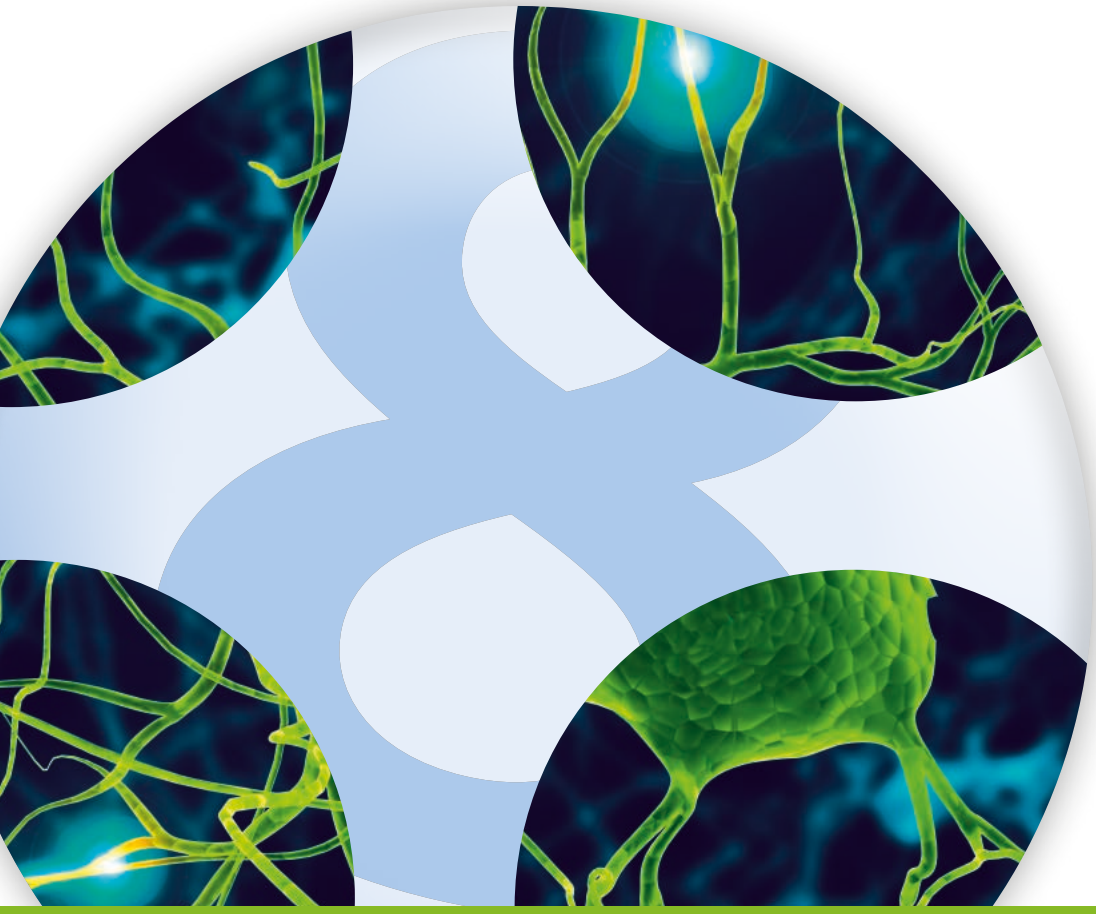
Stem Cell Network
North Rhine Westphalia



_program & abstracts

# _8th International Meeting

Stem Cell Network North Rhine-Westphalia

## _April 21–22, 2015

_Final Program
_Poster Abstracts
_Company Profiles

Ministry of Innovation, Science
and Research of the German State
of North Rhine-Westphalia

# _8th International Meeting

Stem Cell Network North Rhine-Westphalia

_program & abstracts

# _Program

## Tuesday, April 21st

8:00 – 9:00 am _Registration

**9:00 – 9:30 am** **_Opening of the Meeting**
Oliver Brüstle, Chairman

Svenja Schulze, Minister for Innovation,
Science and Research of the State of North Rhine-Westphalia

**9:30 – 10:50 am** **_Opening Lectures, Chair: Oliver Brüstle**
Fred Gage (La Jolla)
*Neuronal plasticity genomic diversity*

Dieter Sturma (Bonn)
*Stem cell research: the ethical challenges*

10:50 – 11:30 am _Coffee Break, Poster Session

**11:30 – 12:30 pm** **_Session I: Neural Development and Stem Cells, Chair: Andreas Faissner**
Jürgen Knoblich (Vienna)
*Modelling human brain development in 3D organoid culture*

Ronald McKay (Baltimore)
*Using the functional identity of human stem cells*

12:30 – 1:45 pm _Lunch Break, Poster Session

**1:45 – 3:00 pm** **_Session II: Stem Cell Engineering, Chair: Martin Zenke**
Matthias Lutolf (Lausanne)
*Engineering tissues via extracellular matrix-guided stem cell self-organisation*

Adam Cohen (Cambridge, USA)
*All-optical electrophysiology for iPSC neuronal disease modeling*

Judith Kempfle (Boston)
*Sox2 – positive cells in the cochlea function as inner ear progenitors (selected talk)*

3:00 – 3:30 pm _Coffee Break, Poster Session

**3:30 – 4:45 pm** **_Session III: Self Organization of Stem Cells, Chair: James Adjaye**
Hiromitsu Nakauchi (Tokyo)
*"Organ niche" and "developmental complementation"*
*for generation of functional organs in vivo*

Thomas Zwaka (New York)
*Cell competition in pluripotent stem cells*

Laura Stappert (Bonn)
*Transcriptional Regulation & Non-Coding RNAs (selected talk)*

4:45 – 7:00 pm _Poster Session

**7:30 – 10:30 pm** **_Networking Event**

## Wednesday, April 22nd

8:30 – 9:30 am _Registration

8:15 – 9:15 am _Early Career Breakfast – Meet the Experts (registered participants only)

**9:30 – 10:45 am** **_Session IV: Therapeutic Approaches, Chair: Boris Greber**
Shoukhrat Mitalipov (Portland)
*Nuclear transfer and reprogramming*

Clare Blackburn (Edinburgh)
*Inducing a thymus, in vivo and in vitro*

Luna Simone Pane (Munich)
*Antisense-mediated exon skipping: a therapeutic strategy for titin-based dilated cardiomyopathy (selected talk)*

**10:45 – 11:30 am** **_Panel Discussion "Great Expectations"**
Christina Berndt, Clare Blackburn, John Harris, Ralf Müller-Terpitz, Dieter Sturma

11:30 – 12:00 am _Coffee Break

**12:00 – 1:30 pm** **_Session V: Specification of Stem Cells, Chair: Christian Kaltschmidt**
Michael Brand (Dresden)
*Regeneration of the adult zebrafish brain*

Benedikt Berninger (Mainz)
*Brains in metamorphosis: Physiological and forced neurogenesis in the adult brain*

Henrik Semb (Copenhagen)
*From pancreas organogenesis to making beta cells in a dish*

1:30 – 2:30 pm _Lunch Break, Poster Session

**2:30 – 3:30 pm** **_Session VI: Disease Modeling, Chair: Matthias Höhn**
Rick Livesey (Cambridge)
*Mechanistic insights into Alzheimer's disease pathogenesis from human stem cell models*

Ludovic Vallier (Cambridge)
*Modelling liver diseases using human induced pluripotent stem cells*

**3:30 – 4:00 pm** **_Coffee Break**

**4:00 – 5:00 pm** **_Session VII: HSC: Metabolism & Aging, Chair: Peter Horn**
Bertie Göttgens (Cambridge)
*Transcriptional network control of blood cell development*

Sten Eirik Jacobson (Oxford)
*Emergence of fetal immune-restricted lympho-myeloid progenitors prior to definitive hematopoietic stem cells*

**Following** **_Poster Awarding, Closing Remarks**

# _Register

## Bioengineering & Biomaterials



## Disease Modeling

# Ethical, Legal & Social Issues

# Induction & Maintenance of Pluripotency

# Niche & Microenvironment



# Organogenesis & Regeneration

# Other

# Somatic & Cancer Stem Cells

# Stem Cell Differentiation

# Stem Cell Epigenetics

# Stem Cells in Development



# Transcriptional Regulation & Non-Coding RNAs

# Company Profiles



# Contact & Disclaimer

# Bioengineering & Biomaterials

# Differentiation of mesenchymal stromal cells is guided by surface topography

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Mesenchymal stromal cells (MSCs) can give rise to several cell types including osteocytes, adipocytes and chondrocytes. In vivo, the extra cellular matrix provides several topographic features – such as micro- and nanostructures – that may impact on cellular differentiation.In this study we have analyzed the influence of line-and-space patterns on proliferation and differentiation of human MSCs. To this end we have systematically varied width and spacing of grooves in polyimide surfaces. Reactive ion etching and multi beam laser interference were used to structure patterns in the range of micro- or nanometers on polyimide surfaces, respectively. Adipose tissue-derived human MSCs were then cultured on these surfaces, analyzed by scanning electron microscopy, and their differentiation towards adipogenic and osteogenic lineages was quantified. Furthermore, the effect of these structured surfaces on genome wide gene expression profiles (Affymetrix 2.0 microarrays) was analyzed. MSCs aligned parallel to the main axe of micro- and nanogrooves. Albeit surface topography did not affect MSCs proliferation it had significant impact on MSCs differentiation: 15µm spaced microgrooves enhanced adipogenesis and diminished osteogenesis; conversely 2µm spaced microgrooves enhanced osteogenesis and impaired adipogenesis. Nanogrooves with a periodicity of 650 nm enhanced both osteogenic and adipogenic differentiation of MSCs which was also confirmed by quantitative real time PCR. Although these structures clearly influenced the propensity of MSCs to differentiate toward specific lineages, their global gene expression profiles hardly revealed significant changes without induction media.Our results indicate that adipogenic and osteogenic differentiation of MSCs can be enhanced by specific topographic patterns. Particularly nanogrooves enhance differentiation towards both osteogenic and adipogenic lineages.

**Keywords: nanotopography; microgrooves; mesenchymal; stem cells osteogenesis; adipogenesis**
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# Comparative analysis of two webtools for TALEN design

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Background and aims: TALE nucleases (TALENs) have emerged as a valuable tool for fast and reliable gene editing in various cell types, including pluripotent stem cells. Although the mechanisms underlying TALEN/DNA binding are well-understood, little is known about the preferential architecture necessary to achieve optimal cleavage results. The aim of this study was to find a reliable tool for the prediction of TALEN activity. For this purpose we compared two different webtools for TALEN design.

Materials and methods: TALEN sequences were generated using two web tools, the TAL Effector Nucleotide Targeter 2.0 and the scoring algorithm for predicting TALEN activity (SAPTA). The TALEN binding sites targeted the human HNF1b and GATA4 genes respectively. TALENs were selected according to the provider's instructions and were then assembled using the 'Golden Gate TALEN Kit 2.0' with the pC-GoldyTALEN backbone vector for improved TALEN activity. TALEN were transfected into HEK-293 cells and 48 h after transfection successful DNA cleavage was investigated using the T7EI assay. The functionally active TALEN pairs were transfected into human ESCs to further verify their function and to derive modified human ESCs. Since optimal TALEN delivery is key for gene editing approaches transfection efficiencies of different methods (three lipofection reagents, electroporation) were analyzed by flow cytometry using a CMV-GFP construct.

Results: The TAL Targeter design guidelines are basic, whereas the SAPTA method aims to predict TALEN activity using a scoring system. None of the ten different TALEN pairs generated with the TAL Targeter for the HNF1b locus showed successful DNA cleavage in the T7EI assay. In contrast, six out of the 14 TALEN pairs generated according to the SAPTA method showed positive cleavage in the T7EI assay. Although the different TALEN pairs were only 100 bp apart of each other, the SAPTA tool clearly proved to be superior over the TAL Targeter. A similar result was obtained for the GATA4 locus. Using the SAPTA method we obtained two out of eight functionally active TALENs compared to no active pair obtained following the TAL Targeter instructions. Re-analysis of the TAL Targeter designed TALENs with the SAPTA method revealed scores of zero or even negative values although they were selected according to the provider's instructions. Electroporation of human ESCs resulted in high transfection efficiencies (~ 70 %) and showed the lowest cytotoxic effects. All tested lipofection reagents resulted in lower efficiencies (< 50 %) and were highly cytotoxic. This demonstrated that electroporation of human ESCs is the method of choice for TALEN delivery.

Conclusion: The SAPTA algorithm is better suited for the generation of functionally active TALEN pairs than the guidelines provided by the TAL Targeter tool. Using the SAPTA method we obtained functionally active TALEN pairs for two different loci, whereas the TAL Targeter remained unsuccessful. Furthermore, TALEN delivery using electroporation is the method of choice for human ESC transfection due to its high efficiency and low cytotoxicity.

**Keywords: TALE nucleases; genome editing**
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# The Stem Cell Factory – automated production of induced pluripotent stem cells

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Reprogramming of patient cells to human induced pluripotent stem cells (hiPSCs) has tremendously facilitated the study of the molecular and cellular mechanisms underlying human disease pathogenesis and progression. The field of stem cell-based disease modeling is increasingly moving from monogenic diseases to complex disorders, creating an urgent need for standardized and automated processes for reprogramming and expansion of hiPSC lines from large patient cohorts. Industrialization of iPSC generation demands a broad set of expertise. We tackled this challenge by combining know-how in stem cell biology and process automation to establish a large system integration for the automated production of patient-specific hiPSC lines (www.StemCellFactory.de). The StemCellFactory provides automation and standardization of all required cell culture steps, ranging from adult human dermal fibroblast (HF) expansion via feeder-free Sendai virus-based reprogramming to clonal selection and expansion of the obtained hiPSCs. Measurement technologies for quality control, including high-speed microscopy, have been implemented to ensure high fidelity performance via in-process data generation. We developed a fully automated, feeder free, Sendai virus-mediated, E8-based reprogramming protocol that delivers footprint-free hiPSC within 3 weeks with state-of-the-art efficiencies. Evolving hiPSC are automatically detected, harvested and clonally propagated in 24-well plates (CellCelector, ALS). Automatically propagated hiPSCs exhibit normal growth characteristics and pluripotency-associated marker expression profiles. Fully automated well- and plate-based splitting processes are scheduled via image-based confluence measurement. FACS analysis shows sustained Tra1-60 expression across 34 days (10 passages) of automated cultivation (automated 94.4% ± 4.3% vs. manual 96.2% ± 3.5%). We expect the StemCellFactory to advance medical research by providing large numbers of hiPSC lines for disease modeling and drug screening at industrial scale and quality.

**Keywords: reprogramming; iPSC; automation; standardization; liquid handling**
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# A customized 3D myocard model engineered by seeding human iPS-derived cardiomyocytes on small intestinal submucosa scaffolds

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In spite of recent advances in medicine cardiovascular disorders remain major causes of mortality in the world. After myocardial infarction, pathological remodeling processes in the damaged heart can lead to cardiac insufficiency. A main shortcoming of innovative therapeutic concepts represents the lack of ideally autologous cell sources and robust functional integration into the host tissue and the vascularization of the implant. Induced pluripotent stem (iPS) cells might serve as an unrestricted cellular source of patient-specific cardiac cells for future regenerative applications. Here we used a novel protocol of robust cardiac differentiation of human iPS cells by systematically modulating BMP and WNT signaling and metabolic selection by lactate enrichment. We show efficient derivation of beating cardiomyocytes from multiple iPS lines. In particular we demonstrate cardiomyocyte differentiation within 15 days with an efficiency of up to 95% as judged by flow cytometry staining against cardiac troponin T. hiPS-derived cardiomyocytes (iPS-CM) were functionally validated by alpha-actinin staining, transmission electron microscopy as well as electrophysiological analysis. In order to explore the possibility to derive iPS-CM-based cardiac 3D tissues we seeded a single cell suspension of 106 iPS-CM cells onto a decellularized 1cm2 small intestinal submucosa scaffolds patch. To assess the potential beneficial impact of other cells on survival, integration and cellular function of seeded iPS-CM we co-cultured iPS-CM with mesenchymal stem cells (MSC) and fibroblasts (derived from a human heart biopsy) as well. 2 days after seeding we observed recovery of spontaneous beating in various regions on the patches. 3 days later the whole patch exhibited synchronous beating. Hematoxylin and eosin staining (HE) shows agglomerates in the mono-culture whereas the cells are well distributed all over the surface of the matrix in the co culture. In both culture conditions the expression of the cardiac specific markers cardiac Troponin T, cardiac Troponin C and MF20 can be confirmed by immunhistological staining. Moreover, we show that engineered tissue patches responded to β-adrenergic stimulation with changes in the beating rate upon β-adrenergic agonist (isoproterenol) and antagonist (propranolol) treatment. We expect our 3D cardiac model to provide a robust basis for personalized cardiotoxicity studies, disease modeling as well as cardiac cell replacement therapies.

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# Exploration of Nrf2 pathway activation as potential human neurotoxicity reporter

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Human pluripotent stem cells (hPSC) can be triggered to differentiate into any cell type of the body. Thus, they are ideally suited to address the question of predictive tissue-specific toxicity in vitro. In this context, repeated-dose toxicity testing is particularly desirable for predicting possible adverse effects on target tissues/organs, on dose-response relationships and on reversibility of any adverse effects observed. There is strong evidence that various cellular toxicity pathways share the Keap1-Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)-antioxidant response element (ARE) pathway as common denominator for sensing of oxidative stress and for the induction of protective cellular responses. Exploitation of this pathway as an indicator of cell stress-associated events could thus provide a valuable tool for in vitro toxicity assay development. In order to establish an hPSC-based platform for repeated-dose neurotoxicity testing, we made use of hPSC-derived long-term neuroepithelial stem cells (lt-NES; Koch et al. PNAS, 2009). Lt-NES retain a stable neuro- and gliogenic potential even after long-term proliferation (>70 passages) and hence represent a standardized and scalable source for assay development. To assess the toxicological relevance of the Nrf2 pathway we first evaluated its basal and inducible activity in lt-NES and lt-NES-derived neurons of different maturation stages. QRT-PCR analysis of the Nrf2 downstream targets NQO1, SRXN1 and HMOX1 revealed increased expression levels with the progression of neuronal maturation (up to 16 weeks). In order to study the modulation of the Nrf2 pathway in lt-NES-derived neurons we used Rotenone, a mitochondrial complex I inhibitor, as a reference compound. In accordance with the qRT-PCR results, dose-response curves for repeated-dose toxicity revealed an increased resistance of more mature neurons (5 and 10 weeks of differentiation) to Rotenone as determined by Alamar Blue assay. Complete removal of antioxidants from the cell culture medium revealed that Nrf2 pathway activation by low dose treatment with Rotenone (120 nM) for 48 hours is strongly increased in comparison to redox-protected conditions. These observations indicate that setting up an hPSC-based neurotoxicity assay platform demands multiple considerations, including the maturity of the neurons and the cell culture conditions. Furthermore, correlative studies employing functional cell type-specific endpoint analysis will be required to distinguish general vs. tissue-specific toxicity. We are currently extending our studies towards the assessment of a trans-well co-cultivation paradigm with hPSC-derived astrocytes and the generation of an Nrf2-luciferase lt-NES reporter cell line suitable for high-throughput neurotoxicity screening.

**Keywords: Nrf2 pathway; neurotoxicity; in vitro assay; lt-NES cells; human pluripotent stem cells**
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# Highly accelerated differentiation of stem cells by exposure to terahertz radiation

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Objective: Terahertz radiation (THz), with a fundamental period in the pico-second range, is uniquely suited to control cellular functions. Biologically important collective modes of proteins and DNA vibrate at THz frequencies and are therefore THz sensitive. Although the THz photons do not carry enough energy to directly alter chemical reactions, non-linear resonance effect may cause local changes of the breathing dynamics in systems such as DNA, leading to changes in gene transcription and cellular state of differentiation. We hypothesized that a short (8–12 hours) THz exposure of mouse and human mesenchymal stem cell cultures (mMSC and hMSC) would result in altered gene expression, cellular morphology, and highly accelerated differentiation; mMSC in a medium with adipogenic supplements will express adipocyte-specific genes differentiating toward adipocyte-like phenotype; hMSC will differentiate toward endothelial-like phenotype forming capillary-like structures after short term exposure to THz in medium with endothelial supplements. Methods: mMSCs (ScienCell Research Laboratories) were cultured in α-MEM, 5 % fetal bovine serum (FBS) by adding adipogenic supplements 48 hours prior to irradiation. hMSC (LONZA) were cultured in DMEM adding endothelial medium supplement. The mMSC were irradiated with a THz source of high-energy: 35 fs (broadband centered at~10 THz, λ=30 μm) for 8 hours. The hMSC were irradiated: 40 fs (broadband centered at~7 THz, λ=45 μm) for 12 hours. Immediately after THz exposure, cells were harvested for analyses. Parallel cultures without THz exposure were used as negative controls. Affymetrix gene chips (n=8), RT-PCR, and light microscopy were used to analyze the cell culture response to THz. Data was analyzed via the Kruskal-Wallis test; RT-PCR data were analyzed by the comparative Ct method.

Results: Hierarchical clustering based on the 20 differentially expressed genes that are identified by the microarray analysis and RT-PCR results in a clear separation between the THz exposed mMSC and control samples. THz of mMSCs resulted in: (i) statistically significant ($p<0.001$) overexpression of the proadipogenic PPARĐ, adiponectin, GLUT4, FABP4, Nfe212; (ii) suppressed expression of Gem and Slco4a1 ($p<0.001$) that are known to be suppressed in differentiated cells; (iii) no effect on genes encoding heat shock proteins; (iv) undetectable apoptosis; (v) clear cytoplasmic lipid droplet-like inclusions. THz exposure of hMSCs resulted in the formation of vessel-like structures.

Conclusions: THz exposure leads to highly accelerated and medium composition-directed differentiation of mouse and human mesenchymal stem cells. THz induces differentiation of mouse MSC toward adipose phenotype within 8 hours of exposure. The exposure of human MSC resulted in the formation of thin-walled capillary-like structures. The precise gene expression response of hMSC to THz is under investigation. The strictly controlled experimental environment indicates lack of apoptosis, minimal temperature changes and the absence of any discernable response to heat shock and cellular stress implying a non-thermal cellular response to the applied broadband low power THz stimulus.

**Keywords: human mesenhymal stem cells; terahertz radiation; differentiation; endothelial cells; adipocytes**
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# Human engineered heart tissue – a new tool for preclinical drug safety screening

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Objective: Human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes represent a new tool in cardiovascular research with tremendous potential regarding preclinical drug safety screenings. However, these cells are still immature and characterized by poor sarcomeric organization and cellular orientation in 2D cell culture. Therefore, the measurement of contractile force, the most important and best understood function of cardiomyocytes in vivo, is not established for these cells. This project describes the generation of three-dimensional, strip-format, force-generating engineered heart tissues (EHTs) from hiPSC-derived cardiomyocytes and presents a characterization of physiological and pharmacological parameters based on force development.

Methods and results: Cardiomyocyte differentiation of hiPSC was achieved by a growth factor-based three stage protocol. Strip-format EHTs were generated from dissociated cardiomyocytes in fibrin matrix between flexible silicone posts. Coherently beating human EHTs developed within two weeks after casting and displayed a regular beating pattern for several weeks. Contractile function was repeatedly monitored by a 24-well, automated video-based system. Histological analysis revealed a high degree of sarcomeric organization and alignment of cardiomyocytes in EHTs. Functional analysis was performed by measuring force response to calcium concentration (EC50 0.9 mM), pre-load (max. force at 110 % of baseline length; n=9), beta-adrenergic (100 nM isoprenaline) and muscarinic agonists (10 µM carbachol). Ion channel modulators revealed concentration-dependent effects (meanffISEM in % of baseline mean): ATX-II (relaxation time at 10 nM: 161±5 %; n=20), TTX (beating rate at 0.3 µM: 73±5 %; n=16), Bay K-8644 (relaxation time at 0.1 µM: 160±6%; n=8), verapamil (beating rate at 0.3 µM: 51±4 %; n=24), NS1643 (beating rate at 30 µM: 154±3 %; n=19), E4031 (relaxation time at 10 nM: 115±2 %; n=20), ibutilide (relaxation time at 10 nM: 128±4 %; n=10). Comparison with native human heart tissue shows an overall high level of similarity and minor differences.

Conclusions: This study demonstrates the principal suitability of hiPSC-derived EHTs for measuring contractile force and kinetics in an automated multiplex assay. The analysis indicates a high level of similarity between EHTs and native human heart tissue, making EHTs a promising platform for automated toxicology screens in future drug development and for in vitro experiments on human cardiomyocytes.

**Keywords: cardiotoxicity; drug safety screening; engineered heart tissue; hiPSC-CM; cardiac differentiation**
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# StemGraft – A stem cell-based tissue engineered vascular graft

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Tissue engineering might offer the possibility to overcome the diverse disadvantages of synthetic or autologous grafts routinely used in bypass surgery or as dialysis shunts in hemodialysis patients. The aim of the current joint project StemGraft is to engineer a small diameter stem cell-based vascular graft. To this end, adipose-derived mesenchymal stem cells (AD-MSC) and microvascular endothelial cells (mvEC) were isolated from fat tissue obtained from the waste products of patients undergoing elective liposuction. The cells were characterized, expanded, differentiated, molded into a fibrin-based graft and finally this graft was endothelialized. After conditioning in a bioreactor under physiological flow conditions, the grafts were evaluated by testing mechanical properties and ECM production. Here we report the first preliminary results of the project. AD-MSC could be successfully isolated from fat tissue derived from liposuction using collagenase digestion and plastic adherence. The cells were characterized as multipotent MSC according to the minimal criteria of the International Society for Cellular Therapy. The cells express typical surface epitopes such as CD44, CD73, CD90 and CD105. After expansion, the cells were differentiated towards smooth muscle cells with functional properties like collagen contraction. The cells were mold into a fibrin-based vascular graft containing a PVDF warp-knitted mesh to support long-term stability. After 14 days of culture under physiological flow conditions in a bioreactor, grafts were removed and subjected to mechanical testing, histological and immunohistochemical (IHC) analysis. Mechanical properties of the vascular grafts were in the range of native human blood vessels (burst pressure approx. 1000 mmHg). Hydroxyproline content was 4.18 ffl 0.79 µg per mg dry tissue and showed intensive ECM production. Collagen type I and III could be detected by IHC. Existence of an endothelial cell layer at the luminal side of the graft was proved by CD31 staining. First results of the project show the potential of stem cell-based tissue engineered vascular grafts as an autologous approach for different types of bypass procedure. Currently, we focus on adapting the protocols to produce such grafts to conditions that conform to GMP standards for clinical translation.

*This project is supported by the Ministry of Innovation, Science and Research of the State of North Rhine-Westphalia within the research program "Translational stem cell research".*

**Keywords: tissue engineering; vascular graft; dialysis shunt; adipose-derived mesenchymal stem cells; microvascular endothelial cells**
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# Disease Modeling

# Functional correction of X-SCID by targeted gene editing in pluripotent stem cells

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X-linked severe combined immunodeficiency (X-SCID) is a rare, life-threatening immune disorder caused by mutations in the IL2RG locus, which codes for the common gamma chain present in several interleukin receptors. The most frequent loss-of-function mutations are found in exon 5, leading to a block of multiple cytokine signaling pathways important for lymphocyte development and function. To generate a humanized X-SCID mouse model, the murine Il2rg locus was replaced with a human IL2RG version harboring the common G691A mutation in exon 5. The goal of this study was (1) to establish a gene editing approach based on transcription activator-like effector nucleases (TALENs) to correct the underlying mutation in humanized murine ES cells (hmESC) and (2) to create an in vitro disease model by establishing a protocol to differentiate these hmESCs via early hematopoietic progenitor cells to effector T cells. Our TALENs showed high activity at the IL2RG target locus, with about 30 % of alleles being targeted, and minimal nuclease-associated cytotoxicity. The mutation in IL2RG was corrected by TALEN mediated integration of a partial cDNA, encoding exons 5 to 8, into exon 5. Corrected and non-corrected clones were subjected to a robust hematopoietic differentiation protocol. Both uncorrected or corrected hmESC clones produced comparable levels of CD41+ hematopoietic precursor cells that were further differentiated towards mature T cells. CD4+/CD8+ double positive T cells expressing the T cell receptor β-chain (TCRβ) were successfully generated from a corrected but not an uncorrected hmESC clone. Furthermore, mature CD8+ cells were produced by challenging the CD4+/CD8+ double positive cells with antigen presenting cells and a co-stimulatory signal using anti-CD3/28 beads in the presence of IL-2, confirming functional correction of X-SCID immunodeficiency in vitro. In conclusion, our study emphasizes the significance of designer nucleases as a tool in generating isogenic disease models and demonstrates for the first time that pluripotent stem cells can be differentiated into mature T cells, with the prospect to produce genetically modified autologous transplants for various applications in the clinic.

**Keywords: X-SCID X-linked sever combined immunodeficiency; TALENs transcription activator-like effector nucleases; ZFNs Zinc finger nucleases; DN double negative; DP double positive**

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# A fluorophore reporter mouse model for optimization of the one-step CRISPR/Cas9 gene knockout method

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The injection of CRISPR/Cas9 components directly into one-cell embryos allows genome editing in animals in a one-step manner. Here, we present a fluorophore mouse model for establishment and optimization of the one-step genome editing. The model is based on a transgenic mouse line, carrying a monomeric CAGGS-Venus Sleeping Beauty transposon. The CAGGS promoter driven Venus fluorophore is systemically expressed, and Venus expression is not affected by epigenetic or environmental factors. Here, the cytoplasmic injection of Cas9 and sgRNA plasmids into Venus zygotes was assessed. In total, 101 Venus-hemizygous zygotes were flushed, from which 86 were normally developed and displayed two polar bodies. 67 treated zygotes were transferred to 3 surrogate animals, which delivered 18 pups, of which 5 showed a knock out of the Venus phenotype. Thus a knock-out efficiency of 28 % of the born pups was achieved. Importantly, the majority of knock-out pups showed a complete knock-out in all organs. The model allows the direct comparison of different compositions of the injection solution (plasmids, RNAs, ribonucleotide/Cas9 complexes), different injection sites (pronuclear, cytoplasmic), as well as the optimization of other parameters (time point of injection, injection volume, concentration). Depending on the breeding schedule, the efficacy of mono- and bi-allelic knock-outs can be assessed.

