7th International Meeting

Stem Cell Network North Rhine-Westphalia

_April 23-24, 2013

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_Program

Tuesday, April 23rd

8:00 – 9:00 am	_Registration
9:00 – 9:30 am	**_Opening of the Meeting** Svenja Schulze, Minister for Innovation, Science and Research of the State of North Rhine-Westphalia
9:30 – 11:00 am	**_Keynote Lectures, Chair: H. Schöler** Kenneth Chien (Boston) *A map for human cardiogenesis and regenerative therapeutics* Christiane Woopen (Cologne) *Pluripotent ethics? Pluralism and the need for guidance*
11:00 – 11:15 am	_Coffee Break, Poster Session
11:15 am – 12:15 pm	**_Session I: Cardiac Differentiation & Repair** Bruce Conklin (San Francisco) *Patient-specific stem cells and cardiovascular genetics* Christine Mummery (Leiden) *Pluripotent stem cells: the new heart patient?*
12:15 – 1:30 pm	_Lunch Break, Poster Session
1:30 – 2:30 pm	**_Session II: Ectodermal Lineage** Cedric Blanpain (Brussels) *Stem cells and skin cancers* Magdalena Götz (Munich) *A new source of neural stem cells after brain injury*
2:30 – 3:00 pm	_Coffee Break, Poster Session
3:00 – 4:15 pm	**_Session III: Disease Modeling** Karl-Ludwig Laugwitz (Munich) *Human models of cardiac disease* Stefan Hauser (Bielefeld) *Human inferior turbinate stem cells (ITSCs) –* *Novel stem cells for the treatment of Parkinson's disease?* Lorenz Studer (New York) *title to be announced*
4:15 – 5:15 pm	**_Session IV: Cultivating human stem cells** Joseph Itskovitz-Eldor (Haifa) *Cultivation of pluripotent stem cells in suspension* Peter Andrews (Sheffield) *Culture adaption in human embryonic stem cell culture: Oncogenesis in vitro?*
5:15 – 6:30 pm	_Poster Session
7:00 – 10:30 pm	**_Networking event at the Wolkenburg** Address: Mauritiussteinweg 59, 50676 Köln (no transfer provided, walkable distance)

Wednesday, April 24th

8:00 – 9:00 am _Registration

9:00 – 10:00 am **_Session V: Hematopoietic Stem Cells**

Michael Clarke (Stanford)
Regulation of normal and diseased stem cells

David Williams (Harvard)
Somatic cell re-programming as a potential future therapeutic option for genetic correction in Fanconi Anemia

10:00 – 11:00 am **_Panel Discussion (Chair: D. Sturma) "Patents for stem cell derived technologies; the legal situation and its consequences"**

Christine Mummery, Clara Sattler de Sousa e Brito

11:00– 11:30 am _Coffee Break, Poster Session

11:30 am – 12:45 pm **_Session VI: Direct (Re)programming**

Marius Wernig (Stanford)
Direct reprogramming of fibroblasts towards neural lineages

Nils Pfaff (Hannover)
MicroRNA-mediated epigenetic regulation represents a roadblock for the generation of iPSCs

Malin Parmar (Lund)
Direct neural conversion; a short cut to generate disease and patient specific neurons

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

2:00 – 3:15 pm **_Session VII: Epigenetics & Chromatin**

Alexander Meissner (Harvard)
DNA methylation dynamics in stem cells and development

Stefan Barakat (Rotterdam)
rnf12 is essential for x-inactivation in female mouse embryonic stem cells and is required for female mouse development

Kenneth Zaret (Philadelphia)
Programming and reprogramming cell fate

3:15 – 3:45 pm _Coffee Break

3:45 – 4:45 pm **_Session VIII: Cells as Testing Tools**

James Bilsland, (Cambridge)
title to be announced

Petter Björquist (Gothenburg)
Human pluripotent stem cells; a paradigm shift for in vitro testing

Following **_Poster Awarding, Closing Remarks**

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Bioengineering & Biomaterials

Abstract 1

Biomaterial-based bone tissue engineering using novel silk scaffolds in combination with MSC

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Introduction: Autologous bone grafting is still the gold-standard for treating large bone defects. However, this method is limited by several disadvantages such as donor site morbidity, lack of donor tissue, as well as increased pain and operation times. An alternative approach is given by combining (degradable) biomaterials with autologous mesenchymal stem cells (MSC). Although numerous studies have examined osteoconductive effects of biomaterials on MSC, the ideal combination of stem cells and biomaterials for bone tissue engineering is still not found. The aim of the present study is to investigate the in vitro and in vivo performance of novel (hydroxyapatite modified) silk matrices cultured with MSC. These constructs will be introduced in critical-size bone defects in a large-animal model.

Materials/Methods: Silk scaffolds (un-/modified with hydroxyapatite) are provided by Spintec-Engineering GmbH. These materials incorporate essential characteristics required for bone tissue engineering such as appropriate mechanical stability, degradability and biomineral supplementation. The MSC compatibility of these scaffolds was investigated in an in vitro test system analyzing viability/cytotoxicity according to ISO 10993-5, proliferation, and live/dead staining. The performance of the silk MSC combination in vivo will be investigated in a preclinical study involving sheep with critical-size defect induction.

Results: Ovine and human MSC were isolated, expanded and characterized according to the Minimal Criteria of the International Society for Cellular Therapy. All silk scaffolds are non-toxic after 24 hours. The proliferation of ovine and human MSC is enhanced in silk scaffolds without or with only low content of hydroxyapatite. Silk scaffolds with higher content of hydroxyapatite reduce proliferation. Current studies involve stem cell differentiation towards osteoblasts by culture in the silk scaffolds.

Discussion: Silk scaffolds with and without hydroxyapatite were shown to be cytocompatible for ovine and human MSC. The reduced proliferation of MSC on silk scaffolds with high hydroxyapatite content might indicate that MSC undergo differentiation. Our study will contribute to better understanding biomaterial-induced osteogenic differentiation of MSC and their potential to regenerate large bone defects in vivo.

Keywords: biomaterials; silk; bone; tissue engineering; MSC

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Abstract 2

Bioluminescence revised for noninvasive neuroimaging of minimal stem cell numbers in vivo

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Introduction: Bioluminescence imaging (BLI) has become the method of choice for optical cell tracking in vivo. Stem cell-based therapies would benefit from the quantitative readout of cell viability combined with a maximized BLI sensitivity. The type of luciferase and the imaging protocol have not yet been optimized for the mouse brain. Therefore, we compared 4 different luciferases expressed by neural stem cells in vivo and evaluated the prerequisites to obtain BLI with high sensitivity in a transgenic mouse model for imaging neurogenesis - the DCX-Luc mouse.

Methods: The full length cDNA of the luciferin-dependent CBG99 (click beetle, λmax 540 nm), Luc2 (firefly, λmax 615 nm), PpYRE9 (firefly, λmax 630 nm) and the coelenterazine-dependent hRLuc (sea pansy, λmax 480 nm) were cloned into the vector pCDH-EF1-MCS-T2A-copGFP (Biocat, Heidelberg, Germany). Mouse neural stem cells (mNSCs) were transduced and FACS sorted for a homogenous copGFP signal. For in vivo experiments, different cell amounts were stereotactically implanted into the striatum of nude mice. Bioluminescence and emission spectra were acquired with the IVIS Spectrum (Caliper, Mainz, Germany) after i.p. injection of 150 mg/kg D-Luciferin or 1 mg/kg Rediject CTZ-h (Caliper). DCX-Luc mice (n=4) were systematically imaged for brain bioluminescence dependent on 1) substrate concentration (15, 150, 300, 750 mg/kg), 2) injection route (s.c., i.p., i.v.), 3) anaesthesia type (ketamine/xylazine, pentobarbital, isoflurane) and 4) time of injection (pre vs. post anaesthesia). Bioluminescence kinetic was recorded using the Photon Imager (Biospace Lab, Paris, France). The best protocol was tested with nude mice receiving mNSCLuc2 grafts of different amounts (each group n=3) and compared against the standard protocol.

Results: We systematically determined the performance of 4 different luciferases expressed by mNSCs. The emission spectra of Luc2 and PpyRE9 remained almost unchanged, while the emission spectrum of CBG99 became biphasic. Most importantly, luciferase performance decreased in the order of Luc2, CBG99, PpyRE9 to hRluc. With increasing substrate concentration, PEmax exponentially raised in the DCX-Luc mice without saturation effect, but with an increasing delay in time-to-peak. In contrast to literature reports, the PEmax was maximized with isoflurane anaesthesia instead of ketamine/xylazine or pentobarbital. In accordance to the physiological resorption, PEmax increased for s.c, i.p. to i.v. injections. Notably, the injection of substrate before isoflurane anaesthesia leads to approx. 1.5 times higher PEmax, most prominent for 300 mg/kg. This workflow resulted in a consistent 2 times higher PEmax, enabling the robust detection of 3,000 transplanted mNSCs in vivo.

Conclusion: This investigation provides the first complete quantitative comparison of different luciferases and protocol conditions in vivo. It results in a clear recommendation of Luc2 as the best luciferase selection in combination with a novel protocol (300 mg/kg D-Luciferin i.p. pre-isoflurane anaesthesia) for in vivo neuroimaging. The boosted bioluminescence is particularly favorable to assess the location, viability and proliferation of minimal stem cell numbers in neurological disease models.

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Keywords: neural stem cell; transplantation; bioluminescence; imaging
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Abstract 3

Towards vascularized tissue engineered bone for elderly patients

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Due to demographical changes, the need for innovative biomaterials to reconstruct bone defects in elderly patients is increasing. Age specific changes in bones (e.g. osteoporosis) are considered in this project, by manufacturing innovative beta tricalcium phosphate scaffolds which include different concentrations of strontium. Strontium is known to enhance osteoblastic function and to inhibit osteoclastic activity. Tailored 3D-scaffolds with defined pore design and pore size have been developed. The aim of this project is to reinforce the bioactivity of this biodegradable scaffold. For that, human mesenchymal stem cells (MSC) undergo osteogenic differentiation while co-cultered in a static way with human umbilical vein endothelial cells (HUVEC) on the scaffolds. In this co-culture, HUVEC should form vessels inside the pores. Finally, the whole seeding system should be optimized for cultivation in a bioreactor for continuous culture. Therein, mechanical stimuli of the fluid shear stress should further improve osteogenic differentiation of MSC. Cytotoxicity of scaffolds for L929 mouse fibroblasts and human MSC was analyzed according to EN ISO 10993-5 using a fluoresceindiacetate/propidium iodide live/dead staining. Proliferation of MSC and murine L929 was measured by CellTiter Blue viability assay on day 1, 3, and 7 after cell seeding. Osteogenic differentiation of MSC is routineously performed using osteogenic induction medium for 21 days. Osteogenic differentiation on the scaffolds is currently analyzed by real time PCR and alkaline phosphatase assay. For seeding HUVEC inside the scaffold pores we used the technique of dynamic seeding. Scanning electron microscopy is used to visualize HUVEC growing inside the pores. Co-culture of MSC and HUVEC is established in a 2D system on cell culture plastic and has now to be adopted to the three-dimensional scaffolds. Beta tricalcium phosphate scaffolds show no cytotoxic effect on L929 cells and on MSC. MSC are proliferating well on beta tricalcium phosphate scaffolds for at least 7 days. Currently the co-culture system of HUVEC and MSC is optimized. Osteogenic differentiation of MSC on beta tricalcium phosphate scaffolds will be performed with different MSC donors. Co-culture system of MSC and HUVEC will be optimized to perform osteogenic differentiation of MSC and parallel vascularization of HUVEC in a bioreactor.

Keywords: MSC; HUVEC; bone; vascularization; osteogenic differentiation

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Abstract 4

Biofabrication of stem cell-laden hydrogels for tissue engineering applications

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The potential of 3D bioprinting technology in regenerative medicine is enormous. In contrast to traditional, non-automated tissue engineering (TE) approaches, bio–printing allows for controlled spatial positioning of different cell types. Multilayered tissue constructs such as arteries, trachea or oesophagus are assumed to be more easily fabricated with this technique rather than with former TE strategies. Different combinations of hydrogels serving as matrices for cell printing and various printing techniques, such as inkjet and laser guided printing were attempted worldwide. The major drawback of past approaches was the relatively small size of the printed construct. Due to its weak mechanical properties, hydrogel constructs measuring more than a few millimetres in height tend to collapse. Here, we show a promising alternative for printing hydrogel-cell suspensions, submerged into a hydrophobic, inert, high-density supporting fluid. A 3D bioprinter specifically adapted to the needs of submerged printing was designed. The developed device allowed precise deposition of hydrogel droplets inside the hydrophobic, high density liquid. Two different drop-on-demand dispensing techniques, a needle based dispenser and a micro-valve based print head, were examined. Both methods were successfully applied in submerged printing of large hydrogel-cell constructs retaining high cell viability and functionality. Human mesenchymal stem cells were encapsulated into 1.5 % (w/v) agarose hydrogels and submerged printed afterwards. Hollow tubes and cylinders of stem cell-laden hydrogels with an outer diameter of approx. 8 mm as well as 5 mm, and a minimal wall thickness of approx. 650 µm measuring 4.8 mm in height were printed. Live/dead staining showed viable cells 24 hours and 21 days after the printing process. Histological examination after 14 and 21 days of in vitro culture revealed vital mesenchymal cells with marked matrix production and focal mitotic activity as a sign of proliferation. Our concept appears as a simple, reproducible, precise and "printing-supporting" approach allowing for freeform fabrication of polymeric 3D constructs. Our first results are encouraging for future applications in the field of regenerative medicine.

Keywords: biofabrication; hMSC; perfluorocarbon; tissue engineering

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Abstract 5

Culture bag systems for clinical applications of adult human neural crest stem cells

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Cellular therapies hold great promise for treatment of neurodegenerative diseases and replacement of complex tissue containing various cell types. In this regard, adult human neural crest-derived stem cells (NCSCs) feature a great potential as they exhibit a broad differentiation capability. They also offer the possibility to be transplanted autologously, avoiding the need of long-term immunosuppression in patients. We previously reported efficient 3D-cultivation of neural crest-derived inferior turbinate stem cells (ITSCs) using human blood plasma, allowing their autologous cultivation under clinical grade conditions. Considering such crucial in vitro expansion steps before transplantation, the Food and Drug Administration (FDA) recommends good tissue handling in a sterile environment with minimal contamination risk, practically limited to cost-intensive cleanrooms with airlocks needing intensively trained personnel. Addressing these challenges we describe here a novel method for cultivation of ITSCs within a closed culture bag system under cGMP-grade working conditions. In contrast to cleanrooms, application of the here presented culture bag system for ITSC-cultivation allows easy handling and cost reduction. ITSCs growing within culture bags showed no significant changes in morphology, proliferation rate and vitality compared to conventionally cultured cells. We demonstrated genetic stability and maintenance of stemness characteristics of bag-cultured ITSCs by unchanged DNA content as well as unaffected self-renewal capability and expression profile. ITSCs pre-cultured within culture bags differentiated efficiently into ectodermal and mesodermal cell types, including neuronal, osteogenic and adipogenic cells. Taken together the here described culture bag system is suitable for NCSC-cultivation under clinical grade conditions and has a great potential for future medical applications.

Keywords: human neural crest-derived stem cells; clinical-grade cultivation; culture bag systems

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Abstract 6

Induced pluripotent stem cells can be generated in bulk culture

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Induced pluripotent stem cells (iPSCs) are usually clonally derived. The selection of fully reprogrammed cells generally involves picking of individual colonies with morphology similar to embryonic stem cells (ESCs). However, the selection of suitable colonies is difficult to standardize as it is rather based on the experience of the operator than on objective criteria. Given that fully reprogrammed cells are highly proliferative and escape from cellular senescence, it is conceivable that they may outgrow non-pluripotent and partially reprogrammed cells during culture expansion without the need of clonal selection. Reprogramming of somatic cells is generally achieved by ectopic expression of defined transcription factors. Various methods have been described, including transfection with episomal plasmid vectors which enable the generation of integration-free iPSCs. In this study, we have reprogrammed human dermal fibroblasts with episomal plasmid vectors then we compared initial colony-formation upon pluripotency induction on different types of feeder cells (irradiated murine embryonic fibroblasts; MEFs, irradiated human dermal fibroblasts; HDFs, and irradiated human bone marrow derived mesenchymal stromal cells; MSCs). These colonies were either manually picked, or all colonies were harvested for subsequent expansion. Colony frequency was higher and size was larger when using MEFs as stromal support instead of HDFs or MSCs. We have then compared iPSCs which were either clonally derived by manual selection of a single colony, or derived from bulk-cultures of all initial colonies. After few passages their morphology and expression of pluripotency markers was investigated. Furthermore, gene expression profiles were analyzed using PluriTest which is regarded as a sensitive and highly specific, animal-free alternative to teratoma assays for assessing the pluripotency. All generated iPSC lines were indistinguishable from conventional clonally derived iPSC lines with regard to morphology and gene expression profiles. Clonally-derived and bulk-cultured iPSCs revealed similar in vitro differentiation potential towards the three germ layers. Therefore, manual selection of individual colonies does not appear to be necessary for the generation of iPSCs. Additionally, the use of bulk-cultured iPSCs provides new perspectives for automated iPSC applications and may facilitate better standardization than experimenter driven selection of suitable clones. In our StemCellFactory consortium we are aiming for optimized protocols to automate generation, culture expansion and differentiation of iPSCs for drug-screening and analysis of patient-specific iPSCs (www.stemcellfactory.de). Identification of suitable clones and colony picking are difficult to automate and are relatively costly and failure-prone. Therefore, this approach may provide an easier and less costly alternative as it does not require automated identification and picking of successfully reprogrammed colonies.

Keywords:

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Abstract 7

Interaction of iPS cells-derived cardiomyocytes with matrices of physiological stiffness

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Background: Induced pluripotent stem cell derived cardiomyocytes have the ability to actively sense their mechanical environment and respond to substrate stiffness by altering their adhesion, locomotion and morphology. To elucidate the impact of different stiffnesses ranging from embryonic myocardial tissue (6-10 kPa) to polystyrene (3-3.5 gPa) on iPS cell derived cardiomyocytes, we used the polyacrylamide hydrogel model. Since it allows cell attachment through the covalent binding of matrix proteins to the gel surface and at the same time possesses excellent optical properties, the gel matrix with its porous nature provides a more physiological environment than glass or plastic surfaces do. Methods: For this study PA gels (polyacrylamide gels) were used as substrate for iPS cell derived cardiomyocytes attachment. Three values of stiffness were selected (Samples A to C) to represent different stages of cardiac development in comparison to polystyrene elasticity. Elastic modulus of each selected stiffness was measured by AFM (Atomic Force Microscopy) using the Hertz model. To facilitate cell adhesion, a protein was cross-linked to the PA gel surface. For this, three cross-linkers were used, two commercially available and one which was synthetized and characterized using NRM (Nuclear Magnetic Resonance Spectroscopy). NMR spectra were recorded on a Bruker Avance III 500 spectrometer (11.7 T) at 298 K, utilizing a 5 mm BBOF probe with z-gradient coil. 1 H and 13 C{1 H} NMR spectra were recorded in pyridine-d 5 using TMS (Tetramethylsilane) as internal standard. Deuterated solvent from Merck, Uvasol®, was employed. Assignment was accomplished via a combination of 1 H, 13 C{1 H}-DEPTQ 1 , H,H COSY, H,C HMQC and H,C HMBC experiments. Results: AFM measurements were done using two different cantilevers with spring constant values of k = 42N/m, Cone: 18° and k = 1,1N/m, Cone: 20°. The elasticity properties of the gel were obtained by measuring different spots of each gel using a non-contact mode and plotting the force-displacement curves. Sample A had an average value of 12.06 kPa (k = 1,1N/m, Cone: 20°), Sample B an average of 49 kPa (k = 42N/m, Cone: 18°) and Sample C averaged in 165 kPa (k = 42N/m, Cone: 18°). After the stiffness characterization of the inert gel surface, fibronectin was coupled the polyacrylamide matrix by means of a cross-linker. This resulted in the displacement of the N-hydroxysuccinimide group from the cross-linker and the subsequent formation of a stable amide bond between the polyacrylamide matrix and fibronectin. Two commercially available crosslinkers (N-Succinimidyl Acrylate and Sulfo-Sanpah) were tested and compared to the synthetized cross-linker. NMR spectroscopy showed a purity of 99% for the synthetized Compound I (2,5-Dioxopyrrolidin-1-yl 6-acrylamide hexanoate) with the resulting 1% being water and ethanol. Our results indicate that the attachment of iPS cell derived cardiomyocytes was improved for the Compound I, with the possibility of long term culture (+6 weeks).

Keywords: iPS cell; cell attachment; stiffness; cardiomyocytes; NMR spectroscopy

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Abstract 8

Functional in vitro delivery of synthetic mRNA and recombinant protein by isolated and artificially loaded exosomes

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Exosomes are small, membrane-coated vesicles (70 - 140 nm diameter), that are secreted by a variety of cell types in vivo and in vitro. They originate from the invagination of the membrane of endocytic compartments, called multivesicular bodies (MVBs), and subsequent fusion of these MVBs with the plasma membrane of the cell. This leads to a release of the intraluminal vesicles into the extracellular space as exosomes. Because of this mechanism, exosomes contain an amount of cytosol from their parental cells in their lumen, which contains a variety of proteins, mRNAs and microRNAs. The internalized mRNAs, proteins and microRNAs can be transferred to other cells, thereby suggesting that exosomes play a role in cell-to-cell communication. Exosomes were found to be secreted by most eukaryotic cell types and can be purified from cell culture supernatants or biological fluids. Recently, it was shown that exosomes isolated from dendritic cells could be artificially loaded with siRNA by electroporation. The loaded exosomes were able to transfer their functional content to target cells in vitro and in vivo. Here, we present the functional delivery of synthetic mRNA and recombinant protein artificially loaded into exosomes. Exosomes were isolated from a CD63-EGFP-HEK293T cell line by ultra–centrifugation and loaded with synthetically produced mRNA encoding Cre-recombinase and Cre-protein by electroporation. Synthetic mRNA was generated by using in vitro transcription (IVT). To ensure efficient translation and increase half-life of the RNA in the cell as well as to minimize immune recognition of the synthetic mRNA a 5' guanine cap and modified ribonucleoside bases were included in the RNA, respectively. Recombinant protein was expressed in E.coli and purified by Ni(II) affinity chromatography. A variety of electroporation as well as buffer conditions were tested to load the exosomes with the synthetic mRNA or protein. The exosomes were then applied on a HEK293T Cre-reporter cell line in cell culture conditions. The generated Cre-reporter cell line is characterized by red fluorescence in the absence of Cre (encoded by DsRED), whereas it switches its fluorescence to green (encoded by EGFP) upon Cre-mediated recombination. Herewith, we could show that the Cre-mRNA and -protein were able to enter the target cells, and that the mRNA was efficiently translated and fully functional in the target cell line. With this, we provide proof-of-principle data for a new method to deliver functional mRNA and recombinant proteins into cells. In the future, this procedure could be expanded to inserting different types of mRNAs or proteins in cells, and by their ectopic presence a change in cell fate could be achieved.

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Abstract 9

Evaluation of dynamic aggregate-based suspension cultures for the expansion of human pluripotent stem cells

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Human pluripotent stem cells (hPSCs) provide a fascinating tool for conducting compound screening and pharmaceutical drug development in an authentic human cellular system and for generating donor cells for regenerative medicine. These biomedical prospects have created an urgent need for generating large quantities of stem cells under standardized conditions in scalable systems. Here we present a bioreactor-mediated (BioLevitator, Hamilton) protocol for the dynamic aggregate-based cultivation of hPSCs utilizing mTeSR medium. Our data show that human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) can be extensively expanded, yielding a maximum overall calculated fold increase of 4,79E+6 across 10 consecutive passages. The average cell yield per ml in the dynamic system was up to 3,5 times higher than in adherent control conditions initiated with same seeding densities. Irrespective of the final cultivation volume (up to 45 ml), a cell yield around 9,00E+5 cells / ml was reached consistently after the first passage. HPSCs maintained their proliferation capacity, pluripotency-associated marker expression and differentiation potential after expansion in the BioLevitator. Recently we further established a bioreactor-based protocol for the expansion of hPSCs utilizing a commercially available GMP-compliant medium. This system yielded an overall calculated fold increase of 8,95E+7 after 10 passages, emphasizing the suitability of such a process for the expansion of clinical grade hPSCs. We expect this technology to facilitate the standardized and automated scale-up of hPSCs and their derivatives for further downstream applications including potential cell replacement therapies.

Keywords: human pluripotent stem cells; suspension cultures; bioreactor

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Abstract 10

Matrix elasticity has only short-term effects on mesenchymal stromal cell function

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Mesenchymal stromal cells (MSCs) raise high hopes for tissue engineering. It has been shown that matrix elasticity supports differentiation towards specific lineages. The question arises if these functional changes require immediate contact to elastic matrices or if they are long-lasting – potentially even epigenetically controlled – and hence maintained upon harvesting of MSCs for use in cellular therapy. In this study, we used elastic polydimethylsiloxane (PDMS) substrates (1 kPa; 7.7 kPa, 15 kPa; 50 kPa and 100 kPa) in comparison to tissue culture plastic (TCP) for culture of adipose tissue-derived MSCs over serial passages until they reached a senescent state. We analyzed the fibroblastoid colony-forming unit (CFU-f) frequency, the proliferation potential and the in vitro differentiation capacity towards adipogenic- and osteogenic lineages. Furthermore, DNA-methylation (DNAm) profiles of MSCs cultivated on 1 kPa, 50 kPa and TCP were compared using the InfiniumHumanMethylation450 BeadChip. The fibroblastoid colony forming unit (CFU-F) frequency was particularly impaired on substrates with intermediate stiffness (15 kPa) whereas the proliferation rates of MSCs increased continuously with increasing stiffness. Although late-passage cells did not show the typical fried-egg morphology of senescent cells on soft substrates, we observed no significant differences in the maximal number of cumulative population doublings (cPD) on elastic substrates. Osteogenic differentiation was clearly increased with increasing stiffness, whereas adipogenic differentiation was promoted by soft substrates. However, when MSCs were isolated and culture expanded on soft matrices and then reseeded to TCP, they revealed the same differentiation potential as those which were continuously cultured on TCP indicating that there is no cellular memory for the matrix-effect on differentiation. Furthermore, we analyzed DNAm profiles of MSCs which were continuously cultured on 1 kPa, 50 kPa and TCP. Notably, we did not observe any significant DNAm changes evoked by matrix elasticity. Taken together, we demonstrated that the differentiation potential of MSCs differs considerably on elastic substrates although there are no significant effects on DNAm profiles. This indicates that MSC-priming by matrix elasticity does not have long-lasting effects upon harvesting for cellular therapy.

Keywords: mesenchymal stromal cells; matrix elasticity; DNA-methylation; replicative senescence; epigenetics

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Abstract 11

Synthetic mRNAs as a tool for manipulation of human stem cells

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Human stem cells represent both, promising cellular models for early developmental studies and a virtually unlimited cell source for therapeutic applications. Deep understanding and efficient targeting of differentiation processes requires modulation of transcription factor activities, which is usually performed by genetic manipulation. Conventional genetic manipulation is poorly achievable in stem cells and furthermore involves permanent modification making clinical applications difficult. We address this problem by the use of synthetic mRNAs. Synthetic mRNAs are a recently described tool to manipulate cells. They bare a low risk for mutagenesis and can be transfected into various cell lines including stem cells as well as somatic cells. This study aims at investigating whether human pluripotent stem cells (hPSC) and long-term self renewing neuroepithelial-like stem cells (lt-NES) can be transfected efficiently with synthetic mRNAs. mRNA synthesis usually requires modified nucleotides for reducing toxicity and increasing the stability in transfected cells. Notably, we show that human stem cells can be manipulated by unmodified synthetic mRNAs presumably due to a lack of cell-autonomous immunity. This enables a strong reduction of costs of mRNA synthesis. Moreover, our data suggests that these naïve mRNAs are translated with a higher efficiency. We could show that hPSC can be transfected with synthetic eGFP-mRNA with an efficiency up to 70%. Further we plan to investigate whether functional synthetic mRNAs of transcription activator-like effector nucleases (TALENs) can be generated. These nucleases could be a promising tool for targeted gene editing in human stem cells. Another application to verify functionality of synthetic mRNAs will be the directed differentiation of lt-NES into various neuronal subtypes with neurogenic factors.

Keywords: synthetic mRNA; human pluripotent stem cells; human lt-NES cells

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Abstract 12

Osteoblastic differentiation of human stem cells by topographic features in the nano-scale range

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Recent studies [1; 2] showed that osteogenic differentiation of stem/progenitor cells can be induced without any osteogenic cues, by nanostructured titanium layers only. Nevertheless mechanisms of stem cells differentiation into an osteogenic lineage, dependent on topographically nanostructured surfaces are not yet understood completely. Hence the aim of this study is to elucidate the cellular mechanisms leading to the differentiation of stem cells into osteoblastic lineage and how they are triggered by topographic features in the nano scale range. Here we used a simple model consisting of titanium coated nano-pores. For this means polycarbonate membranes with 30nm as well as 100nm nano-pores in diameter were covered with a 5nm titanium layer. Recently we characterized stem cells from the human nose as neural crest derived cells. Since neural crest derived cells generate the human skull, we tested human nose stem cells on topographically structured titanium layers. Titanium was chosen due to its biocompatibility and its widespread use in orthopedic surgery. Here we observed that after 21 days of culture in a medium containing no osteogenic cues formation of calcium deposits were visible. Using high resolution scanning electron microscopy we observed filopodia. They were able to penetrate through the 100nm holes while they tethered to the 30nm holes with membrane protrusions having a diameter between 10nm and 20nm. We conclude that this interaction might transmit osteogenic signaling. Future studies will examine the signal transduction involved in this process.

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Keywords: neural crest derived stem cells; osteogenesis; differentiation; nanostructures

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Abstract 13

‘Programmable cells of monocytic origin’ for bone tissue engineering strategies

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Critically sized bone defects are still a challenge for traumatologists in their daily clinical routine. Mesenchymal stem cells (MSC) seeded on biomaterials are regularly used for bone-healing setups but they are difficult to isolate in high amounts for autologous cell transplantations. Programmable cells of monocytic origin (PCMO) which are derived from peripheral blood mononuclear cells after minimal invasive intervention and are dedifferentiated to a multipotent cell type (Ruhnke 2005) might provide a promising alternative for bone-tissue engineering strategies. Since pluripotent cells as well as monocytic cells were shown to have regenerative capacities (Gurtner 2007, Nicolaidou 2012) we are investigating the ability of PCMOs to contribute to bone repair in vitro and in vivo. Mononuclear cells were dedifferentiated for 6 days with IL-3 and MCS-F and FACS analysis of monocytic surface protein CD14 revealed a reduction of monocytic characteristics as described previously (Ungefroren 2010). Firstly we tried to differentiate PCMOs into osteoblast-like cells with a vitamin D3 containing differentiation medium and tested the expression of osteogenic marker genes as runx2, osteocalcin and collagen 1A by quantitative real-time-PCR (qRT-PCR). To check if PCMOs accelerate bone formation in vitro we isolated MSCs from trabecular bone of people after total hip replacement and seeded them in 1:1 co-cultures with PCMOs under osteogenic differentiation conditions. The enzyme activity and relative expression of alkaline phosphatase (ALP) a known marker for early osteogenesis was tested photometrically or with qRT-PCR, respectively. Osteogenic matrix production of mono- or co-cultured cells was tested with alizarin red which stains calcified depositions and can be solubilized for quantification. To study the regenerative capacity of PCMOs in vivo we are establishing a critically sized femur defect model in rats where defects are treated with cell-seeded 3x5 mm cylindrical polyurethane scaffolds. A pico-green based DNA-quantification procedure and histologically stained sections of cell-seeded scaffolds were performed and determined an amount of 1.5 million cells that have to be seeded previous to operation. Although the significant reduction of CD14 on PCMOs confirmed their monocytic dedifferentiation the following osteogenic differentiation failed. This was assumed as the expression of runx2, osteocalcin and collagen 1A stayed low when relatively compared to osteogenic differentiated MSCs. However, PCMO/MSC-co-cultures revealed a significant increase in alkaline phosphatase (ALP) enzyme activity in the first week compared to mono-cultures, but only when the osteogenic differentiation medium was applied. Similar results were shown in qRT-PCR experiments of ALP expression under the same conditions. Nevertheless, since our method to quantify matrix calcification with alizarin could not yield significant results so far the ability of PCMOs to improve the osteogenic differentiation of MSCs still remains questionable. Microcomputed tomography analysis and histological staining of the callus region from the critically sized femur defect model will provide an answer to the question if PCMOs can be confirmed as an alternative regenerative cell for bone tissue engineering strategies in humans.

Keywords: programmable-cells-of-monocytic-origin; co-culture; osteogenesis; tissue engineering

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Chromatin & Epigenetics

Abstract 14

Prevention of epigenetic transgene silencing in murine and human pluripotent stem cells utilizing ubiquitous chromatin opening elements (UCOEs)

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Introduction: Pluripotent stem cells (PSC) represent a promising target population for gene therapy approaches. However, epigenetic transgene silencing, especially during the differentiation of PSCs, constitutes a major obstacle for this strategy. One way to overcome this problem is the use of ubiquitous chromatin opening elements (UCOE). We recently demonstrated that the defined 1.5 kb A2UCOE derived from the human heterogenous nuclear riboprotein A2/B1/ chromobox homolog 3 (HNRPA2B1/CBX3)- locus used in combination with the truncated elongation factor-1 alpha (EFS) promoter effectively prevents CpG-methylation-associated silencing of lentiviral transgene expression during hematopoietic differentiation of murine (m)PSC. Based on these results we here investigate the transgene promoting effect of the A2UCOE (i) in combination with other (viral) promoters, (ii) during differentiation into cells of other germlayers such as hepatic (endoderm) and neuronal (ectoderm) cells, as well as (iii) in human (h)PSCs and their differentiated progeny.

Methods/Results: (i) Also when combined with the spleen focus forming virus (SFFV) promoter the A2UCOE effectively stabilized transgene expression in murine iPSCs (~80% vs. ~3% dTomato+ in passage (p)3) as well as thereof differentiated CD41+ hematopoietic progenitor cells (~80% vs. 1-3% dTomato+ cells). Similar to the EFS promoter, bisulfit sequencing revealed protection of the SFFV promoter from CpG-methylation to be associated with this effect. (ii) To analyse transgene stabilization by the A2UCOE during hepatic and neurogenic differentiation the physiological EFS promoter was utilized. Similar to the effect observed during hematopoietic differentiation, the A2UCOE effectively reduced silencing of the EFS-promoter and allowed for sustained transgene expression in ~97% of hepatically (EFS-driven controls: ~3%) and ~65% of neuronally (EFS-driven controls: ~3%) differentiated cells, respectively. (iii) When we investigated transgene expression in human PSCs profound silencing of EFS-driven transgene expression was observed in undifferentiated H9 hESCs. This was prevented by the A2UCOE (~23% vs. 83% dTomato+ cells in p6, respectively). Again, the effect was associated with reduced EFS promoter methlyation in A2UCOE transduced cells (~33% vs. 90% methylated CpGs). Similar efficiency of the A2UCOE was noted in pluripotent hES3 ESCs and in hiPSCs derived from CD34+ hematopoietic cells. Furthermore, analysis of hES3 cells during non-directed differentiation revealed sustained, A2UCOE-mediated transgene expression in ~80% of Tra-1-60 negative progeny cells harvested on day 13 (EFS-driven controls ~10%). Even more important, similar effects were observed during the directed differentiation of hPSCs into cardiomyocytes or monocytes/macrophages.

Discussion: We here prove efficacy of the A2UCOE in murine PSC when combined with viral and physiological promoter elements as well as during differentiation into cells of all three germlayers. In addition we demonstrate the A2UCOE to stabilize transgene expression in human PSC in the undifferentiated status and during mesodermal differentiation. Thus our data introduce UCOEs as a generalized concept to stabilize transgene expression during the generation of PSC-derived transgenic cell therapy products.

Keywords: gene therapy; pluripotent stem cells; differntiation; epigenetic silencing; UCOE

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Abstract 15

RNF12 is essential for x-inactivation in female mouse embryonic stem cells and is required for female mouse development

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X-chromosome inactivation (XCI) in placental mammals is a mechanism which equalizes dosage of X-linked genes between sexes. This crucial process for female development is first initiated around the 2-4 cell stage in the pre-implantation embryo in an imprinted way, in which the paternal X-chromosome is inactivated. In the inner cell mass (ICM) of the developing blastocyst, the inactivated X-chromosome is reactivated, allowing a short window in time in which a double dosage of X-linked gene products is tolerated. Upon further development, random XCI is initiated in the post-implantation embryo, thereby coupling initiation of XCI to differentiation. Female mouse embryonic stem (ES) cells, derived from the ICM, are characterized by two active X-chromosomes, and undergo XCI upon differentiation, making them a unique in vitro model. Initiation of chromosome wide silencing of the X-chromosome is regulated by the up-regulation of the non-coding RNA Xist. In undifferentiated cells, the core pluripotency factor network indirectly suppress Xist up-regulation. We have previously shown that Xist up-regulation is triggered by a stochastic mechanism, in which the X-linked gene Rnf12 acts as an X-linked activator of XCI, allowing XCI initiation only in females. The encoded RNF12 is an E3 ubiquitin protein ligase, which activates Xist by targeting the pluripotency factor REX1 for proteasomal degradation. RNF12 over expression results in ectopic XCI, and homozygous Rnf12-/- ES cells fail to undergo XCI upon differentiation, showing that RNF12 is a key factor in XCI initiation. Here we further decipher the XCI initiation mechanism, by showing that RNF12 is continuously required for maintaining XCI. To address the role of X-chromosome pairing in XCI initiation, we generated ES cell lines harbouring deletions of all known X-pairing elements. Surprisingly, these cells show normal XCI kinetics upon differentiation. In addition, in experimental XX-XY heterokaryons, XCI initiation is found in the male nucleus upon differentiation with normal kinetics, showing that X-pairing is indeed not functional required for XCI in ES cells. By creating a variety of knockout alleles of other genes located in the vicinity of Xist, we provide evidence that the non-coding RNAs Jpx, Ftx and the Xpr region are important for the cis-regulation of the Xist gene, but cannot act as trans-activating factors, in contrast to RNF12. The generation of an Rnf12 knockout mouse model confirms that RNF12 is crucial for XCI initiation in vivo. Whereas male Rnf12-/Y are viable, female Rnf12-/- mice fail to undergo XCI leading to lethality in the post-implantation embryo. Rnf12-/+ animals inheriting the maternal knockout allele are lethal due to silencing of the paternal Rnf12 allele upon imprinted XCI. Rnf12+/- females inheriting the paternal knockout allele are viable but surprisingly show an XCI defect, with cells which have failed to undergo XCI properly. These animals for the first time show that it is possible to live with cells with failure of dosage compensation in various tissues, thereby challenging a more than 50 year old dogma. This has important implications for understanding and treating X-linked diseases in the future, as it might become possible to reactivate a silenced X-chromosome.

Keywords: X chromosome inactivation; xist; rnf12; ES cells; female mouse development

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Abstract 16

Histone acetylation impacts on dendritc cell lineage commitment and differentiation

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Hematopoietic stem cells (HSC) develop into all cells found in blood and lymphoid tissues. Dendritic cells (DC) are professional antigen presenting cells and thus crucial for immunity and tolerance induction. DC comprise various subsets, such as conventional DC (cDC) and plasmacytoid DC (pDC). DC derive from HSC through consecutive steps of lineage commitment and differentiation. HSC first develop into multipotent progenitors (MPP). MPP subsequently undergo DC lineage commitment by progressing into common dendritic progenitors (CDP). CDP eventually differentiate into cDC and pDC. With accumulating knowledge of epigenetic modifications, their function as regulators of transcription is becoming increasingly clear. One such epigenetic modification is histone acetylation, which is generally associated with active gene expression. In this study, we investigated whether histone acetylation impacts on DC lineage commitment and DC differentiation by using the histone deacetylase inhibitor trichostatin A (TSA). We observed that TSA had a major impact on the DC lineage commitment step as it reduced the propensity of MPP to transit into CDP, indicating that TSA keeps progenitor cells in a more primitive state. Additionally, TSA inhibited the differentiation of CDP into cDC and pDC and instead promoted cell proliferation. To understand how TSA affects DC differentiation, we performed gene expression profiling on a set of genes, which are known to promote DC development, such as Flt3, PU.1, E2-2, IRF-8 and Id2. We found that TSA inhibited the upregulation of these DC-related factors during DC differentiation. Conversely, TSA treatment maintained expression of MPP-related genes, like Gfi1, which are normally downregulated during DC differentiation. Thus, an altered gene expression explains the notion that TSA treatment keeps the cells in a more primitive state. Importantly, pulse-chase experiments showed that the inhibitory effect of TSA on DC differentiation was reversible on the cellular and molecular level. Altogether, we show that histone acetylation impacts on DC lineage commitment from MPP to CDP and on differentiation of CDP into cDC and pDC, indicating that the acetylation status underlies the cell's choice between differentiation and stemness.

Keywords: hematopoietic stem cells; epigenetics; lineage commitment; dendritic cells; histone acetylation

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Abstract 17

Analysis of protein-protein interactions using BAC-transgenic human pluripotent stem cell-derived neural stem cells and their neuronal progeny

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While contemporary approaches in cell-based disease modeling have been focusing mostly on the effects of defined mutations on the cellular phenotype, the assessment of underlying alterations in the interactomes of disease-relevant proteins has faced several technical challenges. First, experiments were typically conducted using overexpression paradigms resulting in unphysiologically high protein levels and thus unspecific interactions. Second, such studies have been relying mostly on transformed cell lines, which enable mass production of cells but do not represent a tissue-specific proteomic environment. We aimed at addressing these issues by BAC-based expression of tagged proteins in pluripotent stem cell-derived long-term self-renewing neuroepithelial stem cells (lt-NES cells), a stable and robust cell population, which generates authentic human neurons with high fidelity. Tagged proteins were found to be expressed at endogenous levels, and FISH analyses revealed an average integration rate of one copy per genome. Correct size and compartmentalization of the tagged proteins could be confirmed by high-resolution live cell imaging and Western blot analysis. Employing this approach, we generated multiple cell lines, which harbor tagged proteins exhibiting altered expression in human developmental disorders, cancer and neurodegeneration. including PCNA, AURKA, CDK2AP1, RUVBL2, the Methyl CpG Binding Protein 2 (MECP2) and the Alzheimer's disease-associated proteins Nicastrin (NCSTN) and Valosin-containing protein (VCP). Using a label-free, quantitative affinity purification-mass spectrometry approach, we identified numerous novel interaction partners of these proteins. Direct comparison of the interactomes of proliferating lt-NES cells and their neuronal progeny further revealed changes in the composition of several chromatin remodeling complexes, suggesting that our system suffices to identify developmental switches in such complexes.

Keywords: protein-protein interaction; protein complexes; differentiation; neural stem cells; chromatin remodeling

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Abstract 18

Functional analysis of histone demethylases during hematopoietic development

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The discovery of the first histone demethylase in 2004 (LSD1/KDM1) opened new avenues for the understanding of how histone methylation impacts cellular functions. A great number of histone demethylases have been identified since, which are potentially linked to gene regulation as well as to stem cell self-renewal and differentiation. KDM6A/UTX and KDM6B/JMJD3 are both H3K27me3/2 specific histone demethylases, which are known to play a central role in regulation of posterior development, by regulating HOX gene expression. So far nothing is known about the role of histone lysine demethylases (KDMs) during early hematopoiesis. We are studying the role of KDM6A and KDM6B on self-renewal, global gene expression and on local and global chromatin states in ESCs and during hematopoietic differentiation. To this end we employ the shRNA Mission Vector system (Sigma-Aldrich) for targeted knock down of KDM6A and KDM6B. Additionally we analyze KDM6A knockout ESCs. Our results so far show that the knockdown of KDM6A or KDM6B has no influence on ESC proliferation. KDM6B ESCs showed normal proliferation and EB formation while in vitro hemangioblast differentiation was reduced. Similar results were obtained with KDM6A KD and KO ESCs. Knockdown of KDM6B in KDM6A knockout cells further decreased hemangioblast differentiation. In order to completely abrogate KDM6 demethylase activity in ESCs we employed a specific inhibitor (GSK-J4, Kruidenier et al. 2012). Treatment of ESCs with GSK-J4 had no effect on viability and proliferation. However, ESC differentiation in the presence of GSK-J4 was completely abrogated. In conclusion our results show that both KDM6A and KDM6B are involved in early ESC hematopoiesis. Further we show that ESC differentiation is completely blocked in the absence of any H3K27 demethylase activity.

Keywords: KDM6A; KDM6B; ESC; hematopoisis

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Abstract 19

DNA methylation somatic memory shared among different human fibroblasts reshapes the distal/promoter regulation balance in induced pluripotent stem cells

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Many studies have focused on the search for the remaining fingerprint of somatic memory of the induced pluripotent stem (iPS) cells reprogrammed by transcription factors. Such studies have been performed based on high throughput data analysis of transcriptomics or methylomics data, using microarrays or next generation sequencing (NGS) technologies. The conclusions of these studies are controversial. The transcriptomics based studies from somatic, iPS and embryonic stem (ES) cells usually identify very few somatic memory, and several concerns exist on whether the detected remaining fingerprint of somatic origin at transcriptomics level is due to poor quality of iPS cells. This poor quality can be confused between several sources, i.e., due to partially reprogrammed cells or due to the remaining memory at low cell passages of well reprogrammed cells. Several works point out that an enough number of cells passages erase the transcriptomics fingerprint of the original somatic cells. However, on methylomics level, there are studies disclosing differences between iPS and ES cells, thus, indicating that some fingerprints of such memory is retained during the reprogramming process. Such studies however do not take into consideration the methylomics state of the original somatic population. We have developed algorithms for searching differentially methylated sites from NGS data, and applied them not only for comparing methylomics data from iPS and ES cells as previous studies do, but we have also integrated in the analysis the methylomics data from the original somatic cell lines from which reprogrammed cells were derived. Using human iPS cells reprogrammed from several fibroblast lines (adipose-derived stem cells, IMR90 lung fibroblasts and foreskin fibroblasts), we have found more than 30000 sites identified as somatic memory loci shared among the DNA methylomes profiles of all the iPS cell lines. Surprisingly, more than 70% of these differently methylated sites are in the distal regulation regions and the leftovers are in promoters.

Keywords: somatic memory; next generation sequencing; DNA methylome; induced pluripotent stem cells; distal/promoter regulation

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Abstract 20

DNA-methylation changes upon in vitro expansion of hematopoietic stem and progenitor cells

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Hematopoietic stem and progenitor cells (HPCs) can be maintained in vitro, but the vast majority of their progeny loses stem cell properties upon culture expansion. In this study, we analyzed DNA-methylation (DNAm) profiles of either freshly isolated or expanded CD34+ cells cultured with or without mesenchymal stromal cells (MSCs). DNAm profiles of the faster proliferating progeny – which downregulates CD34 expression - reflect hematopoietic differentiation such as hypermethylation of CD34 and CD133. Culture expansion of CD34+ cells either with or without stromal support has relatively little impact on DNA-methylation profiles although MSCs have been shown to mimic components of the hematopoietic niche and greatly increase the proliferation of HPCs. Notably, all cultured HPCs - even those which remained CD34+ - acquired extensive DNA-hypermethylation within seven days of in vitro culture, particularly in shore-regions of CpG islands, up-stream promoter regions and binding sites for PU.1, HOXA5 and RUNX1, which are essential transcription factors for hematopoietic development. The vast majority of these DNAm changes were not related to senescence-associated DNAm changes but they were rather located in relevant developmental genes. Furthermore, DNAm changes were associated with differential expression of hematopoietic genes and aberrant splicing of DNMT3A. Low concentrations of demethylating agents (zebularine or epigallocatechin-3-O-gallate) slightly increased the frequency of colony-forming unit initiating cells indicating expansion of progenitor cells by inhibiting DNA-methylation. Taken together, our results demonstrate that culture expanded HPCs - even those which maintain a primitive immunophenotype - acquire significant DNA-methylation changes. These epigenetic modifications may reflect the inefficient self-renewal of HPCs upon in vitro culture. Therefore, control of epigenetic modifications during culture expansion may prevent loss of stemness.

