the induction gene trap technology to mouse NSCs grown as free-floating neurospheres, which serve as a widespread model system for NSCs maintenance and neural differentiation. The pt1 geo gene trap vector was electroporated into cells derived from 3rd passage neurospheres with an transfection efficiency of 56±9% as determined 1 d after electroporation. Upon integration into the genome, transfected neural stem cells were selected with G418 (neomycin) giving rise to clonal neurospheres. Using our library we have detected 2 integrations that responded to Tnc treatment and identified the trapped genes using 5`-RACE as a guanidyl exchange factor (GEF) and a dynein light chain, respectively. The regulation of the identified candidates was confirmed in non transfected cells via RT-PCR, which also shows that the lacZ expression of the induced clones reflects the endogenous expression of the trapped gene in response to Tnc in vitro. In situ hybridisation analysis of the GEF showed that it is expressed throughout the telencephalon with most prominent signals in the ventricular and subventricular zone of the developing mouse brain. Corresponding analysis will be carried out for the other candidate and for both gain and loss of function approaches will be performed using overexpression or RNAi techniques, respectively. In summary, we successfully applied the induction genetrap technology to mouse NSCs and developed a screening strategy for Tnc responsive target genes, which led to the identification of reasonable and promising candidates. The discovered candidates are likely to be important for understanding the cell biology of NSCs, which will help to evaluate their function in development and disease to generate new improved therapeutic concepts. (supported by DFG, SPP 1109 and GRK 736) Correspondence: alexander.vonholst@ruhr-uni-bochum.de

Keywords: neural stem cell; extracellular matrix; induction gene trap; forebrain; development

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Regulation of stem cells and lineage differentiation in skin and skin tumorigenesis: a role for the ,-catenin/Lef1 signalling pathway

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The canonical Wnt/,-catenin pathway plays a crucial role during embryonic development, homeostasis, morphogenesis and cell fate decision in various tissues. In the skin, ,-catenin functions as the key regulator of stem cell maintenance and lineage commitment. Transgenic mice over-expressing an active mutant of ,-catenin in the skin have an unscheduled de novo formation of hair follicles (Gat et al., 1998; Van Mater et al., 2003; Lo Celso et al., 2004). Conversely, conditional deletion of ,-catenin in embryonic or postnatal skin results in failure of hair follicle development and hair loss, respectively (Huelsken et al., 2001). Previously, we have generated transgenic mice in which ,-catenin signalling is repressed by expressing a dominant negative transcription factor Lef1 (K14ΔNLef1 mice). These animals develop epidermal cysts with sebocyte and interfollicular differentiation (Niemann et al., 2002). Thus, the level of ,-catenin activation determines whether keratinocytes differentiate into hair, interfollicular epidermis or sebocytes (Niemann and Watt, 2002). Aberrant activation of Wnt signalling occurs frequently in a range of tumours (Vogelstein and Kinzler, 2004). Transgenic mice over-expressing a stabilised mutant form of ,-catenin in the epidermis develop hair follicle tumours (Gat et al., 1998) and activating ,-catenin mutations are found in the corresponding human tumours (Chan et al., 1999). Surprisingly, K14¢NLef1 mice develop spontaneous skin tumours. Consistent with the role of ,-catenin activation levels in controlling lineage selection, the tumours in K14¢NLef1 transgenic mice show sebaceous and squamous differentiation rather that hair follicle differentiation. Recently, Takeda et al. found mutations in the ,-catenin binding domain of Lef1 in human sebaceous tumours. These mutations lead to an inactivation of Wnt/ ,-catenin signalling confirming our initial finding that mutated Lef1 and repression of ,-catenin signalling indeed is involved in skin tumour formation. To investigate how mutated Lef1 contributes to tumorigenesis, we performed two-stage chemical carcinogenesis experiments on K14¢NLef1 transgenic mice. K14¢NLef1 mice developed sebaceous tumours whereas littermate controls developed squamous cell carcinomas. Transgenic mice developed more tumours, more rapidly than controls, and without exposure to any tumour promoter indicating that in the skin, ¢NLef1 can function as tumour promoter itself. Furthermore, K14¢NLef1 epidermis failed to upregulate p53 and p21 proteins, most likely as a result of failure to upregulate ARF and was acutely sensitive to UV irradiation. From these results we conclude that mutated Lef1 plays a dual role in skin cancer, acting as a tumour promoter and specifying the differentiation program in developing tumours. From the chemical carcinogenesis experiments we learned that in K14¢NLef1 transgenic mice, skin tumours can be induced very early in high frequency after application of a single subthreshold dose of the carcinogen (within 4-5 weeks). Therefore, this inducible tumour model enables us to study early steps of tumour formation. We now investigate which cell populations are contributing to skin tumorigenesis. In particular, we would like to find out if the sebaceous tumours originate from keratinocytes possessing a stem cell/progenitor phenotype.

Keywords: skin; lineage; differentiation; stem cells;,-catenin signalling

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Long term culture of human neural progenitor cells derived from the adult brain

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Multipotent precursors, able to generate neurons, astrocytes, and oligodendrocytes have been isolated from human brain embryos in the past and from neurogenic regions of the adult brains more recently. The isolation of neural precursors from adult human brains has opened new perspectives in the study of adult neurogenesis and brain repair potentials. The present study describes the in vitro isolation, proliferation, and differentiation of neural progenitor population from the adult human brain. Tissue, isolated from surgical samples taken during stereotactic surgeries or partial temporal lobotomy from epilepsy patients, was enzymatically digested and propagated into substrate-free culture flasks. Cells were proliferated as neurospheres in growth medium supplemented with mitogens. Differentiation of expanded cells was achieved by plating dissociated spheres, onto polyornithine coated cover-slips in mitogen-free medium. After 14 days, the cultures were fixed and processed for immunocytochemistry against ,III-tubulin, microtubule associated protein 2 (MAP-2), glial fibrillary acid protein (GFAP) and neuroepitheliale precursor cell marker–nestin. The proliferate activity of the cultures was investigated with BrdU incorporation assay – 62 hours incubation of the respective cultures with medium containing 10nM of BrdU, followed by 14 days of differentiation. 61 samples were evaluated for this study. 12 samples obtained during stereotactic biopsy with an average patient's age of 55.5 years. These samples were very small and showed no proliferation capacities. 36 samples were obtained during epilepsy surgery with an average patient's age of 33, 4 years. 45, 1% from these samples were obtained from female and 54, 9% from male patients. 18 tissue probes were resected due to dysplasia, 15 following epilepsy induced by sclerosis and 3 probes could not be classified precisely. These samples contained cells that could proliferate (up to 1 year) and differentiate in vitro. Immunostaining of these cultures revealed ,III-tubulin, MAP-2, GFAP, Nestin and BrdU positive cells. The present study suggests that adult human neural progenitor cells can be isolated and expanded in vitro, without loosing their potential to differentiate into cells with neuronal or glia features. Therefore, these cells might serve as a useful source of human neurons for neuroregenerative strategies in neurological diseases as wells as a cell model to investigate the regenerative potential of endogenous neural stem cells. Further characterization at molecular, cellular and morphological level are needed in order to fully analyse these cells and discover their true potential during proliferation and differentiation.

Keywords: neurodegeneration, neuroregeneration, transplantation

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Mathematical model for NF-kappaB driven proliferation of adult neural stem cells

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Neural stem cells (NSCs) are early precursors of neuronal and glial cells. NSCs are capable of generating identical progeny through nearly unlimited numbers of cell divisions, producing daughter cells committed to differentiate. This crucial property is defined as proliferation. Nuclear factor kappa B (NF-kappaB) is an inducible transcription factor detected in neurons, glia and neural stem cells. Recently several evidences for a central role of NF-kappaB in NSC proliferation control have been provided. Here we propose a novel mathematical model for NF-kappaB driven proliferation of NSCs. In this study we reconstruct the molecular pathway of activation and inactivation of NF-kappaB and its influence on cell proliferation by a system of nonlinear ordinary differential equations. We use the combination of analytical and numerical techniques to study the model dynamics. The obtained results are illustrated by computer simulations and in general accordance with biological findings reported by several independent laboratories. The model is able to explain and predict experimental data. Understanding of proliferation mechanisms in NSCs may provide novel outlook in both –potential use in therapeutic approaches and basic research as well.

Keywords: neural stem cell, Nuclear Factor-kappaB, IkappaB, proliferation, mathematical model

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Building up the NRW Stembase: Global Gene Expression Analysis of osteogenic Differentiation from Unrestricted Somatic Cord Blood Stem Cells (USSC) and Mesenchymal Bone Marrow Stem Cells (MSC)

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Osteogenesis is a strictly controlled developmental process in which numerous extrinsic factors activate osteoblast-specific signaling proteins and transcription factors required for osteoblast differentiation. To identify regulatory factors specifically involved in the commitment of USSCs to osteoblasts global gene expression profiling was carried out. Microarray analysis was performed using 70mer-oligo DNA microarrays comprising ~16000 genes including technical replica for each time point. For comparative hybridization RNA isolated from uninduced USSCs and USSCs induced to the osteogenic lineage by dexamethasone, ascorbic acid and b-glycerol phosphate were used. Differentiation of cells was phenotypically monitored by Alizarin red staining showing initial calcium phosphate deposition after 1 week. Furthermore, sequential increased expression of transcription factors Runx2 and Osterix as well as of matrix protein Osteocalcin confirmed differentiation along osteogenic lineage as revealed by RT-qPCR. The global gene expression analysis revealed a common expression pattern of 388 genes >1.4-fold differentially expressed upon induction on day 1, 3, 5, 7 or 14 in USSCs (2 donors). Global gene expression analysis of osteogenesis from BM derived MSCs revealed a pattern of 533 genes >1.4-fold differentially expressed upon induction on day 1, 3, 5, 7 or 14. Both USSCs and MSCs specific pattern overlap for 154 genes representing specific gene expression changes independent of the stem cell type used for differentiation. The microarray expression data were corroborated by RT-qPCR for genes exhibiting differential expression and unchanged expression. Besides transcriptional regulation of gene expression interaction of mRNA transcripts and microRNAs - a class of short non-coding RNAs that have been shown to be involved in a wide variety of biological processes including cell differentiation - can lead to cleavage and digestion of transcripts resulting in altered mRNA levels. Therefore, correlation of known/predicted microRNA targets displaying altered mRNA-abundance and microRNA profiles is helpful to identify regulatory factors potentially involved in differentiation along the osteogenic lineage (see also poster presentation of Trompeter et al.). Within this context the NRW StemBase is linked to the smiRNA database from the Tuschl-Lab in New York. Together, these gene expression data and microRNA data sets serve to build up the NRW StemBase within and for the Stem Cell Network North Rhine Westphalia.

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Hematogenous macrophage recruitment is regulated by the chemokine receptor-2 after transient cerebral ischemia in mice

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Cells resident within the central nervous system and a multitude of different hematogenous cells contribute to the pathogenetic cascade following cerebral ischemia including regulatory, neuroprotective and cytotoxic functions as well as an inflammatory-like reaction, which are all substantially responsible for infarct development. Activation of resident microglia and recruitment of blood-borne macrophages play an important role but distinguish from a high complexity of interacting beneficial or even harmful mechanisms which are presently not fully understood. The chemokine Monocyte chemoattractant protein-1 (MCP-1) is considered as one of the main effectors driving postischemic infiltration of monocytes into the brain parenchyma and MCP-1 overexpression results in larger cerebral infarcts. Due to this facts we wanted to further investigate the role of chemokine receptor-2 (CCR-2), the high-affinity MCP-1 receptor, in hematogenous macrophage recruitment as the individual contribution of the different chemokine receptors in these mechanisms is largely unknown. On morphological grounds activated microglia and blood-borne macrophages were indistinguishable, since both cell types were able to take up a ramified as well as an activated bloated phenotype. To distinguish these cells, in the first step we generated radiated bone marrow chimeric mice by transplanting bone marrow from CCR-2 knockout and green fluorescent protein (GFP) transgenic mice into irradiated CCR-2 knockout and GFP-negative recipients. After three months of convalescence we compared the recruitment of hematogenous, GFP-positive macrophages in CCR-2 deficient mice and littermate controls after transient focal cerebral ischemia. Following 30 minutes of middle cerebral artery occlusion, in CCR-2 deficient mice occasional hematogenous macrophages, positive for monoclonal antibody F4/80 and transgenic for GFP, were first seen at day 2 mainly in the core of the ischemic area. Their number rapidly increased to maximum numbers at day 4 and decreased again until day 7. In contrast, in littermate controls we found quantitative minor numbers of hematogenous macrophages on day 4 but a proceeding increase until day 7, when maximum numbers were reached. In both, CCR-2 deficient and control mice, the vast majority of macrophages remained GFP negative during the first week after cerebral ischemia indicating their derivation from resident microglia. Our results indicate that there are no differences in microglial activation in CCR-2 deficient mice and their littermate controls. Microglial activation precedes over macrophage infiltration by several days, and the majority of macrophages following mild ischemic brain injury are derived from resident microglia rather than hematogenous macrophages. Furthermore we found different kinetics of hematogenous macrophage recruitment. In CCR-2 deficient mice hematogenous macrophage numbers reached an earlier an quantitative minor maximum than in CCR-2 littermate controls. These results demonstrates that the hematogenous macrophage recruitment is at least partially regulated by the CCR-2 receptor and that this receptor might be involved in the harmful MCP-1 mediated mechanisms. An inhibition of this system could be a new acute treatment approach to limit infarct size after stroke.

Keywords: cerebral ischemia; bone-marrow; macrophage; microglia; CCR-2

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Pharmacological and functional characterization of murine CM7/1-ESC-derived cardiomyocytes

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Cardiac muscle cells are notoriously difficult to cultivate. Moreover, contaminating non-muscle cells often interfer with the cardiomyocytes. Therefore cardiomyocytes derived from the recombinant murine embryonic stem cell line CM7/1 might have the advantage to be used as a highly reproducible cell culture model sytem in cardiovascular research. We have analysed pharmacological and mechanical properties of the recombinant murine cell line CM7/1, which allows selection of pure cardiomyocytes in vitro. Moreover, these cells are upscalable and can be cultivated in a bioreactor. ESC-line CM7/1 was cultivated and differentiated in spinner cultures. Selection with geniticin leads to formation of beating cardiobodies and isolated cardiomyocytes can be derived by trypsinization. Selection was possible due to stable integration of a neomycine-resistance gene under the control of the alpha myosin heavy chain promotor. Isolated cardiomyocytes were analysed on chronotropic effects of isoprenaline, clenbuterol, terbutaline and endothelin 1. Hypertrophic effects of isoprenaline and endothelin 1 was measured by microscopy as the circumferential diameter. Effects of mechanical strain and catecholamines were analysed in a flexer cell apparatus on silicon membranes. Gene expression changes were recorded from isolated RNA by real time RT-PCR using Taqman and LightCycler chemistry. Isolated cardiomyocytes derived from CM7/1 showed stimulation of chronotropy by isoprenaline, endothelin 1, terbutaline and clenbuterol with pEC50 of 8.24, 8.37, 5.23 and 3.12, respectively. Isoprenaline appeared to be the most potent stimulator for chronotropy analysed so far. Incubation with 10-6 M isoprenaline for 110h lead to downregulation beta1 adrenoceptor (b1AR) mRNA, whereas the beta2 receptor (b2AR) mRNA was upregulated. Incubation with 10-6 M clenbuterol, downregulated the b2AR but not b1AR. Hypertrophic effects could not be recorded for isoprenaline, endothelin 1 or clenbuterol. The incubation of 10-6 M isoprenaline reduced the circumferential diameter ($p<0.05$). However, myocyte diameters increased per se during cell culture. Mechanical stress increased BNP- and ANP-mRNA. Concomittant incubation with isoprenaline had no effect on BNP- and ANP-mRNA, respectively. However, combined effects of isoprenaline and mechanical stress were found for the myosin heavy chain beta mRNA.CM7/1 derived cardiomyocytes respond to positiv inotropic substances. Isoprenaline was the most potent stimulator for chronotropy. Stimulation by the b2AR-selective agonist terbutaline was also possible. Hypertrophic effects of isoprenaline, clenbuterol and endothelin 1 could not be documented due to sponanteous hypertrophy of cardiomyocytes during culture. Mechanical stretching allowed differentiation between neurohumoral and mechanical influences. CM7/1 derived cardiomyocytes appear to be a source for pharmacological analysis in cell culture.

Keywords: cardiomyocytes, embryonic stem cells, pharmacology, mechanical stress

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Differentiation of mouse embryonic stem cells into functional pancreatic and hepatic cells

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The organogenesis of pancreas and liver are highly interwoven with the pancreas emerging from the dorsal and ventral regions of the foregut, while the liver develops solely from the ventral endoderm. However, little is known about the signaling mechanisms that control early differentiation and specification of these cell types. Therefore, we established strategies for the in vitro differentiation of mouse ES cells via embryoid bodies (EBs), which spontaneously differentiate into a population of multilineage progenitor cells after plating. Addition of tissue-specific hepatic or pancreatic differentiation and extra-cellular matrix factors led to the development of committed pancreatic or hepatic progenitors and terminally differentiated cell types of the hepatic and pancreatic lineage, respectively. Hepatic differentiation generated glycogen- and albumin-producing hepatocyte-like cells with induced TTR, TAT, and Cytochrome P450 expression. At intermediate stages, ES-derived cells transiently co-expressed nestin/albumin, nestin/AFP and AFP/albumin. Differentiation into the pancreatic lineage resulted in islet-like clusters that showed glucose-responsive insulin release and normalized blood glucose levels of diabetic mice. Here intermediate stages displayed induced pancreatic transcription factors and transient co-expression of nestin/islet-1, nestin/C-peptide and C-peptide/CK19. Further, we performed transcriptome profiling via oligonucleotide array (Affymetrix) and quantitative RT-PCR analysis to study gene expression levels of intermediate and terminal stages of ES cell differentiation. A comparison of transcript levels with fetal and adult tissue of liver and pancreas suggests that ES-derived intermediate cell types represent fetal cellular stages. In conclusion, we propose that our data of in vitro ES cell differentiation may have general implications for the characterization of pancreatic/hepatic progenitors and the processes of adult tissue regeneration and transdifferentiation.

Keywords: Embryonic stem cells, in vitro differentiation, pancreatic/hepatic cells

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Generation and Characterization of Functional Cardiomyocytes from Rhesus Monkey Embryonic Stem Cells

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Mouse and human embryonic stem cells (ESCs) have been shown to be able to efficiently differentiate towards cardiomyocytes (CMs). As murine and human ESCs do not allow for establishment of preclinical allogeneic transplantation models, it was aim of our study to generate functional CMs from rhesus monkey ESCs (RESCs). Although formation of ectodermal and neuronal / glial cells appears to be the default pathway of the rhesus monkey embryonic stem cell line R366.4, we were able to change this commitment and to direct generation of endodermal / mesodermal cells and further differentiation towards cardiomyocytes. Differentiation of RESCs resulted in an average of 18 % of spontaneously contracting embryoid bodies (EBs) from RESCs. Semi-quantitative RT-PCR analyses demonstrated expression of marker genes typical for endoderm, mesoderm, cardiac mesoderm and CMs including brachyury, goosecoid, Tbx-5, Tbx-20, Mesp1, Nkx2.5, GATA-4, FOG-2, Mlc2a, MLC2v, ANF and alpha-MHC in RESC-derived CMs. Immunohistological and ultrastructural studies showed expression of CM-typical proteins including sarcomeric actinin, troponin T, titin, connexin 43 and cross-striated muscle fibrils. Electrophysiological studies by means of multi electrode arrays (MEA) revealed evidence of functionality, electrical coupling and b-adrenergic signaling of the generated cardiomyocytes. This is the first study demonstrating generation of functional cardiomyocytes derived from RESCs. In contrast to human ES-cells, RESCs allow for establishment of preclinical allogeneic transplantation models. Moreover, RESC-derived cardiomyocytes represent a cell source for the development of high-throughput assays for cardiac safety pharmacology.

Keywords: Embryonic stem cells; differentiation; cardiomyocytes; primates

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Functional analysis of the mammalian Pumilio genes, Pum1 and Pum2 in stem cell maintenance and differentiation

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The mammalian Pumilio genes, Pum1 and Pum2 are members of the Puf protein family, a group of RNA-binding proteins acting as translational repressors. Puf proteins are characterized by their consensus RNA binding motif, the Pum-homology domain (Pum-HD). They are key regulators in stem cell and germ cell maintenance in diverse organisms from invertebrates to vertebrates. In drosophila, Pumilio has also been shown to be involved in neuronal excitability, formation of long-term memory as well as dentritic arborization. Two Pumilio homologues, Pum1 and Pum2, have been identified in mice and humans. However, their functions remain unclear. We show that the Pumilio genes are widely expressed in human and mouse tissues and in ES cells. To further investigate the role of these genes in vivo, we generated Pum1 and Pum2 knockout mice. While Pum2 knockout mice are viable and fertile, inactivation of Pum1 leads to embryonic lethality. To study the role of Pumilio in early embryonic development in a more controlled manner, we developed a siRNA-based approach for silencing PUM in ES cells. In vitro differentiation of these ES cells will provide a tool to study PUM function in stem cell maintenance, germ line development and neural induction in vitro.