**Keywords: genome editing; microinjection; disease model**
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# Modeling Takotsubo cardiomyopathy using human induced pluripotent stem cell-derived cardiomyocytes

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Purpose and Aim: Takotsubo Cardiomyopathy (TTC) is characterized by apical dyskinesia of the left ventricle that cannot be explained by an occlusive coronary lesion. The pathogenic mechanisms leading to TTC are still not fully understood, but it has been suggested that the heart can be damaged by excessive catecholamine spill-over in the absence of a protective mechanism. The ability to generate induced pluripotent stem cells (hiPSCs) provides a unique opportunity for creating a patient-matched human heart disease model. We aimed to investigate the effects of high catecholamines on iPSC-derived cardiomyocytes (iPSC-CMs) with regard to the cardiac TTC phenotype. The establishment of a human in vitro TTC-model would offer deeper insights into the mechanisms and potential treatments of this disease.

Methods and Results: Human dermal fibroblasts of three healthy control probands were reprogrammed to patient-specific iPSCs (ps-iPSCs). These cells were directly differentiated into pure cardiomyocytes (CMs). After two months, iPSC-CMs were treated with increasing dosages of the different catecholamines isoprenaline (β1/β2-adrenoceptor agonist), epinephrine (β1/β2/α-adrenoceptor agonist), or phenylephrine (α-adrenoceptor agonist) to induce a TTC-specific phenotype. We investigated the mRNA expression of cardiac stress related genes including atrial natriuretic peptide (ANP) and Myosin light chain 2v (MLC2v) and observed a dose-dependent increase for all three used catecholamines. Since TTC is often described with an higher susceptibility to myocardial apoptosis induced by stressful trigger events, we analyzed the expression of the apoptosis-related gene nuclear receptor subfamily 4 group A member 1 (NR4A1) and found a 3-fold increase of expression, even after low catecholamine concentrations. Severe arrhythmias frequently occur in TTC patients, which could stem from damaged biomembranes caused by excessive intracellular lipid accumulation. Therefore, we analyzed cardiac lipotoxicity by Oil red O staining in TTC-induced iPSC-CMs and observed a slight increase in lipid droplet accumulation when stimulated with isoprenaline or epinephrine. In contrast, sarcomeric organization was not influenced by increasing amounts of isoprenaline or epinephrine in iPSC-CMs. Bioelectrical features were analyzed by the usage of multi electrode arrays (MEA) showing an isoprenaline dose-dependent increase in beating frequency, which slightly reverts if the dosage exceeds a certain threshold.

Conclusion: This is the first demonstration that catecholamine-treated hiPSC-CMs recapitulate some abnormalities that were found in individuals with TTC. We show evidence for a catecholamine-dependent increased expression of stress-and apoptotic-related genes as NR4A1, which was shown to be increased in rat hearts after restraint stress and might contribute to the TTC phenotype by triggering apoptosis. Furthermore, lipid accumulation and beating frequency could be induced by catecholamines. HiPSC-CMs should therefore be used as an in vitro model of TTC to investigate the native genetic background of TTC-patients for the development of therapeutic and protective strategies on a patient-specific level. However, more experiments are needed to validate the model and increase its relevance.

**Keywords: disease modeling; iPSCs; Takotsubo**
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# A standardized human cell model enabling rapid analysis of HSP-associated phenotypes

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Hereditary spastic paraplegia (HSP) is characterized by progressive spasticity in the lower limbs caused by axonal degeneration of corticospinal motoneurons. Spastic paraplegia 4 (SPG4) makes up 40 % of all HSP cases and is the most frequent, autosomal dominant subtype. Affected patients carry mutations in the SPAST gene encoding the microtubule-severing enzyme spastin. So far, no curative treatment for HSP is available, and drug discovery screens are hampered by the lack of suitable model systems. While SPG4-associated phenotypic alterations have been described in iPSC-derived neurons, development of these in vitro phenotypes typically requires several weeks of in vitro differentiation, which limits their exploitation for high-throughput assays. We became interested in developing a SPG4 model enabling rapid phenotypic analyses within a few days. IPSCs from three patients carrying heterozygous SPAST nonsense mutations were differentiated into highly enriched neuronal cortical cultures comprising >80 % glutamatergic neurons expressing the layer V/VI markers CTIP2 and TBR1. We found that axonal swellings, a hallmark of the HSP pathology, can be reliably detected already 5 days after plating of SPG4 iPSC-derived cortical neuronal progenitors. Swellings were 1–7 µm in diameter and stained positive for the axonal markers tau1, acetylated tubulin and neurofilament. In an in vitro regeneration assay SPG4 neurons exhibited a 40 % reduction in overall neurite length compared to controls already 24 hours after plating. At that time point we also observed enlarged growth cones suggestive of cytoskeletal imbalance. We transferred the regeneration assay to an automated 96-well-setup and achieved low experimental variability and good separation between populations with a z-factor of 0.5 within 24 hours. We expect these fast phenotypic assays to accelerate the study of pathomechanisms underlying HSP and to promote the identification of therapeutic compounds counteracting HSP-associated neuronal degeneration.

**Keywords: iPSCs; disease modeling; spastin; cortical differentiation; phenotypic assays; automation**
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# Purification of cardiomyocytes from iPS cells with a non-clonal strategy enables automated planar patch clamp analysis of long QT-syndrome 3

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Induced pluripotent stem (iPS) cells can be generated from patients with cardiac disease and differentiated into functional cardiomyocytes. The aim of this study was to establish a straight-forward selection strategy to obtain pure cardiomyocytes for characterization of a cardiac disease using automated electrophysiological analysis. We therefore designed a novel non-clonal purification approach using a lentivirus to express a puromycin resistance gene under the control of a cardiac-specific promoter. With this virus we infected our previous reported wild-type and long QT syndrome type 3 (LQT3)-specific mouse iPS cells. Because the lentivirus construct also expressed a neomycin resistance under the stem cell promoter Rex1, iPS cells were selected without time-consuming picking of individual clones and could be stably propagated in bulk culture without virus silencing. Application of puromycin during differentiation gave rise to a nearly pure cardiomyocyte population (wild-type iPS cell line: 92.8±6.2%, n=5 and LQT3-specific iPS cell line: 87.7±9.7%, n=4). Using conventional patch clamp experiments we found that purified cardiomyocyte from the non-clonal LQT3 iPS cell lines preserved the phenotype indicated by prolonged action potential duration (ADP) at slow pacing rate and early after depolarizations which both were not seen in wild-type controls. The slope of APD restitution (APD versus pacing period) in purified LQT3 cardiomyocytes (7.94±4.05 ms/s, n=18) was significant different (1.85±0.73 ms/s, n=10) than in wild-types. Importantly these values were similar to previous reported values using non-purified cardiomyocytes form clonal LQT3 and wild-type iPS cell lines (Malan et al. Circ. Res. 2011). Cardiomyocytes differentiated form early (P12–P18) and late (P19–P35) passages of non-clonal iPS cells showed identical action potential parameters (resting potential, APD, Vmax, APD restitution slope) proving the stability of the non-clonal iPS cell lines. For automated electrophysiological analysis we used planar patch clamp recordings (Patchliner, Nanion) of purified cardiomyocytes. Using this method the LQT3 phenotype with positive APD restitution slope and early after depolarizations could be automated and reliably detected. Also we found no significant differences in action potential parameters compared with the manual patch clamp data.

**Keywords: induced pluripotent stem cells; purification of cardiomyocytes; long QT syndrom 3; disease model**
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# Defective cilium disassembly in a human iPS cell model of Seckel syndrome

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Mutations in centrosomal proteins cause Seckel syndrome characterized by dwarfism, low birth weight, microcephaly, intellectual disability and premature aging. Studies have suggested that decline in respective stem cell pool underlies the disorders associated with both pre-and postnatal Seckel syndrome defects. Microcephaly-related disorders associated with Seckel syndrome is thought to be due to a decline in the neural stem cell pool. However, the mechanisms of neural stem cell maintenance remain unclear. Here, we report an unexpected role for primary cilium in the pathophysiology of Seckel syndrome and identify CPAP as a negative regulator of ciliary length independent of its role in centrosome biogenesis. The cilium is a microtubule-based organelle that is dynamically regulated, with assembly occurring during cell cycle exit, and disassembly coinciding with cell cycle re-entry. In wild type fibroblasts, we found CPAP protein levels to be low during cilium assembly and elevated during disassembly. At the onset of cilium disassembly, CPAP provides a scaffold for the recruitment of Nde1, AuroraA, OFD1 and HDAC6, which are required for cilium disassembly (Cilium-Disassembly-Complex; CDC). In contrast, Seckel fibroblasts, which contain numerically and structurally normal centrosomes and mitotic spindle poles, lack the dynamic CPAP localizations required for efficient CDC recruitment. As a result, Seckel fibroblasts display unusually long cilia, the length of which is correlated with delayed cell cycle re-entry. Importantly, overly long cilia and delayed cell cycle re-entry was also observed in neural progenitors derived from Seckel patient-induced pluripotent stem (iPS) cells. Interestingly, this resulted in premature differentiation of neural progenitors. To model Seckel syndrome in disease relevant tissues, we developed human Seckel iPS-derived cerebral organoids and demonstrate that aberrant CDC function also promotes premature differentiation of neural progenitors, providing an explanation for the reduced neurogenesis and brain size in Seckel patients. Taken together, our results suggest a previously unknown role for cilium disassembly in fate determination during neurogenesis and brain size control.

**Keywords: Seckel syndrome; microcephaly; primary cilium; neurogenesis, stem cell maintenance**
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# Non-alcoholic fatty liver disease can be modelled with ES-cell derived hepatocyte like cells

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Non-alcoholic fatty liver disease (NAFLD) is an increasingly common diagnosis in the Western Hemisphere. It is defined by an accumulation of lipid droplets in more than 5 % of hepatocytes. In the beginning the disease is rather benign, but later on patients develop steatohepatitis, cirrhosis and up to 27 % of these patients end up with hepatocellular carcinoma. The molecular mechanisms underlying this disease are still questioned, but it is well-recognised that NAFLD is highly associated with obesity and insulin resistance. In this metabolism-based field of research, results obtained from rodent model systems cannot be easily extrapolated to humans as both organisms differ in their metabolisms. Unfortunately, liver cells from steatosis patients are very rarely available and not suitable for longer experiments as hepatocytes rapidly dedifferentiate in culture. To bypass this deficiency, we have established a human model system for NAFLD based on hepatocyte like cells (HLCs) generated from embryonic stem cells. We are able to induce the accumulation of lipid droplets (LDs) in these cells by adding oleic acid (OA) into the medium. LD formation has been documented by staining with Oil Red O or BODIPY. We thoroughly investigated the consequences of LD accumulation at the level of gene and microRNA expression. We found that our HLCs express, amongst others, various Cytochromes and Sulfotransferases which are important for detoxification processes and characteristic for mature hepatocytes. Unexpectedly, almost all liver specific microRNAs were predominantly down regulated after steatosis induction indicating profound changes in gene regulation. Here, the microRNAs hsa-miR-106b, -122, and -27a are to be highlighted as they have already been described to play a role in steatosis. After fat induction with OA the expression of PLIN2, a protein associated with LDs, was consistently up-regulated. As PLIN2 knockout mice are protected against the development of steatosis, we selected PLIN2 expression as a molecular marker for the successful induction of steatosis. In order to get deeper insights into the molecular basis of the disease, we investigated the functional consequences of an siRNA mediated PLIN2 knockdown in cultured hepatocytes. Although the cells still accumulated fat in LDs, they showed pronounced differences in their gene expression, for example the family of insulin growth factor binding proteins (IGFBP). Overall, our HLC-based model system of NAFLD offers many opportunities for the analysis of the molecular basis of this common disease and will be a valuable tool for the development of pharmaceuticals targeting the accumulation of lipids in hepatocytes.

**Keywords: non-alcoholic fatty liver disease; hepatocytes; PLIN2; Lipid droplets**
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# Generation of a new hiPSC-derived vascular model of Marfan syndrome for drug screening

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Marfan Syndrome is a multisystem disorder, caused by mutations in Fibrillin-1, the major constituent of extracellular microfibrils. Defective Fibrillin-1 causes increased matrix metalloproteinase (MMP) expression, elastic fibre degeneration and loss of aortic medial smooth muscle cells (SMCs) leading to aortic dilatation/dissection. Although the precise mechanisms underlying aortic degeneration are still poorly understood, previous studies have suggested that Fibrillin-1 mutations perturb local TGF-β signalling. To provide new insights into this mechanism, we have generated hiPSCs (human induced pluripotent stem cells) derived from patients with Marfan syndrome, which have been then differentiated into populations of SMCs of distinctive developmental origin. Here, we show that Marfan hiPSC-derived SMCs mimic the vascular phenotype observed in Marfan patients, by displaying altered fibrillin-1 deposition/degradation and an abnormal proliferative and contractile phenotype. In this model, TGF-β levels/activity and MMP expression were found to be upregulated in Marfan-SMCs versus wild type-controls as expected. In addition, we have dissected TGF-β downstream pathways and identified P38 as new key component in Marfan phenotype. Finally, we have used Marfan hiPSC-derived SMCs as a model to test drugs currently in use, such as Losartan and a newly identified TGF-β receptor inhibitor (IDT-1) by measuring their efficiency in rescuing Fibrillin-1 phenotype. In summary, we have established an in vitro hiPSC model that recapitulates many of the key vascular features of Marfan syndrome and that is suitable for testing potential therapeutic agents.

**Keywords: Marfan Syndrome; Fibrillin-1; smooth muscle cells; TGF-β pathway; hiPSC**
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# Synaptic connectivity of iPSC – derived neurons from patients with schizophrenia

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Approximately 1 % of adults worldwide suffer from schizophrenia, a CNS disease of neurodevelopmental origin. Positive and negative symptoms as well as impaired cognitive functions signify this complex and heterogeneous disease. The appearance of impaired cognitive functions correlates with aberrations in synaptic connectivity. We have analyzed induced pluripotent stem cell (iPSC) – derived neurons to understand basic mechanisms of cognitive dysfunction in schizophrenia. Fibroblasts of patients with schizophrenia as well as of healthy individuals were reprogrammed into iPSCs via retroviral transduction and characterized by the analysis of stem cell markers (e. g. SSEA4 and Tra-1-81). Subsequently, iPSCs were further differentiated into SOX1 and PAX6 expressing neural progenitor cells and finally into neurons. Successful terminal differentiation of iPSCs into functional neurons was assured via immunostaining for neuronal marker proteins and assessment of electrophysiological properties. The amount of presynaptic proteins like VGluT and VGaT as well as postsynaptic proteins like PSD95 and Gephyrin were quantified by means of immunocytochemical stainings. In comparison to the control group of healthy donors, neurons derived from patients diagnosed with schizophrenia exhibit a diminished density of pre- and postsynaptic proteins that implies a reduced synaptic connectivity. In conclusion the results indicate that this in vitro system is applicable to the investigation of synaptic deficits and underlying signalling contributing to schizophrenia.

**Keywords: schizophrenia; synapse; iPSC**
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# Mice differ from rats: Characterization of tyrosine hydroxylase mRNA-expressing neurons in the dopamine depleted mouse striatum

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Parkinson's disease (PD) is the second most abundant neurodegenerative disorder with a rising clinical and social relevance due to demographic aging. PD is mainly characterized by a loss of dopaminergic neurons in the substantia nigra pars compacta leading to a dopaminergic deprivation in the striatum. Since the late 60ies of the 20th century the unilateral 6-hydroxydopamine (6-OHDA) rat model of PD has mainly been used in preclinical studies. However, currently it is of great interest to transfer the well characterized 6-OHDA rat model to mice. By using transgenic or knock-out mice we would obtain more possibilities to study neuroprotective or neuroregenerative therapeutic strategies. However, in contrast to rats a high quantity of tyrosine hydroxylase-containing (TH) neurons can be observed in the mouse striatum after dopamine depletion. This is of interest, because TH is the rate limiting enzyme of dopamine synthesis. Darmopil et al. (2008) suggests that these neurons emerge from a phenotypic shift of neurons, which are already present in the striatum prior 6-OHDA-injection. Therefore, our current study aimed to further characterize the phenotype of TH-mRNA expressing neurons in the striatum by using a transgenic mouse producing GFP under the control of the TH-promoter (TH-GFP+). Mice received a unilateral injection of 6-OHDA into the medial forebrain bundle. Successful lesions were evaluated by apomorphine-induced rotations. Animals were perfused 3 days or 3 months postlesion. TH-GFP+ cells with a mature neuronal morphology possessing various dendrites appeared in the striatum as early as 3 days after lesion. By 3 month post lesion TH-GFP+ neurons were present throughout the entire striatum and these cells were never immunoreactive for the astrocytic marker GFAP. Though, the vast majority of the TH-GFP+ cells were detected in the rostral part of the striatum. In comparison to other studies, three days postlesion, we also did not find any newly generated cells containing doublecortin, indicating a phenotypic shift. Less than 1% of TH-GFP+ neurons expressed calretinin, identifying this subpopulation as interneurons. Comparing lesioned and non-lesioned hemispheres, there were no obvious differences in the quantity of calretinin expressing cells. TH-GFP+ neurons were never immunoreactive for choline acetyltransferase (ChAT). In our study only 10% of GFP+ neurons were co-localized with calbindin, indicating them as projection neurons. We conclude, that unknown factors lead to a phenotypic shift of striatal neurons into TH-mRNA expressing neurons due to dopaminergic deprivation in mice. Such a phenotypic shift was not observed in rats. This is of great interest, because in mice there seem to exist an endogenous program to push GABAergic neurons into a molecular dopaminergic phenotype. By further characterization of these TH-GFP+ neurons in the striatum we hope to identify mechanisms leading to therapeutic strategies by modulating the molecular phenotype of striatal neurons in patients suffering from PD.

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**Keywords: Parkinson's disease; neuronal phenotypic shift; 6-ohda; tyrosine hydroxylase**
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# Generation of disease-specific induced pluripotent stem cells for cell-based disease modeling and drug screening of hereditary spastic paraplegia type 5

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Hereditary spastic paraplegia (HSP) refers to a group of rare monogentic disorders which are characterized by a progressive axonal degeneration of corticospinal motor neurons, leading to spasticity and weakness of the lower limb. Spastic paraplegia gene type 5 (SPG5) is an autosomal recessive subtype of HSP caused by mutations in CYP7B1, a gene encoding for the cytochrome P-450 oxysterol 7-α-hydroxylase, essential for the liver-specific alternative pathway in bile acid synthesis. Mutations within CYP7B1 lead to a decreased enzyme activity and consecutively to an accumulation of oxysterol substrates (e.g. 27-hydroxycholesterol) in plasma and cerebrospinal fluid (CSF) of patients. In cultures of motor neuron like cells (NSC-34) and iPSC-derived neurons a neurotoxic effect of 27-hydroxycholesterol could be demonstrated and supports the hypothesis that the accumulation of oxysterols leads to progressive axonal degeneration. Research into molecular pathogenesis of HSP is limited by the restricted access to primary neurons and hepatocytes from patients. Derivation of disease-specific induced pluripotent stem cells (iPSCs) provide an unlimited cell population which can give rise to any somatic cell type. We reprogrammed primary fibroblasts of five SPG5-patients and two age-matched controls using non-integrative episomal plasmids and characterized genetic integrity and pluripotency of the generated iPS cells. Differentiation into iPSC-derived neurons and hepatocyte-like cells could be established, leading to an in vitro human cell model genetically identical with our patients. We aim to use this cell model to study further molecular mechanisms of SPG5 and to screen compounds for potential disease-specific biochemical or functional effects. These studies will improve our insight in pathogenesis of SPG5 and may help to develop new therapeutic approaches for the treatment of HSP.

**Keywords: hereditary spastic paraplegia; SPG5; induced pluripotent stem cells; cortical neurons; Hepatocytes**
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# Restoration of membrane-bound Nav1.1 levels in Dravet syndrome neurons via β2-ICD overexpression

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Dravet syndrome (DS) is a congenital disorder diagnosed in up to 1.5 % of juvenile epilepsies. Myoclonic seizures develop within the first 3–18 months of life, coinciding with the beginning expression of voltage-gated sodium channel 1.1 (Nav1.1) in developing GABAergic interneurons. In most DS patients, monoallelic loss-of-function mutations in the SCN1A gene, encoding the Nav1.1 α-subunit, lead to reduced Nav1.1 levels. Results from previous studies in primary neurons and tumor cell lines suggest that the Nav1.1 β2-subunit, a type I membrane protein that covalently binds Nav α-subunits, is a substrate for BACE1 and γ-secretase, and that the cleaved intracellular domain of Nav subunit β2 (β2-ICD) can increase mRNA and Nav1.1 protein levels (Kim D.Y. et al., Nature Cell Biol. 2007). Based on these observations we set out to explore whether β2-ICD overexpression could restore Nav1.1 α-subunit levels in neurons derived from DS patients. Induced pluripotent stem (iPS) cells from DS-patient fibroblasts (SCN1A+/mt) were differentiated into long-term self-renewing neuroepithelial stem (lt-NES) cells, a stable intermediate that efficiently gives rise to GABAergic neurons (Koch et al., PNAS 2009). lt-NES cell-derived DS neurons exhibit voltage-dependent in- and outward currents, action potential generation, and spontaneous synaptic activity. qPCR analysis of these neurons revealed that 50 % of SCN1A mRNA originates from the mutant allele. Western blot analysis confirmed a 50 % loss of Nav1.1 protein, while no truncated protein was detected. We next set out to compensate for reduced neuronal Nav1.1 levels by raising transcription from the functional SCN1A allele. To this end, the intracellular domain of Nav subunit β2 (β2-ICD) was fused to GFP via a self-cleaving 2A peptide and incorporated into a lentiviral, inducible construct. Subsequently, a DS lt-NES cell line was transduced with this vector and sorted for GFP+ cells to near purity. Autocatalytic construct cleavage was confirmed by Western blot analysis, and transgenic β2-ICD could be detected in cell nuclei by 3D microscopy. β2-ICD transgenic lt-NES cells maintained the expression of typical neural stem cell markers and could differentiate into GABAergic neurons. After doxycycline-induced transgene expression, a 4–8-fold increase in Nav1.1 protein levels was detected in 6-weeks-old neuronal cultures by Western blot analysis. Prior to transport to the plasma membrane, Nav1.1 and two β-subunits must form a functional, heterotrimeric complex, while free Nav α-subunits remain in the Golgi apparatus. Plasma membrane-bound proteins were therefore tagged with biotin and purified by affinity chromatography to distinguish these states. Indeed, subsequent Western blot analysis demonstrated a significant increase of Nav1.1 α-subunit levels on the cell surface. Our results indicate a regulatory activity of β2-ICD on Nav homeostasis in authentic human neurons. Furthermore, our data suggest that upon β2-ICD overexpression additional Nav1.1 α-subunits are transported to the plasma membrane and might be integrated into functional channel complexes, which could eventually be exploited for counteracting Nav1.1 deficiency in DS-specific neurons.

**Keywords: Dravet; SMEI; epilepsy; SCN1A; Nav1.1**
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# Safety improved SIN-lentiviral vectors for gene and cell therapy of pulmonary alveolar proteinosis

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Hereditary pulmonary alveolar proteinosis (PAP) caused by mutations in the granulocyte/macrophage – colony stimulating factor (GM-CSF- alias CSF2-) receptor α or β chain (CSF2RA or -B) represents a rare, life-threatening lung disease characterized by the accumulation of phospholipids and proteins in the lungs due to functional insufficiency of alveolar macrophages. Whereas current therapeutic options for this disease are limited, recent data suggest, that a gene therapy approach based on the endotracheal application of gene-corrected autologous macrophages may be feasible. To this end, we evaluated the efficiency and safety of improved 3rd generation SIN-lentiviral vectors for the genetic correction of Csf2rb as well as CSF2RA deficiencies in the murine and human system respectively. Initial studies were performed in a Csf2rb deficient PAP-mouse model (Csf2rb-/-). Using a lentiviral construct expressing the murine Csf2rb-cDNA from a shortened elongation factor 1 α (EFS1α) promoter (EFS.Csf2rb), transduction of Csf2rb-/- bone marrow derived hematopoietic stem/progenitor cells (HSPCs) revealed efficiencies of 15–30 % as evaluated by FACS analysis and restored the defective colony formation potential in response to mGM-CSF administration in these cells. As a safety feature unperturbed differentiation and colony formation (as compared to healthy-controls) was detected in the presence of IL3/IL6/SCF/EPO. Subsequently, gene-correction strategies were investigated in a human scenario using lentiviral constructs expressing a codon-optimized human CSF2RA-cDNA either in combination with eGFP (EFS.CSF2RA.iGFP) or with the inducible suicide gene iCaspase9 (EFS.CSF2RA.iCASP). Here, following the transduction of cytokine-dependent murine BaF3 cells with the EFS.CSF2RA.iGFP vectors, stable and longterm ( >3 month) expression of CSF2RA (CD116) was demonstrated by flow cytometry. Furthermore, transduced cells survived even at low concentrations of GM-CSF (5 ng/ml) confirming the formation of functional hybrid receptors with the murine GM-CSF receptor ß-chain by the transgene. Further characterization of GM-CSF receptor downstream signalling revealed 5- to 6-fold increased STAT5 phosphorylation by Western blot analysis in response to hGM-CSF (over control-transduced cells). Moreover, when BaF3 cells were transduced with the safety improved EFS.CSF2RA.iCASP vector, administration of a chemical inducer of dimerization (AP20187) activating the iCaspase9 suicide switch led to robust, time- and concentration dependent apoptosis of transduced cells. Even more important, transfer of these vectors to CSF2RA-deficient CD34+ patient derived HSPCs rescued hGM-CSF dependent colony formation as well as GM-CSF induced differentiation along the monocyte/macrophage lineage as demonstrated by typical morphology and surface phenotype. Again unperturbed colony formation of transduced cells in the presence of non-CSF2R associated cytokines such as G-CSF was observed. Furthermore, healthy CD34+ samples overexpressing CSF2RA exhibited no aberrations in growth and differentiation capacity including colony formation and in vitro differentiation towards CD14+, CD11b+, CD68+, CD163+ macrophages. In summary, we here established clinically applicable vectors for a cell-based gene therapy approach to human PAP disease addressing the CSF2RA or CSF2RB deficiencies in the human or the murine context, respectively, to broaden the therapeutic options for this extremely rare though life threatening disease. Addition of the iCaspase9 safety switch in the CSF2RA.iCASP construct may increase vector safety in first clinical approaches.

**Keywords: Pulmonary alveolar proteinosis; lentiviral vectors; gene therapy; iCaspase9; monocytes/macrophages**
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# Generation of human pluripotent stem cell-derived sensory neurons for toxicity testing

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Human pluripotent stem cell (hPSC) technology offers great potential in its application for human cell-based drug and toxicity screening. Chemotherapeutic induced peripheral neuropathy is the major dose-limiting side effect of commonly used anti-cancer drugs. To test for such interferences there is a need for human cell-based assays that cover cell type specific physiological and damage-induced response. We aimed to generate identical lots of human sensory neuronal precursors from PSCs that can be differentiated into functionally active sensory neurons and show cell-type specific response to adverse effects. Therefore, a 2-step differentiation protocol was established based on a recently published method to generate PSC-derived sensory neurons. Within 8 days, dorsal root ganglia-related cultures were generated and cryopreserved. After thawing, a pure neuronal population arose that formed a dense neurite network. Whole genome analysis, calcium signaling and immuno-staining confirmed peripheral neuronal identity. Sensory neurites were affected by several chemotherapeutic drugs like cisplatin and bortezomib. Moreover, we generated whole transcriptome data sets of the differentiation track and constructed a principle component analysis (PCA) based "cell feature map" comprising data from the new 2-step protocol, CellNet data from liver and brain samples, primary human dorsal root ganglia as well as data from hPSC-derived sensory neurons (original 1-step protocol) and further data from in vitro neuro-differentiations. The map suggests that the additional freezing and thawing step leads to an acceleration of the sensory neuron differentiation. Additionally, the new 2-step differentiation strategy facilitates the application of hESC-derived sensory neurons for drug and toxicity screenings.