Keywords: hematopoietic stem cells; expansion; epigenetic; DNA-methylation

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Development and Differentiation

Abstract 21

bHLH protein ATOH8 is involved in the adult myogenesis transcriptional network

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ATOH8 is a member of the Atonal family of basic Helix-Loop-Helix transcription factors. Although the protein is well characterized in relation to neurogenesis, its involvement in other developmental contexts is not well understood. Previous work in our laboratory has shown that ATOH8 is expressed in the somite of chicken embryos and downregulation of ATOH8 in the lateral somite at the trunk level results in a blockage of differentiation and causes cells to be maintained in a predetermined precursor state. In the present study, we show the expression profile of ATOH8 during adult myogenesis. The satellite cells in vitro dynamically express ATOH8 while the cells undergo the process of differentiation, as evidenced by the co-expression of the protein together with Pax7 and Myogenin. We also report the re-appearance of the ATOH8 expression in skeletal muscle undergoing regeneration, which is induced in vivo, and also in the normal regeneration process during myopathy, which all together point towards an important role for ATOH8 in the regeneration process. Furthermore, ATOH8 is also expressed in cultured C2C12 mouse myoblasts and dramatically decreases in differentiating myoblasts. Hence we propose a role for ATOH8 during the transition of myoblasts from the proliferation to the differentiation phase. We also demonstrate that ATOH8 expression in the somite is regulated by Notch signaling. In conclusion, we speculate that ATOH8 is a bHLH protein, which could be required to fine regulate the balance between skeletal myogenesis and self-renewal of satellite cells and of the myogenic progenitors during embryonic myogenesis.

Keywords: myogenesis; skeletal muscle regeneration; myopathy; satellite cells; bHLH

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Abstract 22

Immunological properties of murine pluripotent stem cell-derived cardiomyocytes in vitro

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Cardiomyocytes (CM) derived from pluripotent stem cells (PSCs) represent an attractive cell source for applications in regenerative medicine. However, for successful clinical translation of PSC-based therapeutic approaches it is important that cells functionally engraft into the recipient tissue without inducing the host immune response and rejection. Despite a number of reports on immunogenicity of undifferentiated PSC and their derived teratomas, very little is known about immunological properties of pure populations of specific types of PSC-derivatives. We previously reported that despite expression of very low levels of major histocompatibility complex (MHC) class I molecules, murine embryonic stem cells-derived cardiomyocytes (ESC-CM) were neither recognized nor lysed by activated syngeneic NK cells in vitro (Frenzel et al., Stem Cells 2009). The aim of this study was to analyze the interaction of PSC-derived CMs with cytotoxic T cells in vitro and to assess their immunogenicity in vivo. To gain highly purified CM populations, we used a murine ESC line, aPIG, stably expressing a green fluorescence protein (GFP) and puromycin resistance under the control of the promoter of the cardiac gene alpha-myosin heavy chain. In addition, we have generated a double transgenic derivative, the aPIG-OVA cell line, in which chicken egg ovalbumin (OVA) is ubiquitously expressed under the control of the cytomegalovirus promoter. B3Z T cell hybridoma assay revealed that OVA is proteolitically processed in undifferentiated ES cells and purified CMs to yield the antigenic peptide SIINFEKL presented on the cell surface in the complex with H-2Kb MHC class I molecules. Cytotoxicity analyses revealed that cytotoxic allogeneic CD8-positive T cells were not capable of lysing purified wild-type ES-CMs in vitro. In addition, purified OVA-expressing ES-CMs were also not lysed by syngeneic cytotoxic CD8-positive T cells isolated from OVA-immunized OT-I mice that exclusively express T cell receptors (TCR) specific for the SIINFEKL/H-2Kb complexes. After strong upregulation of MHC class I molecules on CMs by interferon gamma (IFNg) treatment, only weak susceptibility of aPIG-OVA-CM towards activated OT-I T cells as compared to control OVA-expressing fibroblasts was observed. Interestingly, when CMs were externally loaded with the SIINFEKL peptide, the OT-I T cell killing was similar to that observed with positive control fibroblasts. The same effect was also observed with the PSCs. This finding indicates the important role of antigen density on the cell surface for efficient T cell mediated killing of target cells. To determine whether the serine protease inhibitor serpine-6 (SPI6) protects CM from T cell killing under conditions of low antigen density, SPI6-specific siRNA was used to downregulate this granzyme B inhibitor in IFNg-untreated CMs. This treatment did not lead to increased susceptibility of CMs towards lysis by T cells, suggesting that under this condition SPI6 does not act as protective molecule. These data indicate that transplanted ES cell-derived CMs may be less susceptible to rejection than reported for ESC-derived teratomas containing mixed populations of cells. Further studies are being performed to elucidate the mechanism of protection, to assess the engraftment capacity and to determine the immunogenicity of ESC-CMs in vivo.

Keywords: cardiomyocytes; murine; embryonic stem cells; immunology

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Abstract 23

Generation and maintenance of neural progenitor cells from human pluripotent stem cells using the STEMdiff™ neural induction system with aggregate or monolayer culture based methods

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Neural progenitor cells (NPCs) generated from human pluripotent stem cells (hPSC), including human embryonic stem (hES) cells and induced pluripotent stem (hiPS) cells, are extensively used for studying human central and peripheral nervous system development, modeling neurological disorders, and screening for therapeutic molecules. We have previously reported that central nervous system (CNS)-specific NPCs can be produced efficiently in our STEMdiff™ Neural Induction Medium (NIM) from multiple human ES and iPS lines when used in conjunction with AggreWell™800. In this system, aggregates are first formed in AggreWell, and then plated onto adherent culture where morphologically distinct neural rosettes form, indicative of neural induction. Recently, several studies have shown that neural induction from hPSCs can also be achieved in a monolayer-based culture system, which is simpler in that it does not require aggregate formation and multi-step protocols. The study presented here has two goals: (1) to use STEMdiff™ NIM in a monolayer-based neural induction system, and (2) to test a new serum-free and defined medium to support expansion of NPCs generated with STEMdiff™ NIM from either the aggregate or monolayer-based cultures. For the first goal, hPSCs previously maintained in mTeSR1™ were seeded at 250,000 cells/cm2 onto poly-L-ornithine and laminin (PLO/L)-coated plates, and cultured in STEMdiff™ NIM for up to 10 days. Cells were assessed at different time points for neural induction by the emergence of PAX6 expression with concomitant downregulation of OCT4. In the hES cell lines (H1 and H9) tested, approximately 25 - 30% of the cells were PAX6+OCT4- by day 3 and all cells were PAX6+OCT4- by day 6 (n = 5). In the hiPS cell lines (WLS-4D1, WLS-1C, and A13700) tested, neural induction was slightly slower, with just 30 - 40% of cells PAX6+OCT4- on day 5 but complete induction to PAX6+OCT4- cells by day 9 (n = 4). These data show that CNS-specific NPCs can be efficiently produced in a single-step from hPSC using STEMdiff™ NIM with the monolayer-based protocol. Toward the second goal, we generated NPCs using STEMdiff™ NIM and the standard AggreWell™800-based system described previously or monolayer-based protocol above. These NPCs were then dissociated into a single cell suspension, re-plated at 100,000 cells/cm^2 onto PLO/L plates containing our optimized STEMdiff™ Neural Progenitor Medium (NPM), and cultured for 7 days. Cells were passaged every 7 days for multiple passages and cell expansion was calculated at each passage. Cultures were also processed separately for immunostaining to evaluate expression of CNS-specific NPC markers such as PAX6, SOX1, ZO-1, and NESTIN. Overall NPCs propagated in STEMdiff™ NPM exhibited 3 5 fold expansion (n = 8) over the first 3 passages, with low spontaneous neuronal differentiation (< 10 %). These NPCs could be maintained for more than 10 passages and importantly, they expressed CNS-type NPC markers (PAX6, SOX1) and not neural crest cell marker such as SOX10. Thus, with the incorporation of our new STEMdiff™ NPM, we now have an integrated system, which is flexible for aggregate and monolayer based culture protocols for the generation and maintenance of hPSC-derived NPCs.

Keywords: neural; induction; differentiation; human; pluripotent

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Abstract 24

Monitoring differentiation of pluripotent stem cells to cardiomyocytes by measuring cardiac Troponin T release into cell culture medium

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Cardiovascular diseases are the major cause of death in the world. Since endogenous self-healing after myocardial damage is limited novel regenerative medicine approaches based on cellular replacement therapy with cardiomyocytes derived from embryonic stem (ES) and induced pluripotent stem (iPS) cells provide new perspectives for patient- and disease-specific therapies and drug testing. In order to achieve reliable and reproducible cardiomyocyte production, suitable strategies for monitoring the kinetic and the efficiency of stem cell differentiation bioprocess are mandatory. In this study we detected by ELISA rapid cardiac Troponin T (cTnT) release into the medium supernatant from purified viable cardiomyocytes derived from transgenic murine iPS cell line in which the expression of enhanced green fluorescence protein (eGFP) and puromycin N-acetyl-transferase is controlled by the cardiomyocytes-specific promoter of alpha-myosin heavy chain gene (MYH6p). Robust cTnT release was also detected in the medium supernatant of puromycin selected cardiomyocytes derived from a corresponding transgenic murine ES cell line and in medium of native cardiomyocytes isolated from murine neonatal hearts, as well as in cardially differentiating cultures of unselected murine germline-derived pluripotent stem (gPS) cells and human iPS cells. In spontaneously differentiating cultures of murine iPS and ES cells cTnT was detectable in the medium concomitant with the first appearance of GFP-positive cardiomyocytes in embryoid bodies and the cTnT concentrations in these cultures correlated well with the proportion of GFP-positive cardiac muscle cells in the total cell population. Moreover, the enhancement of cardiac differentiation efficiency in murine ES cell cultures by ascorbic acid treatment was accompanied by higher cTnT concentrations in the medium as compared to non-treated cultures, indicating good correlation between cTnT medium content and cardiomyocyte yield in embryoid bodies. Measurements of lactate dehydrogenase (LDH) activity in the medium and of caspase activation in purified cardiomyocytes revealed that cTnT was not released from apoptotic or necrotic cells. Rather, cTnT appeared to be secreted as a full length protein as revealed by immunoprecipitation with cTnT-specific antibody. Collectively, these observations provide a potential means for monitoring cardiomyocyte production in the course of bioprocess optimization or screening of novel cardioinductive substances that is highly specific, simple and broadly applicable to any cell line without requirements for a specific genetic cell modification. The biology and mechanism behind the cTnT secretion by viable cardiomyocytes remain to be elucidated.

Keywords: pluripotent stem cells; cardiomyocytes; differentiation; monitoring; Troponin T

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Abstract 24a

pH influences cardiac differentiation of murine induced pluripotent stem cells in controlled stirred tank bioreactors

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Induced pluripotent stem (iPS) cell-derived cardiomyocytes represent promising source of cells for implementations in cardiac regenerative medicine and drug testing. Successful clinical translation of iPS cell technology will greatly depend on the availability of efficient, reproducible, scalable and economically affordable culture systems capable of supporting the production of clinically useful quantities of therapeutic cells. Stirred tank bioreactors offer a platform for generation of required quantities of cardiomyocytes under controlled conditions insuring the reproducibility, scalability and GMP compatibility. In this study, we aimed at developing an integrated bioprocess to afford both the cardiac differentiation and cardiomyocyte purification in controlled stirred tank bioreactors. A transgenic murine iPS cell line stably transfected with a vector encoding for an antibiotic resistance and fluorescence reporter under the control of cardiac specific alpha-myosin heavy chain promoter was used for establishing this bioprocess. iPS cells were allowed to form EBs for 2 days when the bioreactors were inoculated with 30000 EBs into 200 ml medium. Spontaneous differentiation was induced by supplementation of ascorbic acid until day 9 followed by antibiotic selection for 7 additional days. The cells were cultured under hypoxic conditions (4% dissolved oxygen) with controlled (pH 7.2) and non-controlled medium pH. Flow cytometric analysis revealed that significantly more iPS cells differentiated to cardiomyocytes when a decrease of pH during the differentiation process was permitted under non-controlled pH conditions. In these cultures the pH continually dropped from average 7.4 down to 6.8 between day 2 and day 9 of differentiation. At day 9, the proportion of cardiomyocytes among total cell population was on average 2.2-fold higher compared to bioreactor cultures with controlled pH. Additionally, the total cell mass in bioreactors without pH control was on day 9 on average 70% higher compared to controlled pH conditions. Metabolite analysis demonstrated that, even though a higher cell proliferation was detected in cultures with decreasing pH, glucose consumption and lactate production were lower compared to cultures with constant pH. Finally, the decrease of pH during differentiation under hypoxic conditions led to up to 5.3-fold higher cardiomyocyte yield compared to controlled pH and brought up to 27 million cardiomyocytes after antibiotic selection out of one 200 ml bioreactor culture. We observed high cardiomyocyte purity after selection under both conditions with few contaminating iPS cells occasionally detectable in cardiomyocyte populations generated under pH controlled conditions. Immunocytochemical stainings revealed that cardiomyocytes produced under both conditions possess characteristic alpha-actinin cross-striations and electrophysiological analysis of these cells revealed that almost all purified cardiomyocytes were of atrial-like phenotype and functionally intact. In conclusion, our results demonstrate a beneficial effect of decreasing pH on cardiac differentiation of murine iPS cells in controlled stirred tank bioreactors under hypoxic conditions that can be utilized for further optimization of iPS cell-derived cardiomyocyte production in vitro.

Keywords: induced pluripotent stem cells; cardiomyocytes; differentiation; bioreactor; pH
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Abstract 25

Wnt/β-catenin signaling regulates sequential fate decisions of murine cortical precursor cells

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The fate of neural progenitor cells (NPC) is determined by a complex interplay of intrinsic programs and extrinsic signals, very few of which are known. β-catenin transduces the extracellular Wnt signals, but also participates in adherens junctions as a bridging component. We identify here for the first time the contribution of β-catenin transcriptional activity as opposed to its adhesion role in the development of the cerebral cortex combining a novel β-catenin mutant allele with a conditional inactivation approach. In agreement with previous reports, Wnt/β-catenin signaling ablation leads to premature NPC differentiation, but, in addition, to a change in progenitor cell cycle kinetics. Contrary to published data, however, Wnt/β-catenin signaling does not affect the dorso-ventral patterning of the cortex and proper lamination. Strikingly, the absence of Wnt/β-catenin signaling affects the sequential fate switch of progenitors, thus leading to shortened neurogenic period with decreased number of both deep and upper-layer neurons and later, to premature gliogenesis. Currently, we are investigating the mechanisms downstream of Wnt/β-catenin signaling which account for the described phenotype.

Keywords: Wnt signaling; cerebral cortex; neurogenesis; astrogenesis

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Abstract 26

Revision of the human hematopoietic tree: Granulocyte subtypes derive from distinct hematopoietic lineages

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The classical model of human hematopoiesis predicts a dichotomous lineage restriction of multipotent hematopoietic progenitors (MPP) into common lymphoid and common myeloid progenitors (CLPs and CMPs respectively). However, this idea has been challenged by the identification of lymphoid progenitors retaining partial myeloid potential (LMPPs), implying that granulocytes can arise within both the classical lymphoid and myeloid branches. Here, we resolve this issue by studying the lineage potentials of cell populations separated by cell surface CD133 expression, i.e. CD34+CD133+ (CD133+) and CD34+CD133low (CD133low) cells, in vitro and in vivo. We show that: i) basophils and eosinophils derive from a common progenitor (EoBP); ii) EoBPs derive in turn from CD34+CD133low erythro-myeloid progenitors that have no neutrophil potential, but do give rise to erythrocytes and megakaryocytes; iii) these erythro-myeloid progenitors (EMPs) are presumably produced together with LMPPs which hold lymphoid, neutrophil and macrophage but no eosinophil, basophil, megakaryocytic or erythroid potential from common CD34+CD133+ MPPs and iv) at least two different qualities of CD133+CD34+ cells can engraft into NSG mice as multipotent progenitors either retaining or lacking erythro-myeloid potential. These findings, which challenge the concept of a common myeloid progenitor, let us propose a new model of early human hematopoiesis. This model restores the dichotomic character of the classical hematopoietic model and includes the existence of LMPPs in human hematopoiesis.

Keywords: human hematopoiesis; granulocytes; lineage specification; hematopoietic differentiation; hematopoietic hierarchy

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Abstract 27

Signaling framework governing cardiac specification in human pluripotent stem cells

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Significant progress has been made during the last few years in improving the directed differentiation of human pluripotent stem cells (hPSCs) along desired lineages. In case of promoting cardiac differentiation, however, multiple diverse protocols and culture conditions have been formulated suggesting that the roles played by the different signaling pathways involved have not been clearly defined so far. We seek to gain a better understanding of the action of signaling pathways driving cardiac fate in hPSCs, which may subsequently be utilized for further improvement and finetuning of directed differentiation approaches. To this end, we have first rigorously tested a number of cell culture parameters potentially impacting cardiac differentiation efficiency. This resulted in a greatly simplified, serum and animal product-free basal differentiation media formulation. Subsequently, we have systematically optimized signaling factor requirements with regards to compositions, activities, and timing in the differentiation process. This protocol enables high-efficiency cardiac differentiation across independent hPSC lines and is applicable to both suspension and adherent culture conditions. We are now using this platform to analyze the specific roles of the manipulated signaling pathways at their respective time windows of action. Preliminary results based on genome-wide expression analysis allow novel insights into the specific downstream effects of signaling pathways driving cardiac fate at different stages in the process.

Keywords: human pluripotent stem cells; directed differentiation; cardiomyocytes; signaling pathways

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Abstract 28

The activating KIT D816V mutation causes hyperplastic expansion of erythroid precursors in mice

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The KIT receptor tyrosine kinase plays a crucial role in the regulation of proliferation, migration and apoptosis in a variety of cell lineages, including primordial germ cells and hematopoietic precursors. Constitutive activation of the KIT/SCF signaling cascade by mutations of the KIT receptor has been implicated in the molecular pathogenesis of several neoplastic diseases originating from those lineages. Activating mutations in the kinase domain of KIT can be found in bilateral testicular germ cell tumors, mastocytosis and core-binding factor acute myeloid leukemia with high incidence. One of the most frequent mutations is the D816V substitution. In order to prove its oncogenic potential and to find out more about the disease mechanisms, we established the R26-GFP-KIT-D816V mouse line with a conditional Knock In of KIT-D816V into the ROSA26 genomic locus. To visualize transgene expression, a GFP-reporter was fused to the mutated KIT by a self-cleaving 2A-peptide. R26-GFP-KIT-D816V mice were mated to Sox2-Cre mice, which mediate Cre-excision in the embryo proper, as well as to Vav-iCre mice, which restrict Cre-expression to the hematopoietic lineage. In R26-GFP-KIT-D816V:Sox2-Cre embryos functional expression of the GFP-KIT-D816V cassette was validated by Western-Blot analysis and GFP-fluorescence. On day E13.5 these embryos exhibit hyperplasia of the fetal liver, which comprises the main hematopoietic organ at that stage of development. Flow cytometric analyses revealed a proportional shift in the erythroid lineage to more immature precursor cells. A similar phenotype was found when analyzing R26-GFP-KIT-D816V:Vav-iCre embryos, demonstrating that this phenotype originates in the hematopoietic system. We hypothesize that KIT-D816V causes increased proliferation of erythroid precursor cells and blocks their terminal maturation. Currently, we are working to identify downstream signaling pathways and genes affected by KIT-D816V to find out more about the molecular mechanisms leading to this phenotype.

Keywords: KIT; D816V; hematopoiesis; erythropoiesis; leukemia

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Abstract 29

Developing an in vitro test system based on the differentiation of human embryonic stem cells towards sensory neurons to assess neurotoxicity

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During development of the nervous system, ventrally migrating neural crest cells (NCC) give rise to dorsal root ganglia (DRG). A temporally and spatially coordinated sequence of signals from adjacent somites and spinal cord leads further to the generation of multiple types of sensory neurons in the DRG. Key events during neurodevelopment are proliferation, differentiation, migration, axonogenesis and synaptogenesis. Even small disruptions in these processes may result in severe impairment of nervous system function, neuropathogenesis and developmental disabilities. Due to uncertainties in the extrapolation of results based on animal tests to humans there is a need for alternative methods to detect human-specific developmental neurotoxicity. Human embryonic stem cell (hESC) technology provides a tool to recapitulate relevant aspects of early human neurodevelopment. To establish a new test system, SOX10+ NCCs were generated from hESCs and further differentiated towards sensory neurons. At day 8 of differentiation, cells were cryopreserved to produce large batches of identical cells. Freshly thawed cells developed neurites and formed a dense neurite network. Here, we present first data on establishment of functional endpoints that maybe used for toxicity testing in this system. We quantified the development of a dense neurite network by live cell imaging. The differentiation process was characterized on mRNA and protein level, showing a progressive upregulation of markers involved in sensory neuron development. The sensory neurons showed a strong calcium response upon depolarization, which could be inhibited by the specific L-type calcium channel blockers verapamil and nifedipine as well as enhanced by the voltage gated calcium channel agonist Bay K8644. This system mimics critical events during nervous system development in vivo that are sensitive to adverse effects of chemicals. This differentiation system could be used as an in vitro test system to detect potential peripheral neurotoxic compounds. Quantification of neurite growth and of calcium responses on single cell level appeared as suitable endpoints that will be further validated with positive and negative control toxicants.

Keywords: hESC-derived sensory neurons; peripheral neurotoxicity; neurite growth; calcium response; in vitro test system

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Abstract 30

Cytokine-directed differentiation and in vitro expansion of hepatic hiPSC- and hESC-derivatives

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Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) hold great promise in regenerative medicine. Hepatic differentiated pluripotent stem cells might serve as hepatic transplants for metabolic or acute liver diseases. So far, hepatic differentiation efficiencies of hiPSCs and hESCs are well studied, but enrichment of functional active hepatic derivatives and a sufficient homogenous population for transplantation purposes need further attention. In our study, we aimed for an efficient protocol that is applicable to differentiate both, hESCs and hiPSCs into an immature expandable endodermal progenitor lineage for further terminally hepatic differentiation. We were using a cytokine– and small molecule–based protocol stimulating the WNT pathway for direct differentiation of hESC and hiPS cells into a definitive endoderm progenitor population. Following the in vivo developmental process we activated the WNT pathway with the GSK3ß inhibitor CHIR99021 resulting in a robust and efficient differentiation towards an endodermal lineage. For further purification prior to expansion we sorted these cells by flow cytometry using the surface antigens CD117 (c-Kit) and CD184 (CXCR4). The sorted double positive cells could be replated into a co-culture system with mouse fibroblasts resulting in an expansion up to 20 doublings. The differentiation status was determined by immunocytochemistry and qRT-PCR for the endodermal markers SOX17 and FOXA2 and by flow cytometry for the cell surface markers CD117 and CD184. To enhance the hepatic specification of the endodermal cells, the WNT pathway was subsequently blocked with sFRP-5 and early hepatic specification markers (hHEX, GATA4, AFP) were analysed by qRT-PCR. Subsequent hepatic differentiation was performed and late hepatic markers (HNF4, Albumin, CYP1A1) were analysed by qRT-PCR. In conclusion, our modified protocol allowed the differentiation of human ESCs and iPSCs into a homogenous and proliferative endodermal progenitor cell population. Activation of the WNT pathway by the small molecule CHIR99021 gave rise to a cell population expressing endodermal marker genes. After purification of these cells, further propagation for four passages resulted in a maintained expression of endodermal marker genes and in a decrease of pluripotency-associated gene expression. Subsequent inhibition of the WNT pathway led to an improved hepatic specification of the endodermal cells showing an increased expression of early hepatic specification markers. Further experiments need to reveal if the cells differentiated under these conditions were suitable for cell transplantation approaches in vivo.

Keywords: ES cells; endodermal progenitor; hepatic differentiation; expansion

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Abstract 31

Mice with thymic epithelial Vhl deletion are lacking a functional thymus

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Early thymic progenitors enter the thymic niche and are exposed to regional hypoxia while they develop in a step-wise manner to mature functional T-cells. Therefore, hypoxia is considered to play an important role for T-cell development. On the molecular level the oxygen-sensing pathway controls the cellular adaptation to hypoxia. In this pathway, the von-Hippel-Lindau protein (pVHL) continuously mediates the destruction of the transcription factor hypoxia-inducible factor-1 alpha (HIF-1 alpha) under normoxic conditions. Under hypoxia HIF-1 alpha degradation is inhibited leading to the activation of HIF-1 alpha target genes. Thymic epithelial cells (TECs) represent a functionally central cell type of the thymic niche. TECs produce key thymopoietic factors, including the Notch ligand DLL4 and the cytokine IL-7 and are characterized by a high turn-over rate in vivo. The highly specialized transcriptional profile of TECs is governed by the transcription factor Foxn1. Two functionally distinct TEC-subtypes, cortical and medullary TECs (cTECs and mTECs), are derived from a bipotent TEC progenitor compartment. cTECs regulate commitment, expansion and positive selection of CD4+CD8+ doublepositive (DP) thymocytes whereas mTECs mediate negative selection of autoreactive CD4+ or CD8+ single-positive (SP) thymocytes. Here, we used a Foxn1-driven Cre-recombinase transgene to specifically delete the Vhl gene in the entire TEC compartment (Foxn1-Cre;VhlloxP;loxP mice). Strikingly, none of n=34 analyzed Foxn1-Cre;VhlloxP;loxP mice developed a macroscopically visible thymus. Flow cytometric analysis of the residual upper mediastinal tissue (with antibodies against CD4, CD8, TCRbeta and CD25) revealed the complete absence of developing T-cells. Additionally, we analyzed stromal cells isolated from upper mediastinal tissue in order to assay for the presence of TECs. In line with the lack of developing T-cells we were neither able to detect mature cortical (EpCAM+CD45-BP1+UEA-MHCII+) nor medullary (EpCAM+CD45-BP1-UEA+MHCII+) TECs in Foxn1-Cre;VhlloxP;loxP mice. In order to confirm the absence of a functional thymus in Foxn1-Cre;VhlloxP;loxP mice we quantified mature CD4+ and CD8+ T-cells in the spleen. Indeed, we detected only minimal numbers of splenic CD4 and CD8 T-cells in Foxn1-Cre;VhlloxP;loxP mice (CD4+TCRbeta+ cells: control 22.3+/-2.6x10ˆ6 [n=4]; Foxn1-Cre;VhlloxP;loxP 0.12+/-0.04x10ˆ6 [n=4]; CD8+TCRbeta+ cells: control 14.9+/-1.9x 10ˆ6; Foxn1-Cre;VhlloxP;loxP 0.32+/-0.20x10ˆ6).

In summary, our data demonstrate that thymic epithelial pVHL is indispensable for thymic organogenesis. Therefore, the oxygen-sensing pathway might be essential for emergence and maintenance of TEC progenitors.

Keywords: T cell development; oxygen-sensing pathway; pVHL; Foxn1; TEC

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Abstract 32

Definitve endoderm formation from plucked human hair-derived induced pluripotent stem cells and SK channel regulation

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Pluripotent stem cells present an extraordinary powerful tool to investigate embryonic development in humans. Essentially, they provide a unique platform for dissecting the distinct mechanisms underlying pluripotency and subsequent lineage commitment. Modest information currently exists about the expression and role of ion channels during human embryogenesis, organ development and cell fate determination. Of note, small and intermediate conductance, calcium-activated potassium channels have been reported to modify stem cell behavior and differentiation. These channels are broadly expressed throughout human tissues and are involved in various cellular processes, such as the after-hyperpolarization in excitable cells but also in differentiation processes. To this end, iPS cells generated from plucked human hair keratinocytes have been exploited in vitro to recapitulate endoderm formation and concomitantly, used to map the expression of the SK channel subtypes over time. We report the successful generation of definitive endoderm from human iPS cells of ectodermal origin using a highly reproducible and robust differentiation system. Furthermore, we provide the first evidence that SK channel subtypes are dynamically regulated in the transition from a pluripotent stem cell to a more lineage restricted, endodermal progeny.

Keywords: definitive endoderm; iPS cells; SK-channel

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Abstract 33

Somatic cells reprogrammed to a pluripotent state by fusion with embryonic stem cells give rise to functional cardiomyocytes in vitro

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The formation of tetraploid cells by fusion of bone marrow cells and cardiomyocytes (CMs) in injured heart has been proposed to contribute to tissue regeneration. In addition, most CMs in the adult heart are binucleated. In this study we used a fusion-based reprogramming to investigate the functional properties of fusion-derived CMs. We have generated pluripotent stem cells by fusion of murine embryonic stem (ES) cells and somatic cells isolated from bone marrow or spleen. These fusion-derived cells expressed markers of pluripotency, exhibited robust demethylation of CpG dinucleotides within promoter regions of pluripotency-associated genes Oct4 and Nanog, formed teratomas in vivo and differentiated to cells of all three germ lineages in vitro. They contained a near-tetraploid genome according to propidium iodide staining, multi-color fluorescence in situ hybridization (mFISH) of metaphase chromosomes and single-nucleotide polymorphism (SNP) analysis, and expressed cell surface major histocompatibility complex (MHC) molecules that were encoded by both fusion partners. The ploidy and marker expression was also retained during differentiation of fusion-derived hybrid cells, confirming the stability of the near-tetraploid genotype in the course of development. Notably, pseudotetraploid fusion-derived pluripotent cells gave rise to CMs, which were similar to their diploid ES cell counterparts in terms of expression of typical cardiospecific markers, sarcomeric organization, action potential parameters, response to pharmacologic stimulation with various drugs and expression of functional ion channels. These data suggest that the state of ploidy does not significantly affect the structural and electrophysiological properties of CMs or their maturity.

Keywords: reprogramming; fusion; embryonic stem cells; ploidy; cardiomyocytes

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Abstract 34

Enhancement of cardiac differentiation of murine embryonic and induced pluripotent stem cells by combined treatment with ascorbic acid and a small molecule agonist of Wnt3a

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Various cardiogenic substances have been reported to enhance the cardiac differentiation of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), however the yield of cardiomyocytes (CMs) in these protocols vary from cell line to cell line. Therefore, we aimed at establishing a cardiac differentiation protocol that would be more efficient and reproducible by evaluating the effect of previously reported cardiogenic small molecules dorsomorphin, cAMP, cardiogenol C, cyclosporine A and ascorbic acid (AA) on cardiac differentiation of murine ESCs and iPSCs. Among these compounds we found that only AA had a robust cardiopoietic effect when applied during the first 2 days of differentiation in murine ESCs line CGR8. However, this effect was not consistent in murine ES cell line aPIG44 and murine iPS cell line AT25. For further enhancement in cardiac differentiation we screened a small molecule agonist of Wnt3a (QS11) and found that addition of QS11, during day 4-8 of differentiation after 2 days treatment with AA produced 5 fold increase in CMs number in comparison to untreated group and 2 fold higher percentage of CMs than AA or QS11 alone in CGR8 and AT25 cell lines. This combined treatment of AA and QS11 also up-regulated cardiac-specific genes as compared to untreated cultures. In order to gain insight into the mechanism leading to superior cardiac differentiation by AA treatment we have compared global gene expression profiles of AA-treated (during days 0-2 of differentiation) and non-treated CGR8 ESCs. Despite clearly evident enhancement of cardiomyocyte yield as assessed in parallel cultures on days 8 and 10 of differentiation in AA-treated group we were not able to detect any significantly differentially expressed transcripts in differentiating cells on days 3, 4 and 5 of differentiation. The reason for this lack of effect on the transcript level in early stages of differentiation is not known. Using Seahorse XF Extracellular Flux Analyzer, we performed functional assay of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) that represents glycolysis in undifferentiatiated CGR8 ESCs cultured in presence of AA for 2 hours or 4 day. At both conditions level of oxygen consumption, ATP production and OCR/ECAR ratio were similar between AA-treated and non-treated groups. Although the direct mechanism of QS11 on cardiac differentiation was not investigated here, it is suggested that QS11 promotes differentiation by activating WNT signaling in ESCs. In conclusion, we have demonstrated that different murine pluripotent stem cell lines exhibit dramatically different capacity to respond to small molecule inducers of cardiogenesis and that the combined use of AA and QS11 enhanced cardiac differentiation of murine ESCs and iPSCs although still with high batch-to-batch variability in CM yield. Combined use of small molecules acting on different signaling pathways may allow production of sufficient amounts of CMs for research, drug screening and potential therapeutic applications in regenerative medicine.

Keywords: cardiomyocyte; embryonic stem cell; induced pluripotent stem cell; cardiac differentiation

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Abstract 35

Characterization of cardiomyocytes derived from induced pluripotent stem cells from a patient with an arrhythmogenic right ventricular cardiomyopathy (ARVC)

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Human iPS cells can be derived from patients with complex genetic defects to create in vitro disease models and thus represent an opportunity to study disease pathophysiology, develop new drugs and test methods for delaying disease progression or reversing its phenotype. We have generated iPS cells from a patient suffering from arrhythmogenic right ventricular cardiomyopathy (ARVC) carrying a novel spontaneous heterozygous autosomal dominant mutation in the gene desmin (N116S). The mutation affects filament formation leading to protein aggresomes in ventricular myocardium in vivo and upon overexpression of exogenous desmin in various types of cells in vitro. Lentiviral overexpression of combination of four transcription factors Oct4, Sox2, cMyc and Klf4 from a single vector (gift of Gustavo Mostoslavsky, Boston University, USA) was used to induce pluripotency in the patient-derived dermal fibroblasts. These iPS cells show a human ES cell-like colony morphology, express pluripotency markers at the protein (alkaline phosphatase, Tra-1–81, Tra-1–60, OCT4, NANOG, and SSEA4) and transcript level (OCT4, SOX2, NANOG, REX1), and exhibit the methylation pattern in promoter regions of OCT4 and NANOG genes, which is undistinguishable from that of conventional ES cells. In addition, these iPS cells carry the same genotype and disease-specific mutation as parental somatic cells, possess normal karyotype, form teratomas in immunodeficient animals and differentiate to spontaneously beating cardiomyocytes in vitro. Immunocytochemical analyses with desmin-specific antibodies revealed that, independently of time in culture (30 versus 100 days), rare (2%) cardiomyocytes express endogenous desmin that forms microscopically detectable aggregates without apparent detrimental effects on their electrophysiological properties. Further analyses are being carried out to assess in more detail the localization and distribution of desmin in disease-specific cardiomyocytes and the mechanism by which it may cause the disease. Thus, the ARVC-specific iPS cells generated in this study may serve as a replenishable source of cardiomyocytes for disease modelling and drug discovery.

Keywords: induced pluripotent stem cells; disease modeling; cardiomyopathy; arrhythmia; cardiomyocytes

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Abstract 36

p57kip2 regulates glial fate decision in adult neural stem cells

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We have previously shown that suppression of p57kip2 leads to accelerated maturation of myelinating glial cell progenitor cells such as immature Schwann cells of the PNS as well as oligodendroglial precursor cells of the CNS. These studies revealed that p57kip2 encodes an intrinsic differentiation inhibitor. Uncommitted, multipotent adult neural stem cells (NSC) of the CNS have been described as an additional source of myelinating glial progenitor cells and we therefore investigated the role of p57kip2 in the glial fate decision process. We found that adult NSC derived from subgranular as well as subventricular zones express p57kip2 in vivo. In cultured adult NSC short hairpin RNA mediated suppression of p57kip2 was shown to induce oligodendroglial characteristics at the expense of astrogenesis. This was revealed by morphological changes and cell-lineage marker expression. Moreover, the anti-astrogenic effect of p57kip2 suppression was also observed under strong astrocyte promoting conditions, such as stimulation of NSC with bone morphogenetic proteins. Finally, when p57kip2 suppressed NSC were transplanted into the adult spinal cord, less GFAP-positive cells were generated and oligodendroglial markers were inducesd when compared to control cells, demonstrating an effect of in vivo relevance. Our results therefore indicate that p57kip2 plays a crucial role in the glial fate decision process, which is of interest regarding the promotion of remyelination activities and the prevention of gliosis in the context of demyelinating diseases such as multiple sclerosis.

Keywords: astrocyte fate; oligodendroglial differentiation; Multiple Sclerosis; regeneration

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Abstract 37

Tbx3 directs cell fate decision towards mesendoderm

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To date, the mechanisms governing transition from the pluripotent state to the onset of differentiation are poorly understood. Recent reports demonstrated additional functions of pluripotency-associated factors during early lineage commitment. Interestingly, the T-box transcription factor Tbx3 has been implicated in the regulation of embryonic stem cell (ESC) self-renewal and cardiac differentiation. In the current study, we report previously unappreciated roles of Tbx3 in early lineage commitment. We show that Tbx3 is dynamically expressed during specification of the mesendoderm lineages in differentiating ESCs in vitro and in developing mouse and Xenopus embryos in vivo. Our results demonstrate that Tbx3 exerts dual cell autonomous and non-cell autonomous effects, directly activating key lineage specification factors while also activating a Nodal/Smad2 signaling signature. Finally, we establish that complex compensatory mechanisms are at play such that there is a functional redundancy between Tbx3 and the closely related family member Tbx2, presumably to protect the progression of normal development. Taken together, we define novel facets of Tbx3 actions and map Tbx3 as a central upstream regulator of the mesendoderm transcriptional program.

Keywords: T-box factors; mesendoderm; pluripotency; gastrulation

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Abstract 38

Red blood cell generation from human induced pluripotent stem cells

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Generating red blood cells (RBC) from human induced pluripotent stem cells (iPS cells) offers the potential to generate large quantities of patient-specific RBC for transfusion purposes from an unlimited source. However, recapitulation of the different steps of RBC generation from pluripotent cells including mesoderm induction, generation of hematopoietic precursor cells, which undergo erythroid maturation, hemoglobin switching and enucleation remains a challenge. Furthermore, epigenetic memory in iPS cells in regard to their donor cell type of origin could lead to variations in their differentiation capacities. We have generated iPS cells from human neural stem cells (NSC-iPS) and human cord blood derived CD34+ hematopoietic stem cells (CD34-iPS) and evaluated their differentiation potential into hematopoietic precursor and mature red blood cells. For hematopoietic induction, iPS cells were allowed to form embryoid bodies (EBs) under cytokine stimulation for 21 days. Thereafter, EBs were dissociated and single cells were applied to a three-step protocol for human erythropoiesis for additional 18-25 days. We have found a similar hematopoietic induction potential among our cell lines. After EB dissociation on day 21, hematopoietic commitment, measured by CD43 expression was about 20% for all cell lines. Colony-forming unit assays demonstrate a similar distribution of myeloid (CFU-M/CFU-GM), erythroid (BFU-E/CFU-E) and more immature mixed (CFU-GEMM) colonies among iPS cell lines. Hematopoietic cells further developed into erythroid precursors as determined by >90% expression of glycophorin A, followed by maturation into normoblasts and partially enucleated RBC. All human iPS cell derived mature erythrocytes predominantly present fetal hemoglobin (> 85%), some embryonic and only a minor amount of adult hemoglobin as demonstrated by HPLC. In summary, we were able to recapitulate the development of RBC from all human iPS cell lines evaluated. This contributes as a first step towards the large scale ex vivo generation of patient-specific RBC from human iPS. Furthermore, our data hint at a similar erythrocyte induction potential of iPS cell lines, independent of their donor cell type of origin.

Keywords: human iPS cells; hematopoiesis; erythroid differentiation; ex vivo generation of RBCs

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Abstract 39

Distinct properties of unrestricted somatic stromal cells (USSC) and cord blood multipotent stromal cells (CB MSC)

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Multipotent stromal cells (MSC) can be isolated from a variety of different tissues in the body, but in contrast to stromal cells from the adult bone marrow (BM) or adipose tissue, cord blood (CB)-derived stromal cells are biologically younger. For these cells, delta-like 1 homologue (DLK-1) and the expression of the homeobox (HOX) genes were determined as discriminating factors between two distinct subpopulations: the unrestricted somatic stromal cells (USSC; DLK-1+ and HOX-), which lack adipogenic differentiation capacity, and the BM MSC-like CB MSC (DLK-1- and HOX+). The aim of this study was to further assess specific properties of USSC and CB MSC populations in regard to a potential biological diversity which might also have impact on future clinical use. Therefore previous data of our group were re-analyzed. Interestingly, significant differences in the haematopoietic supportive capacity of USSC and CB MSC were detected. In co-cultures experiments with isolated CD34+ cells from cord blood, USSC led to a stronger increase of total cell numbers as well as significant expansion of CD34+ cells and colony-forming units. The analysis of haematopoietic relevant growth factor expression revealed a significant higher expression of Insulin-like growth factor binding protein 1 (IGFBP1) in USSC cell lines. IGFBP1 is also involved in the regulation of adipogenic differentiation. The interaction complex of IGFBP1 and insulin-like growth factors can bind to the protease-target region of DLK-1, thereby inhibiting the adipogenic differentiation potential of a cell. Recent data of our group confirmed a high DLK-1 mRNA expression and the intracellular localization of the protein in vesicles in USSC. However, USSC lack the expression of extracellular DLK-1 as well as its secretion, questioning the function of DLK-1 as an inhibitor for adipogenic differentiation in USSC. This observation needs further investigation especially since our group was able to document that the regulations induced by DLK-1 in human stromal cells are different from what is described for mice. In the murine system, DLK-1 leads to an activation of ERK1/2 required to induce SOX9 expression, thereby inhibiting adipogenic specific genes. By contrast, the addition of soluble DLK-1 did neither activate the ERK1/2 in DLK-1 negative CB MSC and BM MSC nor did overexpression of DLK-1 result in an upregulation of SOX9 expression in human MSC. In summary, cord blood-derived stromal cells can be distinguished by their expression of DLK-1, HOX and IGFBP1. Moreover, the data presented here confirm the biological diversity of USSC and CB MSC as demonstrated by the differences in adipogenic differentiation potential and haematopoietic supportive capacity. Interestingly, the molecular changes induced by DLK-1 in human MSC analyzed here are not consistent with the mechanisms described for several murine model systems (ERK1/2 phosphorylation, SOX9 upregulation), providing evidence for fundamental differences on molecular level between both systems and further experiments have to clarify whether the regulations in human and mice are subject to the same molecular mechanisms.

Keywords: MSC; USSC; cord blood; differentiation; adipogenesis

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Abstract 40

Embryonic stem cells carrying a transgenic BMP-reporter construct: A useful tool for the identification and analysis of teratogenic compounds in vitro

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Embryonic stem cells (ESC) are used as a tool for the identification of teratogenic activities in the analysis of chemicals or pharmaceuticals. In particular, the embryonic stem cell test (EST) has been scientifically validated some time ago and is now used for screening purposes. The differentiation of embryonic stem cells in various tissues is regulated by a set of essential signal transduction pathways, including the TGFß, Wnt, and Shh pathway as well as tyrosine kinase receptors mediating FGF or EGF signaling. For some of these essential signal transduction pathways, transgenic reporter mice have been developed that allow the in vivo analysis of pathway activity during embryonic development. We have isolated and characterized embryonic stem cells from transgenic mice that carry a GFP transgene under the control of a BMP response element that has been shown to nicely recapitulate in vivo BMP activity. Our results show that the activity of the reporter gene can be used for the detection of teratogenic activities in vitro. In addition these cells provide a useful tool to characterize the molecular mechanism underlying the activity of chemicals or pharmaceuticals on the differentiation process during early embryonic development.

Keywords: differentiation; BMP; EST;

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Abstract 41

Generation of hiPS reporter cell lines by zinc-finger-nuclease (ZFN) assisted gene targeting and recombinase mediated cassette exchange (RMCE)

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Human induced pluripotent stem cells (hiPSC) hold great promise in the field of regenerative medicine, pharmacological and basic research. They are characterized by their capability of indefinite self-renewal and their potential to differentiate into cells of all three germ layers of the human body. Of particular importance are transgenic hiPSC as reliable reporter systems which are needed to monitor differentiation events in order to allow the development of stable differentiation protocols, identification of differentiation related surface markers and to analyze the function of a single gene. In that context reporter gene based molecular imaging in vitro has shown to be an authentic technique for effective cell investigation during proliferation and differentiation periods. To prevent undesirable random integrations during the generation of transgenic reporter cell lines, we performed genome editing by the use of zinc-finger-nucleases (ZFN) which target the AAVS1-locus, a published „safe harbor" locus in hiPSC. The vector for the initial targeting of the hiPSC consists of two elements, a gene trap cassette and a constitutively expressed reporter cassette which together are flanked by heterospecific flippase recognition target sites (FRTs). Several clones could be established that were shown to be targeted on both alleles based on PCR and Southern blot analyses and had a normal karyotype. These entry cell lines could subsequently be modifed by recombinase-mediated-cassette exchange (RMCE) allowing zinc-finger independent introduction of alternative constructs that carry compatible FRT sites. The RMCE could be monitored via a switch of specific fluorescent marker expression. The initial targeting vector contained a cytomegaly-virus-early enhancer chicken β-actin promoter (CAGG) driven red fluorescent protein (TagRFP) and a surface marker (Δ-LNGFR), linked by a 2A-element. Successfully targeted entry clones showed homogenous and coordinated expression of RFP and Δ-LNGFR. In contrast, the reporter cassette present in the RMCE-exchange vector carried a CAGG-driven green fluorescent protein (GFP) and an artificial surface marker (ArtEp). Consequently RMCE resulted in a genetic modification of the homozygous entry cell line (RFP+/+) to either RFP+/GFP+ or GFP+/+ genotype that could be tracked by live cell imaging as well as flow cytometry. Finally, we were able to generate homozygous (GFP+/+) and heterozygous clones (RFP+/GFP+) after RMCE. In summary we generated generic gentically modified entry cell lines which could be further modified through RMCE. The color coding introduced into the entry cell lines allowed us to track the RMCE events and facilitated the identification of recombination. The concept will also enable us to visually select these events even when tissue-specific reporters are to be introduced via RMCE. Future studies will focus on the generation of reporter cell lines carrying tissue specific promoters that may be utilized for monitoring differentiation events in hiPSC.

Keywords: human induced pluripotent stem cells; gene targeting; recombinase mediated cassette exchange

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Abstract 42

Physiological tracking of differentiation time series using large scale gene expression analysis

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In vitro differentiation of pluripotent stem cells into diverse somatic cell types is increasingly studied in order to obtain a molecular understanding of embryogenesis, to build disease specific in vitro models, and to develop new options for regenerative medicine and drug development. An important task in this context is the detailed determination of cell fate and differentiation dynamics. Specification of cell fate requires comparison of in vitro differentiated cell lines to primary cells or biopsy material. Such a comparison is challenging due to culture effects as well as heterogeneity in clinical samples, affecting whole-genome gene expression. Differentiation dynamics are usually monitored based on the expression of a few cell type specific marker genes, disregarding genome wide gene expression changes. We present a method that determines phenotypic changes in differentiation time series in the context of large-scale gene expression patterns, derived from clinical tissue phenotypes as a reference frame. The so called PhysioSpace method complements single-marker based monitoring by consideration of genome-wide changes and deals with large heterogeneities using correlation based data normalization in combination with robust statistical methods. The method leads to stable results, even in the case of small sample sizes and across microarray platforms, enabling to reduce experimental efforts. We applied the PhysioSpace method on publicly available datasets from differentiation of pluripotent stem cells towards neural cells, trophoblasts, and cardiomyocytes. These analyses led to results that were highly consistent with a priory known biological information showing the robustness and biological relevance of the method even in the case of small sample sizes. We were able to detect a rapid change in differentiation dynamics in terms of physiological patterns, suggesting kind of a cell fate decision point in the analyzed cardiomyocyte differentiation time series. Analyses of the trophoblast differentiation time series revealed a proper differentiation until day 10 and a complete switch of the cell culture from day 10 to day 12 of differentiation. Finally, our analyses of neural differentiation data suggests that ESC-derived neural epithelial cells are more similar to fetal brain tissues than to adult brain tissues, while ESC-derived mature neural cells show also high similarities to adult brain tissues. These results demonstrate the validity of the PhysioSpace method for tracking of cell fate dynamics in wet lab experiments compared to clinical data sets.

Keywords: gene expression; large scale patterns; differentiation; dynamics

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Abstract 43

Bioluminescence in vivo imaging of genetically selected iPS cell-derived cardiomyocytes after transplantation into infarcted heart

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Cell loss after transplantation is a major limitation for cell replacement approaches in regenerative medicine. To efficiently assess cell retention and facilitate the optimization of procedures for enhancing the cell survival without extensive use of animals, tools for longitudinal in vivo tracking of viable transplanted cells are needed. Here, we have generated a transgenic murine induced pluripotent stem (iPS) cell line which, in addition to cardiomyocyte (CM)-specific expression of puromycin N-acetyl-transferase and EGFP, also expresses firefly luciferase (Fluc) under the control of the constitutive ubiquitin C promoter. Genetic modification of cells did not affect their cardiac differentiation capacity as demonstrated by comparable yields and cardiac specific gene and protein expression to the parental cell line. In vitro comparison of luciferase activity between the lysates of Fluc-iPS cells and their derived Fluc-iPSCM revealed a 4-fold decrease of bioluminescence (BL) in Fluc-iPSCM. This could be at least partly attributed to a decrease of luciferase transcript levels of 44% in Fluc-iPSCM as detected by RT-qPCR. Measurements of BL signal intensity in intact Fluc-iPS cells and Fluc-iPSCM by in vivo imaging system (IVIS) confirmed that luciferase activity in purified Fluc-iPSCM was strongly reduced. To investigate the engraftment potential of Fluc-iPSCM in vivo, puromycin selected Fluc-iPSCM were transplanted into native or cryoinfarcted hearts and hind leg muscles of syngeneic mice and the engraftment of viable cells was monitored by in vivo BL imaging and histological analysis of explanted hearts. Although luciferase activity in purified Fluc-iPSCM was lower than in undifferentiated Fluc-iPS cells it was sufficient to detect the transplanted Fluc-iPSCM in vivo with the threshold of $5x10^4$ cells in the heart and $2.5x10^4$ cells in skeletal muscle. Transplants of identical numbers of Fluc-iPSCM yielded different BL signals, depending on the injection site, and were on average 71-fold higher in the leg than in the heart. 24 hours after transplantation into the healthy heart and hind leg muscle the signal decreased by 44% and 71%, respectively. Similar decline in amount of surviving Fluc-iPSCM was also observed after transplantations into cryoinjured heart, where BL signal was decreased by 54% on day 1 and by 84% on day 28 after transplantation. Histological analysis of hearts 28 days after transplantation of Fluc-iPSCM confirmed the presence of small areas of EGFP positive Fluc-iPSCM, showing expression of cardiac α-actinin in alignment with host tissue. We conclude that in vivo BL imaging proved is a powerful tool for non-invasive monitoring of delivery, retention and distribution of viable firefly luciferase-expressing iPSCM in the host tissues and can be used to develop procedures for enhancing cell survival after delivery and investigate immunogenicity of transplanted cells in syngeneic and allogeneic recipients.