Keywords: Pumilio; translational repressor; ES cells; stem cell maintenance; germ cells

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Chondroitin Sulphate Glycosaminoglycans are reqired for the development of Telencephalic neural stem/progenitor cells

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The germinal regions of the embryonic cerebral cortex contain stem cells whose development is driven by the combination of intrinsic programmes and extracellular signals from the ECM. The chondroitin sulphate proteoglycans (CSPGs) as ECM component are present in the ventricular and subventricular zones of the developing forebrain. The DSD-1-PG/Phosphacan is a CSPG and represents a splice variant of the receptor protein tyrosine phosphatase (RPTP)-beta gene. It is thought to be involved in the regulation of many developmental events. The DSD-1 epitope is a defined but complex cell surface-associated chondroitin sulphate-glycosaminoglycan (CS-GAG) motif recognized by the monoclonal antibody 473HD. We have isolated 473HD-positive cells by immunopanning and characterized them as actively cycling, BLBP-positive neurogenic radial glia during cortical development. When the 473HD-positive cells were cultivated under neurosphere-forming conditions an increase in the number of neurospheres compared to the non-selected cell population was observed at all stages examined. This describes a significant aspect of the biology of embryonic neural stem cells – a subset of early multipotent neural progenitor/stem cells express DSD-1-PG/RPTPß, which aids their identification and enrichment. To address the functional importance of CS-GAGs for development and differentiation of telencephalic neural stem/progenitor cells in vitro and in vivo we used GAG-lyases for deglycanation of CSPG and investigated the functional consequences of CS-GAG removal on neural stem/progenitor cell behaviour using several cell biological assays. Our results show that removal of CS-GAGs from neural stem/progenitor cell-surface caused: (1) reduction in proliferation of these cells and their capacity to generate neurospheres in presence of mitogen factors EGF and bFGF and (2) changes of the composition of the precursor cell pool by a shift from neurogenic radial glia towards gliogenic radial glia after treatment. These findings revealed an important role of CS-GAGs for proliferation of neural stem/progenitor cells and suggest that this specific class of carbohydrates has a pro-neurogenic as well as an anti-gliogenic role during forebrain development.

Keywords: stem cells, proliferation, differentiation, ECM, neurogenesis

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NUMB, a cell fate determinant known from Drosophila melanogaster, inhibits maintenance of primitive human hematopoietic cell fates

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Hematopoietic stem cells (HSC) are the most investigated mammalian stem cells. Like other stem cells they are undifferentiated cells that can self-renew over a long period of time and give rise to progenitor cells which will reconstitute the whole immune and blood system. Although the mechanisms regulating the decision process self renewal versus differentiation remain largely unknown, there is good evidence that a combination of both extrinsic and intrinsic factors as well as the Notch signaling pathway are involved in controlling the cell fates of primitive hematopoietic cells and their arising daughter cells. Similarly, the Notch signaling pathway, its extrinsic ligands and its intrinsic modulators (specify) regulates the cell fates of the four cells of developing external sensory organs of Drosophila. In this system, the so called sensory organ precursor cells (SOPs) divide asymmetrically and give rise to IIa and IIb daughter cells. Mechanistically, the cell fate determinant NUMB, an antagonistic protein of NOTCH, segregates mainly into IIa daughter cells and inhibits the signal transduction of NOTCH, being activated by its ligands DELTA and SERRATE (the homolog of mammalian JAGGED). Depending on the transduction of the Notch signal, daughter cells are either specified as IIa (no transduced signal) or as IIb cells (transduced signal). Since NUMB is conserved during evolution and NOTCH activity seems to be required to maintain primitive hematopoietic cell fates, we wondered whether NUMB is involved in cell fate specification during early hematopoiesis as well. First of all, we found that NUMB is expressed in primitive human hematopoietic (CD34+) cells. To functionally analyse the effects of enforced NUMB expression on the cell fate of primitive hematopoietic cells, we have optimized the non-viral nucleofection technology to efficiently transfect primary human CD34+ cells. Using this technology we have over-expressed any of four different isoforms of NUMB in comparison to the constitutive active variants of Notch1 and Notch2. As it will be presented, effects on the cell fate of successfully transfected CD34+ cells were analyzed in LTC-IC and CFU-GEMM assays.

Keywords: stem cells; Notch; Numb; cell fate; differentiation

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Interaction of mesenchymal stem cells with the endothelium: Role of endothelial phenotype and VLA-4/VCAM-1

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Human adult pluripotent bone marrow derived mesenchymal stem cells are in the focus of scientific interest because they differentiate into various mesenchymal tissues. Mesenchymal stem cells (MSCs) are not only used in the field of tissue engineering but also as a potential therapy for the regeneration of infarcted myocardial tissue. After an intravascular application, MSCs have to interact with the endothelium before passing through the blood vessel wall to enter their target tissue. To date, the underlying mechanisms remain to be elucidated. Therefore, to asses how MSCs interact with the endothelium two different approches were emploied. First, to characterise the direct interaction of MSCs and endothelial cells, co-cultivation experiments have been performed. Second, to identify cell adherence molecules involved in the interaction, blockade experiments were carried out. The co-cultivation indicated that a direct interaction between MSCs and endothelial cells occures within less than one hour. Moreover, an integration of stem cells into the endothelial monolayer goes along with strong morphological changes of MSCs. In addition, co-cultivation experiments with different types of endothelial monolayer were conducted to identify differences of the interaction depending on the endothelial phenotype. Therefore, venous, arterial, aortic and microvascular endothelial cells were employed. To summarize, our results infere that the time course of integration and modification of shape of MSCs is depending on the type of endothelial cells and varies between 60 to 240 minutes. Blockade experiments with anti-VLA-4 (integrin alpha4/beta1) and anti-VCAM-1 (CD106) resulted in a significant slow down of integration of MSCs into the endothelial monolayer. Thus, we assume that the VLA-4/VCAM-1 interaction plays a key role in the transmigration of stem cells across the endothelial barrier. Taken together, our data suggest that MSCs are able to integrate in endothelial monolayer in vitro and thereby transmigrate across the endothelial barrier in vivo. The time course of this process varies depending on the vascular section and requires the interaction of VLA-4 and VCAM-1.

Keywords: mesenchymal stem cells; endothelium; VLA-4; VCAM-1

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Senescence and functional failure in mesenchymal stem cells

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The role of adult mesenchymal stem cells (MSC) in tissue maintenance and regeneration has received significant attention of late. Our research investigates to what extent adult stems cells are subject to, or causes of organismal aging, whether age-related changes in MSC are due to intrinsic factors or induced by the somatic environment, and the implications for tissue engineering. MSC from rats and humans of different ages were investigated for a range of parameters, including: age factors (p21, p53, beta-galactosidase), stress levels (actin, ROS, NO, TBARS, carbonyls, lipofuscin, SOD, GPx), cellular processes (apoptosis, proteasome activity), heat shock proteins (HSP27, 60, 70, 90), receptors (Notch-1, vitaminD, glucocorticoid), morphology, as well as differentiation range and proliferative ability. Conclusions are drawn about the role of adult stem cells in regeneration and aging. Our research suggests that age-related changes in MSC can be partially reversed ex vivo, by comparatively simple measures that could be of use in tissue engineering especially where aged donors and elderly patients are concerned.

Keywords: Mesenchymal stem cells, aging, heat shock proteins, differentiation, notch

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Involvement of the MCP-1/CCR-2 pathway in hematogenous cell influx after transient cerebral ischemia in mice

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At present specific stroke treatment, which includes thrombolysis with recombinant tissue plasminogen activator (rt-PA), is only applicable in a minor fraction of patients due to a limited time window after onset of symptoms. The development of additional strategies in stroke therapy is of great importance, which requires a profound knowledge of cellular and molecular mechanisms following cerebral ischemia. The identification of molecular pathways directing stem cell migration to the damaged region in the brain might be an advanced approach for improvement of therapy in neurovascular diseases. Cells of resident origin as well as a multitude of different hematogenous cells contribute to the pathogenetic cascade following cerebral ischemia. Although most intention has been directed to the possible role of neutrophiles after ischemic stroke there is evidence that T-lymphocytes also accumulate in the postischemic brain. Cytotoxic T-cells can contribute to tissue damage by several mechanisms including release of inflammatory cytokines that might recruit other hematogenic cells into the injured brain parenchyma. The chemokine Monocyte chemoattractant protein-1 (MCP-1) is considered as one of the main effectors driving postischemic infiltration of monocytes, memory T-lymphocytes and natural killer cells. As in vitro experiments showed that MCP-1 activates the migration capacity of neuronal stem cells and the chemokine receptor-2 (CCR-2), the high-affinity MCP-1 receptor, is involved in these mechanisms, we investigated the regulatory capacity of CCR-2 in neutrophile and T-cell recruitment in an in vivo model of transient focal cerebral ischemia in CCR-2 deficient mice and littermate controls. After 30 minutes of transient middle cerebral artery occlusion, we found only few neutrophiles within the infarct 24 hours after ischemia but a rapid increase of cell number at day 2 and proceeding increase with a maximum at day 4. After one week neutrophiles decrease to a similar level compared to day 2. In CCR-2 deficient mice we found the same profile of neutrophile recruitment to the ischemic area, but the absolute cell counts were reduced compared to littermate controls. Furthermore recruitment of CD-3 positive T-cells was investigated. While neutrophiles were distributed over the entire infarct area CD-3 positive lymphocytes accumulated in close vicinity to blood vessels. In control mice we found single lymphocytes on day 1 and also only few CD-3 positive cells 48 hours after MCA-occlusion. At day 4 a massive increase of cell number was seen followed by a rapid reduction at day 7. In CCR-2 deficient mice the number of lymphocytes detected in the ischemic area was quantitatively minor but revealed the same temporal pattern than in littermate controls. Our results indicate that the CCR-2 receptor is involved in neutrophile and T-cell recruitment after transient cerebral ischemia but the absence of a nearly complete reduction of neutrophile and lymphocyte infiltration supports the assumption of the participation of additional receptors than CCR-2 in the recruitment of hematogenous cells after cerebral ischemia. Furthermore also neuronal stem cell migration might be controlled by a network of receptors after brain damage in vivo

Keywords: MCP-1/CCR-2 pathway; cerebral ischemia; neutrophiles, lymphocytes

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ES cell vs. bone marrow: Analogy of macrophages from different derivation

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Macrophages are cells of the mononuclear phagocyte system with pleiotropic functions. They can be found in all tissues where they recognize and eliminate own cells as well as foreign cells, particles, viruses and bacteria. Macrophages are also key regulators in inflammatory processes. In a complex series of events involving stroma cells secreting cytokines they differentiate from hematopoietic stem cells in the bone marrow. During embryonic development of mice the first hematopoietic cells were found in the blood island of the yolk sac from day 7.5 on. Macrophages can be identified before monocytes are present in the embryo. So, macrophages arise from fetal precursor cells without exposure to bone marrow which lacks at this point of development. Our aim was to generate macrophages directly from embryonic stem (ES) cells and compare them to macrophages derived from the bone marrow precursor cells concerning their morphology, function and expression pattern. Similarities were found in phagocytosis, one of the main functions of macrophages, and in expression of macrophage specific markers both on protein level and at the genetic level. Significant differences were found in cell proliferation and size. A DNA-Array-Analysis showed analogy in 90% of the expression pattern but also differentially expressed genes involved in differentiation. Of these at least ten genes known to play a role in myeloid lineage differentiation processes were identified i.a. Csf1, the macrophage-colony stimulating factor. This data demonstrates that embryonic stem cell derived macrophages show typical properties of macrophages but at the same time can be characterized as a special type of macrophages because of the differences specified above.

Keywords: ES cells; macrophages; differentiation

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Identification and functional analysis of transcription factors specifically expressed in primitive hematopoietic cells

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Somatic stem cells have been found in a number of different tissues. They are required to generate differentiated cells that replace cells which are permanently lost during the lifetime of a multi-cellular organism. To fulfil this function over a long period of time, it is essentially required that the pool of stem cells remains more or less constant. 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 controlled. The understanding of such mechanisms will not only be essential for the clinical use of these cells in regenerative medicine but should also increase our understanding of certain aspects of tumor formation and degenerative diseases. At the example of the hematopoietic system, a few transcriptionfactors, 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 leukaemia. With the aim to identify additional transcription factors required for the self-renewal process if primitive human hematopoietic cells, we have performed geneome wide GeneChipTM analyses of different cell fractions, containing either primitive or more mature hematopoietic cells. Indeed, we could identify a number of transcription factor encoding genes which are specifically expressed in the most primitive hematopoietic cell fractions tested, whose function has not been associated with hematopoiesis so far. In order to characterise the early hematopoietic function of some of these candidates we decided to perform over expression as well as RNAi mediated knock down experiments. As it will be presented, 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 successfully transduced cells in different functional read out systems.

Keywords: Stem cells; self-renewal; transcription factor; gene transfer

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Epithelial-mesenchymal transition (EMT) is a regular process in rhesus monkey ES cell colonies growing on mouse feeder cells

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Epithelial-mesenchymal transition (EMT) is a regular process in rhesus monkey ES cell colonies growing on mouse feeder cells. A characteristic of embryonic stem (ES) cells is their ability to self-renew as well as the ability to give rise to all cell types that are derived from the three primary germ layers of the embryo. Germ layer formation involves and depends on epithelial-to-mesenchymal transition (EMT) and a cascade of gene expressions reminiscent of verterbrate gastrulation. However, knowledge about details of the processes of germ layer (derivative) formation, EMT and cell migration in ES cell colonies is still fragmentary. In the present investigations the rhesus monkey ES cell line 366.4 (rhESC; WiCell) was used to study in detail processes of epithelialisation and EMT in colonies grown on feeder cells, colonies maintained on laminin-coated plates and colonies re-plated from laminin to feeder co-culture. Experiments were performed with multiple antibody labelling (E-cadherin, ZO-1, Oct-4), and three-dimensional colony structure was analysed with confocal laser scanning microscopy. Additionally marker RNA expression was confirmed with RT-PCR analysis. Undifferentiated rhES cells growing on mitotically inactive mouse embryonic fibroblast (MEF) feeder layers form distinct multilayered colonies the majority of which demonstrate pit-like depressions between day two and four after plating. A characteristic downregulation of E-cadherin and ZO-1 expression occurs in the cells undergoing ingression and EMT as we have already shown previously. The present series of experiments revealed that slight variation of culture conditions, i.e. by changing pre-treatment of the feeder cells, or the thickness of the feeder cell layer leads to altered colony morphology and incomplete ingression or even the loss of ingression. This correlated with imperfect EMT and a disturbed E-cadherin and ZO-1 pattern. Thus a stringent definition of the conditions of feeder cell layer preparation is important for reproducible pit formation in the colonies. We further tested the same marker expressions in rhESC grown alternatively on laminin-coated plates in medium conditioned by MEFs (Xu et al., Nature Biotechnology. 19, 2001). Surprisingly, these cells showed an incomplete ZO-1 pattern and the E-Cadherin signal was totally missing in the CLSM, even though these cells were Oct-4 positive and retained the capability to form embryoid bodies, i.e. maintained characteristics of pluripotent ES cells. When these laminin-grown ES cells were placed back on MEF layers they failed to form pit-like ingressions within the colonies. This seemed to be related to the initial lack of E-cadherin expression and the incomplete ZO-1 pattern. Thus the described altered marker pattern may be causally related to the loss of ability to undergo a complete EMT, which is a prerequisite for ingression and pit formation in cells under three-dimensional culture conditions.

Keywords: EMT; rhesus monkey; E-cadherin; ZO-1; gastrulation

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Demonstration of Pluripotency of Unrestricted Somatic Stem Cells from Cord Blood on the Clonal Level

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A given single stem cell within a population must be able to differentiate into the distinct cell types of all three germ layers. Unrestricted somatic stem cells (USSC) have recently been described by our laboratory as a CD45-negative pluripotent population from cord blood. Upon induction, osteogenic, hepatic, and neural differentiation could be observed from USSC in vivo. To determine whether this pluripotency can be tracked down to a single pluripotent cell type within this population, cells were labeled by a lentivirus-based genomic integrate expressing a nuclear localization signal-coupled lacZ reporter gene from the constitutively active spleen focus forming virus promoter. Transfected cells were seeded by limiting dilution to a statistical density of 0.5 cells/well and checked for homogenous nuclear lacZ expression. Seven independent putative clones obtained in this manner were then tested for lentiviral genomic integration sites by ligation-mediated PCR (LM-PCR). To this end genomic DNA from the putative USSC clones was digested with HaeIII and a lentivirus-LTR-specific 5'-biotin-labeled primer was hybridized to the LTRs flanking each integrate. The subsequent primer extension then stops at the next HaeIII site within the viral integrate or the genomic DNA respectively. After purification using streptavidin-coupled magnetic beads, an adaptor was ligated to the 3'-ends of the single strand PCR products and the products were specifically amplified by PCR followed by a nested PCR and evaluation on an agarose gel. All seven putative clones tested showed the lentiviral contol band of appr. 380bp solely derived from the lentiviral integrate and certain different fragments representing the individual integrate-genomic DNA borders. Numbers of integrations varied from one to four and southern blotting using LTR probes revealed the lentiviral content of all fragments seen. The putative USSC clone 4 revealed only one genomic integration fragment beside the virus-internal control fragment in the LM-PCR thus pointing to its potential clonality. Consecutively, sequencing of 8 subclones generated from this band was performed and revealed identical sequences demonstrating the lentiviral integration into an ALU repeat on human chromosome 17. Thus, with only this single integration site found, USSC clone 4 must be viewed as truly clonal. USSC clone 4 was successfully differentiated in-vitro into osteogenic as well as into neural cells. LM-PCR performed for osteogenic cells differentiated from USSC clone 4 revealed identical band patterns to the undifferentiated clone 4 cells. Taken together, the homogeneity of clone 4 in lacZ expression, the finding of only a single lentiviral integration site, and its ability to differentiate into osteogenic as well as into neural cells clearly demonstrate that USSC indeed possess pluripotent potential on the clonal level. Lentiviral labeling to identify clonal USSC has not only served as an ideal marker to follow cell fate during diffentiation. Most importantly, this appoach clarified the critical issue of clonality with regard to USSC pluripotency. As a consequence of this result a solid cellular platform for molecular analyses like global expression comparisons of mRNAs or microRNA and further identification of functionally important genes is established.

Keywords: Somatic Stem Cells; Clonality; Pluripotency; Lentiviral Labeling

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Neurogenesis in the human adult epileptic brain: in vitro and in vivo study of human temporal lobe epilepsy resection probes as a potential source for multipotent progenitor cells

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The evidence of neuronal precursor generation in the hippocampal dentate gyrus and the subventricular zone throughout adulthood has given rise to novel therapeutic strategies for CNS disorders by either stimulating neurogenesis in vivo or by transplantation of propagated and differentiated neuronal-determined precursors. It has been shown that in epilepsy disorders, increased neurogenesis takes place though it remains unclear whether these mechanisms are reparative or pathogenic. The principal discussion is focused of whether the epileptogenic source of these cells should be seen as a focally stem cell infiltrated region or whether the potential of these cells is diminished by the notion that seizure- or injury-induced neurogenesis triggers the onset of the epileptic hippocampal formation characterized histologically by aberrant neuronal cytoarchitecture. Our aim is to facilitate the understanding of neuronal development, maturation and function of human adult neuronal precursors (hNP) by in vitro and in vivo analysis of resected probes derived from temporal lobe epilepsy patients. Temporal lobe tissue probes were enzymatic dissociated and propagated as neurospheres in proliferation medium containing EGF, bFGF and PDGF. Proliferating cultures were treated with BrdU and then differentiated according to various protocols including cytokine withdrawal or addition of retinoic acid, ascorbic acid, fetal calf serum (FCS) or BDNF for two, three, four, six and eight weeks at low oxygen (5% O2) or atmospheric O2 levels. After fixation, immunocytochemistry analysis followed to determine proliferation and differentiation outcome. In parallel these cultures were transplanted into not immunosuppressed post-natal day five (P5) neonatal rats which were either bilaterally lesioned using 6-hydroxydopamine (6-OHDA) at P1 or healthy controls. Grafts were evaluated by amphetamine and apomorphine drug-induced rotations before being perfused after six months. Additionally, proliferating cells were transplanted into either the hippocampus, striatum or substantia nigra of Ciclosporin immunosuppressed healthy adult rats. Animals were perfused after three and six months. All brains were sectioned and stained against anti-human nuclei and anti-BrdU for localization of grafted cells as well as population markers. Preliminary evaluation of cell cultures during proliferation conditions showed heterogeneous cell mixtures containing ß-tubulin III neurons, cells positive for the intermediate filament markers nestin and vimentin and glial fibrillary acidic protein (GFAP) as well as proliferating cells positive for Ki67 and BrdU. Counting and evaluation of cell cultures after differentiation and stereological analysis of grafted animals is ongoing and will be presented during the conference.