**Keywords: hPSC-derived sensory neurons; differentiation strategy; peripheral neurotoxicity; in vitro screening**
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# Patient-specific iPS cell-based modeling of transthyretin-related familial amyloid polyneuropathy

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Human pluripotent stem cells hold great promise in regenerative medicine and are a valuable tool for research on disease models and drug screening. Since somatic cells can be reprogrammed to induced pluripotent stem (iPS) cells, disease-relevant cell types can be obtained by directed in vitro differentiation of iPS cells. Therefore, iPS cells might serve as a source for modeling multisystemic diseases. In our study, we aimed for modeling Transthyretin-Related Familial Amyloid Polyneuropathy (TTR-FAP) using patient-specific iPS cells. TTR-FAP is a rare autosomal dominant disease caused by the aggregation of mutated Transthyretin (TTR) protein, thereby forming amyloid fibrils. These are accumulating as amyloid deposits especially in neuronal and cardiac tissues, leading to an impairment of autonomic organ function. Importantly, TTR-FAP shows genotype to phenotype variations between the affected patients, making iPS cell-based disease modeling to a promising tool for this mutisystemic disease. By lentiviral transduction of the four reprogramming factors OCT4, SOX2, KLF4 and C-MYC we successfully reprogrammed fibroblasts from two different TTR-FAP patients, both being heterozygous for the most common TTR-FAP causing mutation Valin30Methionin. These patient-specific iPS cells showed pluripotency characteristics like expression of OCT4, SOX2, NANOG, SSEA4, TRA1-60 and Alkaline Phosphatase. Upon hepatic in vitro differentiation of TTR-FAP-iPS cells, mRNA expression levels of hepatic marker genes (Albumin, HNF4α, AFP and TTR) were investigated and comparable to expression levels obtained from differentiated human ES cells and control iPS cells. The TTR-FAP hepatic cells also showed functional characteristics like secretion of TTR and Albumin measured by Western Blot and ELISA, respectively, as well as Cytochrome P450 1A1 activity, analyzed by EROD-Assay. By Mass Spectrometry analyses TTR protein with wildtype sequence as well as protein with Valin30Methionin substitution was found in supernatants of TTR-FAP hepatic cells, thereby verifying the heterozygous mutation detected in the patients fibroblasts as well as in reprogrammed iPS cells. Most importantly for the disease modeling approach, the supernatants of TTR-FAP hepatic-differentiated cells showed the presence of TTR in an insoluble form. This insoluble TTR could be identified as amyloid fibrils by staining with suitable intercalating dyes such as Congo Red and ThioflavinT. In conclusion, reprogramming of fibroblasts from two different TTR-FAP patients resulted in iPS cells capable of being differentiated towards hepatocyte like cells that express and secrete mutated TTR protein. Importantly, we were able to investigate the disease-causing amyloid fibrils formed by the mutated TTR in our in vitro system.

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# Therapeutic effects of lentiviral-mediated gene transfer and vitamin B3 in an induced pluripotent stem cell model of severe congenital neutropenia

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The generation of pluripotent stem cells (iPSC) from patients with genetic disorders provides new opportunities for the investigation of disease-related molecular dysfunctions and the evaluation of new therapeutic treatment options. In our current studies, we focused on glucose-6-phosphatase 3 (G6PC3) deficiency, which has been described to cause severe congenital neutropenia (SCN) associated with an increased susceptibility of granulocytes for apoptosis. "Factor-free" iPSC from a SCN patient with a nonsense-mutation in the G6PC3 were generated and exhibited the capacity to differentiate into hematopoietic cells of the myeloid lineage. In particular, we identified cytokine conditions for in vitro SCN modeling, in which highly reduced numbers of CD66b-positive granulocytes (0.1–4 %) were produced from G6PC3-deficient iPSC compared to control cells (10–35 %). Additionally, we observed a 2–3 fold increase of CD14-positive monocytes (up to 85 %) from the SCN iPSC. Most importantly, the transfer of the functional curative gene via lentiviral vectors as well as the addition of vitamin B3 (nicotinamide) induced granulocytic differentiation and increased the number of granulocytes from SCN iPSC to similar levels as obtained from healthy control iPSC. In summary, we established an iPSC-derived SCN disease model for SCN for testing the potency of small molecules and gene therapeutic vectors for the development of alternative treatment options for SCN patients in future.

**Keywords: primary immunodeficincy; induced pluripotent stem cells; gene therapy; small molecule drug**
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# Modulation of intracellular calcium-release and autophagy induce ataxin-3 positive nuclear inclusions in Machado-Joseph disease-specific neurons

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Machado-Joseph disease (MJD) or spinocerebellar ataxia type 3 is the most frequent form of inherited spinocerebellar ataxia worldwide. Expansion of a polyQ repeat increases the aggregation propensity of ataxin-3, leading to the formation of ataxin-3-positive inclusions, a hallmark of MJD. Our previous studies employing patient-specific MJD neurons showed that excitation by glutamate or NMDA and subsequent Ca2+ entry via voltage-gated calcium channels activates calpain-mediated cleavage of ataxin-3, which initiates the generation of SDS-insoluble ataxin-3 microaggregates (Koch et al., Nature 480:543-46, 2011). Here we set out to explore whether and to what extent intracellular calcium release contributes to this disease-initiating step. We investigated the early expressed purinergic receptor family for the induction of cytosolic calcium increase and found that ATP-stimulation of P2Y receptors enables induction of microaggregates as early as 6 days after initiation of neuronal differentiation. Microscopically visible neuronal intranuclear inclusions (NIIs) positive for ataxin-3 and ubiquitin could be detected selectively in ATP-stimulated MJD cultures. Since both glutamate and ATP-mediated aggregation rely on receptor types with largely variable spatio-temporal distribution pattern, we also explored the potential contribution of ubiquitously expressed ryanodine receptors as well as sarco/endoplasmic reticulum calcium-ATPase (SERCA) for their capacity to modulate aggregate induction. Both activation of ryanodine receptors and SERCA inhibition resulted in ataxin-3 NIIs after only 8 days of differentiation with SERCA inhibition yielding >80 % MJD neurons carrying ataxin-3-positive aggregates. However, these paradigms still showed quantitative fluctuations of NII induction between independent experiments. We reasoned that this variability might be due to fluctuations in proteasomal degradation and autophagy. Indeed, media conditions resulting in autophagy inhibition yielded highly efficient NII formation in MJD neurons even in the absence of additional stimuli. Our data indicate that dysregulation of intracellular calcium homeostasis and autophagy may play important roles in the process of protein aggregation in MJD neurons.

**Keywords: iPS cells; disease modeling; Machado-Joseph disease; neurodegeneration**
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# Oncogenic KIT D816V causes stem cell activation and mobilization in mice

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The KIT tyrosine kinase, the receptor for stem cell factor (SCF), has a prominent function in hematopoiesis, including self-renewal and survival of hematopoietic stem cells (HSC). KIT D816V represents a constitutively active version of KIT and causes hematopoietic malignancies, such as acute myeloid leukemia and mastocytosis. Here we used a double-transgenic R26-GFP-KIT D816V mouse line to study the pathological activities of unrestrained KIT signaling on hematopoietic cell development. R26-GFP-KIT D816V mice contain a conditional knock-in of a humanized KIT D816V receptor into the ROSA26 locus. KIT D816V receptor expression is induced by tamoxifen (tmx) mediated Cre activity under control of the Scl promotor, which restricts Cre expression to hematopoietic stem and progenitor cells. To visualize transgene expression a GFP reporter is fused to the mutated KIT by a self-cleaving 2A peptide. We found that tmx-induced KIT D816V activation in mice enhanced stem differentiation into cells of the myeloid and erythroid lineages. Accordinlgy, KIT D816V mutant animals acquired a myeloproliferative neoplasia (MPN) marked by a massive increase in peripheral blood red cells and monocytes as well as severe splenomegaly caused by excessive extramedullary erythropoiesis. We also found a prominent activation of long-term HSC from the quiescent niche. KIT D816V activation also caused mobilization of short-term HSC into the circulation and spleen, leading to a shift of stem cells, progenitors and differentiated cell populations to spleen. Splenectomy prior to KIT D816V induction prevented expansion of red cells, but rapidly lead to a state of aplastic anemia, rapid stem cell loss and bone marrow fibrosis. In summary, we developed a model of KIT D816V induced MPN including myelofibrosis. The KIT D816V tyrosine kinase is insensitive to conventional tyrosine kinase inhibitors, such as Imatinib (Gleevec/Glivec) used for treating chronic myeloid leukemia (CML). As a result, KIT D816V MPN patients are essentially left without effective therapy, rendering this a fatal disease. Thus, the humanized KIT D816V mouse model presented here should pave the way towards understanding the pathological mechanisms of KIT D816V MPN and developing novel treatment strategies.

**Keywords: hematopoietic stem cells; KIT D816V; oncogenic; myeloproliferative neoplasia; mouse model**
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# iPS cell-based gene therapy for pulmonary alveolar proteinosis using TALEN and CRISPR/Cas technology

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Human pluripotent stem cells, such as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), represent an innovative cell source for regenerative medicine. In this respect, genetic correction of disease- and patient-specific iPSCs holds great promise for personalized medicine. However, clinical application of iPSC-based gene therapy with integrating vector systems is hampered by a number of substantial obstacles, such as transgene silencing or the potential risk of insertional mutagenesis. Targeted genetic modification of PSCs with so-called designer nucleases, such as Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 technology can avoid these problems. Here, we investigate the feasibility of precise genome editing using induced pluripotent stem cells from a patient suffering from hereditary Pulmonary Alveolar Proteinosis (herPAP) due to CSF2RA deficiency. Patient-specific iPSCs from a granulocyte/macrophage colony stimulating factor (GM-CSF) 2 α-chain (CSF2RA)-deficient individual were generated using an all-in-one third-generation self-inactivating (SIN) lentiviral reprogramming vector (Lv.SFFV.OSKM.dTom), expressing the four Yamanaka factors OCT4, SOX2, KLF4 and c-MYC. Expression of SSEA4, TRA-1-60, endogenous NANOG, OCT4 and SOX2 as well as teratoma formation were used to validate pluripotency of the generated iPSCs. Moreover, the patient-specific point mutation in exon 7 (R199X) of the CSF2RA gene was confirmed. Myeloid differentiation of PAP-iPSCs resulted in monocytes/macrophages (Mo/MΦ), showing perturbed GM-CSF-dependent characteristics, such as GM-CSF clearance, STAT5-phosphorylation and CD11b up-regulation. For genetic correction we pursued two different strategies, either aiming at the precise nucleotide correction of the patient-specific mutation or making use of the human AAVS1 safe harbour locus to reconstitute the expression of normal CSF2RA. In order to specifically repair the R199X mutation in the PAP-iPSCs, suitable TALENs & single-guide RNAs (sgRNA) for the CRISPR/Cas9 platform have been designed, and single-strand oligodeoxynucleotides (ssODN) are currently investigated to correct the C>T point mutation by means of Homologous Recombination (HR). Efficiency of the approach was evaluated in a HEK293T-based reporter cell line, harbouring part of the CSF2RA exon 7 within an inactive eGFP reporter gene. Gene correction, measured as reconstituted eGFP expression, showed a 25- or 100-fold increase using five sgRNAs and two TALEN pairs, respectively, as compared to the donor alone.

No off-target sites were predicted bioinformatically for 2 sgRNAs, and designer TALENs showed homology scores of 20/100 and lower, indicating a relatively low chance for off-target cleavage. Genetic correction of CSF2RA-deficient PAP-iPSC clones is currently ongoing by integrating a therapeutic CSF2RA gene into the putative safe harbour site AAVS1. Initial targeting studies were performed in CD34+-derived iPSC lines using a proof-of-concept donor containing an eGFP reporter gene. PCR analysis revealed AAVS1 targeting in 9/10 clones (90 %) with an integration profile of mono-allelic to bi-allelic frequency of 55 %. In summary, we have developed TALEN and CRISPR/Cas9 tools for the genetic correction of CSF2RA-deficient herPAP-iPSC lines. We hypothesise that genetic correction of the R199X point mutation in the patient-specific iPSCs and subsequent intrapulmonary transplantation of differentiated monocytes/macrophages derived from the corrected iPSCs may represent an innovative, cause-directed and safe iPSC-based cell replacement therapy.

**Keywords: iPSCs; genetic correction; TALEN & CRISPR/Cas9; Pulmonary Alveolar Proteinosis (PAP); AAVS1**
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# Characterization of an isogenic disease model of Alzheimer's disease from human iPS cell-derived neurons

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Objectives: Mutations in the gene encoding beta amyloid precursor protein (APP) have been linked with the progression of Alzheimer's Disease (AD). Leveraging our ability to produce previously inaccessible human neurons from iPS cells, we made biologically relevant AD disease models incorporating two APP mutations of interest as part of our disease and diversity products that represent an array of both healthy and disease-specific backgrounds.

Methods: Using a TALEN-mediated SNP alteration, we introduced APP A673V or APP A673T gene mutations into a "control" iPS cell line (01279.107) from an apparently normal healthy Caucasian male donor (no family history of neurological disorders). Cortical neurons were differentiated from these three unique isogenic iPS cell lines. Gene expression was analyzed by target- and disease-focused PCR arrays. Levels of AD-related biomarkers (i.e., sAPPα, Aβ 1-40, and Aβ 1 42) were quantified using various HTS-compatible assays (i. e., HTRF and AlphaLISA). The network-level activity of neurons from each background was evaluated on multi-electrode array (MEA).

Results: Here, we present data characterizing the gene expression and functionality of these neurons. Results from the HTS-compatible assays and MEA showed differences in functionality between the allele variants. Additionally, we present their individual responses to pharmacological modulation.

Conclusions: These data illustrate how human neurons derived from isogenic iPS cell lines provide a biologically relevant disease model that can be used to study AD in a dish.

**Keywords: Alzheimer's Disease; APP; human iPSC-derived neurons; mutations**
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# Frequency-dependent QT-analysis in human induced pluripotent stem cell derived cardiomyocytes in vitro using optogenetics

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The long QT syndrome (LQTS) is a severe inherited or drug-induced cardiac disorder that is characterized by abnormal long QT durations, arrhythmia and sudden cardiac death. LQTS are occuring because of prolonged action potential durations (APD) and there is a strong dependence of APD on beating frequency. Cardiomyocytes obtained from human pluripotent stem cells are a new and promising tool to screen for drug-effects by recording extracellular field potential durations (FPD) that provide an indirect measure of the APD using micro-electrode arrays (MEA). Unfortunately, electrical stimulation on MEA has technical limitations such as corrosion of electrodes, electrolysis and stimulation artifacts. Therefore we have established an optogenetic stimulation method for frequency-dependent investigation of FPD in human cardiomyocytes. Purified cardiomyocytes derived from human induced pluripotent stem cells were transfected with an adeno-associated virus of the serotype 2/1 (6.6x10^4 particles/cell) to express Channelrhodopsin-2, a light-gated cation channel in fusion with mCherry. FACS sorting revealed 70.8±9.6 % (n=3) of the cells expressing mCherry and in these patch clamp recordings showed light-induced inward currents and action potentials. Importantly, APD turned out to be very similar using 1 ms light pulses (147.3±42.6 ms, n=13), compared to electrical (2 ms) stimulation (135.4±45.1 ms, n=13). Transfected cardiomyocytes were plated on MEA and global stimulation at 1–2.5 Hz with 1 ms long light pulses (0.92 mW/mm^2) induced synchronized field potentials on all 60 electrodes without conduction delay. This allowed averaging of field potentials from all electrodes and from multiple beats to obtain one global field potential for the analysis of FPD. We found a clear frequency-dependence with significant shortening of FPD at higher beating rates (2 Hz and 2.5 Hz compared to 1Hz: p=<0.05, Bonferroni 1-Way ANOVA). Drug-induced LQTS was evoked by application of the hERG potassium channel blocker Sotalol (100 µM). Interestingly this prolonged FPD only at low (1–1.5 Hz) pacing rates (1Hz: +30.4±3.5 %, n=6, p=0.0003; 1.5Hz: +9.1±2.4 %; n=10; p=0.0059) but not at high frequencies (2 Hz). The specific hERG channel blocker E4031 and the cardiac sodium channel and hERG channel blocker Quinidine showed similar results. In addition Quinidine led to a significant reduction in downstroke velocity of the initial sodium component of the field potential at all frequencies (1Hz: 123.4±36.6mV/s to 21.5±6.3mV/s, n=5, p=0.0395; 1.5Hz: 134.0±39.0mV/s to 8.4±2.3mV/s, n=5, p=0.031). Furthermore the calcium channel blocker verapamil (100 nM) significantly shortened FPD at all frequencies (e.g. at 1Hz: -42.8±8.9 %, n=5, p=0.0087). In summary we present a new method for optogenetic pacing of human cardiomyocytes on MEA, which enables spatial averaging of synchronized field potentials. This novel tool was used to measure frequency-dependent effects of drugs on potassium, sodium and calcium channels and is therefore suited for drug screening and to investigate inherited LQTS in vitro.

**Keywords: stem cells; cardiomyocytes; optogenetics; micro-electrode array; drug screening**
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# Electrophysiological characterization of human induced pluripotent stem cell derived cardiomyocytes with long QT syndrome type 3

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The long QT syndrome type 3 (LQT3) is an inherited heart disease caused by gain of function mutations of Na+ channels. It is characterized by long QT intervals in the ECG because of prolonged action potentials in cardiomyocytes especially at slow heart rates. Affected patients have a high risk of torsades de pointes ventricular tachycardia and sudden cardiac death during rest or sleep. To investigate the phenotype of patient-specific human cardiomyocytes with LQT3, induced pluripotent stem (iPS) cell lines were generated from a LQT3 patient with the R1644H mutation (mutant) in the Ð-subunit of the Na+ channel and from a not affected control person. To this aim fibroblasts were obtained by skin biopsies and transduced with retroviruses to express the reprogramming factors Oct4, Sox2, KLf4 and c-Myc. The resulting iPS clones had ES cell-like morphology and global transcription profiles, expressed the pluripotent marker SSEA4, transgenes were fully silenced and cells had normal karyotypes. In addition these cells could be differentiated in various cells of the three germ layers, including cardiomyocytes. Electrophysiological characterization of cardiomyocytes from the LQT3 and control human iPS cell lines was performed using classical whole-cell patch clamp recordings. Peak Na+ current density was not different, but recovery from inactivation of Na+ current was significantly faster in LQT3 (3.6±0.6 ms, n=5) compared to control cells (10.6±2.3 ms, n=13). Action potential duration (APD) was recorded at various pacing frequencies and showed a prolongation at slow heart rates in LQT3, but not in control cells. Although APD at 1 Hz was not significant different overall, however, analyzing only cardiomyocytes with ventricular-like action potentials showed significant longer APD in cells from LQT3 iPS cells (234.9±35.2 ms, n=8) compared with controls (137.3±15.2 ms, n=5). To quantify the frequency-dependent APD prolongation, the slope of APD restitution (APD versus pacing period) was determined for each individual cell by linear regression analysis. This yielded a negative slope (-3.3±3.5 ms/s, n=5) for control cells, whereas a clearly positive slope (19.3±5.4 ms/s, n=8) was observed in LQT3 iPS cell-derived cardiomyocytes. Application of the Na+ channel blocker mexiletine (100 µM) reduced APD prolongation only in LQT3 cardiomyocytes (-25.8±6.7 %, n=5), but not in control cells (+3.2±4.8 %, n=8). Thus, iPS cell-derived cardiomyocytes from a LQT3 patient showed known biophysical features of LQT3 and allow patient-specific pharmacological screening in the future.

**Keywords: long QT syndrome hIPS-derived cardiomyocytes; action potential; drug screening**
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# Generation of reprogramming-vector-free iPSCs from an Angelman syndrome patient

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The Angelman syndrome is a rare neurodevelopmental disorder caused by lack of the E3 ubiquitin ligase UBE3A in neurons. Clinically, the disease is characterized by seizures, frequent laughter, microcephaly, movement disturbances, hyperactivity, developmental delay, and an absence of speech and language. In neurons of healthy individuals, the UBE3A gene is expressed only from the maternal chromosome due to silencing of the paternal copy during neuronal development. Silencing is caused by allele-specific expression of an antisense, long non-coding RNA, which overlaps the entire paternal UBE3A gene. In neurons of Angelman syndrome patients, the maternal UBE3A locus is defective thus resulting in the complete absence of the encoded enzyme. Because primary neurons from the brains of patients are inaccessible, we generated induced pluripotent stem cells (iPSCs) from an Angelman patient carrying a defined 3bp deletion in the maternal copy of the UBE3A gene (AS_3bp-iPSCs) for subsequent neuronal differentiation. Fibroblasts from a skin biopsy of this patient and of a healthy control person were reprogrammed by SIN-lentiviral expression of the four so called 'Yamanaka'-factors OCT4, SOX2, KLF4 and MYC. The vector contains FRT-sites at the deleted U3 position of the SIN-LTR allowing for excision of the reprogramming vector by FLP recombinase. Selected clones containing a single lentiviral integration were selected for excision. Flp-Recombinase was delivered into iPSCs by a VSV-G pseudotyped lentivirus. Succesful excision was analysed by PCR. The resulting iPSCs were free of the reprogramming vector; only one SIN-LTR containing one Frt-site is left in the genome. To evaluate pluripotency of generated iPSCs prior to and after vector excision, we determined alkaline phosphatase activity, surface expression of SSEA4, TRA1-60 and TRA1-81, expression of endogenous, pluripotency-associated genes by qRT-PCR and immunocytochemistry. Furthermore, their functionality was proven as they formed cells of all three germ layers during embryoid body differentiation, in vitro. All analyzed iPSC clones displayed pluripotency characteristics.

**Keywords: Angelman Syndrome; iPSC**
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# Antisense-mediated exon skipping: a therapeutic strategy for titin-based dilated cardiomyopathy

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Frameshift mutations in the TTN gene encoding titin are a major cause for inherited forms of dilated cardiomyopathy (DCM), a heart disease characterized by ventricular dilatation, systolic dysfunction, and progressive heart failure. To date, there are no specific treatment options for DCM patients but heart transplantation. Here, we show the beneficial potential of reframing titin transcripts by antisense oligonucleotide (AON)-mediated exon skipping in human and murine models of DCM carrying a previously identified autosomal-dominant frameshift mutation in titin exon 326. Correction of TTN reading frame in patient-specific cardiomyocytes derived from induced pluripotent stem cells rescued defective myofibril assembly and stability and normalized sarcomeric protein expression. AON-treatment in Ttn knock-in mice improved sarcomere formation and contractile performance in homozygous embryos and prevented the development of the DCM phenotype in heterozygous animals. These results demonstrate that disruption of the titin reading frame due to a truncating DCM mutation can be restored by exon skipping in both patient cardiomyocytes in vitro and mouse heart in vivo, indicating RNA-based strategies as a potential treatment option for DCM.

**Keywords: dilated cardiomyopathy; exon skipping; induced pluripotent stem cells; titin**
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# Osteopontin mediates survival, proliferation and migration of neural stem cells through the chemokine receptor CXCR4

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Introduction: Osteopontin (OPN) is a phosphoglycoprotein with important roles in tissue homeostasis, wound healing, immune regulation, and stress responses. It is expressed constitutively in the brain and upregulated during neuroinflammatory responses, e.g. after focal cerebral ischemia. To date, its effects on neural stem cells (NSC) remain to be elucidated and are, accordingly, subject of this study.

Method: Primary fetal rat NSC were cultured as homogenous monolayers and treated with different concentrations of OPN. Fundamental properties of NSC were assessed following OPN exposure, including proliferative activity, survival under oxidative stress, migration, and differentiation potential. To elucidate a putative action of OPN via the CXC chemokine receptor type 4 (CXCR4), the latter was blocked with AMD3100. To investigate effects of OPN on endogenous NSC in vivo, recombinant OPN was injected into the brain of adult rats, followed by 7 days of systemic injections of bromodeoxyuridine (BrdU) to label proliferating cells. Effects of OPN on NSC proliferation and neurogenesis in the subventricular zone (SVZ) were studied immunohistochemically.

Results: OPN dose-dependently increased the number of NSC in vitro. As hypothesized, this effect was mediated through CXCR4. The increase in NSC number was due to both an enhanced cell proliferation and survival, and was confirmed in vivo. Additionally, OPN dose-dependently stimulated the migration of NSC via CXCR4. Moreover, in the presence of OPN, differentiation of NSC led to a significant increase in neurogenesis.

Conclusion: Data show positive effects of OPN on survival, proliferation, migration, and neuronal differentiation of NSC. At least in part these effects were mediated via CXCR4. Results suggest that OPN is a promising substance for the targeted activation of NSC in future experimental therapies for neurological disorders such as stroke.

**Keywords: neural stem cells (NSC); osteopontin (OPN); proliferat ion; migration; CXC chemokine receptor type 4 (CXCR4); AMD 3100; oxida tive stress; neuroprotection; neuroinflammation**

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# Turn back the clock – gene expression analysis of iPS-derived neural cells from a sporadic Alzheimer's disease patient to understand the onset of neurodegenerative disorders

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The dramatic increase in average life expectancy resulted in an increase of age-related dementia, including the neurodegenerative Alzheimer´s disease (AD). Unfortunately, the disease process of neuronal loss starts a few years back after the onset of the cognitive dysfunction and almost nothing is known about the cellular and molecular mechanism during this period. Cortical inhibitory (GABAergic) interneurons which control the firing of glutamatergic neurons and synchronize brain activity are affected by Amyloid-beta peptide and Tau protein, two major hallmarks of AD. The axonal Tau protein regulates the dynamic stability of the dendritic microtubules and axonal transport. During AD, Tau becomes hyperphosphorylated and forms aggregates called neurofibrillary tangles. We have derived and characterized neuronal cells differentiated from dermal-fibroblast-derived induced pluripotent stem (iPS) cells of an 82-year-old female patient diagnosed with sporadic AD (sAD) (late onset of AD)1. Here we exploit our established iPS-based in vitro model to study the recapitulation of the human pathology within the context of the emergence of GABAergic interneurons and the neurodegenerative disorder AD. We used three distinct protocols for the neuronal differentiation of the iPS cells derived from the sAD patient (AD5). The expression of GABAergic (GAD1/GAD2), glutamatergic (vGlut1/vGlut2) and interneuron-specific (Somatostatin, Calbindin, Calretinin) markers highlights that the neuronal cells featured distinct subtypes in a time- and growth factor-dependent manner. The common feature of all AD5-differentiated neuronal cells was the missing expression of Tau. Remarkably, human-foreskin-fibroblast-derived iPS cells that were differentiated in the same matter for 4 weeks displayed Tau (and Somatostatin) gene expression. In addition, commercially bought RNA (Amsbio®) from fetal, adult and AD brain were used as reference. As expected, Tau as well as GABAergic and interneuron-specific markers were down-regulated in the Alzheimer-affected brain. Our iPS-based model displays the retrograde step towards the onset of disease – the neural cells from this aged-donor diagnosed with sAD were able to differentiate towards inhibitory interneurons, but failed to produce Tau mRNA. Our preliminary findings illustrate the correlation and complexity of the onset and progression of AD. The iPS-based model reflects early stages of neuronal development and will help to understand the basic pathological mechanism underlying this debilitating neuronal dysfunction.

*Amir M Hossini, Matthias Megges, Alessandro Prigione, Bjoern Lichtner, Mohammad R Toliat, Wasco Wruck, Friederike Schröter, Peter Nuernberg, Hartmut Kroll, Eugenia Makrantonaki, Christos C Zouboulis and James Adjaye (2015) Induced pluripotent stem cell-derived neuronal cells from a sporadic Alzheimer's disease donor as a model for investigating AD-associated gene regulatory networks. BMC Genomics, doi:10.1186/s12864-015-1262-5*

**Keywords: Alzheimer; iPS-based model; neuronal differentiation; inhibitory interneuron; Tau**
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# Modeling IRF8 deficient dendritic cell development and function with engineered human induced pluripotent stem (iPS) cells

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Induced pluripotent stem (iPS) cells provide excellent opportunities for knockout models to study gene function in human hematopoiesis. Interferon regulatory factor 8 (IRF8), also known as interferon consensus sequence-binding protein (ICSBP), is a transcription factor, which acts as tumor suppressor and lineage determining factor for myeloid cells, including dendritic cells (DC). Loss of IRF8 function in patients causes severe monocytic and DC immunodeficiency. Here we generated a human IRF8 knockout model based on IRF8 deficient iPS cells by employing RNA guided CRISPR/Cas genome editing. IRF8 knockout cells were induced to differentiate into hematopoietic stem/progenitor cells and further into DC to study the impact of IRF8 on human DC development and function. DC are professional antigen presenting cells with a pivotal role in immunity and maintenance of immune tolerance. DC comprise different subsets: classical/conventional DC (cDC), which capture, process and present antigens to T-cells, and plasmacytoid DC (pDC), which produce large amounts of interferon alpha in response to pathogens. We demonstrate that human iPS cells can be differentiated into BDCA1+ and BDCA3+ cDCs and BDCA2+ pDCs in a GM-CSF/FLT3L/IL-4/SCF culture system. Differentiated cells express DC markers, such as MHC class II and CD11c, and DC specific markers, like Clec9A for BDCA3+ cDCs or CD123 for pDCs. They express DC genes, including PU.1, BATF3, ID2 or TCF4. iPS cell-derived DC are fully functional and effectively upregulate costimulatory molecules and chemokine receptors upon Toll-like receptor stimulation. Initial data show that development of BDCA3+ cDC and pDC from IRF8 knockout iPS cells is impaired whereas BDCA1+ cDC are unaffected. Taken together, we engineered a human IRF8 knockout model that allows studying molecular mechanisms of human DC development in vitro, including the pathophysiology of IRF8 deficient DC.

**Keywords: engineered iPS/ES cells, dendritic cells, IRF8**
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# Human induced pluripotent stem cells with KIT D816V mutation for modeling leukemia

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Induced pluripotent stem cells (iPS cells) provide unique opportunities for disease modeling and drug screening. KIT D816V myeloproliferative disorders are a class of devastating diseases with a constitutively activated stem cell factor (SCF) receptor tyrosine kinase KIT. KIT D816V cells are resistant to the tyrosine kinase inhibitor Imatinib (Gleevec/Glivec®), which is successfully used for treating chronic myeloid leukemia (CML). Thus, KIT D816V patients in the terminal phase of disease are essentially left without effective therapy, rendering this a fatal disease. In the present work we report on the generation of leukemia patient derived iPS cells carrying the KIT D816V mutation. CD34+ cells of peripheral blood from patients were cultured for 2 days in the presence of Imatinib and reprogrammed with Sendai virus vectors. Additionally, we used the CRISPR/Cas9n technology to introduce the KIT D816V mutation in human ES cells to generate isogenic pairs with and without the mutation. The presence of the KIT D816V mutation was confirmed by allele specific PCR, restriction fragment length polymorphism (RFLP) and Sanger sequencing. We also established culture conditions for efficient differentiation of iPS cells and ES cells into hematopoietic stem cells (HSC) via a mesodermal commitment step. Such HSC are CD34+ CD43+ and exhibit a HSC specific gene expression repertoire. In ongoing work, KIT D816V HSC are obtained from iPS cells and ES cells and analyzed for KIT downstream signaling and inhibition by novel tyrosine kinase inhibitors. In summary, KIT D816V iPS cells and ES cells, and the KIT D816V HSC derived thereof, are expected to provide new insights into the molecular pathways of disease pathophysiology and eventually development of new disease specific therapies.