Keywords: bioluminescence imaging; iPSC derived cardiomyocytes; cardiac cell transplantation; transgenic reporter cell line; in vivo cell tracking

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Abstract 44

BMP10, a heart-specific cytokine, induces differentiation of human pluripotent stem cells with a much higher potency than members of the BMP7 subgroup

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Bone morphogenetic proteins (BMPs) are a large family of biologically active cytokines. BMP ligands are present within a broad range of animals where they are highly conserved. In humans they are known to have pleiotropic functions during all stages of life. With respect to human pluripotent stem cells, it has been shown that BMP2, 4, 6 and 7, either as homodimers or heterodimers, can induce differentiation into embryonic (mesoderm, endoderm) and extraembryonic (trophoblast) lineages. In differentiation protocols applying more stringent cell culture conditions, these BMPs have also been successfully used to differentiate human pluripotent stem cells into vascular progenitor cells, chondrocytes and primordial germ cells. However, these ligands only represent a minority of the BMP family, which consists of more than 20 members and can be divided into several subgroups, depending on sequential and structural homology. In addition, it has been shown for certain human adult stem cells and progenitor cells, that one and the same cell type can respond differently to distinct ligands, even if they belong to the same subgroup (e.g. BMP5, 6 and 7). This prompted us to investigate the effect of distinct members of the BMP family – that have to our best knowledge not been tested yet - in terms of their effect on human pluripotent stem cells. For this purpose, we chose BMP5, BMP13 (GDF6) and BMP10, all representing members of distinct subgroups, with BMP5 belonging to the BMP7 subgroup, as well as the well-researched BMP7 as a reference. Furthermore, we successfully reprogrammed human chorionic villi cells to induced pluripotent stem cells (hiPSCs). In our in vitro experiments we treated these hiPSCs - as well as human embryonic stem cells (hESCs lines H1 and H9) as a reference, with equal concentrations of these human recombinant BMPs in chemically defined medium (without addition of any other exogenous cytokines or inhibition of autocrine signalling pathways). We observed that hESCs as well as hiPSCs respond identically to all the four BMPs tested, namely they first differentiated to mesendoderm cells and, predominantly to early trophoblast cells (indicated by the expression of primitive streak markers T and MIXL1 and early trophoblast markers CDX2 and HAND1), followed by cells belonging to the definite mesoderm/endoderm and trophoblast lineage (indicated by cell fusion markers and as latest, secretion of hCG as marker of multinuclear syncytiotrophoblast cells). However, as we found out on mRNA (microarray, qPCR) and protein level (Western Blot, immunocytochemistry, ELISA), the kinetics and potency of these ligands differ immensely. Interestingly, we discovered that BMP10, whose expression in vivo is restricted to specific parts of the heart for a short time frame during heart development, induces differentiation with by far the highest kinetics/potency, and BMP13 being the weakest. Detailed comparative kinetic studies of earliest treatment responses - when differentiation of the cells had not occurred, revealed a significantly higher level of phosphorylated SMAD1/5 for BMP10 at any time point investigated, whereby signaling via non-SMAD-pathways (such as MAPKs and Akt) appears to play only a minor role. In summary, our studies have unveiled additional cytokines that could be applicable for lineage specific differentiation of hiPSCs/hESCs to derive donor cell types useful for cellular regenerative therapies in the future.

Keywords: BMPs; human pluripotent stem cells

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Abstract 45

Rab-GTPases are involved in erythroid and megakaryocytic differentiation of human hematopoietic stem and progenitor cells

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An important aspect of stem cell biology is the understanding of mechanisms controlling the decision of self-renewal or differentiation. In this context stem cell niches and asymmetric cell divisions have been demonstrated to control sizes of somatic stem cell pools in different organisms. Previously, we confirmed the long lasting conjecture that human hematopoietic stem and progenitor cells (HSPCs) have the capability to divide asymmetrically. In our studies we demonstrated that in a proportion of dividing HSPCs endosomal proteins segregate differently into arising daughter cells, pointing towards a relationship between organization of the endosomal compartment and cell fate specification processes in HSPCs. Rab-GTPases are key regulator proteins of endosomal trafficking. We analyzed by means of ectopic expression impacts of wild type (WT), constitutive active (CA) and dominant negative (DN) Rab5 and Rab7 variants on the development of human CD34+ cells in vitro. Subcellular localization studies of the different Rab-GTPase variants showed that ectopic expression of Rab5CA promotes formation of giant endosomal structures, demonstrating that manipulation of Rab-GTPase function can affect organization of the endosomal compartment. In terms of cell fate analyses, we comprehensively analyzed the erythro-myeloid differentiation capacity of genetically manipulated CD34+ cells in vitro. Expression of Rab5WT, Rab5CA and Rab7DN led to an increased proportion of CFU-Mix and BFU-E colony formation in CFC-assays. Next, we confirmed in erythroid and megakaryocytic read out systems that Rab5WT, Rab5CA and Rab7DN enhance megakaryocytic and erythroid differentiation in vitro. In summary, our results demonstrate essential roles of the Rab-GTPases in erythropoiesis and megakaryopoiesis.

Keywords: Rab-GTPases; erythroid differentiation; HSPC

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Abstract 46

Early and late effects of densely ionizing radiation in mouse embryonic stem cells

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ESCs are a powerful tool in disease modeling, tissue engineering and drug testing. Furthermore, they might be very useful in the field of radiation research, where many questions concerning the radioprotection of the early embryo are not yet solved. Embryos are unavoidably exposed to natural background radiation. Additionally, in utero exposure to ionizing radiation (IR) can occur due to accidental exposure of the expectant mother, or due to medical diagnostics and radiotherapy treatment of women unaware of their pregnancy status. The biological response to IR exposure severely depends on the radiation quality: While X and gamma-rays (sparsely IR) exhibit a random spatial distribution of comparably small energy depositions, charged particles (densely IR) deposit the energy along their track. This leads to a more clustered and complex damage in the cells compared to X-rays. Appropriate radioprotection guidelines can be obtained only when the effects after radiation exposure are fully understood. So far, most of the information comes from data obtained after sparsely IR exposure. Hence, additional studies investigating the biological effects of densely IR are required. To investigate the effect of densely IR on the early embryonic development, we chose the mouse ESC line D3 as a model system. Cells were irradiated with carbon ions with doses ranging from 0.5 to 5 Gy. Exposure to X-rays was performed for comparison. Endpoints like clonogenic cell survival, cell cycle progression delay, chromosome aberrations, presence of pluripotency markers as well as differentiation capacity into beating embryoid bodies (EBs) were investigated. Our data show that exposed cells undergo a transient G2 block that is more pronounced after particle exposure. Furthermore, a dose dependent cell killing is observed. Cells reaching the first post-irradiation mitosis exhibit a significant increase in the frequency of structural chromosome aberrations compared to the control. However, 8 days after X-ray exposure, this number is similar to the control value, while the progeny of carbon ion exposed cells still exhibit a significantly higher aberration frequency than control cells attributable to transmissible aberrations. Despite radiation exposure, pluripotency markers are detected in surviving cells 17 days post irradiation. Additionally, the capability of differentiating into cardiomyocytes was analyzed. Beating EBs are observed in all samples. Nevertheless, their fraction is lower in carbon ion exposed samples compared to control. In summary, for all endpoints investigated except for the presence of pluripotency markers, carbon ions show a higher biological effectiveness than X-rays. However, surviving cells maintain genomic stability. These in vitro results suggest that in vivo exposure to charged particles might have a higher impact on the developing embryo compared to sparsely IR. Further studies are needed to confirm these observations.

Keywords: embryonic stem cells; radiation; pluripotency; genomic stability

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Abstract 47

Derivation and characterisation of endoderm progenitors from integration-free episomal plasmid based- iPSCs generated from human fetal foreskin fibroblasts

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Human embryonic stem cells (hESCs) have two fundamental characteristics. First is pluripotency, i.e. the ability to differentiate to all cell types of the three germ layers endoderm, ectoderm and mesoderm in vitro (formation of embryoid bodies) and in vivo (teratoma formation in immunodeficient mice) Second, hESCs have the capability to self-renew indefinitely. Embryonic stem cells express pluripotency associated markers such as OCT4, NANOG and SOX2 also the surface markers SSEA-4, TRA-I-60, TRA-1-81 and TRA-2-49 but not SSEA-1. Induced pluripotent stem cells (iPSCs) are embryonic-like cells and can be generated from somatic cells derived from individuals with known genetic characteristics by the over-expression of OCT4 and SOX2 in combination with either KLF4 and c-MYC or NANOG and LIN28. We have generated episomal-derived and integration-free E-iPSCs from human fetal foreskin fibroblast cells (HFF1) as described by Yu et al. (2009) and compared the transcriptome to that of retro-viral derived HFF1-cells (V-iPSCs) generated in our laboratory. The transcriptome of E-iPSCs are closer to that of hESCs (R2 = 0.9363) in comparison to V-iPSC (R2 = 0.8176). This viral-free method has the advantage over viral-based protocols because of the lack of integrations which otherwise leads to chromosomal re-arrangements of the host genome. Using the E-iPSC line we have derived and characterized hepatocyte-like cells (HLCs) as described by Sullivan et al. (2010) and endodermal progenitors (EPs) as described by Cheng et al. (2012). We detected in HLCs the expression of AFP, Albumin, HNF4α, E-Cadherin and Glycogen. In EPs we detected the expression of LGR5, CXCR4, ALCAM, Albumin, E-Cadherin and HNF4α. Further studies are planned involving the use of the E-iPSCs derived EPs to generate hepatocyte and pancreatic cells. These studies will enable uncovering the genes and associated pathways that specify a bipotential EP to differentiate to either liver or pancreas. Additionally, these E-iPSCs and derived EPs provide unique resources for disease modeling, developmental studies, drug screening and toxicology studies.

Keywords:

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Abstract 48

Genetic manipulation of neural progenitor cells derived from human induced pluripotent stem cells by using zinc finger nucleases

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Objective: Induced pluripotent stem cell (iPSC)-derived neural progenitor cells (NPCs) represent a valuable source of neural cell lineages for research, drug testing and potential therapeutic applications. Optimal utilization of NPCs in these areas requires improved protocols for their genetic manipulation. Random insertion of transgenes into the NPC genome is frequently accompanied by transgene silencing and may lead to insertional mutagenesis. Here, we tested the utility of zinc finger nuclease (ZFN) technology for targeted insertion of selectable markers into the genomic safe harbor locus of NPCs without gene silencing and deleterious effects on cell function.

Methods: Long-term self-renewing NPCs capable of differentiation into neurons, astrocytes and oligodendrocytes were generated from human iPSCs using published protocols. ZFNs were used to target the insertion of a gene cassette in which EF1α promoter drives the expression of a puromycin resistance gene and a reporter marker GFP into AAVS1 locus in NPCs. Stable transgenic NPCs were selected and characterized.

Results: DNA sequencing confirmed that the transgene cassette was successfully integrated into the targeted AAVS1 locus. Transgenic NPCs were indistinguishable from parental NPCs in regard to proliferation rate, marker expression and potential for in vitro differentiation into neurons, astrocytes and oligodendrocytes. Moreover, ZFN-engineered NPCs integrated into the neonatal mouse brain cortex, differentiated toward neural cell lineages retaining the GFP expression and did not form teratoma two months after transplantation.

Conclusion: These data indicate that ZFN can be successfully employed for gene manipulation in human iPSC-derived NPCs without causing gene silencing and perturbing the cell function.

Keywords: neural progenitor cells; zinc finger neuclease; gene manipulation; human induced pluripotent cells

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Abstract 49

SMAD, NF-kappaB and GSK3-beta dependent signalling intersect to induce and pattern mesoderm formation in human embryonic stem cells

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Mesoderm and endoderm are formed in a defined order during gastrulation in response to diverse signalling environments in distinct regions of the primitive streak. How these signalling networks act to pattern mesoderm and distinguish it from endoderm is still poorly understood. Here, we compared SMAD-dependent (ACTIVIN-A and BMP4) and GSK3-beta/WNT-dependent mechanisms of primitive streak formation using human embryonic stem cells. We demonstrated that NFkappaB is required for SMAD-mediated but not GSK3-beta/WNT pathway-mediated mesoderm induction. We found that high levels of CDX2 and BRACHYURY achieved by GSK3-beta inhibition were sufficient to block endoderm and anterior lateral mesoderm differentiation, thereby favoring paraxial mesoderm formation. We used this human model of mesoderm patterning to develop a robust and chemically defined 2D cardiac differentiation method with broad translational potential. Accordingly, separate induction mechanisms interact to pattern mesoderm into therapeutically relevant sub-types and to distinguish it from endoderm.

Keywords: hESC; mesoderm; cardiomyocites; patterning

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Abstract 50

Generation of rat induced pluripotent stem cells using a non-viral inducible vector and differentiation towards mesodermal and ectodermal lineages

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Current methods of generating rat induced pluripotent stem cells are based on retro- or lentiviral transduction of pluripotency inducing genes (Oct4, Sox2, c-myc and Klf4) into somatic cells. Establishment of a true self-sustaining pluripotent state, independent of exogenous reprogramming factor expression, requires epigenetic silencing of the exogenous genes. Virally transduced genes are frequently silenced in the host cell, but persistence or reactivation of factor expression can interfere with differentiation. Next generation iPS cells must therefore incorporate tight control of transgene expression. To gain more control over the expression of exogenous reprogramming factors, we used a novel reprogramming vector. The plasmid consists of a bidirectional doxycycline-inducible promoter controlling the expression of exogenous Oct4, Sox2, c-Myc and Klf4. In addition all regulatory components of the Tet-On system are present on the plasmid. Because transfected DNA integrates into the host genome less frequently than infecting retro- or lentiviruses, we equipped the reprogramming vector with a bacteriophage ΦC31 attB site and used a ΦC31 integrase expression plasmid to mediate vector integration. In total we generated 64 induction-independent rat iPS cell lines by plasmid based reprogramming. They expressed endogenous rat pluripotency genes and markers such as Oct4, Rex1, Nanog, FGF4, SSEA1 and alkaline phosphatase. Bisulfite sequencing analysis of the rat Oct4 promoter showed complete demethylation, consistent with a pluripotent state. The rat iPS cells also formed teratomas in immunosuppressed mice demonstrating pluripotency in vivo. The reprogramming efficiency with our safe and simple non-viral approach was between 0.0027% and 0.0078% which compares favorably with methods based on viral vectors. We established protocols to robustly generate embryoid bodies (EBs) from our rat iPS cells. RT-PCR analysis and immunocytochemistry of rat EBs confirmed the expression of marker genes for all three germ layers. Published protocols for neuronal differentiation of mouse ES cells were adapted to rat iPS cells and resulted in a highly pure population of cells positive for the neuronal marker beta-III tubulin. Electrophysiological analysis showed functionality of these neurons. Moreover, we established EB based differentiation to beating cardiomyocytes, skeletal muscle and adipocyte-like cells. All cell types were characterised by a variety of methods and showed characteristic gene expression or function. We have generated rat iPS cells that show similar characteristics to rat ES cells. Our iPS cells expressed pluripotency markers and showed the ability to generate cell types of all three germ layers in vitro and in vivo. We developed protocols to obtain differentiated cells such as cardiomyocytes, neurons, skeletal muscle and adipocyte-like cells. Therefore our rat iPS cells promise to be a useful source of differentiated cells for in vitro toxicological analysis, drug discovery or compound screening.

Keywords: rat; iPS cells; differentiation; mesoderm; ectoderm

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Abstract 51

A single amino acid residue on HOXB4 controls the transition from hemogenic endothelium to earliest hematopoietic progenitors, in vitro

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Ectopic expression of the homeodomain transcription factor HOXB4 enhances the formation and expansion of hematopoietic stem and progenitor cells (HSPCs) from differentiating mouse embryonic stem (ES-) cells, in vitro and in vivo. However, the underlying mechanisms how this transcription factor mediates its hematopoiesis-promoting activities are still ill-defined. To date, neither the crucial posttranslational modifications of HOXB4 nor the qualitative composition of its protein interactions influencing the selective activation or repression of gene loci involved in hematopoietic development and expansion have been investigated sufficiently. Because Lysine residues are common targets for modifications such as acetylation or ubiquitination, we asked whether a certain Lysine (K16) can alter the molecular and biological properties of HOXB4. An exchange of Lysine16 for Arginine (K16R) enhanced its stability in undifferentiated mouse ES-cells. Functional analyses of the mutants uncovered an essential role of Lysine 16 during development of definitive hematopoietic stem and progenitor cells from differentiating ESCs, in vitro. Whilst ectopic expression of wildtype (wt) HOXB4 and the K16R mutant both promoted the formation of hemogenic endothelium structures, earliest hematopoietic CD41+ suspension cells were only generated when wildtype HOXB4 was expressed. In contrast HOXB4 (K16R) completely blocked further progression of hematopoietic development. Noteworthy, expression of Runx1, which is an absolute prerequisite for definitive hematopoiesis, was not altered by any of the HOXB4 proteins. Because Runx1 directly controls transcription of other crucial hematopoietic genes such as PU.1, Gfi1, Flk1 or CD41, HOXB4 possibly acts as a gatekeeper controlling Runx1 activity and, thus, hematopoietic fate decisions. In particular, Lysine16 on HOXB4 appears to act as a switch determining the developmental fate of Runx1+ hemogenic endothelium cells.

Keywords: HOXB4; embryonic stem cells; hematopoiesis; hemogenic endothelium; Runx1

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Abstract 52

Efficient generation of definitive endoderm from human embryonic stem cells by GSK3 beta inhibition and nodal signaling

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The generation of insulin-producing cells from embryonic stem cells (ES cells) requires the stepwise transfer of developmental principles onto in vitro differentiation protocols. It is accepted that treatment of human ES cells with high doses of activin A by activation of the Nodal/Tgf-beta pathway is the biochemical reason for differentiation of ES cells into cells reminiscent of definitive endoderm (DE). However, the thesis that Nodal/Tgf-beta is solely responsible for in vitro differentiation of ES cells into DE appears as a simplification of developmental mechanisms in particular when comparing with early in vivo events during gastrulation. Consequently, the differentiation protocols used so far have not yet yielded DE-like cells with 100% purity. The inhibition of the GSK3beta was recently discovered as a promising method to generate DE-like cells in vitro. We analyzed the role of the GSK3beta inhibitor CHIR-99021 in a comparative study against a classic endoderm differentiation protocol. Hues8 human ES cells were differentiated with a control protocol comprising the treatment with wnt3a (25 ng/ml) and activin A (100 ng/ml) for 3 days and a protocol designed to initiate a primitive-like stage by a 24h GSK3 inhibition with 5 µM CHIR-99021 (Chir) followed by a 48h treatment with activin A (100 ng/ml). The differentiation into DE was monitored by FACS-analysis of the DE-surface markers CXCR4 and CD49e, qPCR, and immunofluorescence staining. The control protocol yielded 22±3.5% CXCR4/CD49e-positive cells and was therewith clearly inferior to cells in which Chir was added initially for 24h followed by a further 48h treatment with activin A (57±7.9%). Analysis of typical DE-related genes revealed an expression pattern similar to developmental kinetics with T and MIXL1 expressed after induction with wnt3a and activin A for 24h from where it decreased. SOX17 and FOXA2 were induced and reliably detectable after 3 days of differentiation. In Chir/activin A treated cells T expression was 17.3 fold higher expressed and MIXL1 7.4 fold higher after 24h. Upon change of the medium to activin A, both genes were downregulated and SOX17 and FOXA2 mRNA transcripts were detectable 3.6 and 6 fold higher (for FOXA2 and SOX17, respectively) compared to control cells. In immunofluorescence stainings, Chir/activin A treatment resulted in predominantly brachyury-positive cells after the 24h GSK3beta inhibition with distinct outgrowth of SOX17/FOXA2 double-positive cells later in the protocol. SOX17/FOXA2 double-positive were not detected before day 2 from where they increased until near homogeneity at day 3. Generally the wnt3a/activin A protocol was less efficient, thereby confirming the FACS results. A dilution of activin A after the induction with Chir showed that activin A concentrations can be significantly lowered to 25-50 ng/ml to yield the same results. Our analyses revealed the inductive effect of CHIR-99021 which directs human ES cells into cells reminiscent of the primitive streak independent of the Nodal/Tgf-beta pathway. By addition of low doses of activin A, these cells can be effectively differentiated into definitive endoderm. This demonstrates that human ES cell differentiation into DE is initially Wnt-pathway dependent and subsequently Nodal/Tgf-beta dependent.

Keywords: embryonic stem cells; differentiation; definitive endoderm; GSK3 beta inhibition

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Abstract 53

Generation and functional analysis of engineered proteins in neural reprogramming

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Meso-diencephalic dopaminergic (mdDA) neurons play an important role in motor control and cognitive functioning. Their dysfunction or loss is associated with several neuropsychiatric disorders, including Parkinson's disease (PD), addiction, bipolar disorder and depression. In clinical trials to treat PD, fetal mdDA tissue has been shown to be beneficial and has been further extended in animal models using DA neurons derived from embryonic stem cells. Due to ethical issues involved in using human ES cells for cell replacement therapy, Caiazzo et al., showed that mouse and human fibroblasts can be directly converted to functional dopaminergic neurons by viral-based expression of the three transcription factors Lmx1a, Mash1 and Nurr1 (Caiazzo, M., et al., Nature 476, 224–227, 2011). However due to their high risk of adverse effects and clinical hindrances, viruses cannot be used in therapies for humans. An alternative approach would be protein transduction, in which heterologous expressed protein of interest can be taken up by cells with the help of a fused cell penetrating peptide (CPP). One of the prominent examples of CPPs is the basic peptide TAT (trans-activator of transcription) derived from human immunodeficiency virus type 1 (HIV-1) (Fawell, S., et al., PNAS 91, 664-8, 1994). Here, we report the cloning of the three human transcription factors Lmx1a, Mash1 and Nurr1 as N-terminal fusion proteins to a His-tag, the TAT-domain and a nuclear localization sequence (NLS) into the pTriEx-vector system thereby obtaining HTN-Lmax1a, HTN-Mash1 and HTN-Nurr1. Functional characterization of these proteins was investigated by cellular uptake assays, where the proteins were labelled with the fluorescent dye Rhodamine for visualization upon cellular uptake by fluorescence microscopy. Furthermore, we verified the biological activity of the transduced transcription factor proteins by quantifying the mRNA levels of their respective downstream targets by real-time qPCR in secondary cell lines and their functional efficacy in promoting fate changes in primary neuronal and neural stem cell cultures. Taken together, we show the generation of cell penetrating and physiologically relevant transcription factors proteins for engineering and induction of neural fate changes.

Keywords: Parkinson's disease; reprogramming; transcription factors; protein transduction; induced neurons

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Abstract 54

TRIM32 dependent transcription in adult neural progenitors regulates neuronal differentiation and olfactory learning

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In the adult mammalian brain neural stem cells in the subventricular zone continuously generate new neurons for the olfactory bulb. As a defining feature of all stem cells, also these adult NSCs have the ability to simultaneously self-renew and generate more fate committed daughter cells. This fate commitment is regulated cell fate determining proteins. Here, we show that the cell fate determinant TRIM32 is upregulated during maturation of adult neural stem cells into olfactory bulb neurons. This upregulation is accompanied by neural progenitor cell divisions which show an asymmetric distribution of TRIM32 during mitosis. We further demonstrate that TRIM32 is necessary and sufficient for neuronal differentiation in adult neural stem cells. Interestingly, TRIM32-deficency induced overproduction of new olfactory bulb neurons leads to a clear impairment in olfactory learning processes. Thus, we provide evidences for a function of TRIM32 for neuronal differentiation of adult neuroblasts and we correlate the cell fate determinant dependent activity of adult neurogenesis with complex learning behaviour.

Keywords: TRIM32; adult neurogenesis; neuronal differentiation

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Abstract 55

Immune-semaphorin expression in stem cells of the common marmoset (Callithrix jacchus)

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Semaphorins are originally known to be crucial for neural development, but a subgroup of these membrane-bound proteins, the so called, has also immunological functions. Molecules such as semaphorin 3A, 3E, 4A, 4B, 4D, 6D and 7A belong to this group and are investigated as regulators of immune cell activation or differentiation and play a significant role in immune cell trafficking, monocytes and macrophages activation (Takamatsu and Kumanogoh, 2012). These immunomodulatory and even immunosuppressive properties of semaphorins could be demonstrated especially in mesenchymal stem cells (MSCs) resulting in enhanced immunotolerance (Marigo and Dazzi, 2011). Since MSCs are the most widely used stem cell type in the field of medical application, in our laboratory MSCs from our animal model, the common marmoset (Callithrix jacchus), have been derived from bone marrow and placenta and were long term cultured and even reprogrammed (Wiedemann et al., 2012). To investigate possible expression of immune-semaphorins in the non-human primate model, we utilized database homology analysis for human and marmoset semaphorins in silico. Interestingly, although the chromosomes of human and marmoset are not fully homologue (Sherlock et al., 1996), the semaphorins of human and marmoset are located on the same chromosomes and regions. Furthermore, our analysis revealed high similarity between the human semaphorins and the 'semaphorin-like' sequences in the marmoset genome, reaching to maximal identity of 97 %. In contrast to the other semaphorins, Sema 7A shows the lowest analogy with just 72 % identity. If this high diversity leads to a difference concerning structure and functionality of the protein has to be examined by analysing the sequences of the marmoset semaphorins. In RT-PCR experiments with specific oligonucleotides expression of six out of seven immune-semaphorins could be detected in different kinds of marmoset tissues, induced pluripotent stem (iPS) cells, embryonic stem cells (ESCs) and MSCs. If the level of semaphorin expression bears immunological advantages for marmoset MSCs is determined in the future by real-time PCR, immunohistochemistry stainings and as functional assay mixed lymphocyte cultures.

Keywords: common marmoset; MSCs; semaphorins

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Abstract 56

Investigating molecular mechanisms of mesoderm induction in human embryonic stem cells

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In human embryonic stem cells (hESCs), OCT4, SOX2 and NANOG are at the core of an intrinsic gene-regulatory network controlling self-renewal and maintaining the undifferentiated stem cell state. However, these individual transcription factors may also actively participate in promoting transitions into specific cell fates. We aim to study the molecular mechanisms of mesoderm formation, focusing primarily on the role of BMP and WNT signaling pathways in the process and to identify downstream targets of these cascades which positively or negatively control the transition from the pluripotent state into the mesodermal lineage. We found that that these pathways act synergistically to induce mesoderm while specifically repressing SOX2 in hESCs. Furthermore, over-expression of SOX2 in hESCs interfered with spontaneous as well as directed differentiation into mesoderm derivatives. In ongoing work, we are studying the mechanisms of SOX2 repression by the two signaling cascades and the inhibitory role of SOX2 in mesoderm induction. This will result in a better understanding of mesoderm formation, with implications for improving protocols for directed differentiation.

Keywords: human embryonic stem cells; pluripotency; mesoderm; SOX2

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Abstract 57

BMP4 promotes human embryonic stem cells to undergo EMT and mesodermal commitment via SLUG and MSX2

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The transforming growth factor beta (TGF-beta) affects most cell types throughout the body and plays a crucial role in both self-renewal and differentiation of hES cells. Bone morphogenetic proteins (BMPs) are one of the key members of the TGF-beta superfamily that lead to activation of Smad1/5/8 through BMP type I and type II receptors. In addition, BMPs have been shown to mediate differentiation in human embryonic stem cells (hES cells) but by what mechanisms remains unknown. We investigated the role of the TGF-beta superfamily, specifically BMP4, in hES cell early differentiation. The hES cells were stimulated with bFGF and different members of the TFG-beta superfamily and their inhibitors or were adenoviral infected with different constitutively active TGF-beta superfamily receptors. Our microarray expression analysis demonstrated that BMP4 induces differentiation of hES cells by upregulating the well-known epithelial-mesenchymal transition (EMT) transcription factors SLUG and MSX2. Our observations indicate that BMP4 induced differentiation is likely to happen via direct binding of phospho-Smad1/5/8 with SLUG which in turn results in morphological changes of hES cells leading to EMT-like differentiation. Moreover, we showed a co-localization of Smad1/5/8 phosphorylation and SLUG on the edges of the colonies after BMP4 treatment where hES cell undergo differentiation using immunofluorescent staining. We analyzed the migratory behavior of BMP4 treated hES cells in transwell inserts to depict the biological relevance of BMP4 inducing SLUG expression. Indeed, knock-down of SLUG expression prevented BMP4 induced hES cell differentiation, acquisition of migratory properties and therefore EMT on transwell inserts. In the next step, we induced embryoid body formation to analyze cardiomyocyte differentiation. We exhibited that BMP4 is a potent inducer of cardiomyocyte differentiation of hES cells in comparison to TGF-beta and ActivinA which had no effect on the number of beating cardiomyocytes compared to untreated cells. Furthermore, we examined the role of MSX2 in early hES cell differentiation using adenoviral overexpression and lentiviral knock-down constructs. We showed that BMP4 induced MSX2 expression and led to differentiation of the mesodermal lineage. In addition, knock-down of MSX2 in BMP4 treated hES cells resulted in reduced expression of early mesodermal marker BRACHYURY. Our results strongly indicate that BMP4 mediates early human ES cells commitment to the mesodermal lineages via the EMT mediators SLUG and MSX2.

Keywords: human embryonic stem cells; TGF-beta superfamily; mesodermal commitment; SLUG; MSX2

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Abstract 58

Low density lipoprotein receptor-related protein 1 (LRP1) is expressed on radial glia cells and controls their differentiation towards oligodendroglia

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In the developing and adult CNS multipotent neural stem cells reside in distinct niches. Specific carbohydrates and glycoproteins are expressed in these niche microenvironments that are important regulators of cell fate determination and stem cell maintenance. In previous studies we described LewisX (LeX) glycan as a specific marker of neural stem precursor cells (NSPCs) in developing brain and showed it to be involved in NSPCs migration and proliferation. Using LeX glycosylation as a biomarker we aimed to identify the LeX carrier glycoproteins which could have a functional relevance for CNS development. In addition to already known LeX carriers we have found LRP1 as new major LeX carrier and for the first time showed it's expression in NSPCs and developing brain. LRP1 is essential for the normal neuronal function in adult CNS, whereas the role of LRP1 in development is still unclear, mainly due to the lethality of the LRP1 knock-out in mice. In the current study we investigated the basic properties of LRP1 knock-out NSPCs created by means of Cre-loxp mediated recombination. The elimination of LRP1 in vitro was induced by the addition of cell permeable Cre-recombinase to NSPCs derived from embryonic brain of LRP1flox/flox mice. The functional status of LRP1-deficient cells was subsequently studied using proliferation, migration and differentiation assays. LRP1 knock-out cells maintained as neurospheres, retained the ability to migrate and differentiate. Interestingly, LRP1 knock-out NSPCs generated 3-times less OPCs in comparison to LRP1wt/wt cells. This suggests that LRP1 is involved in the control of the oligodendroglial differentiation.

Keywords: LRP1; LewisX; neural Stem Cells; oligodendrocytes; brain development; conditional knock-out

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Abstract 59

Tcfap2c target genes in mouse primordial germ cells

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Transcription factor Tcfap2c (AP-2γ) is a member of the activating protein 2 family of transcription factors. During mouse embryogenesis Tcfap2c is expressed in primordial germ cells (PGCs) from embryonic day (E) 7.25 until E12.5. Lack of Tcfap2c leads to sterile animals, in which PGCs are specified but lost around E8.0. Tcfap2c has been identified as a downstream target of Blimp1/Prdm1, a key determinant of primordial germ cell development. We could demonstrate that in vitro differentiated Tcfap2c-/- PGCs show down-regulation of germ cell markers and an up-regulation of the somatic gene program. To investigate the function of transcription factor Tcfap2c in the transcriptional network of PGCs we used an in vitro differentiation protocol to generate PGC-like cells (PGCLCs). Mouse embryonic stem (ES) cells harboring a Blimp1-mVenus transgene were differentiated by embryoid body (EB) formation. Upon differentiation in EB culture, PGCLCs were identified and sorted by the germ cell specific reporter-signals. Using these in vitro generated PGCLCs we performed whole genome expression analysis and discovered 455 genes which are differentially expressed in Tcfap2c-/- PGCLCs (208 genes down- and 247 genes up-regulated). Interestingly, genes involved in germ line and spermatogenesis (e.g. Stella, Kit, Dmrt1, Rhox4a/ 5/ 6/ 9) are down-regulated and genes known to maintain pluripotency (e.g. Fgf4, Klf4, Eras), epigenetic modifications (e.g. Dnmt3l, Dnmt3b, Mbd2), cell cycle regulation (e.g. Ccnd1, Ccnt2, Cdk6) and somatic differentiation (e.g. Hoxa5, Neurod1, Robo1) are up-regulated in Tcfap2c deficient PGCLCs. These data suggest that Tcfap2c regulates and reinforces the genetic and epigenetic network that is utilized to establish and maintain PGC identity. The experiments indicate that Tcfap2c is an important regulator of PGC maintenance by regulating the cell cycle, epigenetic modifications, activation of germ cell specific genes and inhibition of somatic differentiation.

Keywords: primordial germ cell; Tcfap2c; differentiation; pluripotency

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Abstract 60

Neurogenic potential and HLA expression of human parthenogenetic embryonic stem cells (ESCs)

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Human parthenogenetic (PG) ESCs (hpESCs) are established from blastocysts derived from chemical-activated unfertilized oocytes followed by blockage of meioisis II. hpESCs carry two maternal genomes. Based on substantial cell autonomous differences in brain development of androgenetic and PG mouse ESCs in chimeras (Keverne et al., Developmental Brain Research, 1996) and the high frequency of imprinting in the brain (Wilkinson et al., Nature Reviews Neuroscience, 2007), our question is whether the lack of a paternal genome influences ESC-derived neurogenesis. To directly address this question we differentiated side-by-side human PG and biparental (N, normal) ESCs (Revazova et al., Cloning and Stem Cells, 2007) in vitro into neural stem cells (NSCs). Our analyses so far demonstrated that despite the lack of a paternal genome, hpESCs generate proliferating NSCs that are capable of differentiation into physiologically functional neuron-like cells and maintain allele-specific expression of imprinted genes (Ahmad et al., PLoS ONE, 2012). As miRNAs are known to play important roles in the control of self-renewal, pluripotency and differentiation of hESCs, we performed a global miRNA expression analyses to detect possible differences between PG and N NSCs. As expected, C/D box snoRNAs of the paternally expressed transcription unit SNURF-SNRPN-UBE3A AS (HBII-13, HBII-85, HBII-438A, HBII-438B) were not detected in PG NSCs. Out of 262 differentially expressed miRNAs, 127 (48 %) showed lower expression in PG NSCs. For example, miR-152 and miR-145, which regulate HLA gene expression, are lower expressed in PG compared to N ESC-derived NSCs. To address whether HLA expression is changed in PG NSCs, we performed global gene expression analysis. We observed that the HLA genes HLA-DOB, -DRB5, -DOA, -DOB, -DPB2, -DRA, -DQB2 and -G as well as the antigen-processing machinery components TAP2 and ERp57 are lower expressed in PG NSCs. Despite these differences, FACS and immunocytochemistry showed that hpNSCs present HLA-class I molecules on their surface while no HLA-DR molecules were detected. Functional analyses of HLA-induced proliferation of peripheral blood mononuclear cells (PBMNCs) in a mixed lymphocyte reaction revealed that the HLA molecules on PG similar to N NSCs evoke proliferation of PBMNCs. In conclusion, PG ESCs similar to N ESCs can be differentiated into NSCs and further into functional neurons. miRNA array confirmed the uniparental origin of PG ESCs and their derivatives. Interestingly, mRNA analyses revealed differently expressed HLA family genes in PG and N NSCs, even if both express HLA class I but not class II molecules and are immunoreactive.

Keywords: human parthenogenetic embryonic stem cells; in vitro neurogenesis; miRNA; HLA

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Abstract 61

Comparison of hepatocyte differentiation protocols for human induced pluripotent stem cells

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A variety of hepatocyte differentiation protocols has been published during the last years with the aim to achieve hepatocyte-like cells that resemble mature human hepatocytes. Different efficacies of hepatocyte-like cell protocols, as determined by hepatic markes, e.g. albumin expression, have been reported by individual authors. However, as efficacy may depend on several factors, including the iPS cell clones used for differentiation, a side-to-side comparison of in vitro several differentiation protocols using a fixed set of identical iPS cell clones is needed. We compared three recently reported differentiation protocols using either monolayer cells, embryoid bodies, or monolayer cells cultivated in a matrigel sandwich. Cells were in vitro differentiated for 14 days. In the first differentiation phase up to day 5, various concentrations of Activin A (5-100 ng/ml), FGF2 (10-100 ng/ml), BMP4 (0-10 ng/ml), and DMSO (0-1%) were added. Thereafter, HGF (100 ng/ml) and dexamethosone (0.1 µM) were added for all cells up to day 11, followed by a 3-day incubation with HGF and Oncostatin (15 ng/ml). To analyze the efficacy, we determined marker expression at two time points, at day 5 and day 14. Cells were stained by endodermal marker SOX17 and hepatic marker albumin. We also included gene expression analysis of different genes by quantitative real time PCR (e.g. SOX17, CK18, AFP, albumin, and TTR). iPS cells incubated via the embryoid body protocol seemed to induce endodermal differentiation quite efficiently (>72%) and also expressed increased levels of albumin mRNA as compared to undifferentiated iPS cells. Results will be compared between different iPS cell clones that were derived from patients having inherent liver disease. Theses findings present one step towards informing further strategies to ultimately aid regenerative efforts in the understanding of pathogenesis or hepatic replacement therapies.

Keywords: iPS cells; hepatocyte; differentiation; SOX17; albumin

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Abstract 62

iPS derived neutrophil granulocyte-like cells from the common marmoset (Callithrix jacchus)

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In research of regenerative medicine and cell replacement therapy, induced pluripotent stem (iPS) cells bear high therapeutical hopes. In our lab, somatic cells of a preclinical non-human primate model, the common marmoset (Callithrix jacchus), were recently successfully reprogrammed into induced pluripotent stem (iPS) cells (Wiedemann et al., 2012). Human iPS cells could be already used in the human not only for autologous but also for allogenic transplantations approach with donors presenting highly compatible major antigen combinations. In transfusion medicine, iPS cells are promising candidates for therapies of haematological diseases of the neutrophilic and myeloid cell production like the myelodysplastic syndromes (MDS) or as intermediate immune defence tool for patients which undergo chemotherapy treatment during acute myelogenous leukemia (AML). This purposely disrupts both patients´ hematopoiesis and formation of immunological relevant lymphocytic and myeloid cell types. Rodent models of congenital and acquired diseases are helpful for human disease modelling but due to different pathophysiology they often do not faithfully mimic the situation in the human. Therefore, in this study, we differentiated iPS cells from the common marmoset into neutrophil granulocyte-like cells based on the protocol of Morishima et al. (2010). The first differentiation into CD34+ cells was achieved by cell treatment with differentiation medium containing human embryonic stem cell medium (hESM) and vascular endothelial growth factor (VEGF). Surprisingly, we obtained cell numbers of approximately 35-50% CD34+ cells which were separated by sorting in adequate amount ($5.8x10^5$ +/- $2.0x10^5$ CD34+ cells of $1.4x10^6$ +/- $1.0x10^6$) and high purity at day 13. During further procedure, these cells were cultured in medium containing IL-3, Thrombopoietin (TPO) and Stem Cell Factor (SCF) for 25 days. After the last differentiation step with Granulocyte-Colony Stimulating Factor (G-CSF) for 10 days, the cells displayed high morphologic analogy to native neutrophil granulocytes not only in size (13.71 µm +/- 1.07) and shape of nucleus. Also expression of granulocyte specific surface markers like Interleukin-8-Receptor (CD128), Fcγ-Receptor1 (CD64) and Integrin αM (CD11b) could be detected. Forming specific intracellular granules could be additionally verified by immunohistochemistry and electron microscopy. The differentiated cells showed granulocyte specific function like phagocytosis in a functional assay demonstrating skills similar to native neutrophil granulocytes. Future steps of our research include the large scale production and transplantation of these functional cells into the living animal.

Keywords: common marmoset; iPS cells; granulocyte; differentiation

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Abstract 63

Developmental regulation of L-type calcium current by intracellular magnesium in murine iPS cell-derived cardiomyocytes

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Background: Intracellular magnesium levels have been extensively studied in adult cardiac cells and shown to play a crucial role in heart physiology function (e.g. modulating the activity of L-type Ca2+ channels in mature cardiomyocytes (CMs)). Because mammalian heart undergoes tremendous changes during development, we investigated the concentration-dependent effects of intracellular Mg2+ via pipette solution ([Mg2+]p) on the phosphorylation and dephosphorylation processes of L-type Ca2+ channels during CM-differentiation.

Methods: For this study, an in vitro model of cardiomyocytes based on induced pluripotent stem (iPS) cells was used. The L-type Ca2+ current (ICaL) was recorded in the presence and absence of well-known stimulators or inhibitors of L-type Ca2+ channel pathways. CMs of early and late developmental stage were investigated by whole-cell patch-clamp technique with patch pipettes containing different Mg2+ concentrations.

Results: Our data demonstrate that increasing [Mg2+]p from 0.2 mM to 5 mM markedly reduce the peak of ICaL density, and alter both activation and inactivation properties in early (EDS) and late developmental stage (LDS) of cardiomyocyte differentiation. This observation was accompanied by acceleration and a shift of the steady-state activation and inactivation to more negative membrane potentials in stage-dependent manner. Raising the [Mg2+]p from 0.2 to 2 mM in the presence of cAMP-dependent protein kinase A (PKA) significantly decreased ICaL in EDS (36%) and in LDS (70%) CMs. In addition, the effect of the adenylyl cyclase (AC) stimulator forskolin was greatly attenuated in the presence of 2 mM [Mg2+]p in LDS but not in EDS cardiomyocytes. In the presence of ATP-γ-S the effect of forskolin on EDS and LDS cardiomyocytes was enhanced. The exposure of both EDS and LDS cardiomyocytes to 2 mM [Mg2+]p considerably reduced the effect of isobutylmethylxanthine (IBMX) and okadaic acid (OA) on ICaL. However, no inhibition effect was observed with 0.2 mM [Mg2+]p.

Conclusion: In this study, we not only demonstrate the physiological relevance of cytosolic [Mg2+] affecting the L-type Ca2+ channel activities (phosphorylation/dephosphorylation balance) during cardiomyocyte development, but we also confirm the important interaction between normal intracellular Ca2+ and Mg2+ concentrations in heart function. Therefore, the negative regulation effect of intracellular Mg2+ concentration on L-type Ca2+ channels may, at least in part, explain some of the pathophysiological changes observed in heart failure patients with magnesium deficiency.

Keywords: stem cells and development; cardiomyocytes; L-type Ca2+ channel; intracellular magnesium; phosphorylation/ dephosphorylation

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Abstract 64

The role of Tcfap2c in the development of murine placenta and trophoblast lineage differentiation Tcfap2c - A regulator of embryonic demand vs maternal resources

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In humans and mice, the TCFAP2 (AP-2) family of transcription factors consists of five proteins, which have unique functions during mammalian development and act by controlling the balance between proliferation and differentiation. Deficiency of Tcfap2c causes early embryonic lethality on day 7.5 dpc due to a defect in the extraembryonic compartment. We demonstrated that Tcfap2c is required to specify and maintain the undifferentiated compartment of the extraembryonic lineages and the undifferentiated state of trophoblast stem cells. However, the role of Tcfap2c in placental development has not been studied so far. To this end, we used a conditional Tcfap2c mouse model established by us which was mated to Tpbpa-Cre transgenic mice. This resulted in the loss of Tcfap2c protein in trophoblast derivatives. Preliminary analyses have shown a deficiency in the developing placenta resulting in embryonic death late in gestation. Tpbpa-Cre mediated loss of Tcfap2c leads to arrested growth of the junctional zone in the developing placenta starting from E11.5. Furthermore, the proliferation and terminal differentiation of Glycogen trophoblast cells within the junctional zone seems affected. There is a significant reduction of glycogen content in the transgenic placenta. Trophoblast stem cell lines from these mice have been generated in order to analyze this defect on a molecular level in vitro. Interestingly a transgenic gain of function model developed by us indicates, that several imprinted genes (H19, Phlda2, Ddc, Impact, Slc38a4) might be targets of Tcfap2c. Additionally, H19 and Phlda2 are involved in placental biology displaying a phenocopy of our model when deregulated. Taken together, the crucial role of Tcfap2c might be exerted by a set of imprinted genes in placental development further strengthening the kinship theory by Haig and colleagues. Interestingly, in human pre-eclamptic placentae, deregulated levels of Tcfap2c have been reported. Therefore, our mouse model with conditional deletion of Tcfap2c will further help in understanding the role of Tcfap2c in placental development. We envision that Tcfap2c-levels in the developing placenta serve as a molecular rheostat of placental growth acting as a regulator of embryonic demand vs maternal resources. Low levels would favor a small placenta and restricted resources for the embryo whereas high levels would favor placental overgrowth and overshooting of fetal development.

Keywords: Tcfap2c; tpbpa; placenta development; glycogen; trophoblast stem cells

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Abstract 65

Human multipotent progenitors – a promising cell model for assessing developmental osteotoxicity in vitro

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To assess potential adverse effects of substances on bone development in man, animal studies are usually conducted. However, in vivo testing is labor and cost intensive and requires a high number of laboratory animals. Until today, there exists no validated alternative method to assess developmental bone toxicity in vitro. The derivation of multipotent progenitors with mesenchymal characteristics from human embryonic stem cells (hES-MP, Cellartis) constitutes one promising strategy in regenerative medicine to work with a cell source that exhibits a low risk of tumor formation after transplantation. hES-MP display the typical morphology of primary human mesenchymal stem cells and show a similar gene expression pattern. In addition, they are highly proliferative and have the capacity to differentiate into specialized cell types of mesenchymal origin in vitro (adipocytes, chondrocytes, osteoblasts). Notably, their experimental use in Germany is exempt from regulatory approval enforced by the German Stem Cell Act. Therefore, hES-MP appear to be an attractive and promising human-based cell model to screen for potentially osteotoxic substances. Crucial stages during osteogenesis include the proliferation of progenitor cells, followed by their gradual differentiation into functional osteoblasts, the maturation of the extracellular matrix (ECM) and, ultimately, the mineralization of the ECM. Initial work to study the osteogenic differentiation process of hES-MP has already been accomplished by another research group (de Peppo et al., 2010). Based on their findings, we further characterized the differentiation process regarding the influence of different inducer cocktails, the delineation of the developmental stages and the expression of lineage-specific protein markers. A diverse range of biochemical and molecular biological methods, e.g. cell viability & proliferation assays, colorimetric assays, cytochemical stainings, flow cytometry and western blotting, were employed to monitor the underlying molecular processes. Additionally, we performed initial experiments to investigate the sensitivity of the differentiating cells toward developmental toxicants. In summary, the hES-MP cell model might prove to be a valuable tool for assessing compound mediated adversity on human bone development in vitro.