Keywords: neurodegeneration, neuroregeneration, transplantation

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TNF triggers proliferation of adult neural stem cells via IKK/NF-kappaB signalling

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Brain inflammation has been recognized as a complex phenomenon with numerous related side aspects. Beside of the very well described neurodegenerative effect of chronic inflammation, several studies suggest a potential positive influence of inflammatory signals on neural stem cell proliferation, migration and differentiation. Tumor necrosis factor alpha (TNF-alpha) is one of the best characterized mediators of inflammation. In addition, TNF secretion into adjacent tissue was described in several neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. Here we describe for the first time the TNF mediated signal transduction cascade in neural stem cells (NSCs) resulting in increased proliferation. Moreover, we demonstrate IKK-alpha/neta depending proliferation and highly up-regulated cyclin D1 expression after TNF treatment. TNF treated cells showed significantly increased proliferation, shown as increased neurosphere volume, an increase in bromodeoxyuridin (BrdU) incorporation and higher total cell number. Furthermore, TNF strongly activated nuclear factor-kappa B (NF-kappaB) as measured by reporter gene assays and with an activity specific antibody. Proliferation of control and TNF-treated NSCs was strongly inhibited by expression of the NF-kappaB superrepressor IkappaB-AA1. Comparably, pharmacological blockade of the IKK-alpha/beta-complex led to decreased NF-kappaB activity and reduced proliferation. IKK-beta gene product knock-down via siRNA led to diminished NF-kappaB activity, attenuated cyclin D1 expression and finally to decreased proliferation. In contrast TGF beta associated kinase 1 (TAK-1) is dispensable only in part for TNF mediated and endogenous proliferation. Understanding proliferation of stem cells is crucial for future regenerative and anti-tumor medicine.

Keywords: neural stem cell, TNF, proliferation, NF-kappaB, signal transduction

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Differentiation of murine embryonic stem cells into alveolar type II epithelial cells

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Introduction: Alveolar type-II (AT2) epithelial cells have important functions, including the production of surfactant and regeneration of lost alveolar type I (AT1) epithelial cells. The ability of in vitro production of AT2 cells would offer new therapeutic options in treating pulmonary injuries and diseases, including genetic disorders and ischemia-reperfusion-injury after lung transplantation. Methods and Results: Embryoid bodies (EBs) were generated from feeder-free cultures of murine embryonic stem cells (mESCs). Differentiation towards mesendodermal progenitors was optimized by means of a 'brachyury-eGFP-knock in' mESC line. After initial suspension culture, EBs were plated to tissue culture discs and were cultured in small airway growth medium (SAGM) or, were cocultured with E11.5 murine embryonic lungs in a transwell setting for up to 30d. Brachyury-dependent eGFP expression with a maximum at d4 after EB-formation demonstrated efficient generation of mesendodermal progenitors. An increasing expression of a series of endodermal markers as well as markers for AT2 cells was shown by semiquantitative RT-PCR in SAGM as well as in transwell cocultures. Immunohistological analyses confirmed SPC-expression in mESC-derived AT2-like cells. Electronmicroscopical studies to demonstrate lamellar bodies as a unique feature of AT2 cells are ongoing. Further studies will be supported by the use of a novel lentiviral vector mediating GFP expression under control of the human SP-C promoter. This vector will allow for detection and FACS-based purification of ESC-derived AT-2 cells. Conclusion: Our results demonstrate that optimised endoderm differentiation is of importance for further efficient differentiation towards AT2 cells. Our data will serve as a basis to identify key regulators mediating differentiation of ESCs towards alveolar epithelial cells.

Keywords: pneumocyte; ES cell; transwell; differentiation; lentivirus

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Consequence of Cdx2 knockdown by RNA interference in mouse embryos

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Caudal type homeobox 2 (Cdx2), a Class I homeobox gene, is required for fate specification and differentiation of the trophectoderm in the mouse; its deficiency results in peri-implantation lethality. We assessed whether Cdx2 is involved in establishing and/or maintaining the pluripotent phenotype of cells of the embryo. To this end, Cdx2-specific siRNA duplex or control molecules were injected into pronucleate ova recovered from oviducts of B6C3F1 mice, and subsequent gene expression and ES cell derivation efficiency was evaluated. Similar to the phenotype observed in embryos with Cdx2 deficiency caused by gene targeting, all blastocysts that developed after Cdx2 siRNA treatment failed to hatch and implant. None of the 109 embryos transferred into 8 recipients implanted, in contrast to 60 of the 108 control embryos that were transferred into 6 recipients. Moreover, the pluripotency markers Nanog and Oct4 were expressed ectopically in the putative trophectoderm obtained from Cdx2 siRNA–treated oocytes. ES cell lines were derived from such blastocysts with low efficiency (2.0%) compared with control blastocysts (19.1%). Using quantitative real-time PCR, there was greater than 95% reduction in Cdx2 expression in these blastocysts compared with control blastocysts. While Oct4 levels increased, Eomes, Hand1, and Fgfr2 levels were drastically reduced in Cdx2-depleted blastocysts, which is consistent with the absence of functional trophectodermal cells. Since the ES cell derivation efficiency of cdx2 knockdown blastocysts were reduced in our experiments, we assessed 8-cell stage embryos for their ability to give rise to ES cells. The efficiency of 8-cell stage control embryos was similar to that of control blastocysts (22.4% vs. 19.1%). In contrast, the efficiency of ES cell derivation drastically differed after Cdx2 siRNA treatment of pronucleate ova (33.4% for 8-cell stage embryos vs. 2.0% for blastocysts). Interestingly, Cdx2 siRNA treatment increased the efficiency of ES cell derivation by 50% over that of the controls. As shown with germline-specific Oct4-GFP and ubiquitously expressed lacZ transgenes, injection of these ES cells into Cdx2-depleted blastocysts contributed to both somatic- and germ-cell lineages, and even to the intestine. Because Cdx2 siRNA treatment is of transient nature in mammals and does not involve manipulations of the genome, we postulate that the derivation of patient-specific ES lines for therapeutic purposes can be accomplished in a straightforward and safe manner.

Keywords: Cdx2; Embryos; RNA interference; Stem cells

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Stem Cells and Cancer

The side population of umbilical cord blood is enriched for stem cell function and maintained during cryopreservation, but is not associated with ABCG2 expression

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The side population (SP) phenotype resulting from efflux of Hoechst 33342 has been associated both with multidrug resistance and with populations of high quality stem cells from both haemopietic and non-haemopoietic organs. Although the SP phenotype of bone marrow stem cells is commonly believed to be associated with expression of the transporter protein ABCG2, the precise relationship between ABCG2 expression, SP phenotype, the expression of surface markers and stem cell activity remains incompletely characterised. In order to define in more detail the properties of SP cells from umbilical cord blood (UCB), we have performed multiparameter FACS analysis, quantitative gene expression studies and CAFC (Cobblestone Area Forming Cell) assays from both fresh and cryopreserved UCB samples. We found that the SP cells consistently accounted for ~0,2% of fresh mononuclear cells and that the absolute yield of SP cells per unit volume UCB was unaffected by cryopreservation. As expected, the SP was highly enriched for 12 week CAFCs (the equivalent of repopulating cells). Although the majority of CD34+ and CD133+ cells had a distinctive Hoechst staining profile bordering the side population, only a minority were actually in the SP itself. SP cells generally expressed the panleukocyte marker CD45. ABCG2+ cells were readily detected in ~0,03% of mononuclear cells. Importantly, neither FACS nor real-time PCR analysis revealed any evidence of increased expression of ABCG2 in the SP compared to the rest of the mononuclear cells. Furthermore, MACS-purified ABCG2+ cells failed to grow in media known to support proliferation from stem cells. We conclude that SP cells from umbilical cord blood are enriched for stem cell function, show heterologous expression of stem cell markers with most cells being negative for CD34 and CD133, and appear to be undamaged by cryopreservation. In contrast to reports of murine bone marrow-derived cells, the side population phenotype of UCB is not associated with increased ABCG2 expression.

Keywords: abcg2; side population; human; cord blood; cafc;

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Direct isolation of Prominin-1+ cells from postnatal mouse brain and hematopoietic tissue using MACS® Technology

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Prominin-1, the mouse structural homolog of human CD133, is known to be a marker for multipotent neural stem cells in the developing and postnatal central nervous system, epithelial cells, and cells of the hematopoietic system. We have developed specific tools for the efficient isolation of prominin-1 positive cells from neural tissue for studying, e.g. their phenotype, their cell differentiation potential and their gene expression profile. In a two-step, mild enzymatic tissue digestion procedure using the MACS® Neural Tissue Dissociation Kit, a single-cell suspension with a high yield of living cells is obtained. Magnetic activated cell sorting (MACS® Technology) is then used for the isolation of prominin-1+ cells. Prominin-1+ cells from postnatal cerebellum were enriched to a purity of up to 90% with the use of our novel Anti-Prominin monoclonal antibody (clone MB9-3G8). The purity of enriched prominin-1+ cells was assessed by co-staining with two anti-mouse Prominin monoclonal antibodies clone 13A4 and clone MB9-3G8 recognizing different epitopes of the prominin-1 antigen. We analyzed the phenotype of prominin-1+ cells in the subventricular zone (SVZ) of adult mice, as well as in the cerebellum of P1 mice, using markers such as PSA-NCAM (neuronal progenitor cells), CD24 (neuronal precursors, ependymal cells), and CD15 (neural stem cells) by flow cytometry. Prominin-1+ cells isolated from both postnatal cerebellum as well as adult SVZ were CD15–, PSA-NCAM– and partially CD24+. In cell culture experiments, Prominin-1+ cells isolated from P1 cerebellum formed neurospheres when cultivated in an in-house–developed medium containing B27, EGF and bFGF, whereas the Prominin– fraction failed to do so. In a second set of experiments, we assessed Prominin-1 expression on cells of the blood and bone marrow of adult mice. A population of less than 0.01% prominin-1+ cells was detected in blood as well as in bone marrow. After immunomagnetic enrichment using MACS® Anti-Prominin-1 MicroBeads, a highly enriched Prominin-1+ cell population was detectable. Further characterization revealed that the prominin-1+ population is CD90+ and CD117 (c-kit)–. 50% of the prominin-1+ population also expresses Sca-1, a marker for mouse hematopoietic stem cells. 15% of the cells display no markers of lineage commitment. These experiments demonstrate a fast and simple way to efficiently purify prominin-1+ cells from different tissue sources, including neural and hematopoietic tissues.

Keywords: prominin-1; CD133; neural stem cells; MACS Technology

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Adult bone marrow derived stem cells: Characterization and cell therapy approach

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Discovered by Friedenstein in 1976, mesenchymal stem cells (MSCs), plastic adherent cells from bone marrow, have been shown to differentiate into osteoblasts, chondrocytes and adipocytes. Over 30 years, many groups have been working on the characterization and differentiation of MSCs, but possible pluri- or multipotency of MSCs is still debated. In our study, we isolated murine bone marrow cells and cultured them in LIF containing medium according a protocol recently described (Jiang, Y et al., Nature 418, 41-49, 2002). The cultured cells expressed gene transcripts of the pluripotency genes Oct4, Nanog, Rex1 as determined by RT-PCR. Furthermore, they were positive for the stage-specific embryonic antigen 1 (SSEA1) as determined by flow cytometry. Differentiation of these cells to adipocytes, osteocytes and , tubulin III expressing neuron-like cells was achieved in vitro. We performed aggregation experiments between bone marrow derived cultured progenitor cells of GFP+ mice and morulas (8-cell stage embryos) to analyze the in vivo differentiation potential of these cells. Contribution of genetic GFP material from the cultured progenitor cells was detected in E12.5 embryos in different tissue types including muscle tissue and neuronal tissue. The contribution of the GFP+ cells to the embryo was relative low (1-3%). In order to enrich the “multipotent” population from the bone marrow, we used a transgenic mouse model, in which GFP is expressed under the Oct4 promoter (Oct4 Prom GiP; Ying, Q et al., Nature 416, 545-548, 2002; kindly provided by A. Smith) to select and expand the Oct4 expressing stem cells in culture. Preliminary results suggest that there is evidence for a “multipotent” Oct4 expressing sub-population of the bone marrow derived cultured mesenchymal cells. Consequently, we are currently defining the optimal culture conditions for the enrichment of this sub-population in the otherwise highly heterogeneous bone marrow derived progenitor cell population. As an application in cell or gene therapy approach we asked whether we can use the properties of chemokine receptors in targeting the bone marrow derived mesenchymal cells. We took advantage of the CXCR4 chemokine receptor and its ligand SDF-1 to address this question. CXCR4 receptor was overexpressed in murine bone marrow derived mesenchymal cells by gene transfer with retroviral vectors. Cells transduced with CXCR4 or a control vector derived form GFP+ mice were injected i.v. into lethally irradiated mice. At 3 weeks after injection CXCR4 transduced GFP+ cells were detected in the bone marrow, and after 5 weeks a high contribution of CXCR4 transduced GFP+ cells was observed in the bone marrow. The results demonstrate that CXCR4 gene transfer of chemokine receptors can target mesenchymal stem cells into the bone marrow opening new avenues for tissue specific gene delivery.

Keywords: adult stem cells, Oct-4, multipotent, cell therapy

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Murine tissue-derived stromal cells show progenitor cell plasticity

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Adult stem/progenitor cells were isolated from different mesodermally derived organs such as bone marrow, adipose tissue, muscle and peripheral blood. Having the potential to show multilineage differentiation, high proliferation and ease of extraction, these progenitor or stem cells offer a promising system for tissue engineering. A characteristic feature of stem/progenitor cells is their ability to differentiate into multiple cell types including osteoblasts, chondrocytes and adipocytes. During this study we posed the question whether stem/progenitor cells isolated from different murine mesodermal tissues show the potential to differentiate into the above mentioned lineages and express tissue specific genes and proteins. Cells isolated from brown adipose tissue and mediastinal connective tissue showed a fibroblast-like morphology, plastic and glass adherence and could be maintained in vitro for extended periods. Plating of bone marrow aspirates led to the formation of colonies termed as colony-forming unit-fibroblast (CFU-F). Analysing and comparing the cellular properties of the different cell lines, we used histological and fluorescent immunostaining as well as RT-PCR to describe multilineage differentiation. Stem/progenitor cells derived from all three stromal tissue resources show the capability to differentiate into chondrogenic, adipogenic and osteogenic cells in the presence of lineage-specific induction factors. Sudan III staining was used to demonstrate adipocyte differentiation and alkaline phosphatase staining to demonstrate osteogenic cells. The cells also expressed specific marker molecules such as aP2 and osteocalcin further characterizing them as adipopcytes and osteocytes, respectively. On the cellular level we identified the expression of osteopontin and bone sialo protein in osteogenic cells via immunostaining. Interestingly, the three different cell isolates differ in their differentiation efficiencies. While the bone marrow-derived cells preferably show osteogenic potential, brown adipose tissue-derived cells differentiate predominantly into adipogenic cell types. These results indicate that the analyzed cells although capable to differentiate into several lineages show a certain restriction depending on their source tissue.

Keywords: murine progenitor cell; mesenchymal differentiation; adipose tissue; mediastinal tissue

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Differentiation of mouse embryonic stem cells to microglia

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Microglia play detrimental as well as beneficial role in neurological diseases. They can act by regulating pro-inflammatory cytokines production and inflammatory reaction, eliminating damaged cells by phagocytosis or supporting the survival of cells by release of numerous growth factors. There is recent evidence that microglia derived from embryonic stem (ES) cells migrated into the central nervous system after intravenous injection in healthy mice (Tsuchiya et al., 2005). We aimed here to differentiate mouse C57/BL6 ES cells to microglia for further use as a therapy of neurological diseases like multiple sclerosis and Alzheimer disease. For differentiation of ES cells to microglia a modified standard protocol for differentiation of ES cells into neurons was applied (Lee et al., 2000; Tsuchiya et al., 2005). At different stages of differentiation cultures were analysed by immunocytochemistry and flow cytometry. At the beginning of differentiation embryonic bodies (EBs) were formed from ES cells. Thereafter Nestin+ neuronal precursors were selected by cultivation of EBs for 6 days in DMEM/F12 medium supplemented with Insulin-Transferrin-Selenin-Fibronectine. Expansion of Nestin+ cells in N2 medium with addition of bFGF resulted in increase in numbers of neuronal precursor cells and formation of neuronal network. At this stage IBa1+ cells, which showed a ramified morphology were detected. The IBa1+ cells incorporated into neuronal network and were found between neuronal processes. When N2 medium was switched to serum containing RPMI medium supplemented with granulocytes macrophages - colony stimulating factor (GM-CSF) IBa1+ cells had an ameoboid shape that represented activated cell morphology. In contrast, in cultures which were kept in N2 medium supplemented with GM-CSF, IBa1+ cells had a ramified microglial-like morphology. Flow cytometry analyses showed a small percentage of CD11b and CD45 positive cell in the cultures. Thus, we conclude that either presence of neurons or neuronal network seems to be involved in the development of microglia from ES cells.

Keywords: embryonic stem cells, microglia, neurological diseases

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Evaluation of the expansion and differentiation potential of CD271+ (LNGFR) marrow stromal cells (MSCS) versus MSCs isolated by plastic adherence

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Background: Marrow stromal cells (MSCs), with the ability to strongly adhere to plastic, comprise a heterogeneous population of nonhematopoietic (NH) stem cells with broad plasticity. Many attempts have been made to define the most efficient marker for the isolation of these MSCs, using markers such as CD271 (LNGFR), CD133, CD117, CD105, anti-fibroblast antigen, or Stro-1. CD271 (LNGFR)+ was reported to be the best known marker for the isolation of MSCs from bone marrow [1], furthermore showing a 1 to 3 log greater expansion rate and a greater capacity to differentiate to adipocytes and osteoblasts [2]. Therefore CD271 (LNGFR) has become one of the most promising and distinct marker for MSC isolation. Methods: MSC isolation and cultivation were performed using the MSC Research Tool Box - CD271 (LNGFR) (Miltenyi Biotec) containing CD271 (LNGFR)-APC antibody and Anti-APC MicroBeads for the separation of CD271+ cells from BM, and NH Expansion Medium supplemented with CytoMix - CD271 (LNGFR) for the expansion of MSCs. The potential of CD271 (LNGFR)-isolated cells was compared with MSCs isolated by plastic adherence (PA) using the same cultivation conditions. The cumulative population doubling (CPD) rate was determined during 41 days of cultivation. Following expansion, cells obtained by the different procedures were differentiated into adipocytes (NH AdipoDiff Medium), chondrocytes (NH ChondroDiff Medium) and osteoblasts (NH OsteoDiff Medium). Results: Starting from 2x10*7 human BM cells, CD271+ cells were isolated with a purity of 73-94%, yielding 2x10*5 CD271+ cells on average (n=3). Comparing the different methods, the best CPD rate was achieved using the MSC Research Tool Box - CD271 (LNGFR). All MSCs obtained showed similar potential to differentiate into adipocytes, chondrocytes and osteoblasts. Conclusion: Our results support the finding that CD271 (LNGFR)+ cells show a greater expansion rate compared to MSCs isolated by PA, and maintain the ability to differentiate into at least mesenchymal lineages. Therefore, CD271 (LNGFR) isolation helps to establish efficient and reproducible procedures for the isolation of a homogeneous population of MSCs for the implementation of reliable therapies.

Keywords: MSCs; CD271; LNGFR; MSC Research Tool Box

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Doxorubicin treatment selects for cells with a stem cell-like phenotype in a bladder carcinoma cell line

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It is commonly thought that tumour cell lines contain a small fraction of tumour stem cells which are responsible for long-term proliferation. These cells are enriched in compact cell colonies (holoclones) and exhibit a natural chemoresistence due to their constitutive expression of several ABC membrane transporters. In an attempt to characterize the population with stem cell characteristics in bladder carcinoma cell lines, we exposed BFTC905 cells to increasing concentrations of doxorubicin, a component of common chemotherapy regimes in bladder cancer, ranging from 100 nM to 250 nM. This resulted in widespread cell death and chemosenescence, followed by restoration of cell growth after a period of ~ 2 month originating from small clusters of small, compactly arranged cells. Strikingly, whereas there is a pronounced variability of morphologies in colonies of the parental BFTC905 cell line seeded at clonal density (41 % holoclones, 33% meroclones, 26% paraclones), all the colonies appearing after the doxorubicin selection displayed holoclone morphology. One such colony was successfully established as a resistant cell subline (BFTC905-DOXO-II), which is able to withstand doxorubicin concentrations as high as 550 nM without any immediate widespread cell death. Immunohistochemical analysis of the parental BFTC905 cells selected by 150 nM doxorubicin and BFTC905-DOXO-II cells selected by 400 nM doxorubicin, respectively, revealed the presence of clusters of small densely packed cells, which were positive for cytokeratin 14 and p63. We conclude that the doxorubicin treatment led to selection of cells with a stem cell-like phenotype.
*Jiri Hatina is a recipient of a teaching professor fellowship by the German Academic Exchange Service (DAAD).