**Keywords: iPS cells; KIT D816V; CRISPR/Cas9; disease modeling; leukemia**
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# MicroRNAs and metabolites in serum change after chemotherapy: Impact on hematopoietic progenitor cells

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Hematopoietic regeneration after high dose chemotherapy necessitates activation of the stem cell pool. There is evidence that serum taken after chemotherapy comprises factors stimulating proliferation and self-renewal of CD34+ hematopoietic stem and progenitor cells (HPCs) – however, the nature of these feedback signals is yet unclear. Here, we addressed the question if specific microRNAs (miRNAs) or metabolites are affected after high dose chemotherapy. Serum taken from the same patients before and after chemotherapy was supplemented for in vitro cultivation of HPCs. Serum taken after chemotherapy significantly enhanced HPC proliferation, better maintained a CD34+ immunophenotype, and stimulated colony forming units. Microarray analysis revealed that 23 miRNAs changed in serum after chemotherapy – particularly miRNA-320c and miRNA-1275 were down-regulated whereas miRNA-3663-3p was up-regulated. miRNA-320c was exemplarily inhibited by antagomiR, which seemed to increase proliferation but did not maintain CD34 expression. Metabolomic profiling demonstrated that 44 metabolites were less abundant, whereas three (including 2-hydroxybutyrate and taurocholenate sulphate) increased in serum upon chemotherapy. Nine of these metabolites were subsequently tested for effects on HPCs in vitro, but none of them exerted clear concentration dependent effect on proliferation, immunophenotype and colony forming unit formation. Taken together, serum profiles of miRNAs and metabolites changed after chemotherapy. Rather than individually, these factors may act in concert to recruit HPCs into action for hematopoietic regeneration.

**Keywords: hematopoietic stem cells; leukemia; metabolomics; micro RNA**
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# IPSC-based Parkinson model enabling rapid phenotypic read-out of LRRK2 mutant-associated neuronal alterations

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Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting about 2 % of the population above the age of 60 years. It is characterized by progressive loss of A9 midbrain dopamine (mDA) neurons in the substantia nigra pars compacta (SNpc). Current therapeutic approaches for PD provide only symptomatic relief but cannot change the disease course. Although most of PD cases are idiopathic and occur sporadically, a minority of 5–10 % can be linked to monogenetic causes. Notably, the autosomal dominant G2019S mutation in the large, multifunctional kinase LRRK2 leads to clinical symptoms typically found in common sporadic PD cases. Thus, in principle, mDA neurons derived from induced pluripotent stem cells (iPSCs) of LRRK2G2019S patients should be highly valuable for drug discovery screens. However, assessment of LRRK2G2019S-associated phenotypes in iPSC-derived mDA neuronal cultures typically requires weeks to months of in vitro differentiation, which makes these cell systems unsuitable for high-throughput applications. Here we set out to establish a culture system based on a standard mDA progenitor population amenable to replating and phenotypic read-out within a 48 hour time period – a time frame acceptable for scale-out and HTS. Our standard cell population is pre-differentiated for 3–5 weeks and shows expression of characteristic mDA markers such as FOXA2, LMX1A, PITX3, NURR1 as well as TH. Replating of these cells enables read-out of a 20 % reduction in the length of TH+ neurites in LRRK2G2019S neurons already after 24 hours. Cell survival assays following autophagy inhibition with 3-methyladenine yield a LRRK2G2019S-specific increase in neuronal cell death already 48 hours after treatment. We expect these rapid phenotypic assays to expedite the study of PD-related pathomechanisms and enable the identification of therapeutic compounds via high-throughput drug screening applications.

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# Cardiac disease phenotypes and pharmacological rescue in human iPS cell models of Jervell and Lange-Nielsen and LQT3 syndrome

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Long QT syndrome (LQTS) is a rare inherited or acquired heart condition in which delayed repolarization of the heart causes high risk of ventricular arrhythmia, which may lead to sudden cardiac arrest. LQTS can be inherited in an autosomal dominant or autosomal recessive fashion. Here, we generated human induced pluripotent stem cell (hiPSC) models of the autosomal recessive LQTS, the Jervell and Lange-Nielsen syndrome (JLNS), as well as of a dominant LQTS variant, LQT3, by reprogramming of patient fibroblasts. In cardiomyocytes of both human induced pluripotent stem cell (hiPSC) models, typical LQTS disease features were recapitulated at the celular level. This included significant prolongation of the QT-like interval on multielectrode arrays (MEAs), as compared to wild-type controls, as well as increased propensity for developing stress-induced arryhthmia and/or early afterdepolarisations (EADs) in the ground state. Interestingly, these LQTS phenotypes were caused by distinct mechanisms at the molecular level. While the JLNS hiPSCs were deficient in the potassium channel-encoding KCNQ1 gene, LQT3 hiPSCs harboured a missense mutation in the sodium channel-encoding SCN5A locus. Specifically, JLNS hiPSC-derived cardiomyocytes (hiPSC-CMs) displayed severely increased field potential durations while showing arrhythmic spontaneous beating at low supplied dosages of the proarrhythmic compound cisapride. In contrast, LQT3 hiPSC-CMs showed EADs, known triggers of arrhythmia, already in the ground state at high probability. Based on the distinct underlying disease mechanisms, drug testing was performed to correct these specific phenotypes pharmacologically. JLNS hiPSC-CMs could be rescued using an activator of the hERG potassium channel in that induced arrhythmias were fully suppressed. In LQT3 hiPSC-CMs, in comparison, EADs could be dose-dependently repressed by means of a pharmacological SCN5A inhibitor, balancing the gain-of-function phenotype in this model. In sum, we demonstrate successful pharmacological disease phenotype correction in two distinct models of LQTS. Our data hence highlight the utility of patient-specific hiPSCs for personalized drug evaluation in case of inherited cardiac channelopathies.

**Keywords: disease modeling; long QT syndrome; pharmacological rescue; human induced pluripotent cell; cardiac differentiation**
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# Urine cell-derived iPS cells differentiate to hepatocyte-like cells and serve as a tool for evaluation of RNAi in metabolic liver disease

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Various types of somatic cells have been reprogrammed to induced pluripotent stem cells (iPSC) followed by differentiation into hepatocyte-like cells (HLC). Recently, cells that shed from the renal epithelial system were shown to be a suitable and convenient source for iPSC reprogramming. In the current study, renal epithelial cells were isolated from urine donations of patients having familial amyloid polyneuropathy (FAP), a complex, neurodegenerative disease caused by mutation of the transthyretin (TTR) gene that is mostly (>95%) expressed by liver. More than 100 TTR mutations and diverse phenotypes are known. FAP is characterized by misfolded TTR protein, extracellular tissue deposition followed by dysfunctions of tissues or organs, including heart and the peripheral nervous system. Therapy of FAP has been established recently in a portion of patients by pharmaceutically affecting the stability of TTR complexes present in the blood. However, reduction of the overall TTR expression is believed to further improve therapeutic outcome. Novel antisense oligonucleotides (ASOs) and small interfering RNAs (siRNA) that target TTR expression are currently evaluated in the clinic. In this study, FAP patient-specific HLCs were generated from urine cell-derived iPSC. Fresh urine (250–500ml) from FAP patients was processed for isolation of renal epithelial cells, followed by reprogramming into iPSCs using integration-free vectors having episomal (EBNA) elements. Five days after cultivation of urinary cells, typical cell colonies emerged from FAP patients that could be propagated (n=9). After characterization of iPSC that expressed high levels of pluripotency markes like OCT4 and NANOG, a 3-step hepatocyte differentiation protocol was performed. iPSCs were subjected to a treatment with growth factors (activin A, Wnt3a, FGF2, HGF) for 14 days. HLCs were characterized by analysis of typical hepatic markers via RT-PCR, and immunocytochemistry. TTR mRNA expression of HLCs was characterized. For the study of TTR gene silencing, ASOs and siRNAs were introduced into cells and an assay for TTR inhibition using real-time PCR analysis was established. Taken together, the results indicate that our iPSC protocol using urine cells and transient reprogramming is excellently suited to model FAP. The noninvasive methodology will allow the evaluation of novel RNAi using primary cells of FAP patients with diverse phenotypes.

**Keywords: iPS cells; hepatocyte; disease model; RNA interference**

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# Ethical, Legal & Social Issues

# Is the human embryo more than just a carrier of developmental totipotency?

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In Germany developmental totipotency is used as a normative criterion in defining the moral and legal status of the human embryo. On this basis, assumed that iPS cells may develop short-term stage of totipotency during cellular reprogramming there would be a need to define the moral and legal status of reprogrammed cells and iPS cells. However, do we perceive any human cell which undergoes a transient stage of totipotency being a human embryo? If we focus only on the criterion of developmental totipotency we ignore other characteristics of the human embryo like fertilisation or its way of coming into existence. Defining an embryo solely by way of its totipotency would seem like a reduced meaning of what we usually call a human embryo. A definition based only on developmental totipotency would probably also determine reprogrammed cells or iPS cells, if having undergone transient totipotency, as human embryos. From an ontological perspective this limitation seems implausible. Ontology considers all objects of our reality and divides them into different classes. These different classes are as well reflected in our lifeworld (Lebenswelt). Aristotele defines the different objects of our reality as persons, animals, plants, tables etc. as substances. Substances have manifold but not arbitrary properties. We understand the sum of their relatively stable characteristics as their nature. It is not necessary for a substance to have all the characteristic properties, but if it has none of these properties, it can be no longer recognized as a member of a given class. Aristotele distinguishes the objects of our reality with regard to the way of their creation. To be able to say what something is, one has to know how it is created. With the help of this distinction an object can be assigned to the class of either natural entities or artifacts. This difference is also crucially manifested in the lifeworld and, therefore, should be considered innormative considerations. If we think about what we do understand as being a human embryo, its way of origin is of importance. It makes a difference whether it was produced naturally or artificially, i. e. whether it owes its existence only to diverse manipulative procedures. Usually we evaluate natural things differently to artificial things. Therefore it does not seem justified to reduce the definition of the human embryo solely to the criterion of its developmental totipotency. However, even if we should not take into account developmental totipotency as the only criterion in the normative debate, this criterion has to be considered, among others, as a criterion in defining a human embryo. Unlike artifacts, we attribute both physical and mental characteristics to a person. A reductionist view of the human person and the human embryo as expressed by focusing only on the criterion of developmental totipotency is, therefore, not very convincing. Content of the poster will be a criticism of the naturalistic definitions of the human embryo and to elucidate their limits.

**Keywords: iPS cells; developmental totipotency; ethics; ontology; legal**
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# No patent, no therapy: The implication of the unpatentability of human embryonic stem cell based inventions in the European Union on the justification of human embryonic stem cell based therapies

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In the aftermath of the European Court of Justice's (CJEU) decision case of Brüstle v. Greenpeace of October 2011 that patent claims encompassing human embryonic stem cells (hESCs) were patent-ineligible in the European Union on public order and morality grounds a rash of stories predicted the exodus of hESC research in Europe. Irrespectively, whether this predictions are justified, it has not been examined, whether this decision has an implication on the justification of hESC based therapies in Europe. The ruling follows a challenge by Greenpeace over a patent granted for the technique to derive nerve cells from hESCs. The CJEU ruling held that processes requiring the prior destruction of human embryos, or their prior use as base material, cannot be patented. This holding applies even if - as is the case with the questioned patent - the patent application describing the process does not refer to the use of hESCs, but where the implementation of the invention necessarily requires the destruction of human embryos. The court based its decision on art. 6 (1) and (2) of the European Biopatent Directive, which stipulate that patents may not be granted for inventions whose commercial exploitation would be contrary to the European ordre public, and that, in particular, patents may not be granted for uses of human embryos for industrial or commercial purposes. The court reasoned that the purpose of the Biopatent Directive was to eliminate the possibility of patentability where respect for human dignity could thereby be affected. Finally, the court came to the result, that a patent is always connected with an industrial or commercial purposes since this is the nature of a patent. Therefore, the court reasoned, that the use of human embryos within the patent claims cannot be separated from the patent itself and the rights attaching to it. This project brings forward the argument, that market approval for hESCs based therapies has to be linked to the CJEU's decision in the Brüstle case since both legal questions are governed by law of the same lawmaker. Therefore, it should be expectable, that the same lawmaker expresses the same (moral) understanding in its statutes. Since stem cell based pharmaceuticals need market approval by an authority to enter the market one must ask whether this authority can have a different opinion on the European ordre public than the CJEU. The market approval of therapies based on hESCs is equivalent with the permission to use these cells for industrial or commercial purposes. Therefore, it must also be asked, if this correlates with an ineligible industrial or commercial use of those embryos which were needed to obtain the stem cells for therapies. If the use of hESCs for therapies is permissible by official market approval, there would be a discrepancy between the value of human embryos in patent law and pharmaceutical law. On the one hand, techniques using hESCs could not be patented, on the other hand, (same) techniques used to produce pharmaceuticals based on hESCs could get official market approval.

**Keywords: human embryonic stem cells; ordre public; moral; therapy; patent**
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# Are parthenotes embryos? – On the biological, legal and ethical qualification of human parthenotes

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Parthenogenesis occurs as common reproductive strategy in arthropods and some insects but is rare in vertebrates (e.g. some lizards, the Prussian Carp, the Burmese python, the wild turkey). In mammals, parthenogenesis does not occur as reproductive strategy, but parthenogenetic activation of oocytes can result in non-malignant ovarian teratoma. Nevertheless, artificial parthenogenetic activation of oocytes in vitro result in entities that form a blastocyst-like structure, which can implant after transfer into wombs of pseudopregnant recipients, but which fail to fully develop into viable, full-term offspring. Recent studies suggest that misregulation of maternally and paternally imprinted genes cause major developmental aberrations. However, major genetic modification mimicking appropriate expression levels of genes (such as H19 or Igf2) allow for generation of viable and fertile mice from bi-maternal embryos. Whether this approach would result in developing offspring from human oocytes is highly questionable, but it is not too unlikely that human parthenotes would exhibit a similar develomental potential compared to nonmanipulated murine parthenotes and would reach a mid-fetal developmental stage. Thus, the question whether human parthenotes qualify as embryos under the (German) Embryo Protection Act (Embryonenschutzgesetz, ESchG) and the (German) Stem Cell Act (Stammzellgesetz) is highly contentious. In particular it is questionable whether parthenotes have the "capability of development", i. e. the ability to develop into an "individual". As stated above, human parthenotes are unable to develop to term. Their ability to nidate seems likely, the creation of organs or the neural tube is at least not impossible. Due to crossing over the parthenote and the egg donor are not genetically identical, therefore Paragraph 6(1) ESchG's ban on cloning is not violated. Exemplified on Swiss and UK legislation, it gets clear that foreign legal systems also show a non-uniform approach towards parthenogenesis. In Brüstle v. Greenpeace e.V. (2011) the CJEU declared that an entity is a human embryo in terms of the EU Biopatent Directive (Directive 98/44/EC) if it is "capable of commencing the process of development of a human being". Irritatingly the court thought parthenotes would be covered by this definition. However, in ISCO v Comptroller General of Patents (2014) the CJEU allowed for national authorities and courts to exclude parthenotes from the scope of the Directive's embryo definition, demanding "the inherent capacity of developing into a human being" to be fulfilled in order to qualify as an embryo under the Directive. In the light of the current legal debate about human parthenotes the ontological and ethical status of these entities has to be taken into account. The similarities between human embryos and parthenotes concerning the first developmental steps cannot be denied but it must be assumed that the human parthenote has per se no potential to develop into later stages. What are the consequences of this for the ethical status of human parthenotes? To answer this question it has to be analyzed what kind of entity a human parthenote is and whether the suggested biological classification of human parthenotes as embryos is sufficient to justify their protection under law.

**Keywords: parthenogenesis; embryo; (German) Embryo Protection Act (Embryonenschutzgesetz); patent law; developmental potential**

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# From "totipotency" to "qualified capability of development"

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## 1. From "totipotency" to "qualified capability of development"

Totipotency is the central criterion in German sub-constitutional law for qualifying as an embryo worthy of protection, e.g. in the German Embryo Protection Act or the German Stem Cell Act.

§ 3 No. 4 StZG: "For the purpose of the present Act (...) embryo means any human totipotent cell which has the potential to divide and to develop into a human being if the necessary conditions prevail."

§ 8 section 1 ESchG: "For the purpose of this Act, an embryo already means the human egg cell, fertilised and capable of developing, from the time of fusion of the nuclei, and further, each totipotent cell removed from an embryo that is assumed to be able to divide and to develop into an individual under the appropriate conditions for that."

However, due to the lack of conceptual clarity, totipotency should not be utilized as legal criterion but should be replaced by the new criterion of "qualified capability of development": Qualified capability of development is the capability of a cell or an entity to develop under the necessary preconditions to a human being in terms of the constitution.

## 2. Constitutional protection of prenatal entities

The starting point of determining an entity to be worthy of constitutional protection is the definition of the subjective scope of protection of Article 1 (1) (the protection of human dignity) and Article 2 (2) (the right to life and physical integrity) of the Basic Law of the Federal Republic of Germany:

a) Thesis 1: There is a need to differentiate between *present* humans and *potential* humans. *Present* humans are humans in terms of the constitution and without any restrictions subjects of fundamental rights, whereas *potential* humans are only able to develop to this status.
b) Thesis 2: The protection of *present* humans extends by means of an advance effect to *potential* humans. Hereby, the criterion of "qualified capability of development" constitutes the necessary connection between the *potential* and the *present* human. However, the intensity of *potential* humans' constitutional protection has to be gradated according to the developmental level. Only a present human being possesses absolute fundamental rights.

## 3. Concrete reform proposal for the sub-constitutional law

The current paragraph 8 German Embryo Protection Act should be changed as the following:

*(1) Any human cell with the qualified capability of development is an embryo in terms of this law. Pre-nucleus stages and stages, in which the qualified capability of development has not been stabilised yet, shall be excluded.*
*(2) Qualified capability of development is the ability to develop under the necessary preconditions at least to the beginning of the stage when the neural tube develops.*

This definition establishes clarity and legal certainty in comparison with the previous wording: The new criterion – in contrast to "totipotency" – is able to include multicellular entities, to set a specific point of time for the capability of development (the beginning of the stage when the neural tube develops), it defines the external preconditions to be assumed and does explicitly not differentiate according to the naturalness of formation. Furthermore, the outdated term of "nuclear fusion" is given up with the reform.

**Keywords: totipotency; German Embryo Protection Act; qualified capability of development; embryo; the protection of human dignity (Art. 1 GG)**
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# Induction & Maintenance of Pluripotency

# Marmoset monkey embryonic stem cell lines derived from morula and blastocyst stages

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Embryonic stem (ES) cells are generally derived from the inner cell mass of preimplantation embryos at the blastocyst stage. If cultured under appropriate conditions, ES cells can proliferate indefinitely while maintaining a pluripotent state, meaning that they can give rise to all somatic cell types deriving from the embryonic germ layers, i.e. ectoderm, mesoderm and endoderm, as well as germ cells. ES cells are very useful for the study of embryonic development. They are also particularly useful for in vitro drug testing and toxicology. Importantly, human ES cells hold promise for curative cell replacement approaches. However, important issues concerning safety and efficacy of such ES cell-based treatments need to be carefully evaluated. Due to the close phylogenetic relationship, non-human primate (NHP) ES cells represent an excellent alternative to human ES cells particularly in preclinical and translational ES cell research. Moreover, NHP embryo and ES cell technology allow experimental studies not possible in humans. The common marmoset monkey (Callithrix jacchus) is a new world monkey endemic to Brazil. It has several significant advantages over the Old World monkey species like the Rhesus monkey (Macaca mulatta) and the Long-tailed macaque (Macaca fascicularis), which are also frequently used in biomedical research. The marmoset monkey is small, easy to handle and free of zoonoses. These characteristics contribute to relatively low housing costs. Additionally, common marmosets have a significantly shorter generation time as well as a higher fecundity than macaques. For these reasons, the common marmoset is the NHP model of choice for specific applications. This is reflected by different studies performed in the fields of stem cell research, reproductive biology and neurobiology. Only very few ES cell lines of the common marmoset monkey are currently available to the scientific community. We already established several marmoset monkey iPS cell lines from postnatal skin fibroblasts by non-viral means. Here, we report the generation and characterization of four novel marmoset monkey embryonic stem cell lines derived from natural preimplantation embryos. Importantly, three of the ES cell lines were derived from morula stages. The derivation of primate ES cell lines from natural morula stages has, to our knowledge, not been reported so far. In summary, we have derived four novel marmoset monkey ES cell lines, which are now available for experimental and preclinical testing of cell replacement therapies in a translational NHP model.

**Keywords: embryonic stem cell; marmoset monkey; non-human primate; pluripotency; pre-implantation embryo**
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# Scalable monolayer PSC culture on LN-521 with robust single cell passage and free weekends under xeno-free and defined conditions

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The lack of defined, xeno-free, easy and robust methods for efficient expansion of human pluripotent stem cells (hPSC) has hindered both the advancement of basic research, due to high experimental variation and poor quality cells with phenotypic and genetic changes, and human cell therapy requiring absolute safe methods and large numbers of low passage cells. By using a human recombinant protein naturally expressed by hPSCs, LN-521, we can culture hPSCs for over 80 single cell passages without any abnormal genetic aberrations and with maintained expression of pluripotency markers. Cells cultured on LN-521 grow twice as fast compared to other matrices and can be split 1:20 or up to 1:30 as single cells without the addition of artificial ROCK inhibitor (Rodin et al, Nature Communication 2014). The simplicity and reliability of the procedure, speed of cell amplification and the genetic stability of the cells make LN-521 suitable as reagent in clinical trials for PSC-based therapy. Furthermore, true clonal growth, important for cell fate tracking, gene function analyses and editing, without inhibitors of anoikis, is possible by using LN-521 and E-cadherin (Rodin et al, Nature Protocols 2014). The same authors have also demonstrated chemically defined and xeno-free derivation of new clinical hESC lines from single blastomers. In essence this circumvents the ethical dilemma of destroying the surplus embryos donated by couples going through fertility treatments. LN-521 provides a biorelevant niche for hPSCs and we are now able to show that hPSCs can be cultured without the need of daily feeding. Data from 10 consecutive passages on three different lines reveal no significant differences in cell morphology, proliferation or the expression of pluripotency markers. The pluripotency after 10 passages was evaluated with immunocytochemistry for Oct4 and Nanog. The results show that the expression levels are similar to both the daily fed group and starting samples. Chromosome analysis after 10 passages indicated normal karyotype for all groups. In conclusion, we show that LN-521 is an optimal matrix for hPSC culture due to its biological relevance allowing derivation, clonal cultivation and robust long-term pluripotent cell growth. The robust method allows minimum culture maintenance and standardized protocols, which can easily be adapted to automation platforms, making LN-521 a suitable reagent choice for human cell therapy trials.

**Keywords: pluripotent stem cells; laminin; single cell passage; clonal culture; automation**
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# Patient-specific iPSC-derived hepatocyte-like cells generated from archived PBMC samples

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The reprogramming of somatic human cells to induced pluripotent stem cells offers great possibilities for regenerative medicine as well as for disease modeling and drug screening purposes. This is due to the capability of iPS cells to be both expanded virtually indefinitely and differentiated towards any cell type possible, including cell types relevant for certain diseases. Directed differentiation towards hepatocyte-like cells might be of particular importance for drug screenings irrespective of the drug's purpose, since the liver serves as the major drug-metabolising organ in the body. In this study, we intended to establish a facilitated protocol for generating patient-specific hepatocyte-like cells for use in disease modeling and drug screening. Peripheral blood mononuclear cells represent one of the easiest accessible cell sources and can be readily stored. We used small numbers of archived PBMCs as a starting cell source and successfully generated iPS cells by transduction of polycistronic lentiviral vectors encoding codon-optimized cDNAs of OCT4, SOX2, KLF4, and C-MYC. Here, 500,000 long-term stored PBMCs and as little as 100,000 short-term stored PBMCs proved to be sufficient cell numbers to obtain high quality iPS cell lines. In the derived iPS cell clones, pluripotency characteristics, like expression of SSEA4, TRA1-60, OCT4, SOX2 and NANOG, could be observed. The differentiation potential of the iPS cells towards all three embryonic germ layers was examined in vitro using the ScorecardTM assay. Also, their amenability to hepatic in vitro differentiation was confirmed by analysing the expression of hepatic marker genes, such as ALB, HNF4α, AFP and TTR. With this, we established a protocol for obtaining patient-specific iPS cells from minimal numbers of easy-access blood cells by making use of frozen PBMC libraries. Upon directed differentiation towards hepatocyte-like cells, these iPSCs can now be used for modeling of liver diseases as well as drug screening. Furthermore, this approach can also be useful for modeling diseases affecting organs other than the liver.

**Keywords: patient-specific iPS cells; hepatic differentiation; disease modeling**
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# A versatile and robust xeno- and serum-free cultivation system for human pluripotent stem cells

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Pluripotent stem cells (PSC) have traditionally been cultivated on mouse embryonic feeder (mEF) cells, which contribute to maintenance of pluripotency and deposit extracellular matrix components mediating cell attachment. However, the xenogeneic nature of mEF cells and commonly used media components is not compliant with current efforts to establish clinically compatible protocols for maintenance and differentiation of PSC. Different compositions have been devised in order to maintain pluripotency in feeder-free conditions, but most media require extensive adaption periods. We have optimized a xeno- and serum-free media formulation that allows rapid adaption to feeder-free conditions and rapid culture initiation after cryopreservation. The formulation enables robust and efficient expansion of PSC and supports episomal reprogramming of human fibroblasts. Furthermore, the medium supports PSC expansion in suspension culture. The formulation will allow a rapid translation into a clinical-grade medium designed following the recommendations of USP <1043> on ancillary materials and will be suitable for clinical-grade PSC expansion.

**Keywords: media; clinical; GMP; tranlsation; feeder-free**
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# Pluripotency potential and MHC class I expression changes during long-term cultivation of the amnion and bone marrow MSCs of human and non-human primate

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Multipotent stromal cells (MSCs) are the most reliable candidates for stem cell therapies because of their plasticity, immunoregulatory features and availability. Unlike the other promising candidates, such as ESCs and iPS, MSCs do not face ethical, legislative and clinical safety issues. MSCs are conventionally defined as a class of cells with potential to self-renewal and certain 'stemness' abilities to differentiate into multiple cell lineages within the same germ layer, displaying a spindle-shaped morphology, adherence to plastic and expression of certain surface markers, such as CD105+, CD90+ and CD73 while being negative for hematopoietic markers, such as CD34- and CD45-. Placental MSCs particularly attract attention in the field of research and clinical application, due to the virtual absence of ethical concerns and ease of obtaining, but reports about possible culture duration of MSCs, expression of immune-relevant molecules, such as MHC class I, and pluripotency potential represented by markers like Oct4, Sox2, Nanog, Klf4, c-Myc, Lin28 remain controversial. To address these topics, we performed a comprehensive long-term characterization of MSCs derived from amnion and bone marrow from the human and our preclinical non-human primate model, the common marmoset monkey (Callithrix jacchus). While ESC and iPS lines from Callithrix jacchus are well established and characterized, there is little known on marmoset MSCs, especially in comparison with the human. Cells from both species and both origins showed typical adherent spindle-shaped fibroblast-like morphology and typical marker combinations, CD44+, CD73+, CD90+, CD105+, CD106+, CD166+, Snail1+ and Bra+, but absence of CD34-. During long term culture expression of surface markers CD73 and CD105 remained unchanged in the amnion MSC, but was significantly reduced in late passages in the bone marrow samples. Interestingly enough, expression of MHC class I complex was significantly reduced in amnion MSCs in early vs. late passages (P3–P12), whereas in bone marrow MSCs samples MHC class I presence was close to 90 % from the beginning. Pluripotency genes Oct4 and Nanog were significantly expressed in early passages, but decreased from P3 on. Methylation status of the Oct4 promoter also indicates capability for expression, though high heterogeneity of cells with various levels of methylation in primary cultures was observed. Sox2 was significantly higher expressed in the human than in the marmoset, while the other way round for Lin28 expression, whereas c-Myc and Klf4 were barely detectable in all samples. From our findings we speculate about the existence of a small "true" pluripotent population of cells in the amnion; their extraction and determination of developmental origin is one of our future goals.

**Keywords: marmoset; MSC; amnion; bone marrow**
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# Dissecting the 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 could also actively participate in promoting transitions into specific cell fates. Acting upstream to these transcription factors, signaling pathways play a key role in directing the specification and differentiation of pluripotent embryonic stem cells into distinct lineages. This interplay of signaling pathways and the pluripotency gene-regulatory network is not well understood. We study the molecular mechanisms of mesoderm formation, focusing primarily on the role of BMP and WNT signaling pathways in the process. An emphasis is put on identifying downstream targets of these cascades that could positively or negatively control the transition from the pluripotent state into the mesodermal lineage. We found that these pathways act in a highly synergistic manner to induce primtive streak genes while specifically repressing SOX2 in hESCs. Furthermore, controlled over-expression of SOX2 in hESCs specifically during primitive streak formation interfered with differentiation into the cardiac lineage, implying that SOX2 repression is a first key event in mesoderm induction. Furthermore, our mechanistic investigation suggests that while the two signaling cascades synergistically repress SOX2, they do so by employing distinct mechanisms. Our data thereby provides insights into key events underlying the transition from the undifferentiated pluripotent cell state into mesoderm, using hESCs as a model system.