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Keywords: bone development; developmental toxicity; human stem cells; in vitro test method

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Abstract 66

Reprogramming human somatic cells towards pluripotency and their differentiation to hematopoietic stem and progenitor cells

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At present, somatic gene therapy of hematopoietic stem and progenitor cells (HSPCs) is typically conducted by retroviral vector-based complementation of underlying recessive genetic defects. However, this procedure is associated with a significant risk of therapy-associated leukemogenesis caused by random vector integration into the genome and subsequently altered oncogene expression. Induced pluripotent stem (iPS) cells may represent an attractive alternative to somatic stem cells as they allow for homologous recombination, clonal expansion and differentiation towards HSPCs, in vitro. For iPS cell generation, we transduced human umbilical vein endothelial cells (HUVECs) with a lentiviral vector co-expressing the cDNAs for human transcription factors OCT4, KLF4, SOX2 and MYC linked to a fluorescent reporter protein. Flanking FRT sites allow for the removal of the reprogramming cassette by Flp-recombinase thus preventing reactivation of reprogramming during differentiation. Formation of colonies with embryonic stem (ES-) cell-like morphology was observed approximately 15 to 20 days after gene transfer with an efficiency of about 1%. Upcoming colonies of sufficient size were passaged and expanded similar to human ES cell line H1 (huESC-H1) and silenced the reprogramming vector over time, detectable through the absence of fluorescence. They showed hallmarks of pluripotency such as alkaline phosphatase activity, surface expression of glycolipid antigen SSEA-4, keratin sulfate antigens TRA-1-60 and TRA-1-81, down regulation of major histocompatibility antigen class I (HLA-A, B, C) expression as well as a re-expression of the endogenous transcription factors NANOG, SOX2, OCT4, LIN28A and DNMT3B. Global transcriptome analysis by RNA-seq revealed significant similarity of two iPS clones to the human embryonic stem cell line H1 (huESC). For establishing the conditions for vector excision in human cells by virus particle-mediated protein transduction of Flp-recombinase, a novel huESC reporter cell line was generated and evaluated. As differentiation of human pluripotent stem cells towards HSPCs capable of long-term repopulation in immunodeficient recipient mice has not been established yet, we aimed at optimizing the conditions for hematopoietic differentiation, using two huESC lines (H1 and H9). The huESCs were differentiated using either a two-step protocol with a first Embryoid Body (EB) differentiation phase for 8 days in hBMP4, hActivin A, hbFGF with or without fetal calf serum, dissociation and subsequent co-culture on OP9 stromal cells in medium containing hSCF, hTPO, hFlt3-L, and hVEGF. Alternatively, the huESCs were differentiated directly on OP9 stroma. To this end, the appearance of hemogenic endothelium-like sheets and presumable hematopoietic suspension cells was supported by the two-step protocol and only when fetal calf serum was present continuously.

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Keywords: reprogramming; iPS; pluripotency; embryoid bodies; hematopoiesis

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Abstract 67

Ectopic HOXB4 expression in differentiating pluripotent stem cells promotes hematopoiesis at the hemogenic endothelium stage

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Hematopoietic differentiation of pluripotent stem cells and subsequent expansion of stem and progenitor cells (HSPCs), in vitro, are strongly enhanced by ectopic expression of the homeodomain transcription factor HOXB4. However, to optimize this process and to redundantize the necessity of ectopic HOXB4 expression, a thorough understanding of its activities during embryonic stem cell (ESC) differentiation is necessary. Thus, we investigated its influence on the major fate decision stages of mesoderm specification and hemangioblast/hemogenic endothelium formation during ESC differentiation. For this purpose, we retrovirally introduced HOXB4 into different reporter ESC-lines for constitutive expression or inducible, Tamoxifen-dependent activity. To follow mesoderm specification, we used an ESC-line containing the cDNA for green fluorescent protein (eGFP) integrated into the brachyury gene locus (BryGFP/+; Hans Jörg Fehling). Since Runx1 (AML1) plays a key role in the generation of definitive hematopoiesis at the hemogenic endothelium stage, we also employed an ESC-line with a Venus gene reporter under the control of the proximal P2 promoter of one Runx1 allele (Runx1Venus/+; Shin-Ichi Nishikawa). We differentiated these ESCs as embryoid bodies (EBs) for 6 days and cultured the dissociated cells on OP9 stroma cells under hypoxic growth conditions (3% O2) with appropriate cytokine support (10% FCS, mSCF, hFlt3-L, mTPO, hVEGF). Under the employed culture conditions, constitutive HOXB4 expression inhibited the formation of Bry+ mesoderm between d3 and d4 of EB differentiation. In contrast, after dissociation Runx1Venus reporter EBs on d6 and coculture with OP9 cells for further 5 days, HOXB4 mediated a significant increase in the number of circular, sheet-like structures expressing CD201 (Endothelial Protein C Receptor, EPCR), CD144 (VE-Cadherin), CD31 (PECAM) and being capable of DiI-Ac-LDL uptake. Some of those structures generated Runx1/Venus+ CD41+ suspension cells, thus representing true hemogenic endothelium layers. Noteworthy, HOXB4 did not induce initial Runx1 expression, but promoted the later appearance of a subpopulation expressing increased levels of Runx1, known to correlate with the formation of definitive HSCs. Actually, only this subpopulation contained CD45+ hematopoietic cells. Finally, the combination of conditional HOXB4 activity (Tamoxifen-inducible HOXB4-ERT2) and Runx1 expression (Doxycyline inducible) in a Runx1(-/-) ESC-line (iRunx1; Georges Lacaud) revealed that HOXB4 and Runx1 activities appear to be strongly interdependent during hematopoiesis. Taken together, our results suggest that the homeodomain transcription factor HOXB4 (or possibly any HOX4 paralogue) together with Runx1, licences the transition from hemogenic endothelium to earliest CD41+ hematopoietic cells, at least during pluripotent stem cells differentation, in vitro.

Keywords: HOXB4; hematopoiesis; embryonic stem cells; hemogenic endothelium; Runx1

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Abstract 68

Non-invasive tracking and fate specification of human neural stem cells

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Introduction Stem cells are a promising new therapeutic approach for acute and degenerative disorders of the brain. However, before stem cell-based therapy can be translated to the clinic the fate of these cells needs to be understood in more detail. Cell specific promoters in combination with imaging reporters provide the possibility to track stem cells and to study their cell fate by non-invasive techniques in real-time. Therefore, human neural stem cells (H9 hNSC) were stably transduced to express imaging reporters under the control of either the early neuronal promoter doublecortin (DCX), the mature neuronal promoter synapsin (Syn), or the astrocyte marker (GFAP). Methods Imaging reporters were cloned in a lentiviral vector system under the control of the cell specific promoter DCX, Syn or GFAP. As reporters ferritin (Fer) - for magnet resonance imaging (MRI), firefly luciferase (Luc2) - for bioluminescence imaging (BLI) and copGFP - for fluorescence imaging (FI) were chosen to generate a trimodal vector system. The H9 HNCs were transduced by lentiviral gene transfer, and a stable cell line was selected by the background signal of copGFP via FACS. The newly generated cell lines were named H9 DCX-Fer-P2A-Luc2-T2A-copGFP, H9 Syn-Fer-P2A-Luc2-T2A-copGFP and H9 GFAP-Fer-P2A-Luc2-T2A-copGFP. To prove BLI and FI in vitro, 7.5 x 10ˆ4 cells were seeded in a 4-well plate. 48 hours later, neural differentiation was induced by a specific medium and the withdrawal of growth factors. The expression of Luc2 and copGFP was investigated at day 0, 7, 11, 14 and 18 of neural differentiation by a bioluminescence system (Biospace photon imager) and fluorescence microscope (Keyence BZ-9000), respectively. Results Upon stimulation of neuronal differentiation, the human DCX promoter becomes active and drives the expression of Luc2 and copGFP. Already after 7 days of differentiation an increase of the BLI signal was measured. In total, the BLI signal increased 31-fold during the differentiation period. The copGFP signal was clearly detectable after 7 days of differentiation. These results were in agreement with histological analysis. In case of the H9 cells overexpressing the reporters under the control of the Syn promoter, the BLI signal increased 5-fold from day 0 to 18. A 15-fold signal increase was measured for the cells transduced with the GFAP-Fer-P2A-Luc2-T2A-copGFP construct. In these two cell lines the BLI signal was much lower compared to the H9 DCX-Fer-P2A-Luc2-T2A-copGFP cells. No clear signal from copGFP could be detected during the entire differentiation procedure for the Syn and GFAP promoter cell line versions. Conclusions By the use of a combination of BLI and FI neural differentiation of transduced H9 HNSCs could be studied in real-time. These results generate important information about the structural and functional status of human stem cells. Finally, this improvement is a step forward for cell-mediated functional recovery in acute and degenerative disorders of the brain.

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Keywords: human neural stem cells; doublecortin; synapsin; GFAP; MRI; BLI

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Abstract 69

TGF-beta1 stimulates proliferation and promotes replicative senescence of mesenchymal stromal cells

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Growth factors are required for culture expansion of mesenchymal stromal cells (MSC). Transforming growth factor (TGF)-β1 is a prominent cytokine in mammalian tissues and present in human or animal sera used as cell culture media supplements. It has been shown that TGF-β1, in combination with other growth factors, promotes proliferation and effects differentiation of MSC. On the other hand, several studies indicated, that TGF-β1 induces cell-cycle arrest and cellular senescence in MSC. Furthermore, it is unknown, whether the response to TGF-β1 differs in MSC of early and late passages. In this study, we cultured MSC from human bone marrow with 1 ng/mL TGF-β1 and compared their morphology, in vitro differentiation, proliferation and replicative senescence upon long-term culture to un-treated controls. Treatment with TGF-β1 induced a network-like growth-pattern and impaired adipogenic and osteogenic differentiation. Proliferation of MSC was significantly enhanced by TGF-β1 for the first passages. Typical signs for senescence, such as "fried egg morphology", senescence-associated β-galactosidase activity or cell-cycle arrest, could not be observed in early passages. However, due to the growth promoting effect, the cells entered replicative senescence earlier than the controls. This was also evident when we used our Epigenetic-Senescence-Signature (Koch et al., Aging Cell 2012; Volume 11, Issue 2; pages 366 – 369, April 2012) DNA-methylation analysis at six relevant CpG sites per pyrosequencing reflected advanced cellular aging upon treatment with TGF-β1. To further analyze the molecular sequel of TGF-β1, gene expression profiles were analyzed with Affymetrix 1.0 ST arrays after 0, 1, 4 and 12 h of TGF-β1 treatment on MSC at early (p3-5) and late (p10) passage. Overall, un-treated MSC of early and late passages revealed considerable differences in gene expression, which is in line with our previous data. Nevertheless, TGF-β1 treatment induced very similar gene expression changes in MSC of early and late passage. Notably, gene Gene Onthology and KEGG pathway analysis indicated that the gene expression changes upon replicative senescence and TGF-β1 treatment are related. Our results demonstrate that TGF-β1 has a major impact on MSC function as it stimulates proliferation and accordingly enhances replicative senescence. On the other hand, TGF-β1 has very similar effects on gene expression in MSCs of early and late passages.

Keywords: mesenchymal stromal cells; TGF-beta1; senescence; gene expression

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Abstract 70

Expression profiling of cardiomyocyte surface markers during murine embryonic development and embryonic stem cell differentiation

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In order to establish a method for transgene-free enrichment of pluripotent stem cell-derived cardiomyocytes, we set up a proof-of-concept study to characterize the expression of cardiomyocyte surface markers during embryonic mouse heart development and their relevance for identification of embryonic stem cell (ESC)-derived cardiomyocytes. Based on previously published data we chose three surface markers to detect mouse embryonic cardiomyocytes: CD166 (Alcam), CD106 (VCAM-1) and ErbB2. In contrast to published data on immunofluorescence or transcript analysis, we re-evaluated the expression pattern of CD166, CD106 and ErbB2 between embryonic day (E) 11.5 and postnatal day 3 by flow cytometric analysis of dissociated hearts. At E11.5, CD166 is exclusively expressed on cardiomyocytes, by E17.5 it was down-regulated in cardiomyocytes, but additionally found on non-cardiomyocytes. Furthermore, we confirmed expression of CD106 on embryonic and neonatal cardiomyocytes, which progressively declined during development. The antibody against ErbB2 labeled cardiomyocytes at all investigated developmental stages. Next, we evaluated the expression of these surface markers on mESC-derived cardiomyocytes at day 10 of differentiation. Corresponding to the E13.5 stage, CD166 expression was found on a distinct subpopulation of mESC-derived cardiomyocytes. In contrast to this, ErbB2 was expressed on a wide range of mESC-derived cell types, including cardiomyocytes. CD106 expression was not detectable on mESC-derived cardiomyocytes. As none of the described surface markers displayed exclusive expression on mESC-derived cardiomyocytes, we developed a non-myocyte depletion strategy and were able to significantly enrich mESC-derived cardiomyocytes and to remove residual Oct4-positive cells. Next steps include the translation of magnetic cell separation strategies to antibiotic-free differentiation protocols enabling an in-depth characterization of enriched mESC-derived cardiomyocytes.

Keywords: cardiogenesis; cardiomyocyte differentiation; surface markers

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Abstract 71

Metabolic regulation of C2C12 myoblasts by inhibiting the pro-myogenic effect of p38 activation

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Skeletal muscle stem cells, so called satellite cells (SCs), lie resting under the basal membrane of muscle tissue and play a major role in skeletal muscle adaptation. Once activated, SCs are called myoblasts and proliferate before initiating the differention program resulting in muscle hypertrophy and/or hyperplasia. Therefore, SCs are a putative target for the treatment of atrophic muscle diseases. Lactate (La) has long been considered as a waste product of energy metabolism causing a decrease in muscle pH and hence muscle fatigue. Now it is known that La is rather an intermediate of glucose metabolism and has been termed Lactormon for its signalling properties inducing gene expression for skeletal muscle adaptation. It was shown to increase MCT content as well as mitochondrial biogenesis. Understanding the effects of La on proliferation and differentiation will add important knowledge to the possible treatment of muscle wasting diseases with physical as well as pharmaceutical interventions that target the involved signalling pathways. The MAPK p38 is a major signalling molecule. In SCs it has been demonstrated that p38 has a pro-myogenic effect by inhibiting Pax-7 expression via epigenetic modification of histone 3 (H3K4me3 and H3K27me3), activating muscle regulatory transcription factors (e.g. Myf5, MyoD, myogenin) and inducing the expression of muscle specific genes (e.g. myosin heavy chain [MHC]). METHODS: C2C12 mouse myoblasts were incubated intermittently for 2 hours daily with differentiation medium containing La over a time period of 5 days with relevant La concentrations (10 mM and 20 mM) simulating a training situation. After the treatment cells were PFA-fixed for staining for Ki67, activated Caspase-3, F5D (myogenin), Mf-20 (MHC), H3K4me3, and H3K27me3. Additionally, cell lysates were made for Western Blot analysis using the same markers as well as Pax7, Myf5, and MyoD. Furthermore, cells were incubated with La for up to 24h to investigate the acute effects of La on intracellular p38 activation. Moreover, cells were treated for 2h with La and RNA was isolated 6h after the end of treatment. Using RT-PCR, gene expression of Myf5, MyoD, myogenin, and MHC I and II was determined. RESULTS: Analysis of cells stained for Ki67 showed a significant decrease of myoblast proliferation if treated with La. Vice versa, apoptotic induction by activated caspase-3 was increased. Terminal differentiation was shown to be delayed if cells received the La intervention as protein content of myogenin and myosin heavy chain was decreased in treated samples. This finding is supported by the down-regulation of myogenin and MHC gene expression after La treatment. Gene and protein expression of Myf5 and MyoD seem unaffected. p38 activation was reduced by La. H3K4me3 is strongly increased after La treatment, and H3K27me3 was only detectable in treated cells. CONCLUSION: Lactate seems to influence the proliferation and inducing apoptosis in C2C12 myoblasts. Furthermore, terminal differentiation seems to be delayed. These results suggest an important role of La in the regulation of muscle hypertrophy and/or hyperplasia. p38 is at least partly responsible, possibly via epigenetic modification of muscle specific genes.

Keywords: myoblasts; myogenesis; lactate; p38 MAPK

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Abstract 72

Connexin expression is required for the differentiation of mouse embryonic stem cells in vitro

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The Connexins (Cx) 43 and Cx45 are coexpressed during pregastrulation development, form gap junctional channels and contribute to the establishment of defined communication compartments in the early mouse conceptus. However, an essential function of gap junctions could not yet be demonstrated at this developmental stage. Here we describe the generation of Cx43/Cx45 double deficient mouse embryonic stem cells (mESCs), which were differentiated into embryoid bodies (EBs), an in vitro model for pregastrulation and early gastrulation. While Cx deficient mESCs did not show any obvious phenotypic abnormalities regarding cell proliferation or apoptosis, we found that the expression of Cx43 and Cx45 is required for the establishment of primitive endoderm. Lentiviral overexpression of either Cx43 or Cx45 rescues the observed phenotype in Cx43/45 deficient mESCs, indicating a redundant function of these isoforms during the process. Defective formation of primitive endoderm leads to a block in subsequent differentiation events e.g. germ layer specification. Viral overexpression of the gap junction channel inactive mutant Cx43G138R in Cx43/Cx45 deficient ESCs did not rescue the differentiation block suggesting that functional gap junctional communication is required for the proper differentiation of mESCs in 3-dimensional cell aggregates.

Keywords: connexin; gap junction; primitive endoderm; differentiation; embryoid body

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Abstract 73

P2 receptor signalling controls human mesenchymal stem cells differentiation towards vascular cell lineages

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Human mesenchymal stem cells (hMSCs) are an interesting source for Regenerative Medicine due to their multipotency. They are particularly attractive for cardiovascular tissue engineering owing to the recent findings showing their ability to differentiate towards endothelial and smooth muscle cell lineages. Our recent publication has shown for the first time that purinergic 2 (P2) receptors are key players during hMSC differentiation towards adipocytes and osteoblasts. P2 receptors can bind to extracellular nucleotides and participate in a series of cellular processes e.g. cell proliferation and migration. However, their functional role in endothelial and smooth muscle cell differentiation is still unknown. Endothelial and smooth muscle-like cells were differentiated from human MSCs and characterized by specific markers via RT-PCR, Western blot and immunochemical stainings. Interestingly, some P2 receptor subtypes were found to be differently regulated during these specific lineage commitments. The administration of natural and artificial P2 receptor agonists and antagonists had a direct influence on these differentiations. Moreover, a feedback loop via exogenous extracellular nucleotides on these particular differentiations was shown by apyrase digest. Taken together, P2 receptors play a crucial role during the differentiation towards endothelial and smooth muscle cell lineages. Some highly selective and potent artificial P2 ligands can control hMSC differentiation, which might improve the use of adult stem cells in cardiovascular tissue engineering in the future.

Keywords: human mesenchymal stem cells; purinergic receptors; endothelial cell differentiation; smooth muscle cell differentiation

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Disease Modeling

Abstract 74

The Parkinson's disease associated LRRK2 mutation R1441G inhibits neuronal differentiation of neural stem cells

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Mutations in the leucine-rich repeat kinase 2 (LRRK2) gene cause familial as well as sporadic Parkinson's disease that is characterized by an age-dependent degeneration of dopaminergic neurons. LRRK2 is strongly expressed in neural stem cells (NSCs), but yet the exact molecular function of LRRK2 in these cells remains unknown. By performing a systemic analysis of the gene expression profile of LRRK2 deficient NSCs we found that the expression of several Parkinson's disease associated genes, like chaperone formation as well as oxidation & reduction in mitochondria, are deregulated upon LRRK2 absence. Furthermore, the lack of LRRK2 leads to an upregulation of neuronal differentiation inducing processes, including the TRIM32/ Let-7a pathway. On the other hand the constitutive mutant of LRRK2(R1441G), known to cause Parkinson's disease, leads to downregulation of the same pathway. In agreement with the function of TRIM32 and Let-7a during neuronal differentiation, LRRK2 deficient NSCs differentiate faster than wild type cells, while LRRK2(R1441G) expressing NSCs show impaired neuronal differentiation. These results might help to better characterize the molecular mechanisms underlying the role of LRRK2 in NSCs and would further improve potential cell replacement strategies as well as drug discovery approaches.

Keywords: NSCs; Parkinson's Disease; LRRK2; differentiation; Let-7a

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Abstract 75

Automated proliferation and differentiation of small molecule neural stem cells (smNPCs) as platform technology for high-throughput neurodegenerative disease modeling

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The discovery of induced pluripotent stem cells (iPSCs) and the generation of in vitro disease models are poised to revolutionize drug discovery for neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD). However, these models are based upon differentiated neuronal subtypes, and, currently, directed differentiation protocols for these lineages are cumbersome, time consuming, involve frequent splitting or manual handling steps, and often result extremely heterogeneous cultures. Because drug discovery using high-throughput screening (HTS) requires a minimum of variation to obtain suitable signal-to-noise ratios, an improved technology platform is essential for the automation and scale-up of iPSC-based models of neurodegeneration. Recently, we derived human neural progenitor cells using only small molecules (smNPCs), which can be very efficiently differentiated into peripheral neurons, midbrain dopaminergic neurons (mDANs) and motor neurons (MNs). We demonstrate that smNPCs derived from patients with mutant LRRK and mutant SOD1 recapitulate aspects of PD and ALS, respectively. We demonstrate that smNPCs are robust, exhibit immortal expansion, form homogeneous cell-line-like adherent cultures, and do not require extensive manual culture and selection steps. These characteristics make smNPCs ideally suited for scale-up and HTS. We demonstrated this by conducting an automated screen for factors modulating smNPC proliferation and differentiation in 96 as well as 384 well plates. In addition, we were able to differentiate smNPCs into mature MNs and mDANs using only automation. Therefore, smNPCs are a platform technology that uniquely enables automated screening on iPSC-based models of neurodegenerative diseases such as PD and ALS.

Keywords: ALS; Parkinson's Disease; disease modeling; high throughput screening; dopaminergic neurons; motor neuron differentiation; neural precursor cells

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Abstract 76

Direct differentiation of patient IPS cells into self-renewing neural progenitors by small molecules to model mitochondrial diseases

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Here, we report a rapid and feasible method to derive self-renewing neural progenitor cells (NPCs) from human pluripotent stem cells (PSCs). With an approach adapted from Li et al. (PNAS, 2011), similar results could be obtained for human embryonic stem cells (ESCs) and human induced pluripotent stem cells (iPSCs) generated from fibroblasts of three patients and control individuals. All patients carry a mitochondrial DNA (mtDNA) mutation in MT-ATP6. Mutations in this gene -encoding a mitochondrial ATP synthase subunit- are associated with neurodegenerative disorders ranging from adult-onset NARP (Neurogenic weakness, Ataxia, and Retinitis Pigmentosa) to severe infantile Leigh syndrome (LS). Importantly, we were able to generate multiple iPSC clones employing episomal plasmid-based reprogramming and thus avoiding traditional viral-mediated transgene delivery. All patient iPSCs still displayed the same mutational load in mtDNA as their parental fibroblasts, even upon prolonged cultivation. Our protocol exhibits major advantages in comparison to standard methods of obtaining NPCs. First, it does not require the formation of embryoid bodies (EBs). Second, it is operator-independent, as it bypasses the need for tedious manual isolation of neural rosettes. A combination of human leukaemia inhibitory factor (hLIF), a GSK3ß inhibitor (CHIR99021), and a TGFß inhibitor (SB431542) in chemically defined media was sufficient to induce the conversion of iPS cells to highly proliferating SOX2 and NESTIN-positive NPCs (98 %). The obtained NPC monolayer population shows distinct morphological changes within a very short time (7-10 days) and could be cultured over several passages without loss of proliferation. Moreover, NPCs derived from various patient iPSC lines underwent in-depth characterization and were capable of differentiating into neural as well as glial subtypes, which was confirmed by expression of markers like TUJ1, HB9, ISL1, GFAP, and CNP. Additionally, patient-derived NPCs were subjected to metabolic analysis, e.g. ATP quantification and Seahorse-based bioenergetic profiling. In these assessments, we could observe responsiveness of NPCs to dihydrolipoic acid (DHLA), an oxygen radical scavenger currently being tested for the treatment of NARP patients (Couplan, PNAS, 2011). DHLA was capable to improve respiratory activity in patient NPCs to nearly 2-fold and to restore mitochondrial functionality upon conditions mimicking glucose shortage, which represents a potential cause of decompensation in NARP/LS patients. Thereby, a novel method of determining the metabolic state of neural tissue could be established, potentially enabling the study of mitochondrial encephalopathies and unravel their underlying molecular mechanisms. Overall, NPCs from patient iPSCs represent an inexhaustible source of neurogenic tissue. We previously found that retroviral-mediated reprogramming may result into mtDNA sequence rearrangement (Prigione et al, Stem Cells 2011). Hence, non-viral generation of iPSCs, followed by small molecule-based derivation of NPCs may represent an advantageous strategy to establish faithful neuronal disease models with positional effect-free phenotypes, thus guarantying reproducibility and genomic stability.

Keywords: iPSCs; neurodegenerative disease; NPCs; mitochondrial disease; neuronal differentiation

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Abstract 77

A human iPS cell-based model for autosomal dominant hereditary spastic paraplegia

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Hereditary spastic paraplegia (HSP) is a rare, heterogeneous group of genetic disorders with progressive spasticity in the lower limbs caused primarily by axonal degeneration of corticospinal motor neurons. The most frequent type of autosomal dominant paraplegia, spastic paraplegia 4 (SPG4), is found in 40% of all HSP cases. SPG4 is caused by mutations in the SPAST gene, which codes for the protein spastin. Research on HSP is complicated by limited access to patient-derived primary neurons. This obstacle may be overcome with the use of induced pluripotent stem cells (iPSCs). iPSCs generated by transcription factor-mediated reprogramming of, e.g., adult skin fibroblasts have the potential to proliferate continuously and to give rise to any somatic cell type, including neurons of the central nervous system. Thus, in principle, iPSCs could provide an unlimited source of patient-specific cells for in vitro disease modeling. Here we report the generation of motoneurons from SPG4 patients. To this end, fibroblasts of family members carrying a specific SPAST nonsense mutation were reprogrammed to a pluripotent state employing retroviruses or non-integrating Sendai viruses encoding OCT4, KLF4, SOX2 and c-MYC yielding several fully validated, SPG4 iPSC lines. Upon neuralization, the cells can be further differentiated into spinal motoneuronal cultures containing up to 50% HB9-positive cells. These iPSC-derived motoneuronal cultures exhibit expression of the short spastin isoform and the disease-related long spastin isoform. Motoneuronal cultures express overall a higher spastin protein level compared to fibroblasts and iPSCs. Surprisingly, there is no significant difference of spastin expression levels in SPG4 fibroblasts and iPSCs compared to controls. However, SPG4 motoneuronal cultures fail to up-regulate spastin protein to the level of control cultures. To gain more insight into the HSP phenotype, iPSCs were differentiated into telencephalic cortical cultures. These cultures contain >74% glutamatergic pyramidal-like neurons, express the layer V and VI markers CTIP2 and TBR1 and should thus permit assessment of the SPG4 phenotype in corticospinal motoneurons. Overall, we expect these neuronal culture systems to provide insight into the pathomechanisms underlying neuronal degeneration in HSP and to eventually enable the identification of therapeutic compounds for the treatment of this disease.

Keywords: HSP; iPS; neuronal differentiaton

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Abstract 78

Deciphering the role of α-synuclein in the pathogenesis of multiple system atrophy using induced pluripotent stem cell-derived neural cultures

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Multiple system atrophy (MSA) is a neurodegenerative disease characterized by oligodendroglial cytoplasmic inclusions (GCIs) mainly composed of α-synuclein (SNCA). SNCA is ubiquitously expressed in all regions of the brain, accounts for up to 0.1% of total brain proteins and is most abundantly located in presynaptic nerve terminals. The factors promoting expression and aggregation of SNCA are considered important contributors to the pathogenesis of MSA. To analyze possible pathomechanisms of MSA directly in human neural cell types, we reprogrammed fibroblasts from patients suffering from MSA and their healthy siblings into induced pluripotent stem cells (iPSC) and further differentiated these cells into gliogenic neural stem cells (glioNSC). GlioNSC express markers characteristic of neural stem/radial glial cells including nestin, SOX2, BLBP, GLAST, 3CB2, musashi-1 and vimentin. They can differentiate into neurons and glia and exhibit prominent expression of region-specific transcription factors typically found in the posterior hindbrain and the anterior spinal cord such as GBX2, EGR2, IRX3 and several HOX genes. They further express NKX6.1/NKX6.2, OLIG2 and SOX9, which are important transcription factors for oligodendrocyte specification and differentiation. SNCA protein was found in the cytoplasm of glioNSC-derived neurons and oligodendrocytes. RT-PCR analysis, however, demonstrated that neurons but not oligodendrocytes express SNCA mRNA, suggesting a passive uptake of SNCA by oligodendrocytes. Presence of SNCA protein in the absence of SNCA transcripts was observed in both MSA-specific and control oligodendrocytes. To elucidate the mode of SNCA transfer we are currently investigating several cellular transport mechanisms, i.e. endosome/exosome formation and clathrin-mediated exocytosis. To mimic possible pathomechanisms of SNCA aggregation we employed induction of oxidative stress as well as mitochondrial and proteasome inhibitors, which lead to an increase in intracellular SNCA aggregation in patient-specific and control cells as demonstrated by Thioflavin S staining. We further developed a transplant paradigm, which enables the long-term analysis of iPSC-derived patient-specific human neural cells in adult immunodeficient mice with the aim to detect disease-associated phenotypes in the context of native brain tissue. We expect these iPSC-based experimental tools to provide insight into the pathomechanisms underlying MSA-associated SNCA aggregation and oligodendrocyte death.

Keywords: Multiple System Atrophy; alpha synuclein; aggregates; oligodendrocyte; disease modelling

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Abstract 79

An efficient TALEN-based system for generating knock-out human pluripotent stem cell lines and disease models

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Human pluripotent stem cells (hPSCs), present an important model system for developmental biology and disease modeling. However, various experimental approaches require targeted genome editing, which remains a difficult and inefficient process in the cells. Transcription activator-like effector nucleases (TALENs) have emerged as a novel tool to facilitate gene targeting and have the potential to overcome these drawbacks. The modular architecture of TAL effectors theoretically enables targeting of any genomic locus and several cloning systems for custom TALEN assembly have recently been established. However, there is a lack of versatile TALEN expression systems applicable to hPSCs. We have extended an existing TALE assembly system by a dual set of new expression vectors for efficient application of TALEN technology in hPSCs. Our system is characterized by improved TALEN architecture as well as antibiotic resistance and fluorescent reporter cassettes, thus enabling enrichment for transfected cells. We demonstrated the functionality of our improved system by targeted disruption of the HPRT1 gene to create isogenic disease models of Lesch-Nyhan-Syndrome. Using female hPSCs, homozygous disruption of HPRT1 occurred at efficiencies of up to 15%. Differentiating knock-out cells both into central (CNS) as well as peripheral nervous system (PNS) neurons recapitulated previously described phenotypes based on patient-specific induced PSCs and extended these findings to the PNS system, respectively. Our combined vector system hence allows for the flexible generation of knock-out hPSCs lines, thus enabling studies of developmental processes as well as the generation of isogenic disease models without the need for patient material.

Keywords: human pluripotent stem cells; TALE nucleases; targeted genome editing; gene disruption; disease modeling

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Abstract 80

In vivo imaging of neurodegeneration, neuroinflammation and adult neural stem cell properties in a 6-OHDA Parkinson's disease mouse model

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Introduction: Parkinson's disease (PD), the second most common neurodegenerative disorder, is caused by a slow degeneration of dopaminergic neurons in the substantia nigra (SN). The subventricular zone (SVZ) of the adult brain contains adult neural stem cells and therefore could serve as a source for endogenous regeneration. However, neurodegeneration (ND) is accompanied by neuroinflammation (NI) affecting regeneration processes. This study aims to non-invasively monitor ND, NI, and stem cell migration in a mouse model of PD by multi-modal molecular imaging employing small animal single photon emission computed tomography (SPECT), positron emission tomography (PET), magnetic resonance imaging (MRI), and in vivo bioluminescence imaging (BLI).

Materials & Methods: Degeneration of the nigrostriatal system was induced by unilateral 6-hydroxydopamine (6-OHDA) injection into the left SN of C57Bl6 mice. Controls were infused with NaCl. Two to three weeks post injection, neuroinflammation was assessed by [18F]DPA-714-PET followed by [123I]Ioflupane-SPECT to determine severity of nigrostriatal degeneration. T2-weighted MRI was performed in order to obtain anatomical information. A lentiviral-based reporter construct carrying the firefly luciferase and mCherry genes under the control of the cytomegalovirus promoter were injected into the SVZ of FVB mice in order to follow progenitor cell migration using BLI (n=5). Reporter constructs driven by differentiation-stage specific promoters have been constructed. Activity and specificity of promoter regions is currently being tested in mouse neural stem cells under maintenance conditions, as well as under conditions inducing neuronal differentiation.

Results: Three weeks after neurotoxin injection, SPECT scans revealed a strong decrease in dopamine transporter ligand accumulation in the left striatum, compared to the normal right striatum (n=15). PET with the TSPO ligand [18F]-DPA-714 indicates an increase in tracer accumulation in the 6-OHDA-lesioned SN two weeks post injection compared to the control region (n=16). Ionized calcium-binding adapter molecule 1 staining verified the inflammatory response. Three weeks after reporter vector injection into the SVZ, first progenitor cell migration towards the OB could be observed using BLI.

Conclusion: Induced neurodegeneration of the nigrostriatal system leads to acute neuroinflammation, which both can be assessed by multi-modal molecular imaging. Migration of neural progenitor cells towards the olfactory bulb can be followed using BLI in the living animal. In the future, stem cell differentiation-stage specific promoters will be used to visualize the influence of ND and NI on the interplay of stem cell proliferation, migration and differentiation in vivo.

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Keywords: Parkinson's disease; neuroinflammation; neurodegeneration; migration; in vivo imaging

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Abstract 81

Spinal cord regeneration: Immune response after transplantation of human umbilical cord blood-derived unrestricted somatic stem cells (USSC)

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Transplantation of stem cells into the injured spinal cord has been considered as a potential therapy for repair due to their ability for cell replacement. However, beneficial effects have mostly been demonstrated without any lineage-specific differentiation or obvious cell replacement. Transplanted stem cells are discussed to release growth promoting factors or modulate the inflammatory response, providing neuroprotection or a permissive environment for regenerating axons. Unrestricted somatic stem cells (USSC) are a well defined stem cell subpopulation derived from human umbilical cord blood (hUCB) which can be isolated under GMP conditions and which has no tumorigenic potential after transplantation [1]. Recently, it has been shown that transplantation of USSC into an acute rat spinal cord injury (SCI) model significantly enhances spinal cord repair including long-term improved functional locomotor recovery, axonal regeneration and enhanced tissue preservation [2]. Interestingly, USSC are known to modulate T-cell proliferation as well as dendritic cell differentiation and function in vitro [3]. We are interested in the immunomodulatory role of USSC after transplantation into the injured rat spinal cord as USSC express pro- and anti-inflammatory cytokines which, in consequence, can lead to tissue repair and stimulation of axon regeneration. We could demonstrate that local transplantation of USSC into the injured spinal cord leads to upregulation of IL-1β, TNFα and TGF-β1 evaluated in short term experiments by quantitative real time-PCR. It is already known that these molecules are regulators for macrophage and lymphocyte responses after spinal cord injury. Interestingly, macrophages can promote regeneration by expression of growth factors thus stimulating axon regeneration and remyelination [4,5]. We suggest that USSC regulate the macrophage polarisation after transplantation into the injured spinal cord as the markers for alternatively activated M2 macrophages (Arginase 1, CD206) are preferentially upregulated compared to classically activated pro-inflammatory M1 macrophage markers (CD86, CD16). Next step now is to investigate different immune cell populations isolated from injured spinal cord after USSC transplantation by FACS analysis. Specific expression profiles will be used to characterize the immunomodulatory role of USSC in the injured spinal cord.

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Keywords: immunomodulation; somatic stem cells; spinal cord injury; transplantation; USSC

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Abstract 82

Human inferior turbinate stem cells (ITSCs) - novel stem cells for the treatment of Parkinson's disease?

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Adult human multipotent neural crest-derived stem cells (NCSCs) harbor a great potential for autologous cell replacement therapies since they have a broad potential and persist into the adulthood within the human body. The ideal adult stem cell source for cell replacement therapies has to satisfy at least the criteria of easy accessibility, a high yield and purity of the isolated stem cells and cultivation without animal-derived medium components. We isolated and characterized a novel human neural crest-derived stem cell population within the respiratory mucosa of inferior turbinates (located within the nasal cavity). High amounts of source material could be isolated even in older patients using minimally-invasive surgery methods. Within their endogenous niche, inferior turbinate stem cells (ITSCs) expressed high levels of specific stem cell markers like nestin. Immune transmission electron microscopy using anti-p75 antibodies identified ITSCs as non-myelinating Schwann cells of glial origin. In vitro differentiation assays demonstrated the extraordinary plasticity of this stem cell source. ITSCs could be differentiated into cells with neuro-ectodermal and mesodermal phenotype, including adipogenic, chondrogenic and osteogenic cell types as well as neurons. Whole genome microarray analysis showed pronounced differences to human ES cells in respect to pluripotency markers Oct4, Sox2, Lin28 and Nanog, whereas expression of Klf4 and c-Myc was nearly similar. Therefore a 4-factor (Oct4, Sox2, Klf4 and c-Myc) reprogramming approach was performed. iPS cells could be picked and expanded after 12 days. First in vivo transplantation experiment using chicken embryos as host showed that ITSCs were able to survive and performed neural crest typical chain migration. In a current approach the therapeutically effect of ITSCs in a Parkinson rat model is under investigation. Therefore, ITSCs were transplanted into the striatum of unilateral lesioned 6-hydroxydopamine (6-OHDA) rats. Preliminary results showed improved motor functions after transplantation determined by rotational analyses. After 12 weeks ITSCs could be detected within the striatum and the substantia nigra demonstrating the survival, integration and migration of these cells. Further experiments will show if ITSCs can be an alternative cell source for cell replacement strategies for the treatment of Parkinson's disease. Additionally, the combined transplantation of ITSCs and ITSC-iPS cells may be an interesting research project.

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Keywords: neural crest-derived stem cells; inferior turbinate stem cells; 6-OHDA rat model; Parkinson's disease

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Abstract 83

Modulation of sodium channel expression in human Dravet syndrome-specific neurons

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Dravet syndrome (DS) is a severe neurological disorder characterized by congenital epilepsy, which is mostly caused by haploinsufficiency of Nav1.1, a pore-forming α-subunit of voltage-gated sodium channels. To model the disease in patient-specific neurons, we have generated DS-specific human induced pluripotent stem cells (hiPSCs) and differentiated into long-term self-renewing neuroepithelial stem (lt-NES) cells to establish a stable expandable neural precursor population. Upon growth factor withdrawal, DS lt-NES cells differentiate into neurons that express significantly less Nav1.1 protein in comparison to unaffected controls. The results of recent studies suggest that the intracellular domain of an accessory channel subunit (β2) might act as a positive regulator of Nav1.1. The aim of our study was to explore whether ectopic β2-ICD expression can induce upregulation of Nav1.1 in human DS-specific neurons. To this end, a patient-derived lt-NES cell line was transduced with a lentiviral doxycycline-inducible construct encoding β2-ICD fused to GFP (green fluorescent protein) via a 2A peptide for co-translational cleavage. The polyclonal line was sorted for GFP+ cells to near purity, and autocatalytic 2A cleavage could be confirmed by Western blot analysis. Localization of transgenic β2-ICD to the nucleus could be verified by 3D microscopy using specific antibodies against V5-tagged β2-ICD. The β2-ICD transgenic cell line maintained the expression of typical neural stem cell markers such as Dach1, Nestin, PAX6, PLZF, SOX1, SOX2 and ZO-1 and was able to differentiate into GABAergic neurons, the most relevant neuronal subtype in DS. After doxycycline-induced transgene expression a significant increase in Nav1.1 protein levels was detected in six week-old neuronal cultures by Western blot analysis compared to control cultures. These results indicate that β2-ICD acts as regulator of cellular sodium channel homeostasis in primary human neurons and that its ectopic expression may compensate for Nav1.1 deficiency in DS-specific neurons.

Keywords: Dravet syndrome; sodium channel; neural differentiation

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Abstract 84

Induced neural stem cells (iNSC) accomplish long-term survival and integration in the adult brain

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Neural stem cells have the ability for self-renewal and for the generation of differentiated cells like neurons or astrocytes. Therefore, neural stem cells have an enormous potential for regenerative therapies for neurodegenerative diseases like Parkinson's disease. We recently have shown that mouse fibroblasts can be programmed into neural stem cells; these cells are called induced neural stem cells (iNCS). Importantly iNSCs are generated without passing through a pluripotent stage. However, in order to be relevant for cell replacement therapies long-term survival, differentiation and integration of iNSCs and their progeny is of crucial importance. To address this issue we transplanted GFP labeled iNSCs into the rostral migratory stream (RMS) and the hilus of the dentate gyrus and conducted their analysis six month after transplantation. In order to minimize immune rejection we used NOD.SCID mice. Firstly, we analyzed the transplanted iNSCs for different stem cell and cell cycle markers. Immunohistological analysis revealed that after six month none of the transplanted cells express stem cell markers. Moreover, overgrowth of the graft did not occur, although a solid survival of the graft was observed. Therefore, we conclude that the tumorigenic potential of iNSCs is minimal. Next we analyzed their differentiation potential. Neural stem cells have the ability to differentiate into neurons, astrocytes and oligodendrocytes. Consequently, for the iNSCs we were able to detect transplanted cells expressing the neuronal marker NeuN, the astrocyte marker GFAP and the oligodendrocyte marker Olig2. Interestingly, the morphology of differentiated neurons was as complex as of endogenous cells by showing a typical neuronal appearance with branched dendrites and elongated axons. These cells perfectly integrated into the host tissue as demonstrated by the formation of synaptic connections with endogenous cells. In summary, long-term transplanted iNSCs demonstrated in vivo differentiation and migration behavior similar to endogenous NSC. Therefore, these cells might be interesting tools for future personalized cell replacement therapies.

Keywords: direct reprogramming; induced neural stem cells; long-term transplantation

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Abstract 85

A stem-cell-based phenotypic assay identifies compounds that protect human neurons from degeneration

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Because the population of the world is living longer than before, the prevalence of neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS) and Parkinson's disease, is expected to increase. However, the treatments currently available for these disorders are generally ineffective. Consequently, better therapeutics are urgently needed. Aberrant activation of microglial cells, the resident immune cells of the central nervous system, plays an important role in the pathogenesis of neurodegenerative diseases including ALS. We generated an in vitro model of microglial-induced neurodegeneration using stem cells. Because stem cells are able to self-renew and differentiate, they can produce theoretically limitless numbers of functional neural cells. As a result, our model is scalable and can potentially be used in high-throughput screening (HTS) campaigns. To validate our model in preparation for HTS, we performed a pilot screen of more than 10,000 small molecules. We identified 12 hit compounds, which acted through diverse mechanisms including the inhibition of nitric oxide production by microglia, activation of the Nrf2 pathway in microglia and astrocytes, and direct neuroprotection. We confirmed that one class of compounds directly protected human neurons from degeneration in response to nitric oxide. In addition, we validated that NRF2 activation in human midbrain dopaminergic neurons protected them from Parkinsonian neurodegeneration. These results indicate that our hit compounds could be ideal starting points for the development of new drugs to treat various neurodegenerative and neurological diseases.

Keywords: stem-cell-based phenotypic screening; microglial neurotoxicity; hit compounds acting through multiple pathways; Nrf2 pathway activation; confirmation on human neurons

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Abstract 86

Restoration of macrophage function by gene correction of CSF2RA deficient patient-derived induced pluripotent stem cells as a therapeutic model for Pulmonary Alveolar Proteinosis

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Introduction: Pulmonary Alveolar Proteinosis (PAP) due to a deficient GM-CSF/IL-3/IL-5 receptor (CSF2R) constitutes a severe lung disease caused by the functional insufficiency of alveolar macrophages, which require GM-CSF signalling for terminal maturation and intracellular processing of phospholipids. Standard treatment options for PAP are limited, however, recently intratracheal (i.t.) transplantation of healthy monocyte/macrophages (M/M) has been suggested as an effective therapy. Thus, we here have evaluated the suitability of patient-specific, iPSC-derived M/M for functional disease modelling and, following gene correction, as a donor source for i.t. transplants.

Methods & Results: Patient-specific PAP-iPSC were generated from CD34+ bone marrow cells of a GM-CSF α-chain (CSF2RA)-deficient PAP patient utilising lentiviral, OCT4/SOX2/KLF4/c-Myc-based reprogramming. Three bona fide iPSC clones were obtained characterized by SSEA4/Tra1-60 expression, endogenous OCT3/4, SOX2, and NANOG reactivation, OCT3/4-promoter demethylation, three germlayer differentiation capacity, as well as lack of chromosomal abnormalities on fluorescence-R banding and array-CGH analysis. Hematopoietic differentiation of these PAP-iPSC clones yielded M/Ms of typical morphology and surface phenotype (CD14+, CD11b+, CD45+). While basic inflammatory functions such as IL-6 secretion remained intact in these M/Ms, GM-CSF dependent functions such as CD11b activation, GM-CSF uptake, phagocytosis, and CSF2R-downstream signalling (STAT5 phosphorylation) were markedly impaired when compared to control M/Ms derived from H9-ESCs. This phenotype faithfully reproduced the defects observed in M/Ms derived from the peripheral blood of CSF2R-deficient PAP patients. Furthermore, when PAP-iPSCs were transduced with SIN-lentiviral vector expressing a codon-optimized CSF2RA-cDNA from a combined ubiquitous chromatin opening element/ EFS1a-promoter sequence (A2UCOE/EFS1a), stable and longterm (> 5 weeks) CSF2RA-expression was observed in pluripotent cells, while iPSC growth, pluripotency, and differentiation capacity remained unaffected. CSF2RA transgene expression was maintained during hematopoietic differentiation to the M/M state, and functional analysis of these gene corrected M/Ms demonstrated almost complete restoration of GM-CSF dependant functions (CD11b activation, GM-CSF uptake, phagocytosis, CSF2R-downstream signalling) when compared to control M/Ms derived from H9-ESCs or non-corrected PAP-iPSCs.

Discussion: In summary, we here not only established iPSC-lines from CSF2RA-deficient PAP patients and established M/M differentiation of PAP-iPSCs as a functionally relevant disease model, but also introduce gene corrected M/Ms obtained by myeloid differentiation of PAP-iPSCs transduced in the pluripotent state with the CSF2RA gene as a highly promising source for cell and gene therapy in CSF2R-deficiency PAP to be delivered by the intratracheal route.

Keywords: induced pluripotent stem cells; gene therapy; PAP; Lentivirus

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Abstract 87

Keratinocyte derived IPS cells from 22Q13.3 deletion syndrome patients

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Plucked human hair represent a valuable source for keratinocytes which can be efficiently reprogrammed to hiPS (human induced pluripotent stem) cells. Our group has successfully used this method to collect samples from several 22q13.3 deletion syndrome (also known as Phelan-McDermid Syndrome) patients. To generate virus free stem cell lines the loxp site containing reprogramming cassette was excised by addition of recombinant Cre protein to the cells. These iPS cells showed all pluripotency hallmarks and were confirmed to carry the deletion on chromosome 22. Subsequently, PMS iPS cells were used for neural differentiation into various neuronal subtypes to study the role of ProSAP2/SHANK3 in vitro and the disease mechanism in detail.

Keywords: iPS cell; 22q13.3 deletion; neurons; disease model

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Abstract 88

Functionally immortalized primary cells

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A major limitation of current research is the shortage of physiologically relevant cells. To generate cells in sufficient numbers in vitro cell expansion is an attractive alternative. Cell expansion can be achieved upon expression of immortalizing genes. We developed an immortalization regimen allowing the efficient and reproducible establishment of novel cell lines. In a proof of concept study we have established novel endothelial cell lines with in vivo like properties. For this purpose, we identified genes that fully retain the functions of endothelial cells and induce their immortalization. Primary endothelial cells from the umbilical cord and from the skin were transduced with these immortalizing genes which led to the establishment of endothelial cell lines with a robust proliferation phenotype. The resulting cell lines were cultivated for more than nine months which corresponds to more than 120 cumulative population doublings. An in-depth characterization of these cell lines was performed side-by-side with primary cells and demonstrated that the established cell lines retained the expression of endothelial specific marker proteins as well as endothelial specific functions. Importantly, this phenotype was stable throughout the whole cultivation period. We envision this system to provide biological relevant cells in sufficient numbers needed e.g. for drug discovery, ADME/Tox testing and in the long term for regenerative medicine approaches.

Keywords: physiologically relevant cells; immortalization; drug discovery; disease models; personalized medicine

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Abstract 89

IPS-based modeling of Nijmegen Breakage Syndrome

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Nijmegen breakage syndrome (NBS) is a rare, autosomal, recessive genetic disorder first described in 1981 in patients living in Nijmegen, Holland. NBS patients display a characteristic facial appearance, microcephaly and a range of symptoms including elevated sensitivity to ionizing radiation, chromosome instability, a high frequency of malignancies, accelerated shortening of telomeres, abnormal cell cycle checkpoints, growth retardation and immunodeficiency. The clinical features overlap with those of Ataxia-telangiectasia, Ligase IV syndrome, Non-homologous end-joining factor 1 (NHEJ1) syndrome and Fanconi anemia. The NBN protein, Nibrin, is part of a DNA repair, DNA double-strand break (DSB) sensor and damage signaling system and forms a trimeric complex together with MRE11 and RAD50. Mutations in this gene result in truncated proteins that maintain part of their function, however null mutation of the homologous gene in mice is lethal. DSBs, the main target for NBN, result from exogenic stresses like ionizing radiation (IR) and chemical compounds or from endogenous mechanisms like reactive oxygen species (ROS), stalled replication forks and recombination in cells of the immune system. As ROS are generated constantly as by-products of mitochondrial respiration, ROS may probably be detrimental for NBS cells under physiological conditions. Interestingly, pluripotent cells, both ESCs and iPSCs rely on glycolysis rather than oxidative phosphorylation as the main source of energy (Prigione 2010, stem cells). Based on this finding, we hypothesized that inducing pluripotency in NBS fibroblasts might by-pass ROS-mediated genome instability. To test this, we performed the following, a) Reprogrammed fibroblasts, derived from NBS patients, into iPS-cells (NBS-iPSC) as an in vitro model of the disease. b) Compared the transcriptomes of four NBS patient derived dermal fibroblasts to healthy foreskin fibroblasts (HFF1) in order to uncover molecular features and etiology of the disease. c) Compared the transcriptomes and stress responses of undifferentiated ES cells (H1 and H9), NBS-iPSCs and HFF-iPSCs.