Keywords: tumour stem cells; bladder carcinoma; doxorubicin

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Cytokines highly influence the migratory activity of ex-vivo expanded murine hematopoietic stem cells

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Hematopoietic stem cells (HSCs) are characterised by their ability to long term reconstitute large numbers of all blood cell lineages. The potential of HSC to undergo self-renewing cell divisions has stimulated investigations of ex-vivo stem cell expansion, which would be valuable to improve the quality of stem cell transplants by increasing the number of stem cells. HSC can be expanded in culture with a variety of cytokines. The cytokines thrombopoietin (TPO), Flt3-Ligand (FL), stem cell factor (SCF) and Interleukin-11 (IL-11) have previously been identified as being able to stimulate amplification of the most primitive murine hematopoietic cells in-vitro. Recent studies showed that ex-vivo expansion failed to augment or even impaired engraftment potential of hematopoietic progenitors. Efficient engraftment requires homing of transplanted HSCs to the bone marrow, depending on directed migration of HSCs along a gradient of stromal cell-derived factor-1 alpha (SDF-1 alpha). It has been observed before by our group that the migration pattern of human HSCs can be highly influenced by the culture conditions. Therefore, we used different combinations of FL, TPO, SCF and IL-11 for expansion of murine HSCs to elucidate their effect on the migratory capacity of these cells. Murine c-kit+ lin- HSCs were cultivated for 5 days in the presence of various cytokine combinations and their locomotory behaviour was investigated in a 3D collagen matrix using time lapse video microscopy and computer aided analysis. Remarkably, the results show that the cytokine combination used for ex-vivo expansion has a profound impact on the migratory response to SDF-1 alpha as well as the spontaneous migratory activity of murine HSCs. The highest migratory capacity was observed on cells expanded with SCF and Flt3-Ligand. Surprisingly, the further addition of either IL-11, TPO or both to this combination was associated with a reduced migratory activity as well as with a reduced SDF-1 alpha induced migration. Furthermore, IL-11 abolished the ability of HSC to respond to SDF-1 alpha if added to medium containing SCF or FL alone or in combination. Together with SCF, TPO had the same effect while acting synergistically with IL-11. With certain cytokine combinations, SDF-1 alpha even has an inhibitory effect on the migratory activity of the cells. Thus, we determined whether these effects correlate with changes in the expression level of CXCR4, the only known receptor for SDF-1 alpha. No correlation between receptor expression and migratory response towards SDF-1 alpha was observed. TPO is able to induce megakaryocytic (MK) lineage differentiation in HSCs, and IL-11 can promote MK development in early hematopoietic progenitors. MK differentiation has been shown to be accompanied by a suppression of migratory activity in HSCs. We were able to show that a higher proportion of cells belonging to the large MK (>4N) fraction tends to correlate with a reduced migratory response towards SDF-1 alpha. These results demonstrate that usage of IL-11 and TPO for in-vitro expansion of HSCs compromises their migratory capacity. This has to be taken into consideration for clinical applications that require efficient engraftment.

Keywords: hematopoietic stem cells;migration;ex vivo expansion;SDF-1 alpha

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Comparison of different Retroviral Gene Transfer Systems allowing stable Gene Transfer into Normal and Leukemic Hematopoietic Stem Cells

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Lifelong normal hematopoiesis originates from a limited number of self-renewing stem cells which differentiate via multipotent and lineage restricted progenitor cells and ultimately give rise to all mature blood cells. It has been postulated, that also in acute leukemias cells are derived from a "leukemic" stem cell population with cell biology organized in a similar hierarchical structure as physiological hematopoiesis. Retroviral marking of leukemic stem cells and xenotransplantation of these cells into immunodeficient mice thus may give new insight into the biology of acute leukemias. We here have utilised three retroviral gene transfer systems, i.e. foamy-, lenti- and gammaretroviral vectors, and compared their potential for stable gene transfer into leukemic cell lines as well as normal and leukemic primary hematopoietic cells. Foamy- and lentiviral vector preparations were produced using a transient transfection system, while gammaretroviral preparations were generated from a stable packaging line. While high titer lentiviral (0,5-4x10^6 particles/ml) and gammaretroviral (1-3x10^5 particles/ml) preparations were successfully generated, foamyviral preparations in our hands, in contrast to other investigators, only achieved low titers (3-5x10^4 particles/ml) when tested on HT1080 cells.When lenti- and gammaretroviral preparations were used to transduce a T cell lymphoma cell (Jurkat) and an ALL-derived cell line (RS), analysis of eGFP transgene-expression by flow cytometry analysis revealed high transduction efficiencies ranging from 50% to 90% for both vector systems. Subsequently lenti- and gammaretroviral constructs were used for gene transfer into primary normal or malignant hematopoietic cells. Non-prestimulated cells (16h protocol) or cells prestimulated with cytokines for 24h (40h protocol) were transduced overnight in the presence of fibronectin and growth factors. For transduction of normal hematopoietic cells also an 72h protocol employing 48h of prestimulation was used. For the lentiviral vector system transduction efficiencies of 6%±1% (mean±SEM, n=5/5) using the 40h protocol on peripheral blood stem cells were achieved, and results could be improved to 26% with the 72h protocol. This compared favorably with gammaretroviral transduction which yielded 7%±3% (n=4/4) transgene expression using the 72h protocol. In primary acute myeloid leukemic (AML) cells lentiviral transduction with the 40h protocol resulted in reproducible gene transfer with an efficiency of 14%±8% (n=9/9). In non-prestimulated cells the gene transfer efficiency was 6%±5% (n=4/5). Surprisingly, even without prestimulation gammaretroviral vectors showed reproducible transduction of AML cells (11%±9%, n = 3/3) which was increased by 24h of prestimulation to 14%±11% (n=8/8). With the foamy viral low titer preparations only low efficient transduction of Jurkat (7%±3%, n=4) and RS (6%±2%, n=4) cells, and even inferior results in primary human hematopoietic cells (1%±1%, n=2/5 samples transduced) were achieved. Thus, in summary, lentiviral as well as gammaretroviral preparations allowed reproducible gene transfer into primary normal and leukemic hematopoietic cells with efficiencies, that may suffice to extend these experiments to marking studies in an NOD/SCID-xenotransplantation model of human AML.

Keywords: Leukemias; Lentivirus; Hematopoietic Cells

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Cytokine secretion of human umbilical cord blood-derived mononuclear cells in vitro

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Intraperitoneal transplantion of human umbilical cord blood-derived mononuclear cells led to the "homing" of these cells specifically to a hypoxic-ischemic lesion in perinatal rats. Motor deficits resulting from the lesion were alleviated upon transplantation (1). However, the molecular and cellular mechanisms underlying the functional improvement are still unclear. There was no evidence for differentiation of incorporated HLA-positive human cells as investigated by expression of standard neural marker proteins (NF 68, TUJ1). We therefore propose secondary events, which might be responsible for the obvious therapeutic effects. One possibility is that the transplanted cells might contribute to a regenerative environment by secretion of cytokines. To identify putative human cytokines involved, we employed an in vitro system of umbilical cord blood mononuclear cells. Cells were cultured according to Sanchez-Ramos et al. (2). Conditioned medium was collected at various time points (2, 7, and 14 days of cultivation) and subsequently assayed for levels of secreted cytokines. With time in culture, the amount and number of cytokines detected in the conditioned medium changed. The initial levels of immunological cytokines (Interleukin-2, 3, 4, 5, 6, 12, 13, 15) decreased with time in culture. In contrast, levels of secreted growth factors (PDGF, GDNF, HGF, BDNF) as well as chemokines (MCP-1, MIP1-beta, Eotaxin) increased substantially. Interestingly, treatment of astrocyte cultures with mononuclear cell conditioned medium resulted in a change of cell morphology. These results are promising in that secreted cytokines of human mononuclear cells might be suitable candidates mediating functional recovery after hypoxic-ischemic brain injury in vivo. (1) Meier et al. (2006) Pediatr. Res. 59:244-249 (2) Sanchez-Ramos et al. (2001) Exp. Neurol. 171:109-115

Keywords: Cytokines; Chemokines; umbilical cord blood; secretion; conditioned medium

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Trophoblastic humeral factors in the regulation of endothelial progenitor cell development

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Hematopoietic stem/progenitor cells (HSPC) have been found in the human villous tissue before fetal circulation is fully established. During this time, villous vessel development occurs in the proximity to and under the influence of the trophoblast. Accordingly, understanding the impact of the trophoblast on placental vessel development may lead an important component in reducing pregnancy-associated morbidity and mortality. Using CD133positive HSPC from umbilical cord blood, we describe a co-culture system with trophoblast-like carcinoma cells (Jeg-3 cell line). The influence of Jeg-3-conditioned medium on the proliferation of HSPC was monitored during a two week culture. No major increase of CD-133positive cells was seen in response to conditioned medium although SCF, TPO or FLt-3-ligand significantly stimulated CD 133 positive cell proliferation. Also the impact of Jeg-3-conditioned medium on the apoptosis was investigated. Flow cytometric analysis demonstrated that Jeg-3-conditioned medium was able to reverse staurosporine induced apoptosis. We have extensively characterised HSPC isolated from umbilical cord blood. The expression of pluripotency, hematopoietic and vascular markers at both the mRNA and protein level was examined. Our data suggest a possible important role of soluble factors in the trophoblast – endothelium interactions during villous placental development.

Keywords: Hematopoietic stem/progenitor cells (HSPC); CD 133+; trophoblast; placenta; endothelial development

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The interplay of haematopoietic and mesenchymal stem cells

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Mesenchymal stem cells (MSC) are known as source for rebuilding of different tissues. But it seems that MSC have the capability for more than only remodelling. In-vivo data suggest that MSC are able to engraft the re-establishing of haematopoietic stem cells (HSC). Other clinical data leads to the assumption that MSC have an immune-modulatory competence. The cellular mechanism between MSC and HSC are unknown. We therefore investigate the interaction between MSC and HSC. MSC of human origin were obtained from bone marrow aspirates as well as bone marrow from femur and knee. After Ficoll-density gradient centrifugation MSC were cultured and adherence isolated. The HSC were isolated from peripheral blood from G-CSF mobilized patients and purified by magnetic bead isolation using CD34. The cell-cultures from MSC as well as HSC were quality controlled using four-color flow cytometry. We investigate the influence onto different cellular parameters such as migration, proliferation, survival and differentiation. Our data indicate that the MSC supply the HSC among other by decreasing there migratory activity and increasing the proliferation. Mesenchymal stem cells seem to have the capability to care for haematopoietic stem cells. The results firstly describe the direct interaction between both stem cell populations. These cellular investigations emphasize the relevance of stem cell crosstalk.

Keywords: MSCs; HSCs; interaction

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Investigations about differentiation of human bone marrow-derived mesenchymal stem cells

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Human hepatocytes find only limited use in cell therapy due to their restricted availability. Mesenchymal stem cells extracted from bone marrow should have the potential to proliferate and differentiate into cells with liver specific functions. Mononuclear cells isolated from adult bone marrow were separated by density gradient centrifugation. The mesenchymal stem cells were then selected by their adherence to plastic culture plates. The cells expressed CD105, CD166, CD44 and did not express CD 45, CD34 or CD14. With the addition of growth factors to suitable medium the cells developed into cells with hepatocytic characteristics. They also showed osteogenic and adipogenic differentiation. Immunocytochemistry and RT-PCR were used to analyse the status of cell differentiation. To prove the functional activity the rate of urea synthesis, the ability to accumulate metabolites and activation of certain promoters were investigated. After supplementation of specific differentiation media the cells lost their mesenchymal characteristics. They underwent morphological changes and expressed specific markers (e.g. osteogenic: osteocalcin; adipogenic: LPL PPAR-gamma; hepatogenic: CD26, CK18, CYP3A4, Cx32, PCK, albumin and HepPar1). The expression of important surface receptors were analysed (HGF-R, EGF-R, TNF-R, Notch and FZD). A higher rate of urea synthesis and accumulation of glycogen were observed in cells with hepatogenic characteristics. After lentiviral infection these cells expressed the liver specific, PCK-Promoter controlled transcript, EGFP. The results showed that mesenchymal stem cells derived from bone marrow have the capacity to differentiate in an osteogenic or adipogenic manner or to gain hepatocytic characteristics. These stem cells could be a good basis for the production of functional hepatocytes.

Keywords: mesenchymal stem cells, differentiation, cell transplantation

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Selection, transplantation and rare engraftment of embryonic stem cells derived hepatocytes

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Selection, transplantation and rare engraftment of embryonic stem cells derived hepatocytes Sharma AD, Wobus AM, Kania G, Iken M, Ott M. Hannover Medical School, Department of Gastroenterology, Hepatology and Endocrinology, Carl-Neuberg-Str. 1, 30625 Hannover Introduction: Embryonic stem (ES) cells have the capability to form all cell types present in the adult body. Hepatocytes derived from in vitro differentiation of the embryonic stem cells may be a valuable source for cell therapy of liver diseases. Contamination of ES-derived hepatic progenitors by non - differentiated embryonic stem cells, however, may pose the risk of teratoma formation in transplantation therapies. Methods: Following embryoid body formation and forced in vitro differentiation murine embryonic stem cell derived hepatic progenitors (ES-HPC) were transduced with a lenti virus expressing the enhanced green fluorescent protein (EGFP) under transcriptional control of the albumin promoter and enhancer. Five days later the cells were harvested and subjected to cell sorting. Cells expressing EGFP-protein were transplanted into recipient NOD/SCID mice and analyzed for the presence of ES-HPC and teratoma tissue eight weeks later. Results: Sorted lentivirus - transduced and EGFP fluorescent cells expressed markers of the hepatic phenotype (albumin, AFP) but not the genes indicating the presence of primitive embryonic stem cells (i.e. Oct-4). After intrasplenic transplantation of sorted ES-HPC in recipient mice, no tumors were seen in the livers of transplanted mice after eight weeks. In contrast, transplantation of unsorted ES cells and cells transduced with a lenti virus expressing EGFP under control of the constitutive phosphoglycerate kinase (pgk) promoter resulted in teratoma in all transplanted animals. Conclusions: Hepatocyte-specific sorting of ES-HPC resulted in a cell population, which comprises cells of hepatic origin but devoid of tumorigenic ES. Although prevention of tumor formation was achieved, a relevant functional engraftment into the livers of different mouse models of liver repopulation should be achieved before considering the therapeutic use of ES derived hepatic progenitors.

Keywords: Embryonic stem cells; Liver; Teratoma

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Stem-Cell Features of Renal Cell Carcinoma Derived Cell Lines

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Mesenchymal-to-epithelial transition contributes to the formation of renal epithelia during kidney development and to the repair of kidney injuries, whereas epithelial-to-mesenchymal transition plays a role in scar formation and renal fibrosis. Thus, distinct renal cell populations appear to maintain developmental plasticity, even in adults. Epithelial-to-mesenchymal transition is also well known in several tumors and has been linked to a migratory and more invasive state. Therefore, tumor cells may still possess a wider differentiation potential, reminiscent of their tissue origin and its developmental plasticity. Furthermore, recruitment of bone-marrow derived stem cells to tumors has been reported and appears to be associated with vascularization. Thus, in tumors stem-cell like activity may be present, originating from the tumor cells themselves as well as from stem cells recruited to the tumor stroma. We have established several primary cell lines from renal cell carcinomas. These cells lines either showed epithelial or mesenchymal morphology. Both types shared a CD13+, CD45-, CD71+, CD73+ and CD105+ consensus phenotype. However, cells with mesenchymal morphology were generally HEA-, CD90+, CD133- and cytokeratin 8/18-, whereas cells with epithelial morphology showed a HEA+, CD90-, CD133+ and cytokeratin 8/18+ immunophenotype. Irrespective of morphology and immunophenotype, however, cells could be differentiated in the presence of dexamethasone, ascorbic acid and glycerol phosphate within 7-14 days along the osteogenic differentiation pathway. They formed bone-nodules, which were stained by Alicarin red, an indication of calcification, and expressed osteocalcin, osteonectin, bone-specific alkaline phosphatase as well as bone sialo protein. Furthermore, global mRNA expression analysis revealed expression of stem-cell associated markers including Oct4, Nanog, Sox-9 and Bmi-1. Thus, the renal cell carcinoma derived cell lines share several characteristics with stem cells.

Keywords: renal cell carcinoma

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Investigation Of The Tropism Of Unrestricted Somatic Stem Cells From Umbilical Cord Blood For Glioma Cells

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Glioblastomas are the most common and most malignant primary brain tumors. Due to the very fast and highly invasive growth pattern of these tumors, often combined with resistance against radio- and chemotherapy, glioblastoma patients usually cannot be cured. The prognosis is poor as indicated by a mean survival of less than 12 months after diagnosis. Several groups have shown that somatic stem cells of different origins display a marked migratory behavior towards malignant gliomas and, therefore, might be useful vehicles to improve therapeutic gene delivery to brain tumors (Aboody et al., Proc Natl Acad Sci U S A. 2000 Nov 7, 97(23):12846-51; Brown et al., Hum Gene Ther. 2003 Dec 10;14(18):1777-85; Tabatabai et al., Brain. 2005 Sep;128(Pt 9):2200-11). Based on these findings, we initiated a research project that aims at the investigation of the potential of a unique type of human somatic stem cells, namely umbilical cord blood-derived stem cells (so-called unrestricted somatic stem cells, USSCs), as new source for stem cell-mediated gene therapy. Pilot experiments were carried out to evaluate the detection and survival of USSCs after transplantation into the brain of adult rats. We also performed in vitro analyses to determine the migratory potential of USSCs towards glioma cells. Our data show that various rat and human glioma cell lines are able to specifically attract USSCs when tested both in an agarose invasion assay and in a transwell assay. Future experiments are aimed to determine the glioma tropism of USSCs in vivo and eventually to test the capacity of these cells as migratory vehicles for targeted glioma therapy.
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Keywords: somatic stem cells; migration; glioma; transplantation; gene therapy

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Intrafemoral transplantation as a strategy to establish a mouse xenograft model to characterize the stem-/progenitor cell compartment in childhood ALL

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The development of new therapeutic strategies against acute lymphoblastic leukemia (ALL) requires the identification of the leukemic stem cell (LSC) as the major target of any curative treatment. The most powerful technique to characterize the LSC is the murine xenograft model where isolated stem and progenitor cells are transplanted onto NOD/scid mice. Successful engraftment indicates the presence of the in vivo clonogenic leukemic cell within the transplanted population. However, there have been some difficulties in establishing sensitive assays for LSC using this xenograft approach: First, leukemic CD34+ CD19- stem and progenitor cells are expected to be extremely rare and probably comprise only 0.01% of the bulk leukemic population. Second, the seeding efficiency following intravenous injection is only 10%, meaning that 90% of the transplanted cells get lost on the way from the peripheral blood into their bone marrow niche. Third, ALL blast cells undergo apoptosis very rapidly and are very sensitive to in vitro handling. Fourth, 75% of the hematopoietic stem cells (HSC) with long-term engraftment capacity are quiescent most of the time, infrequently cycling only once in 30 days. The known similarities between HSCs and LSCs suggest a resembling situation within the latter cells. In order to enhance the efficiency and sensitivity of our NOD/scid model for ALL we directly inject the leukemic cells into the right femur of the mice under general anaesthesia which avoids cell loss due to bone marrow homing. Additionally the intrafemoral approach allows engraftment analyses by aspirating the bone marrow without prematurely sacrificing the mice. Our preliminary results show engraftments (defined as >5% human CD45+ cells) after transplantation of 1.0 -7.0 x 106 unsorted cells from patients with B-precursor ALL, ALL/t(4;11) and ALL/t(12;21), respectively (19 of 68 – 28%). Transplantation of 0.5 -1 x 105 CD34+ CD19- cells from two patients with ALL/t(4;11) resulted in leukemia initiation within primary (3 of 12 – 25%) and secondary (1 of 9 – 11%) NOD/scid recipients. Furthermore we obtained secondary engraftments with 6.0 x 104 – 6.0 x 106 CD34+ CD19+ cells (5 of 12 – 42%) and with 2.0 x 106 CD34- CD19+ cells (3 of 7 – 43%) per mouse. The median time necessary from primary transplantation to the first measured engraftment was 10 weeks in case of unsorted cells and 30 weeks with sorted populations. The secondarily transplanted and sorted cells engrafted after a median of 22 weeks. Because of these long incubation periods we strive to optimize the performance of our model. In current experiments, we administer a CD122-neutralizing antibody to the NOD/scid mice in order to block their residual NK cell activity. Additionally, we will use the IL2RÁnull mice that lack NK cell activity a priori.