**Keywords: human embryonic stem cells; BMP and WNT signaling; SOX2; mesoderm**
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# A dynamic role of Tbx3 in the pluripotency circuitry

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The signalling network defining stem cell pluripotency comprises a fine-tuned pattern of factor activity required for the maintenance of embryonic and induced stemness. Amongst the T-box transcription factor family, Tbx3 is the earliest expressed member and has been reported to be involved in both maintenance and induction of pluripotency. Hence, Tbx3 has been widely believed to belong to the inner core of the pluripotency circuitry, with loss of Tbx3 leading to differentiation. In marked contrast, we report fluctuating Tbx3 levels: "Tbx3-low" cells resemble the gastrulating epiblast in vivo, but retain the capacity to switch back to a Tbx3-high state. Moreover, a series of experimental evidence, including tetraploid complementation assays, clearly show Tbx3 to be dispensable for the induction and maintenance of naïve pluripotency. Taken together, we delineate novel facets of Tbx3 action in pluripotency and show an involvement of Tbx3 in the transition from the naïve embryonic state to the pre-patterned epiblast-like state.

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# Establishment of a non-integrating retroviral vector system for targeted cell fate modification

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The possibility to generate cell types of choice is of significance for regenerative medicine and basic research, potentially serving to replace damaged or degenerated tissue and to perform functional studies of selected disease backgrounds. Based on ectopic expression of transcription factors (TFs), specific cell types can be derived through either the generation and targeted differentiation of pluripotent cells or the direct interconversion of somatic cell states. Transient expression of the respective TFs is sufficient since expression often is required only during conversion, and the sequence and timing of TF expression has a major impact on maturity of generated cell products. Thus, transient delivery modes allowing a precise adjustment of these parameters appear advantageous for fate conversion and additionally exhibit improved safety profiles compared to integrating methods, preventing insertional mutagenesis and permanent expression of the potentially oncogenic TFs. Non-integrating retroviral vectors (NIRV) are efficient gene delivery vehicles that differ from their integrating counterparts in that they are specifically impaired in integration. Non-integrated linear and episomal DNA formed after reverse transcription here provides the template for transient gene expression. We developed a strategy employing repeated administration of NIRV to extend their naturally short expression windows in dividing cells. To easily track expression kinetics for establishment and characterization of the concept, we initially transferred fluorescent proteins. Expression periods of choice, indicated by stable percentages of fluoroprotein-expressing cells over defined time periods, could be generated governed by the number of repeated transductions. Kinetic analyses revealed the cell proliferation rates and the protein half-life as the main determinants of the course of transgene expression and of optimal retransduction intervals, allowing a further control by adjusting these parameters. Retransduction was successful with murine and human cell types and proved more efficient in primary fibroblasts than plasmid transfection while being comparably or less cytotoxic. Furthermore, retransduction showed a stochastic behavior enabling the repeated targeting of individual cells without receptor saturation. Unrestricted retargeting applied to transduction with two commonly employed envelope pseudotypes mediating cell entry.

However, the generation of sufficient numbers of individual cells coexpressing sequentially transferred fluoroproteins required high transduction efficiencies. Nevertheless, as a promising perspective for application, repeated coadministration of several NIRV enabled the generation of high numbers of multiple-positive cells, with up to 40 % of cells coexpressing three individually delivered fluoroproteins. Alternatively, through sequential gene transfer in a defined order at selected time points, complex patterns of expression of different transgenes could be exerted. With prospect of application for targeted differentiation, we could efficiently transduce human induced pluripotent stem cells (iPSC) with NIRV encoding eGFP, yielding transduction efficiencies above 80 % along with high fluorescence intensities. As a proof-of-principle for NIRV-mediated cell fate modification, we repeatedly administered a NIRV expressing the TF Oct4 to human fibroblasts stably expressing Klf4, Sox2 and c-Myc, and demonstrate that NIRV-derived Oct4 was sufficient to support the reprogramming into iPSC. Altogether, the developed repeated NIRV transduction strategy offers to precisely control the sequence and duration of expression of multiple transgenes and thus appears suitable to generate integration-free cell types of choice.

**Keywords: retroviral vector; integration-deficient; transient gene expression; reprogramming; cell fate modification**
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# Epigenetic memory is not intrinsic to transcription factor-mediated reprogramming

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Somatic cells can be reprogrammed to pluripotency using different methods. In comparison to pluripotent cells obtained through somatic nuclear transfer, induced pluripotent stem cells (iPSCs) exhibit a higher number of epigenetic errors. Furthermore, most of these abnormalities have been described to be intrinsic to the iPSC method. Here we investigate whether the aberrant epigenetic patterns detected in iPSCs are specific to transcription factor-mediated reprogramming. We used germline stem cells (GSCs), which are the only adult cell type that can be converted into pluripotent cells (gPSCs) under specific culture conditions, and compared GSC-derived iPSCs and gPSCs at the transcriptomic, epigenetic and functional level. Our results show that both reprogramming methods generate indistinguishable states of pluripotency. GSC-derived iPSCs and gPSCs retained similar levels of donor cell-type memory and exhibited comparable numbers of reprogramming errors. Therefore, our study demonstrates that the epigenetic memory detected in iPSCs is not intrinsic to transcription-factor mediated reprogramming.

**Keywords: iPSC; gPSC; epigenetic memory; reprogramming; germline**
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# Niche & Microenvironment

# The role of glutamine in erythropoiesis

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Haematopioetic bone marrow produces erythroid and myeloid cells at different spatial locations relative to the blood supply, suggesting that the metabolic environment influences the migration of progenitor cells and/or lineage specific patterns of anabolic metabolism. In support of a decisive influence of the metabolic environment on haematopoiesis, it has recently been reported that glutamine concentration governs the commitment of multipotent progenitors to the erythroid lineage (Oburoglu et al., Cell Stem Cell, 2014, 15:1-16). Here, we have studied the effects of glutamine on the migration and colony formation of erythroid-myeloid (EMP) progenitors derived from umbilical cord blood. We first developed a procedure that enables the establishment and maintenance of metabolite gradients under otherwise standard colony assay conditions and found that the application of either glutamine or glucose gradients had no reproducible effect on the distribution of colonies generated by EMPs. However, during the course of this work we noted that the maintenance of 8mM glutamine throughout the medium resulted in the development of myeloid colonies but not of erythroid colonies. Rather than supporting erythroid development, high glutamine levels therefore appear to be inhibitory or damaging to erythroid progenitors under the conditions of the colony assay, possibly due to the accumulation of toxic products. In parallel, studies of metabolite flux during the myelo-erythroid differentiation of the multipotent murine cell line FDCPmix confirmed that differentiating erythroid cells do indeed take up large amounts of glutamine. However, at the same time these cells release large amounts of glutamate and down-regulate the expression of the enzyme alanine amino transferase (ALAT, GPT1) that normally enables glutaminolysis by feeding glutamate (as a-ketoglutarate) into the TCA cycle. This suggests that glutaminolysis actually decreases as the uptake of glutamine increases specifically during erythroid differentiation, and that exogenous glutamine is most likely being directed into another pathway. One possibility is nucleoside synthesis, although nucleoside requirements are unlikely to differ markedly between erythroid and myeloid progenitors. Based on these observations, we hypothesise that exogenous glutamine may be being used during erythroid differentiation for the biosynthesis of polyamines that are required for the chromatin condensation that is specific to this lineage.

**Keywords: metabolism; erythropoieses; metabolic-gradients**
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# Direct visualization and characterization of mesenchymal stem cells and endothelial bone marrow niche cells using a CD73-BAC-EGFP live reporter mouse

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Mesenchymal stem cells (MSCs) are multipotent cells residing in the bone marrow and other tissues. In vitro MSCs are well characterized by their immunophenotype and differentiation potential to mesenchymal lineages. However, the current understanding of their localization and function in vivo remains elusive. We therefore generated a transgenic mouse model in which MSCs are labeled by a live reporter gene. CD73, also known as ecto-5'-nucleotidase, is a membrane-bound enzyme catalyzing the dephosphorylation of AMP to adenosine. It is a well-established marker for MSCs with only moderate distribution in other mesodermal cell types. We confirmed high expression of CD73 in various MSC populations by FACS, immunostainings and qPCR. Next, a reporter vector expressing EGFP under control of the CD73 promoter was created from bacterial artificial chromosomes (BACs), and transgenic mice were generated. As expected from the literature, kidney and lung epithelium showed a strong fluorescent signal, proving specificity of the construct. The expression in other organs like white fat, uterus, and liver showed a typical perivascular distribution pattern as would be predicted for MSCs in vivo. In developing bones EGFP+ cells emerged at the sites of peri- and endochondral ossification, labeling mesenchymal precursor cells. Primary cultures from cortical bone, epiphysis, bone marrow (BM), and white fat gave rise to adherent growing EGFP positive cells that were able to differentiate into mesenchymal lineages, underlining their MSC character. Bone sections also revealed a distinct pattern of EGFP-labeled cells forming elongated structures in the BM. By FACS, RNAseq, and immunohistochemical analysis we identified these as sinusoidal endothelial cells. Interestingly, hematopoietic progenitor cells localized in close vicinity of these EGFP+ structures. In summary, our CD73-BAC-EGFP live-reporter mouse enables the direct visualization of MSCs in various organs. In addition, we also found that it labels endothelial sinusoidal cells in the bone marrow and these cells appear to support the hematopoietic stem cell (HSC) niche. Therefore this mouse model will enable the characterization of specific niche components and potentially also the identification of molecular cues playing a role in HSC development or fate.

**Keywords: mesenchymal stem cells; niche; bone marrow**
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# Angiogenesis controls neural stem cell expansion by regulating oxygenation of the stem cell niche

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The expansion and differentiation of neural stem cells (NSCs) in the developing brain is controlled by their cellular microenvironment, the stem cell niche. Blood vessels are part of this niche, but their functional significance for the regulation of NSC differentiation and the mechanisms involved remain unclear. Here, we report that blood vessel formation in the developing mouse and ferret cerebral cortex coincided with induction of NSC differentiation in time and space. Moreover, selective inhibition of brain angiogenesis in vessel-specific Gpr124 null embryos caused hypoxia and increased NSC expansion at the expense of differentiation. The hypoxia-inducible factor (HIF)-1α mediated this process, as the level of HIF-1α controlled NSC differentiation. Niche blood vessels regulated NSC differentiation at least in part by providing oxygen, as exposure to increased ambient oxygen levels rescued NSC differentiation in Gpr124 deficient embryos. Our findings establish a novel oxygen-dependent mechanism of how blood vessels regulate NSC differentiation.

**Keywords: blood vessel; neural stem cell; stem cell niche; hypoxia; HIF-1 alpha**
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# Organogenesis & Regeneration

# Osteogenic differentiation of human and ovine MSCs from different tissues: A tissue engineering model using alginate beads and scaffold-free self-organizing microspheres

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Introduction: Regenerative abilities of bone enable self-repair and healing of fractures, but large bone defects are unlikely to regenerate due to the significant quantity of bone tissue required. While the use of autologous bone graft is the gold standard in reconstructing of large bone defects, the surgical stress and the low quantity of extracted bone are limiting factors. Hard tissue regeneration is based on a triangular shaped model: osteogenic cell groups, otseoinductive stimulant and osteoconductive matrix. The biological resources appear to be limited; therefore MSCs cell-therapies have the potential to provide an effective alternative for bone regeneration due to their osteogenic potentials. Currently sheep is used as a large animal model in orthopedics studies but ovine (o)MSCs are not well characterized and standardized to date in comparison to the human (h)MSCs.

The aims of the current study are to characterize and compare h- and oMSCs from different tissues, moreover, to investigate their osteogenic differentiation in a tissue engineering model using alginate beads and scaffold-free self-organizing MSCs microspheres.

Methods: Sources for oMSCs: adipose tissue, bone and fat marrow; for hMSCs: adipose tissue, femur head and vertebral bodies. All MSCs were characterized via their tri-lineage differentiation potential, surface-marker expression, proliferation capacity.

Scaffold-free self-organizing MSCs microspheres were generated on agarose. MSCs were encapsulated into alginate beads polymerized in CaCl2 Solution. MSCs were retrieved by dissolving alginate beads and cultured in monolayer. Differentiation toward osteogenic lineage was confirmed via ALP/Alizarin-Red-S staining on generated cryosections and monolayer cultures. Moreover, osteogenic differentiation process was evaluated by measuring the optical density (OD) of monolayer cultures and free phosphate ions in the supernatants.

Results: All investigated MSCs showed typical fibroblast-like morphology, expressed MSCs surface markers, differentiated toward adipo-chondro-osteogenic lineages and showed the same proliferating tendencies. All MSCs were able to self-organize and form microspheres on agarose. Alginate beads were dissolved and retrieved MSCs were able to grow in monolayer cultures. MSCs osteogenic differentiation in microspheres and alginate beads was confirmed via ALP/Alizarin-Red-S specific staining on cryosections and monolayer cultures.

The measured OD of osteogenic induced MSCs was increased in comparison to controls. Induced hMSCs showed double amount of free-phosphate ions released from beta-glycerophosphate in comparison to oMSCs.

Discussion: Tissue-specific stem cells are easy to harvest, do not tend to form tumours and represent no ethical concern. Here we report a successful isolation and characterization of oMSCs and their comparison to hMSCs from three different sources each.

OD measurement as an early indicator of calcification to determine the presence of crystalline deposits indicating calcium deposition. The obtained values of free-phosphate measured demonstrate that ALP activity, which catalyzes the release of phosphate ions, is much lower in oMSCs than in hMSCs.

We report a successful osteogenic differentiation of oMSCs and hMSCs using two different 3D approaches. Alginate gelation is cell friendly and represents an ideal tool for tissue engineering application. Scaffold-free self-organizing microspheres technique offers a better in vivo-like surrounding for MSCs.

Encapsulating microspheres into alginate beads can optimize MSCs seeding efficiency and therefore improve tissue regeneration.

**Keywords: MSCs; differentiation; biomaterials; microspheres; regeneration**
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# A natural product approach to targeting late-stage cardiac differentiation and regeneration

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Background: There is a great medical need for innovative therapies of heart disease. The minimal and clearly insufficient ability of the adult heart to regenerate after ischemic injury is a great appeal for identifying biological mechanisms, substances and factors that improve this process. Two main sources for cardiomyocyte renewal and regeneration have emerged in the field: 1) Adult multipotent progenitor cells and 2) pre-existing cardiomyocytes.(1)

Objective: Natural products frequently serve as an inspiration and attractive starting point for the development of novel pharmacological agents. Based on the many positive effects on the myocardium after infarction and the overall cardiovascular protective activity of Crataegus ssp. (extract WS1442),(2) we aimed at studying whether also mechanisms of cardiac differentiation and regeneration could possibly play a role.

Results: Here, we show that WS1442 efficiently stimulated cardiomyocyte differentiation from murine and human ESCs in a dose-dependent manner after mesoderm was formed. This activity was thoroughly validated in a mESC-based (CGR8-Myh6-GFP) spontaneous differentiation assay. Importantly, bioassay-guided fractionation of the extract allowed identification of the active compound class. Whole extract WS1442 and the active component enhanced cardiomyocyte yields up to 20-fold (20–30% GFP+ cells) which has not been achieved by any cardiogenic agent for directed differentiation in this assay, not even by the well-appreciated Wnt/beta-catenin inhibitors.

Conclusions: According to the observed activity profile, we hypothesize that the identified active components in WS1442 target multipotent progenitors, stimulate their differentiation towards the cardiac lineage but also expand their pool (proliferation). Further elucidation of the underlying cellular and molecular mechanisms will possibly lead to novel targets that can be exploited for ex vivo and in vivo expansion of cardiac progenitor cells. At the same time, a defined extract sub-fraction is already available and can possibly be used as a novel pharmacological tool.

*References: (1) Harvey RP et al. Stem Cell Reseach 2014, p521 (2) Koch E and Malek FA Planta Medica 2011, p1123*

**Keywords: heart regeneration; cardiac differentiation; cardiac progenitors; bioassay-guided fractionation; WS1442**
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# From stem cell screening towards in vivo small molecule tools for heart regeneration

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Background: The development of small molecules that control stem cell fate is of tremendous interest for various regenerative medicine applications as it opens up the druggable space.(1) From a high-content screen of 17.000 compounds in mESCs we discovered in the past a novel class of TGF-beta inhibitors in the context of cardiac differentiation.(2) A specific subclass of 1,4-dihydropyridines (DHPs) stimulated cardiomyogenesis from murine and human ESCs in stages when uncommitted mesoderm specifies towards a cardiac fate.(2,3) Objective: Since TGF-beta is also involved in cardiac remodeling and fibrosis, we propose a dual mode of action for these DHPs as regenerative agents. However, in order to demonstrate proof-of-concept in animal models later in development, the compounds require hit-to-lead optimization. Here, a translational case study illustrates earliest steps in a preclinical drug development campaign for phenotypic, stem cell-based screening-derived small molecules.

Results: We describe the multidisciplinary medicinal chemistry workflow from 'screening hit' to in vivo-suitable pharmacologic tool candidates. Ligand-based (quantitative) structure-activity relationships (SARs), X-ray crystal structure analysis, pathway selectivity and in vitro-pharmacokinetic profiling provided a solid basis for the development of selective, potent and drug-like lead candidates.(4) Key obstacles that typically limit in vivo applicability, such as poor compound solubility and stability, could be addressed. Moreover, we demonstrated efficacy in and an engineered heart tissue (EHT, from neonatal rat cardiomyocytes) model of hypertrophy and fibrosis.

Conclusions: Systematic, medicinal chemistry-driven efforts led to highly attractive small molecules as novel in vivo pharmacology tool candidates to study regeneration and remodeling after myocardial infarction.

*References: (1) Längle D et al. ACS Chemical Biology 2014, p57 (2) Willems E, Teixeira JC, Schade D et al. Cell Stem Cell 2012, p242 (3) Schade D et al. Journal of Medicinal Chemistry 2012, p9946 (4) Längle D et al. (submitted)*

**Keywords: cardiogenesis; cardiac fibrosis; drug development; TGF-beta inhibition; 1,4-dihydropyridines**
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# Enrichment and molecular characterization of epithelial stem cells of the human cornea

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One of the most common causes for blindness worldwide is the loss of a functional cornea. Regeneration and maintenance of the human corneal epithelium is ensured by limbus-resident epithelial stem cells (LESCs). Hence, LESCs are of key relevance for clinical application to rebuild the cornea after substantial damage, such as burns or infections, or for the treatment of degenerative processes caused by genetic defects, leading to LESC-deficiency (LSCD). In clinical routine, stem cell-containing limbus biopsies are commonly used as a source for generating cornea-grafts, in vitro. Long-term engraftment after transplantation is assumed to correlate with their frequency within the graft. However, because their identity is ill-defined, their quantitation in an individual transplant is currently not feasible, making it impossible to predict the success of transplantation and impedes the systematic improvement of graft generation, in vitro. The aim of this project is the identification and characterization of LESCs, because tissue stem cells commonly reside in a subpopulation of slowly cycling cells, we have labeled limbus tissue explants by lentiviral expression of an inducible histone H2B-GFP protein-fusion to allow for enrichment of label-retaining cells by flow cytometry. Isolated cell fractions will be tested for their capability to generate stratified cornea epithelium layers in a routine in vitro culture system. Preliminary results will be presented.

**Keywords: cornea; limbus-resident epithelial stem cells**
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# Identification and validation of neurite growth and regeneration promoting factors secreted by stem cells from human umbilical cord blood by functional proteomics approach

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Stem cell transplantation is a promising therapeutic strategy to enhance axonal regeneration after spinal cord injury (SCI). Unrestricted somatic stem cells (USSC) isolated from human umbilical cord blood is an attractive stem cell source which can be isolated with GMP grade without ethical concerns. It was shown that USSC transplantation into acute injured rat spinal cords leads to axonal regrowth, enhanced tissue sparing and significant locomotor recovery, but without differentiation (Schira et al., 2012). Instead, USSC secrete trophic factors which enhance neurite growth of primary neurons in vitro. With the present study we aimed to characterize the regenerative phenotype of USSC with a workflow comprising secretome analysis by quantitative mass spectrometry and neurite outgrowth in vitro assays. With our proteomic approach, we identified 1156 USSC secreted proteins including 385 proteins secreted classically, 276 non-classically secreted proteins, 8 cell surface proteins and 487 proteins predicted to be localized extracellular. These proteins were assigned to Gene Ontology biological processes including blood vessel formation, regulation of cell motion and cell adhesion. Moreover, USSC express proteins regulating precursor cell differentiation and the immune response. All together, these processes have to be tightly regulated after SCI and could be influenced by transplanted USSC. Additionally, comparison with literature revealed that USSC express at least 31 well-known neurite growth promoting factors, e. g., MANF, neuronal growth regulator 1, NDNF, PEDF and SPARC. Subsequent performed functional validations with primary neuronal cell cultures suggested that SPARC and PEDF are involved in USSC mediated neurite growth which might lead to significant locomotor recovery after transplantation thereby supporting the concept of paracrine functions of USSC. Summarized, secretome analyses with subsequent functional validation reveal a powerful tool to identify factors involved in neurite growth and neural regeneration processes.

*References; Schira, J., Gasis, M., Estrada, V., Hendricks, M., Schmitz, C., Trapp, T., Kruse, F., Kogler, G., Wernet, P., Hartung, H. P., and Muller, H. W. (2012) Significant clinical, neuropathological and behavioural recovery from acute spinal cord trauma by transplantation of a well-defined somatic stem cell from human umbilical cord blood. Brain 135, 431-446*

**Keywords: umbilical cord blood; secretome; nerve regeneration; mass spectrometry; spinal cord**
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# Other

# Optimization of the large-scale expansion of bone marrow-derived mesenchymal stem cells and the production of clinical-grade extracellular vesicles (EV)

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Mesenchymal stem cells (MSCs) have been used in over 400 NIH-registered clinical trials to treat various diseases in humans. Novel data imply that MSCs exert their therapeutic functions in a paracrine manner rather than by the intercalation into damaged tissues as it has been suggested for many years before. In this context extracellular vesicles (EVs), such as exosomes (70–140 nm), derivatives of the endosomal system, have been identified as active paracrine agents. Indeed, we have previously successfully treated a steroid-refractory GvHD patient with MSC-EVs. Due to their therapeutic relevance, we aim to optimize the large-scale MSC expansion and subsequent MSC-EV purification technologies. In this context, conditions have to be established and validated allowing for the long-term storage of MSC-EVs. To this end, a buffer and plastic containers have to be identified which prevent degradation of the MSC-EVs and allow for high MSC-EV recovery rates without affecting the MSC-EVs' biological features. So far, by using the Nano Particle Tracking (NTA) analysis we learned that a high proportion of sodium chloride solved MSC-EVs bind to their containers, finally reducing the recovery rate down to 30 %. Currently, we compare combinations of different buffers, storage containers and storage temperatures. Preliminary data will be presented.

**Keywords: mesenchymal stem cell; extracellular vesicles; nano particle tracking**
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# Characterization of moieties within the A2 ubiquitous chromatin opening element (A2UCOE) that are relevant for the anti-silencing function

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The advancement of pluripotent stem cell (PSC)-based gene therapy is considerably obstructed by epigenetic silencing mechanisms that impede the expression of retroviral transgenes. We previously demonstrated that a 1.5 kb ubiquitous chromatin opening element (A2UCOE) derived from the human HNRPA2B1-CBX3 housekeeping gene locus effectively preserved transgene expression driven by both viral and physiological promoters in murine and human ESCs and iPSCs as well as their differentiated progeny of different germ layers. However, the inclusion of the A2UCOE in SIN-lentiviral vectors has resulted in low titer productions and the dual promoter activity of the A2UCOE may result in the formation of aberrant fusion transcripts. To overcome these limitations and to illuminate which sections of the A2UCOE convey the anti-silencing function, we have identified a minimal 0.7 kb UCOE (CBX3) that comprises the CBX3 region and lacks the A2 region of the A2UCOE. Inclusion of this CBX3 in SIN-lentiviral vectors improved viral titers and effectively sustained the expression from different promoters in mESCs/miPSCs and thereof derived hematopoietic cells without overriding the tissue-specificity of the myeloid-specific MRP8 promoter. In the presence of the CBX3 element, a marked reduction in promoter CpG-methylation and repressive histone marks (e.g. H3K27me3) and an increase in active chromatin marks (e.g. H3K4me3) was observed within the heterologous promoters, clearly demonstrating the chromatin remodeling activity of the CBX3 element. These observations challenge the original concept that divergent transcription from dual promoters is required for UCOE function and instead highlight the relevance of the CBX3 segment. To further elucidate the mechanisms that enable UCOE-mediated transgene stabilization and to define in more detail the regions mediating this function, we generated subfragments of CBX3 and evaluated their function by analyzing the transgene expression in pluripotent mESCs and thereof derived progeny following an eight-day EB-based hematopoietic differentiation procedure. While the CBX3 element retained most if not all functionality of the original A2UCOE, a 0.3 kb fragment from the 5'-end of the CBX3 (CBX3(bp1-339)) only showed 30.8 % ± 7.74 functionality, whereas a 0.5 kb fragment (CBX3(bp1-508)) possessed 60.1% ± 5.02 of A2UCOE functionality. Interestingly, the 0.2 kb region that represents the difference between the two fragments (CBX3(bp340-508)) preserved 58.8 % ± 8.49 of function. Two longer fragments (CBX3(bp170-508) and CBX3(bp85-508)) showed 55.9 % ± 11.4 and 40.4 % ± 11.3 function, respectively. These data highlight the relevance of the CBX3 moiety and particularly the CBX3(bp340-508) region in A2UCOE function. Of note, analysis of the CBX3(bp340-508) sequence predicted a binding site for the transcription factor Sp1 that has previously been linked to the formation of active chromatin hubs and the prevention of DNA methylation in CpG islands. In the future we therefore aim to further characterize such binding sites by performing ChIP-based assays as well as mass-spectrometric analyses.

**Keywords: induced pluripotent stem cells/cell reprogramming; gene expression; lentiviral vectors; ubiquitous chromatin opening elements; epigenetic silencing**
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# Platelets promote CD133+BMSC adhesion to murine micro and hepatic sinusoidal endothelium independent of ADP stimulation under flow conditions

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Introduction: We previously demonstrated the therapeutic potential of hematopoetic CD133+ bone marrow stem cells (CD133+BMSC) to promote clinical liver regeneration and the potential of platelets for CD133+BMSC homing along human micro endothelium under shear stress. In this study, we have established a murine model to gain the option of investigation of null and overexpressing cells for homing relevant factors. Furthermore, we evaluated a putative adhesion promoting effect of platelets for CD133+BMSC-homing along hepatic sinusoidal vasculature.

Methods: Commercially available mouse primary dermal micro vasculature endothelial cells (dMEC-C57BL/6) and mouse hepatic sinusoidal endothelial cells (mSEC) were cultured in capillaries in a live cell imaging system (Bioflux). Primary CD133+BMSC were purified from bone marrow flushed from murine tibiae and femori utilizing FACsorting. dMEC or mSEC were co-cultured under shear stress with CD133+BMSC and platelet rich plasma (PRP) or platelet poor plasma (PPP) as control prepared from murine BMSC donors. To test the effect of pre-stimulation of platelets for adhesion of CD133+BMSC, murine PRP was pre-stimulated with ADP monitored by aggregometry utilizing the Bohr-light-transmission method. Latter prevented over-stimulation with clot-formation.

Results: Murine CD133+BMSC co-infused with PRP demonstrated mean adhesion rates of 144 % (+/ 17 % StdDev; $p<0,005$) along dMEC under flow if contrasted to PPP co-incubation. Pre-stimulation of platelets with ADP demonstrated a further increase leading to improved adhesion of CD133+BMSC compared to unstimulated conditions. Analogue to micro endothelium a boost of mean adhesion rates of CD133+BMSC to 131 % (+/ 9 % StdDev; $p<0,05$) was observed subsequent to unstimulated PRP co-infusion along mSEC.

Conclusion: Our data indicate platelets to trigger adhesion of CD133+BMSC to murine micro vasculature analogue to men in a stimulation independent manner. Further thrombocytes showed the capacity to promote BMSC-adhesion to hepatic vasculature. These data may add to the understanding of mechanisms by which platelets support hepatic generation and offer novel strategies to increase the efficacy of therapeutic BMSC-application in clinical liver disease.

**Keywords: CD133+ BMSC; platelets; liver regeneration; dMEC-C57BL/6; mSEC**
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# Tracking cell number-dependent viability of stem cell grafts in the mouse brain

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Introduction: Engraftment of stem cells is a promising therapeutic strategy for a variety of neurological disorders. However, cell survival upon engraftment is strictly limited which impedes the stem cell-mediated recovery. A previous study focusing on histological analyses revealed that a lower number of engrafted stem cells could increase the survival rate – also in stroke-damaged rats. Invasive tools to study the fate of engrafted stem cells are not appropriate to follow the dynamic time profile of cell viability. Bioluminescence imaging (BLI) is a most common method to track the survival and the location of engrafted stem cells noninvasively. Whit this study, we aim to verify reliability of BLI for determining the number of engrafted viable cells and to study the cell-number dependent survival affected by the immune response in the mouse brain.

Methods: The murine neural stem cell (mNSC) line D3WT_N2Euro was retrovirally transduced with pBABE-Luc2-T2A-copGFP-SV40-Neo, including Firefly luciferase (Luc2) for BLI, green fluorescent protein (copGFP) from copepod Pontellina plumata for histological validation, and Neomycin (Neo) resistance gene for clonal selection. A stable cell line with high copGFP expression was selected via FACS and Neo selection. Differentiation behavior and reporter expression was analyzed in transduced mNSCs via RT-PCR, Western Blot, and immunocytochemistry. For in vivo experiments, four different cell numbers – 1,500; 15,000; 75,000 and 150,000 – were engrafted into the right striatum of nude mice (n=6 or 9 per group). BLI time profile of mNSC survival (300 mg/kg D-Luciferin i. p. pre-Isoflurane anesthesia) was monitored on several days (d) over two weeks (d0, d1, d4, d7, d11 and d14). Animals were perfused at d7 and d14 and processed for histology.