Our findings are: All iPSCs expressed pluripotency associated proteins (Alkaline phosphatase, OCT4, NANOG, TRA1-81, TRA1-60, SSEA4) and pluripotency was further confirmed both in vitro (EB assays) and in vivo (teratoma formation). Comparative transcriptome and associated pathway analyses revealed (a) that NBS fibroblasts compared to healthy HFF1 seem to have a high impact on cell cycle regulation, apoptosis, p53 signalling and the Fanconi Anemia pathway. (b) The comparisons between ES cells, HFF-iPSCs and NBS-iPSCs revealed regulated genes and pathways associated with DNA replication, glycolysis, pyrimidine, fructose and mannose metabolism as well as DNA repair related pathways. Interestingly all of these pathways are known to be associated with ROS homeostasis. (c)NBS-iPSCs retained a set of genes related to B cell receptor signaling pathway, ion transport, cell adhesion and others, not seen in ESCs and HFF-iPSCs. Comparative tests based on sensitivity towards oxidative stress and DNA damaging agents such as hydrogen peroxide and bleomycin, revealed that NBS-iPSCs and NBS-fibroblasts compared to healthy HFF1 were highly sensitive to DSB inducer bleomycin but similar sensitive towards oxidative stress induced by exogenous hydrogen peroxide.

Keywords: NBS; ROS; iPS

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Abstract 90

Investigation of the in vitro differentiation ability of hybrid cell clones derived from spontaneous cell fusion events between murine breast cancer cells and murine mesenchymal stem cells

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We have previously shown that murine breast cancer cell lines can fuse spontaneously with bone marrow derived mesenchymal stem cells in vitro. Hybrid cell clones emerged from such spontaneous fusion events in a co-culture model showed genetic alterations and marked heterogeneity compared to their parental cell lines. With relation to the importance of cell fusion in tumour heterogeneity and progression the objective of this study was to characterize and investigate if hybrid cell clones could maintain their ability to differentiate into the neural, adipogenic and osteogenic lineage. Hybrid cell clones arose from spontaneous fusion events between hygromycin resistant murine 67NR-Hyg mammary carcinoma cells and puromycin resistant murine bone marrow derived stem cells (BMDCs) from Tg(GFPU)5Nagy/J mice. Dual hygromycin/puromycin resistant mBMDC/67NRHyg cells originated by cell fusion were confirmed by a dual drug selection procedure and PCR analysis to examine an overlap of parental markers. BMDCs are generally defined as self-renewable, multipotent progenitor cells with the ability to differentiate into several mesenchymal lineages. Differentiation was performed by cultivating the hybrid cell clones and parental cell lines under appropriate conditions to an established neural, adipogenic and osteogenic differentiation protocol, respectively. Differentiation of hybrid cell clones and mBMDCs into the osteogenic lineage was associated with expression of bone sialoprotein II and collagen type I alpha 1. Furthermore osteocalcin could be detected in mBMDCs but only in hybrid cell clone 3. Parental 67NR-Hyg cancer cells were negative for collagen type I alpha 1 and osteocalcin expression, while an increasing alkaline phosphatase activity was detected in all cells. Differentiation of parental and hybrid cell lines into neuronal lineage was associated with expression of neurofilament M, GAD67 and class III beta-tubulin. Immunocytochemical staining was investigated to confirm PCR analysis results. Adipogenic differentiation was confirmed through Oil Red O staining of intracellular developed lipid droplets, aP2 and PPARγ expression. While parental 67NR-Hyg cells showed accumulation of intracellular lipid droplets only after incubating in differentiation medium for at least one week, moderate to strong lipid droplet staining could already revealed in undifferentiated hybrid cell clones. In contrast, PCR analysis showed mRNA expression of adipocyte markers aP2 and PPARγ before and after differentiation of parental as well as hybrid cell lines. In conclusion, our data show that hybrid cell clones are able to differentiate into specialized cells from either neural, adipogenic or osteogenic lineages. These stem cell properties indicate that cell clones derived from cell fusion events maintain abilities of the parental cell lines.

Keywords: breast cancer; cell fusion; differentiation; hybrid cells

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Abstract 91

Rapid and robust generation of long-term self-renewing human neural stem cells

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Emergence of the iPSC technology holds great promises in the field of regenerative medicine. Pluripotent stem cells have the potential to differentiate into any desired cell type. However, directed and homogenous differentiation into only a single cell type remains a challenge. Particularly for in vitro modeling of neurodegenerative diseases like Parkinson's disease or Alzheimer's disease efficient differentiation into certain neurons or glia cells is needed. Instead of deriving these cell types directly from pluripotent stem cells (which can be patient specific), first more lineage committed neural stem cells (NSCs) could be generated from iPSCs. Additionally, when considering transplantation based cell replacement approaches, NSCs have the huge advantage that their tumorigenic potential is reduced when compared to iPSCs. Here we describe the development of a novel human iPSC based protocol enabling us to generate hNSCs that can be maintained under self-renewing conditions. Furthermore homogenous differentiation in the neuronal or the glial lineage can be induced within these cells. Through a detailed analysis we characterized the stemness as well as the differentiation potential of these cells thoroughly. Based on these results we came to the conclusion that the here presented hNSC cultures have an immense potential for in vitro disease modeling as well as for cell replacement approaches in regenerative medicine.

Keywords: human neural stem cells; stable culture system; disease modeling; neuronal and glial differentiation

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Abstract 92

Genetic correction of a LRRK2 mutation links Parkinsonian neurodegeneration to ERK-dependent changes in gene expression

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The LRRK2 mutation G2019S is the most common genetic cause of Parkinson's disease (PD). To better understand the link between mutant LRRK2 and PD pathology, we derived induced pluripotent stem cells from PD patients harboring LRRK2 G2019S and then specifically corrected the mutant LRRK2 allele using zinc finger nucleases. We demonstrate that gene correction resulted in phenotypic rescue in differentiated neurons, including stress induced apoptosis and decreased neurite outgrowth. Whole genome expression profiling uncovered gene expression changes associated with LRRK2 G2019S, and knockdown experiments demonstrated that four of these genes (CADPS2, CPNE8, MAP7 and UHRF2) contribute to dopaminergic neurodegeneration. Furthermore, LRRK2 G2019S induced increased extracellular- signal-regulated kinase 1/2 (ERK) phosphorylation. We demonstrate that transcriptional dysregulation of three genes was dependent on ERK activity. We showed that PD-associated neurodegeneration and neurite outgrowth were ameliorated by pharmacological inhibition of ERK. Finally, we developed this PD model into a system compatible with high-throughput screening (HTS) using neural precursor cells that are dependent only on small molecules for proliferation (smNPCs). This HTS-compatible model recapitulates PD – relevant phenotypes including the ERK-dependent disease mechanisms.

Keywords: neurodegeneration; disease modeling;neural precursors; drug screening; gene correction

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Abstract 93

The pig as a model for human cancer

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Cancer is a leading cause of human death and morbidity worldwide. Most animal models of solid cancers are in rodents, particularly genetically engineered mice. However, mice differ significantly from humans in size, lifespan, physiology, anatomy and diet, limiting their usefulness for some pre-clinical studies. Pigs are increasingly recognised as a valuable adjunct to biomedical research. Our aim is to provide a series of genetically-defined pigs that model serious and common human cancers. These will allow new diagnostic and therapeutic strategies to be investigated at human scale, and longitudinal studies under conditions that mimic the human patient. Here we report gene-targeted pigs carrying mutations in two key tumour suppressor genes: adenomatous polyposis coli (APC) and tumour protein 53 (TP53). APC plays a vital initiating role in both sporadic colorectal cancer (CRC) (1) and the inherited predisposition to colorectal cancer, familial adenomatous polyposis (FAP) (2). We generated gene-targeted cloned pigs carrying nonsense mutations in APC at sites orthologous to human germline mutations responsible for FAP. At one year old the APC1311 mutation resulted in >60 polyps in the colon and rectum. Histological and molecular analysis showed that the porcine model recapitulates all major features of early stage human FAP (3). Somatic mutations affecting p53 function are present in most human cancers, and germline TP53 mutations are responsible for Li-Fraumeni multiple cancer syndrome. A survey of somatic p53 mutations reveals R175H as the most frequent missense mutation in many sporadic human cancers (4). We created gene-targeted pigs carrying a latent TP53R167H mutant allele orthologous to human mutant TP53R175H that can be activated by Cre recombination to model oncogenic mutant p53 found in sporadic human cancers (5). We are confident that both these pig models will make significant contributions to human oncology.

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Keywords: APC; TP53; gene targeting; porcine models; cancer

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Abstract 94

Systemic feedback signals in myelodysplastic syndromes: increased self-renewal of the aberrant clone suppresses normal hematopoiesis

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Myelodysplastic syndromes (MDS) are heterogeneous hematologic disorders associated with dysplasia in myeloid cells and ineffective hematopoiesis. It is commonly accepted that MDS derives from an aberrant stem cell clone with impaired differentiation potential. However, it is yet unclear how it suppresses normal hematopoiesis. This process may involve systemic feedback signals acting also on the normal hematopoietic stem cell (HSC) pool. In this study, we provide a mathematical model based on ordinary differential equations to simulate different feedback signals for regulation of self-renewal and differentiation. Two signals act on the aberrant clone as well as on normal hematopoiesis: we hypothesized that the feedback signal for proliferation is inversely correlated with the number of mature cells in peripheral blood. Furthermore, we reasoned that self-renewal is the default pathway for stem cells which is down-regulated by an increasing number of primitive cells (including MDS cells) in their niche. The simulations indicated that a single anomalous cell clone can outgrow normal hematopoiesis only if the self-renewal rate is higher than in HSCs. Furthermore, the model indicated that the systemic feedback signal for proliferation increases, whereas the signal for self-renewal declines during disease development. To test this thesis, we isolated serum samples of 57 MDS patients and 12 healthy controls to compare their impact on in vitro expansion of cord blood derived HSCs. In fact, culture medium supplemented with 10% serum of MDS patients slightly increased proliferation of CD34+ cells. On the other hand, expression of CD34 and CD133 after five cell divisions (as determined by CFSE-staining) was moderately down-regulated and this is compatible with reduced self-renewal. Only the number of erythroid colony forming units (BFU-E and CFU-E) increased upon culture with MDS versus control serum, which may be attributed to significant higher levels of erythropoietin, whereas the frequency of other CFUs and concentrations of other growth factors (TPO, SCF and FGF) were not affected. Our mathematical model indicates that MDS clones require higher self-renewal rates than normal HSCs. We suggest that normal hematopoiesis is suppressed by an overall down-regulation of self-renewal due to accumulation of malignant and normal progenitor cells in the bone marrow. These hypotheses are in line with the effects of MDS-serum on proliferation of CD34+ cells. The nature of these feedback signals is yet unknown, but they may play a central role for MDS progression.

Keywords: myelodysplastic syndrome; mathemaical modeling; hematopoietic stem cells; proliferation; differentiation

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Ethical, Legal & Social Issues

Abstract 95

The unpatentability of human embryonic stem cell inventions within the European Union and under the European Patent Convention

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Contrary to public and partly professional opinion, after the Brüstle-Case within the patent law of the European Union and the WARF-Case under the European Patent Convention (EPC) there are, de facto, no patentable methods or products in the field of human embryonic stem cell technology under the patent law of the European Union and within the scope of the European Patent Convention. After the Brüstle- and the WARF-decision it became clear that under both patent legislations patents cannot be granted for inventions concerning human embryonic stem cells from human embryos, if the embryo is destroyed for this purpose. Also no patents can be granted under both legislations for patents which rely on human embryonic stem cells, no matter how they were derived in the past as long as it was necessary to destroy an embryo. Although both patent legislations are not directly depending on each other, nor entirely congruent (as the European Patent Convention covers all states of the European Union, but additional also non-member states) the interpretation of the patentability rules are similar, and connected. Therefore, if an invention based on human embryonic stem cell is not patentable under one of the both European patent legislations; it is very likely that the same invention or a similar invention is not patentable under the other legislation. However, even for those methods for the derivation of embryonic stem cell lines, which do not rely on the destruction of a human embryo, and which have thus been described as "solutions to an ethical dilemma" there is a more factual exclusion from patentability. This is due to the fact that these methods are either banned by national laws, e.g., because the respective approach involves cloning of a human embryo, or seem to be unsuccessful by scientific meanings, and thus no real alternative to the established methods which involve the destruction of an embryo. Thus, although inventions in the field of human embryonic stem cell technology are de facto not patentable any longer within the European Union and within the scope of the European Patent Convention, we neither could detect, when looking into publication numbers of scientific journals, a reduction of research activity in this field nor do we expect such a reduction due to patent limitations. This indicates that the stem cell research field is not solely depending on patent protection.

Keywords: patent; de facto unpatentability; Brüstle; WARF; Cloning

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Homing & Migration

Abstract 96

Platelets and stroma derived factor-1 (SDF-1) demonstrate an independant capacity to attract human CD133+ bone marrow stem cells to micro endothelium under shear stress

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Introduction: We previously demonstrated the therapeutic potential of hematopoietic CD133+ bone marrow stem cells (BMSC) to promote liver regeneration and stroma derived factor 1 (SDF-1) to have a mobilising capacity for those cells in that scenario. The aim of this study was to evaluate the modulating potential of platelets and SDF-1 for the homing of CD133+BMSC along endothelium under flow conditions.

Methods: Human micro vasculature endothelial cells (HMEC-1) were cultured in capillaries in a live cell imaging system. Primary CD133+BMSC were purified from bone marrow aspirates from patients undergoing abdominal surgery utilizing magnetic sorting. Endothelial cells (EC) were co-cultured with CD133+BMSC under shear stress. Platelet rich plasma (PRP) prepaired from BMSC donors was co-cultured with or without 2mM ADP pre-stimulation. Experiments were performed in duplicates testing treatment vs control conditions parallel with the same preparation of BMSC for 1h followed by quantification of adherent CD133+BMSC.

Results: If compared to platelet poor plasma (PPP), PRP-coculture resulted by mean in 3.4 and 2.9 fold increase of CD133+BMSC adherence to EC under shear stress of 1.0 and 0.2 dyne/cm^2 respectively. ADP-pre-stimulation of PRP lead to significantly increased levels of EC-adhering BMSC, however the level was with a factor of 2.9 comparable to unstimulated platelets. SDF-1 coincubation of EC without PRP demonstrated moderately extended rates of platelet adherence (mean 2.2-fold). However PRP-effects were neither augmented by additional SDF-1 co-incubation nor diminished by blockage of the SDF-1-receptor CXCR-4 utilizing antagonist AMD3100.

Conclusion: The fact that the impact of platelets on CD133+BMSC-adherence is not further gradable by platelet pre-stimulation corresponds to our previous findings of CD133+BMSC to control platelet activation subsequent to their co-culture. These data suggest PRP and SDF-1 to bear an independant promoting effect for homing of CD133+BMSC along vasculature in a non-synergistical mode, which may have relevance for BMSC homing along hepatic sinosoids during liver regeneration.

Keywords: CD133+ BMSC; platelets; SDF-1; liver regeneration; HMEC-1

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Abstract 97

Radioprotection of lung endothelial cells by mesenchymal stem cell therapy

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Radiation-induced pneumonitis and fibrosis constitute dose-limiting side effects of therapeutic thorax and whole-body irradiation. Vascular endothelial cells and the damage of these cells by irradiation is a key event in the pathogenesis of radiation-induced damage of healthy tissue, in particular radiation-induced pneumopathy. Although not well understood so far, circumstances exist under which radiotherapy favors metastasis. Thus, in this project we want explore the central of lung endothelial cells within the process of radiation induced tissue damage as well as the use of mesenchymal stem cell-based therapy in order to protect metastasis formation and progression of fibrosis. To this end, C57Bl/6 mice were used to induce lung injury and the intravenously application of GFP-labeled MSCs after lung irradiation and analyzed for the potential to counter endothelial cell injury and influence the metastasis of irradiated lung tissue. Our data show that whole thorax irradiation stimulates tumor cell extravasation and settling to the irradiated lung tissue. Increased formation of lung metastasis in the irradiated mice compared to the sham-irradiated mice by day 14 after tumor cell injection was paralleled by pro-invasive and pro-metastatic cellular activities e.g. upregulation of MMP2, at the time point of tumor cell injection. Of note, metastasis formation was increased in both mouse strains, the fibrosis sensitive C57Bl/6 and the fibrosis-resistant Balb/c mice, suggesting the involvement of fibrosis-independent molecular factors. Therapeutic applications of mesenchymal stem cells (MSC) isolated either classically from the bone marrow (BM-MSCs) or from the adventitia of the aorta (vascular wall-resident multipotent stem cells, VW-MPSCs) counteract the radiation-induced vascular damage and metastasis formation in the irradiated lung tissue. The definition of optimal time points for MSC application and their impact on radiation-induced pneumopathy are subject to current investigations. A thorough analysis of the pro-metastatic effect of thorax irradiation, the underlying mechanisms and potential protective strategies are of potential direct clinical relevance as radiotherapy is an essential part of cancer treatment.

Keywords: cancer research; radiooncology; mesenchymal stem cell; stem cell therapy

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Abstract 98

Pharmacokinetics of human mesenchymal stem cells (hMSC) in mice

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Objective: MSC therapy is currently explored as an emerging new therapeutic modality for various indications, mainly caused by inflammatory dysregulation. MSC are thought to act via immunomodulation and stimulation of endogenous repair mechanisms by secreted paracrine and humoral factors. However, little is known about circulation and homing of MSC in the recipient. In this project, the pharmacokinetics of intravenously administered hMSC was investigated in wildtype mice and a murine type-1 Diabetes (T1D) disease model.

Materials and Methods: Wildtype and NOD (non-obese-diabetic) mice were transplanted with 1 million hMSC into the tail vein and sacrificed five minutes to six weeks after transplantation (10 timepoints in total, n=5 mice per timepoint). Pharmacokinetics of hMSC were quantitatively detected in liver, spleen, heart, kidney, blood, pancreas, bone marrow and lung by Real-Time PCR of a human-specific one copy target gene. The number of human cells per mouse organ was extrapolated accordingly. Immunohistochemistry (IHC) was performed on all tissues for qualitative analysis. Wildtype mice were additionally analyzed using the in vivo imaging approach "Near-Infrared-Fluorescense" (NIRF) to monitor circulation of fluorescently labeled hMSC immediately after systemic transplantation.

Results and Conclusion: RT-PCR data of wildtype mice showed that approximately 80% of transplanted hMSC could be detected in the lung one hour after transplantation, whereas only up to 0,4% of the infused cells could be detected in spleen, liver, kidney, pancreas, blood and bone marrow. Atypical large cells could be identified in subsequent H&E staining of lung tissue, which might be of human origin. One hour up to six weeks after transplantation, the amount of detectable hMSC in mouse organs declined to an amount of ≤ 10 cells per whole mouse organ after six weeks and could not be detected via IHC anymore. Our data showed that hMSC disappear in immuno-competent mice within weeks. It is possible that the cells undergo apoptosis, necrosis or are recognized by the mouse immune system and therefore actively cleared. In vivo imaging of wildtype mice transplanted with fluorescently labeled hMSC showed an immediate accumulation of cells in the chest region after tail vein infusion. These data are consistent to the finding of hMSC accumulation in the lung detected via RT-PCR. The cell count of hMSC detected in the lungs of diabetic mice after transfusion was up to half of the amount of those observed in wildtype mice, whereas no significant difference could be found in the pancreas, which was the proposed target organ. However, the decrease of the amount of hMSC in all organs was similar in both mouse models. Taken together, our data suggests a constitutional accumulation of cells in the lung due to blood flow after i.v. injection and minor random cell appearance in other organs, followed by passive or active clearance over a period of six weeks. We could not determine any active homing or migration processes of hMSC in wildtype or T1D mice.

Keywords: pharmacokinetics; MSC; type 1 diabetes mellitus; cell therapy; NOD mouse

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Abstract 99

Platelets promote homing and extravasation of human CD133+ bone marrow stem cells in a xenogeneic model of the isolated perfused rat liver following warm ischemia

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Introduction: Thrombocytes play a role for liver regeneration after ischemia reperfusion injury. Previously, platelets demonstrated a preclinical impact on homing of transplanted hematopoietic stem cells to bone marrow vasculature. Recently we demonstrated the therapeutic potential of hematopoietic CD133+ bone marrow stem cells (BMSC) to promote liver regeneration. The aim of this study was to evaluate an impact of platelets for hepatic homing of CD133+BMSC to the liver following warm ischemia.

Methods: Primary CD133+BMSC were purified from bone marrow aspirates from patients undergoing abdominal surgery utilizing magnetic sorting. Autologous Platelet rich plasma (PRP) was prepared from BMSC-donors. Rat livers were introduced to an isolated perfused rat liver model (IPRL) and subjected to 30min of warm ischemia. Following a 3h reperfusion period, 1x10E5 human CD133+BMSC were infused to the liver via the portal vein. Extravasation of BMSC was characterised by human specific CD45 immuno fluorescence staining. Quantification of total hepatic homing and the proportion of extravasation of BMSC was realised by fluorescence labelling and in situ imaging of the IPRL.

Results: After 30 min. CD133+BMSC demonstrated extravasation and were in part localised to hepatocytes. The mean total number of homing CD133+BMSC to the liver was not significantly increased following PRP-infusion to the IPRL when compared to non-pre-treated controls. However, the mean ratio of extra sinusoidal to intra-sinoisoidal BMSC was increased in PRP-pre-infused livers with 1.03+/-0.36 in contrast to non-pre-treated livers (0.36+/-0.04; $p<0,05$). The relative number of extra-sinosoidal BMSC was also significantly increased in platelet-pre-infused livers (10.8+/-3.2 vs. 3.6+/-1.5; $p<0.05$).

Conclusion: We were able to demonstrate hepatic homing and extravasation of CD133+BMSC infused to an IPRL subsequent to warm ischemia. Platelets demonstrated to have a positive effect on BMSC homing to the extra-sinusoidal space of the liver. Future investigations will further characterise the interaction of CD133+BMSC and platelets with the hepatic microenvironment.

Keywords: CD133+ BMSC; platelets; IPRL; liver regeneration; hepatic homing

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Abstract 100

Live cell imaging (bioluminescence) of transplanted murine embryonic stem cells in NOD/SCID mice

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Objective: Imaging by bioluminescence is a valuable method to detect and to monitor the distribution of injected stem cells in vivo. In this study, different application routes of luciferase transfected murine embryonic stem cells (mESC) were used and their in vivo engraftment was analysed in a time dependent manner.

Methods: Mouse ESC line 7AC5/EYFP was transfected with a Nucleofection kit from Amaxa using plasmid pGL4.51 from Promega, which contains a luciferase gene. $1x10^2$ to $1x10^6$ transfected 7AC5/EYFP (7AC5/Luc) cells were injected subcutaneously, intrahepatically and intrasplenically into immunodeficient NOD/SCID mice. For intravenous application up to $1x10^7$ cells were used. Luciferase signal of these cells was determined by imaging of the animals at different points in time. Furthermore, the original 7AC5/Luc cells and the derived teratomas were analysed concerning their expression profile of pluripotency and germ layer markers by RT-PCR, immunofluorescence and FACS analysis. Additionally, teratomas were histopathologically assessed with a focus to the three germ layers (ectoderm, endoderm and mesoderm).

Results: Luciferase activity of 7AC5/Luc was stably maintained during four weeks in vitro. The cells expressed typical markers of pluripotency (Oct-3/4, Nanog), while germ layer markers were very low (AFP, Pax6) or moderately (Nodal, beta-3-tubulin) expressed. They disclosed no difference compared to non-transfected 7AC5/EYFP cells concerning gene expression patterns. After transplantation of 7AC5/Luc by different injection routes increasing luciferase signals were measured over time. For subcutaneous, intrahepatic and intrasplenic transplantation a signal only at the application site could be observed, which was cell number-dependent. After intravenous application an enrichment of cells and tumour growth could be detected in the lung and in the bone but only after transplantation of a cell count higher than $5x10^6$ cells. Histological analysis revealed a teratoma histology and germ layer structures at the three transplantation sites (subcutaneous, spleen and liver) and after intravenous application in the lung and bone. Expression profile of teratomas showed markers of the three germ layers and lower levels of pluripotency markers. No significant differences in gene expression could be observed between teratomas grown in different organs and in comparison to teratomas derived from non-transfected cells.

Conclusion: Stable transfection of 7AC5/EYFP stem cells was established. Plasmid integration was without any influence on the pluripotent character of the cells. Using imaging by bioluminescence, the kinetics of cell distribution and settlement of transplanted cells after different application routes can be followed in vivo and be evaluated semiquantitatively.

Keywords: bioluminescence; murine embryonic stem cells; 7AC5/EYFP; different application routes

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Abstract 101

Human bone marrow stromal cells show positive cell biological behavior when stimulated mechanically by extracorporeal shock waves in vitro

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Human bone marrow stromal cells (hBMSCs) are characterized by their capability to differentiate into various mesenchymal tissues, including adipocytes, chondrocytes and osteocytes. hBMSCs are a promising tool for the field of regenerative medicine for several reasons; they can be cultured ex vivo, have endogenous activation potential, can be systemically delivered and have a high self-renewal potential. Their immunosuppressive properties make them a promising tool to counter complications arising from graft versus host rejections in transplantation settings of cells other than hBMSCs. It should be noted that the transplantations of BMSCs have not been highly successful, yet, in order to cure disease tissue, as BMSCs disappear within a short period of time after transplantation. Thus, the therapeutic success of using hBMSCs in tissue regeneration has been limited and falls far behind their potential. Despite these observations, hBMSCs are among the most promising types of stem cells for therapeutic applications and are the subject of intense research. However, the clinical use of hBMSCs has been confounded by limitations in their availability; they are scarce cells cumbersome to isolate and purify. Additionally, they are difficult to target to the site of injury in regeneration experiments. In order to combat the low efficiency of targeting hBMSCs to the disease area and to promote their beneficial cellular response at those desired targets for therapeutic use, novel strategies avoiding genetic manipulation are needed. It is known that the expression of genes involved in differentiation pathways of hBMSCs can be influenced by mechanical stimuli. A mechanical source is known as extracorporeal shock waves (ESW). ESW are transient pressure fluctuations that propagate 3-dimensionally and that are widely applied in the context of therapeutic mechanotransduction with a high success of increased tissue regeneration.

To test the hypothesis that extracorporeal shock waves exert beneficial effects on cell biological parameters of hBMSC, we applied focused ESW (fESW, 0.2/0.3mJ*mm-2) to purified, cultured hBMSCs. fESW (0.2mJ*mm-2) stimulations were found to increase hBMSCs growth rate ($p<0.05$), proliferation ($p<0.05$), migration, cell tracking and wound healing ($p<0.05$, respectively), as well to reduce the rate of apoptosis activation ($p<0.05$) when compared to untreated control hBMSCs and hBMSCs treated with 0.3 mJ*mm-2. The increase in hBMSCs migration behavior was found to be mediated by active remodeling of the actin cytoskeleton as indicated by increased directed stress fiber formations ($p<0.05$). Furthermore, hBMSCs maintain their full differentiation potentials after fESW treatment, whereas 0.2mJ*mm-2 is the most effective application.

In conclusion, our results establish first-timely that hBMSCs behavior can be modified and optimized in response to defined mechanical stimulation. Furthermore, our results demonstrate the existence of a physiological threshold above which mechanical stimulations seem to be counterproductive for the induction of hBMSCs migration. Our findings appear particularly promising as they suggest that mechanical stress preconditions hBMSCs for improved therapeutic performance without genetic manipulations and that mechanically preconditioned hBMSCs will be advantageous for hBMSCs-based tissue regeneration. Therefore, this approach opens the door for exploiting the full potential of these cells in regenerative medicine.

Keywords: human bone marrow stromal cells; regenerative medicine; mechanical stimulation; extracorporeal shock wave

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Induction and Maintenance of Pluripotency

Abstract 102

Transcription factor networks that define two distinct pluripotent states

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Pluripotent stem cells derived from the epiblast of postimplantation mouse embryos, called epiblast stem cells (EpiSCs), display different molecular and cellular characteristics from embryonic stem cells (ESCs). Although they are pluripotent, the developmental capacity of EpiSCs is limited compared to ESCs. EpiSCs hardly contribute to somatic and germ cells in chimeras when injected into preimplantation embryos, or are hardly dedifferentiated into ESCs without genetic manipulation. Importantly, the pluripotent state of EpiSCs is maintained by FGF/Activin signaling in EpiSCs, in contrast to required LIF signaling in ESCs, although downstream core transcription factors, such as Oct4, Sox2 and Nanog, are shared. It remains unclear how these two pluripotent states are defined and maintained downstream of the distinct signaling pathways. To clarify this, we performed a gain-of-function screening for transcription factors that can dedifferentiate EpiSCs into ESCs and identified Esrrb as the most effective factor. Esrrb induced morphological and molecular characteristics of ESCs when cells are cultured in the presence of LIF. This dedifferentiation is achieved in a highly homogenous and synchronous manner in about 5 days. Significantly, these ESC-like cells contributed to the adult tissues in chimeras. These results demonstrate that the pluripotent state of EpiSCs (primed pluripotent state) is efficiently reprogrammed to the pluripotent state of ESCs (naïve pluripotent state) by a single transcription factor. By taking advantage of this system, we mapped genome-wide transcription factor binding sites during dedifferentiation of EpiSCs into ESCs. The results showed that the binding sites of core transcription factors, Oct4, Sox2 and Nanog, are dramatically changed during the 5 days of reprogramming, suggesting that they play context-dependent roles in regulating pluripotency. Further analyses will clarify how intrinsic and extrinsic regulators cooperatively define the two distinct pluripotent states.

Keywords: pluripotency; transcription factor; EpiSC

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Abstract 103

Comparative analysis of the role of USP44 in human embryonic stem cells, retroviral and mRNA-derived amniotic fluid induced pluripotente stem cells

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Human amniotic fluid cells (AFCs) have gained increasing appreciation in the biomedical field over the last decade. Due to the presence of fetal stem cells within this heterogenic mixture of cells, which have multipotent capacities and possess putative immune privilege characteristics, primary human AFCs are believed to be valuable for conducting disease modeling, pharmaceutical and toxicological studies and even cell-based regenerative therapies in the future. Adding even greater value to AFCs, we and others demonstrated very recently that amniotic fluid cells enable fast and efficient induction of pluripotency, whereby senescence of primary human AFC cultures is bypassed and their differentiation potential enhanced. We extensively characterized several retrovirally generated human amniotic fluid-derived induced pluripotent stem cell (AFiPSC) lines and work with these established AFiPSC lines in different follow-up studies. We will present the current status of two of our ongoing AFiPSC-based projects: (I) We focus on the optimization of an improved non-viral, synthetic mRNA-based reprogramming technique. As our retroviral-derived AFiPSC lines harbour several integration events within their genomes with yet unknown effects on the transcriptome it is our goal to generate integration-free AFiPSC lines. We anticipate that the stem cell-like cells present in bulk primary AFC cultures facilitate the realization of this otherwise rather complex, inefficient mRNA reprogramming approach. This will give us the opportunity to compare the transcriptomes and differentiation potential of our retroviral and mRNA-derived induced pluripotent stem cell (iPSC) lines harbouring identical genetic background. (II) We aim to compare the downstream effects of knocking down a gene in the AFiPSC lines and embryonic stem cells (ESCs). For this purpose we chose to knock down the gene encoding the deubiquitinating enzyme USP44. This enzyme is a critical regulator of the spindle checkpoint during cell cycle. It has been shown to prevent premature activation of the anaphase promoting complex. Using an OCT4 ChIP-on-chip approach, we and others identified USP44 as a positively regulated OCT4 target gene in various human pluripotent cell lines. We also demonstrated that the USP44 proximal promoter harbours an evolutionary conserved OCT4 binding site and we were able to confirm OCT4 binding by ChIP-real-time-PCR. Yet, the exact role of USP44 in the maintenance of self-renewal and pluripotency is unknown. Therefore, utilizing our AFiPSC lines as well as the ESC lines H1 and H9, we will aim at deciphering its function. As a result of these studies we expect to be able to attain the following: (I) unveil similarities and differences between viral and in vitro mRNA-based reprogramming methods on the transcriptome, long-term stability and differentiation capabilities of iPSC lines derived from the same genetic background. (II) unravel the role of the pluripotency-associated gene USP44 on self-renewal and the maintenance of pluripotency in human ESCs and iPSCs.

Keywords: iPSCs; USP44; mRNA transfection; reprogramming; amniotic fluid

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Abstract 104

Analysis of reprogramming-associated alterations using an isogenic human stem cell system

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Human induced pluripotent stem cells (iPSCs) could provide a continuous donor source for the generation of specific somatic cell types for disease modeling or neural replacement. Considering the harsh reprogramming procedure and the requirement of epigenetic rearrangement for proper reprogramming, a critical question to be addressed is whether and to what extent alterations caused by the reprogramming process can confound readout parameters in disease modeling or influence clinical safety. An ideal approach to this question would be to reprogram a well-defined somatic cell population into iPSCs, which are subsequently re-differentiated in the very same somatic cell type. Here we used human embryonic stem cell (ESC)-derived neural stem cells (ESC-NSCs) as a well-characterized and highly standardized starting population. ESC-NSCs were reprogrammed by retroviral or lentiviral transduction with the transcription factors (OCT4 and KLF4 with or without cMYC). The resulting iPSC clones were validated by marker expression (e.g. OCT4, NANOG, DNMT3B), their multilineage differentiation potential in vitro as well as teratoma formation in vivo. Subsequently, these iPSCs were differentiated into a second population of neural stem cells (iPSC-NSCs). These iPSC-NSCs and their ESC-derived counterparts showed similar rosette-like morphology, were positive for characteristic neural stem cell markers such as SOX2, Nestin, PLZF and DACH1 and differentiated into neurons and glia with comparable efficiency. Global transcription profiling performed on both neural stem cell populations and their parental, pluripotent cells of origin showed a highly similar transcriptome between ESC-NSCs and iPSC-NSCs, with the exception of 85 transcripts, which exhibited >2 fold changes. Amongst these were two transcripts (DLK1, AL132709.5) of the imprinted DLK1-DIO3 gene cluster and a disproportionate number of genes encoded on the X-chromosome. These results indicate that despite a remarkably close transcriptome and comparable differentiation potential, ESC-NSCs and isogenic iPSC-NSCs show alterations in the stability of imprinted gene expression and X-chromosome gene activation, which might impede readout parameters in disease modeling and the clinical utility of iPSC-derived somatic cell populations.

Keywords: human iPSC; reprogramming; isogenic; neural stem cell; gene expression

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Abstract 105

Recombinant Nanog for modulation of stem cell characteristics

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For the genetic modification of cells and the analysis of the function of proteins, several conventional methods including transfection or viral transduction are available. These methods have certain limitations such as overall poor transfection efficiency, toxic side effects, and the risk of unwanted permanent genetic modification by insertional mutagenesis. Protein transduction represents a promising alternative, which is able to overcome of these limitations. Moreover, this approach provides an additional advantage to provide a control over dose and time to comprehensively assess the function of the protein. Nanog is expressed in embryonic stem (ES) cells and plays a vital role in early development and for natural induction of pluripotency. To elucidate the function of Nanog, we engineered a recombinant cell-permeant mouse Nanog by fusing it with the protein transduction domain TAT as a non-DNA based, non genetic paradigm to modulate cellular function. We demonstrate that mouse Nanog-TAT treatment promotes ES cell proliferation, self-renewal, inhibits endodermal specification in a Stat3-independent manner and promotes pluripotency in the absence of leukemia inhibitory factor (LIF). Mouse Nanog-TAT together with LIF induced synergistic effects as judged by morphology, enhanced clonogenicity and activation of an Oct4-promoter-driven GFP reporter gene. Similarly, we also engineered recombinant human NANOG-TAT by co-expressing it with the E.coli periplasmic chaperone Skp. We expected Skp to stabilize recombinant proteins by complex formation and by that to prevent aggrega-tion of unfolded proteins. We evaluated the potential of NANOG protein transduction to promote ES cell self-renewal and pluripotency by cultivation in 2D and 3D systems in presence of NANOG and analysed for viability, pluripotency and apoptosis at different time-points and under various media conditions.

Keywords: nanog; pluripotency; human stem cells

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Abstract 106

A unique Oct4 interface and its role in reprogramming to induced pluripotency

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In 2006, the field of stem cell research was captivated by a possibility of epigenetic reprogramming of terminally differentiated cells to a pluripotent state by four factors – Oct4, Sox2, Klf4 and c-Myc. Hovewer, six years after this breakthrough, we still lack proper insight into this fascinating process on a molecular level. Among all four factors in the original cocktail, Oct4 evinces a special feature as it is impossible to replace this transcription factor by any other member of its protein family. But what makes the protein so unique? A potential and unbiased approach to answer this question makes use of the recently solved crystal structure of Oct4. This revealed a unique structured linker sequence of Oct4, which is not found in other POU class members. Moreover, this alpha-helical stretch is exposed to surface, creating an attractive interface regarding direct interactions with other components of the reprogramming machinery. A mutation screen along the linker proved several amino acids to play a crucial role in reprogramming. Based on current interactome studies, we conclude that the region is involved in recruiting key epigenetic players. In ongoing efforts, we try to determine the direct interaction partners of aforesaid Oct4 alpha helix. Two different approaches have been chosen: Firstly, a yeast two hybrid screen using wild type-based bait constructs and also baits containing point mutations will be performed. Secondly and for the first time in stem cells, we present the genetic code expansion technology, based on additional orthogonal translation machinery.

Keywords: Oct4; induced pluripotency; protein-protein interactions

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Abstract 107

ES cell-associated microRNAs and hypoxia guide reprogramming of somatic cells

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Objective: Reprogramming of somatic cells into induced pluripotent stem cells (iPS cells) allows the generation of disease- and patient-specific pluripotent stem cells. The iPS cell technology offers a powerful tool to analyse development, progression and treatment of diseases in vitro. Viral mediated expression of transcription factors and miRs was demonstrated, but adverse effects on the integrity of the host genome hinder the analysis of the genomic background of certain diseases and clinical application of iPS cells. In embryonic stem cells (ESCs), gene expression of pluripotency markers is regulated by the sophisticated microenvironment of the stem cell niche, which can be mimicked by ESC-specific culture conditions. Additionally, pluripotency is orchestrated by specific transcription factors and microRNAs (miRs), which in turn can be used to reprogram somatic cells. Episomal expression of transcription factors or the introduction of mature miRs aim at non-viral and non-integrative reprogramming and the generation of disease- and patient-specific iPS cells.

Material and Methods: Human MSCs, fibroblasts, and B-lymphoblasts were analyzed in non-viral and non-integrativ reprogramming procedures in the presence of hypoxia and normoxia. Transfection with minicircles induced expression of Thomson reprogramming factors Oct4A, Nanog, Sox2, Lin28 (ONSL) and other pluripoteny-associated factors analyzed by semi-quantitative PCR, real-time PCR, and IF staining. Additionally, cells were transfected with miRs from the miR-302a-367 and miR-371-373 cluster to induce pluripotency marker gene expression. Delivery was quantified by flow cytometry. Induction of gene expression and the repression of miR targets were demonstrated by real-time PCR, IF-analysis, and western blot. CpG methylation of the Oct4 promoter was analyzed using MSRE-based real-time PCR.

Results and Conclusion: Minicircles were efficiently introduced yielding about 25-35 % ONSL-expressing cells. Endogenous ONSL expression was induced and mRNA levels were elevated by hypoxic conditions. Oct4 and Nanog mRNA expression were preserved in the presence of hypoxia instead of normoxic conditions. Oct4, Sox2 and Nanog were expressed at the protein level. Efficient miR-delivery was shown yielding about 90-95% miR-transfected cells. Transfection of miRs induced endogenous precursor miRs and hypoxia preserved induction instead of normoxic conditions. The mRNA expression of Oct4 and Nanog was induced by miRs and protein expression of Dnmt3b was increased. The repression of predicted target genes in a dose dependent manner (about 50-80 %) demonstrated functionality of miRs. Oct4 promoter DNA demethylation was demonstrated in response to miR-delivery. Hypoxic culture conditions were sufficient to induce reprogramming factors and ES cell-associated miRs without treatment with minicircles and miRs. Further, proliferation was enhanced demonstrated by elevated Ki-67 staining. ESC-specific culture conditions were sufficient to preserve induced pluripotency marker gene expression by mimicking the stem cell niche of pluripotent stem cells. The expression of alkaline phosphatases was demonstrated. In summary, ES cell-associated microRNAs and hypoxia induced or enhanced reprogramming making them a powerful tool for non-viral reprogramming strategies. Such non-viral strategies will be used to generate disease-specific iPS cells from B-lymphoblasts to study neural disorders such as Schizophrenia in an in vitro differentiation model.

Keywords: pluripotency; reprogramming; methylation; microRNA

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Abstract 108

Excision of a viral reprogramming cassette by Cre protein transduction: An efficient protocol for transgene-free human iPS cell derivation

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Emergence of induced pluripotent stem (iPS) cell technology has paved new hopes in the field of regenerative medicine. It offers the possibilities of disease modeling, drug toxicity studies as well as cell replacement therapy by autologous transplantation. Most protocols of iPS cell generation involve integrative retro- or lenti-viral transduction. However, the presence of viral transgenes in iPS cells is undesirable as it raises the possibility of insertional mutagenesis leading to malignant transformation and has also been shown to affect the differentiation potential of reprogrammed cells. Those limitations not only restrict the use of iPS cells for clinical applications but also for obtaining suitable models for disease or toxicity studies. Transgene-free iPS cells have been obtained via introduction of reprogramming factors via protein transduction, mRNA transfection, episomal plasmid transfection as well as non-integrating viruses such as sendai viruses. However, the utility of each protocol remains limited due low efficiency and narrow range of cell specificity. Our study shows the efficient derivation of transgene-free human iPS cells by deleting a loxP-modified reprogramming cassette employing direct delivery of active Cre recombinase protein. Factor-free human iPS cells exhibit appropriate morphological and immunochemical staining characteristics of pluripotent cells, possess a normal karyotype and are capable of differentiating into derivatives of all three germ layers in vivo. We obtained more than 60% of transgene excision efficiency with 2μM of cell-permeant recombinant Cre protein applied to the cells for 4 hours. Unlike electroporation with Cre-encoding plasmid or mRNA transfection, Cre protein transduction provides a simple and robust protocol for the generation of transgene-free iPS cells suitable for disease modeling, personalized medicine and cellular replacement therapies.

Keywords: iPS cells;TAT-CRE; protein transduction; disease modelling;

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Abstract 109

Cre/loxP-mediated fate-mapping identifies stochastic events during early reprogramming

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In their groundbreaking work of 2006 Takahashi and Yamanaka dedifferentiated fibroblast cells back to a pluripotent state by viral introduction of the four transcription factors Oct4, Klf4, Sox2 and c-myc (OKSM). Since then the generation of so-called induced pluripotent stem (iPS) cells has been vastly improved both in matters of pace and efficiacy. Despite these advantages in iPS technology the mechanisms of cellular reprogramming still remain to be investigated and comprehensively understood in order to safely use iPS cells for medical treatments.Here we use a fate mapping system based on a double fluorescence reporter to trace the activation of single genes and identify cellular intermediate states during reprogramming. We infected Nestin-Cre fibroblasts with a lentiviral reporter carrying a loxP-modified red fluorescence (RFP) gene followed by an eGFP sequence and reprogrammed them either with all four factors or with Klf4, Sox2 and c-myc alone. At day 3-5 post transduction we observed several high proliferative cell clusters that changed color from red to green indicating a recombination of the lentiviral reporter and thus an at least transient expression of Nestin. Interestingly no such recombinated clusters appeared when Oct4 was omitted from the reprogramming cocktail. We further reprogrammed fibroblast lines that carried the Cre transgene under the control of the neural crest marker Wnt1 and the smooth muscle gene SM22. Again around day 4 of reprogramming green clusters of small and fast proliferating cells were observed - independent of the Cre- driver gene screened. Our findings indicate that 3 to 5 days after the induction of OKSM cells enter a stochastic phase where genes being originally silent get activated randomly. They further underline the importance of Oct4 in reprogramming since the stochastic phase can seemingly not be entered without destabilization mediated by this transcription factor.

Keywords:

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Abstract 110

Reprogramming to pluripotency through a somatic stem cell intermediate

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In recent years, direct reprogramming and transdifferentiation from one somatic cell lineage into another has become an important area of stem cell research. We recently reported the direct reprogramming of mouse embryonic fibroblasts (MEFs) into induced neural stem cells (iNSCs). In this study, we have reprogrammed iNSCs into induced pluripotent stem cells (iPSCs), which we termed iNSC-derived iPSCs (iNdiPSCs). iNdiPSCs are truly pluripotent, as evidenced by a pluripotent gene expression profile and the ability to differentiate into all three germ layers, both in vitro and in vivo. We have further shown that iNdiPSCs do not retain the epigenetic memory of either NSCs or MEFs. In conclusion, our data provide evidence that iNSCs can give rise to bona fide iPSCs, which are indistinguishable from other pluripotent stem cells.

Keywords: iNSCs; iPSCs; reprogramming; epigenetic memory

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Abstract 111

Reprogrammed bone marrow derived mesenchymal stem cells show differences in epigenetic memory related gene expression and differentiation capacities compared to fibroblast derived iPS cells

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The generation of induced pluripotent stem cells (iPSCs) is a promising approach to alter the short life span in culture and to broaden the restricted differentiation potential of somatic cells. Especially, regenerative applications of human bone marrow derived mesenchymal stem cells (BM-hMSCs) are limited by these features. It has been shown that BM-hMSCs can be reprogrammed into iPSCs via expression of the four factors OCT4, SOX2, KLF4 and c-Myc. Furthermore, it is known that iPSCs retain a donor cell type specific epigenetic memory which is reflected in the features of the respective iPS cell line. However, it is poorly understood which effect retained epigenetic features have on the transcriptome and differentiation capacity of BM-hMSC derived iPSCs (BM-hMSC-iPSCs). In this study iPSCs were generated from BM-hMSCs and human foreskin fibroblasts (HFF). The transcriptomes of the parental cells and the respective iPS cell lines were compared by microarray based gene expression analysis to detect donor cell specific gene expression. Subsequently, BM-hMSC-iPSCs and HFF-iPSCs were comparatively differentiated into Osteoblasts, Chondrocytes and Adipocytes. hMSCs derived from a 74 year old patient could be reprogrammed with retroviruses expressing OCT4, SOX2, KLF4 and c-Myc and addition of SB-431542 (TGF-beta receptor inhibitor), PD-325901 (MEK inhibitor) and a P53 inhibitor. HFF could be reprogrammed without the addition of inhibitors. A high similarity between hESCs and the derived BM-hMSC-iPSCs and HFF-iPSCs could be confirmed by microarray based expression analysis, embryoid body formation, pluripotency marker expression and teratoma formation. In addition, using microarray based gene expression, genes involved in Osteoblast differentiation such as MAP2K7, RPS6KA1 and TGFB1 were found to be expressed in BM-hMSC-iPSCs but not in HFF-iPSCs. BM-hMSC-iPSCs could be differentiated in vitro into Osteoblasts more efficiently compared to HFF-iPSCs. BM-hMSC-iPSCs could not be differentiated into Adipocytes. Chondrocyted differentiation was more efficient when BM-hMSC-iPSCs were used. BM-hMSC-iPSCs indeed retained donor cell type specific epigenetic memory related expression of genes that could be confirmed in parental BM-hMSCs and BM-hMSC-iPSCs but not in HFF-iPSCs. The distinct differentiation capacity between BM-hMSC-iPSCs and HFF-iPSCs towards Osteoblast and Chondrocytes suggest an enhancing effect of the epigenetic memory related gene expression in BM-hMSC-iPSCs towards the lineage of the parental cell type.