Keywords: ALL; leukemic stem cell; intrafemoral transplantation

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The Heterogeneity of Human Mesenchymal Stem Cell Preparations

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Mesenchymal stem cells (MSC) raise high hopes in clinical applications. However, the lack of common standards and of a precise definition of MSC preparations remains a major obstacle in research and application of MSC. Whereas surface antigen markers have failed to precisely define this population, a combination of proteomic data and microarray data provides a new dimension for the definition of MSC preparations. We have analyzed the molecular signatures of human MSC that were isolated from bone-marrow under two different culture conditions, from adipose tissue or from umbilical cord blood in comparison to non-multipotent human fibroblast cell lines (HS68 and NHDF). No phenotypic differences were observed between MSC and fibroblasts by flow cytometry using a panel of 22 surface antigen markers. Gene expression profiles were compared by cDNA microarray analysis (51,144 different cDNA clones of the RZPD3 Unigene Set). Twenty-five genes were overlapping more than two-fold up-regulated in all MSC preparations and these included fibronectin, ECM2, glypican-4, ID1, NF1B, HOXA5 and HOXB6 whereas several inhibitors of the Wnt-pathway (DKK1, DKK3, SFRP1) were higher expressed in fibroblasts. Differential gene expression was verified for 20 genes by RT-PCR. Hierarchical cluster analysis revealed a close relation of four individual donor samples for each of the 4 MSC preparations while pair wise comparison of MSC from different tissues or culture-isolation procedures revealed significant differences. By using two-dimensional gel electrophoresis and MALDI-TOF-MS we have generated a proteome reference map of MSC from bone marrow. 136 protein spots were unambiguously identified most of which play a role in cytoskeleton, protein folding and metabolism. This reference map was used to compare protein expressions of MSC that have been isolated under two different culture conditions. Furthermore, a combinatorial analysis of microarray data and proteomic data revealed a correlation in differential gene and protein expression. Genes that were differentially expressed on mRNA level ($p<0.05$) were differentially expressed on protein level as well (Pearson correlation = 0.83). Interchanging culture conditions for eight days revealed that differential expression was retained in several genes whereas it was altered in others. Expression of a selection of differentially expressed proteins was further analyzed by fluorescence microscopy whereby no subpopulations could be discriminated within cell preparations while marked differences were observed in morphology and organization of MSC isolated under different culture conditions. Our results provide evidence for reproducible isolation of relatively homogeneous MSC preparations under standardized isolation conditions, while MSC from different tissues or culture-isolation procedures display significant differences in their transcriptome, proteome and cellular organizations. Our comparative approach provides foundation for a reliable quality control using genotypic and proteomic analysis.

Keywords: MSC, microarray, proteomics, molecular characterization

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Circulating Endothelial Cells in Malignant Disease

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Circulating endothelial progenitors (CEPs) represent a rare population of peripheral blood mononuclear cells. Studies that involve the identification of endothelial progenitors in peripheral blood have to date delivered little clarity with regard to antigens presented in this population. A momentary consensus is that CEPs cells express CD133, CD34 and KDR. These cells are of therapeutic interest because they may be utilized both as intrinsic markers as well as themselves being targeted in therapy. In our study we aimed to further resolve CEP antigen expression as well as population size of a normal group (n=6) in comparison to cancer patients diagnosed with B cell non Hodgkin Lymphoma (NHL) and osteosarcoma. To this end we performed extensive four colour flow cytometry analyses with peripheral blood samples. Mononuclear cells were isolated via density gradient, and cells were subsequently stained with CD133, CD117, CD45, tie-2, VE-Cadherin, CD11b, CD14, CD33 and KDR. To date, literature has shown that CEPs are part of the CD133 positive population, we therefore deemed CD133 as the population of interest in order to identify further subpopulations. Analysis of samples from the osteosarcoma patient show that the CD133+, tie-2+, CD45- increased from 0.002% to 0.037% (in % PBMCs). The CD133+, tie-2+ CD11b- population increased from 0.0085% to 0.0618% and the CD133+, CD45+, CD11b- population also increased from 0.0595% to 0.2450%. Furthermore the CD133+, tie-2+, CD33+ population increased from 0.0172% to 0.0979%. The KDR positive population as such increased from 0.249% to 0.671% which could in part be identified as KDR+, CD133+, CD33- cells (increased from 0.0303% to 0.11%). In the case of NHL patients we found a two-fold increase in the CD133+, CD14-, CD117- cells. We however found no change in the number of CD133+, KDR+ cells or the CD133+, tie-2+ cells. These comparative results give an indication of a change in the antigen profile of the CD133 population in malignant disease. More extensive analyses are being performed in order to enable to cement these findings and find their relevance as intrinsic factors. From the Department of Medicine / Hematology and Oncology and the IZKF Muenster, Albert-Schweitzer-Strasse 33, D-48129 Muenster. This work was supported by a grant of the Deutsche Krebshilfe (10-2167-Ke1).

Keywords: circulating endothelial progenitors; intrinsic marker

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Transcription factor AP-2Á involved in germ cell development and tumor formation

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Primordial germ cells are the precursors of sperm and eggs and arise from the epiblast around E6.5. They can be detected because of their high level of alkaline phosphatase activity as soon as E7.25 where they are located at the base of the developing allantois. They become incorporated into the developing hindgut around E8.5 and migrate along the gut towards the developing genital ridges and reach the genital ridges which will later become ovary and testes around E10.5-E11.5. AP-2Á belongs to the family of AP-2 transcription factors which are expressed in a variety of tissues during embryogenesis and are also believed to play a role in tumor formation. Besides its expression in placenta, AP-2Á is expressed in germ cells during embryonic development. Mice deficient for AP-2Á are growth retarded at embryonic day 7.5 and die around E9.5 because of malnutrition due to a failure of the trophectoderm cells to proliferate and form a proper labyrinth layer. To overcome the embryonic lethal phenotype we have generated a conditional allel of AP-2Á to address the consequences of loss of this transcription factor in a cell-type-specific manner. Therefore two Cre lines were used, the epiblast-specific Sox2Cre and the germ cell specific TNAP-Cre to ensure the cell specifity of the phenotype. Animals derived from both lines (AP-2Áflox/flox : Sox2Cre and AP-2Áflox/flox : TNAP-Cre) are sterile and show a complete loss of germ cells in both sexes leading to Sertoli-Cell-Only-Syndrom and Leydig Cell hyperproliferation in male mice. Germ cells are lost as soon as embryonic day 8.5 indicating a crucial role for AP-2Á in germ cell migration and proliferation. In addition we could show that AP-2Á is expressed in human fetal germ cells at the gonocyte stage prior to prespermatogonia differentiation. The expression lasts during pregnancy until the 37th week and later vanishes so AP-2Á cannot be found in adult testes. In the gonocytes AP-2Á is found coexpressed with c-Kit, a known target of AP-2. Both genes are also found to be expressed in seminomas, tumors originating from germ cells, and their precursors, so-called intratubular germ cell neoplasias (IGCNU) and can be used as diagnostic markers. Crossed into 129/SV background, male mice heterozygous for AP-2Á develop teratomas with a high frequency. Teratomas are believed to arise from malprogrammed primordial germ cells. Because of the high differentiation potential of germ cells, teratomas may consist of all three germ layers and include a variety of different cell types like for example cartilage and neuronal parts. Taken together, our results suggest an important role for AP-2Á gene dosage during germ cell development as well as germ cell tumor formation as the absence of AP-2Á leads to germ cells loss, the persistence to seminoma formation in humans and a reduced gene dosage to teratoma formation in mice.

Keywords: AP-2; primordial germ cells; germ cell tumors

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Tissues Regeneration/ Clinical Aspects

Induction of Immunotolerance of Xenotransplanted Human Hepatocytic Cell Line into fully Immunocompetent Mice using Anti-CD4 Monoclonal Antibody Induction Therapy

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The main goal of anti CD4 monoclonal antibody therapy is the effective, antigen specific induction of immunotolerance of allogenic and xenogenic transplants in fully immunocompetent hosts. The aims of this project were to 1. Monitor the cellular immune status of C57BL6 mice treated with temporary CD3+CD4+ depleting anti-CD4 monoclonal antibody. 2. Establish and monitor the successful transplantation of human differentiated hepatocytes into the parenchyma of immunocompetent C57BL6 mice. Cultured human SV40LT immortalised hepatocytes were transplanted into immunocompetent, wild type C57BL6 mice. The animals were treated with a single i.p. administration of anti-mouse CD4 monoclonal antibody (verum) or PBS (control) in the presence of hepatocyte antigen at day 0 and day 4. Blood T cell counts were monitored using flow cytometry. Successful transplantation and establishment of human hepatocytes was confirmed by analysis of human albumin expression (ELISA, immunohistochemistry) and the presence of human hepatocytes in the host liver (FISH, in situ hybridisation). Here we provide evidence that anti-CD4 induction therapy enabled the effective induction of immunotolerance of human differentiated hepatocytes into the liver of fully immunocompetent C57BL6 mice in vivo. Thus, anti CD4 induction therapy might provide an alternative to general immunosuppression for allogenic and xenogenic cell therapies.

Keywords: induction therapy, immunotolerance allotransplant, xenotransplant

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Mesenchymal stem cells rescue photo receptor cells in the retina of Rhodopsin knock out mice

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Retinitis pigmentosa belongs to a large group of degenerative diseases of the retina with a heriditary background. It involves loss of retinal photoreceptor cells and consequently peripheral vision. At present there are no satisfactory therapeutic options for this disease. Just recently the use of mesenchymal stem cells has been discussed as one therapeutical option for retinal degenerations, as they have been shown to differentiate into various cell types, including photo receptor cells. In this paper we wanted to investigate the potency of mesenchymal stem cells to induce rescue effects in an animal model for retinitis pigmentosa, the rhodopsin knock out mouse. For the experiments three experimental groups of 10 animals each were formed. The first group consisted of untreated rho-/- animals taken as controls. The second group consisted of rho-/- mice that received an injection of mouse mesenchymal stem cells, which were transduced using an adenoviral vector containing the sequence for the green fluorescent protein (GFP) prior to transplantation. In the third sham group, animals received an injection of medium only. 35 days after transplantation GFP expressing cells were detected in whole-mount preparations of the retinas as well as in cryostat sections. For the detection of rescue effects semi thin sections of eyes derived from all experimental groups were produced. Furthermore, rescue effects were also analysed ultrastructurally in ultra thin sections. Histological analysis reveals that after transplantation cells not only morphologically integrated in the retinal pigment epithelium but also in layers of the neuroretina displaying neuronal and glial morphologies. Furthermore, significant rescue effects as demonstrated by the occurrence of preserved photoreceptor cells could be detected.

Keywords: retinitis pigmentosa; Rhodopsin knock out: adenovira vector; GFP

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Smooth Muscle Differentiation of Bone Marrow Stromal Cells

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INTRODUCTION AND OBJECTIVES: In the field of tissue engineering and cell therapy of the urinary tract system, autologous bladder smooth muscle cells have several limitations, as harvesting is difficult, in vitro-proliferation is insufficient, and they cannot be used in malignant conditions. The presented data report about capabilities of bone marrow stromal cells (BMSC) to differentiate in vitro towards a smooth muscle phenotype. METHODS: BMSC were stimulated in vitro with several cocktails of growth factors in combination with epithelial-mesenchymal interactions. Differentiation stages were analysed by means of expression patterns of smooth muscle marker genes using quantitative PCR. RESULTS: Stem cell character of adherent cell fraction was assesed by differentiation into adipogenic and osteogenic lineages. In myogenic differentiation assays, TGF-beta1 overall upregulated in BMSC the transcription of smooth muscle desmin, actin and myosin heavy chain. Stimulatory effects were significantly increased by coculture with urothelial cells. In contrast, VEGF had no inductive effects. Combination with chromatin altering agents as well as prolonged incubation times further enhanced transcription levels of smooth muscle genes. CONCLUSIONS: TGF-beta1 and epithelial-mesenchymal interactions play important roles in the myogenic differentiation pathway of multipotent adult stem cells. However, expression levels of differentiated BMSC still differ to bladder positive control. This might be due to a mixed population of different competent cells and may depend on their respective cell cycle stages. Since in vivo-myogenic differentiation certainly depends on a complex environment and does not occur in a single step, our data suggest in vitro-predifferentiated BMSC as applicable raw material to substitute smooth muscle tissue in vivo.

Keywords: bone marrow stromal cells, TGF-beta1, epithelial-mesenchymal interactions

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Catheter-assisted Transplantation of Endothelial Progenitor Cells into the Coronary Arteries of Rat

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Coronary Catheter-assisted Transplantation of Endothelial Progenitor Cells into the Arteries of Rat Angelika Blaszczyk, Sandra Goettsch, PhD, Zhaoping Ding, MD, Andreas Wirrwar, PhD, Doris Buchholz, Hans-Wilhelm Müller, MD, Jürgen Schrader, MD Institute for Heart and Circulatory Physiology, Clinic for Nuclear Medicine, Heinrich Heine University Duesseldorf, Germany Background: Transplantation of endothelial progenitor cells (EPCs) may improve vascularisation and left ventricular function and reduce remodeling after experimental myocardial ischemia. Administration of EPCs in these experimental studies was performed either into the tail vein or by direct injection into the heart muscle. To test the engraftment of EPCs into the heart under clinical-like conditions we transplanted radioactive labeled EPCs – either as single cells or crosslinked - into the coronary artery system of rats using a balloon catheter. Methods and Results: EPCs were isolated from human peripheral blood, characterized by the expression of endothelial marker proteins and labeled with 111Indium oxine. Labeling of EPCs did not change cell survival and morphology. Two days after labeling approximately 50% of radioactivity was still bound to cultured EPCs. One million EPCs were injected into the coronary arteries of either healthy rats (n = 6) or rats after ischemia-reperfusion injury (n = 6). In a second set of experiments we crosslinked EPCs with Phytohaemagglutinin (PHA) in order to create small cell clusters of two to four EPCs to enhance the engraftment of the cells in the heart. Two animals additionally received 99Tc-MyoView to locate the infarcted area in vivo. Continuos 3-dimensional SPECT-images were acquired every 5 minutes for one hour and again 48 hours after cell delivery. Animals were then sacrificed and specific radioactivity was measured in the heart. One hour after transplantation of single EPCs, 23 ± 3% of the radioactivity was found over the entire heart of control rats while only 16 ± 4% was trapped in the injured heart. After two days the respective values were 3 ± 0% and 6 ± 1% suggesting a homing effect. A detailed analysis revealed that radioactivity was significantly higher in the infarcted area (12,6 ± 1,9 cpm*103/g*MBq) than in the uneffected left ventricle (6,2 ± 1,1 cpm*103/g*MBq) or the border zone (10,1 ± 3,9 cpm*103/g*MBq). When PHA-treated EPCs were administered the overall radioactivity in the rat heart was about twice as high as in animals receiving single EPCs. In contrast to single cells, crosslinked EPCs were preferentially trapped in the healthy area of the left ventricle (21,8 ± 6,8 cpm*10*/g*MBq) compared to the border zone (19,5 ± 1,2 cpm*103/g*MBq) and the infarcted area (16,2 ± 3,2 cpm*103/g*MBq). Conclusion: Intracoronary delivery of single EPCs resulted in an enhanced deposition of cells in the injured myocardium only after two days, suggesting a homing effect. Crosslinking of EPCs substantially enhanced the engraftment of cells in the heart. This might constitute a promising way for future therapeutic cell delivery.

Keywords: EPC; Myocardial Infarction; SPECT; Homing; PHA

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Mesenchymal stem cells do not show site specific differentiation after transplantation into the lesioned myocardium

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Bone marrow-derived mesenchymal stem cells (MSCs) have been proposed as a promising source for cellular cardiomyoplasty after heart infarction because of their high plasticity and good availibility. We have isolated bone marrow from transgenic mice expressing EGFP and culture-expanded MSCs. The cells showed typical MSC markers, differentiated into osteocytes and adipocytes in vitro and generated to a small extent muscle-like cells after exposure to 5-azacytidine. MSCs were injected into infarcted hearts of syngeneic wild type mice (n=50) and their fate determined 1 to 19 weeks postoperatively. Prominent long term engraftment of EGFP positive MSCs was observed. However, morphological and immunohistochemical analysis did not reveal transdifferentiation into cardiomyocytes or endothelial cells. We rather detected calcifications and bone formation in a large number of mice. Thus, the local microenvironment does not restrict the fate of the transplanted MSCs.

Keywords: myocardial infarction;cardiomyoplasty;mesenchymal stem cells;differentiation

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Experimental autoimmune encephalomyelitis: valuable tool for experimental neural stem cell therapy

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Neural stem cells (NSCs) represent organ-specific stem cells of the mammalian nervous system with the capacity to differentiate into neurons, astroglia and oligodendroglia. They can be isolated from the foetal or adult CNS and maintained in vitro without losing their multipotency. NSCs are promising for stem cell therapy of many CNS diseases, e.g. demyelination disorders. For successful application of stem cell therapy in human medicine it is necessary to study behaviour of NSCs in animal models of diseased nervous tissues. We established model of multiple sclerosis in rodents – experimental autoimmune encephalomyelitis (EAE) and studied behaviour of NSCs and their progeny in vitro and following transplantations. NSCs were isolated from brains of E14 mouse fetuses using a neurosphere assay. Differentiation potential of NSCs was assessed in vitro and in vivo. In in vitro experiments differentiation potential was evaluated using the differentiation assay or in long-term cultivated non-passaged neurospheres. In vivo experiments were based on grafting neurospheres in the intact brains of histocompatible recipients. For EAE induction adult mice or rats were immunized by subcutaneous injections of emulsion consisting of 100 µl of mouse spinal cord homogenate and 50 µl of complete Freund's adjuvant supplemented with 50 µg of Mycobacterium tuberculosis. After 24 h each mouse was injected intravenously with 250 ng of Pertussis toxin in 0.1 ml PBS or on days 7, 8 and 9 following the immunization octreotide was given subcutaneously at a dose of 3 x 5 µg/kg/day. EAE was evaluated clinically daily using a 5 degree scale and changes in CNS of sacrificed animals were examined histopathologically. NSCs cultured as neurospheres differentiated into beta-III tubulin+ neurons, GFAP+ astroglia and generated NG2+ A2B5+ precursors of oligodendroglial cells and O4+ preoligodendrocytes. Double immunofluorescence proved zonal distribution of cells at different developmental stages inside of neurospheres. O4+ preoligodendrocytes occupied central core of the neurospheres while NG2+ cells were localized in the outer belt and nestin+ stem and progenitor cells were distributed in the thin peripheral cell layer. The same potency for production of oligodendroglia was observed after neural grafting of both solid neurospheres and dissociated neurospheral cells. In the latter case, immature oligodendroglial cells appeared mainly in the white matter. In paraffin CNS sections of animals with EAE we distinguished and evaluated extent of demyelination lesions and accompanying changes using luxol blue and immunohistochemical detections of myelin basic protein, non-phosphorylated neurofilaments, amyloid precursor protein and ED1 (marker of tissue macrophages). Our results confirmed multipotency of NSCs in vitro as well as after transplantations and in vivo experiments gave evidence that NSCs represent transplantable cells without any tumorigenic potential. We successfully established and characterised model of multiple sclerosis in rodents – EAE that is now utilizes for experimental stem cell therapy using unambiguously detectable mouse ROSA26 neural stem cells. This work was supported by the grant projects MSM0021620820 from MSMT and NR 7969-3/2004 from IGA MZ of the Czech Republic.

Keywords: neural stem cells; neurospheres; oligodendroglia; EAE; immunohistochemistry

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Isolation and characterization of ectomesenchymal progenitor cells from human tooth

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Neural crest-derived mesenchymal progenitor cells are found in human teeth. However, these ectomesenchymal progenitor cells (EPCs) are still poorly characterized concerning their phenotype and potency. Here, we described a new population of EPCs and studied their capability to differentiate along given cell lineages, i.e. neurogenic, chondrogenic and osteogenic lines. EPCs were enzymatically obtained from the apically situated connective tissue (i.e. apical pad) of surgically removed human immature wisdom tooth, cultured and expanded in FCS-containing growth medium, thereby selecting a distinct population of cells with a fibroblastoid morphology. In passage 2, these cells were reactive for CD90, CD44, and CD49e, while they were negative for CD45, CD14 and CD31 using FACS analysis. To achieve a neurogenic differentiation, EPCs were cultured in neurogenic medium and analyzed by neurofilament and GFAP expression. To achieve chondrogenic differentiation, progenitors were cultured in chondrogenic medium and analyzed by RT-PCR for expression of type II collagen and aggrecan. Bone matrix was formed when EPCs were cultured in osteogenic medium as analyzed by alizarin red staining, osteocalcin and ALP expression. Most important, differentiated osteoblasts started to form bone containing entrapped osteocytes. To evaluate in vivo bone-forming capacity, EPCs were loaded on fibrin-ceramic constructs and implanted in subcutaneous sites of athymic mice. Indeed, direct osteogenesis i.e. forming woven bone could be observed after 5 and 10 weeks. These data indicate not only the multipotency of our tooth-derived EPCs but also their high potential for bone formation. Therefore, dental EPCs which normally function to form supporting structures of the tooth may be used not only to cure tooth damage but also for bone tissue engineering and for treatment of craniofacial and other skeletal defects.