Results: FACS of genetically modified mNSCs results in a cell population with high reporter gene expression compared to Neo selection. Furthermore, mNSCs were not altered in their stem cell behavior during genetic modification, cultivation and high reporter expression. In vivo, the viability of engrafted mNSCs is decreasing over time for all groups. However, high cell numbers featuring a stronger and significantly earlier decrease of photon emission starting at d4 for 150,000 cells per graft ($p<0.001$) and at d7 for 75,000 cells per graft ($p=0.003$) compared to d1. For the low cell numbers – 15,000 and 1,500 cells per graft – a significant decrease of photon emission could not be observed. Furthermore, BLI is appropriate to discriminate 1,500 cells per graft from unspecific background signals till d11 post engraftment with a statistical significance ($p=0.005$). For all cell numbers an accumulation of glial cells around and inside the graft site was observed. Further histological analyses are in progress.

Discussions: Engrafting low numbers of mNSC into the mouse brain results in a better long-term survival rate reflected in a significantly weaker decrease of photon emission over time. The histological validation of BLI results and the immune response as the most likely cause of cell loss is scheduled and will be fully quantitatively analyzed by semiautomatic cell counting.

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**Keywords: neural stem cells; survival; bioluminescence imaging; mouse**

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# Extracellular vesicles from different donor-derived mesenchymal stem cells differ in their immunomodulatory properties

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Human mesenchymal stem cells (hMSCs) were administered in more than 400 NIH-registered clinical trials to patients suffering from various diseases including myocardial infarction, stroke and graft-versus-host disease (GvHD). Initially, hMSCs were thought to replace lost cells in damaged tissues. Despite controversial reports regarding the efficacy of MSC-treatments, MSCs seem to exert their beneficial effects rather by secretion of immunosuppressive factors than by cell replacement. In this context, extracellular vesicles (EVs, 70–140 nm), such as exosomes and microvesicles, were identified to execute the hMSCs' therapeutic effects. Our recent successful treatment of a steroid-refractory GvHD patient with MSC-EVs further highlighted their therapeutic potential. Assuming that hMSCs of different donors contain different immunosuppressive potential, we harvested EVs from cell-culture supernatants of hMSCs of 20 donors and analyzed their immunomodulatory properties in a T cell proliferation assay. Regarding their immunosuppressive capabilities, huge differences were observed, suggesting that not all MSCs release therapeutically effective EVs. For the establishment of a MSC-EV potency assay, we now compare the feasibility of different T cell activation assays and analyze their sensitivity by multicolour flow-cytometry amongst others for the expression of known T cell activation markers. Our preliminary results will be presented.

**Keywords: exosomes; extracellular vesicles; bone marrow; mesenchymal stem cells, immunmodulation**
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# Pre-screening for chromosomal duplications of iPS clones based on qPCR amplification

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Although induced pluripotent stem (iPS) cells are generated from somatic cells, without pathological findings and carrying a diploid genome set, recent studies indicate a high risk of developing chromosomal aberrations during reprogramming and expansion in long term culture (Sommer et. al, 2009; Mayshar et. al, 2010). To prevent tumor development after transplantation karyotyping of iPS clones represents an essential step to ensure the patient's safety, however, karyotyping procedures are time-consuming and cost intensive. Therefore, we developed a simple and efficient 2-step method based on RT-PCR followed by quantitative realtime (qPCR) amplification, to pre-screen chromosome number of our iPS clones. Previous karyotypings of iPS clones from our preclinical non-human primate animal model common marmoset (Callithrix jacchus) indicated different frequencies of trisomies of specific chromosomes, similar to the human, such as duplication of chromosome 6 in nearly 84 % of all compromised clones, often accompanied with trisomy 12 (62 %) and 21 (7 %). Since trisomy 14 was also occurring individually (12 %) our pre-screening method focussed mainly on the detection of this four chromosomes. Specific oligonucleotides of single copy genes for each chromosome were designed respectively (TTC21B Chr. 6, Calcul Chr. 12, CALM2 Chr. 14 and ADAMTS1 Chr. 21) and utilized for RT-PCR by gDNA amplification. The RT-PCR product was further used for qPCR accompanied by respective controls. Duplication of specific chromosomes were clearly depicted by significantly lower cT values representing higher relative gene expression rates; these results were in accordance with selected karyotypings. Utilization of qPCR trisomy detection reduces cultivation time by 90 % and costs by about 76 %. Furthermore, this method can be feasibly adapted for every chromosome, species and region of interest.

**Keywords: karyotyping; qPCR; iPS; trisomy**
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# Mimicked long term cryopreservation of transgenic multipotent stromal cells affects viability but not transgene expression

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Cryopreservation is the only effective approach for prolonged storage of viable biological objects. Widely used in various fields of medicine and biological research, it is especially important for rare, unique, irreplaceable, or patient-specific samples. Moreover, current development and application of methods of genetic engineering boosts the need for storage of transgenic samples. Survival rates of frozen cells significantly vary depending on the freezing protocols and cell types. Cryopreservation protocols for adherent cell lines, e.g. multipotent stromal cells (MSCs), are well established and provide high survival outcome. However, there is a lack of information concerning cell survival and alterations of cellular functions as well as changes in transgene expression after actual long-term storage (>25 years). During cryopreservation biochemical and cellular processes are anticipated to be on hold for infinite time, but even with modern biobanking technology, repeated temperature fluctuations and other interruptions of the cold chain regularly occur during maintenance and handling. This leads to periodic release of unfrozen bound water fractions in the samples, resulting in accumulation of stress, which possibly compromises not only cellular survival and vital parameters, but also expression of transgenes. In order to mimic conditions of temperature fluctuations similar to practical long-term biobanking, we cryopreserved native and transgenic amnion derived human MSCs and exposed the samples to multi cycles in the temperature range between -180 °C and -80 °C. Majority of survival rates specified in cryo protocols are based on Trypan blue, 7-Aminoactinomycin (7AAD) and Propidium Iodide (PI) staining data, indicating membrane integrity and efficiency of efflux pumps. However, early necrotic and apoptotic cells cannot be distinguished by such approach, while vital parameters might be already irreversibly compromised by cryodamage. Therefore, besides the classic coulter counter method of Trypan blue staining, we evaluated the cells by flow cytometry (Annexin V, PI) to differentiate necrotic, apoptotic and intact cells (5). Additionally, 24 hour attachment test was performed after thawing as a functional test. MSC frozen under mimicked long-term storage conditions showed 81.2±8.3% viability according to Trypan blue staining in comparison to 92.1±8.5% viability of the samples stored at the constant temperature of -180 °C. However, when both necrotic and apoptotic populations were taken into consideration, the number of intact cells was significantly lower in mimicked long-term protocol (52.5±0.8%) compared with constant temperature storage (84.7±1.2%, $p<0.05$; $n=4$). Furthermore, this result was reflected by functional attachment assay. Statistically significant decrease was observed already after 30–40 cycles of temperature fluctuations. Interestingly, transgenic vector expression was not significantly compromised in any studied sample. Our study displayed: I) low temperature thermal cycling of cells allows mimicking practical long-term cryopreservation conditions; II) evaluation of only cellular membrane integrity provides misleading survival rates and should be avoided in cryopreservation studies; III) long term cryopreservation does not significantly affect the expression of transgenes.

**Keywords: multipotent stromal cells; transgenesis; cryopreservation; biobanking**
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# Tracking relapse-inducing cells in human glioblastoma

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Glioblastoma is the most malignant brain tumor in adults. At the time of diagnosis, surgery aims for complete macroscopic resection. Yet in every patient, due to their infiltrative nature, some „Residual tumor cells" (RTCs) are left behind. RTCs are thought to play a role in the relapse of disease that inevitably occurs – in most patients even within the 8.5 month-period of primary therapy. To investigate a potential spatio-temporal relationship of recurrent tumor growth and RTCs, we conducted a prospective study at the University Clinic of Bonn between the years 2010 and 2014. Multiple biopsies were obtained from the resection wall after completion of routine neurosurgery in 37 glioblastoma patients. MRI-based monitoring of biopsy sites during the individual clinical courses enabled anatomic correlation of biopsy location with future sites of tumor recurrence. Taking advantage of recently presented methods for the extraction and enrichment of vital RTCs from biopsies of the surgical resection wall, we subsequently conducted molecular profiling and classification studies. Our data indicate that subsets of RTCs bear a signature that allows prospective, personalized identification of glioblastoma recurrence. Thus, our approach may present a previously unrecognized opportunity to predict the site of recurrent growth activity in human disease.

**Keywords: glioblastoma; residual tumor cells; tumor recurrence**
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# Pivotal role of Tfap2c in murine placental development by orchestrating proliferation and differentiation via impinging MAPK signalling pathway

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Embryonic lethality in Tfap2c mutants has been attributed to the developmental defects in the extraembryonic compartment at E7.5. Here, we investigate Tfap2c requirement in the trophoblast progenitor lineage and the underlying mechanisms leading to the failure of correct placental development upon loss of Tfap2c. To this end, we generated TpbpaCre:Tfap2c conditional deletion mouse model resulting in loss of Tfap2c specifically in Tpbpa positive precursor cells within the ectoplacental cone which later develop the murine junctional zone (JZ) and are progenitors to several trophoblast subtypes. Loss of Tfap2c leads to a compromised JZ starting from E12.5 due to impaired proliferation and differentiation leading to deregulation of trophoblast specific genes in JZ (Tpbpa, Prl8a8, Prl3a1, Pcdh12, Gjb3), spiral artery-associated trophoblast giant cells, SpA-TGCs (Prl2c2, Rgs5, Psck6), canal trophoblast cells, C-TGCs (Prl2c2, PL II) and several other genes critical for placental development (H19, Tex19.1, Slc38a4, Ascl2). Interestingly, aberrant differentiation of GCs leads to reduced glycogen deposit in the mutant placenta. These defects point to restriction of placental and embryonic growth from E16.5 onwards resulting in 18–20 % lighter mutant pups. Additionally, we identified the underlying molecular mechanism where Tfap2c impinges on MAPK signalling pathway to orchestrate these effects via altered FGF signaling. We further validate our findings using human choriocarcinoma Jar cells, where knockdown of Tfap2c reduces activation of Erk1/2 and Akt, key players of molecular signaling cascade important for proliferation and differentiation.

**Keywords: placenta; trophoblast cells; spongiotrophoblast; MAPK; Tfap2c**
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# Investigation of TLR-4 as well as -9 signaling in breast cancer cells, breast epithelial cells and their hybrids

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Background: Cell fusion is a biological process and plays an important role in several physiological events, e. g. fertilization and placentation. However, cell fusion is also involved in tumor progression. The fusion of tumor cells between themselves or with other cells results in hybrid cells, which could exhibit new properties such a higher proliferation rate. Tumor progression might also be triggered by expression of Toll like receptors (TLRs). Activation of TLRs leads to secretion of various cytokines for immune and inflammatory responses. In particular, TLR-4 is associated with tumor growth and progression and TLR-9 can mediate metastasis. The aim of this study was to analyze TLR signaling to show if they were up-regulated or not after CpG and LPS application. Furthermore, it would be of clinical importance to identify potential targets for the triggering of tumor cell apoptosis. For this reason, expression analysis of pro-apoptotic and anti-apoptotic genes such as trail, bax as well as bcl-2 were investigated by PCR.

Methods and Results: TLR-4 and -9 expression was analyzed in breast cancer cell lines MDA-MB-231, MDAMB435Hyg and MCF-7, breast epithelial cell line M13SV1eGFP-Neo as well as in hybrids M13MDA435-1 and M13MDA435-3 by RT-PCR and Westernblot. The migratory activity with different concentrations of unmethylated CpG was analyzed by using 3D collagen matrix migration assay. Whereas in both hybrid cells and parental cells a CpG dependent decrease in migration potential was observed, showed MDA-MB-231 and MCF-7 tumor cell lines no effect. Indeed, cell proliferation analysis using XTT assay showed no effect after stimulation with CpG. By contrast, a significant inhibition of proliferation rate by LPS was detected in hybrids M13MDA435-1 and M13MDA435-3, due to induction of apoptosis. Western blot analysis were made for TRAF-6, IRF7 and NF-KB as well as MyD88 and TRIF proteins from TLR signaling. The treatment with unmethylated CpG and LPS seems to induce translocation of NF-κB to the nucleus only in MDA-MB-231 and both hybrid clones, whereas all other cells showed no effect. However, for TRAF6 and IRF7 expression no clear effects were achieved after stimulation. PCR data revealed upregulation of IFNβ and TNFα in LPS-treated M13MDA434-1 and -3 hybrid cells. Addition of neutralizing antibodies revealed that apoptosis was impaired in hybrid cells when IFNβ, but not TNFα, was blocked.

Conlusions: These data show a differential expression pattern of TLRs in parental cells and hybrids. Moreover the tested cells responded differently to the TLR ligands. The obtained data could provide important information about the mechanism of metastasis and proliferation rate in breast cancer and how this results could be positively to use for a new therapy approaches.

**Keywords: cell fusion; breast cancer; toll like receptor; unmethylated CpG; LPS; apoptosis**
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# Identification of transcription factors affecting the self-renewal or lineage specification of human hematopoietic progenitor cells

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Hematopoietic stem cells (HSCs) contain a life-long potential to self-renew and to create daughter cells being committed to differentiate. In this context, the decision between self-renewal and differentiation needs to be tightly controlled. A main goal in stem cell biology is to unravel mechanisms regulating the bias of this decision process. Here, we aim to identify cell fate determinants being involved in this process. Previously, genome wide GeneChip analyses of cell fractions enriched for more primitive and more mature hematopoietic progenitor cells were performed. A number of specifically expressed transcription factors (TFs) have been identified which have not previously been associated with early hematopoiesis. Different TFs have been cloned into IRES-eGFP cassette containing lentiviral vectors, allowing their transduction into different cell types. To test for their functional impact, human umbilical cord blood derived CD34+ cells are transduced with these vectors. Allowing the identification of different hematopoietic progenitor cell subsets the expression of the cell surface antigens CD133, CD34 and CD45RA is monitored via flow cytometry. The functional impact of transgenes affecting the progenitor pool assembly are studied in different read out systems, including colony forming cell (CFC), long-term culture initiating cell (LTC-IC) and natural killer initiating cell (NK-IC) assays. Following the lentiviral driven expression of the different TFs, three effector categories could be defined: ectopically expressed TFs which i) do not affect the relation of the different hematopoietic subpopulations to each other and, ii) which accelerate or iii) reduce the decline of the progenitor pool over time. Currently, we test for the developmental capacity of cells of phenotypically defined subpopulations such as of the multipotent progenitor (MPP; CD133+CD34+CD45RA-), lymphoid primed multipotent progenitor (LMPP; CD133+ CD34+CD45RA+) and erythro-myeloid progenitor (EMP; CD133-CD34+CD45RA-) fractions. We have identified a number of promising TFs, which apparently affect the decision self-renewal versus differentiation and/or lineage specification processes. Within our ongoing study we aim to confirm these effects on a functional level.

**Keywords: hematopoietic stem cells; multipotent progenitors; transcription factors**
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# Valproic acid induces catecholaminergic differentiation of sympathoadrenal progenitor cells

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Chromaffin cells and sympathetic neurons derive from a common sympathoadrenal progenitor cell. Sympathoadrenal progenitor cells have common characteristics with neural stem cells and are considered a potential cell source in the treatment of neurodegenerative diseases. Recently we demonstrated the existence of sympathoadrenal progenitor cells in the adult adrenal and their capacity to derive dopaminergic neurons in vitro. The antiepileptic drug valproic acid (VA) is a histone deacetylase inhibitor that stimulates extended growth of neural stem cells. Here, we studied effects of VA in two culture conditions: suspension conditions aimed to expand adrenomedullary sympathoadrenal progenitors within free-floating chromospheres and adherent cell cultures optimized to derive neurons. Similar to neural stem cells, sympathoadrenal progenitors were cultured as free-floating spheroid clusters, chromospheres (CS). Treatment with VA increased proliferation of chromaffin progenitors and their enrichment within CS accompanied by an increased expression of neural progenitor markers such as nestin, Sox1/10, Hes1. Accumulation of cells in S-phase and their increase in G2 cell cycle phase was pronounced after treatment with VA. Our findings indicate common molecular mechanisms that control maintenance of neural and sympathoadrenal progenitor cells. In adherent neural differentiation conditions, VPA initiated catecholaminergic neuronal differentiation indicated by upregulation of the neuronal marker β-III-tubulin, the dopaminergic transcription factor paired-like homeodomain 3 (Pitx3), and the catecholaminergic enzymes tyrosine hydroxylase (TH) and guanosine triphosphate cyclohydrolase (GTPCH). Furthermore, differentiated catecholaminergic neurons contained significantly elevated nor- and epinephrine levels. These data indicate that VA treatment launches proliferation as well as neuronal differentiation of sympathoadrenal progenitors in CS. The use of sympathoadrenal progenitors as a relevant cellular source in the treatment of neurodegenerative diseases such as Parkinson's disease was discussed. The observed duality in VA action could be valuable in increasing the number of sympathoadrenal progenitor cells and triggering their neuronal differentiation prior to grafting. Thus, data from this study contributes validation and the potential use of sympathoadrenal progenitors in transplantation therapies of neurodegenerative diseases.

**Keywords: sympathoadrenal progenitor; valproic acid; catecholaminergic neurons**
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# P-selectin and its ligand PSGL-1 support human CD133+ bone marrow stem cell adhesion along micro endothelium under shear stress

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Introduction: We previously demonstrated the therapeutic potential of hematopoetic CD133+ bone marrow stem cells (CD133+BMSC) to promote liver regeneration and the potential of platelets for CD133+BMSC homing along human micro endothelium under shear stress. The aim of this study was to evaluate the role of the p-selectin / p-selectin-glycoprotein-ligand-1 (PSGL-1) axis and platelet-endothelial-cell-adhesion-molecule-1 (PECAM-1) for platelet-CD133+BMSC-endothelium interaction under flow conditions.

Methods: Human micro vasculature endothelial cells (HMEC-1) were cultured in capillaries in a live cell imaging system (BIOFLUX). Primary CD133+BMSC were purified from bone marrow aspirates from patients undergoing abdominal surgery utilizing magnetic activated cell sorting (MACS). In all experiments, endothelial cells (EC) under shear stress co-cultured with CD133+BMSC and platelet rich plasma (PRP) prepared from BMSC donors served as control. To detect the platelet effect, platelet-poor plasma (PPP) was used in group A. In groups B and C pre-incubation of CD133+BMSC with PSGL-1-antibody IM2090 and platelets with p-selectin antagonist KF38789 respectively were performed. In Group D and E HMEC-1-cells were pre-incubated with PECAM-1 blocking antibody and recombinant rhCD31/PECAM-1 (ADP6, R&D) respectively.

Results: Platelet-free co-incubation resulted in 34,3 % of CD133+BMSC adherence to EC if contrasted to control PRP. In Group B and C, blocking of CD133+BMSC PSGL-1 and corresponding p-selectin on platelets respectively led to a reduction of BMSC adhesion similar to PPP co-cultures conditions (35,1 %(+/-17,2 %) and 48,3 %(+/-24,4 %) of control). Neither PECAM-1 blockage (group D) nor PECAM-1 pre-incubation with HMEC-1 cells significantly had an effect on CD133+BMSC adhesion under flow when compared to controls.

Conclusion: Our study provides evidence that platelet-triggered adhesion of CD133+BMSC to micro vasculature is largely dependent on CD133+BMCS-PSGL-1 interaction and in part related to platelet p-selectin. However, PECAM-1 seems to play only a subordinate role in platelet adhesion. These data add to the understanding of initial BMSC homing steps along vasculature as discussed for liver regeneration and offer novel strategies to increase the efficacy of therapeutic BMSC-application in clinical liver disease.

**Keywords: CD133+ BMSC; platelets; liver regeneration; p-selectin; pecam-1**
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# Somatic & Cancer Stem Cells

# In vitro expansion of human haematopoietic stem and progenitor cells in a stromal cell-free culture system using Delta-like 4

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Haematopoietic stem and progenitor cells (HSPCs) are an attractive source for cell therapy of various immunological and haematological diseases. The availability of HSPCs from limited sample material such as cord blood can be improved by in vitro expansion and has the potential to broaden the scope of clinical application. However, this is a challenging task as these cells tend to differentiate during cultivation resulting in loss of their stem cell character. Promising approaches to enhance HSPC self-renewal involve intrinsic regulators such as transcription factors but also extrinsic stimulation using signalling molecules. The Notch signalling pathway plays an essential role in HSPC development, proliferation and cell fate determination[1]. Over the last decade, various studies have reported that cultivation of HSPCs in the presence of Notch ligands influences HSPC self-renewal and differentiation. Co-culture with stromal cells expressing the Notch ligand Delta-like 4 (Dll4) results in reduced proliferation of HSPCs but preservation of long-term culture-initiating cells (LTC-IC)[2], as well as expansion of primitive HSCs and delayed emergence of downstream progenitors[3]. We developed a stromal cell- and serum-free culture system for cultivation of HSPCs with bead-bound Dll4 which enables proper dosing of the Notch ligand. Long-term cultivation of human CD133+ HSPCs using this system over three weeks resulted in an up to 3.9-fold higher expansion rate of primitive CD133+CD34+CD38-CD45RA- cells compared to Dll4-free control culture. The proliferation of total nucleated cells was 2-fold decreased by Dll4. Dll4 maintained a higher percentage of CD34+ and CD34+CD133+CD38-CD45RA- cells and this effect was dose-dependent. Cell cycle analysis revealed a higher proportion of cells in the G0 and a lower percentage in the G1 phase. Cells cultured with Dll4 displayed a higher colony forming potential in colony-forming unit assays, particularly for erythroid colonies (BFU-E). Gene expression profiling of cells cultured with and without Dll4 revealed 174 significantly differentially expressed genes that are currently under investigation. Taken together, our data confirm that bead-bound Dll4 improves the maintenance of primitive human HSPCs during in vitro expansion. Our Dll4-culture system facilitates the investigation of Dll4 dosage effects and enables stromal cell- and serum-free HSPC expansion.

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**Keywords: CD133; CD34; Delta-like 4; notch; haematopoietic stem cell**
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# Identification of key regulators of symmetric versus asymmetric cell divisions during human hematopoietic lineage specification

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Hematopoietic stem cells (HSCs) contain lifelong potentials to self-renew and to create progenitors of all mature blood cells. According to the current view, HSC homeostasis is controlled by both, HSC-niches as well as asymmetric cell divisions. Our previous studies linked the process of asymmetric cell division of human hematopoietic stem and progenitor cells (HSPCs) to the expression kinetics of the stem cell surrogate antigen Prominin 1/CD133[1]. Furthermore, by characterizing human HSPCs sub-populations by means of their CD133 surface expression we gained evidence that CD133+ multipotent progenitors (MPPs) create CD133+ lymphomyeloid (LMPP) and CD133low erythromyeloid (EMP) daughter cells. The LMPP lineage was shown to contain lymphoid and neutrophil potentials, while the EMP lineage mainly creates eosinophils and basophils as well as erythrocytes and megakaryocytes[2]. Regarding lineage specification, we showed for the first time that under conventional culture conditions almost all MPPs divide asymmetrically to create a set of LMPP and EMP daughter cells, resulting in a loss of MPPs after the first cell division[3]. Thus, our data suggest that under conventional culture conditions asymmetric cell divisions are rather lineage instructive than self-renewing[4]. Now, aiming to identify key factors regulating the MPP division mode, we study whether conditions reported to promote HSC/MPP expansion interfere with the outcome and symmetry of the HSC/MPP cell division. In this context we co-cultured human MPPs with murine and primary human stromal lines (human bona fide MSCs) and surprisingly observed that LMPPs are maintained and expanded but not MPPs. This contrary finding can be attributed to the former experimental definition of multipotent cells based on the classical model of hematopoiesis, according to which cells with dual lymphocyte and granulocyte (conventionally neutrophil) potentials can insufficiently be considered as multipotent. Currently, we test other culture conditions reported to expand human HSCs/MPPs in vitro. After confirming any of these conditions as HSC/MPP expansion condition, we will analyze its impact on the division mode of HSCs/MPPs using multi-parametric flow cytometry, live-cell imaging and functional differentiation assays at the single cell level.

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**Keywords: human hematopoiesis; hematopoietic stem cells; asymmetric cell division; cell fate regulation**
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# Human mesenchymal stem cells from fresh and cryopreserved umbilical cord tissue: a comparison

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The umbilical cord tissue represents a promising source for various kinds of stem and progenitor cells. Especially the Mesenchymal Stem Cells (MSC) gained a lot of interest in past years and were used in numerous therapy studies, although today the interests are more focused on immunomodulatory properties of MSC rather than their regenerative potential. For autologous applications of human umbilical cord-derived MSC (hUC-MSC) the cryopreservation of the umbilical cord tissue after delivery seems to be an auspicious approach. Nevertheless, only very few is known about the characteristics of hUC-MSC isolated from cryopreserved cord tissue. We addressed this issue by a systematic comparison of hUC-MSC isolated from fresh and cryopreserved cord tissue by enzymatic digestion. For that purpose we used classical CFU-f assay, quantification of trilineage differentiation potential and flow cytometric surface marker analysis. Additionally, we examined the influence of hUC-MSC on mitogen-activated T-Lymphocytes. We were able to isolate up to 2.19 Million viable cells per g of cryopreserved cord tissue. The CFU-f frequency varied in a donor dependent manner from 2.6 % to 21.3 % with a mean at 8.81 %. All classical mesenchymal surface markers were expressed, whereas haematopoetic marker expression was lacking. Furthermore, trilineage differentiation was successfully accomplished. Moreover, both, MSC from fresh and cryopreserved cord tissue, showed potential to effectively suppress mitogen-activated T-Lymphocyte proliferation. Our comparison showed that hUC-MSC from cryopreserved umbilical cord tissue maintain all major hallmarks of MSC. Additionally, the suppression of overshooting or unwanted immune reactions that occur for example in graft-versus-host disease (GvHD) could be successfully modeled in vitro. Therefore, the cryopreservation of the umbilical cord represents a feasible approach for providing high amounts of regenerative cells for both allogenic and autologous therapies.

**Keywords: MSC; umbilical cord; isolation; mesenchymal; cryopreservation**
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# Role of Sdc1 in the tumorigenicity of the stem cell-like subpopulation within colon cancer cell lines

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Syndecan-1 (Sdc1), a transmembrane heparan sulfate proteoglycan binds growth factors, cytokines and ECM components thereby regulating cell motility, proliferation and invasion. During colon cancer development, complex changes occur in the expression pattern of Sdc1 during progression from well differentiated to undifferentiated tumours. Loss of Sdc1 is associated with change in phenotypic plasticity with an increase in invasiveness, metastasis and dedifferentiated cells. Empirical evidence showed that this change in phenotypic plasticity allows cancer cells to dynamically enter into a stem-cell-like state. Therefore, we investigated the ability of Sdc1 to modulate cancer stem cell properties using the human colon cancer cell lines Caco2 and HT-29. We demonstrate that siRNA mediated depletion of Sdc1 increased the stem cell phenotype based on in vitro sphere-forming assays and flow cytometry-based assays (side population (SP), ALDH and CD133). Mechanistically, loss of Sdc1 activates β1 integrin with an increase in focal adhesion kinase phosphorylation (pFAK), suggesting that Sdc1 may be linked to integrin-induced actin remodeling. Importantly, with Sdc1 knockdown Wnt signaling is enhanced which in turn induces TCF4 expression promoting the FAK:WNT signaling axis. Specifically, we demonstrated an increase in SP, CD133 and colonospheres, which could be blocked using a FAK specific inhibitor. We conclude that loss of Sdc1 co-operatively enhances activation of integrins, focal adhesion kinase and WNT, which then generates signals for increased invasiveness and cancer stem cell properties.

**Keywords: proteoglycan; extracellular matrix; cancer stem cells**
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# Isolation of functional satellite cells using automated tissue dissociation and magnetic cell separation

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One of the most used experimental models in tissue regeneration are satellite cells. However, the analysis of their molecular and functional characteristics is frequently hampered by the use of heterogeneous populations after tissue dissociation or culture as observed effects are caused by contaminating cell populations instead of the target fraction. To circumvent this issue, a purification of satellite cells is necessary prior to downstream analysis. A crucial prerequisite for the efficient isolation of solid tissue resident target cell populations are reliable dissociation methods for the tissue of origin. We have screened multiple types of enzymes and enzyme combinations in order to optimize yield and viability of cells after dissociation. After determining optimal enzyme components and concentrations, we automated all mechanical steps based on usage of the gentleMACS device. As a result, a complete dissociation of skeletal muscle tissue with low amounts of debris, viabilities above 90% and improved marker preservation allowing for the downstream identification of lymphocyte as well as muscle and endothelial derived cell lineages were achieved. Subsequently, a full automation was achieved using the gentleMACS Octo device which was developed further by integrating heater modules. This enables automated incubation steps directly on the device. After screening markers to define optimized panels in order to identify satellite cells, we have established a functional cell isolation protocol using magnetic cell separation (MACS). This protocol is based on the depletion of lineage markers (CD31, CD45, CD11b, Sca-1) with an optional downstream step of positive selection based on integrin-alpha-7 expression in order to further increase the purity. Cells isolated by this novel method give rise to pure myoblast cultures, can be easily expanded in vitro, and differentiated into functional myotubes. In addition, we have developed a method to transfect cultured myoblasts with artificial mRNAs allowing for the efficient (>75% transfection efficiency per treatment) modulation of this stem cell population without the risk of permanent genetic modification. In summary, we have developed an easy and fast method for the dissociation of skeletal muscle tissue as well as a cell separation method allowing for an accurate downstream analysis of satellite cells, avoiding bias caused by contamination with non-target cells.