Keywords: induced pluripotent stem cells; epigenetic memory; bone marrow mesenchymal stem cells; osteoblast differentiation; iPS transcriptome analysis

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Abstract 112

Lrrc34, a novel nucleolar protein, is involved in ribosome biogenesis of pluripotent stem cells

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The gene Lrrc34, leucine rich repeat containing 34, is a predicted member of the ribonuclease (RI)-like subfamily. Leucine rich repeats are frequently involved in the formation of protein-protein interactions. Recent immunostaining experiments gave the first hint for the localization of Lrrc34 in the nucleolus. Using the SILAC approach to find interaction partners of Lrrc34 we found many proteins that are known to be localized in the nucleolus and involved in ribosome biogenesis. The interaction with two of these candidate proteins, namely Npm1 and Ncl, could be confirmed by Co-IP and PLA. Furthermore Lrrc34 was found to be exclusively expressed in undifferentiated ESCs, maGSCs and other pluripotent cell lines as well as in the adult testis. After differentiation of these pluripotent cells with RA (Retinoic Acid), the expression of Lrrc34 is strongly downregulated. Methylation studies of the promoter of Lrrc34 showed that it is hypomethylated in undifferentiated stem cells and becomes hypermethylated upon differentiation. Furthermore ChIP- qPCR analyses of histone modifications at the Lrrc34 promoter region were performed and detected an enrichment of activating and a depletion of repressive histone modifications. To further analyze the function of Lrrc34, we performed siRNA experiments in ESCs and found a downregulation of Oct4, Zfp206 and Klf4 but a strong upregulation of differentiation markers like Vimentin and Hnf4. In another approach, we used overexpression of Lrrc34 in ESCs and observed an increased expression of Stra8 and Klf4 and a decreased expression of Oct4, Nanog, Nestin and Gata4. Our results suggest that Lrrc34 is involved in ribosomal biogenesis of pluripotent cells and has an impact on the expression of pluripotency regulators. Further experiments analyzing ribosome biogenesis in ESCs with altered Lrrc34 expression will be performed.

Keywords: Lrrc34; nucleolus; robosome biogenesis; pluripotent stem cells

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Abstract 113

Production of porcine induced pluripotent stem cells using Sleeping Beauty transposons with the porcine reprogramming transcription factors

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The derivation of induced pluripotent stem cells (iPSC) by Yamanaka and co-workers has created new perspectives for developing regenerative therapies in the human medicine. In order to fulfill the iPSC potential in this field, relevant animal models are necessary for method testing and risk assessment. In this regard, the pig is a particularly suitable large animal model due to the fact that physiology, metabolism, and organ size are similar in humans and pigs. Since 1990, putative porcine iPSC have been derived by using mostly lenti- or retroviral vectors with the human or mouse reprogramming transcription factors. Here, we report the derivation of putative porcine iPSC using Sleeping Beauty transposons carrying the porcine OCT4, SOX2, C-MYC, KLF4, and NANOG cDNAs in addition to the human LIN28. Porcine embryo fibroblasts carrying an OCT4-eGFP reporter construct were transfected with two transposons SB-CAG-pOSMK-ires-Tomato and Epi-Ef1a-pNANOG-ires-hLIN28 together with the SBx100 transposase (generously provided by Dr. Zoltan Ivics from Paul Ehrlich Institute, Langen, Germany). Putative iPSC colonies, identified by expression of the eGFP, were manually picked and expanded in stem cell medium (DMEM supplemented with 20% Knockout Serum Replacement, non-essential amino acids, 50 µM beta-mercaptoetanol, penicillin-streptomycin, 10 ng/ml bFGF, and 1000U/ml ESGRO). The resulting cell lines had mouse ESC-like morphology and showed upregulation of alkaline phosphatase, SSEA-1 and SSEA-4. They also expressed the eGFP reporter and the endogenous OCT4, SOX2, NANOG, REX1, CDH1, and TDH. Methylation analysis by bisulphite sequencing showed that the promoters of OCT4 and NANOG were hypomethylated. As verified by Tomato expression and RT-PCR, the pOSMK transgenes were downregulated, but not completely silenced, while the NANOG-LIN28 expression was no longer detected. These cells are currently being tested for in vitro and in vivo differentiation.

Keywords: iPSC; porcine; pluripotency

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Abstract 114

MicroRNA-mediated epigenetic regulation represents a roadblock for the generation of iPSCs

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Introduction: MicroRNAs (miRNAs) have repeatedly been demonstrated to play important roles in the generation of induced pluripotent stem cells (iPSCs). To further elucidate the molecular mechanisms underlying transcription factor-mediated reprogramming and to increase the efficiency of this process, we have established a model which allows screening of whole libraries for miRNAs potentially modulating the generation of iPSCs. In this model, murine embryonic fibroblasts (MEFs) from OG2 mice (Oct4-GFP) were transduced with a polycistronic lentiviral construct expressing Oct4, Klf4 and Sox2 and an IRES-coupled dTomato reporter. One day after transduction, miRNAs from a Pre-miR™ Library (Ambion) containing 379 miRNAs were individually transfected into these MEFs. At day 7 to 10, emerging GFP-positive iPSC-like colonies were counted and further analyzed.

Results: Applying this model, we have identified a miRNA family consisting of miR-130b, miR-301b and miR-721, which strongly enhance iPSC generation at least in part through repression of the homeobox transcription factor Meox2, a transcriptional activator of the tumor suppressor genes Cdkn2a and p21. Our analyses also revealed several miRNAs effectively inhibiting iPSC generation upon overexpression, including miR-132 and miR-212. Intriguingly, repression of these miRNAs during iPSC generation led to significantly increased reprogramming efficiencies. This observation was further confirmed by demonstrating that fibroblasts from miR-132/212 knock-out mice displayed significantly increased propensities to undergo reprogramming compared to wild-type cells. miRNA target identification by qRT-PCR, western blot and luciferase assays revealed two crucial epigenetic regulators, the histone acetyl transferase p300 as well as the H3K4 demethylase Jarid1a (KDM5a) to be directly targeted by both miR-132 and miR-212. We further demonstrated that specific siRNA-mediated knockdown of either p300 or Jarid1a recapitulated the miRNA effects and led to a significant decrease in reprogramming events.

Conclusion: Thus, conducting a full library miRNA screen we identified a novel miRNA family strongly enhancing iPSC generation from MEFs and we could associate this to a repression of Meox2. In addition, we found several miRNAs, which strongly restrain iPSC-generation and upon inhibition in turn enhance reprogramming. These miRNAs, at least in part, exert their functions through repression of the epigenetic modulators p300 and Jarid1a, thereby potentially representing an endogenous epigenetic roadblock for the generation of iPSCs. These observations underline that robust RNAi screening approaches represent a powerful tool to identify novel factors modulating iPSC generation and may substantially add to our understanding of the molecular mechanisms underlying transcription factor-mediated reprogramming.

Keywords: induced pluripotent stem cells; miRNA; reprogramming

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Abstract 115

Human induced pluripotent stem cells exhibit high PKM2 levels and HIF1alpha-driven early reconfiguration of energy metabolism

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Reprogramming somatic cells to a pluripotent state drastically reconfigures the cellular anabolic requirements, thus potentially inducing cancer-like metabolic transformation. Accordingly, we and others have previously shown that somatic mitochondria and bioenergetics are extensively remodeled upon generation of induced pluripotent stem cells (iPSCs), as the cells transit from oxidative to glycolytic metabolism. Here, we sought to identify possible regulatory mechanisms and eventually determine whether metabolic restructuring is a necessary step for the induction of pluripotency. We discovered that iPSCs express high levels of pyruvate kinase M2 (PKM2), whose elevation has been linked to the metabolic switch in tumor cells, and pyruvate dehydrogenase kinase (PDK) 1 and 3, all known downstream targets of hypoxia-inducible factor 1 alpha (HIF1alpha). The up-regulation of HIF1alpha-related transcripts occurred during the initial reprogramming phase, when the genes dictating self-renewal and pluripotency have yet to be turned on, and was associated with corresponding metabolic alterations. Importantly, the ablation of the master metabolic regulator HIF1alpha or its target PDK1 dramatically hampered reprogramming efficiency, while chemical HIF1alpha activation improved cell fate conversion. HIF1alpha-mediated reconfiguration of glucose metabolism may thus represent an early enabling step of cellular reprogramming, a barrier that has to be overcome in order to allow somatic cells to sustain their newly acquired proliferative and biosynthetic needs.

Keywords: iPS cells; metabolism; HIF1alpha; PDK1; PKM2

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Abstract 116

The invertebrate POU transcription factor – Smed-POU5 – maintains the identity of the pluripotent planarian adult stem cells

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The evolutionary origin of pluripotency is an unresolved question. In mammals, pluripotency is limited to early embryos and is induced and maintained by a small number of key transcription factors, of which the class V POU domain protein Oct4 is considered central. Clonal invertebrates, by contrast, possess pluripotent stem cells throughout their life, but the molecular mechanisms that control their pluripotency are poorly defined. Planarians are freshwater flatworms with the ability to regenerate any missing body part. This regenerating ability hinges on a population of stem cells that has been studied for over a century, but was confirmed pluripotent only recently. This pluripotency-based regenerative capability makes planarians a good model system to study stem cell biology as well as regenerative processes in vivo. To address the role of POU domain proteins in planarians, we analyzed the expression pattern and function of the POU domain genes from the free-living flatworm Schmidtea mediterranea. Smed-Pou5 is expressed in the parenchyma – where the planarian adult stem cells are located – and in the brain. During regeneration, it is abundantly expressed in the blastema and in the tissue immediately adjacent to it, called post-blastema, where the stem cells accumulate upon injury. Following Smed-Pou5 RNAi, amputated planarians formed a small blastema, but the regeneration process ended after few days and the animals ultimately died. We also observed re-patterning defects during regeneration when new eyes were formed in the already differentiated tissue. Thus, upon Smed-Pou5 RNAi, the stem cells retain the ability to respond to wound signals and differentiate. By using antibodies against SMEDWI-1, a stem cell marker, and PH3(Ser10), which recognize proliferating cells, we observed that both the total number of stem cells and the number of proliferating stem cells in Smed-Pou5(RNAi) animals gradually decreased. These effects were also observed during homeostasis. Moreover, when we analyzed the expression of Smed-Pou5 in sexual animals we found positive cells located at the basal layer of the planarian testes, where the spermatogonia localize. In addition, preliminary experiments showed that when Smed-Pou5 replaces Oct4 in the so-called Yamanaka cocktail, some mouse fibroblast re-activated the endogenous Oct4; however, iPS cells could not be expanded. Pluripotency outside the Mammalia is less well understood. An Oct4-like gene has been identified in fish, but its role in maintaining pluripotency is unclear. Research in chick and frogs, however, suggested that a similar role that Oct4 plays in mammals is fulfilled in non-mammalian vertebrates by POU domain proteins. Collectively, our results suggest an ancient role of POU proteins as key regulators of pluripotency and provide a notably candidate for the first invertebrate Oct4-like protein capable of sparking the reprogramming in terminally differentiated mammalian cells.

Keywords: planaria; adult stem cells; regeneration; pluripotency; neoblasts

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Abstract 117

A system analysis of translation in stem cells

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Induced pluripotent stem cells (iPSC) offer numerous opportunities for research and medical applications, because of their similarity to embryonic stem cells (ESC). However, little is known about the translational programs of both ESC and iPSC. To address this knowledge gap, I apply ribosome profiling, which allows for high-resolution snapshots of translation based on deep sequencing of ribosome-protected mRNA fragments. To this end, I am using murine and human iPSC and ESC. I have already established polysome profiling to characterize their translational state. Overall, I found global translation of the different stem cells lines to be fairly similar. Furthermore, I established the conditions for ribosome profiling in stem cells and compared different digest protocols by sequencing the sample libraries. Comparing the read output, the RPF length and the rRNA contamination I could show that a slight overdigest is the best possible treatment for the samples. Furthermore I compared the translational state of murine iPSC and mESC using the RPF sequencing data and could find differentially translated targets, which will be examined soon in detail. This will promote our understanding on stem cell biology and in the future will allow to model translational diseases.

Keywords: translation; ribosome profiling; stem cells

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Abstract 118

Exploring the reprogrammome using bioinformatics approaches

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Mammalian somatic cells can be artificially reprogrammed by ectopic expression of transcription factors in an unforeseen straightforward manner. Cellular reprogramming involves for instance the generation of so-called induced pluripotent stem (iPS) cells or multipotent neural stem (iNS) cells from terminally differentiated cells. Although cellular reprogramming holds great potential for biomedical applications such as cell replacement therapy and disease modeling the mechanism underlying the artificial cell conversion is largely unknown. The development of genomic high-throughput technologies such as gene expression microarrays, chromatin immunoprecipitation with microarray technology (ChIP-on-ChIP) and ChIP with massive parallel DNA sequencing (ChIP-seq) enabled a genome-wide profound assessment of cellular conversion and provide researchers with new insights towards the reprogramming process. Microarray technology has become widely used technique for measuring the expression levels of mRNA across different samples. However, the existence of different platforms (Agilent, Affymetrix and Illumina) makes it quite challenging to compare data generated from different experimental settings. Recently, virtualArray (Heider and Alt., 2013), an R/Bioconductor package has been successfully deployed which integrates the data generated from diverse platforms and enables cross platform comparison. In this study, we have demonstrated how bioinformatics approaches can be used to unravel mechanisms involved in cellular reprogramming, particularly the direct conversion of somatic cells into neural stem/progenitor cells. Using virtualArray, we compared the microarray datasets generated in Illumina and Affymetrix platforms from the Gene Expression Omnibus (GEO) with respect to induced Neural Stem Cells (iNSC) and Neural Stem Cells (NSCs). This comparison provided us with the information about key transcription factors that could play an essential role in cellular conversion of somatic cells to iNSCs. We additionally explore the evolutionary relationship between them employing meta-analysis of several experimental data sets. By that we show how novel bioinformatics approaches will enhance our understanding of gene regulatory networks and signal transduction pathways involved in reprogramming.

Keywords: cell reprogramming; bioinformatics; microarray technology; transcription factors

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Abstract 119

Identifying the OCT4 and SOX2 domains required for inducing pluripotency

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The transcriptional factors OCT4 and SOX2 are essential for inducing pluripotency in somatic cells. Many efforts have been made to identify the sequence of the genes that are activated during the reprogramming process. However, little is known about the OCT4 and SOX2 protein domains required to activate target genes. To identify which components of the mouse OCT4 and SOX2 proteins are critical in the reprogramming process, we have generated Oct4 and Sox2 constructs in which the N-terminal, the C-terminal, or both transactivation domains were deleted. We then tested the ability of these mutants to reprogram mouse embryonic fibroblasts into pluripotency. In addition, we have evaluated several point mutations that have been described to affect the OCT4 and SOX2 conformational dimerization. In summary, the results of this study will help us gain insight into the protein interactions required to recruit the transcriptional machinery and to establish a de novo pluripotent network.

Keywords: reprogramming; pluripotency; OCT4 and SOX2 interactions; transcriptional machinery

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Abstract 120

Nanog enhances cellular reprogramming by suppression of reprogramming-induced senescence

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Cellular reprogramming has fascinating prospects in both understanding human disease and regenerative medicine. However, this phenomenon is highly inefficient, laborious and the underlying molecular mechanisms are largely unclear. Combination of reprogramming factors associated with pluripotency has been demonstrated to accomplish this process. Nanog has been shown to be a part of this combination in some studies but has also been demonstrated to be dispensable for cellular reprogramming. However, induction of endogenous Nanog expression has been shown to be essential for successful generation of induced pluripotent stem (iPS) cells. In embryonic stem (ES) cells, Nanog plays an important role in early development and is essential to maintain these cells in an undifferentiated state. In this study, we set out to analyze a putative function of Nanog in somatic cells. For this, we engineered a biologically active recombinant cell-permeant Nanog by fusing it with the protein transduction domain TAT as a non-DNA based, non genetic paradigm to modulate cellular function. We demonstrated that Nanog-TAT treatment promotes ES cell proliferation, self-renewal, inhibits endodermal specification and promotes pluripotency in the absence of leukemia inhibitory factor (LIF). To elucidate the effect of Nanog in somatic cells, we found that Nanog protein transduction enhances proliferation of both, NIH 3T3 as well as primary fibroblasts. Moreover, Nanog induces anchorage-independent growth of NIH 3T3 cells in a dose- and time-dependent manner. Nanog transduction into primary fibroblast results in effective suppression of senescence associated beta galactosidase activity. In a cellular reprogramming paradigm cell-permeant Nanog is able to enhance iPS cell generation by 3-fold when applied at day 10 post infection. We show that transient activation of Nanog correlates with consistent down-regulation of p27KIP1 as well as high level of hyperphosphorylated Rb protein. Moreover, chromatin immunoprecipitation (ChIP) analysis reveals bona fide Nanog binding sites upstream to the p27 gene, establishing a close link between physical occupancy and functional regulation. In conclusion, our data indicate that Nanog acts as a link between the stemness transcriptional network and cell cycle machinery and might be instrumental to overcome reprogramming-induced senescence.

Keywords: cell reprogramming; pluripotency; protein transduction; cell cycle; embryonic stem cell

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Abstract 121

Different somatic cell types present a unique transcriptional response to Oct4 overexpression

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Differentiated cells can be reprogrammed into induced pluripotent stem cells (iPSCs) following the forced overexpression of a specific combination of transcription factors, of which Oct4 is essential. To better understand the mechanism underlying iPSC reprogramming, we investigated the immediate transcriptional response to ectopic Oct4 expression in somatic cells. To this end, we derived different somatic cell types from Oct4-inducible transgenic mice and induced Oct4 expression for different time periods. Using microarray analysis, we have identified early Oct4 transcriptional targets, including genes not previously known to interact with Oct4. Surprisingly, Oct4 induction in somatic cells led to the downregulation of many genes that are expressed in embryonic stem cells and the upregulation of only a few genes expressed in pluripotent cells. Furthermore, genes were differentially regulated among the different cell types. In other words, although some genes were commonly up- or downregulated in more than one cell type, others were oppositely regulated, i.e. upregulated in one cell type but downregulated in another cell type. In summary, our data suggests that Oct4 interferes with somatic transcriptional programs in a cell type–specific manner, thereby conferring general transcriptional plasticity rather than the direct initiation of a pluripotent gene expression network.

Keywords: Oct4; reprogramming; transcriptional regulation; iPSC mechanism; pluripotency

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Abstract 122

"Rainbow reprogramming" of nonhuman primate common marmoset cells towards pluripotency

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Pluripotent stem cells are highly attractive for regenerative medicine because they can, in principle, be differentiated towards any somatic cell type and tissues needed for transplantation. To test the medical applicability of this notion in a relevant animal model, we are currently establishing protocols to reprogram dermal fibroblasts of the nonhuman primate common marmoset (*Callithrix jacchus*) towards induced pluripotent stem (iPS) cells. For this purpose, we employ a lentiviral vector co-expressing the cDNAs for human OCT4, KLF4, SOX2 and MYC (in the order 5´-OKSM-3´). This vector was successfully used for reprogramming human cells (umbilical vein endothelial cells and dermal fibroblasts) and mouse fibroblasts (tailtip and embryonic fibroblasts). However, under comparable conditions it only mediated a partial reprogramming of marmoset skin fibroblasts. The obtained colonies expressed alkaline phosphatase, stained positive for SSEA-3 and SSEA-4 but were negative for TRA-1-81 and TRA-1-60 expression. Furthermore, global transcriptome measurements by RNA-seq and subsequent principle component analysis (PCA) revealed that the generated pre-iPS cells showed comparable gene expression among each other but still differed from the marmoset embryonic stem (ES-) cell line. However, they were much more similar to the ES cell line than to the skin fibroblasts indicating their partially reprogrammed state. To overcome possible epigenetic roadblocks due to remaining DNA-methylation of crucial pluripotency-associated gene loci, the DNA-methyltransferase (DNMT) inhibitor 5-Azazytidine (5-Aza) was added to the stable partially reprogrammed iPSC clones. Because the stoichiometry of the ectopically expressed transcriptions factors is also known to be crucial for complete reprogramming, as has been demonstrated in the mouse model, we transduced each factor together with a different fluorescence protein into marmoset fibroblasts by separate lentiviral vectors. Using this "rainbow reprogramming" system, the stoichiometric ratios of all four transcription factors can be estimated during different phases of iPS cell generation by determining the fluorescence intensities and subsequent additive color mixture. Indeed, upcoming transformed colonies showed a narrow range colors, thus indicating that OCT4, SOX2, KLF4 and MYC expression need to be expressed in stoichiometries other than in human or mouse cells.

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Keywords: Callithrix jacchus; induced pluripotent stem cells; partial reprogrammed cells; stoichiometry; 5-Azacytidine

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Niche & Microenvironment

Abstract 123

Prox1 determines oligodendrocyte cell fate in adult neural stem cells of the subventricular zone

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Adult neural stem cells with the ability to generate neurons and glia cells are active throughout life in both the dentate gyrus (DG) and the subventricular zone (SVZ). Differentiation of adult neural stem cells is induced by cell fate determinants like the transcription factor Prox1. Evidence has been provided for a function of Prox1 as an inducer of neuronal differentiation within the DG. We now show that within the SVZ Prox1 drives cells not into a neuronal cell fate, but into oligodendrocytes. Moreover, we find that loss of Prox1 expression in vivo reduces cell migration into the corpus callosum, where the few remaining cells fail to differentiate into oligodendrocytes. Thus, our work uncovers a novel function of Prox1 as a fate determinant for oligodendrocytes in the adult mammalian brain. This suggests a dual function of Prox1 – one in promoting neuronal fate in the hippocampus and another in determining oligodendrocyte fate in the SVZ.

Keywords: Prox1; oligodendrocytes; neural stem cells; adult neurogenesis

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Abstract 124

Induced germ cell loss is associated with an increase of the chemokine Cxcl12

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Background: Cxcl12 (chemokine (C-X-C motif) ligand 12) and its receptor Cxcr4 (CXC chemokine receptor 4) are constitutively expressed by most organs. It is known that an up-regulation of Cxcl12 expression occurs after adult stem cell loss and facilitates the recruitment of Cxcr4+ stem cells to the injured site, which aids tissue regeneration. Recently it has been shown that Cxcl12 is expressed at the basement membrane of mouse seminiferous tubules and that Cxcr4 is expressed by a subpopulation of undifferentiated spermatogonia (1). However, it remains unknown so far, whether an up-regulation of Cxcl12 can be detected after testicular germ cell loss, which would indicate a role of Cxcl12 and Cxcr4 during testicular regeneration. The aim of this study was therefore to investigate the expression patterns of Cxcl12 and Cxcr4 following an induced germ cell loss in adult mouse testes.

Methods: Adult male NMRI mice were either given a single injection (i.p.) of DMSO (vehicle control) or of busulfan at a concentration of 38 mg/kg bodyweight. On days 1, 3, 7, 21 and 28 after treatment, 10 animals per time point and treatment were killed and the testicular tissue was collected. Relative gene expression analysis was performed using real-time PCR for Cxcl12 and Cxcr4, the germ cell marker genes Ddx4 and Lin28a as well as for somatic marker genes including Amh, Cldn5, Erm, Gdnf and Itgb1. Furthermore, the expression of selected markers was evaluated performing immunohistochemistry.

Results and conclusion: Histological analysis revealed that the majority of seminiferous tubules were devoid of germ cells 28 days after busulfan treatment. This germ cell loss was accompanied by significantly reduced transcript levels of the germ cell marker genes Ddx4 and Lin28a. Interestingly, while expression levels of most somatic marker genes did not change significantly in the course of the study period, the expression levels of Cxcl12 were significantly increased 28 days after treatment compared to the DMSO control group. This finding was supported by immunohistochemical results. Our data therefore demonstrate that germ cell loss is associated with an up-regulation of Cxcl12 expression, indicating a role of the Cxcl12 / Cxcr4 interaction for the recovery of spermatogenesis. (1) Yang et al. CXCL12/CXCR4 signaling is required for the maintenance of mouse spermatogonial stem cells.

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Keywords: Cxcl12; busulfan treatment; testicular stem cells

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Abstract 125

Chemically defined medium for murine trophoblast stem cell culture

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Trophoblast stem (TS) cells are the in vitro equivalents of the precursor cells of the placenta. In the mouse, TS cells can be derived either from polar trophectoderm (TE) of blastocyst outgrowths or from postimplantation extraembryonic ectoderm (ExE), originating from polar TE. Since their first successful derivation in 1998 TS cells are cultured in serum-rich medium in the presence of fibroblast growth factor 4 (FGF4), the cofactor heparin and fibroblast conditioned medium. Here, we tested a simple medium formulation for TS cell culture, previously used for human induced pluripotent stem (iPS) cell derivation and culture by Chen et al., 2011 in which all protein reagents for liquid media are chemically defined. After adapting the growth factor composition for the requirements of TS cells, the defined medium consists of only ten ingredients (TX-medium). TX supports growth and self-renewal of TS cells grown on matrigel-coated dishes over multiple (>10) passages. TS cells cultured under these conditions express key trophoblast markers, maintain their ability to differentiate into all derivatives of the trophectodermal lineage in vitro and give rise to haemorrhagic lesions in nude mice, indistinguishable from cells grown in standard conditions. Global gene expression profiling of cells cultured in both media conditions revealed, that 99.6 % of genes are similarly expressed. Besides the culture of already established TS cell lines in defined media, new TS cell lines could be derived directly in defined medium conditions. The fact that TX media formulation no longer requires fetal calf serum and conditioned medium, facilitates and standardizes the culture of the extraembryonic trophectoderm lineage in vitro.

Keywords: trophoblast stem cells; culture conditions; defined medium

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Abstract 126

Neonatal mesenchymal-like cells adapt to surrounding cells

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Hematopoietic cord blood (CB) transplantations are performed to treat patients with life-threatening diseases. Besides endothelial cells, the neonatal multipotent stromal cell subpopulations CB MSCs (cord blood derived multipotent stromal cells) and USSCs (unrestricted somatic stromal cells) are like bone marrow (BM) MSCs interesting candidates for clinical applications if detailed knowledge is available. Clonal USSCs compared to CB MSCs and BM MSCs lines differ in their developmental origin reflected by a distinct HOX gene expression. About 20 (out of 39) HOX genes are expressed in CB MSCs (HOX+), whereas native USSCs reveal no HOX gene expression (HOX-). Moreover, USSCs display a lineage-specific absence of the adipogenic differentiation potential. As the specific HOX code can be ascribed to topographic bodysites it may be important to match the HOX code of transplanted cells to the tissue of interest. Herein co-culture experiments were performed, presenting a novel approach to modulate the differentiation potency of USSCs towards HOX positive stromal cells. After co-culturing native USSCs (HOX-) with CB MSCs and BM MSCs (HOX+), USSCs adapt a positive HOX code and gain the adipogenic differentiation capacity. These results present for the first time modulation of a lineage-specific differentiation potential by co-culture. Finally, USSCs can be claimed as potential candidates to substitute unique progenitor cell populations in clinical approaches.

Keywords: HOX code; cell fate switch; multipotent stromal cells; unrestricted somatic stromal cells; adipogenic differentiation

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Abstract 127

Effects of nanostructures and mouse embryonic stem cells on in vitro morphogenesis of rat testicular cords

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Morphogenesis of tubular structures is a common event during embryonic development. The signals providing cells with topographical cues to define a cord axis and to form new compartments surrounded by a basement membrane are poorly understood. Male gonadal differentiation is a late event during organogenesis and continues into postnatal life. The cellular changes resemble the mechanisms during embryonic life leading to tubular structures in other organs. Testicular cord formation is dependent on and first recognized by SRY-dependent aggregation of Sertoli cells leading to the appearance of testis-specific cord-like structures. Here we explored whether testicular cells use topographical cues in the form of nanostructures to direct or stimulate cord formation and whether embryonic stem (ES) cells have an impact on this process. Using primary cell cultures of immature rats we first revealed that variable nanostructures exerted effects on peritubular cells and on Sertoli cells (at less than <1000 cells/square millimeter) by aligning the cell bodies towards the direction of the nanostructures. After two weeks of culture testicular cells assembled into a network of cord-like structures. We revealed that Sertoli cells actively migrate towards high density zones. Contractions of associated and underlying peritubular cells pull the dense areas into elongated cord-like structures. The addition of mouse ES cells or conditioned medium from ES cells enhanced and intensified this process. Our studies show that epithelial (Sertoli cell) and mesenchymal (peritubular cells) cells crosstalk and orchestrate the formation of cords in response to external stimuli from the underlying matrix and secretory factors from ES cells. We consider these data on testicular morphogenesis relevant for the understanding of basic mechanisms in arrangement of cords and tubules in other organs. A better understanding of these mechanisms may help to create optimized in vitro tools for artificial organogenesis.

Keywords: nanostructure; testis; tubulogenesis; embryonic stem cell; Sertoli cell

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Abstract 128

Establishment of an extended in vitro assay to analyse the developmental potential of the most primitive human hematopoietic cells at a clonal level

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According to the classical model of human hematopoiesis, hematopoietic stem cells give rise to multipotent hematopoietic progenitor cells (HPCs), which either become restricted to the lymphoid or myeloid lineage. Recent findings of lymphoid progenitors that retain partial myeloid but lack erythroid potential, e.g. lymphoid primed multipotent progenitor cells (LMPPs), have challenged this model. These findings, including results from our group, suggest additional or alternative developmental pathways. In this project, we aim to unravel novel hierarchical relationships by retrospectively analyzing the developmental potential of primitive human hematopoietic cells at a single cell level. For such studies, HPCs need to be expanded without losing lineage potentials within their progeny fraction. Following an expansion phase the developmental potential of arising daughter cells should be analyzed using previously adopted functional read out systems for NK cell, T cell, macrophage, granulocyte, megakaryocyte and erythrocyte differentiation. Here we compared the hematopoietic support of murine stromal cell line cells and primary human stromal cells to maintain and expand multipotent HPCs, i.e. CD133+CD34+ cells, containing long-term myeloid, lymphoid and myelo-erythroid capabilities. We observed that expansion of CD133+CD34+ cells was 2 to 6-fold increased in co-culture with murine stromal cell line cells compared to primary endothelial cells (ECFC, HUVEC, PLVEC), placental-, vein-, artery- and AGM-derived mesenchymal stromal cells (MSCs). Notably, highest expansion rates were achieved in co-cultures with bone marrow (BM) mononuclear cell- and fat-derived MSCs. Functional analysis of expanded CD133+CD34+ cells revealed that the long-term myeloid (LTC-IC) differentiation potential was maintained under all conditions. However, NK cell capabilities were supported best in co-culture with endothelial stromal cells. Remarkably, only 1 out of 3 murine, 2 out of 10 MSC and 2 out of 10 endothelial cell lines supported the expansion of cells with myelo-erythroid potential. Next we will analyze whether these stromal cells also permit B cell, T cell and megakaryocyte development.

Keywords: hematopoietic stem cells; niche; stromal cells; lineage read outs; expansion

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Abstract 129

Evaluation of a protocol for isolation and expansion of murine lacrimal gland mesenchymal stem cells

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Purpose: Dry Eye disease is caused by inadequate quantity or quality of tears. One of the major reasons for severe Dry Eye is aqueous tear deficiency due to lacrimal gland insufficiency. Tools to facilitate regeneration of aggrieved lacrimal glands would be a desirable treatment option for patients suffering from severe Dry Eye. Aim of the present study was to establish a standardized protocol for isolation and expansion of putative mesenchymal stem cells (MSC's) from native murine and rat lacrimal glands to characterize the cells and to assess the differentiation potential in vitro.

Methods: Lacrimal glands were removed from wildtype mice. One part of the tissue was used for cell culture, the other part was embedded for immunostaining. Cultivated cells and lacrimal gland tissue was evaluated for the expression of mesenchymal progenitor cell markers, such as CD73, CD105, nestin, thy 1, PDGFR-β, SMA, desmin, vimentin, musashi-1, CD34 and pan-cytokeratin.

Results: Cells expanded from lacrimal gland tissue explants showed a fibroblast-like cell shape. After eight days of culture a subpopulation of cells showed signs of differentiation into myofibroblasts by demonstrating typical stress fibers. Expression of mesenchymal stem cell markers CD73, CD105, thy-1, PDGFR-β, SMA, desmin, vimentin and musashi-1 was demonstrated by immunostaining. Cells appeared to be negative for CD34. Furthermore cells positive for nestin could be detected in lacrimal gland tissue sections.

Conclusions: Murine lacrimal gland cells can be successfully isolated and expanded in vitro using explant culture techniques. The outgrowing cell population shows characteristics of mesenchymal stem cells, however more studies are needed to evaluate the differentiation potential and regenerative ability of this cell population.

Keywords: mesenchymal stem cells; lacrimal gland; regeneration; isolation; expansion

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Abstract 130

Comparison of enzymatic digestion and mechanical dissociation of human testicular tissue with regard to their effect on germ cell population

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Background: The possibilities for pediatric patients suffering from malignant diseases to protect their fertility are quite rare, because mostly the extraction of spermatogonial stem cells (SSC) of testicular biopsies in sufficient numbers is quite difficult. In the past, either a combination of enzymatic digestion and mechanical dissociation or solely enzymatic digestion has been used to generate single cell solutions from human testicular tissue. However, it has recently been demonstrated that it can be effectively obtained from rodent testes using the Medimachine system for mechanical dissociation. This method is of advantage because it is very fast, provides reproducible results and does not selectively damage certain cell types (1). Our study aimed at elucidating whether the Medimachine System can be used as an alternative for enzymatic digestion using human testicular tissue and whether the number of SSC can be enriched using mechanical dissociation.

Material & Methods: Testicular tissue was obtained after informed consent from patients suffering from Gender Identity Disorder (GID) undergoing Sex Reassignment Surgery (SRS; registered under 2012-555-f-S by the ethics committee, Ärztekammer Westfalen-Lippe). Testicular tissues were obtained from 34 patients by our cooperating clinicians in Osnabrück and Essen. On operation date, testicular tissues containing SSC were selected based on microscopic examination of the epididymal fluid as well as by cryosection assessment. Subsequently, 500 mg of testicular tissue each were subjected to both, enzymatic digestion and mechanical dissociation. Following this procedure, total cell numbers were determined using Trypan Blue. Relative gene expression analysis was performed using quantitative real-time PCR for germ cell (MAGE-4, SALL-4, UTF-1, FGFR-3, VASA) and somatic cell markers (SMA, VIM). Flow cytometric analysis was performed in addition for SALL-4 and VIM.

Results & Conclusion: So far, four human testes have been included into the study to compare effects of enzymatic and mechanical dissociation on the resulting proportion of germ cells. Although more cells were obtained using mechanical dissociation, the number of living cells was lower compared to enzymatic digestion. Interestingly, qPCR analyses showed an increased expression of germ cell specific and decreased expression levels of somatic marker genes following mechanical dissociation compared to enzymatic digestion. This indicates a selective damage of somatic cells. However, in the future, these results still need to be validated at the protein level. In conclusion our preliminary data indicate that the use of the Medimachine system is a reliable method for the dissociation of human testicular tissues, which results in the reduction of somatic cells in the single cell population and simultaneously in an enrichment of the germ cells. The next step would be the definition of culture conditions to be able to grow the spermatogonial stem cells and harvest them.

Literature (1) Rodriguez-Casuriaga et al. (2009) Ultra-Fast and Optimized Method for the Preparation of Rodent Testicular Cells for Flow Cytometric Analysis. Biol. Proced. Online, 11: 184-195

Keywords: spermatogonial stem cells; testicular tissue; mechanical dissociation; enzymatic digestion; germ cell population

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Abstract 131

Extrinsic niche components regulate the differentiation of neural stem cells and their self-renewal capacity

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The embryonic and adult niches in which neural stem cells (NSCs) reside are structured by the cells lying in these specialized microenvironments and by the molecules present there. Soluble factors and extracellular matrix molecules act on the behavior of stem and progenitor cells and drive their differentiation program and cell division modes. In the present study we analyze the influence of extracellular matrix proteins and other external cues on the differentiation and self-renewal capacity of NSCs derived from embryonic forebrains in vitro. Complex matrices produced by different cell lines and conditioned media containing various soluble factors influence the differentiation and proliferating activity of stem and progenitor cells. NSCs differentiate into all three neural cell types when cultured in the presence of fetal calf serum. Nevertheless, some cells maintain their proliferation capacity over several weeks and can generate neurospheres again when proliferative culture conditions are re-established. This self-renewal capacity was studied growing the cells in differentiation medium in the presence of special molecules. The experiments revealed that the extracellular matrix glycoprotein tenascin C and especially distinct domains of this molecule not just prolong, but enhance the self-renewal capacity of murine NSCs. The differentiation behavior of NSCs under the influence of distinct environmental cues was analyzed and revealed that matrix components which keep stem cells in their proliferating status prevent these cells from neuronal differentiation but can favor glial differentiation. The analysis of conditioned media from different cell lines revealed opposing effects for different media leading to a strong enhancement of stem cell self-renewal on the one hand and a drive to astrocytic differentiation on the other. A new cell type with a high self-renewal capacity and proliferation rate resembling NSCs cultured in adherent conditions can be described using medium conditioned by an immortalized glial cell line. This cell type was analyzed for its stem cell characteristics. In contrast, medium derived from a glioblastoma cell line strongly enhanced the differentiation into astrocytes. This study shows that the niche components are important regulators of the proliferation and differentiation of NSCs during embryonic development. In adult stem cell niches it is important that some cells maintain their stem cell potential. This can be accomplished by factors like tenascin C present in the respective microenvironments.

Keywords: neural stem cells; extracellular matrix; maintenance; neurospheres

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Organogenesis & Regeneration

Abstract 132

Biomaterial-based bone tissue engineering using novel silk scaffolds in combination with MSC

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Introduction: Autologous bone grafting is still the gold-standard for treating large bone defects. However, this method is limited by several disadvantages such as donor site morbidity, lack of donor tissue, as well as increased pain and operation times. An alternative approach is given by combining (degradable) biomaterials with autologous mesenchymal stem cells (MSC). Although numerous studies have examined osteoconductive effects of biomaterials on MSC, the ideal combination of stem cells and biomaterials for bone tissue engineering is still not found. The aim of the present study is to investigate the in vitro and in vivo performance of novel (hydroxyapatite modified) silk matrices cultured with MSC. These constructs will be introduced in critical-size bone defects in a large-animal model.

Materials/Methods: Silk scaffolds (un-/modified with hydroxyapatite) are provided by Spintec-Engineering GmbH. These materials incorporate essential characteristics required for bone tissue engineering such as appropriate mechanical stability, degradability and biomineral supplementation. The MSC compatibility of these scaffolds was investigated in an in vitro test system analyzing viability/cytotoxicity according to ISO 10993-5, proliferation, and live/dead staining. The performance of the silk MSC combination in vivo will be investigated in a preclinical study involving sheep with critical-size defect induction.

Results: Ovine and human MSC were isolated, expanded and characterized according to the Minimal Criteria of the International Society for Cellular Therapy. All silk scaffolds are non-toxic after 24 hours. The proliferation of ovine and human MSC is enhanced in silk scaffolds without or with only low content of hydroxyapatite. Silk scaffolds with higher content of hydroxyapatite reduce proliferation. Current studies involve stem cell differentiation towards osteoblasts by culture in the silk scaffolds.

Discussion: Silk scaffolds with and without hydroxyapatite were shown to be cytocompatible for ovine and human MSC. The reduced proliferation of MSC on silk scaffolds with high hydroxyapatite content might indicate that MSC undergo differentiation. Our study will contribute to better understanding biomaterial-induced osteogenic differentiation of MSC and their potential to regenerate large bone defects in vivo.

Keywords: biomaterials; silk; bone; tissue engineering; MSC

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Abstract 133

Rapid heart dissociation and subsequent allocation of purified heart cells for cardiac tissue engineering

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Engineered cardiac tissue offers the possibility to replace infarcted myocardium and to investigate heart development and cardiomyogenesis in a simplified in vitro model. It is generally accepted that tri-culture of cardiomyocytes, fibroblasts and endothelial cells enhances functionality of engineered cardiac tissues in vitro and improves survival in vivo. However, one of the major challenges is the availability of pure heart cells to generate in vivo-like cardiac tissues in a dish. Utilizing an automated tissue dissociator (gentleMACS) we developed a rapid neonatal heart dissociation protocol enabling an efficient immunomagnetic enrichment of cardiomyocytes and cardiac fibroblasts. First, we established an enzyme mix optimal for the dissociation of neonatal mouse and rat hearts (P0-P3) by screening our enzyme library. Next, we optimized the dissociation by utilizing our gentleMACS technology resulting in a fast (1h), robust and automated heart dissociation protocol. Analysis of the dissociated heart cells showed: (i) high cell vitalities (>90%), (ii) high frequencies of α-Actinin-positive cardiomyocytes (60%; P2) and (iii) vimentin-positive non-myocytes with a frequency of 40% (P2). In order to selectively enrich various cell-types from these heterogeneous cell populations, we performed a cell-surface marker screen. We identified several candidates for the composition of antibody cocktails enabling selective enrichment of cardiomyocytes and cardiac fibroblasts with purities of up to 97%. Cultured cardiomyocytes showed spontaneous beating activity and the expression of sarcomeric proteins (α-Actinin and cardiac Troponin) in a cross striated pattern. In summary, we established an automated protocol for the dissociation of neonatal hearts, enabling the subsequent immunomagnetic enrichment of cardiomyocytes and cardiac fibroblasts, which can readily be utilized to generate in vitro heart muscle models and surrogate tissue for myocardial repair.

Keywords: cardiomyocytes; cardiac fibroblasts enrichment; heart dissociation

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Abstract 134

Hepatic stellate cells are mesenchymal stem cells that contribute to liver regeneration

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Hepatic stellate cells are thought to contribute to fibrosis in chronic liver diseases, but their identity and function in normal liver remain enigmatic. This study identifies stellate cells as liver-resident mesenchymal stem cells (MSC) that can support extra-medullary hematopoiesis and contribute to liver regeneration. Stellate cells typically reside in the liver as pericytes between sinusoidal endothelial cells and hepatocytes in the space of Disse, which represents their niche. Hepatic stellate cells store retinoids and express glial fibrillary acidic protein (GFAP) in their quiescent state, but start to express Nestin and other MSC markers during activation, while GFAP expression and retinoid contents decrease. In the fetal liver, stellate cells are associated with hematopoietic sites and are able to support hematopoietic stem cells in vitro. Moreover, isolated hepatic stellate cells can differentiate into adipocytes and osteocytes. This demonstrates that typical functions of bone marrow MSC are fulfilled by hepatic stellate cells. After liver injury, hepatic stellate cells are activated and contribute to liver regeneration. Freshly isolated hepatic stellate cells purified by fluorescence-activated cell sorting differentiate into hepatocytes and integrate into bile ducts after transplantation into rats that underwent partial hepatectomy in the presence of 2-acetylaminofluorene (2AAF). The application of 2AAF inhibits the proliferation of hepatocytes, which otherwise mainly contribute to liver repair through proliferation. Hepatic stellate cells transiently express markers of hepatic progenitor cells also termed oval cells during their differentiation into hepatocytes. Oval cells, which only appear after severe liver injury, express hepatobiliary markers and are generally seen as precursor cells of hepatocytes. Transplanted hepatic stellate cells are also detectable in the bone marrow of host animals, which further confirm their classification as MSC. Similar to neuronal stem cells and bone marrow MSC, hepatic stellate cells can fulfill a dual role as supportive cells for stem/progenitor cell populations such as hematopoietic stem cells on the one hand and as stem cells that can generate new cell types during tissue repair on the other.

Keywords: stellate cells; mesenchymal stem cells; liver regeneration; extra-medullary hematopoiesis

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Abstract 135

β-catenin is a critical regulator of mitochondrial function and energy balance in liver homeostasis and disease

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Background: Hepatocytes are central for toxicology studies and drug screening. As it is impossible to perform large scale studies on primary cell cultures due to rapid dedifferentiation and limited life span, an induced pluripotent stem cells (iPSC)-based strategy may serve as an additional strategy for studying toxin-induced liver injury. To date hepatocyte-like cells have been derived from iPS cells (iPSC-HLCs), however analysis of these have been restricted solely to their biochemical properties. There is therefore a need to dissect and understand liver function in model organisms such as mice and thereafter transfer this knowledge to the characterisation of human iPSC-HLCs . Since the liver is the central organ for regulating systemic energy balance and nutrient metabolism and the Wnt/β-catenin signaling pathway is a critical molecular regulator of hepatic development, regeneration and carcinogenesis, we hypothesized a novel role for Wnt signaling in the liver in regulating mitochondrial function and cellular energy balance.

Material and Methods: Mice with hepatocyte-specific β-catenin deletion (KO) and wild-type littermates (WT) were fed either normal chow or administered acute ethanol intoxication. Energy balance was determined by ATP level, pyruvate and lactate concentration. Mitochondrial function was assessed by mitochondrial content, membrane potential and functional analyses for TCA cycle and oxidative phosphorylation (OXPHOS). Hepatic steatosis was determined by ALT, triglycerides and histology. Intracellular oxygen radicals were detected by DHE staining, MDA assay and NAD+/NADH ratio. Changes in fatty acid β-oxidation (FAO) were determined by mRNA and protein changes in PPAR-α and Sirt1 signaling.

Results: We report that β-catenin-deficient hepatocytes possess basal mitochondrial dysfunction and energy deficit involving impaired TCA cycle, OXPHOS and ATP production. Despite these findings, there is no evidence for redox imbalance in the absence of metabolic stress. In β-catenin-deficient hepatocytes, acute ethanol intoxication leads to significant redox imbalance and further deterioration in mitochondrial functionincluding compromised OXPHOS and ATP production. Ethanol feeding resulted in increased steatosis and oxidative damage in β-catenin-deficient mice along with a disrupted NAD+/NADH ratio. We further demonstrate that KO mice show reduced FAO with a disrupted Sirt1/PPAR-α signaling axis.

Conclusion: Taken together, our results demonstrate that β-catenin plays an important role in the maintenance of mitochondrial homeostasis. These findings demonstrate an important link between mitochondrial function and hepatic regenerative capacity that may have significant implications targeting iPSC-based strategies for hepatic protection, regeneration and carcinogenesis.

Keywords: Wnt signaling; mitochondria; energy metabolism; liver injury

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Abstract 136

Adipose derived stem cells improve migration and proliferation of keratinocytes in acute and chronic wounds in vitro

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Introduction: Chronic Wounds have a high significance in our health system. In the last decade there have been new therapeutic approaches to accelerate the healing of chronic wounds, but often with little success. Adipose derived stem cells (ASCs) are mesenchymal stem cells and can be easily isolated from fat tissue. They are known to have regenerative effects. The aim of this study was to analyse the effects of ASCs on keratinocytes (KCs) in the context of wound healing.

Material and Methods: The environment of acute and chronic wounds was simulated by adding 2% wound fluid (WF) to cell culture medium. Acute WF (AWF) was taken from subcutaneous drains of 7 patients after elective surgery. Chronic WF (CWF) was harvested from seven patients with sacral or trochanteric decubituus that existed for minimally 6 weeks. Samples were processed and pooled in the laboratory. ASCs from one donor were used in passage 2 to 4. HaCaT (human adult low calcium high temperature), an immortalised human KC cell line, was used in passage 45-52. To analyse migration and proliferation of KCs in the presence of ASCs, scratch assay and MTT-Test were performed in an indirect co-culture setting using hanging inserts with a semipermeable membrane (4 µm pores). In three independent experiments KCs were incubated for 24, 48 and 72 (only MTT-test) hours with DMEM and 2% FBS (basal medium, BM), 2% AWF or 2% CWF. KC cell culture without hanging insert served as control.

Results: Under the influence of AWF or CWF, proliferation of KCs significantly increased. After 72 hours proliferation rate was 207% with AWF and 166% with CWF. In co-culture with ASCs proliferation further increased in both groups, whereas no significant difference was seen between AWF and CWF (after 72 hours: AWF+ASC 307%; CWF+ASC 293%). Without WFs KC proliferation was also enhanced by ASC (after 72 hours: BM+ASC 132%), but the effect was less pronounced. Using the scratch assay cell free area (CFA) decreased in every group, but migration was slower with CWF compared to AWF (after 48 hours: AWF 83% CFA vs. CWF 90% CFA). The paracrine impact of ASCs led to a higher migration rate after 48 hours (AWF+ASC 20% CFA vs. CWF+ASC 64% CFA). The highest migration rate was observed with BM, when co-cultured with ASCs. After 48 hours the defect was almost closed (cell free area 6%).

Conclusion: ASCs significantly increased the performance of keratinocytes in vitro. They potentially represent an important source in regenerative medicine for the treatment of chronic wounds. They have shown a great secretory activity and further investigation looks promising, that underlying biology of ASCs can be explained.The understanding of their potential is critical for developing a therapeutic utilization in the future.

Keywords: cocultur; adipose derived stem cells; wound healing

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Abstract 137

Co-transplanted non-cardiomyocytes enhance early persistence of induced pluripotent stem cell derived cardiomyocytes after intramyocardial injection but they also proliferate in loco over time

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Purpose: Induced pluripotent stem cell derived cardiomyocytes (iPS-CM) are suitable for cardiac cell replacement therapy since they can integrate into host myocardium after transplantation and thereby improve heart function. However, highly purification is needed to avoid teratogenic risk but it leads to poor persistence of transplanted iPS-CM which we here tried to improve by co-transplantation of non-cardiomyocytes (non-CM).