Keywords: ectomesenchymal progenitor cells; stem cells; multipotency; tissue regeneration; tooth

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Influence of glycosaminoglycans on the generation of mouse embryonic stem cell-derived cardiomyocytes

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The mammalian extracellular matrix is largely comprised of fibre proteins and complex polysaccharides, called glycosaminoglycans (GAGs). Historically GAGs were considered to be inert materials that hydrate cells and contribute to the structural scaffolds. Recent studies have brought about a dramatic transformation in understanding the numerous biological roles of these polysaccharides. GAGs bind to a wide variety of proteins and signalling molecules in the cellular environment and modulate their activity. By that they participate in cell adhesion, migration, proliferation, apoptosis and differentiation. To investigate the influence of GAGs on the differentiation of mouse embryonic stem (ES) cells a genetically engineered ES cell line was used. The ES cells are transfected with a fusion gene that allows selection of cardiomyocytes via antibiotic addition. During cultivation of differentiating ES cells GAG-degrading enzymes were added to the medium. On the basis of different cultivation parameters and immunohistochemistry the yield of ES-cell derived cardiomyocytes was determined to assess the influence of GAGs on cardiomyocyte-differentiation. It was shown that the degradation of chondroitin sulfate and dermatan sulfate results in a nearly 30% decreased metabolism activity of differentiating ES cells. Furthermore with 1.3·104 cells/ml the yield of ES-cell derived cardiomyocytes was about eight times lower than without adding enzymes to the cultivation process.

Keywords: stem cells, cardiomyocytes, glycosaminoglycans, differentiation

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Transplanted human cord blood derived unrestricted somatic stem cells improve left-ventricular function and prevent left-ventricular dilation and scar formation after acute myocardial ischemia

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Background- Functional improvement after acute myocardial ischemia (MI) has been achieved by transplantation of different adult stem and progenitor cell types. It is controversial, whether these cell types are able to form novel functional myocardium. Alternatively, graft- or immune-related paracrine mechanisms may preserve existing myocardium, improve neovascularization, affect tissue remodeling or induce endogenous de novo formation of functional myocardium. We have applied an alternative somatic cell type, human cord-blood derived unrestricted somatic stem cells (USSCs), in a porcine model of acute MI. Methods and results- USSCs were transplanted into the acutely ischemic lateral wall of the left ventricle. LV dimension and function were assessed by transesophageal echocardiography (TEE) pre-MI, immediately post-MI and 8 weeks after USSC injection. Gender- and species-specific FISH / immunostaining failed to detect engrafted donor cells 8 weeks post-MI. Nevertheless, cell treatment effectively preserved natural myocardial architecture. Global left ventricular ejection fraction (LVEF) prior to MI was 60,4 ± 7,4 %. Post-MI, LVEF decreased to 33,8 ± 8,2 %. After 8 weeks, LVEF had further decreased to 27 ± 5 % in the control group and recovered to 52 ± 2 % in the USSC group ($p < 0.01$). Left-ventricular end-diastolic volume (LVEDV) prior to MI was ± ml. 8 weeks post-MI, LVEDV had increased to 77 ± 4 ml in the control group. No LV dilation was detected in the USSC group (LVEDV: 26 ± 2 ml, $p < 0.01$). Conclusions- Transplantation of USSCs significantly improved LV function and prevented scar formation as well as LV dilation. Since engraftment and differentiation was excluded as underlying mechanism, paracrine effects are most likely to account for the observed effects of USSC treatment.

Keywords: somatic stem cell;progenitor cell;neovascularization, myocardial ischemia

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Role for prolactin in gliogenic wound response and neuroblast migration after ischemic cerebral injury

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It had been recently reported that the pituitary hormone prolactin stimulates neurogenesis in the pregnant rat (Shingo et al., Science 299: 117-120). This prompted us to investigate whether the lactogenic axis is involved in neuroprotection and neurogenesis after hypoxic ischemic (HI) injury. 21 day old rats underwent ligation of the right common carotid artery and following 1 hour recovery, were exposed to 8% oxygen at 34°C for 60 minutes. From one day post-injury cortical prolactin immunostaining increased parallel to the severity of the injury ($p<0.001$). Initially prolactin and its receptor protein were increased on penumbral neurons and astrocytes. Neuronal staining progressively decreased whilst reactive astrocytes remained strongly immunopositive, suggesting involvement in gliogenic wound response during recovery from injury. Concomitant to the increase of prolactin surrounding the infarct, five days after severe unilateral injury, co-localization of prolactin receptor and doublecortin was found on an increasing number of neuroblasts. The upregulation of prolactin in the penumra might therefore have served as a chemoattractive clue for the migration of newly generated neuroblasts towards the cortical lesion. We then went on to substantiate these observations with in vitro experiments. Our first finding was that prolactin protected cortical primary cultures from neuronal death induced by growth factor withdrawal. More significantly for the suspected role in neurogenesis and migration after HI injury, prolactin induced a more than two-fold increase of proliferation in primary cortical cultures, quantified by BrdU incorporation ($p<0.001$). Further, it more then doubled the number of migrating neural precursors from neurosphere cultures in a Boyden chamber migration assay ($p<0.001$). Together this data suggests that prolactin and its receptor may play a role in endogenous recovery after injury in the cerebral cortex.

Keywords: Stroke; prolactin; proliferation; migration; neuroblast

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Neotendon Formation Induced by Manipulation of the Smad8 Signalling Pathway in Mesenchymal Stem Cells

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Tendon and ligament present a major clinical challenge to orthopedic medicine. Until the present time, therapeutic options used to repair torn ligaments have consisted of autografts, allografts, and synthetic prostheses but none of these alternatives has provided a successful long-term solution. We used murine adult mesenchymal stem cells (MSCs) as a platform for tendon healing. Tissue regeneration requires the recruitment of adult stem cells and their differentiation into mature committed cells. In this study, a novel approach for tendon regeneration based on a specific signalling molecule, Smad8, which mediates the differentiation of mesenchymal stem cells (MSC) into tendon-like cells, is described. A biologically active Smad8 variant was transfected into an MSC line that co-expressed the osteogenic gene, bone morphogenetic protein 2 (BMP2). The engineered cells demonstrated the morphological characteristics and gene expression profile of tendon cells both in vitro and in vivo. In addition, following implantation in an Achilles tendon partial defect, the engineered cells were capable of inducing tendon regeneration demonstrated by double quantum filtered MRI. The results indicate a novel mechanism in which Smad8 inhibits the osteogenic pathway in MSCs, known to be induced by BMP2, while promoting tendon differentiation. A further novel finding is that after intramuscular transplantation and dependent on the mode of adenoviral infection we are able to either generate ectopic tendon or entire ectopic tendon–bone insertions with a seemingly intact osteotendinous junction exhibiting a fibrocartilage enthesis. Such a model of ectopic tendon formation mimicking the formation of an entire bone-tendon-muscle unit in an ectopic environment may significantly contribute to the investigation of the factor-dependent stem cell repair of tendon disorders. These findings may have considerable importance for the therapeutic replacement of tendon or ligaments and for engineering other tissues in which BMP plays a pivotal developmental role. The study was funded in part by the integrated project of the EU "GENOSTEM". Reference: Hoffmann,A., Pelled,G., Turgeman,G., Eberle,P., Zilberman,Y., Shinar,H., Keinan-Adamsky,K., Winkel,A., Shahab,S., Navon,G., Gross,G., and Gazit,D. (2006). Neotendon Formation Induced by Manipulation of the Smad8 Signalling Pathway in Mesenchymal Stem Cells. J. Clin. Invest. in Press.

Keywords: Mesenchymal stem cells; MSCs; tendon; ligament; regenerative medicine; signalling; Smad8

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Gene Therapy of ,c-deficient Pulmonary Alveolar Proteinosis (,c-PAP): Studies in a Murine Knockout Model

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PAP due to deficiency of the common beta chain (,c) of the IL3/IL5/GM-CSF receptor superfamily is a monogeneic disease characterized by functional insufficiency of pulmonary macrophages. While hematopoietic stem cell gene therapy has been advocated as a potentially curative approach the defect is not functionally manifested on the stem cell level and stable enrichment of genetically corrected cells in vivo will require an additional selection system. Thus, we have generated a retroviral vector construct (SF91-m,c-IRES-MGMTP140K) expressing the murine ,c (m,c) cDNA in combination with the in vivo selectable drug resistance gene MGMTP140K coding for an O6-benzylguanine (BG) resistant point mutation of the DNA-repair-protein O6-methylguanine-DNA-methyltransferase and utilized this construct to transduce hematopoietic progenitor and stem cells in a murine model of m,c-deficient (m,c-/-) PAP. Our construct functionally restores m,c activity as demonstrated by significantly enhanced colony formation in the presence of GM-CSF upon m,c transduction of m,c-/- bone marrow cells (1±0.5 vs. 19±5 CFU-C/1x10^5, p=0.002; n=11). Control cultures set up with G-CSF, EPO and SCF indicated the GM-CSF specificity of the restored cytokine sensitivity. The GM-CSF sensitivity profile of "repaired" m,c-/- cells matched that of non-transduced but also of m,c-transduced wildtype bone marrow cells. Functional expression of MGMTP140K was demonstrated by increased resistance of progenitor cell-derived colonies to 10µM BG plus 25 to 200 µg/ml TMZ following SF91-m,c-IRES-MGMTP140K transduction. Significant in vitro enrichment of genetically corrected m,c-/- cells was shown when SF91-m,c-IRES-MGMTP140K transduced cells were exposed to 10µM BG plus 0, 50 or 100 µg/ml TMZ before clonogenic culture (11.4±4.3% (0 µg/ml TMZ) versus 36.4±9.6% (50 µg/ml TMZ, p=0.034) or 73.7±19.2% (100 µg/ml TMZ, p=0.015) (n=6)). To analyse the effect of m,c gene transfer in our in vivo PAP model, SF91-mßc-IRES-MGMTP140K transduced ßc-/- cells were transplanted into lethally irradiated ßc-/- recipient mice which upon hematopoietic recovery were treated with BG/TMZ for 5 weeks. When animals were sacrificed at the end of the experiment, dramatic improvements in lung pathology were noted. Lung sections showed reduction of PAS positive regions in gene corrected animals to near normal levels and, furthermore, a radical reduction of lymphoid infiltration. GM-CSF-dependent colony function was restored by bone marrow cells and functional as well as flow cytometric analysis demonstrated substantial enrichment of genetically corrected cells in the bone marrow by BG/TMZ application (from 10-20% to 50-90%). Thus, in summary, our data demonstrate functional correction of the ,c-PAP phenotype by therapeutic mßc gene transfer, and MGMTP140K may serve as a suitable selection marker in this context.

Keywords: retroviral gene transfer, PAP, MGMTP140K

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Functional and behavioural restoration after transplantation of fetal dopaminergic grafts in an animal model of Parkinson's disease depends on specific motor training

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Sensorimotor impairments are the leading symptoms of Parkinson's disease (PD), which is caused by the degeneration of the dopaminergic (DAergic) cells within the nigrostriatal pathway. To substitute the loss of these cells and their functions cell replacement strategies have been established in the past, but no optimal therapeutical protocol has been achieved yet, neither in animal models nor in clinical trials. Physical exercise has been proven as beneficial in terms of increased adult neurogenesis and in terms of improved survival of foetal, ectopic, and DAergic grafts (e.g. with increasing training up to 62% enhanced cell survival after transplantation). Additionally, intermittent and repeated physical exercises improved not only the survival rate but also the functional integration of grafted cells within the host tissue. In this study, we examine which specific motor training enhances functional recovery after transplantation of DAergic cells (E14 VM derived) in hemiparkinsonian rats. Five groups of rats were lesioned and transplanted: 1. enriched environment housed rats, 2. rats trained in spontaneous behaviour, 3. rats trained in the paw-reaching-task, 4. rats trained only in the forced choice paw-reaching-task, 5. rats that had voluntary access to a running wheel. There were three control groups with standard housing and no behavioural tests: 1. healthy rats, 2. lesioned but sham-transplanted rats, 3. lesioned and transplanted rats. Lesion and graft effects were evaluated by drug-induced rotation. Graft survival, graft volume, fibre density and neurogenesis will be assessed by immunocytochemistry and BrdU labelling. Preliminary results revealed that all animals showed overcompensation in amphetamine rotation and that the enriched environment conditions were able to reduce the overcompensatory response compared to the other housing methods. Intensive training in the spontaneous behaviour test and the paw reaching task could significantly improve the motor performance. The main focus of this ongoing study is to find out if there are specific motor tests which promote best the functional recovery in hemiparkinsonian rats, or if general exercise is sufficient for convalescence after transplantation of E14 progenitor cells. Together with our previous results the outcome of this study may be taken into consideration for future clinical trials and prove that physical exercise can promote the functional recovery induced by cell replacement strategies.

Keywords: skilled limb movements, ventral mesencephalon, E14, progenitor cells

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HOXB4 Expression levels critically determine the competence of ES-CELL derivatives to mediate hematopoietic long-term repopulation, in vivo

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After in vitro differentiation of mouse embryonic stem (ES-) cells, long term hematopoietic repopulation, in vivo, is especially efficient when the homeodomain transcription factor HOXB4 is ectopically expressed. We have recently shown that HOXB4-ES-cell derivatives behave similar to bone marrow cells also expressing this transcription factor ectopically, both in vitro and in vivo. Here we demonstrate that long term repopulation (>6 months) in Rag2(-/-)gammaC(-/-) recipient mice can also be achieved with ES-cell derived hematopoietic cells (ES-HCs) obtained from single ES-clones, but only when HOXB4 is expressed above a certain threshold level. Increased expression led to a high extent of chimerism in the bone marrow of transplanted mice (average 75%; range 45-95%, n=4) whereas ES-HC clones expressing lower levels only repopulated with very low efficiency (average 2.5% chimerism, range 1-4%, n=6 mice). These results suggest that HOXB4 can enforce the development of HSCs from ES-cells, but their long term repopulation capabilities are crucially dependent on the expression levels of HOXB4. Furthermore, mice reconstituted with ES-HC clones expressing high amounts of this protein recapitulated the morpho-histological phenotype observed in polyclonally reconstituted mice, including the bias towards myelopoiesis, “benign” myeloid proliferation in spleen and the incompatibility of HOXB4 expression with T-cell poiesis.

Keywords: Embryonic Stem Cells; Hematopoetic Stem cells; HOXB4; Repopulation; Transplantation

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The transdifferentiation capacity of transplanted bone marrow-derived cells in the retina of a mouse model of oxygen induced retinopathy

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Background and Purpose: A number of reports have highlighted the potential for bone marrow–derived endothelial progenitor cells (EPCs) to participate in the neovascularization of many adult tissues under both physiologic and pathologic conditions. We wanted to evaluate the transdifferentiation capacity of transplanted bone marrow-derived cells in the retina of a mouse model of oxygen induced retinopathy (OIR). Methods: C57BL/6J mice were exposed to 75% oxygen from postnatal day 7 (P7) to day 12 (P12) and recovered at room air thereafter. Bone marrow was harvested from C57BL/6J mice expressing GFP under control of an actin promoter. At postnatal day 13 one million cells were injected into the sinus cavernosus. The retinal vasculature was examined after perfusion with Rhodamine-Concanavalin A, the number of GFP(+) donor cells in the retina was counted, and the cells identified by immunofluorescence analysis. Results: About 21 days after transplantation at least 70 % of the blood leukocytes in the recipient mice were determined to be GFP+ by FACS analysis. The first GFP(+) cells were detected 6 days (on P18) after transplantation. Most of the mice were analyzed between P23 and P28. The oldest mice were analyzed at P79. The GFP(+) cell numbers varied extremely from virtually zero to nearly 2500 per retina. A statistically analysis (Mann-Whitney-U) revealed that there were significant ($p = 0.004$) more GFP(+) cells in oxygen treated retinae (median: 163, min: 4, max: 952) than in the retinae of the normoxic controls (median: 42, min: 12, max: 121). There was no association of GFP(+) cells with the edges of the avascular zones. The morphology of the GFP-positive cells varied greatly. The majority of the cells exhibited a microglia-like morphology and was positive for the macrophage/microglia marker F4/80. In addition to these expected cells, a large number of cells tightly associated with large/medium-size vessels and with the microvasculature was identified. GFP(+) donor cells incorporated into the vasculature were demonstrated on P54. They had a cobble-stone like morphology and were preferentially integrated into the walls of blood vessels with a diameter of 3 to 11 µm. Conclusions: Injection of bone marrow cells into the sinus cavernosus of young mice is a suitable method for successful engrafting. Bone marrow-derived cells can be incorporated into the endothelial layer of the retinal vasculature. Another cell type exhibits a subendothelial location and shows a variable number of cellular processes, but the majority of the cells transdifferentiate into microglia cells and is not associated with the edges of the avascular zones in a mouse model of oxygen induced retinopathy.

Keywords: Retina; angiogenesis, endothelial cells; bone-marrow cells; oxygen induced retinopathy (OIR)

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G-CSF/SCF Reduces Susceptibility to Ventricular Arrhythmias in the Infarcted Heart by Increasing Regional Connexin43 Expression and Promoting Arteriogenesis and Cardiomyocyte Growth

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Granulocyte-colony stimulating factor (G-CSF), alone or in combination with stem cell factor (SCF), can improve hemodynamic cardiac function after myocardial infarction. Apart from impairing the pump function myocardial infarction causes an enhanced vulnerability to ventricular arrhythmias. Therefore, we investigated the electrophysiological effects of G-CSF/SCF and the underlying cellular and molecular events in a murine infarction model. Mice underwent ligation of the left anterior descending artery on the third day of G-CSF/SCF treatment (G-CSF, 200µg/kg/day and SCF 50µg/kg/day s.c.) or placebo. Some of these were subjected to bone marrow transplantation 10 weeks prior G-CSF/SCF using bone marrow isolated from transgenic EGFP-expressing mice, from which a subset was sacrificed one day after induction of myocardial infarction. The hearts of all other animals were investigated 5 weeks after coronary artery ligation. G-CSF/SCF improved cardiac output after myocardial infarction. Although, G-CSF/SCF led to a twofold increased, potentially proarrhythmic homing of bone marrow-derived cells to the area of infarction, less than 1% of these cells adopted a cardial phenotype. Inducibility of ventricular tachycardias (VT) during programmed stimulation was reduced five weeks after G-CSF/SCF. G-CSF/SCF increased cardiomyocyte diameter, arteriogenesis, and expression of connexin43 in the border zone of the infarction. Whereas one day after coronary artery ligation G-CSF receptor was predominantly expressed by small interstitial cells and cells of the vessel walls, 5 weeks later it was also strongly expressed by cardiomyocytes. In addition to paracrine effects that may stimulate cardiomyocyte and vessel growth and increase intercellular electrical connections, potentially caused by an increased homing of bone marrow-derived cells, an enhanced expression of the G-CSF receptor in cardiomyocytes and other cell types of the infarcted myocardium might contribute to the therapeutic effects of G-CSF by sensitizing the heart to direct influences of this cytokine.

Keywords: myocardial infarction, ventricular arrhythmias, bone marrow stimulation, connexins, granulocyte-colony stimulating factor

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Comparative characterisation of differentiated adult mesenchmal stem cells in a collagen-1-matrix with autologous chondrocytes in a collagen-1-matrix as a cartilage tissue implant

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Introduction: Adult Bone marrow mesenchymal stem cells (MSCS) are candidate cells for cartilage tissue repair. This is due to the fact that these cells are thought to be multipotent and able to replicate as undifferentiated cells and have the potential do differentiate to lineages of mesenchymal tissues, including bone, cartilage, fat, tendon etc.. Additionally it is assumedthat they have the ability of heterologous transplantation because of their lack of antigenicity. Question: In the present study we compare the chondrogenity of autologous chondrocytes in a collagen-I-matrix with with the chondrogenity of chondrogenic differentiated adult stemm cells in a collagen-I-matrix. Methods: Human chondrocytes and bone marrow cells were derived from 10 patients undergoing operation of the knee joint. Chondrocytes were cultivated as a monolayer culture and after being harvested mixed with Collagen-I. The adult stemm cells were cultivated as a monolayer and then – for the purpose of chondorgenic differentiation – 3x105 cells were stimulated for 6 days, in a first step with TGFß1 at a concentration of 5ng/ml and then for another 14 days with IGF-1 at a concentration of 200ng/ml. To evaluate the chondrogenic effect, histological and immunhistochemical examinations have been performed. Results: It was shown that adult stem cells show to have the capacity for in vitro chondrogenic differentiation. Nevertheless in comparison to autologous chondrocytes they show to have less conformity to hyaline cartilage, especially in specific stainings. Conclusion: Above demonstrated results showed that stem cell – matrix implants reveal a good possibility for cartilage repair, bur still do not reach the results of autologous chondrocytes – matrix implants. Nevertheless the advantage of quite unlimited sources of adult stem cells and the missing of antigenicity should lead to further investigation to improve chondogenic differentiation of adult stem cells.