**Keywords: satellite cells; skeletal muscle; myoblast; myogenesis; adult stem cells**
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# Quantification of cell fusion events between breast cancer cells and breast epithelial cells exhibiting stem cell characteristics

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Hybrids of cancer cells and normal cells can arise from spontaneous cell-cell fusion processes in vitro and in vivo. Recent data suggest that hybrids of cancer cells and BMDCs or even progenitor or stem cells could harbor a more aggressive phenotype compared to parental cells. Beside extensive genetic alterations cell-cell fusion can contribute to the adoption of properties provided by both merging cells and lead inevitably to an altered behavior and cell shape. Cell fusion processes in cancer development could thereby reveal an often underestimated process promoting tumor progression. While an influence of the (chronic) inflammatory microenvironment as cause for cell-cell fusion as well as cancer progression is increasingly being recognized, less is known about forces particularly triggering cell fusion processes between breast cancer cells and normal tumor surrounding cells. To get more information on how and which components could affect cell-cell fusion and thereby disease outcome remains an important question and need reliable tools to investigate. An available facility to quantitate these cell-cell fusion processes in vitro could provide a novel method to identify cancer promoting components of the tumor stroma. In the present study cell-cell fusion was measured by using a cre/loxP reporter system. Therefore, four breast cancer cell lines were stably transfected with a double fluorescence reporter vector. As fusion partner a breast epithelial cell line exhibiting stem cell characteristics stably expressing the cre recombinase was generated and spontaneous cell fusion was promoted by co-culturing cells under appropriate conditions. Regarding the complex nature of tumor milieu the potential influence of several pro-inflammatory and anti-inflammatory cytokines as well as hypoxia was tested and revealed alterations in fusogenic behavior of cell lines. Increased fusion activity could be observed while stimulation with the pro-inflammatory cytokine TNF alpha or IL-1 beta as well. Moreover, hypoxia and TNF alpha stimulation together intensified the impact on spontaneous cell fusion likely through a TNFR1 dependent mechanism of MDA-MB-435-pFDR and MDA-MB-231-pFDR cells with M13SV1-Cre, whereas fusion capacity of HS-578T-pFDR and MCF-7-pFDR cells was not or no more than partly increased. The present study establishes an efficient tool for the quantification of cell-cell fusion processes in vitro by cre mediated recombination of a loxP reporter system. Once stimulating or repressing factors are detected, possible pathways involved in cell fusion can be further investigated. This highlights a new opportunity to identify potential components involved in priming and triggering cell-cell fusion in the context of cancer.

**Keywords: cancer; cell fusion; inflammation; Cre loxP recombination**
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# Effect of MSC-derived extracellular vesicles on the development and function of dendritic cell subtypes

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In more than 460 clinical trials mesenchymal stem/stromal cells (MSCs) have been administered to a variety of different patient cohorts. Apart of pro-regenerative effects, MSCs exert immune modulatory functions and are able to suppress the function of a variety of different immune effector cells including dendritic cells (DCs). Against the initial dogma that MSCs act in a cellular manner, novel findings suggest that extracellular vesicles (EVs), such as exosomes (70-140 nm), mediate a huge proportion of the MSCs therapeutic effects. After showing that MSC-EVs indeed suppressed symptoms in a graft-versus-host disease (GvHD) patient the MSC-EVs' target cells should be identified. Here, we aim to investigate the impact of MSC-EVs on the development and maturation of human DCs, which are divided in CD1c+, CD141+, CD303+ and monocyte-derived (MoDCs) subtypes. We decided to establish an in vitro DC differentiation assay allowing for the in vitro generation of all DC subtypes first. To this end, human hematopoietic stem and progenitor cells (HSPCs) were raised under different conditions known to promote DC development. Arising immature DCs (iDCs) are identified and purified via multi-color flow cytometry. The maturation of purified iDCs is stimulated by the addition of different TLR-ligands (LPS, R848, polyI:C and CpG) and confirmed by flow cytometric analyses of the expression of co-stimulatory (e.g. CD80, CD86 and CD40) and co-inhibitory (e.g. PD-L1) molecules and by functional assays, e.g. T cell activation assays. By adding purified MSC-EVs to the differentiation cultures or the maturation conditions, respectively, the functional effects of MSC-EVs on DC development will be studied. So far, the efficacy of different differentiation and maturation protocols has been compared. Furthermore, 9 color panels to discriminate the different DC subtypes have been set up. Provided MSC-EV will severally affect DC development (differentiation/maturation), the corresponding assay(s) should be qualified as potency assay(s) to compare the immune modulating capabilities of independent MSC-EV fractions.

**Keywords: mesenchymal stem/stromal cells; extracellular vesicles; hematopoietic stem and progenitor cells; dendritic cells**

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# Identification of RUNX1 key target genes leading to clonal dominance in MDS

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Myelodysplastic syndromes (MDS) are a heterogeneous group of disorders of the hematopoietic system characterized by clonal expansion of stem cells and inefficient hematopoiesis due to partial inhibition of differentiation. Mutations of the RUNX1 gene, encoding a key hematopoietic transcription factor, occur at a high rate (>10%) in MDS patients. However, the mechanisms underlying mutant RUNX1-driven MDS pathogenesis are still unknown. Here we show the development of a mouse model to identify the pathomechanism of RUNX1 mutations. We reconstituted the hematopoietic system of recipient mice using a transduction/transplantation approach with hematopoietic stem and progenitor cells (HSPCs) expressing a dominant-negative form of RUNX1 (dnRUNX1) and a green fluorescent protein (GFP). Control animals were reconstituted with HSPCs modified with a GFP-only expression cassette. Of note, only 10–20 % of the transplanted cells were genetically modified while the majority was unaltered (competitive reconstitution). HSPCs expressing dnRUNX1 displayed a clear growth advantage over non-modified cells, as revealed by the significant expansion of GFP+ cells (>45 % of the bone marrow cells) after 6 months. Moreover, the frequency of differentiated cells was skewed towards the myeloid lineage. Conversely, HSPCs expressing only GFP did not show any clonal advantage (<5 %). The complete blood count analysis of the peripheral blood showed signs of leucopenia (WBC: 4x103/mm3 vs. 10x103/mm3, $p < 0.05$) and anemia (RBC: 7x106/mm3 vs. 9x106/mm3, $p < 0.05$) in experimental mice compared to controls. DnRUNX1-expressing cells were able to engraft secondary recipients with an expansion phenotype. Interestingly, the onset of anemia occurred significantly earlier in those animals compared to primary transplanted mice (six weeks vs. six months). Histological examination of the spleen and the bone marrow of these secondary recipients showed an increased extramedullary hematopoiesis, myeloid hyperplasia and pronounced dysmegakaryopoiesis, all indications of an abnormal bone marrow hematopoiesis. Hence, the mouse model we successfully generated presented numerous MDS-like features. In the system we developed, dnRUNX1 expression was maintained by doxycycline, constantly provided in the diet. Withdrawal of doxycycline led to a 1000-fold decrease of dnRUNX1 expression within 48h. Hence, secondary recipients that had been transplanted from a single donor and harbored pre-malignant cells of the same oligo-clonal origin were divided into two groups, one kept under a doxycycline-enriched diet and the other fed with normal food. Currently, we compare global gene expression profiles of HSPCs obtained from each group. In summary, our model will allow to identify RUNX1 molecular targets that play a key role in MDS pathogenesis.

**Keywords: MDS; hematopoiesis; mouse model; RUNX1**
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# Functional analysis of glioblastoma subclones enables predictions on therapy-related alterations to the tumor cell composition

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The malignant brain tumor glioblastoma is a prime example for the examination of intra-tumor heterogeneity. Recent studies have revealed a substantial degree of intra-tumor cellular diversity on a genetic and non-genetic level. Notably, even patient stratification markers can be nonuniformly distributed among co-existing cells of individual tumors. However, little is known on the consequences of this heterogeneity in respect to pharmacological intervention. The investigation of drug-related changes to the clonal diversity may be key to understanding mechanisms of therapeutic failure in human cancer. In this study, we used 33 single cell-derived subclones generated from five clinical glioblastoma specimens for exploring intra- and inter-individual spectra of drug resistance profiles. Subclones from individual tumors exhibited a remarkable heterogeneity of endogenous resistance to a compound library of potential anti-glioblastoma drugs. In a personalized setting, stable genetic and phenotypic characteristics of co-existing subclone identities could be correlated with distinct drug sensitivity profiles. The data obtained from differential drug response analysis could further be employed to predict clonal population shifts within the naïve parental tumor in vitro and in vivo. Together, our data provide a previously unrecognized strategy for revealing functional consequences of intra-tumor heterogeneity by enabling predictive modeling of treatment-related subclone dynamics in human glioblastoma.

**Keywords: cancer stem cells; cellular heterogeneity; glioblastoma**
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# Cell cycle-dependent gene expression by Mybl2, a tumor suppressor in MDS

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MYBL2 has been identified as a dosage-dependent tumor suppressor in myelodysplastic syndromes (MDS). The gene is ubiquitously expressed and encodes a transcription factor which is strictly required for cell cycle progression. However, downregulation of Mybl2 expression levels to 30 % (sub-haploinsufficiency) provides a clonal advantage to hematopoietic stem and progenitor cells (HSPCs) in vivo. Yet, the molecular mechanism of this phenotype is unknown. Here we show that RNAi-mediated knockdown of Mybl2 to sub-haploinsufficient levels allows freshly isolated HSPCs to proliferate and expand in vitro. While untransduced or with a control short hairpin RNA-expressing vector transduced HSPCs died within 14 days of culture, the cells with reduced Mybl2 levels maintained their growth. The re-expression of wildtype Mybl2 abrogated the growth of these cells within a few days. This reversion of the growth phenotype revealed the specificity of the RNAi-mediated knockdown. We exploited this experimental set-up to identify genes affected by reduced levels of Mybl2 using global gene expression analysis. In addition, as Mybl2 is an established cell cycle regulator, we aimed to analyze Mybl2-dependent gene expression differences at the cell cycle phase level. Thus, we established the expression of a cell cycle phase-dependent indicator protein that allowed the undisturbed separation of cells at the different cell cycle phases by flow cytometry. We expected that gene expression differences induced by sub-haploinsufficient changes in Mybl2 levels remain difficult to determine while they would become significant when analyzed at the cell cycle phase level. Indeed, the cell cycle-dependent expression of Cyclin B1 (Ccnb1), a well-established Mybl2 target gene, supported our hypothesis and showed significant expression differences in the G2 cell cycle phase upon Mybl2 downregulation. Our approach will identify genes that are strongly affected by Mybl2 gene dosage changes as opposed to well-known target genes that are barely regulated under this sub-haploinsufficiency condition. The strongly affected genes are candidates for the tumor suppressor function of Mybl2 and will be tested functionally in additional experiments to define the Mybl2-dependent tumor suppressor pathway.

**Keywords: Mybl2; MDS; HPSC; tumor suppressor; cell cycle**
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# Towards a molecular understanding of chemoresistance in glioblastoma

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Glioblastoma multiforme (GBM) represents the most common and most aggressive primary brain tumor in humans. Along with excessive cellular and molecular heterogeneity, development of therapy resistance in recurrent tumors represents one of the major reasons for the poor prognosis (as evident from the mean overall survival time of below 15 months). Current treatment options, including surgery, radiation and chemotherapy with DNA alkylating agents (such as Temozolomide, TMZ), have only marginally prolonged the patients' life expectancy. In order to gain insight into the molecular events associated with establishment of therapy resistance, we have developed an In-Vitro-Recurrence (IVR) assay using primary GBM cell cultures which have been expanded under cancer stem cell – enriching conditions. Using this assay and a candidate gene approach which covers several potential target mechanisms (the AKT/PKB pathway, stemness associated factors, detoxifying enzymes, DNA repair pathways) we identified a transcriptional profile associated with acquired chemoresistance. Functional follow up studies assessed the implications of dysregulated pathways for establishment of resistance towards alkylating agents. Our data emphasize the causative role of aldehyde dehydrogenase 1A1 (ALDH1A1) as a marker for therapy-resistent subpopulations of tumor cells that are responsible for recurrence of the disease. In addition, hyperactivation of the AKT pathway in ALDH1A1/pAKT-double positive cells is evident in both TMZ resistant cell cultures (in vitro) and in patient tumor tissues (in situ). Functional implications of these findings in combination with high expression of specific stemness factors in the chemoresistant subpopulation are discussed. Our study furthers our understanding of therapy resistance in GBM and thus opens novel avenues for therapy of this fatal disease.

**Keywords: glioblastoma; therapy resistance; cancer stem cells; ALDH1A1; AKT/PKB**
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# Identification of new therapeutic targets by the analysis of residual glioblastoma cells

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Glioblastoma accounts for one of the most aggressive types of cancer and is characterized by a diffuse infiltrative phenotype. Despite macroscopically complete resection of the tumor followed by combined radio- and chemotherapy a small residual population of cells escapes leading to tumor recurrence ~7 months after diagnosis. Surprisingly little is known about this cell population that migrates away from the tumor bulk into the surrounding native brain parenchyma. Understanding the molecular and functional architecture of these Residual tumor cells that remain behind after complete neurosurgical resection is critical for the development of successful therapies. We previously demonstrated that Residual cells can be derived via experimental biopsy from the surgical resection margin, enriched, and investigated under controlled conditions in vitro. In the present study we performed drug-screenings on residual tumor cells from glioblastoma patients and compared data with paired samples from the respective tumor core. The center and respective Residual cells showed distinct drug response profiles. Applying an array of compounds used in clinical trials for glioblastoma, we consistently observed a more resistant phenotype in the Residual tumor samples. Molecular fingerprinting of 12 respective, paired patient samples identified a set of “druggable” molecules. Their differential expression could be confirmed by qRT-PCR, western blot analysis, and by immunhistochemistry in the original patient tissue. Using pharmacological and siRNA-mediated approaches two particularly suited candidates emerged as potential future targets for selective interference in situ. In summary, probing the biological and functional features of Residual tumor cells exposed unique cellular targets for cancer therapy. Future strategies employing combined and region-specific tumor cell targeting may open entirely new avenues for the diagnosis and therapy of glioblastoma.

**Keywords: glioblastoma; residual tumor cells; drug target discovery**
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# Role of toll-like receptor 4 in neural stem cells and astroglioma cells

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In *Drosophila melanogaster,* Toll proteins are transmembrane receptors responsible for the establishment of the dorso-ventral polarity. Beside of their function in the developmental context, Toll receptors in *Drosophila* play a pivotal role in the innate immune response of the fly via recognition of pathogens. In mammals, the Toll-like receptors (TLR) are a group of pattern recognition receptors (PRR) co-operating in detection of different pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (PAMPs) released after pathological evens such as infection-mediated tissue damage or injury.

One of the most intensely studied TLR/ligand pairs is TLR4 and its ligand (PAMP) bacterial lipo-polysaccharide (LPS). Notably, TLR4 is the only known TLR able to activate both: the MyD88 – dependent, pro-inflammatory signaling pathway culminating in NF-κB activation as well as the MyD88-independent pathway leading to activation of IRF3. Remarkably, in stem cells and tumor cells both transcription factors seem to exhibit opposing effects in regard to migration, proliferation, apoptosis and differentiation. Most TLR4-ligands have the intrinsic ability to activate NF-κB as well as IRF3, but the balance of both factors differs depending on the ligand.

In our study, we investigated the impact of different TLR4 ligands on the downstream signaling events in human astroglioma cell lines and rodent neural stem cells (NSCs). We were able to show that exposure to different TLR4 ligand culminates in a shift of balance between IRF3 and NF-κB and affects their dynamics and kinetics in a ligand dependent manner. Moreover we demonstrate that this shift of balance significantly affects proliferation of NSCs and human astroglioma cells.

**Keywords: TLR4; inflammatory balance; neural cells**
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# Alpha-Catenin is a dosage-dependent tumor suppressor gene in MDS

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Myelodysplastic syndromes (MDS) are a heterogeneous group of hematological disorders characterized by clonal expansion of hematopoietic stem and progenitor cells (HSPCs). Cytogenetic and high-resolution genomic profiling studies of a large patient cohort have defined two commonly deleted regions (CDRs) on chromosome 5q. However, the identity of the predicted haploinsufficient tumor suppressor gene within the proximal 5q CDR is still obscure. Here we employ a transduction/transplantation experiment to establish a mouse model of this specific genetic aberration using lentiviral-mediated RNA interference (RNAi). As donor cells, we used HSPCs obtained from Tp53 knockout mice to implement the frequent co-occurrence of inactivating TP53 mutations with 5q deletions in patients. We first tested the knockdown of alpha-Catenin (Ctnna1) and found a competitive advantage of HSPCs with loss of Tp53 and reduced expression of Ctnna1 leading ultimately to a myeloid malignancy and an earlier onset of death compared to control animals. To further specify the observed phenotype and the downstream effects of low Ctnna1 levels, we cultivated primary wildtype bone marrow cells with RNAi-mediated knockdown of Ctnna1. Surprisingly, this genetic modification was sufficient for the survival, proliferation and immortalization of the cells in vitro. After 8 weeks in culture, the cells mainly showed a myeloid immunophenotype, and the culture contained a lineage-negative subpopulation of progenitor cells that appeared to maintain its growth. The re-expression of Ctnna1 reversed this growth phenotype in vitro revealing the specificity of the RNAi-mediated effect. Currently, we perform a gene expression analysis to define the molecular pathway engaged by reduced expression of Ctnna1. Our studies determined the role of Ctnna1 for the pathogenesis of MDS with 5q deletions and will uncover potential functions of Ctnna1 in hematopoiesis.

**Keywords: Myelodysplastic syndromes; Ctnna1; RNAi; competitive bone marrow reconstitution**
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# Stem Cell Differentiation

# Searching for deep-intronic ABCA4 mutations in Stargardt patients by using induced pluripotent stem cell-derived photoreceptor progenitor cells

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We previously found that ~40 % of persons with autosomal recessive Stargardt disease (STGD1) carry only one, and ~10 % no ABCA4 mutations. We hypothesized that the missing mutations either constitute heterozygous deletions that are missed by the use of exon PCR and Sanger sequencing, or are located deep in the introns, most likely affecting the ABCA4 mRNA. Using multiplex ligation-dependent probe amplification-based deletion scanning, we identified heterozygous deletions in three of 45 probands with STGD1 or related cone-rod dystrophy. We analysed the same cohort for the presence of five deep-intronic variants published elsewhere, and identified deep-intronic variants in seven probands. We have shown previously that ABCA4 mRNA is expressed at very low levels in lymphoblasts and fibroblasts but is expressed robustly in photoreceptors that had been differentiated from human stem cells. We therefore propose to perform sequence analysis of ABCA4 mRNA isolated from photoreceptor progenitor cells (PPCs) that were differentiated from reprogrammed patient-specific induced pluripotent stem cells (iPSCs). Fibroblasts were cultured from skin biopsies from eight STGD1 patients and reprogrammed into iPSCs. iPSCs were differentiated into PPCs, and the presence of the ABCA4 transcript was evaluated by quantitative RT-PCR and immunocytochemistry. ABCA4 transcript analysis by RT-PCR and Sanger sequencing to identify insertions or deletions is ongoing.

**Keywords: iPSCs; photoreceptors; ABCA4; Stargardt**
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# Xeno-free differentiation of human adipose derived and bone marrow derived mesenchymal stem cells towards myocytes for applications in tissue engineering

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Tissue engineering is a growing field which is dealing inter alia with bio-functionalization of synthetic materials using stem cells. Examples for applications are the coating of bone replacement materials to improve ingrowth in critical size defects or the coating of stents in cardiovascular tissue engineering. Most tissue engineered constructs fail on their way to the market because of several limitations. A common known limitation is that a huge amount of cells is needed to produce an autologous tissue engineered construct. Human mesenchymal stem cells (MSC) derived from bone marrow are widely used for tissue engineering settings, but there is only a limited amount of cells available based on the characteristic of the tissue source. Another limitation of tissue engineered constructs on their way to the market is the usage of fetal calve serum (FCS) in cell culture. A promising tissue source for MSC and FCS-free culture media need to be investigated. Lipoaspirate is such a promising tissue source for MSC. In this study, human adipose-derived stem cells (ADSC) are isolated from tissue lipoaspirates and compared to bone marrow derived MSC (BM-MSC). The ADSC are characterized by flow cytometry using the typical MSC marker panel (CD73, CD90, CD104, CD44, CD34, CD45) as well a series of other markers known to be present in adipose derived MSC (AD-MSC) (CD49d, CD29, CD106, CD166). Cells from both sources are differentiated under xeno-free conditions towards myocytes following a cytokine-induction protocol. Functional analysis of myocytes is performed by using lifetime imaging with a carbachol contraction assay. AD-MSC and BM-MSC are further compared under xeno-free and FCS-based medium expansion conditions for their proliferation potential. In the present study we pave the way for a myogenic differentiation of AD-MSC and BM-MSC under xeno-free conditions for future tissue engineering applications.

**Keywords: adipose-derived mesenchymal stem cells; myogenic differentiation; xeno-free cell culture**
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# Specific transcripts of DNMT3A are important for differentiation of hematopoietic stem and progenitor cells

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DNA-methyltransferase 3A (DNMT3A) is a de novo DNA-methyltransferase that is alternatively spliced in a tissue- and disease-specific manner, but little is known about the function of its transcripts. DNMT3A is frequently mutated in acute myeloid leukemia (AML) and a recent study from our group indicated this mutation can be mimicked by epigenetic dysregulation within the DNMT3A sequence: about 40 % of AML samples revealed aberrant hypermethylation at this region and this was associated with shorter overall survival. In analogy to DNMT3A mutations, this "epimutation" seems to interfere with normal expression of DNMT3A transcripts. In this study we aim to elucidate the functional role of individual DNMT3A splice variants in the development of AML. Single DNMT3A transcripts were knocked down by lentiviral expression of short-hairpin RNAs in cord blood derived hematopoietic stem and progenitor cells (HSPCs). Knockdown efficiency of individual transcripts was validated by qRT-PCR. Notably, the knockdown of transcript 2 caused an upregulation of transcripts 1+3 and transcript 4. We also examined the potential of colony formation with a CFU-assay. The overall number of colonies formed in the CFU-assay was significantly decreased in HSPCs with downregulated transcripts 1+3 compared to control HSPCs. Downregulation of transcript 4 leads to a bias towards erythroid progenitors. Subsequently, we analyzed the proliferation and immunophenotype of infected HSPCs. Interestingly, HSPCs with downregulation of transcript 2 and 4 tend to proliferate slower whereas HSPCs with downregulation of transcript 2 maintain higher CD34 expression for more cell divisions. Our results indicate that the different DNMT3A transcripts, especially transcript 2, have specific regulatory functions in HSPCs that may be relevant for disease progression in AML patients, which carry either a mutation or epimutation in DNMT3A.

**Keywords: DNMT3A transcripts; hematopoietic stem and progenitor cells; differentiation**
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# Custom-tailored cardiomyocytes: a directed in vitro differentiation of human pluripotent stem cells into defined cardiomyocyte subtypes

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Cardiomyocytes derived from human induced pluripotent stem cells (iPS cells) are a promising tool for regenerative applications by cardiomyocyte transplantation, for the study of cardiac development and disease modeling as well as for drug discovery and cardiotoxicity screenings. The generation of relatively homogenous populations of subtype-specific cardiomyocytes in large numbers and high purities is crucial for potential clinical applications in cell based therapies as well as for a better understanding of the predominantly cardiac subtype-restricted disease mechanisms and their therapeutic approaches. The goal of our study is to develop a reproducible, efficient and cost-effective method for a directed in vitro differentiation of human iPS cells into a defined cardiomyocyte subtype in feeder-free culture conditions. Our findings indicate that the ratio of heterogeneous populations of ventricular, atrial and pacemaker-like iPS cell-derived cardiomyocytes can be directed into a more homogeneous cardiac atrial subtype by temporal treatment with distinct small molecules. Taken together, this study will provide an efficient tool of a directed in vitro differentiation of iPS cells into defined functional cardiomyocyte subtypes for a more specific cardiac disease modeling as well as for potential clinical and therapeutic approaches.

**Keywords: cardiomyocytes; subtype; differentiation; iPS cells; disease modeling**
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# A reliable and efficient protocol for human pluripotent stem cell differentiation into the definitive endoderm based on dispersed single cells

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Background and aims: Formation of definitive endoderm (DE) is the initial step to differentiate pluripotent stem cells (PSCs) into cell types derived from the endoderm germ layer, such as liver or pancreatic cells. Differentiation of primed PSCs is usually initiated from colonies, a suboptimal starting material due to different colony sizes, colony numbers and their potentially altered sensitivity to media supplements. The aim of this study was the development of a reliable and highly efficient protocol for the differentiation of human PSCs into the DE from dispersed single cells and their further differentiation into the pancreatic lineage.

Materials and methods: Three human ESC lines and one human iPSC line were passaged as dispersed single cells, seeded in a defined cell number and used the next day for differentiation. Multiple DE inducing protocols were tested by combining various concentrations of Wnt3a, CHIR-99021, LY-294002 and ActivinA for four days. Subsequent differentiation into the pancreatic lineage was performed with FGF10, retinoic acid, Dorsomorphin and SB-431542. The different steps during the differentiation were analyzed by flow cytometry, IF and qPCR.

Results: The combined treatment with CHIR-99021 plus ActivinA for the first 24h and subsequently ActivinA alone (CA-A protocol) resulted in the most efficient DE formation for all tested human PSC lines. Additional PI3K inhibition had no beneficial effect under this condition. The expression of the DE-marker genes SOX17 and FOXA2 were significantly increased compared to a classical reference protocol, ActivinA treatment alone or random differentiation. Quantification of DE-committed cells, by IF and flow cytometry, revealed efficiencies of >70% (HUES4, HES3, hCBiPS2) or >80 % (HUES8) for the CA-A protocol, a >2-fold increase compared to the reference protocol (~33–40 %). Treatment with ActivinA alone was not successful to induce substantial numbers of DE-committed cells. Additionally, the CA-A condition exhibited the highest proliferation rates during the differentiation and allowed a reduction of the required ActivinA concentration up to 4-fold compared to the reference protocol. DE-cells obtained with the CA-A protocol were able to further differentiate into PDX1-positive progenitors (~40 % after 10 days) and subsequently into NGN3-positive endocrine precursor cells (~10 % after 14 days). High expression levels of the marker genes FOXA2, HNF6, MNX1 (HB9), NKX2.2, NKX6.1 and NGN3 were detected in a manner similar to the in vivo pancreatic development indicating a maturation of the pancreatic precursor cells.

Conclusion: The CA-A protocol was able to differentiate several human PSC lines highly efficient into the DE. Therefore only low initial cell numbers and reduced concentrations of expensive growth factors were required without growth limitations. In addition, these cells were able to differentiate further into the pancreatic lineage.

**Keywords: definitive endoderm; differentiation; pluripotent stem cells**
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# Direct Nkx2-5 transcriptional repression of Isl1 controls cardiomyocyte subtype identity

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During cardiogenesis most myocytes arise from cardiac progenitors expressing the transcription factors Isl1 and Nkx2-5. Here, we show that a direct repression of Isl1 by Nkx2-5 is necessary for proper development of the ventricular myocardial lineage. Overexpression of Nkx2-5 in mouse embryonic stem cells (ESCs) delayed specification of cardiac progenitors and inhibited expression of Isl1 and its downstream targets in Isl1+ precursors. Embryos deficient for Nkx2-5 in the Isl1+ lineage failed to downregulate Isl1 protein in cardiomyocytes of the heart tube. We demonstrated that Nkx2-5 directly binds to an Isl1 enhancer and represses Isl1 transcriptional activity. Furthermore, we showed that overexpression of Isl1 does not prevent cardiac differentiation of ESCs and in Xenopus laevis embryos. Instead, it leads to enhanced specification of cardiac progenitors, earlier cardiac differentiation, and increased cardiomyocyte number. Functional and molecular characterization of Isl1-overexpressing cardiomyocytes revealed higher beating frequencies in both ESC-derived contracting areas and Xenopus Isl1-gain-of-function hearts, which associated with upregulation of nodal-specific genes and downregulation of transcripts of working myocardium. Immunocytochemistry of cardiomyocyte lineage-specific markers demonstrated a reduction of ventricular cells and an increase of cells expressing the pacemaker channel Hcn4. Finally, optical action potential imaging of single cardiomyocytes combined with pharmacological approaches proved that Isl1 overexpression in ESCs resulted in normally electrophysiologically functional cells, highly enriched in the nodal subtype at the expense of the ventricular lineage. Our findings provide an Isl1/Nkx2-5-mediated mechanism that coordinately regulates the specification of cardiac progenitors towards the different myocardial lineages and ensures proper acquisition of myocyte subtype-identity.

**Keywords: Isl1; Nkx2-5; cardiac differentiation; cardiac progenitors; embryonic stem cells**
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# Heart, brain, eye and pancrease cells thrive on biologically relevant defined and xeno-free laminins

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Laminins are a group of 16 heterotrimeric glycoprotein isoforms found in the basement membrane in the extracellular matrix and are composed of α, β and γ chains. Laminins are the only tissue-specific proteins in the basement membrane and therefore one critical factor that differentiates one niche from another. Laminins contribute to the structure of extracellular matrix and influence the behavior of associated cells, such as adhesion, differentiation, migration, phenotype stability, and resistance to anoikis. The use of specific laminins for tissue culture and cell therapy applications have been hampered by lack of access to most laminin isoforms. Tissue-purified laminins have been available for years but often result in poor quality due to protein degradation, lot-to-lot variation and impurities, resulting in variable and unreliable research results. We have now solved these problems by successful production of human recombinant laminins and have shown that individual laminin isoforms drastically improve the functional properties of different cells. Pluripotent stem cells: By using LN-521, which is naturally expressed by human pluripotent stem cells, we can culture stem cells for over 130 single cell passages at split ratios of 1:10-1:30, without any abnormal genetic aberrations and with maintained expression of pluripotency markers. Cardiomyocytes: By using heart specific laminins, LN-211, LN-221 and LN-521 in the natural combination of adult heart cell expression, the differentiation can now be fully controlled in a defined and xeno-free context and the number of hPSC-derived beating cardiomyocytes is significantly increased. Neurons and glia: Neural stem cells prefer LN-521, different neuronal subtypes require primarily LN-511/521 and LN-111 for full maturation, and glia cells express LN-111, LN-211 and LN521. RPE cells: Robust retinal pigmented epithelium cell culture has been reported on LN-521/511, LN-111 and LN-332. In conclusion, cell culture of primary cells and stem cells is reliable and robust when growing cells on the natural human recombinant laminin that match the in vivo niche. Almost all cells grow on specific laminins in the human body and as these laminins are now available as recombinant laminins it makes cell culture in a physiologically relevant environment possible, making production of clinically relevant cells possible.