Methods: Male iPS-CM were derived from transgenic murine induced pluripotent stem cells and highly purified using an antibiotic resistance under a cardiac specific promoter. Intramyocardial injection of 300,000 iPS-CM with or without 300,000 male wild-type murine non-CM (embryonic fibroblasts [+MEF] or adult mesenchymal bone marrow cells [+MSC]) was performed into healthy hearts of syngeneic female wild-type mice. Immediately (0h) or after 24h, 48h or 7 days (each n≥4 per group), hearts were harvested and the number of persisting transplanted cells was determined by quantitative real-time PCR with specific primer for SRY or transgene. One additional cell aliquot was mixed with an explanted native heart ex vivo as control (=100%) for every surgery day.

Results: Immediately after transplantation of iPS-CM alone (0h), 28.4±4.0% of transplanted cells were detected in recipient hearts and the number decreased to 2.6±0.9% at 6h ($p<.001$ vs. 0h) and 0.6±0.2% at 24h ($p<.0001$ vs. 0h). After co-transplantation with non-CM the number of detectable male cells (iPS-CM+non-CM) at 24h was more than 4-fold increased to 2.7±1.5% (+MEF) and 2.8±1.0% (+MSC, $p=.06$ vs. iPS-CM alone) and it increased over time in both groups up to 15.8±5.6% (+MEF) or 4.7±1.7% (+MSC) at 48h and 12.3±5.2% (+MEF) or 6.2±1.6% (+MSC) at 7d (p for linear trend <.01 in both groups). The number of detectable transgenic cells (iPS-CM) after co-transplantation was more than 2-fold higher than in iPS-CM alone at 24h with 1.3±0.7% (+MEF) and 1.5±0.2% (+MSC, $p<.05$ vs. iPS-CM alone), and it remained stable over time in +MEF (48h: 2.4±1.2%, 7d: 2.4±0.9%) and it tended to decrease in +MSC (48h: 0.3±0.1%, 7d: 0.7±0.4%, $p=.1$ vs. 24h). Accordingly, the fraction of iPS-CM within the persisting transplanted cells decreased over time.

Conclusions: Persistence of highly purified iPS-CM at 24h after intramyocardial injection into syngeneic mouse hearts is poor (<1%) but it is increased by co-transplantation of non-CM (MEF or MSC). While this higher persistence remains stable over time only with co-transplanted MEF, proliferation of non-CM is seen in both co-transplantation groups and must be cautiously monitored.

Keywords: persistence of iPSCM; co-transplantation with MSC & MEF; real-time PCR

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Abstract 138

Heat shock treatment prior to intramyocardial injection into syngeneic mouse hearts increases early engraftment and persistence of murine induced pluripotent stem cell derived cardiomyocytes

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Purpose: Induced pluripotent stem cell derived cardiomyocytes (iPS-CM) can integrate into host myocardium and thereby improve function of failing hearts as cardiac cell replacement therapy. While highly purified iPS-CM are needed to avoid teratogenic risk, this therapy's effectiveness is limited by their poor persistence after transplantation. Thus, we tested heat shock treatment of cells prior to transplantation to improve their persistence.

Methods: iPS-CM were derived from transgenic murine iPS and highly purified using an antibiotic resistance under a cardiac-specific promoter. Intramyocardial injection (CTx) of 300,000 iPS-CM with (hs-iPS-CM) or without (iPS-CM) heat shock treatment (30min at 42°C 2 days prior to CTx, each group n≥4) was performed into healthy hearts of wild-type recipient mice. Hearts were harvested immediately (0h) or after 6h or 24h and the number of transplanted cells was determined by quantitative real-time PCR with transgene-specific primers. As control (=100%), one cell aliquot was mixed with an explanted native wild-type heart ex vivo each surgery day.

Results: For non-treated iPS-CM, we detected 28.4±4.0% of transplanted cells immediately after CTx and numbers decreased over time to 2.6±0.9% at 6h ($p<0.001$ vs. 0h) and 0.6±0.2% at 24h ($p<0.001$ vs. 0h, $p<0.0001$ for linear trend). For hs-iPS-CM, values were 37.7±9.7% of transplanted cells at 0h, 26.9±4.6% at 6h and 1.8±0.6% at 24h ($p<0.05$ vs. 0h and vs. 6h, $p<0.01$ for linear trend). In comparison, numbers of hs-iPS-CM were significantly higher than those of untreated iPS-CM at 6h ($p<0.05$) and in trend also at 24h ($p=0.1$).

Conclusions: Heat shock treatment prior to transplantation increased engraftment and persistence of highly purified iPS-CM, interestingly already at 6h after transplantation. Therefore, this strategy might also enhance adhesion of treated cells in addition to anti-apoptotic effects and should thus be further investigated, maybe in combination with other methods, to optimize the effectiveness of cardiac cell replacement therapy and to facilitate its possible therapeutic benefit.

Keywords: persistence of iPSCM; heat-shock; real-time PCR

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Abstract 139

Improved recovery of heart function by co-transplantation of iPS cell-derived cardiomyocytes with mensenchymal stem cells

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Objective: The effects of co-transplanting mesenchymal stem cells (MSC) with cardiomyocytes derived from induced pluripotent stem cells (iPS-CM) in a murine model of myocardial infarction were examined.

Methods: Immediately after cryo-induction of myocardial infarction one the following suspensions was injected intramyocardially into the peri infarction area: A) EGFP and luciferase expressing iPS CM, B) magnetically labeled MSC, C) a combination of both or D) saline. Transplanted cells were tracked by either in vivo bioluminiscent imaging (iPS CM) or magnetic resonance imaging (MSC) weekly for four weeks. Left ventricular ejection fraction (LVEF) was assessed by magnetic resonance imaging. Size of infarction scar and structural integration of transplanted cells were analyzed histologically.

Results: Relative luminiscence intensity of transplanted iPS CM was reduced to 43±7% on day 1 and further to 18±2% on day 28 for iPS-CM and to 49±11% on day 1 and 19±2% on day 28 for iPS CM/MSC. Transplanted MSC remained in the peri-infarct region of the left ventricular wall for four weeks. LVEF increased significantly in A) iPS-CM: 51.8±3.3% and C) iPS CM/MSC: 55.7±2.3%, compared to B) MSC: 47.6±1.9%; both $p<0.001$ and D) saline: 44.2±2.6%; both $p<0.001$ after four weeks. Infarction scars were significantly smaller in A) iPS-CM: 2.77±1.35mm^2 and C) iPS-CM/MSC: 2.73±1.18mm^2 compared to B) MSC: 4.9±1.79mm^2, both $p<0.05$ and D) saline: 5.73±2.68mm^2, both $p<0.05$. Structurally organized expression of cardiac α-actinin 2 and connexin 43 was detected in transplanted iPS CM.

Conclusion: Co-transplantation of iPS CM and MSC into infarcted hearts improved recovery of heart function and reduced infarct scar size, demonstrating the potential of this approach for regenerative therapies.

Keywords: iPS; cardiomyocytes; MSC; transplantation; myocardial infarction

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Abstract 140

Altered extracellular matrix produced by glial cells after cortical laser lesions

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The mammalian central nervous system (CNS) shows a limited capacity to regenerate after lesion. Extracellular matrix (ECM) molecules are critical factors for regeneration as they modulate survival, proliferation, migration, differentiation, and integration of cells as well as neurite outgrowth. Therefore, an understanding of the ECM composition and of the cell types that produce this matrix after lesion is essential for effective treatment of the lesioned CNS in the future. The visual cortex represents an appropriate model system for regeneration and plasticity studies due to its convenient accessibility and the extensive knowledge about synaptic plasticity and reorganization in this region. Laser lesions in the rat visual cortex are well described, whereas the mouse system used in this study offers the advantage of various knockout models. The lesion site was characterized by immunohistochemistry in terms of the different glial subtypes involved in reactive gliosis and of the ECM composition. Three days after lesion, glial subtypes expressing the markers nestin, glial fibrillary acidic protein (GFAP), vimentin or S100beta were not equally distributed. Nestin was restricted to a small area adjacent to the lesion, GFAP showed a more widespread up-regulation, whereas vimentin was found in an intermediate pattern. Subpopulations of cells expressed either one of these markers alone, or coexpressed different markers. Also 14 days after lesion, a similar pattern persisted. The ECM was altered after lesion: The stem cell-related DSD-1-epitope was detected on the surface of astroglia-like shaped cells near the lesion core. Tenascin-C, a glycoprotein that regulates differentiation and axon growth and that is expressed in the CNS during development and after injury, was found up-regulated in the lesioned cortex in GFAP-positive astroglia. Differences in the spatial distribution of the markers nestin, GFAP, vimentin, and S100beta indicate a complex composition of glial subtypes instead of a uniform type of activated astrocytes. The postlesional ECM contains the DSD-1-epitope and tenascin-C, expressed by astroglial cells. GFAP and vimentin expression is typical of reactive astrocytes, whereas nestin and the DSD-1-epitope are also characteristic of radial glia and progenitor cells during development. Therefore, these cells are candidates for neurosphere-forming progenitors that had been observed post-lesionally in the rat model. In the light of a neurogenic potential described for cells in the penumbra, this suggests a model where some astroglial cells, probably expressing the stem cell-related DSD-1-epitope, provide a potential intrinsic source of newborn neurons. At the same time, these cells produce an ECM that can influence regeneration by regulating cell differentiation or neurite outgrowth.

Keywords: extracellular matrix; Tenascin-C; gliosis; regeneration; visual cortex

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Abstract 141

The Integrator complex is required for stem cell maintenance in planarians

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Planarians exhibit outstanding regenerative capacities, enabling tiny fragments from almost everywhere of their body to give rise to entire new animals. This ability requires a population of adult stem cells called neoblasts, of which a subpopulation was shown to be pluripotent. Hence, planarians are a powerful model organism to study stem cell biology and regeneration in vivo. A recent proteomic screen identified two subunits of the Integrator complex, IntS3 and IntS9, as novel putative regulators of stem cell maintenance and regeneration in Schmidtea mediterranea. The Integrator complex is a multiprotein mediator of spliceosomal snRNA 3' end processing. Here, it is shown that the Integrator is expressed in the parenchyma, a mesenchymal tissue between organs, where neoblasts reside. Its expression is reduced after elimination of neoblasts by irradiation. IntS3 and IntS9 RNAi animals develop tissue regression during homeostasis and amputated fragments initiate blastema formation during regeneration but fail to complete the regenerative process. Immunofluorescence analysis for Smedwi-1, a neoblast marker, and flow cytometry of regenerating IntS3 and IntS9 RNAi fragments displayed a neoblast loss over time. However, no characteristic changes in neoblast proliferation and apoptosis could be detected in Integrator knockdown animals. Expression level analysis for early and late neoblast progeny markers in Integrator knockdown animals indicated an unimpaired ability of neoblasts to differentiate, but increased amounts of snRNAs. These data show that after Integrator knockdown neoblasts are able to proliferate and differentiate, yet animals fail to maintain the stem cell pool. Thus, our study indicates that the Integrator complex is required for the maintenance of the neoblast pool during homeostasis and regeneration in Schmidtea mediterranea. Furthermore, it suggests that the regulation and function of the splicing machinery is essential for the control of stem cell self-renewal.

Keywords: stem cells; regeneration; planarians; Integrator complex

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Abstract 142

Enhanced engraftment but poor persistence of highly purified murine embryonic stem cell derived cardiomyocytes after intramyocardial injection in clusters together with murine adult mesenchymal bone marrow cells

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Objective: Ischemic heart disease and resulting heart failure are the leading causes of death world wide and cell replacement therapy is a promising therapeutic option. Embryonic stem cell derived cardiomyocytes (ES-CM) can integrate into host myocardium and improve cardiac function in heart failure but their low persistence after cell transplantation limits this therapy's effectiveness. Here, we tested transplantation of in-vitro grown clusters of ES-CM together with adult mesenchymal bone marrow cells (MSC) as a novel approach to facilitate early cell retention.

Material and methods: Male transgenic murine ES-CM were generated and highly purified (>97%) using antibiotic resistance under cardiac specific promoter and male MSC were isolated from syngeneic wild-type mice. Both cell types were then grown in co-culture on special dishes enabling gentle detachment of the in-vitro grown mixed clusters which were then intramyocardially injected into healthy hearts of syngeneic wild-type female mice. Hearts were harvested immediately (0h) or after 24h or 48h (each n≥6) while native explanted hearts with ex-vivo added cluster aliquots served as controls (ctrl=100%). Genomic DNA was isolated from all samples and numbers of male and transgenic cells were determined using quantitative real-time PCR with specific primers for Y-chromosome and transgene.

Results: Immediately after intramyocardial injection of in-vitro grown mixed clusters, we detected 21.7±5.0% of male cells (i.e. ES-CM+MSC) injected and numbers decreased to 8.6±2.7% at 24h and 3.7±0.9% at 48h ($p<0.05$ vs. 0h, $p<0.01$ for linear trend). With selective quantification of transplanted ES-CM alone, we detected 23.4±4.4% of transgenic cells (i.e. ES-CM) injected at 0h, 4.9±2.2% at 24h ($p<0.01$ vs. 0h) and 1.0±0.4% at 48h ($p<0.01$ vs. 0h, $p<0.001$ for linear trend). The fraction of ES-CM within the detectable cells was 59.2±4.6% in ctrl, 86.6±27.3% at 0h and then decreased over time to 38.6±8.0% at 24h and 18.1±7.1% at 48h. Although similarly low at 48h, detection of injected ES-CM at 0h and 24h was remarkably higher than in control experiments with intramyocardial injection of single cell suspensions of ES-CM alone (13.3±2.8% at 0h and 0.8±0.3% at 24h).

Conclusion: Intramyocardial injection of ES-CM in in-vitro grown mixed clusters together with MSC seems to delay the massive early loss of transplanted single cells presumably by increasing volume, surface and adhesiveness of injected particles and by providing a beneficial milieu within the injected clusters and should therefore be further investigated.

Keywords: ES-CM clusters; cardiac differentiation; intramyocardial injection

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Abstract 143

Reactive glia in the injured brain acquire stem cell properties in response to Sonic hedgehog

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The reaction of glial cells toward brain injury comprises a proliferative response with some reactive astrocytes even reacquiring stem cell potential as indicated by forming long-term self-renewing and mutlipotent neurospheres (for review: Robel et al., 2011). Here we addressed first the question to which extent the type of injury selectively affects the proliferative response of NG2 glia and astrocytes and to which extent the proliferative reaction of astrocytes may be linked to the stem cell response. Interestingly, while both astrocytes and NG2 glia mounted a profound proliferative response after acute invasive injury, such as stab wound and MCAo, non-invasive injury models, such as amyloid plaque deposition or widespread neuronal death could not activate the proliferation of these macroglial cells, while microglia proliferated actively in all these lesion models. Intriguingly, the proliferative response of astrocytes correlated to the activation of their potential to form self-renewing, multipotent neurospheres with a steep decline with age. Then we set-out to determine the signals that regulate reactive astrocyte proliferation and neurosphere formation after invasive injury and demonstrate that invasive lesions result in entrance of Sonic hedgehog (SHH) into the brain from extraneural sources, such as the cerebro-spinal fluid, and activate astrocyte proliferation and neurosphere formation. This response can be blocked by systemic injection of the SHH-signaling antagonist cyclopamine as well as inducible astrocyte-specific deletion of the receptor smoothened by GLASTCreERT2. Interestingly, SHH-agonists can boost the proliferative response of astrocytes in vivo and their subsequent neurosphere-forming capacity, thereby providing a new approach how to activate this response in conditions where it is limited, as e.g. in age or non-invasive injury conditions. Taken together, our work highlighted for the first time differences in the proliferative and stem cell response of reactive astrocytes in different injury paradigms and identified the SHH signaling pathway as a key signal in this response.

*Robel S, Berninger B, Götz M. The stem cell potential of glia: lessons from reactive gliosis. Nat Rev Neurosci. 2011 Feb;12(2):88-104.

Keywords: reactive gliosis; neural stem cells; sonic hedgehog; amyloid; cerebral injury

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Abstract 144

„Cross-talk“ between natural killer (NK)-cells and mesenchymal stem cells (MSCs): MSCs enhance the activation of NK-cells

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Injuries of the bone induce an inflammatory reaction accompanied by the recruitment of immune cells to the affected tissue. Amongst others these cells comprise natural killer (NK)-cells. After stimulation with Interleukin (IL)-12 and IL-18 NK-cells secrete the pro-inflammatory cytokine Interferon (IFN)-gamma. IFN-gamma plays a crucial role in the infection defense. During the process of bone regeneration mesenchymal stem cells (MSCs) are accumulated and differentiate into osteoblasts. In consideration of close proximity of NK-cells and MSCs at the affected bone the aim of this study was to investigate if MSCs could influence the ability of NK-cells to produce IFN-gamma. To investigate the mutual influence of NK-cells and MSCs co-culture experiments with both cell types were performed under conditions of direct cell-cell contact or by separating both cell types with a membrane in a transwell culture system. Therefore, the human cell line NK92 was used as well as human primary NK-cells isolated from the peripheral blood of healthy probands. Human MSCs were isolated from bone marrow of patients undergone a total hip replacement surgery. NK-cells and MSCs were co-cultured at different proportions (1:1 to 20:1). The activity of the NK-cells was determined by the production of IFN-gamma after stimulating the cells with IL-12 and IL-18. The amount of IFN-gamma production was measured by ELISA assay and intracellular flow cytometry. Preliminary results show an increased IFN-gamma secretion of NK-cells if they were stimulated in the presence of MSCs. At the rate of 20:1 NK-cells to MSCs the IFN-gamma production is augmented more than 200%. With increasing number of MSCs this effect is further enhanced up to 350%. Interestingly, this activation is additionally increased by preincubating the NK-cells with MSCs for 24 hours prior to stimulation with IL-12/IL-18. This enhanced activation of NK-cells due to the presence of MSCs is also observed in a co-culture system where both cell types are separated by a membrane permeable for soluble molecules. In conclusion, MSCs enhance the ability of NK-cells to secrete IFN-gamma. This modulatory activity of MSCs seems to depend to some extent on soluble factors. The aim of the current work is to resolve the molecular mechanism which accounts for this effect. As MSCs and NK-cells come in close contact within the injured tissue an enhanced reactivity of NK-cells could have a positive impact on the infection defense.

Keywords: MSC; NK; interferon; co-culture; activation

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Somatic & Cancer Stem Cell

Abstract 145

Pharmacological interference with the stemness-associated Notch-signaling pathway exerts an antiproliferative effect on the endometriotic 12Z cell line

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Adult stem cells are thought to participate in endometrial regeneration during the reproductive phase of women. We have previously demonstrated dysregulated stem cell activity in endometriosis, a benign disease associated with unspecific pain symptoms and reduced fertility due to ectopic endometrial growth. We hypothesize that the unlimited proliferative potential of stem cells, along with their high differentiation capacity, contributes to the pathogenetic process. In the present study, we evaluated the effects of interference with the stemness-associated notch pathway on the properties of an epithelial endometriotic cell line (12Z). Pharmacological inhibition of notch signaling was achieved by in vitro treatment with a gamma-secretase inhibitor (GSI), which blocks proteolytic activation of the receptor. Furthermore, we employed siRNA-mediated depletion of two modulators of notch endocytosis, Msi-1 and Msi-2. Both GSI-treatment and Msi-knockdown rinhibited cell proliferation as determined by MTT assay, and ALDH1 activity, a surrogate marker of stem cell activity. Flow cytometric annexin V assays demonstrated increased apoptosis in 12Z cells subjected to notch inhibition. TaqMan low density PCR array analysis of GSI-treated endometriotic cells revealed a dysregulation of several stemness-associated factors. Among these, qPCR-analysis confirmed a significant downregulation of the stemness-associated factors LIFR, SOX2, PODXL and IFITM1 upon notch inhibition. Our data support the stem cell concept of endometriosis and suggest that pharmacological interference with the notch pathway may be a worthwhile future strategy for its treatment.

Keywords: musashi 1 and 2; notch pathway; ALDH activity; GSI

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Abstract 146

Investigation of signal pathways in hybrid cells from breast cancer and breast cells with stem cell properties

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Cell fusion is a process, which occurs not only during fertilization but also in many physiological and pathophysiological processes, including cancer. Hybrids of cancer cells and other cells exhibit novel properties, for example an increased migratory behaviour. In this study the activation of the RAS-RAF-MAPK, the PI3K-AKT, and the PLC-β3/γ1 pathways in hybrid cell lines M13HS-2 and M13HS-8 was analysed. Both hybrid cell lines were in vitro obtained from spontaneous fusion events between M13SV1-EGFP-Neo cell line exhibiting stem cell characteristics and HS578T-Hyg breast cancer cell line. Previous studies have shown that all cell lines express the CCR7 receptor, but only the hybrid cell lines showed an increase of migratory activity by treatment with the CCR7 receptor ligand CCL21. A 3D collagen matrix migration assay was applied to investigate the migratory activity. Western blot analyses were applied to investigate the activation of different proteins. The inhibitors U73122 (PLC-β3/γ1 inhibitor), Ly294002 (PI3K inhibitor), and PD98059 (MAPK inhibitor) were used to test the influence of PLC-β3/γ1, PI3K, and MAPK on the signal transduction. CCR7 siRNA was applied to knock down the CCR7 receptor. Calcium measurements with FACS were made to test CCL21 and / or EGF depending calcium influx. Both parental and both hybrid cell lines responded to EGF stimulation with an increased migratory activity and an increase in phosphorylation of MAPK and AKT. In contrast, only the hybrid cell lines responded to CCL21 with an increased migratory activity and MAPK and AKT phosphorylation. Calcium influx occurred only in hybrid cells after CCL21 and / or EGF treatment. Knock down experiments with siRNA for CCR7 showed that the CCL21 mediated migratory activity increase depended on CCR7 expression. Incubation with Ly294002 leads in CCL21 and / or EGF treated hybrid cells to a slight decrease of migratory activity. In EGF treated parental cell lines Ly294002 leads to a decrease in migratory activity. Treatment with Ly294002 resulted in decrease of AKT phosphorylation. Also hybrid cell lines treated with PD98059 showed after CCL21 and / or EGF treatment only a slight decrease of migratory activity. PD98059 treatments have no effect of EGF induced migratory activity in parental cell lines. Western blot analyses showed that PD98059 leads to a decrease of MAPK phosphorylation. Treatment with U73122 leads to inhibition of CCL21 and / or EGF depending Calcium influx in hybrid cells, but it had no effect on the migratory activity. In conclusion these results show that mainly the PI3K-AKT pathway has a contribution to the CCL21 mediated increase of migratory activity in M13HS hybrid cell. The RAS-RAF-MAPK pathway shows a lower contribution to the CCL21 mediated increase of migratory activity in these hybrid cell lines. The PLC-β3/γ1 pathway has no effect on the migratory activity of hybrid cell lines. In parental cell lines EGF mediated migratory activity increase depends only on the PI3K-AKT pathway. The investigation of these artificial cancer / stem cell hybrids can make a contribution to the understanding of invasiveness and the metastatogenic behaviour of cancer.

Keywords: cell hybrids; MAPK / AKT signaling

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Abstract 147

Impact of simultaneous deletion of Caspase-3 and p21 on hematopoietic stem cells homeostasis

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Specialized blood cells are generated through the entire life of an organism by differentiation of a small number of hematopoietic stem cells (HSC). There are strictly regulated mechanisms assuring a constant and controlled production of mature blood cells. The mechanisms controlling HSC are not completely understood but some factors regulating cell cycle and differentiation have been identified. We have previously shown that Caspase-3 is an important regulator of hematopoietic stem cell (HSC) homeostasis and cytokine responsiveness. HSC derived from the Caspase-3 knock-out mouse have accelerated proliferation and altered differentiation, whereas at the molecular level they show increased sensitivity to cytokine stimulation. p21cip1/waf1 is a known cell cycle regulator, however there is some controversy regarding its role in stem cell homeostasis. Several reports indicate an interaction between p21 and Caspase-3. Therefore we decided to investigate how the simultaneous depletion of both factors affects HSC regulation. Analysis of the peripheral blood of the Caspase-3/p21 double mutant mouse (DKO) shows no differences in any cell lineage compared to WT or single knock-out. However, analysis of the primitive bone marrow compartment revealed that depletion of both Caspase-3 and p21 resulted in an even more pronounced increase in a population enriched in HSPC (LKS: lineage- cKit+ Sca1+) compared to the already augmented pool found in the Caspase-3-/- mice. Nevertheless, such difference in the LKS population size was not due to an increased pool of the most primitive hematopoietic cells (lineage- cKit+ Sca1+ CD48- CD150+) but to an augmented pool of multi-potential progenitors (lineage- cKit+ Sca1+ CD48+ CD150-). In addition, deletion of both genes revealed an even more accelerated cell cycle activity compared to single knock-outs and WT control mice. No differences in apoptosis were found in any of the studied genotypes. Upon transplantation, both p21-/- and DKO show increased LKS populations in the BM compared to WT or Caspase-3-/- controls, most likely due to an increased cell cycling activity as shown by BrdU incorporation assay. However, upon serial transplantation, bone marrow cells from the four genotypes (WT, Caspase-3-/-, p21-/-, DKO) showed long term reconstitution capacity, giving rise to cells from the different hematopoietic lineages without sign of exhaustion. At the molecular level, LKS from the DKO show altered sensitivity to cytokine stimulation in a fashion that resembles the Caspase-3-/- phenotype. Taken together, simultaneous deletion of both Caspase-3 and p21 reveal little changes in the phenotype of the HSC additional to the deletion of Caspase-3 and p21 alone. We found no strong evidence of an interaction of Caspase-3 and p21 in the primitive hematopoietic compartment.

Keywords: caspase-3; p21; hematopoietic stem cells; cell cycle

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Abstract 148

Myeloprotective properties of inducible (Doxycycline-mediated) deoxycytidine-kinase (dCK) knock-down in human hematopoietic cells

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Introduction: Drug resistance (CTX-R) gene transfer has been advocated to protect the lymphohematopoietic system from the side effects of anti-cancer chemotherapy. In the context of leukemias cytidine deaminase (CDD), MDR1, and DHFR-mutants protecting from cytidine analogs, anthracyclines and antifolates, respectively, appear of interest. However, resistance genes for other clinically relevant nucleotide analogs with anti-leukemic properties such as fludarabine or cladribine are missing, so far. As reduced expression of deoxycytidine kinase (dCK) has been linked to nucleotide analog resistance in cell line models and clinical studies of acute and chronic leukemias, we here investigated dCK-knockdown as a novel approach to protect normal hematopoietic cells from nucleotide analogue induced myelotoxicity.

Methods/Results: We here introduce a novel Doxycycline (Dox)-regulated lentiviral vector harbouring a T11 Pol(II) promoter and an exchangeable shRNA (embedded in a miRNA-30 backbone) to mediate specific dCK knock-down and nucleotide analog resistance in hematopoietic cells. Robust and Dox-dependent knock-down of 80% of dCK expression within 2-3 days of Dox administration was demonstrated in human promyelocytic HL-60 cells by western blot and quantitative real-time-PCR analysis. In addition, dCK knock-down in these cells conferred more than 10-fold increased Ara-C resistance in comparison to control cells with LD50 values of 700nM and 60nM, respectively, and titration assays revealed Dox concentrations > 0,2µg/ml as sufficient for this effect. Furthermore, dCK knock-down allowed for highly efficient selection of transduced cells by 200nM Ara-C (from 10 to > 95% within 7 days). Most importantly, dCK knock-down also protected HL-60 cells effectively from fludarabine and cladribine as evident by a 6-10- or 5-fold increased LD50 concentration, respectively. Similar results were obtained for human primary CD34+ hematopoietic progenitor cells.

Discussion: We here describe a novel Dox-inducible, lentiviral "knock-down" vector expressing a dCK-specific shRNA in a miRNA-30 backbone from a T11 Pol(II) promoter. This construct not only induces resistance to Ara-C in hematopoietic cell lines and primary cells and allows for efficient in vitro selection of transduced cells, but also provides profound resistance to fludarabine and cladribine, thus introducing a novel CTX-R-gene for myeloprotective gene therapy strategies.

Keywords: hematopoiesis; gene-therapy; deoxycytidine-kinase; lentiviral vectors

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Abstract 149

7-epi-nemorosone induces apoptosis, androgen receptor down-regulation and disregulates PSA production in LNCaP prostate carcinoma cells

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Prostate cancer is the most frequently diagnosed malignancy and the second-leading cause of cancer death in men. Despite therapeutic options for this cancer entity, many patients die due to metastases in distant organs and acquired chemotherapy resistance. Thus, new approaches to provide improvements in outcome and quality of life for such patients are urgently needed. 7-epi-nemorosone, a small molecule isolated first from Clusia rosea floral resins, has been described in the last years to be a potent cytotoxic agent in cancer models. Here, we present for the first time the analysis of the activity of this small molecule in prostate carcinoma cell lines of different molecular biology. The cytotoxicity of 7-epi-nemorosone was assessed in human prostate carcinoma cells. Interestingly, MDR+ etoposide-resistant cell lines which show cross-resistance to cytostatics employed as chemotherapy standard like mitoxantrone, taxanes, adriamycin, vinblastine turned out to be sensitive to 7-epi-nemorosone. Molecular analyses of the influence of 7-epi-nemorosone were performed in LNCaP (human lymph node carcinoma of prostate), a well known androgen dependent cell line which produces PSA at quantities detectable in cell culture supernatants and in the serum of xenograft mouse models. Thus, LNCaP is appropriate for use in mouse tumor growth models allowing for the evaluation of the therapeutic index. Cell cycle analyses revealed a significant increase in the sub-G0/G1 and G1 populations, a depletion in the S phase, and a concomitant down-regulation of cyclins D1/D3 and CDK 4/6 in LNCaP cells. 7-epi-nemorosone induced apoptotic processes, as assessed by both fluorescence microscopy and the cleavage of caspase-3. Major signal transduction elements such as p38 MAPK and Akt/PKB as well as androgen receptor and PSA production were down-regulated. Although ERK1/2 protein levels were up-regulated, ERK1/2 proteins were hypophosphorylated, indicating an inhibition of their immediate upstream kinases MEK1/2 showing for the first time that 7-epi-nemorosone exerts cytotoxicity in an androgen-dependent prostate carcinoma entity by targeting the MEK1/2 signal transducer. Additionally, Akt/PKB enzymatic activity was effectively inhibited by 7-epi-nemorosone at a similar concentration as for MEK1/2.

Keywords: 7-epi-nemorosone; prostate cancer; chemotherapy resistance; cell cycle; MEK1/2 and Akt/PKB inhibition

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Abstract 150

Strategy for development of second generation of antitumoral compounds against chemotherapy refractory cancer stem cells

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Relapse of cancer occurs months or even years after apparently successful treatments. Probably, the reason for the appearance of chemotherapy refractory tumors is the survival of cancer stem cells (CSCs) due to their intrinsic biological characteristics like dormant periods, expression of multidrug resistance (MDR) phenotypes and radioresistance. We have established a strategy for screening antitumoral compounds aimed at selectively targeting chemotherapy refractory CSCs-like tumor entities. In our screening of natural compounds for antitumoral activity, several compounds isolated from Caribbean Propolis were shown to be highly active against CSC-enriched human tumor entities in vitro. CSCs-like cells were obtained by selection chemotherapy refractory subpopulations of cell lines from different tumor entities including neuroblastoma, glioblastoma, retinoblastoma, prostate carcinoma and colon carcinoma. Their molecular characterization of surface markers was performed applying immunotechniques and qPCR. The isolation and production of the active Propolis compounds were carried out using sequential HPLC-techniques. Cytotoxicity was monitored by MTT proliferation assay in a panel including the above mentioned human tumor cells lines enriched in CSCs. Since the majority of cytostatics currently employed in the therapy of cancer do not target CSCs, which are presumably the cause of untreatable resistant and secondary tumors, we have established a system for the identification and characterization of new antitumoral agents from natural sources which might be employed in the therapy of remanent chemotherapy resistant metastatic CSCs. This system enabled us to identify compounds which selectively target highly resistant CSCs-like tumor cells. At present four compounds isolated from Propolis were selected for a full study of mechanism of action and target definition. Thus, Propolis has been found to be a promising source of second generation of anticancer therapeutics, specifically against CSCs.

Keywords: cancer stem cell; therapeutics of second; chemotherapy resistance; propolis; multidrug resistance (MDR)

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Abstract 151

Acquired resistance cytostatics triggers cancer stem cell (CSC) phenotype and enable to find rare cell populations that show a novel form of intramural cell division

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Acquired resistance to cytostatics is a major problem in the failure of chemotherapy in malignant diseases. Thus, relapse of cancer occurs months or even years after apparently successful treatments. Probably, the reason for the appearance of chemotherapy refractory tumors is the survival of cancer stem cells (CSCs) due to their intrinsic biological characteristics like dormant periods, expression of multidrug resistance (MDR) phenotypes and radioresistance. Our studies revealed that chemotherapy refractory tumor cell lines displayed significant differences in the expression of stem cell markers compared to their parentals. Based on this knowledge we have selected CSCs-like populations via etoposide exposure in order to employ these cells in our antitumoral screening program. For leukemia, neuroblastoma, prostate and glioblastoma, intrinsic stem cell markers (CD44, CD117, CD133, CXCR4 and p75NTR, among others) were found to be significantly and sustainably upregulated in the resistant sublines, which also showed cross-resistance to many cytostatics and high radioresistance. Interestingly, we found within chemotherapy refractory glioblastoma and other tumors cells with a spiral nuclear projection and a novel form of intracellular replication which generates new cells in an intramural-like mechanism, as corroborated by ICC, IHC and EM. These cells were observed in i) cultured tumors, ii) after being xenografted into mice and iii) also found in human glioblastoma paraffin sections, suggesting a natural mechanism of survival strategy. It appeared that these cells build a capside to protect themselves from the cytostatic injury and might use it as a "niche" in which highly metabolic and replicative processes take place. Cells with induced CSC-phenotype generate more metastatic colonies in disseminated prostate carcinomas and neuroblastoma. Moreover, in leukemia, we found a rare cell type which apparently take care of clusters of small cells and named these cells "Hirten Zellen". These cells change their morphology extending large pseudopodia-like prolongations which closely interact with the small clustered cells. Currently, our efforts are focused on revealing the biological roll of CSC-like entities in the heterogeneity of human tumors and developing antitumoral therapeutics of second generation to target these cells.

Keywords: cancer stem cell; chemotherapy resistance; multidrug resistance (MDR); intramural cell division; glioblastoma & neuroblastoma

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Abstract 152

Exploring novel pathways of neural reprogramming by instructive factors and pharmacological intervention

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Transcription factor-driven reprogramming of fibroblast cells has been shown to yield neurons, cardiomyocytes and neural as well as hepatocyte progenitors, demonstrating that direct conversion of somatic cells may develop into a new paradigm for both regenerative medicine and disease modeling. Recently, we demonstrated the direct derivation of induced neural stem (iNS) cells from mouse fibroblasts. Retroviral transduction of Sox2, Klf4, c-Myc and timely restricted activation of Oct4 was used to initiate dedifferentiation of fibroblast cells. 19 days post infection we observed neurosphere-like colonies that could be readily isolated and clonally expanded both as sphere and adherent cultures. iNS cells are able to differentiate into all three neural lineages, neurons, astrocytes as well as oligodendrocytes. Fibroblast-derived iNS cells exhibit clonal growth and maintain their marker expression profile and differentiation capability over prolonged expansion (>50 passages). Additionally, the transduction of several neurogenic transcription factors as well as the application of small molecule compounds were tested to explore alternative strategies for neural conversion. By this, we generated neural progenitor populations with enhanced plasticity. Putative mechanisms and therapeutic values of reprogrammed cells will be discussed. We expect directly converted somatic stem cells such as iNS cells to provide a safe and robust, virtually unlimited source of patient-specific cells for future applications in regenerative medicine and disease modeling.

Keywords: direct conversion; lineage reprogramming; neural stem cells; POU; SOX

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Abstract 153

Role of toll-like receptors in breast epithelial / breast cancer hybrid cell clones and parental cells

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Events of cell fusion are omnipresent in physiological processes and also are involved in tumor progression. In cancer, tumor cells can fuse among each other or fuse with other cells evolving new unpredictable characteristics concerning changed drug resistance or metastatogenic capacity. In addition, the progression of cancer might also be determined by expression patterns of toll-like receptors (TLRs), which basically play a fundamental role in innate immunity, on tumor cells and mononuclear inflammatory cells promoting inflammation and cell survival in the tumor microenvironment. Persistent TLR triggered inflammatory response, including cytokine induction, can induce cancer formation promoting angiogenesis, metastasis, and subversion of adaptive immunity. However, concerning the great heterogeneity of breast cancer the activation of TLRs might result in totally different effects such as enhancing tumor proliferation or suppressing tumor growth. In this study the expression levels of TLRs were analyzed in M13SV1-EGFP-Neo breast epithelial cells exhibiting stem cell characteristics fused with MDAMB435-Hyg breast cancer cells. Both hybrid clone cell lines M13MDA435-1 and -3 exhibited expression of TLR1-6, TLR9 and TLR10 at mRNA levels. By contrast, parental cell lines were found to express only TLR4, TLR6 and TLR9, in addition to lower expression levels of TLR2 in M13SV1-EGFP-Neo cells. In each cell line the expression of TLR4 and TLR9 could be verified on protein levels, whereas TLR5 and TLR3 were just weakly expressed. The migratory activity was analyzed by using the 3D collagen matrix migration assay after application of TLR ligands. Hybrid clones as well as parental cells responded to non-methylated CpG-DNA (TLR9 ligand) with a significant decrease of migratory activity. Stimulation of TLR4 receptor with LPS and TLR5 receptor with flagellin exhibited no distinct impact on migration levels in hybrid clones and parental cells. XTT assays revealed a significant inhibition of proliferation in both hybrid clones by LPS. Compared to parental cells, there was no effect in M13SV1-EGFP-Neo breast epithelial cells and even a slight increase in proliferation in MDAMB435-Hyg tumor cells. Flagellin showed no effect or only tendencies to increase proliferation levels in each cell line. To investigate if treatment with LPS triggers apoptosis flow cytometry experiments were performed. TLR4 has been shown to be directly involved into apoptosis in hybrid cells, but not in parental cell lines correlating with XTT assays. These investigations might be helpful to understand metastatogenic capacity and tumor aggressiveness in breast cancer and several characteristics of hybrid cells suggesting toll-like receptors as targets for a new therapy.

Keywords: toll-like receptors; human breast cancer; hybrid cells; tumor progression

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Abstract 154

Successful treatment of therapy-refractory acute Graft-versus-Host Disease with mesenchymal stem cell-derived exosomes

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Background: Graft-versus-Host Disease (GvHD) is a major cause of morbidity and mortality after allogeneic stem cell transplantation. A number of studies reported positive impacts of systemically applied mesenchymal stem cells (MSCs) for preventing or treating acute GvHD. In contrast to the initial paradigm that MSCs intercalate into injured tissues and thus reduce tissue damage, it is now widely assumed that MSCs secrete a number of immune-modulatory factors, which impair inflammation and thus help to suppress GvHD. Exosomes are secreted cell organelles, which exert immune-modulatory properties. These small membrane vesicles are released by a huge variety of different cell species, including MSCs.

Methods: Here, we enriched exosomes from bone-marrow derived MSCs of four different unrelated stem cell donors and compared their immune modulatory properties in vitro. Next, we administered immunosuppressive MSC-derived exosomes in escalating doses into a 22-years female GvHD patient. This patient suffered a severe and therapy-refractory cutaneous and intestinal GvHD grade IV. We monitored the clinical effects on an in-hospital basis and correlated this with the levels of inflammatory cytokines measured in the patient's plasma.

Results: We show that even though all propagated MSC lines released exosomes, exosome-enriched fractions differed in their potential to modulate immune responses in vitro. Administration of the exosome-enriched fraction with the strongest immune suppressive in vitro effect into the GvHD patient was well tolerated and appeared to be safe. During the course of the exosome therapy a clear reduction of the pro-inflammatory cytokines IL-6, IL-8 and IL-17 was observed in the patient's plasma. In line with that, the clinical cutaneous and intestinal GvHD symptoms improved significantly and the dosage of the immunosuppressive agents – particularly of steroids – could be reduced. In total the patient was stable for 5 months.

Interpretation: MSC exosome-enriched fractions exert immune suppressive functions in vitro and in vivo. Since the in vivo administration seems to be safe, MSC exosome administration appears as a promising new treatment option for steroid refractory GvHD patients.

Keywords: MSCs; exosomes; microvesicles; bone marrow transplantation; graft versus host diesease

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Abstract 155

A human bone marrow stroma cell line with inducible proliferation and supportive potential for hematopoietic stem and precursor cells

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Interactions of normal and malignant hematopoietic stem or progenitor cells with their surrounding microenvironment or stroma in the bone marrow (BM) are increasingly considered highly important for the regulation of proliferation and differentiation. The stroma consists of a variety of different cell types such as fibroblasts, endothelial cells, and macrophages. One approach to investigate these interactions and the cells involved is the isolation and culture of stroma cells and, moreover, co-culturing them with primary normal hematopoietic stem or precursor cells, primary leukemic cells, or leukemic cell lines. Often, the heterogeneity of primary stroma cell cultures and their limited lifespan pose major challenges for the generation of consistent data. Cell lines conserving as much as possible of the typical characteristics of their cell of origin but enabling work with defined populations might be of value to circumvent these hurdles. Although a limited number of human stroma cell lines, e.g. HS-5, are available, numerous studies are performed with murine stroma cells including MS-5, M2-10B4, and OP-9 cells, rendering possible biases by the species difference. We generated a conditionally immortalized human BM stroma cell line from BM stroma of a patient suffering from a B cell lymphoma without BM infiltration. Two immortalizing genes, hTERT and SV40T, were expressed under the control of a tetracycline-responsive element (Tet-on system). These cells can be expanded in media containing doxycycline (dox) and used for experiments after withdrawal of the drug. Such cells are considered to represent a widely normal phenotype in the absence of proliferation. Polyclonal cells were cultured for more than 80 passages. Their surface marker phenotype was concordant with that of mesenchymal stem cells. In addition, they could be differentiated into osteoblasts but not into adipocytes, and osteogenic differentiation potential decreased with higher passage numbers (Koch et al, Genome Res 23: 248-259, 2013). Here, we report that in presence of dox they could be cloned by limiting dilution with high efficiency but not at all in the absence of dox. The subclones conserved the strict dependence on dox for proliferation. Moreover, the cells supported the differentiation of CD34+ stem and progenitor cells into colonies of hematopoietic lineages including erythroid cells. In comparison with the established OP-9 stroma cell line these cells supported colony formation of CD34+ cells to about comparable levels. This novel human cell line and its subclones may be valuable tools for the study of stem cell- and precursor cell-stroma interactions in vitro avoiding a species barrier and the need of e.g. irradiation to arrest the stroma cells for long-term co-cultures.

Keywords: bone marrow stroma; conditional immortalized cell line

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Abstract 156

Knock-down of lysine specific demethylase-1 alters hematopoietic H3K4 methylation and interferes with terminal differentiation

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The hematopoietic system is comprised of multiple different blood cell types with diverse functions. The production of mature blood cells from self-renewing hematopoietic stem cells is a tightly regulated process referred to as hematopoiesis. Covalent histone modifications such as methylation were shown to play a critical role in controlling hematopoiesis. However, the specific functional roles of histone modifiying enzymes in hematopoiesis have so far not been studied in great detail. We used a conditional in vivo knockdown model of the histone lysine (K)-specific demethylase-1 (LSD1/KDM1A) to study the impact of altered histone modification on hematopoiesis. In vivo LSD1 knockdown (LSD1-kd) expanded progenitor numbers by enhancing their proliferative behaviour. LSD1-kd led to an extensive expansion of granulomonocytic, erythroid and megakaryocytic progenitors. In contrast, terminal granulopoiesis, erythropoiesis and platelet production were severely inhibited. The only exception was monopoiesis, which was promoted by LSD1 deficiency. Importantly, we showed that peripheral blood granulocytopenia, monocytosis, anemia and thrombocytopenia were reversible after LSD1-kd termination. Extramedullary splenic hematopoiesis contributed to the phenotypic reversion. LSD1-kd was associated with the upregulation of key hematopoietic genes, including Gfi1b, Hoxa9 and Meis1, which are known regulators of the hematopoietic stem and progenitor compartment. Next, we performed chromatin immunoprecipitation sequencing (ChIP-seq) analysis and demonstrated that within hematopoietic progenitors the promoter regions of Gfi1b and Hoxa9 histone H3K4-dimethylation marks were dramatically increased. We demonstrated that this H3K4 hypermethylation abrogated Gfi1b-negative autoregulation by crossing LSD1-kd with Gfi1b-GFP knock-in mice. Another critical hematopoietic regulator previously shown to be regulated by LSD1 in vitro is SCL/tal1. Therefore, we crossed SCL-LacZ knock-in mice with LSD1-kd mice to analyze the effect of LSD-kd on SCL-expression within different stem and progenitor populations by flow cytometry. Surprisingly, we observed differential regulation of SCL/tal1 by LSD1 within different hematopoietic subpopulations: in hematopoietic stem cells LSD1-kd did not alter LacZ reporter gene expression while in CFU-E erythroid progenitors LacZ reporter gene expression was increased twofold. Taken together, our finding that LSD1 controls the histone code of important hematopoietic genes distinguishes LSD1 as a critical regulator of hematopoiesis.

Keywords: hematopoietic stem cells; hematopoiesis; lysinespecific demethylase-1; H3K4 methylation

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Abstract 157

Syndecan-1 (CD138) modulates breast cancer stem cell properties via regulation of IL-6-mediated STAT3 signaling

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Syndecan-1 (CD138), a heparan sulfate proteoglycan, acts as a coreceptor for growth factors and chemokines and is a molecular marker associated with epithelial-mesenchymal transition during development and carcinogenesis. Resistance of Syndecan-1-deficient mice to experimentally induced tumorigenesis has been linked to an alteration in Wnt-responsive precursor cell pools suggesting a potential role of Syndecan-1 in breast cancer cell stem function. However, the precise molecular mechanism is still elusive. In this study, we decipher the functional impact of Syndecan-1 knockdown using RNA interference on the breast cancer stem cell phenotype of human MDA-MB-231 and MCF-7 cells in vitro employing an analytical flow cytometric approach. Successful Syndecan-1 siRNA knockdown was confirmed by flow cytometry. Side population measurement by Hoechst dye exclusion and Aldehyde dehydrogenase-1 activity revealed that Syndecan-1 knockdown substantially reduces putative cancer stem cell pools by 60% and 27%, respectively, compared to controls. Moreover, the CD44(+)CD24(-/low) phenotype decreased by 6% upon siRNA-mediated Syndecan-1 depletion. Intriguingly, IL-6, its receptor sIL-6R, and the chemokine CCL20, implicated in regulating stemness-associated pathways, were downregulated by >40% in Syndecan-1-silenced cells, which showed a dysregulated response to IL-6-induced epithelial-to-mesenchymal transition. Furthermore, activation of STAT-3 and NFkB transcription factors and expression of a coreceptor for Wnt signaling, LRP6, were reduced by >45% in Syndecan-1-depleted cells compared to controls. At the functional level, Syndecan-1 siRNA reduced the formation of spheres and cysts in MCF-7 cells grown in suspension culture. Our study demonstrates the viability of flow cytometric approaches in analyzing cancer stem cell function. As Syndecan-1 modulates the breast cancer stem cell phenotype via regulation of the IL-6/STAT3 and Wnt signaling pathways, it emerges as a promising novel target for therapeutic approaches.

Keywords: cancer stem cell; ALDH; side population; CD44; CD24; CD138; heparan sulfate proteoglycan; IL-6; STAT; Wnt

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Abstract 158

Expression of the neural stem cell marker Nestin and of other pluripotency markers in conjunctival melanoma

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Conjunctival melanoma is a rare malignant tumor of the ocular surface that shares the same developmental origins as cutaneous melanoma. Both types of melanoma result from the proliferation of neural-crest derived cells, the melanocytes. Recent studies have revealed a strong expression of the embryonic neural stem-cell marker Nestin in cutaneous melanomas, making it an interesting marker of melanocytic neoplasms. This study investigates the transcriptional levels of Nestin and other biomarkers of pluripotency in melanomas of the conjunctiva. Five samples of human conjunctival tissue identified as primary conjunctival melanoma were analyzed. Six samples of healthy limbal and six samples of healthy fornical conjunctiva served as controls. Expression of Nestin, SOX2, OCT4, NANOG, c-Myc, ABCG2, P63, and cKIT was measured using semi-quantitative real-time polymerase chain reaction. Expression of Nestin was significantly higher in melanoma than in limbal (p=0.008) and fornical (p=0.02) conjunctival tissue. The expression of C-Myc and SOX2 tended to be higher in melanoma than in limbal conjunctiva but without reaching statistical significance. Expression of other markers was not statistically different between melanoma and normal tissue from either limbal or fornical conjunctiva. In conclusion, conjuctival melanoma is associated with overexpression of the neural stem cell marker Nestin. The expression of other biomarkers of pluripotency appearing less prominent and unsignificant. Further studies are needed to evaluate whether this transcription factor could help in the diagnostic or therapeutic strategy of conjunctival melanomas.