Keywords: stemmcells; chondrogenic differentiation; cartillage repair; matrix;bone marrow mesenchymal stem cells

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A New Chance for Cell Therapy: Isolation & Characterisation of Cells from Umbilical Cord Tissue

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Tissue engineering is a promising approach for therapy of many diseases, but currently limited by need of high cell numbers. Additionally, the tissue used as cell source must be easily available at low risk for the donor. Usually discarded after the birth of a child, the umbilical cord can be regarded as a candidate cell source. Recently, so-called Wharton's jelly cells (WJC) have been isolated from the connective tissue of the umbilical cord by Mitchell et al. (2003) and shown to possess properties of mesenchymal stem cells (MSC). In this study, the isolation procedure was simplified and standardised, giving the method the potential for automation. Two phenotypes of cells were obtained, one identified as endothelial cells by characteristical morphology and surface marker expression profile. These were not found in all umbilical cord samples, and if present, their frequency was rather low, thus they were not used for further investigation. The other cell type, having a fibroblast-like morphology as described by Mitchell, was isolated at much higher frequencies. Fibroblast-like cells were cultured from every sample tested and showed rapid growth. Calculations demonstrated that in a 7 week cultivation, from 1 cm of umbilical cord 2 x 10^6 - 1 x 10^8 fibroblast-like cells may be derived. Unlike MSC, WJC showed no clonogenic growth in CFU-F assay. They were negative for a wide variety of blood cell markers and MHC-II, but expressed MHC-I, CD54, CD71, CD90, CD95, CD105, CD117 and CD166, thus their surface marker profile was similar but not identical to MSC. For further characterisation, DNA chips designed for analysis of stem cell differentiation were used to determine gene expression profiles of WJC. Cells from three donors were included, using two independently isolated and cultured samples per donor. 30 genes were found positive, i.e. they were expressed at least in 2 out of these 6 samples. 60 % of the positive genes showed donor variability and 20 % variability between independent samples from one donor. Among the expressed genes were muscle-specific genes like those encoding myoD and myosin heavy chain and mesoderm-specific gene products including vimentin and cbfa1. Genes normally assigned to cells of the nervous system were also expressed, like GFAP and synaptophysin. Additionally, the liver-specific gene product fatty acid transporter was found. Thus, WJC combined features of cells from all three germ layers. Interestingly, several genes characteristic of early mesodermal progenitor cells were also expressed, e.g. brachyury or GATA-4 genes. These data may indicate that WJC either, in spite of uniform morphology, comprise several cell types, or that they resemble an early mesodermal cell type. This, in addition to the ease of their isolation and their growth properties, makes them powerful candidates for use in cell therapies and tissue engineering. Future studies should address the differentiation potential of WJC to investigate their potential therapeutic application.

Keywords: MSC; umbilical cord; Wharton's jelly; gene expression

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In vitro und in vivo differentiation potential of unrestricted somatic stem cells from human cord blood into cardiomyocytes

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Recently an unrestricted somatic stem cell (USSC) with a broad differentiation potential from human cord blood has been described from our laboratory. For any therapeutical use of stem cells assessment of their biological potential is important. The chicken embryo is a well established model system in developmental biology of vertebrates because of its direct visibility and accessibility to manipulations during practically all stages of development. In addition it is a very time saving and inexpensive in vivo model. Thus our objective is to analyze the differentiation behaviour of USSC in the developing chicken embryo. For this purpose several injection methods have been established and the USSC has been transduced with a lentiviral vector expressing LacZ or GFP in order to identify easily labelled USSC and their progeny after injection even in whole mount embryos. Embryos were incubated up to ten days. Injection into blastoderm stage embryos resulted in only a very small number of normally developed embryos (about 20%) with a modicum of USSC. The injection in the gastrulation stage (18/19h of incubation) caused a lower malformation rate (60%) and an increase in the number of transduced cells detectable. Injection next to the somites, or into the extraembryonic blood vessel system, or directly into the heart of 48h old embryos affected the USSC either to be mobilized to or remain in the heart. In both instances these USSC became integrated into heart tissue. The injection next to the somites also resulted in randomly distributed human cells over the whole embryo. When USSC are injected into the wing bud they did not migrate but integrated into sceletal muscle tissue of the embryo. Stimulated by these findings we elucidated the cardiac differentiation pathway of USSC further by co-cultivation with cardiomyocytes from two day old chicken embryos for up to five weeks. Differentiation of USSC was analyzed by selective gene expression studies. RT-PCR´s for genes coding for embryonic as well as for adult cardiac tissue showed that atrial natriuretic factor (ANF), atrial (MLC2a) and ventricular (MLC2v) myosin light chain, Troponin I and the transcription factor Nkx2.5 become expressed. These results taken together suggest that USSC can be differentiated into heart muscle cells in vitro by co-cultivation with chicken cardiomyocytes as well as during chicken embryogenesis in vivo.

Keywords: USSC, cardiomyocytes, chicken embryo

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Effects of USSC on CB-CD34 engraftment in NOD/SCID mice

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Recently we were able to show that cord blood-derived unrestricted somatic stem cells (USSC) produce functionally significant amounts of hematopoiesis supporting cytokines (Kögler et al., 2004 and 2005) and were superior to BM-MSC in expansion of immunomagnetically enriched CD34+-cells from cord blood (regarding CFU and LTC-IC). USSC are therefore a suitable candidate for stroma-driven ex-vivo-expansion of hematopoietic CB cells for short term constitution. To determine the supportive potential of USSC on HSC in vivo, CD34+-cells were transplanted into NOD/SCID-mice alone or co-transplanted together with USSC or BM-MSC, respectively, either intra femoral (i.f.) in cooperation with the group of John Dick at the Toronto General Research Institute, University Health Network, Toronto, Canada, or intra venous (i.v.) in cooperation with the group of Thomas Moritz at the Department of Internal Medicine, University of Essen. In the experiments, mostly 1x104 CD34+-selected HSC and 1x106 USSC or BM-MSC were applied. The cells were injected into the right femur or the tail vein directly (for i.f.) or 24 h (for i.v.) after 300cGy (sublethal) total body irradiation. After 5-6 weeks mice were sacrificed, organs of interest harvested and frozen at –80°C for further analysis. Screening for human cells in tissue was performed by immunohistological staining against human nuclei and FACS, respectively. Since i.f. injection resulted mainly in local repair of the damaged bone tissue by the USSC and no influence on the engraftment of CD34+ CB cells was observed, further experiments using only i.v. injection were performed. Preliminary experiments with i.v. co-transplantation of CD34+-cells with USSC or BM-MSC showed that 2-24 h after transplantation, human cells were found exclusively in the lungs, independent of whether the mice received USSC or BM-MSC alone or in combination with CD34+ HSC. Five weeks after transplantation, human cells were found in very low numbers in the lungs and, in higher numbers ,either in the liver and spleen or in the bone marrow. Since it is known from the migration of HSC that i.v. injected cells home first into the liver and spleen and later on to the bone marrow, 5 weeks might have been too short a time for final engraftment: In some animals, human cells were found in high amounts in liver, spleen or outer regions of the femur, but not in the bone marrow. This might also explain why only in those animals which showed engraftment of human cells within the bone marrow, a significant impact of stromal cells could be observed by FACS-analysis. While an engraftment of only 3-4% in control animals was observed, co-tranplantation of BM-MSC or USSC led to an engraftment of 7-9%. Analysis of migration of USSC, MSC and CD34+ cells to organs other than bone marrow is ongoing. Additional experiments with an evaluation time of 6-8 weeks are currently running.

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Keywords: USSC;Cord-blood;Hematopoiesis;NOD/SCID;Engraftment

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Transplantation of arylsulfatase A overexpressing ES cell-derived glial precursors in an animal model of metachromatic leukodystrophy

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The availability of embryonic stem cells (ES) offers exceptional opportunities for combining cell and gene therapy and provides attractive prospects for neural transplantation. Their extensive capacity for self-renewal, their potential to differentiate into all somatic cell types and their accessibility to genetic modification make ES cells a virtually unlimited and highly versatile donor source for a large variety of tissues. In this study we explored whether glial precursors derived from arylsulfatase A (ASA)-overexpressing ES cells can be used for enzyme substitution in an animal model of metachromatic leukodystrophy (MLD). To that end, ES cells were stably transfected with constructs encoding human ASA. Expression of hASA from a beta-actin promoter yielded ES cells with an up to 30-fold increase in ASA activity. High expression of ASA was maintained throughout all stages of neural differentiation. ASA overexpressing glial precursors could be further expanded in the presence of FGF2 and EGF. Upon growth factor withdrawal, they efficiently differentiated into astrocytes and oligodendrocytes. Following transplantation into the brain of neonatal ASA-deficient mice, they incorporated into a variety of host brain regions. Immunofluorescence analyses with antibodies to hASA and sulfatide revealed an up to 30% reduction of immunoreactive sulfatide deposits in the vicinity of the engrafted cells. These findings suggest that ES cell-derived glial precursors may serve as a potential donor source for cell-mediated enzyme delivery in MLD.

Keywords: ES cells, arylsulfatase A, metachromatic leukodystrophy, sulfatide reduction, transplantation

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Discovery of Stem Cell Selective Peptides for Targeted Therapy of CNS Diseases

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Adult neural stem cells (aNSCs) are defined by their ability to self-renew, to differentiate into cells of all glial and neuronal lineages throughout the neuraxis, and to populate developing or degenerating central nervous system (CNS) regions. The recognition that NSCs propagated in culture could be reimplanted into mammalian brain, where they might integrate appropriately throughout the mammalian CNS and stably express foreign genes, has unveiled a new role for neural transplantation and gene therapy and a possible strategy for addressing the CNS manifestations of diseases that heretofore had been refractory to intervention. To potentiate basic stem cell research and cell replacement strategies, we take advantage of the combination of gene and stem cell therapy. Due to the low pathogenicity of adenovirus (Ad) for humans, its large delivery capacity, and long-term transgene expression, Ad-vectors are a perfect means to deliver foreign genes (e.g. therapeutic genes, differentiation-inducing genes) into stem cells. However, analysis of Ad receptor expression in primary NSCs revealed a complete lack of the coxsackie-adenovirus receptor (CAR) and no or low expression of an- and b5-integrins. To direct Ad-vector mediated gene transfer efficiently and specifically to aNSCs, we searched for stem cell selective peptides using phage display technology. Adult NSCs specific ligands were identified by performing three rounds of subtraction using phage display. Selected phages showed a higher binding efficiency for aNSCs when compared to wild-type M13 phage and other murine or human cells. Homing to adult NSCs of the best binding phages were clearly blocked in the presence of specific peptide. The both identified peptides mediate efficient internalization of the phages into aNSCs in vitro. Importantly, specific binding and internalization was also mediated by the identified peptides within the adenoviral context. Our results indicate that the identified peptides should be suitable to improve the efficiency and selectivity of adenoviral gene transfer to aNSCs in vivo. This work was supported by BMBF grant FKZ 01ZZ0108 to B.M.P and by Forschungsförderung Universität Rostock (FORUN) to A.S.

Keywords: adult NSC; adenoviral vectors; CNS diseases; phage display

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Plasticity of human mesenchymal stem cells: Is the epidermal fate included?

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Human mesenchymal stem cells (hMSC) can differentiate in vivo and in vitro into mature cells of multiple mesenchymal tissues. It has also been reported that hMSC might differentiate into cells of ectodermal origin. This indication of plasticity makes hMSC a promising donor cell source for cell-based therapies in various human diseases with pathological cell death. However, this plasticity could partly be attributed to cell fusion and not to direct differentiation. Here, we investigate the in vitro three-dimensional growth and potential epidermal differentiation of hMSC on so called dermal equivalents under the influence of air-contact. Therefore, hMSC are grown under similar conditions used for the organotypic generation of skin equivalents with keratinocytes. Collagen embedded dermal fibroblasts on membrane inserts (0.4 µm pore size) serve as dermal stroma and hMSC are cultured instead of keratinocytes at high density on top of these dermal equivalents. Constructs are lifted to the air-liquid-interphase after four days by lowering the medium at the bottom of the collagen gel. Culture conditions are modified by pretreating the cells with the demethylating agent 5-azacytidine or supplementing the medium with all trans-retinoic acid. Potential epidermal differentiation is analysed with morphological and immunohistochemical methods. To determine whether hMSC maintain their multipotency in contact to dermal equivalents on one side, and to the air on the other side, adipogenic and osteogenic differentiation were additionally induced in parallel experiments using standardized protocols according to Pittenger et al. We could demonstrate that hMSC are still capable of differentiating into adipocytes and osteoblasts, even under influence of air-contact. In all experiments concerning the epidermal differentiation fate of hMSC, the cells survived and grew in organotypic manner with air-contact. Further, hMSC were building a compact multilayer on the collagen surface with morphological features of early epidermis, like flattening of the superficial cells and elongation of the basal regions. Immunohistochemical analyses revealed, however, a persistent expression of mesenchymal markers as vimentin, but not of cytokeratins, suggesting no epidermal (trans) differentiation. Besides, hMSC were seen to migrate into the collagen-gel causing a contraction when cultured on cell-free collagen scaffolds. These results point out that hMSC stimulated with differentiation factors for the 3D-growth of keratinocytes (collagen matrix, dermal fibroblasts, growth factors and air contact) retained their mesenchymal phenotype and failed to differentiate into epidermal cells

Keywords: human mesenchymal stem cells, epidermal differentiation, dermal equivalents, stromal cells

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Up-regulation of nestin, four and half LIM domains 2 (FHL2) and thioredoxin-like 1 (TXNL1) in injured mouse and human myocardium potentially indicates spontaneous stem cell-based regeneration

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Background: Alterations in protein expression probably constitute the molecular basis underlying regeneration in the ischemic and infarcted myocardium. Due to its reproducibility and defined etiology the mouse model of myocardial infarction should allow for the reliable identification of such changes. Methods and results: Myocardial infarction was induced in CD-1 male mice at 6 weeks of age by ligation of the left descending coronary artery (LAD). Using proteomic analysis (2 D gel electrophoresis in combination with mass spectrometry) to screen for proteins involved in regenerative processes in the injured mouse myocardium nestin, four and half LIM domains 2 (FHL2) and thioredoxin-like 1 (TXNL1), proteins known to be associated with tissue repair and regeneration, were found to be up-regulated in the experimentally infarcted myocardium at 3 weeks after ligation of the LAD. Corresponding changes were evident for the mRNAs encoding these proteins not only in the infarcted mouse myocardium but in infarcted and failing human hearts as well as revealed by quantitative real time RT-PCR. Nestin, a known marker of stem cell differentiation was localized by immunofluorescence predominantly in the border-zone between the infarcted and non-infarcted area in various differentiated heart cell types including cardiomyocytes, endothelial cells and fibroblasts. In infarcted hearts of chimeric mice transplanted with bone marrow from enhanced green fluorescent protein(EGFP)-transgenic mice only a very small proportion of nestin-positive cells (< 1 %) were found to coexpress EGFP although EGFP-positive cells were abundant in these hearts. Conclusions: The enhanced expression of nestin, FHL2 and TXNL1 in the injured mouse and human myocardium probably reflects the activity of spontaneous regenerative processes supposedly based on the activation of resident cardiac stem cells. To definitely identify the origin and the fate of nestin-expressing cells further co-immunolocalizations with antibodies against stem cell as well as lineage markers of other differentiated heart cells (e.g. smooth muscle cells) are being performed.

Keywords: heart; infarct; regeneration; nestin

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Modulation of the spontaneous and SDF-1· induced migratory activity of adult CD133+ HSCs

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The application of haematopoietic stem cells (HSCs) and their migratory behaviour play a critical role in modern therapeutic uses such as bone marrow homing after systemic application in leukaemia therapy and after local application for tissue regeneration. For this purpose the aptitude of HSCs to extravasate and the mechanisms regulating cell migration in the connective tissue are of crucial interest. The chemokine stromal cell-derived factor 1· (SDF-1·) is the only known potent chemoattractant for HSCs so far. However, although mechanisms initiating HSC migration are well understood, considerably less is known about the signals modulating the SDF-1· induced HSC-migration. In this study, interleukin-8 (IL-8) and the neurotransmitter gamma-aminobutyric-acid (GABA) were investigated for their influence on the migratory activity of unstimulated and SDF-1· treated adult CD133+ HSCs obtained from aphaeresis.IL-8 is released by immune cells at inflammatory or necrotic tissues and has been shown to inhibit the migration of leukocytes under certain conditions. Likewise, recent studies have shown an inhibitory effect of GABA on the migratory activity of lymphocytes. Additionally, both IL-8- and GABAB-receptor are expressed by adult CD133+ HSCs. Computer-assisted analysis and a three-dimensional collagen matrix assay enabled us to analyze single-cell migratory activity of CD133+ HSCs whereas the migration was modulated by application of SDF-1· alone or in combination with IL-8 or GABA, respectively. CD133+ HSCs showed a spontaneous migration rate of about 50% that was increased up to 70-75% after application of SDF-1· and 61% in the presence of IL-8 respectively. Interestingly, coapplication of SDF-1· and IL-8 on CD133+ HSCs showed no additive effect, but a migration rate of 63% similar to the migration rate of IL-8 treated CD133+ HSCs. Thus, IL-8 might be a regulator of the SDF-1· induced migration of CD133+ HSCs. In contrast to IL-8, GABA inhibited effectively both the spontaneous and the SDF-1· induced migration of CD133+ HSCs (GABA: 35%; SDF+GABA: 46%). Flow cytometry based analysis revealed that SDF-1· treated cells showed an increased intracellular calcium level whereas coapplication with SDF-1· and GABA decreased the calcium level to or even below the level in unstimulated CD133+ HSCs. These observations lead us to the hypothesis that in CD133+ HSCs the SDF-1· induced increase of intracellular calcium is similar to lymphocytes not solely attributed to the release from intracellular stores but is also mediated by extracellular calcium-influx via calcium-release-activated-calcium-channels (CRACs). Mimicking the GABA-effect after treatment with the CRAC-specific inhibitor BTP-2 we not only recommend a plausible signalling of GABA in CD133+ HSC-migration, but the results also indicate that extracellular calcium might be necessary for an effective migratory signalling in CD133+ HSCs. In summary our results show that CD133+ HSC-migration can be modulated by IL-8 and GABA, which might be of certain relevance in stem cell therapy.

Keywords: Hematopoietic stem cells; Cell migration; SDF-1; Cytokines

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Glial conversion of SVZ-derived committed neuronal precursors after ectopic grafting into the adult brain

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In the adult mouse forebrain, large numbers of neuronal precursors, destined to become GABA- and dopamine-producing interneurons of the olfactory bulb (OB), are generated in the subventricular zone (SVZ). Although this neurogenic system represents a potential reservoir of stem and progenitor cells for brain repair approaches, information about the survival and differentiation of SVZ-derived cells in ectopic brain regions is still fragmentary. We show here that ectopic grafting of SVZ tissue gave rise to two morphologically distinguishable cell types displaying oligodendrocytic or astrocytic characteristics. Since SVZ tissue contains neuronal and glial progenitors, we used magnetic activated cell sorting to deplete A2B5+ glial progenitors from the dissociated SVZ and to positively select cells that express PSA-NCAM. This procedure allowed the purification of neuronal precursors expressing TUJ1, DCX and GAD65/67. Transplantation of these cells led again to the generation of the same two glial cell types, showing that committed interneuron precursors undergo glial differentiation outside their normal environment.

Keywords: stem cell, transplantation, cell therapy, neurogenesis, gliogenesis

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Bone-marrow-derived stem cells in TNFRp75-/- knock-out mice with experimental choroidal neovascularization

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Choroidal neovascularization (CNV) is responsible for the severe visual loss in ARMD. Additionally, an inflammatory component has been shown to be involved in the pathogenesis of ARMD. Macrophage-derived TNF-· increases the expression of vascular adhesion molecules (ICAM-1, VCAM-1, E-selectin) and VEGF. In current study, we combined the transplantation of whole bone marrow cells and laser-induced CNV in TNFRp75 -/- mice to examine the development of CNV. Lethally irradiated adult TNFRp75 -/- mice as well as C57BL/6 mice were used as recipients for the transplantation. Whole bone marrow mononuclear cells from gfp(+) transgenic mice were injected into the tail vein of recipient mice. Additionally, whole bone marrow from C57BL/6 and TNFRp75 -/- mice was transplanted to lethally irradiated C57BL/6 and TNFRp75 -/- mice, respectively. CNV was induced by 4 separate laser burns in the choroid. Two weeks later, mice were perfused transcardially with rhodamine-conjugated concanavalin-A for visualization of the blood vessels. CNV was then examined by dual-fluorescence scanning confocal microscopy of flatmounts. The area of CNV, as well as, colocalization of gfp(+) to the vasculature was measured. One month after transplantation, FACS analysis showed 70% gfp(+) circulating cells in both C57BL/6 and TNFRp75 -/- mice. Gfp(+) cells were localized predominantly within and around the edge of CNV and within the optic nerve head. Some gfp(+) cells with branching processes (F4/80-positive) were observed to infiltrate the overlying neurosensory retina. The area of neovascularization was significantly reduced in TNFRp75 -/- in comparison to C57BL/6 recipients. Gfp(+) fluorescence areas within the CNV were significantly reduced in TNFRp75 -/- in comparison to C57BL/6 recipients. Many gfp(+) cells in both groups appeared to be integrated to the neovascular tissue (co-localization of gfp(+) and rhodamin-ConA fluorescence within the laser burn). However, no significant difference in co-localization areas (merged green and red images) was found between the both groups. Laser injury induced bone marrow-derived stem cell recruitment to the CNV lesions in both TNFRp75 -/- and C57BL/6 mice. CNV injury was reduced in TNFRp75 -/- mice in comparison to C57BL/6 recipients. It remains to be determined whether decreased CNV formation in TNFRp75 -/- recipients is due to decreased involvement of transplanted cells to the formation of new vessels or to other mechanisms associated with the absence of TNFRp75.