Keywords: conjunctival; melanoma; nestin; stem cell

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Abstract 159

Tightly regulated Doxycycline (Dox)-inducible lentiviral vectors for human myeloprotective gene therapy: in vitro and CD34+ xenotransplant studies

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Introduction: Myeloprotective gene therapy using drug resistance (CTX-R) genes such as cytidine deaminase (CDD), Multi Drug Resistant Gene 1 (MDR1) or O-6-methylguanine-DNA methyltransferase mutants (mutMGMT) has proven an effective way to protect lymphohematopoietic cells from anti-cancer chemotherapy. However, administration of anticancer cytotoxic agents usually is only transient and constitutive overexpression of transgenes such as CDD and MGMT has been associated with side effects such as lymphoid or stem cell toxicity. Thus, we here describe a Doxycycline inducible "all in one" lentiviral vector construct for induced CTX-R gene expression.

Methods/Results: In our construct the CDD-c-DNA is expressed from the T11-promoter and the reverse transactivator protein is provided by a PGK promoter in the same construct. CDD-mediated drug resistance was evaluated in K562 and primary human CD34+ cells. Both, transduced K562 and CD34+ cells showed robust transgene induction within 24-48h of Dox-application at doses of >0.2µg/ml. In K562 cells this resulted in protection from apoptosis and cell-cycle arrest for Ara-C doses of 2000nM and 600nM, respectively, whereas control cells were affected at 10-fold lower doses. Primary human CD34+ cells were protected from Ara-C doses of up to 600nM versus 25nM for non-transduced control cells and no adverse effect of CDD-overexpression was noted. Furthermore, xenotransplantation of hCD45+ cells into immunodeficient NOD/SCID/IL2Rγc-/- (NSG) mice not only showed efficient engraftment with 5–20% human CD45+ cells in the peripheral blood but also revealed robust transgene expression in hCD45+ cells within 4 to 7 days of Dox administration 8 weeks after transplantation of transduced hCD34+ cells. Among the human CD45+ cells, 20-40% CD19+ peripheral blood B-, 5-20% CD3+ T- and 5-15% CD11b+ myeloid cells could be detected. Furthermore, 25-50% GFP marker gene distribution was detected equally throughout all lineages. In addition, no significant background transgene expression was noted in non-treated animals as in the K562 or CD34+ in vitro models.

Discussion: These data demonstrate Dox-inducible vectors as an elegant tool for robust and rapidly controlled transgene expression in human hematopoietic cells which effectively avoids background expression. The improved "all-in-one" lentiviral vector system appears particularly suited for gene therapy approaches requiring only transient transgene expression such as myeloprotective strategies in the context of anticancer chemotherapy.

Keywords: hematopoiesis; gene therapy; doxycycline; lentivirus

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Abstract 160

Syndecan-1 modulates colon cancer stem cell properties via the Wnt signaling pathway

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Syndecan-1 (Sdc1), a cell surface heparan sulfate proteoglycan has important roles in cell cytoskeletal organization and regulates both cell-cell and cell-extracellular matrix interactions. Sdc1 is highly expressed in by normal epithelial cells, whereas loss of expression has been associated with epithelial-mesenchymal transition (EMT) during development and tumorigenesis. Sdc1 can function as either tumor suppressor or tumor promoter depending on tumor type and specific location of the proteoglycan (either at the cell surface or shed into the microenvironment). Resistance of Sdc1 deficient mice to experimentally induced tumorigenesis has been linked to an alteration in Wnt-responsive precursor cell pools, demonstrating the essential role of Sdc1 in cancer stem cell function. We investigated the functional impact and mode of action of Sdc1 in human Colon carcinoma cells (Caco2 and HT29) using an siRNA approach. Western blot analysis revealed a decrease in E-cadherin expression suggestive of EMT induction. Consistent with this finding, Sdc1-depletion dramatically increased Caco2 cell invasiveness in matrigel chamber assays. Side population (SP) analysis by Hoechst dye exclusion and Aldehyde dehydrogenase- 1 (ALDH-) activity revealed significantly reduced SP and ALDH-1 in Sdc-1 knock down cells compared to controls. Western blot analysis showed decreased levels of β-catenin and interestingly, upon irradiation both β-catenin and TCF4 levels were increased in Sdc1-depleted cells compared to controls. Taken together, Sdc1 emerges as novel player in colon carcinogenesis as reduced expression leads to a decrease in the cancer stem cell population via modulation of the Wnt signalling pathway. Interestingly, the function of Sdc1 is apparently different under irradiation therapy conditions, with potential implications for future Sdc1-centered therapeutic approaches.

Keywords: Syndecan-1; EMT; cancer stem cells; Wnt signaling

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Abstract 161

Characterising the invasive margin in GBM

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Glioblastoma Multiforme (GBM) is the most common and most lethal type of brain malignancy. While the origin of this disease is still not confirmed, it most likely stems from glial progenitors. Despite constantly updated and optimised therapy, the median survival span of patients does not exceed 15 months. Thus, novel findings, furthering the knowledge about the biology of this disease, are much needed. The high rate of recurrence especially represents a major problem in fighting GBM, caused by tumour cells that are left in the apparently healthy tissue margin after resection. These cells are likely to possess traits different from the bulk of the tumour, rendering them more resistant to radio- and chemotherapy and awarding them with an elevated invasive potential. Though after reaching their destination, they are able to switch back to a proliferative phenotype, due to a yet unknown intrinsic and or extrinsic signal, thus giving rise to a new malignancy. Thus, more information about these elusive cells has to be found by a variety of different methods to gain more knowledge on their innate biology to be enabled to slow down or even prevent recurrence. It was possible for the first time, by introducing changes to the standard protocol in our laboratory, to derive cell lines from the invasive margin and the expression of different molecules related to invasion and proliferation was tested throughout different human glioma cell lines, derived in our laboratory from patient samples. Moreover, tissue sections of mass and margin from GBM and healthy brain were stained for several interesting targets. Thereby, molecules that might play an important role for resistance and invasion in GBM have been found. Especially the elevated expression of the L-glutamate/ L- cystine antiporter system xc throughout all cell lines and in tissue suggests a role of system xc in invasion and resistance of GBM- A role that has been proposed by several laboratories already, but as of now has not been investigated more closely and not with having access to many fresh samples as we have in our laboratory. In future, the role of system xc will be investigated in detail by different means.

Keywords : invasion; glioblastoma multiforme; cancer stem cells; system xc; tumor margin; marker

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Abstract 162

The role of vascular wall-resident progenitor cells (VW-EPCs) in tumor vascularization

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Purpose: To evaluate the role of VW-EPCs in tumor vascularization and the disintegration of the vasculogenic zone (VZ) in contrast to BM-EPCs.

Methods: We examined human urothelial tumors of different staging using immunohistochemical staining for CD34 to judge the pattern of CD34+ cells in the VZ of blood vessels. We evaluated the number of blood vessels with and without intact VZ with respect to their distance to tumor tissue. Also a mouse model was used evaluating migration and differentiation of VW-EPCs during tumor vascularisation and distinguishing VW-EPCs from BM-derived EPC. Therefore, C57Bl/6-mice were lethally irradiated and intravenously transplanted with $2x10^6$ murine EGFP-expressing BM cells from EGFP-donor mice. After hematopoietic reconstitution, tumor cells were subcutaneously transplanted into the flank of the mice. Tumor and surrounding tissues were removed and analyzed.

Results: Even in early stages of tumor growth VW-EPCs could be found in tumor tissue. The degree of disintegration varied in dependence to the distance between blood vessels and tumor tissue. Within the tumor tissue all blood vessels had no VZ. Blood vessels in close vicinity to tumor tissue exhibited a partially disintegrated VZ. Almost all large and middle sized blood vessels far from the tumor tissue displayed an intact VZ. Comparable results were obtained for glioblastoma, sarcoma, and testicular tumors.

Conclusions: Our results suggest that VW-EPCs are apparently involved directly in new vessel formation and may serve as a local source for tumor vascularization. Thus, VW-EPCs have to be considered in future strategies for anti-angiogenic tumor therapy.

Keywords: VW-EPCs; tumor vascularization; vasculogenesis

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Abstract 163

Highly efficient neuronal differentiation of human neural crest-derived stem cells

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Neurodegenerative diseases represent a major challenge in the field of regenerative medicine. For treating such neurodegenerative disorders, the application of cell replacement therapy provides a highly promising approach, thereby particularly adressing Parkinson´s and Alzheimer´s disease. In this regard, adult stem cells represent a promising source for clinical applications. We previously reported the successful isolation of adult neural crest-derived stem cells from the inferior turbinate of the human nose. In this study, the potential of such inferior turbinate stem cells (ITSCs) to generate neurons and brain tissue was investigated. Using a neuronal induction medium (NIM) ITSCs were differentiated into neuronal cells, as shown by a subset of specific neuronal markers. Maturation of such neuron-like cells was further evidenced by expression of respective mature markers as well as their ability to recycle synaptic vesicles. Chemical stimulation of neurons resulted in repeating calcium spikes, suggesting their functionality. Transplanted ITSCs successfully integrated into murine pre-existing neuronal tissue, thereby demonstrating the ability of peripheral ITSCs to give rise to cell types of the central nervous system. Emphasizing the extraordinary plasticity of ITSCs, our findings underline their great capability to give rise to neuroectodermal cell types with particular respect to potential future clinical applications.

Keywords: adult stem cells; neural crest-derived stem cells; neuronal differentiation

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Abstract 164

Oct4-EGFP transgenic pig model for isolation and characterisation of spermatogonial stem cells

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We had previously reported the production of germ line transgenic pigs carrying the entire 18 kb genomic sequence of the murine Oct4 gene fused to the enhanced green fluorescent protein (EGFP) cDNA (OG2 construct) (Nowak-Imialek et al., 2011; Stem Cells and Development). Expression of the EGFP reporter construct was confined to the inner cell mass and trophectoderm of blastocysts, genital ridges and testicular cells. Information on SSCs in pigs has been hampered by the inability to isolate these cells from the complex cell population of the testis. Thus isolation of SSCs from OG2 transgenic pigs based on Oct4-EGFP expression and additionally surface markers could be used to facilitate characterization of these cells. Here, we used Oct4-EGFP transgenic pigs as model to isolate and characterize Oct4-EGFP cells in the adult porcine testes. Fluorescence microscopy of testicular tissue isolated from transgenic piglets revealed a very low number of EGFP positive cells, whereas testicular tissue isolated from adult transgenic boars contained a high amount of EGFP fluorescent cells. Northern blot analysis confirmed stronger EGFP expression in the testis of adult transgenic pigs than in the testis from transgenic piglets. Time course and signal intensity of EGFP expression in transgenic testis paralleled mRNA expression of the endogenous Oct4 gene. We report the identification of surface antigenic markers (THY1, CD49f, CD29, SSEA-4) in porcine testis successfully used in other species for enrichment of SSCs. Flow cytometry revealed distinct populations of THY1 (14%), CD49f (68%), CD29 (24%) and SSEA-4 (5%) positive cells in the adult porcine testes. Thereafter fluorescence-activated cell sorting (FACS) based simultaneously on Oct4-EGFP and THY1 expression was successfully used to purify specific testicular cell populations. Four cell populations, i.e. EGFP+/THY1-, EGFP+/THY1+, EGFP-/THY1+ and EGFP-/THY1- could be isolated. Real-time PCR analysis revealed expression of germ cell specific markers in both EGFP+ (14%) and THY1+ (4.7%) cell populations. Further characterization of THY1 and EGFP expressed cells in the porcine testis sections using immunohistochemistry is currently underway.

Keywords: spermatogonial stem cells; transgenic pig; Oct4

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Abstract 165

Zeb2 deficiency in the adult murine hematopoietic precursor cells leads to differentiation defects in multiple hematopoietic lineages and a myeloproliferative-like phenotype

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The life long replenishment of highly specialized blood cells by a small number of hematopoietic cells (HSC) requires a strict regulation between self-renewal and differentiation in the immature compartment of the bone marrow. Perturbation of this equilibrium can result in stem cell loss or hematologic malignancies. This balance is at least in part controlled by a network of transcription factors. Zeb2 is a transcriptional repressor and plays an important role during the embryonic development as a modulator of the epithelial to mesenchymal transition (EMT) as well as tumor progression and metastasis. We have previously identified the essential role of Zeb2 in murine embryonic hematopoiesis, where selective Zeb2 deficiency in the hematopoietic stem cells resulted in early lethality around day 12.5. The aim of this study was to analyze whether Zeb2 plays a specific role in the regulation of homeostasis in the adult hematopoietic system. Using the Mx1-Cre based inducible Zeb2 conditional knock out mouse model we analyzed the impact of Zeb2 loss on adult hematopoietic stem cell function. Upon the induction of Zeb2 deletion we found a significant decrease in most cell lineages of the peripheral blood, except the neutrophil granulocytes. However, the reduction of mature cells in the blood was not accompanied by reduced bone marrow cellularity, as the cellularity was similar between Zeb2Δ/Δ Mx1-Cre (Zeb2 conditional KO) mice and the control animals (Zeb2 +/+ Mx1-Cre). However, in the bone marrow of the Zeb2Δ/Δ Mx1-Cre animals the granulocytic lineage was dominating, whereas other lineages e.g. red blood cell precursors and B-lymphoid precursors were drastically reduced. Histological sections of the bone marrow cavity revealed megacaryocytes with abnormal morphology reflecting maturation defects and an increased production of reticular fibers in the BM of Zeb2Δ/Δ Mx1-Cre mice. In addition Zeb2Δ/Δ Mx1-Cre mice displayed a two to three fold increase in spleen size compared to control animals due to an extramedullary hematopoiesis. Analysis of the primitive hematopoietic compartment in the bone marrow and spleens revealed that Zeb2 deletion resulted in a pronounced increase in the most immature hematopoietic cells, defined as Lin-Sca1+cKit+CD48-CD150+ population, and perturbation in different lineage restricted progenitor subpopulations. No difference in cell cycling or apoptotic rate in the stem cell enriched bone marrow population (Lin-Sca1+cKit+CD48-CD150+) was detectable between the genotypes. Upon transplantation into lethally irradiated wild type recipients, Zeb2 deficient stem cells demonstrated significantly reduced ability to differentiate into multiple hematopoietic lineages indicating a niche independent effect of Zeb2 in promoting differentiation of hematopoietic stem cells. These data indicate that Zeb2 is involved in the regulation of the balance between self-renewal and differentiation at multiple stages of hematopoietic cell maturation. Furthermore the lack of Zeb2 in the hematopoietic compartment leads to a phenotype that resembles the features of human myeloproliferative disorders, especially the early stages of primary myelofibrosis with dominant granulopoiesis, production of reticular fibers in the bone marrow, and morphological abnormalities in megacaryocytes, accompanied by extramedullary hematopoiesis.

Keywords: Zeb2; hematopoiesis; differentiation; myeloproliferative syndrome

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Abstract 166

DNA damage in mammalian neural stem cells leads to senescence-associated secretory phenotype and Bmp2/ JAK-STAT mediated astrocytic differentiation

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Recent research demonstrates the necessity to understand the impact of DNA damage on somatic stem cells. Especially for neural stem cells (NSC), this impact is poorly elucidated. We predominantly employed wildtype and gene-deficient embryonic stem cell-derived NSC and have uncovered that NSC, when exposed to X-ray induced DNA damage, lose their self- renewal potential by exiting the cell cycle and downregulating the expression of stem cell markers such as Nestin, Sox2 and Pax6. Consequently, irradiated NSC undergo astrocytic differentiation and upregulate GFAP while still cultured in conditions promoting self-renewal. We elucidated the mechanism of this process and show that irradiated NSC rapidly enter a novel kind of irreversible cellular senescence, associated with a loss of DNA damage response (DDR) signalling and increased cytokine secretion. Of these cytokines, we show Bmp2 to be responsible for expression of GFAP in irradiated NSC via its non-canonical signalling through JAK-STAT. We show that DDR genes control this process of cytokine-induced differentiation: while ATM plays a supportive role, this process can be efficiently potentiated through p53 deficiency. We recapitulate our observation of irradiation-induced NSC differentiation in adult NSC and murine glioblastoma cells in vitro and in vivo. Using a Sox2—Cre reporter mouse model, we demonstrate that cranial irradiation results in astrocytic differentiation of Sox2-expressing NSC residing in the adult subventricular zone.

Keywords: neural stem cells; DNA damage; differentiation; senescence

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Abstract 167

Towards the establishment of a stem cell line from conjunctival squamous cell carcinomas

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Background: According to the cancer stem cell theory the proliferation and evolution of tumors arise from stem cells within the tumorous tissue, the cancer stem cells (CSC). Those cells were identified in a variety of cancer types, but to date no CSCs were located in tumours of the ocular conjunctiva. Our current research project examines the existence of CSCs in tissues of conjunctival squamous cell carcinoma. It aims at the identification, isolation and the establishment of a cell line of CSCs.

Material and Methods: Tissue from a conjunctival squamous cell carcinoma was lysed and the cell suspension seeded on 24 Well plates. Cultures were screened for cells of epithelial morphology which were separated from the other cells. Those separated cells were cultivated over several passages and analyzed in regard of properties of adult stem cells. The growth characteristics were examined. The expression of markers for adult epithelial stem- and progenitor cells (ABCG2, p63 and OCT4) and differentiation (K19, CX43) was examined by means of semi-quantitative real-time polymerase chain reaction and Immunohistochemical stainings.

Results: A fraction of the isolated cells from the squamous cell carcinoma had epithelial morphology. Those cells had colony forming ability and expressed the examined markers on the RNA and protein level. The characteristics were stable over more than 20 passages.

Conclusion: The cells isolated from the tumor tissue displayed some features of tissue specific stem/progenitor cells of the ocular surface and maintained those over several passages. The cells are possible candidates for CSCs, but further examination is needed to verify their identity.

Keywords: somatic stem cells; cancer stem cells

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Abstract 168

Quantification of primary CFU-f from umbilical cord tissue

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Introduction: Mesenchymal stromal cells (MSC) can be obtained from a broad range of organs and tissues such as adipose tissue, bone marrow and umbilical cord (UC) tissue. The expansion potential of UC-derived MSC (UC-MSC) has been shown to be higher than that of MSC derived from more mature tissues like e.g. bone marrow. Various techniques have been applied to isolate MSC from UC tissue: expanding these out of large UC tissue segments in culture medium, the mechanical dissociation by cutting UC tissue in small pieces with subsequent cultivation and a broad range of different protocols for direct cell isolation after enzymatic dissociation of UC tissue. The yield of cells differs significantly between the reported protocols both in quality, and in quantity.

Objectives: To further the use of UC-MSC in regenerative medicine, we are aiming to develop a more efficient, reliable, and standardized method for the isolation of UC-MSC by enzymatic digestion. Here we report the establishment of robust methods to determine the precise number of viable, nucleated, and functional cells within a given population of freshly prepared UC-derived cells. We will use these methods to thoroughly compare different isolation protocols, and to optimize enzyme composition, digestion time, and digestion conditions.

Results: The enzymatic dissociation of UC tissue yields large amounts of cell debris and clumps of undigested tissue fragments that spoil the reliable quantification of viable dissociated cells by simple microscopy. To overcome this problem, we stained the cells with acridine orange (AO, nucleated cells) and propidium iodide (PI, dead cells) and FACS-analysed the crude tissue homogenates. Living nucleated cells we gated as AO+PI- and quantified using TruCount tubes (BD). Based on this reliable and precise quantification of cells in an UC preparation, we further made use of a colony-forming unit (CFU-f) assay in a limiting dilution type format to determine the proportion of proliferative progenitor cells within the tissue homogenate. The limiting dilution format in 96-well plates is not influenced by secondary colonies and offers both, a higher resolution and a broader range of measurement compared to conventional bulk CFU-f assays in tissue culture flasks or dishes. Both methods allow a robust quantification of nucleated, viable and functional cells derived from an enzymatic dissociation of UC tissue. Using the established assay, we next performed screens of various batches of fetal calf sera from different vendors in order to identify the most effective fetal calf serum batch. Furthermore, we tested the effects of the cultivation in hypoxic (1%-5%) and normoxic environments on the proliferation of UC-MSC. 3% pO2 yielded the highest number primary CFU-f, and thus proved optimal for the expansion of hUC-MSC.

Conclusion: Here we report the establishment of reliable and robust methods for the quantification of primary nucleated, living, and functional mesenchymal cells derived from enzymatically digested UC tissue. We will employ these methods on order to systematically optimize the digestion protocols for UC tissue.

Keywords: umbilical cord; CFU-f assay; tissue dissociation; mesenchymal stem cells; hypoxia

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Abstract 169

Identification of dosage dependent tumor suppressor genes in hematopoietic stem and progenitor cells

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Malignant transformation of cells is caused by alterations in oncogenes and tumor suppressor genes. Classical tumor suppressor genes require a bi-allelic inactivation to entail functional consequences in the cell. Recently, this "two-hit hypothesis" has been replaced by a continuum model of tumor suppression. This novel concept implicates gene dosage dependency and encompasses haploinsufficiency and sub-haploinsufficiency of tumor suppressor gene expression. Here we report a novel approach to identify such genes using a series of shRNA vectors to knockdown the expression of candidate genes to various levels. To test whether gene dosage effects of these candidates provide a clonal advantage for hematopoietic stem and progenitor cells (HSPCs), we performed competitive reconstitution experiments in vivo. We first tested alpha-Catenin (CTNNA1), a proposed tumor suppressor gene that is located within a common deleted region of chromosome 5q and suggested to be affected in the transformation of HSPCs leading to myeloid malignancies. In a transduction/transplantation experiment, we found that RNAi-mediated knockdown of Ctnna1 in HSPCs using a series of 8 different Ctnna1-targeting vectors led to marked expansion of the transduced cells compared to controls in two out of 8 animals. One animal showed a strong dominance of cells with reduced Ctnna1 expression within the HSC compartment including a decreased contribution to differentiation and a bias towards the myeloid lineage. This phenotype is highly reminiscent of a myeloid malignancy in humans. Further in vivo studies are underway to validate our initial finding suggesting a functional role of Ctnna1 gene dosage in tumor suppression in HSPCs. We propose that our approach to model gene dosage insufficiency will identify novel tumor suppressor genes involved in hematopoietic stem cell transformation.

Keywords: MDS; tumor suppressor gene; RNAi; hematopoiesis

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Transcriptional Regulation & Micro-RNAs

Abstract 170

Combinatorial regulatory motifs indicate pluripotency

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To know the map between transcription factors (TFs) and their binding sites is essential to reverse engineer the regulation process. Only about 10%-20% of the transcription factor binding motifs (TFBMs) have been reported. This lack of data hinders understanding gene regulation. To address this drawback, we propose a computational method that exploits never used TF properties to discover the missing TFBMs and their sites in all human gene promoters. The method starts by predicting a dictionary of regulatory DNA words. From this dictionary, it distills 4098 novel predictions. We hypothesized that when a motif appears together with the same set of motifs in multiple promoters, the motif is more likely to play a relevant role in gene transcription regulation. We denote this arrangement of TFBM co-occurrence as combinatorial binding patterns (CBPs), i.e., CBPs are motifs of motifs. To discover such patterns, we developed a computational method that identifies combinations of motifs that bind to more than one promoter. Thus, allowing us to disclose the crosstalk between motifs creating a collection of TF regulatory syntactic rules. Using these rules, we narrowed down a list of 504 novel motifs that appear frequently in syntax patterns. We tested the predictions against 509 known motifs confirming that our system can reliably predict de novo motifs with an accuracy of 81% - far higher than previous approaches. Our algorithm is able to predict not only individual TFBMs but also possible combinations, such as the OCT4+SOX2 pair, which is well known in embryonic stem cells ESCs. We found that on average, 90% of the discovered combinatorial binding patterns target at least 10 genes, suggesting that to control in an independent manner smaller gene set, supplementary regulatory mechanisms are required. A high percentage of our CBP and TFBM predictions can be functional annotated with statistical significance with gene ontology terms. Such annotation reveals that the CBP and TFBM predictions are strongly related to transcription activity and development. The TFs show a trend to target the promoter of other TFs, thus, the TFBMs and their combinatorial patterns convey biological meaning, targeting TFs and genes related to developmental functions. This reveals a recursive arrangement in the transcription regulation process where among all the possible available targets in the genome, the TFs tend to regulate other TFs and genes involved in developmental functions. Since genes involved in pattern formation, morphogenesis and development are regulated by our TFBMs and CBPs with high significance. Our method identifies development as one of the most demanding regulatory processes, in agreement with the pivotal role of transcription regulation mechanisms during development. We provide a comprehensive resource for regulation analysis that includes a dictionary of DNA words, newly predicted motifs and their corresponding combinatorial patterns. Combinatorial patterns are a useful filter to discover TFBMs that play a major role in orchestrating other factors and thus, are likely to lock/unlock cellular functional clusters. These rules are a valuable tool to understand the pluripotent networks and to disclose the mechanisms of cellular reprogramming.

Keywords: regulatory networks; pluripotency network; transcription factor binding site prediction. binding motives; human ESC

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Abstract 171

Gene expression profiling during cardiomyocyte-specific differentiation of murine embryonic stem cells infers transcriptional regulation network

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Embryonic stem cells (ESCs) are defined by infinite proliferation potential and the ability to differentiate into all the cell types of the adult body. For this reason, ESCs are of interest in a variety of research fields since decades. On molecular level differentiation of ESCs is defined by gene expression patterns, which are precisely regulated in response to diverse stimuli. In this study, murine ESCs were specifically differentiated to cardiomyocytes with over 99% purity. Gene expressions of undifferentiated ESCs (day 0) and ESC-derived cardiomyocytes in different maturation stages (day 12, day 19, and day 26) were profiled using Affymetrix GeneChip® Mouse exon arrays. Differentially expressed genes between ESC-derived cardiomyocytes and undifferentiated ESCs were identified and then subjected to further functional analysis. Besides gene expression pattern, which represents a static picture of cell, the regulation of gene expression is obtaining more interest in biological and medical research. Transcripts of cells are regulated on different levels via diverse regulatory factors. One important group of regulatory molecules in ESC differentiation is represented by transcription factors. Therefore, we paid intensive attention to transcription factor expression profiles and the expression of their target genes during cardiomyocyte differentiation. To study gene expression regulation network, in silico modeling is a powerful tool compared to comprehensive experimental methods. In our study, an undirected Gaussian graphical Markov model is applied to gene expression profile data in purpose of inferring transcription factor regulatory network involved in cardiomyocyte-specific differentiation of murine ESCs. Key transcription factors with their target pathways discovered with this method not only enhance the understanding of transcript regulatory network during cardiomyocyte-specific differentiation but also provide candidates for experimental verification.

Keywords: gene expression profiling; ESC; cardiomyocyte-specific differentiation; transcriptional regulation; transcription factor

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Abstract 172

Regulatory feed-back loop between TP73 and TRIM32

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The p73 transcription factor is one of the members of the p53 family of tumor suppressors with also unique biological functions in processes like neurogenesis, embryonic development and differentiation. For this reason, p73 activity is tightly regulated by multiple mechanisms, including transcription and posttranslational modifications. Here, we identified a novel regulatory loop between TAp73 and the E3 ubiquitin ligase tripartite motif protein 32 (TRIM32). TRIM32, a new direct p73 transcriptional target in the context of neural progenitor cells, is differentially regulated by p73. While TAp73 binds to the TRIM32 promoter and activates its expression, TAp73-induced TRIM32 expression is efficiently repressed by DNp73. TRIM32 in turns, physically interacts with TAp73 and promotes its ubiquitination and degradation, impairing p73-dependent transcriptional activity. This mutual regulation between p73 and TRIM32 constitutes a novel feedback loop, which might have important implications in CNS development as well as relevance in oncogenesis, thus emerging as a possible therapeutic target.

Keywords: p73; TRIM32; ubiquitination; neural differentiation; E3-ligase

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Abstract 173

microRNA miR-145 modulates endometriotic stem cell properties via regulation of pluripotency-associated transcription factors and Msi2

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Endometriosis is a benign disease characterized by ectopic growth of endometrial tissue. It is associated with pain symptoms and infertility in reproductive-age women. An involvement of adult stem cells has been postulated as a contributing factor for the pathogenesis of this disease. In the present study, we have investigated the role of the microRNA miR-145, predicted to target several stemness-associated gene products, in an in vitro model of endometriosis. The human endometriotic cell line 12Z and primary eutopic and ectopic endometrial stroma cells were transiently transfected with miR-145 precursors or anti–miR-145 inhibitors and investigated for posttranscriptional regulation of predicted target genes and changes in cell behavior. Predicted target expression was measured by quantitative reverse transcription–polymerase chain reaction and Western blotting, and altered cell behavior was monitored by cell proliferation assays. The 12Z cells were additionally investigated by Matrigel invasion assays, cell cycle analysis, side population analysis, and aldehyde dehydrogenase activity assays. In all cells investigated, miR-145 overexpression inhibited cell proliferation and induced down-regulation of FASCIN-1, SOX2, and MSI2. In 12Z cells, miR-145 upregulation increased Matrigel invasiveness and reduced side population and aldehyde dehydrogenase-1 activity. Additional down-regulated targets in 12Z cells included OCT4, KLF4, PODXL, JAM-A, and SERPINE1/PAI-1. ACTG2 and TAGLN were up-regulated upon pre–miR-145 transfection. JAM-A, FASCIN-1, and PAI-I down-regulation in 12Z cells were confirmed by Western blotting. We conclude that miR-145 inhibits endometriotic cell proliferation, invasiveness, and stemness by targeting multiple pluripotency factors, cytoskeletal elements, and protease inhibitors, marking this miRNA as a potential therapeutic target in endometriosis.

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Keywords: pluripotency factors; musashi-2; microRNA; miR-145; endometriosis

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Abstract 174

In silico molecular analysis reveals unique properties of the transcription factors defining the stem cell pluripotency

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Oct4 and Sox2 are in the core of the transcription regulation network that is essential for the maintenance and induction of pluripotency. Oct4 cannot be replaced by any other member of its family in the process of reprogramming to pluripotency, whereas only a few other Sox factors are able to substitute for Sox2 in this process. Oct4 is a member of the POU family of transcription factors that have a bipartite DNA-binding domain (POU domain) composed of two subdomains: the POU specific domain (POUS) and the POU homeodomain (POUHD), both binding in the major groove of the DNA. Sox2 has one DNA-binding domain (HMG domain) that binds the minor groove and induces a sharp kink in the DNA. It has been shown that Oct4 cooperates with Sox2 to bind regulatory regions that are responsible for the regulation of pluripotency-defining genes. The DNA elements bound by Oct4 and Sox2 differ in the number of the base pairs that separate the binding sites of the POUs and HMG domains. Furthermore, one DNA element has been identified on which Oct4 cooperates with Sox17 and not Sox2. A mechanism in which Oct4 changes partners between Sox2 and Sox17 to interpret a different enhancer code during early differentiation into primitive endoderm has been proposed.

What are the unique properties of Oct4 and Sox2 that confer them the functional specificity towards regulating stem cell pluripotency? We use computational structural biology methods to reveal these properties. First, we will present a comparative analysis of the electrostatic potential of Oct4 orthologues that explains the ability or inability of these orthologues to replace the mouse Oct4 in the induction of pluripotency in mouse fibroblasts and predicts potential protein-protein interaction interfaces on the surface of Oct4. Then, we will show how we are using molecular dynamics simulations to reveal the potential differences in the DNA recognition mechanisms between Oct4 and Oct1 (another member of the POU family). Finally, we will show how molecular modeling and simulations successfully predict the amino acids that are responsible for the different functions of the Oct4/Sox2/DNA and Oct4/Sox17/DNA complexes. These predictions lead to the design of a functional switch between Sox2 and Sox17 that was validated experimentally.

Keywords: Oct4; Sox2; transcription regulation; cooperativity; DNA recognition

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Abstract 175

Changing the microRNA-375 expression profile during pancreatic differentiation of human embryonic stem cells

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Micro RNAs (miRs) play an important role in beta cell differentiation and function. We analyzed the expression profile of the pancreas-associated miR-375 during in vitro-differentiation of murine and human embryonic stem (ES) cells. In mouse and human ES cells expression of miR-375 increases during development into definitive endoderm (DE) followed by a continual decrease at later stages. MiR-375 is essential for early definitive endoderm development in both species since low expression at this stage leads to ineffective production of pancreatic progenitors. The observed expression profile of miR-375 in murine and human ES cells underlines the successful development of pancreatic and endocrine progenitors using our differentiation protocol. Changing the miR expression profile is an important tool for the development of terminal differentiated insulin producing cells. We transfected human ES cells with a miR-375-inhibitor and measured the miR-expression profile during DE development as the first step of pancreatic differentiation. Inhibition of miR-375 results in decreased expression of endodermal marker genes like SOX17 and FOXA2 indicating a correlation between miR-expression and differentiation efficiency. Moreover, the induction of miR-375 at later differentiation stages leads to more efficient formation of endocrine pancreatic progenitors shown by increased expression of markers such as PDX1 and NGN3. This observation is supported by increased expression of endogenous pre-miR-375 after transfection of miR-375. Currently, we investigate if prolongation of our differentiation protocol together with induction of miR-375 leads to increased expression of endogenous miR-375 at later differentiation stages followed by increased expression of more terminal differentiation marker genes. In summary, miRs are a powerful tool to direct the developmental potential of pluripotent cells and can be used to generate pancreatic cells and to support the establishment of in vitro test systems for the analysis of T2DM.

Keywords: microRNA-375; human ES cells; pancreatic differentiation

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Abstract 176

A novel mathematical model to investigate NF-kB feed-back loops after treating stem cells with TNF-alpha

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Many biological processes such as proliferation, protection and apoptosis can be coordinated by the transcription factor NF-kB. This triggered our interest to investigate its role in previously characterized multipotent stem cells from the human nose called Inferior Turbinate Stem Cells (ITSCs) after treatment with Tumor Necrosis Factor alpha (TNF-a), a well-known inducer of the canonical NF-kB signaling pathway. After degradation of the inhibitor-kB (IkB), NF-kB/RelA (p65) is activated, liberated and translocate to the nucleus. Here we show that TNF-a activates NF-kB in tissues specific stem cells from the human nose. The optimal concentration (10ng/mL) of TNF-a necessary to activate NF-kB was determined by luciferase assay. The combination of bioinformatics and biological results suggested simplified scheme of the NF-kB pathway including some interesting NF-kB target genes as p65, IkB-alpha, IkB-beta, IkB-epsilon and p50, anti-apoptotic genes as cIAP1, cIAP2 and c-FLIP and pro-apoptotic genes like Bcl-xL and Bcl-2. Real time PCR results demonstrate the involvement of NF-kB in stem cells proliferation and protection. This takes place via an up-regulation of anti-apoptotic genes additional to slightly down-regulation of apoptotic genes. Immunohistochemistry and Western Blots provide an image to translocation of p65 into the nucleus. p65 reaches the highest concentration within 20 min after TNF-a treatment and that it accumulates in the nucleus more than one hour, whereas the termination of stimulation was illustrated after about 120 min. In contrast to previous studies, our results do not show any signs of oscillation of p65 between the nucleus and cytoplasm within the first 120 min of TNF-a stimulation. Furthermore, we conclude that TNF-a functions as a growth factor in these stem cells as previously shown in the immune system.

Keywords: mathematical model; NF-kB feed-back loop; NF-kB/RelA (p65); adult stem cells; inferior turbinate stem cells

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Abstract 177

Impact of microRNAs on the generation of human dopaminergic neurons

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MicroRNAs (miRNAs) control gene expression at the post-transcriptional level and are emerging as key regulators of neural cell proliferation, differentiation and fate choice. Recent studies have demonstrated that miRNA activity is required for the generation and maintenance of midbrain dopamine (DA) neurons, which are the prime target of Parkinson's disease. Gaining more insight into the role of miRNAs during human DA neuron specification may help to improve the generation of this neuronal subtype for regenerative purposes. So far, two miRNAs have been associated with this neuronal subtype, i.e. miR-133b and miR-132, which both inhibit the development of murine midbrain DA neurons. We used a population of long-term self-renewing neuroepithelial-like stem (lt-NES) cells derived from human pluripotent stem cells to identify novel miRNAs associated with human neuronal differentiation and fate specification. Based on gain- and loss-of-function analyses we show that miR-181a as well as other candidate microRNAs are required for the generation of neurons from lt-NES cells and contribute to the transition from self-renewal to differentiation. However their impact on the generation of specific neuronal subtypes differs. While overactivation of miR-181a in lt-NES cells induces an increased yield of TH positive neurons other candidates impair the generation of this population. Thus, modulation of miRNAs might provide a tool for further enhancing the derivation of medically relevant neuronal subtypes from human pluripotent stem cells.

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Abstract 178

Depletion of dicer during reprogramming results in a self-renewing, highly proliferative state

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The discovery of micoRNAs has added a new paradigm of genetic regulation to our understanding of numerous cellular processes. They play a pivotal role in functional maintenance, proliferation, and differentiation of cells. Furthermore micoRNAs have also been implicated to be involved in disease pathogenesis, and more recently, cellular reprogramming including induction of pluripotency in somatic cells. In particular the overexpression of microRNAs mir-200c, mir-302 s and mir-369 s have been shown to enhance the generation of induced pluripotent stem (iPS) cells and can even substitute for the reprogramming factors Oct4, Sox2, Klf4, and c-Myc. However, the role of microRNAs during the reprogamming process remains largely unclear. To address this question we used Dicer conditional knockout fibroblasts in a reprogramming paradigm. Dicer-floxed mouse embryonic fibroblasts were transduced with a doxycycline (dox)-inducible, polycistronic reprogramming cassette. Cre-mediated recombination was used to delete Dicer, thereby depleting the fibroblast for almost all functional microRNAs. 5 days later the reprogramming process was initiated by application of dox. Intriguingly, Dicer-null fibroblasts failed to produce iPS cells (Kim et al., PLoS ONE (2012)). Here we show that comprehensive analysis of this reprogramming paradigm revealed an unexpected, stably reprogrammed cell type. This was accomplished by sustained transgene activity through continous dox-application. PCR confirmed the Dicer-less genotype of these reprogrammed cells. Interestingly, the cell population is dependent on the sustained activity of reprogramming factors. In presence of dox the cells are viable, highly proliferative and show expression of pluripotency-associated markers. Whereas dox-withdrawal results in down-regulation of pluripotency markers, increased cell-death and senescence. By that we show for the first time that Yamanaka-type reprogramming of Dicer-less fibroblasts results in a self-renewing, highly proliferative cell population. Our study demonstrates the importance of micro-RNAs for the reprogramming process, emphasizing their role in stabilizing the pluripotent niche as well as their importance for proliferation and cell viability.

Keywords: reprogramming; microRNA; iPS

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Abstract 179

MicroRNAs MiR-17, MiR-20a, and MiR-106b act in concert to modulate E2F activity on cell cycle arrest during neuronal lineage differentiation of USSC

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Unrestricted somatic stem cells (USSC) from human cord blood constitute a rare CD45-negative population capable of inducible in-vitro differentiation into cells from all three germinal layers. During induction into neuronal lineage differentiation, USSC undergo a strong cell cycle arrest. MicroRNAs, short (~22 nt) non-coding regulatory RNAs that negatively control gene expression at the post-transcriptional level by sequence-specific binding to 3'-UTRs of target genes, have gained emerging attention as potent regulatory molecules. Here we have analyzed the impact of microRNAs on the cell cycle arrest of USSC during neuronal lineage differentiation. qPCR-based expression profiling of the 377 most abundant microRNAs revealed a strong downregulation of microRNAs hsa-miR-17, -20a, and -106b in USSC differentiated into the neuronal lineage but not in USSC differentiated into the osteogenic lineage. Ectopic overexpression of miR-17, -20a, and -106b using microRNA mimics followed by Ki67 immunostainings demonstrated that each of these three microRNAs was able to promote proliferation of native USSC and to prevent in part cell cycle arrest during neuronal lineage differentiation of USSC. In turn ectopic inhibition of miR-17, -20a, and -106b using microRNA inhibitors led to a significant reduction of USSC proliferation. Bioinformatic target gene predictions followed by experimental target gene validations based on a firefly/renilla luciferase reporter assay revealed that miR-17, -20a, and -106b act in a common manner by downregulating an overlapping set of target genes which is involved in regulation and execution of G1/S transition. Interestingly, target genes of pro-proliferative nature (cyclinD1 (CCND1) and E2F1) as well as anti-proliferative targets (CDKN1A (p21), PTEN, RB1, RBL1 (p107), RBL2 (p130)) were both experimentally validated as common targets for miR-17, -20a, and -106b. Furthermore, these microRNAs also downregulate WEE1 which is involved in G2/M transition. Most strikingly, miR-17, -20a, and -106b were found to promote cell proliferation by increasing the intracellular activity of transcription factor E2F, despite the fact that miR-17, -20a, and -106b directly target the transcripts that encode for this protein family. In summary, hsa-mir-17, -20a, and -106b downregulate a common set of pro- and anti-proliferative target genes to impact cell cycle progression of USSC and their expression increases intracellular activity of E2F transcription factors to govern G1/S transition.

Keywords: microRNA; unrestricted somatic stem cells; cell cycle regulation; neuronal lineage differentiation

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Company Profiles

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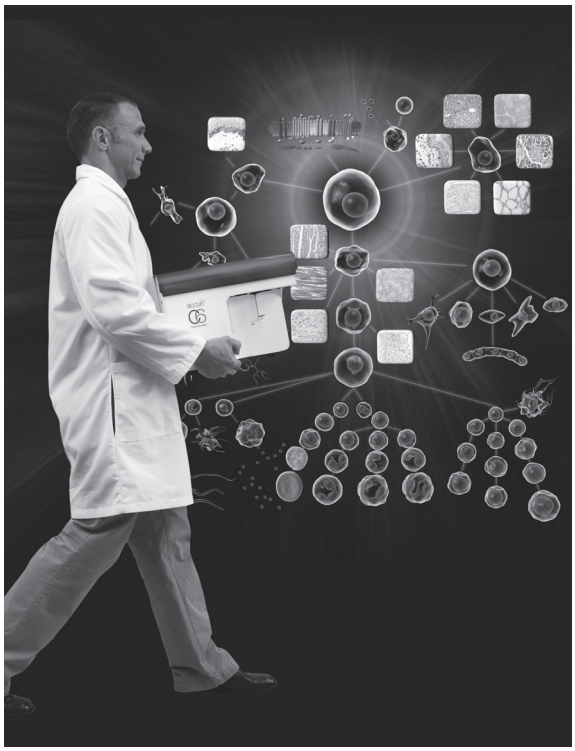

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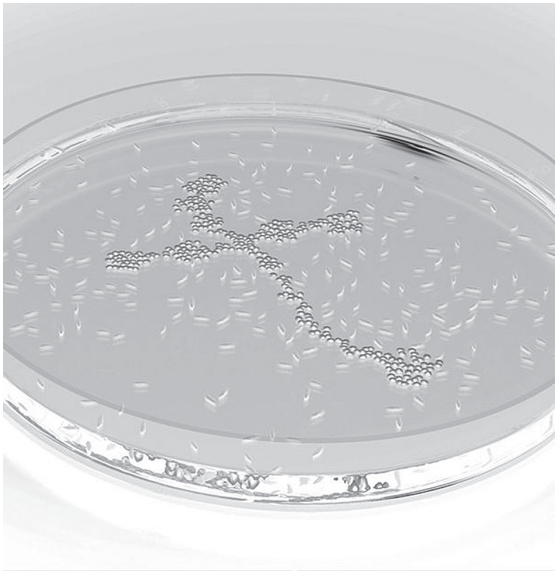

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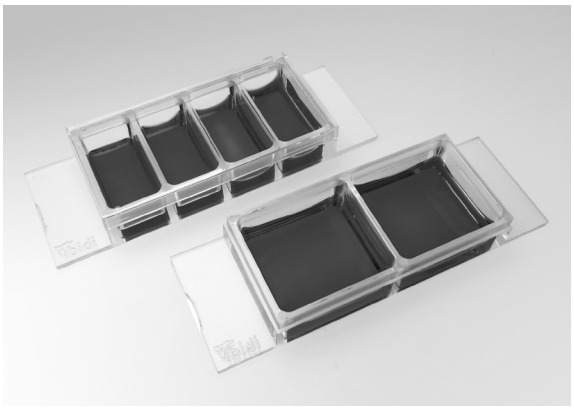
ibidi – cells in focus



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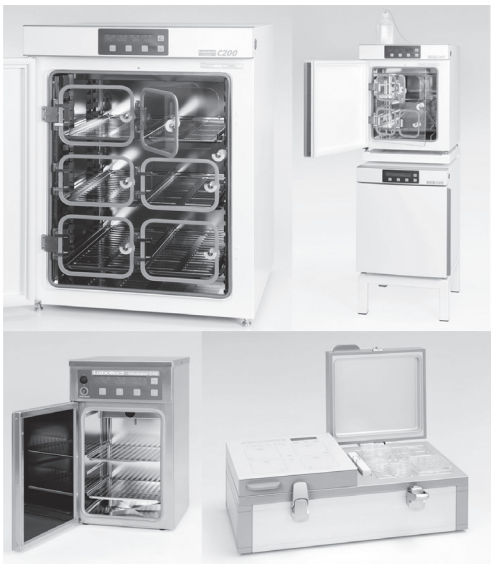

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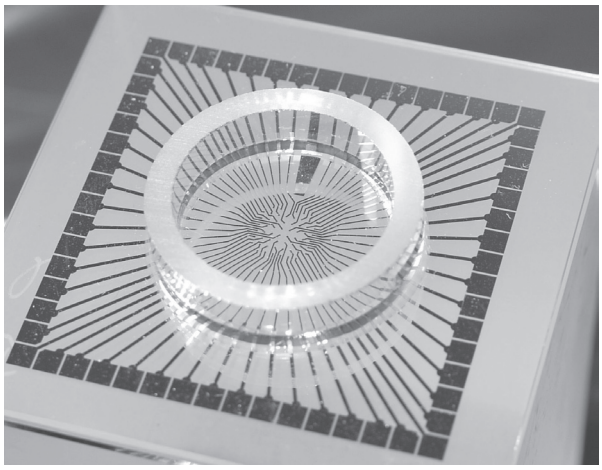

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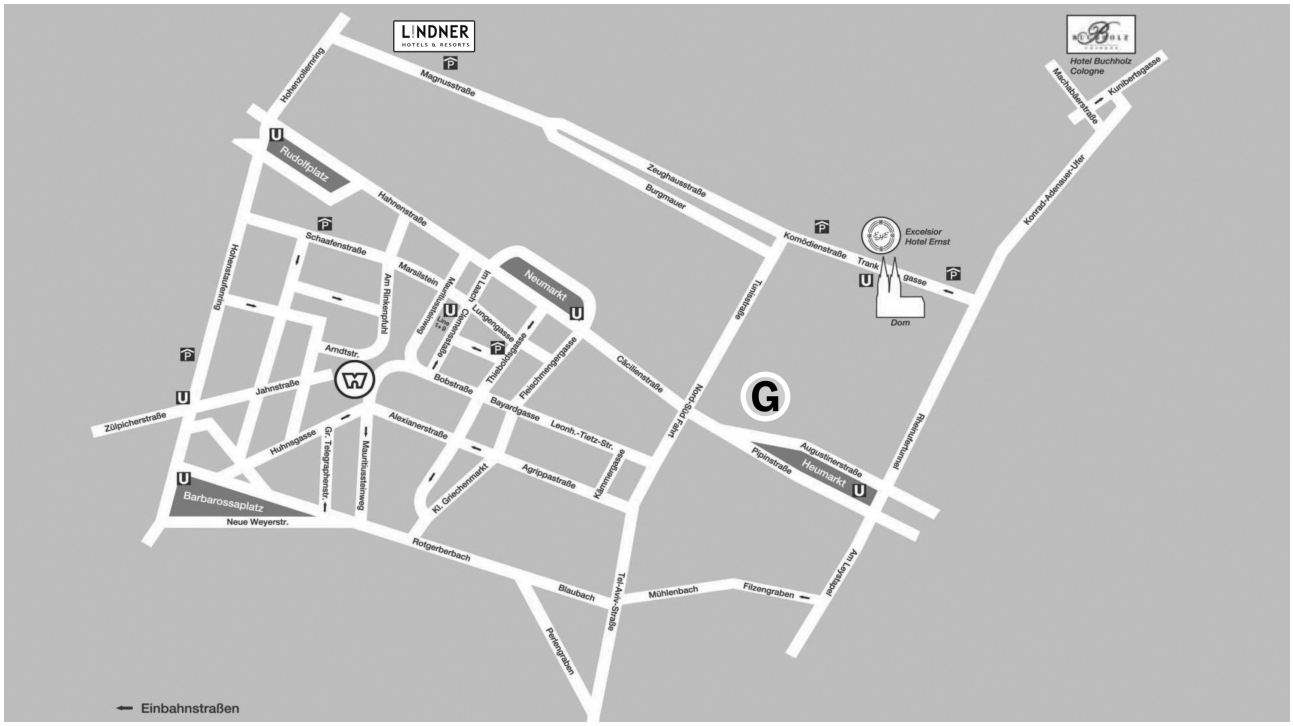

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