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Pluripotent Unrestricted Somatic Stem Cells From Cord Blood Generate An Immature Endodermal Precursor Cell Type In Vitro

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USSC are unresticted somatic stem cells from cord blood with an intrinsic pluripotent differentiation potential (Kogler et al. 2004; 2005). Immunophenotyping of USSC showed differences towards MSC from bone marrow. Moreover, telomere length assays revealed longer telomeres for USSC in comparison to MSC. General gene expression and varying differentiation potentials may allow the conclusion that USSC represent an earlier stem cell type than MSC. As shown recently by our group, transplantation of USSC in a non-injury model, the preimmune fetal sheep, resulted in more than 20% albumin-producing human parenchymal hepatic cells with absence of cell fusion. Here we examined whether USSC can be differentiated in vitro into the endodermal linage by use of different protocols described for both embryonic and adult stem cells. Different matrices, growth factors and organic substances were applied to change the gene expression of USSC. Best results were achieved by using retinoic acid, Matrigel matrix and a special differentiation protocol with HGF and OSM that was introduced for MSC by Lee (Lee et al. 2004) and modified by Hong (Hong et al. 2005). To examine the influence of the biological niche, USSC were further cocultivated with cells of endodermal origin, such as primary rat hepatocytes and tissue from sheep organs. Coculture of USSC with different endodermal cells or conditioned medium was performed either through a transwell system or with direct coculture of the cells. 15 different USSC lines were differentiated for 1, 2, 3, 4 and 6 weeks. Primers were designed with the strategy to define stages of endodermal development on the basis of the embryonic cell development from mouse and human. Accordingly the following markers were established: GATA4, HNF1, HNF3b and HNF4a to define the embryonic and visceral (extra-embryonic) endoderm, a common precursor phenotype for both liver/exocrine pancreas development. To further assess differentiation into liver cells, alpha-fetoprotein, albumin, Cyp2B6, Cyp3A4, Gys2 and PDX-1 were utilized. PAX4, ISL-1, Nkx6.1, NeuroD and insulin were used to determine differentiation into the pancreatic linage. Both factor and coculture approaches resulted in a changed endodermal gene expression for USSC. In total 11 of the 15 used markers were seen positive throughout the differentiation procedures. Expression of the early endodermal markers HNF1 and HNF3b as well as PAX4 and insulin for late beta-cell development were never observed. Expression of all other markers revealed that USSC can be differentiated towards an endodermal lineage, but as yet cannot be directed to a specialized cell-type. Differentiation results showed frequently a mixture of early and late as well as pancreatic and hepatic gene expression. Kogler, G., S. Sensken, et al. (2004). "A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential." J Exp Med 200(2): 123-35. Kogler, G., T. F. Radke, et al. (2005). "Cytokine production and hematopoiesis supporting activity of cord blood-derived unrestricted somatic stem cells." Exp Hematol 33(5): 573-83. Lee, K. D., T. K. Kuo, et al. (2004). "In vitro hepatic differentiation of human mesenchymal stem cells." Hepatology 40(6): 1275-84. Hong, S. H., E. J. Gang, et al. (2005). "In vitro differentiation of human umbilical cord blood-derived mesenchymal stem cells into hepatocyte-like cells." Biochem Biophys Res Commun 330(4): 1153-61.

Keywords: USSC; cord blood; somatic stem cell; endodermal differentiation; liver

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Immunomodulating Activity of Cord-Blood Derived Unrestricted Somatic Stem Cells

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Unrestricted somatic stem cells (USSC) from cord blood have the potential to differentiate to tissues of all three germinal layers. Thus, they hold promise in regenerative therapies. Furthermore, they have stromal activity supporting haematopoiesis and may, therefore, also be used in haematopoietic co-transplantations to enhance engraftment. Both applications of USSC would put the cells or their progeny in an allogeneic environment, which may cause rejection or modulation of GvHD. Therefore, we have studied the immunogenicity of USSC and have determined their interactions with components of cellular immunity. USSC revealed very limited expression of immunorelevant molecules. They only expressed the adhesion molecules CD49b, CD49e, CD54 and CD58 and were HLA-class I positive. Expression of the adhesion and co-stimulatory molecules CD11a, CD11b, CD11c, CD18, CD40, CD49d, CD50, CD80 and CD86 as well as of HLA-class II was absent. Consistent with this immunophenotype, USSC showed no activity as antigen-presenting cells in an allogeneic MLR, irrespective of stimulation with IFNg, which upregulated HLA-class I and II as well as CD54. When co-cultured with an allogeneic MLR, USSC caused only limited inhibition. However, following pre-stimulation with IFNg and TNFa, USSC suppressed T-cell alloresponses dose-dependently (66.9±4.1% inhibition (n=5) for 30,000 USSC). This inhibition was mediated by indoleamin-2,3-dioxygenase (IDO). Tryptophan in the MLR medium had been completely catabolized to kynurenine by IDO activity and T-cell responses could be restored by tryptophan supplementation. Next, the influence of USSC on dendritic cell (DC) differentiation and maturation was studied. Differentiation of monocytes to immature DC was not affected. They showed a normal phenotype (CD14-/CD83-). Pinocytosis as an indication of functional competency was comparable or slightly increased. In contrast, in co-cultures a USSC-dose dependent inhibition of DC maturation was observed: Expression of typical mature DC markers (CD83, CD86) and T-cell stimulatory activity were reduced. Normal DC maturation could not be restored by neutralizing prostaglandin E2 or TGFb-activity – potential inhibitors of DC differentiation/maturation, which are expressed by USSC - consistent with the observation that inhibition was only detected in co-cultures but not in transwell-cultures or in the presence of USSC-conditioned medium. Thus, USSC interfere with T-cell responses either directly in an IDO-dependent fashion or indirectly by inhibiting maturation of dendritic cells, which may not only protect them and possibly also their progeny from rejection, but which may also be exploited in allogeneic haematopoietic stem cell transplantation, to prevent GvHD. However, when this immunosuppressive activity was evaluated in an ex-vivo skin explant model of GvHD, no reduction of GvHD was observed.

Keywords: Immunosuppression, T Cells, Dendritic Cells, Cord Blood

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Characterization of two distinct proliferating liver epithelial cells isolated from liver tissue after ischemic treatment

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Introduction: It is generally accepted that there are progenitor cells residing in the adult human liver but little is known about their niche and their identity. Isolation of these adult human liver progenitor cells is difficult because there is uncertainty about specific markers characterizing the cells. Here we describe the characterization of two distinct epithelial cell types with progenitor characteristics isolated from human liver tissue after ischemic treatment. Methods and materials: Human liver tissue pieces utilized for this study were obtained from partial hepatectomies. The resected liver pieces (n=30) were placed in culture medium at 4°C and kept under hypoxic conditions for 7 days until cell isolation. The liver tissue was mechanically minced and incubated for 30 min at 37°C in a buffer solution containing 0.1% Pronase E and 0.025% DNase I. Then the cells were filtered through sterile gauze and remaining erythrocytes were removed by lysis. Finally, cells were seeded into collagen coated culture vessels. Cultured cells characterized by their morphology, immunofluorescence staining and reverse transcription polymerase chain reaction using markers for hepatocytes, biliary epithelial cells, non-parenchymal cells and progenitor cells. Results: In cultures of the isolated cells two distinct types of epitheloid colonies were observed that also showed different culture behaviour. Cell type 1 appeared about two days after cell isolation, forming sharply bordered colonies of densely packed cells with a low cytoplasm-to-nucleus ratio. The cells in the colonies proliferated with a low frequency during the first 7 days of culture. Afterwards a change of the morphology of the cells was observed: the cytoplasm was growing in size and cells started to spread. This change in morphology was followed by detachment and death of the cells. Cells of type 2 were not detected in culture until culture day seven. Initially the cells appeared in loose colonies and had a round to oval outer shape and a convex cytoplasm. The cells showed constant proliferation rates and when reaching confluency, changed their morphology to polygonal-shaped, densely packed cells. Analysis of markers by immunofluorescence staining and RT-PCR showed that both cell types expressed cytokeratins 8, 18, 7 and 19, vimentin and cytochrome P450 1B1, but no alpha-fetoprotein. The hepatocyte-specific markers albumin, -1-antitrypsin, gamma-glutamyl transferase and cytochrome P450 2B6, were only detected in the cells of type 1. Conclusion: The characterization of the two cell types by immunofluorescence staining and RNA expression analysis showed the expression of both hepatocyte and biliary epithelial cell markers in cell type 1, which suggests a bipotent character of the cells as described for adult liver progenitors. Cell type 2 expressed markers described for biliary epithelial cells. Additional studies have to be performed to further validate the progenitor character of type 1.

Keywords: adult human liver progenitor cells; characterization; isolation

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Transplantation of adenosine-releasing ES cell-derived neural precursors: An experimental approach for seizure control

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Adenosine is a potent anticonvulsive and neuroprotective agent, but strong adverse side effects preclude its systemic application. To develop a cell-based and focal therapeutic tool for the treatment of mesial temporal lobe epilepsy murine ES cells were engineered for adenosine delivery by a targeted disruption of adenosine kinase (ADK), the major adenosine-metabolizing enzyme. ADK-/- ES cells were subjected to an established in vitro differentiation protocol to derive neural precursors. Disruption of the ADK gene resulted in elevated release levels of adenosine in differentiated neuronal cultures, without affecting the cells' potential to differentiate into neurons and glia. To evaluate the therapeutic potential of these cells, we transplanted 2 x 106 GFP labeled neural precursor cells via a transverse trajectory in and adjacent to the hippocampus of fully kindled rats. Histological analysis revealed that cell suspensions grafted in the vicinity of the hippocampus differentiated into neurons and glia but showed only limited spread into host tissue. The transplanted neurons gave rise to an axonal network extending far into adjacent brain regions. Partial seizure suppression developed within two to four weeks after transplantation of ADK -/- cell suspensions. Our data depict ES cell-based adenosine delivery as a potential strategy for seizure control.

Keywords: ES cells, Adenosine, Epilepsy

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Apoptotic neurons attract cord blood-derived unrestricted somatic stem cells

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Endogenous as well as transplanted stem cells have the capacity to migrate towards lesions in the adult central nervous system and may have the therapeutic potential to enhance regeneration after brain injury initiated by stroke or trauma. To investigate whether unrestricted somatic stem cells (USSC) that were recently isolated from human cord blood have the potential to migrate towards injured brain tissue we established an agarose invasion assay that allows to study and characterize migration in vitro. As model system for chemoattraction we used: 1.) homogenate of brain tissue of mice in that cerebral ischemia was induced by occlusion of the middle cerebral artery 2.) murine clonal hippocampal neurons (HT22 cell line) in which apoptotic pathways were initiated by incubation with staurosporine. Homogenate of post-ischemic brain tissue strongly attracts USSC while homogenate of healty brain hemispheres only has a moderate effect. To analyse whether lesioned neurons themselves secret factors that attract USSC we studied apoptotic murine hippocampal cells in the agarose invasion assay and found that neurons in which apoptosis was induced strongly attract USSC whereas healthy neurons do not. Investigating the expression of growth factors and chemokines in lesioned brain tissue and neurons and of their respective receptors in USSC revealed an expression of hepatocyte growth factor (HGF) in injured tissue and of the HGF receptor c-met in USSC, suggesting a potential role for HGF in attracting USSC to sites of brain injury. Our data demonstrate that USSC have the capacity to migrate towards apoptotic neurons and injured brain tissue and this together with their proven neural differentiation potential suggest a neuroregenerative potential of USSC.

Keywords: Cerebral Ischemia; Hepatocyte Growth Factor; Migration; Neuronal Apoptosis; Somatic Stem Cell

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The number of early and late differentiated progenitor cells increases in the early phase of acute myocardial infarction

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The availability of circulating progenitor cells (CPC) is important for the recovery of heart function after acute myocardial infarction (AMI). Therefore, the aim of the present study was to quantify the CPC in patients with AMI early after onset of symptoms and acute PCI. Circulating CD34+ cells were phenotypically analyzed in patients with AMI at different time points after onset of symptoms: T0: prior to primary PCI; T1: 4 hours after PCI; T2: day 2 post AMI; T4: day 4 post AMI. Data were compared with results obtained in patients with stable coronary artery disease (CAD) and healthy controls (CTR). The number of circulating CD34+ cells was higher in patients with AMI at admission (T0) compared to CTR and CAD ($p<0.01$, and $p<0.001$, respectively). Interestingly, this even increased at T1. However, a tendency towards a decreased CD34+ cell number was seen on T2 and T4, but this still remained higher than in CTR and CAD ($p<0.05$ and $p<0.04$, respectively). Subpopulation analysis of CD34+ cells showed that the number of early CD34+/CD133+ CPC, as well as late CD34+/CD133- CPC, increased rapidly after AMI (T0) and PCI (T1), but decreased afterwards. Interestingly, at T2 and T4 the number of late CPC was still significantly higher than in CTR and CAD ($p<0.05$ and $p<0.02$, respectively). In contrast, the number of early CPC reached comparable levels to those of CTR and CAD. Similar evolution was observed for CD34+/VEGF receptor-2-positive/CD133+/- cells, including the group of endothelial progenitors. No significant differences were documented between patients with CAD and CTR. Thus, mobilization of CPC takes place within a few hours after onset of AMI. Post-AMI inflammatory conditions, PCI-associated mechanical, or drug-related stimuli may contribute to the increase of CPC pool in circulation. A different evolution of early and late CPC number has to be further analyzed with respect to the recovery of myocardial function.

Keywords: progenitor cells, acute myocardial infarction

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ELSI

Interdependency of public attentiveness and scientific progress – the case of stem cell research

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Since the first successful derivation and cultivation of human embryonic stem cells (hESC) in 1998, a controversial debate aroused worldwide. The debates concerned the use of human embryos for scientific purposes as having potential benefits for regenerative medicine on the one hand and the implied ethical problems on the other hand. If research disregarded moral values – as with the case of hESC the dignity of the embryo in vitro – it would be difficult to ascertain its legitimation in society. Establisment of this legitimation is necessary for science not only in order to get federal funding, but also in affecting governmental regulations. This controversy on hESC was battled in the media to a great extent. In modern society, mass media plays the role of representing and structuring a public debate. Public debates about research on hESC led to various national regulations where Germany formulated quite a restrictive law on hESC whereas Great Britian devised the most liberal legislation on stem cell research in Europe. The aim of my PhD-project is to investigate the communicative strategies for legitimising the scientific progress on hESC both by science and the national press, set against the background of diverse regulation mechanism of hESC research in Germany and Britain. The empirical data consists of the landmark papers on stem cell research from 1998-2006 and the news coverage about these scientific milestones in the elite press in Germany and Great Britain. Furthermore, focus is placed on the decisive role faced by scientific journals like Nature and Science as an intermediated unit between science and the media which frames the media coverage on science by stating editorial comments on new findings using news values like sensationalism, moralisation, etc. The leading questions are: How could legitimation be established for controversial research? Which role, in the discourse of legitimation, do the scientists themselves, the scientific journals and the media (which are often critisised as rather censorius towards biomedical science) play? To answer these questions I am accomplishing a qualitative content analysis of the argumentative pattern in scientific and press articles as described above. My assumption is that in the scientific publications an increasing orientation towards the media can be observed already, which is in line with the high visibility of scientists in the press. Under the global stress of competition in the biomedical field, there are not only individual argumentative strategies, but also methodological approaches which are identifiable beyond the moral dilemma. Some scientists operate instead of hESC with adult stem cells, others circumvent the moral arguments, e. g. within the human nuclear transfer by knocking out a key development gene out that is responsible for organising an embryo and named this method: "altered nuclear transfer". Not every scientist is in agreement as indicated by Daley (2004): „Doing a scientific experiment without a scientific reason but to quell an ethical debate" is not the idea of science. (Science, 306)

Keywords: mass media, science journalism, moralisation, legitimation

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Conflicts of interest in stem cell research

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As the debates on the regulation and ethics of stem cell research mature, the focus shifts from the grand struggles of competing ideology to issues of good governance. This presentation takes up some themes first addressed during the 2004 congress by highlighting practical legal and ethical issues in stem cell research, in particular the handling of potential conflicts of interest and of consent management. Such conflicts can arise along the spectrum of innovative endeavour in stem cell research: from the application for funding to the procurement and handling of biomaterials, in biobanking, anonymisation, data abstraction and data mining, in clinical and pre-clinical trials, within the framing of intellectual property and other commercialisation initiatives, during the involvement with spin-out ventures and in the interaction with the media and society. Drawing on case studies from Biobank UK, the California Stem Cell Initiative, and Stem Cell Research at the University of Sheffield, and the hands-on experience from European framework projects such as "PRIVIREAL" and "@neurIST" this presentation reviews the potential for conflicts of interest as well as strategies of consent management and good governance. Best practice recommendations for policy makers, research administrators, clinicians, and scientist conclude this brief overview.

Keywords: bioethics; consent; biobanking; integrity

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Company Profiles

abcam

Abcam plc located in Cambridge, U.K. was founded in 1998 by
Dr. Jonathan Milner, then a researcher at Cambridge University, U.K.

"Like many life science researchers, I was frustrated by the time it took to locate and select antibodies essential for my research. This was largely due to poor information and out-of-date catalogs from the vast range of suppliers who were spread across many countries. In some cases, I also experienced difficulties with companies whose products were unreliable and whose customer service was slow and unhelpful. My vision was to build a company that offered reliable cutting-edge products and great customer service". It was a tough vision – Abcam was created to sell the best antibodies in the world with the most comprehensive, honest and up-to-date datasheets, fast delivery, helpful customer service and comprehensive technical support. Seven years on and Abcam plc is providing cutting edge primary and secondary antibodies to researchers worldwide. Abcam's catalogue of over 21,000 reagents includes tools for neuroscience, chromatin, stem cells, immunology, cancer, subcellular markers and nuclear signalling.

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amaxa currently provides ready-to-use kits for a huge variety of primary cell types, including most difficult-to-transfect cells such as dendritic cells, neurons and mesenchymal stem cells. The recently introduced Nucleofector® 96-well ShuttleTM System now also allows high throughput transfection of such demanding-to-transfect cell types in 96-well format.

With direct sales teams in the EU and US, as well as distribution partners in almost 30 countries, the company covers >90% of the global market. Founded in 1998, amaxa currently employs 135 people and is based in Cologne, Germany, and Gaithersburg, MD, USA.

AVISO GMBH

AVISO GmbH with its 60 employees worldwide, manufactures not only pipette systems and positioning units for blood analysis but also assemblies for the drug research as well as components for automated lab-streets for medical diagnostic canters, digital photo-printer-systems and others. AVISO is also your partner for your demands in any cases of liquid handling with example our Platform technology TheOnyx.

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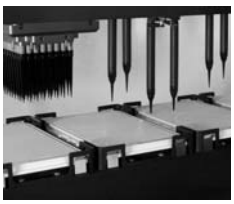
Hamilton Robotic Systems



In den letzten Jahren führte die rasche Beschleunigung der Foschungszyklen in den Life Sciences zu einer wachsenden Nachfrage nach automatisierter Liquid Handling Technologie. HAMILTON kombiniert sein Know-how in Liquid Handling Technologie und Automation mit seiner jahrzehntelangen Erfahrung in der Entwicklung kundengerechter Lösungen, um mit flexiblen, modular aufgebauten Pipettier-Workstations den Kundenbedürfnissen optimal gerecht zu werden. Der MICROAB® STAR workstations sind durch den Einsatz vollständig unabhängiger, asymmetrisch spreizbarer Pipettierkanäle die flexibelsten Automationslösungen. Höchste Prozessstabilität und überlegene Pipettierresultate für die Forschung im Pharma- und Biotech-Bereich werden durch Innovationen wie "Dual Liquid Level Detection" oder das revolutionäre "Monitored Air Displacement" Pipettierprinzip erreicht.

HAMILTON hat sich zum Ziel gesetzt, durch "Innovation for Automation" zufriedene Kunden zu gewinnen und sich dadurch als führendes Unternehmen für automatisiertes Liquid Handling in den Life Sciences zu etablieren.

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LIFE & BRAIN GmbH is a development-stage biomedical enterprise located in Bonn, Germany. Our mission is to discover and develop novel strategies for the diagnosis and therapy of nervous system disorders. Interacting closely with the University of Bonn Neurocenter, LIFE & BRAIN integrates a unique set of expertise in Genomics, Transgenics, Cellomics and NeuroCognition. Dedicated to the advancement of translational neuroscience, we aim at delivering the next generation of products for disease modeling and prediction, compound development and tissue regeneration.

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_Contact

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