**Keywords: laminins; xeno-free; defined; cell niche; controlled differentiation**
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# Multipotent glia-like stem cells in adult adrenal medulla and their involvement in stress adaptation

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The neural crest derived adrenal medulla is closely related to the sympathetic nervous system; however, unlike neural tissue, it is characterized by high plasticity and its ability to adapt to highly demanding situations, such a stress; this suggests the involvement of stem cells. Our study, by using a transgenic nestin-GFP mouse model, defines a population of glia-like nestin expressing progenitor cells in the adult adrenal medulla. These glia-like cells have features of adrenomedullary sustentacular cells; they are multipotent and in vitro differentiate into chromaffin cells and neurons. For lineage tracing and to study the progenitors' role in the adrenal medulla in vivo, an inducible mouse model, Nestin-CreER:Rosa26YFP, was used. This mouse model revealed the differentiation of the nestin-expressing cells into the three main cell types in the adrenal medulla, chromaffin cells, neurons and glia. Furthermore, the adrenal gland plays a central role in the body's stress response, providing the proper adaptation to regain and maintain homeostasis. Our results from stress experiments in vivo show that nestin progenitor cells are prompted to differentiate into new chromaffin cells, when the stress becomes chronic. In summary, we describe a glia-like population of multipotent stem cells that is involved in adrenal tissue adaptation. This study sheds light on the contribution of stem cells in the adaptation of adult tissue function and might be extended to other neuroendocrine tissues.

**Keywords: nestin-GFP; adult stem cells; glia; stress**
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# 40+ multi-parameter phenotyping of human hematopoietic stem and progenitor cells

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Like other somatic stem cells, hematopoietic stem and progenitor cells (HSPCs) are able to balance four states – quiescence, cell migration (homing), proliferation (self-renewal), and differentiation – in dependence of widely unknown regulation mechanisms. Especially, mechanisms controlling cell polarity are discussed to influence cell fate decision processes, e.g. by regulating the symmetry plane of occurring cell divisions (symmetric vs. asymmetric cell division) and the interaction of HSPCs with their niches. Multi-parameter phenotyping with 40+ specificities on a single cell level could significantly improve our understanding of the underlying mechanisms. To do this, a novel imaging technology, the so called Multidimensional in situ Cytometry Survey (MICS), was established. This technology is based on subsequent repetitive antibody staining – and fluorochrom wiping cycles. Within every cycle images with a fluorescence microscope are taken. A crucial aspect in case of suspension cells like HSPCs is that cells have to get immobilized. In contrast to standard protocols for suspension cell microscopy (e.g. dehydration, Cytospin), a novel system allowing single cells trapping in microcavities was developed. A combination with a special automated pipetting permits recovery of more than 90 % of individually captured cells. Thus, the cells itself and especially the cell morphology is not influenced by physical processes and fixation artifacts are avoided. Additionally, a new analysis software was developed which fulfills the special requirements of multi-parameter phenotyping using the novel MICS technology. Among other processing steps the software enables cell identification via supervised machine learning. In a next step the generated mask is transferred to the microscopic images and cell specific pixel intensities are extracted for each antibody-stain. Finally, correlation and clustering methods may facilitate promising new findings. For the selection of antigens, initially the expression of 198 proteins was examined via a full-automated screen using a liquid handling system and standard flow cytometry technology. Then, the subcellular distribution of 26 selected proteins was studied using an image flow cytometer. In this context, analysis strategies for the identification and characterization of morphological and molecular cell phenotypes were generated. Well described cell polarity markers, e.g. CD50, CD133 and CD43, were used as controls and novel cell polarity markers identified. Currently, 48 proteins including standard HSPC markers, i.e. CD34, CD133, CD45RA and CD38, and promising new markers from the previous screens are being tested using the MICS technology. In focus of these analysis is the influence of unidentified serum components on cell fate decision in the HSPC compartment.

**Keywords: HSPC;multi-parameter phenotyping; novel technology**
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# Investigating the signaling rationale underlying cardiac induction of human pluripotent stem cells

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Understanding the mechanisms underlying differentiation of human pluripotent stem cells (hPSCs) into specific fates may enable improved protocols for the generation of desired cell types and also allow insights into early human development. However, gene regulatory events underlying directed differentiation protocols are poorly understood in many cases. We have developed a directed differentiation procedure for converting hPSCs into cardiomyocytes at high efficiency. Through systematic optimization, this allowed us to define the minimal signaling requirements driving this process. One key requirement for efficient cardiac induction of hPSCs is inhibition of the WNT pathway, following the initial induction of mesoderm. The WNT inhibition step is evolutionary conserved and shared with other reported protocols but the underlying rationale is not understood. Using global time-course gene expression analysis, we have elucidated the gene-regulatory consequences underlying WNT inhibition for promoting cardiac induction in our protocol. Unexpectedly, WNT inhibition does not seem to promote the activation of procardiac factors in the cells. Rather, we show that it serves to suppress anti-cardiac regulators that would otherwise, i. e. without extrinsic WNT inhibition, become strongly upregulated by default. Several such candidate regulators were identified using unbiased subtractive filtering of global gene expression data. Their overexpression in the presence of a WNT inhibitor compromised cardiac induction and promoted differentiation into alternative fates, demonstrating their anti-cardiac functions. The CRISPR/Cas9 system was used to simultaneously knock out these genes in hPSCs. Triple knock-out hPSCs displayed facilitated cardiogenesis at limiting concentrations of the WNT inhibitor, thus demonstrating that suppression of anti-cardiac regulators is necessary – and partially sufficient – for promoting cardiomyocyte formation. Our data hence reveal an unexpected repressive roadblock in the human cardiac induction lineage, which might have universal validity.

**Keywords: human pluripotent stem cells; cardiac induction; directed differentiation; gene regulation**
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# The P2Y1 receptor is controlling both, osteo- and angiogenes is required for vascularized bone tissue engineering

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Introduction: The repair of non-healing bone fractures is not only relevant for elderly people suffering from aging-associated diseases but might also affect the young generation e.g. due to traffic or sports accidents. For treatment of critical size bone defects besides ossification, new blood vessel formation is required. Adult stem cells have the capacity to differentiate towards osteoblasts and endothelial cells, the two main cell types needed. In addition, we have shown that the expression of specific purinergic receptor subtypes plays an important role in the commitment of these cell lineages. Based on our pioneering findings, this project aims to use this knowledge for the development of a new bone repair strategy. Material and

Methods: Human adult stem cells were isolated from particulate jawbone material, dental follicle and liposuction material from belly followed by differentiation towards the osteogenic and endothelial cell lineage confirmed by lineage specific staining. In addition, functional tests for endothelial cell differentiation such as Dil-Ac-LDL uptake and tube formation in Matrigel® assay were performed. Afterwards highly selective artificial purinergic ligands have been tested for their influence on both, the extracellular calcium deposition during osteogenesis within a collagen matrix and the endothelial cell sprouting behavior. Results: Jawbone- and dental follicle-derived stem cells (ectomesenchymal) displayed a strong osteogenic differentiation potential which could even be enhanced by the P2Y1 receptor antagonist MRS2500 submerged into the biomaterial. Moreover, the agonist MRS2365 of the same receptor subtype increased the endothelial cell differentiation and sprouting of adipose tissue-derived stem cells (mesenchymal).

Conclusion: Here we demonstrate that jawbone, dental follicle and fat tissue contain adult stem cells. Due to their different embryological origin ectomesenchymal stem cells possess a stronger commitment towards hard tissues compared to mesenchymal stem cells. This makes the former ones an ideal source for osteogenic differentiation, whereas the latter ones are suitable candidate cells for endothelial cell differentiation. Our findings indicate the functional role of the P2Y1 receptor subtype in both, osteogenesis and angiogenesis of adult stem cells. Thus, it is a promising target to control stem cell fate for future bone fracture treatment approaches.

**Keywords: purinergic receptor; (ecto)mesenchymal stem cells; critical size bone defect; osteogenesis; angiogenesis**
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# Sox2- positive cells in the cochlea function as inner ear progenitors

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The vestibular and auditory organs have a limited ability to replace damaged cells, but our laboratory has recently identified cells in the cochlear sensory epithelium with stem cell properties. Located in the organ of Corti, this epithelium consists of hair cells, the receptor cells for sound, and surrounding supporting cells. These supporting cells gave rise to hair cells and acted as putative stem cells since they formed neurospheres that could self renew and differentiate to hair cells. Sox2-expressing cells isolated by flow cytometry expressed several other stem cell markers that played important roles in the progenitor activity of these cells. Thus the sorted Sox2 cells showed expression of Lgr5 and Pou3f1. Lgr5 conferred Wnt sensitivity to these cells as shown by overexpression studies both in vitro and in vivo, where stimulation of β-catenin expression increased proliferation of supporting cells. The Sox2 cells that expressed Lgr5 were shown to be the precursors for hair cells by lineage tracing. Sox2 played a crucial role for hair cell specification from the inner ear progenitors. Sox2-expressing cells could be induced to differentiate to hair cells by inhibition of Notch signaling. Loss of Sox2 disrupted hair cell differentiation. We identified Pax2 as a novel binding partner for Sox2 in inner ear progenitors. Sox2 and Pax2 directly activated transcription of Atoh1, which stimulated their differentiation to hair cells. Manipulation of signaling pathways, after inner ear trauma could lead to hair cell replacement and hearing restoration.

**Keywords: inner ear; regeneration; hair cells; Sox2; Lgr5**
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# Approaches for in vitro differentiation of primate germ cells in an organ culture system

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Introduction: Spermatogonial stem cells (SSCs) are able to self-renew and to produce spermatozoa by meiotic differentiation throughout adulthood. Spermatogenesis is initiated in puberty. Immature prepubertal testes contain progenitors of SSCs. During chemo- or radiotherapies, SSCs and their progenitors are often damaged and depleted from the testis resulting in infertility. Thus, young prepubertal boys, who cannot overcome this problem by cryopreserving a semen sample, often lose the chance to father own offspring after cancer therapies. To abolish this side effect, protocols are needed to differentiate progenitor cells from immature testes into spermatozoa. In 2011, Sato and colleagues succeeded in producing spermatozoa from neonatal mouse testes in an organ culture system. However, in vitro production of primate spermatozoa has not yet been achieved. Since the SSC system in non-human primates and men differs from that in mice, we used a non-human primate model (Marmoset, Callithrix jacchus) to evaluate the feasibility of organ culture systems for differentiating immature primate testicular cells into spermatozoa.

Materials & Methods: Small testicular fragments (1 mm3) from two prepubertal (4-months-old) and two pubertal (8-months-old) marmosets were distributed on polycarbonate membranes of culture inserts. Fragments were cultured at the interphase of medium (DMEM + NEAA + P/S) and atmosphere at 35 °C for 3, 7, 14, 28, 49 and 65 days with and without adding follicle-stimulating hormone (FSH) (500 IU/l) and chorionic gonadotropin (CG) (500 IU/l). At each time point qPCR and immunohistochemical (IHC) analyses were performed to evaluate the differentiation status of germ cells within the cultured fragments.

Results: In testicular fragments from 4-months-old marmosets, the expression of MAGE-A4, a spermatogonial marker gene, was detected for up to 28 days of culture. Meiotic (BOLL) and post-meiotic marker gene (CREM) expression remained low over the entire culture period. IHC analyses for MAGE-A4 and CREM confirmed the presence of spermatogonia and the absence of post-meiotic cells in cultured fragments. In fragments obtained from 8-months-old marmosets MAGE-A4 expression was also still detected at 28 days of culture. The meiotic (BOLL) and post-meiotic marker gene expression (CREM) was initially higher than for the 4-months-olds, but was hardly detectable by day 14. In IHC analyses post-meiotic cells were detected up to 14 and spermatogonia up to 28 days in the fragments. No difference was seen with regard to sex hormonal supplementation.

Conclusion: In our organ culture setting (pre-) pubertal marmoset spermatogonia were maintained for up to 28 days. However, FSH and CG were not able to stimulate germ cell differentiation. Further factors and serum components, which are provided in vivo, are likely essential to trigger gem cell differentiation. Thus, we will analyze the effect of retinoic acid and knock out serum replacement on germ cell differentiation in future experiments. In addition, the somatic environment of the cultured fragments will be examined in more detail to reveal whether impaired lacking somatic support hinders germ cell differentiation. Performing such experiments, we will hopefully provide options for childhood cancer patients to overcome infertility in adulthood.

**Keywords: in vitro spermatogenesis; marmoset monkey; non-human primate; organ culture; spermatogonial stem cell differentiation**

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# Unraveling the role of Cdk1 in postnatal neurogenesis

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Age-related neurological disorders, including stroke and neurodegenerative diseases are becoming a major health care problem in many countries as average life expectancy is increasing. One appealing therapeutic strategy to treat neurological disorders would consist of recruiting endogenous neural precursor cells (NPCs) to replace the lost neurons. NPCs are present in two specific areas of the postnatal brain, the adult dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles. These NPCs give rise to neurons throughout life, a phenomenon termed postnatal neurogenesis. A prerequisite for the development of new therapeutic strategies involving postnatal NPCs is a better understanding of their molecular regulation. In this context, both extrinsic factors and intrinsic factors have been identified. For instance, the family of cell cycle regulators cyclin-dependent kinases (Cdks) are key regulators of postnatal neurogenesis since genetic invalidation of Cdk2 or Cdk6 leads to a decrease of NPCs proliferation. Here, we wanted to unravel the role of Cdk1 in postnatal neurogenesis using a mouse model that allows the specific deletion of Cdk1 in postnatal NPCs in vivo.

**Keywords: adult neurogenesis; neural stem cells; cell cycle**
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# The role of retinoic acid and Wnt/beta-catenin signaling during anterior-posterior patterning of definitive endoderm derived from human embryonic stem cells

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The in vitro differentiation of pluripotent stem cells (PSCs) into the definitive endoderm (DE) is nowadays robustly performed. The next differentiation step requires anterior-posterior (AP) patterning of the endoderm into the broad primitive gut tube domains foregut and hindgut. Subsequently specific domains are patterned, which give later rise to the organ primordia. The activities of Wnt/FGFs and BMPs in the posterior half and all-trans-retinoic acid (ATRA), TGF-β-ligands, Wnt- and BMP-inhibitors in the anterior half of the endoderm sheet are thought to be responsible for A-P patterning. However, it is currently unclear how these complex interactions can be translated into a differentiation protocol for PSCs. Two PSCs lines were differentiated into DE-like cells applying a single cell based protocol. Next, the effects of Wnt/beta-catenin-, TGF-β-, ATRA-, and FGF2-signaling were tested by various combinations of ligands and inhibitors. Differentiated cells were analyzed by qPCR and immunofluorescence. The treatment of DE-cells with 5 µM CHIR-99021, 25 ng/ml BMP4, 100 nM ATRA and 100 ng/ml bFGF for 48 h resulted in a midgut/hindgut population positive for CDX2. Additionally, these cells expressed a specific combination of HOXC5, HOXC6, and HOXB8 indicating their midgut/hindgut identity. If Wnt- and BMP4-signalling were chemically suppressed, ATRA could induce and posteriorize foregut cells in a concentration-dependent manner. Neither activin A nor FGF-2 alone induced the expression of domain-specific posterior foregut markers. Specifically 0.5/1/2 µM ATRA resulted in a cell population that highly expressed HNF6, HNF1B, and FOXA2 on the gene and protein level typical for foregut cells of the foregut/midgut boundary. The gene expression of MNX1, SHH, HOXC5 and HOXA3 was also detected whereas the anterior foregut endoderm markers TBX1 and HEX1 were suppressed. This study shows that the foregut/hindgut identity is controlled by ATRA- and Wnt/beta-catenin signaling. The treatment of DE-cells with 0.5-2 µM ATRA resulted in a cell population reminiscent of the posterior foregut; a domain which gives rise to liver and pancreas primordia during further human development.

**Keywords: embryonic stem cells; differentiation; anterior-posterior patterning; all-trans retinoic acid; wnt-signaling**
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# Novel tools for generation, purification, and analysis of pluripotent stem cell derived cardiomyocytes

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Pure and well-characterized cardiac cells derived from human pluripotent stem cells (hPSCs) are of high interest for cardiovascular disease modeling, drug safety studies and development of cell replacement strategies. Although several protocols for cardiac differentiation of hPSCs have been developed, major limitations are clone-to-clone variations in differentiation efficacy as well as heterogeneity of generated cardiomyocyte populations. Therefore, we have developed novel tools for cardiomyocyte differentiation, magnetic cell sorting-based purification and cell analysis using new antibody-conjugates enabling for flow cytometry- and immunofluorescence-based identification of cardiomyocytes and subtypes. hPSCs were maintained under xeno-free conditions in our recently developed StemMACS iPSbrew XF medium to keep pluripotency for more than >20 passages and enable for efficient cardiac differentiation. We chose a monolayer differentiation protocol based on the timely regulated activation and inhibition of Wnt signaling by small molecules like CHIR, IWP2 or IWR1. In this setting, first contractions were observed at day 9 of differentiation. In order to identify antibodies suitable for cardiomyocyte enrichment or depletion of non-myocytes, we performed a surface marker screen with more than 400 antibodies between days 10–20 of differentiation. Besides identification of new surface markers, our screen confirmed expression of recently published markers like Sirpα (CD172a) and VCAM-1 (CD106). In order to evaluate kinetics of their expression and correlation with intracellular markers of cardiomyocytes and subtypes, we performed a flow cytometry-based analysis of marker expression until day 45 of differentiation. Our data indicate a dynamic expression pattern for both Sirpα and VCAM-1, either completely or partially overlapping with intracellular cardiomyocyte marker expression. Based on these data we developed magnetic cell separation procedures enriching up to 97 % pure VCAM-1 or Sirpα-positive cell populations. Magnetically enriched cardiomyocytes initiated contractions after replating and could be stably maintained in culture. Taken together, we have developed novel tools supporting the workflow for efficient generation of PSC-derived cardiomyocytes, magnetic purification and flow cytometry or immunofluorecence-based characterization of cardiomyocytes.

**Keywords: stem cells; cardiomyocytes; differentiation**
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# Molecular mechanism involved in cardiac differentiation from embryonic stem cells

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Numerous studies have identified presumed heart progenitors from fetal, postnatal and adult mouse, rat and human hearts. These progenitor cells can differentiate into functioning cardiomyocytes, but in some cases the progenitors only differentiated into immature cardiac cells. Nevertheless, recent clinical assays have indicated the improvement of heart function and life quality of patients treated with autologous cardiac progenitor cells. Hence, understanding the molecular mechanisms that rule the fates and differentiation of the diverse muscle and non-muscle cell lineages of the heart is essential to improve the current therapies and to design novel heart stem cell-based strategies. On the other hand, embryonic stem (ES) cells have the greatest capacity for cell differentiation, including cardiac cell lineages. ES cells long-term self-renewal and survival make them a virtually unlimited supply of cells for regenerative therapy. However, the low efficiency of cardiac differentiation process from ES cells, the teratogenic nature of ES cells and the lack of reliable protocols to purify the desired cells are holding back the use of ES cells-derived cells for clinical application. In vitro ES cell differentiation is a complex, progressive and highly regulated process that relies on the interplay between several signaling pathways and transcriptional network. During the initial phase of cardiac differentiation, mesodermal progenitors express a core and essential proteins which further promote the expression of cardiogenic transcription factors, which will define cells as multipotent cardiac progenitors. Guiding ES cells differentiation towards multipotent cells, controlling their expansion, as well as directing their further differentiation into distinct cardiac lineages remain fundamental challenges in stem cell biology. Using loss- and gain-of-function approaches, we have established that the transcriptional co-activator, Cited2 which is critical for normal heart embryonic development, is required for the optimal mouse ES cells differentiation towards cardiac cell lineages. We have also established that Cited2 function is required for the early steps of ES cells differentiation towards cardiac progenitors.

**Keywords: cardiac differentiation; murine ES cells**
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# Dissection of the regulatory network governing cardiac differentiation in human embryonic stem cells

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Human embryonic stem cells (hESCs) can be differentiated into beating cardiomyocytes with prospects for disease modeling, regenerative medicine and developmental biology. However, a precise understanding of the complex temporal changes in gene expression patterns that have to unfold during this process is missing. By manipulating (knockout/overexpression) putative cardiac differentiation related genes we aim to dissect this network. As a starting point, we chose the T-box transcription factor Eomes (Tbr2) that plays a pivotal role in multiple developmental processes including trophoblast differentiation, gastrulation, and epithelial-mesenchymal transition. Moreover, in mouse, Eomes has recently also been implicated in cardiac development. However, whether this function is conserved in human and if it might be cell-autonomous is not clear. We therefore generated EOMES knockout hESC lines (EO-KO) using the CRISPR/Cas9 method. EO-KO cells formed neuronal structures indicating a failure to differentiate along the mesendodermal lineage (spontaneous differentiation). Next, we used a directed 2D cardiac differentiation protocol that allows the generation of near-homogeneous CTNT positive, beating cardiomyocytes within 7 days. This protocol, in contrast to EB based methods, does not lead to possible signaling gradients and spatial differences across the cells and therefore non-cell-autonomous processes are highly unlikely. Morphological differences between hESCs and EO-KO cells were apparent from as early as day 2 and the EO-KO cells completely failed to differentiate into cardiomyocytes. Further, most genes that display pronounced upregulation during the first days of normal cardiac differentiation, including T (Brachyury), Mesp1, Mixl1 and Cer1, failed to become induced in the absence of EOMES. Interestingly, after 6–8 days of subjecting EO-KO cells to our cardiac differentiation protocol we observed the expression of marker genes that are characteristic of subtypes of placental cells, such as trophoblast giant cells. With these data we demonstrate for the first time that, while its ablation is tolerated for the induction of some lineages, EOMES is, besides its well-known role in endoderm induction, absolutely required for cardiac specification in human in a cell-autonomous manner.

**Keywords: hESCs; mesoderm; cardiomyocytes; EOMES**
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# Investigating the cardiac progenitor stage during cardiomyocyte induction of human pluriopotent stem cells

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During embryogenesis two different lineages of cardiac progenitors originate from mesoderm and contribute to heart development: the First Heart Field (FHF), which gives rise to left ventricle and parts of atria, and the Second Heart Field (SHF), which gives rise to parts of atria, right ventricle and outflow tract. LIM-homeodomain Isl1 protein is a highly conserved component of the transcription factor network driving cardiac development during embryogenesis. Isl1 was for long considered a SHF marker, and in vivo studies show that this transcription factor is required for completing cardiac chamber formation, in part by promoting survival, proliferation and migration by SHF progenitors. Recent evidence, from several animal models, however, suggests that Isl1 may be a more general factor involved in cardiogenesis. In human, the role of ISL1 during cardiac development is not clear. Here we generated an ISL1 knockout model, using the CRISPR/Cas9 system, and an overexpression model, using the Tet-On system, on a human embryonic stem cell (hESC) background. Using a novel 2D cardiac differentiation protocol, through which we are able to differentiate hESCs into cardiomyocytes with high efficiency under defined conditions, we show that ISL1 knockout cells present a delay in terminal differentiation and a delay in cardyomyocyte maturation. Moreover the observation of different features in cardiomyocytes obtained from wild-type, ISL1 knockout and ISL1 overexpressing cells may account for different cardiac populations depending on ISL1 activity. These findings highlight the role of ISL1 during human cardiogenesis using hESCs as a model.

**Keywords: hESCs; cardiomyocytes; CRISPR/Cas9; ISL1**
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# Osteogenic differentiation is negatively influenced by hydrogen peroxide

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Introduction: The treatment of bone defects with mesenchymal stem cells (MSCs) is becoming increasingly important in regenerative medicine. In this context, multipotent stem cells of adipose tissue (ASCs) are gaining increasing importance, since they can be obtained minimally invasive and in sufficient quantity. Furthermore the use of these cells allows autologous reimplantation, which consequently minimizes the risk of rejections. It is known, that an inflammatory reaction is correlated with an increased generation of reactive oxygen species (ROS) in the affected tissue. Regarding osteogenic differentiation, literature describes inhibiting as well as promoting effects of oxidative stress. Therefore it was already shown, that weak oxidative stimulus promotes the differentiation potential. In contrast, supraphysiological oxidative stress was shown to cause a decrease in osteogenic differentiation which was accompanied by increased cell death. In this work we examined the impact of antioxidative substances and of hydrogen peroxide at physiological as well as pathological concentrations on in vitro osteogenic differentiation of ASCs.

Methods: Human ASCs were isolated from fat tissue obtained by liposuction according to Coleman´s protocol. Mesenchymal stem cell identity was proved by plastic adherence, the expression of specific surface antigens and the differentiation capacity into osteoblasts, chondrocytes, and adipocytes. Osteogenic differentiation was initiated by maintaining cell cultures in medium containing dexamethasone (500 nM), ascorbate (50 µM) and beta-glycerophosphate (10 mM). After three weeks, osteogenic differentiation was characterized by the quantification of calcified matrix, alkaline phosphatase-expression and the expression of osteoblast-specific proteins. Oxidative stress during the differentiation was quantified by Dichlorodihydrofluorescein Diacetate (DCFDA). In order to demonstrate the role of antioxidants during the differentiation process, cells were treated with glutathione (1 mM), N-Acetyl-Cysteine (NAC, 1 mM), catalase (500 U), the synthetic superoxide dismutase and catalase mimetic EUK134 (50 µM), ascorbate (0,5 mM) and the water soluble vitamin E derivate Trolox (1 mM).

Results and discussion: During osteogenic differentiation, ROS-Expression continuously increased. Exogenous addition of hydrogen peroxide caused increased cell death and decreased osteogenic differentiation. The antioxidants, we treated the cells with, exerted different effects on osteogenesis: catalase led to a significantly stronger expression of calcified matrix, even in “non-responder” cell preparations that are characterized by a failed potency to differentiate under standard condition, whereas the presence of ascorbate significantly decreased osteogenic differentiation of ASCs. The other antioxidants mentioned above showed no significant effects on induced osteogenic differentiation of ASCs. Our Results reveal, that a specific intracellular depletion of hydrogen peroxide has a promotional effect on the osteogenic differentiation. This highlights, that hydrogen peroxide negatively affects osteogenic differentiation, while the presence of ascorbate-sensible oxygen radicals seems to play an essential role as a stimulus for osteogenic differentiation.

**Keywords: osteogenic differentiation; reactive oxygen species; adipose derived stem cells; antioxidants; hydrogen peroxide**

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# Human induced pluripotent stem cell-derived neural progenitors generate functional glutamatergic and gabaergic neurons

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Neurodegenerative and psychiatric diseases are often associated with genetic variations. Specific single nucleotide polymorphisms (SNPs) and copy number variations (CNVs) presumably affect the neural development, resulting in modified gene expression patterns up to malfunctioning neural cells. Not least the lack of appropriate animal models complicates the investigation of altered biological pathways. Non-viral reprogramming enables the generation of induced pluripotent stem cells (iPSCs) with patient-specific genetic information. Thus, iPSCs provide a useful tool for the in vitro modeling of diseases by analyzing early and late neural development. Here, we induced well-established human iPSCs into neuroepithelial rosette-like structures to obtain three-dimensional neural tube-like structures within the rosettes. The 3D structures contained self-renewing neural stem cells (NSCs), which were initially cultured in non-adherent neurospheres to improve cell proliferation. Within 3 weeks, we received stable populations of NSCs. The induction of neural cell fates was demonstrated through transcript analysis of specific markers (MSI-1, S100B, PAX6). Proliferation and neural stem cell identity was also analyzed in double immunostaining of Ki67, SOX2 and NESTIN. Since NSCs retain the potential to generate the three main neural cell lineages (neurons, astrocytes and oligodendrocytes), we subsequently performed an in vitro neurogenesis to acquire functional neurons. Within 8 weeks, NSCs generated both glial progenitors (GFAP, O4) as well as neuronal fated cells (NeuN, TUBB3, NEFL). The regional identity of the neurons was confirmed through the expression of the telencephalic and hippocampal markers FOXG1, OTX2, EMX1 and PROX1 as well as the rhombencephalic markers NKX2.2 and OLIG2. Protein analysis, transcript analysis and whole-cell patch-clamp recordings verified the presence of specific postmitotic neuron subtypes such as inhibitory GABAergic (GABRA1, GABA) and excitatory glutamatergic (GLUR2, vGLUT1, GAD1/2) cortical cells. Additionally, the differentiation model and the culture conditions were transferred to a 3D culture system allowing the generation of cerebral organoids. In order to mimic brain regions including the cerebral cortex, human NSCs were cultured as free-floating neurospheres in bioreactors to improve nutrient absorption. Within 4 weeks, the neurospheres differentiated into cerebral organoids (approximately 1.5 mm in diameter). Early neural development was confirmed through transcript and protein expression of specific markers such as PAX6, SOX2 and NESTIN. Immunostaining of Ki67 as well as NeuN and TUBB3 demonstrated the presence of post mitotic neural cells. Different subtypes such as GABAergic neurons (GABA) and motoneurons (HB9) with forebrain and hindbrain identities (FOXG1/NKX2.2) were also detectable within the cerebral organoids. Together, these results confirmed the efficient neural differentiation of human iPSCs using two different culture systems. These culture systems enable functional studies of healthy and diseased human cortical development. Thus, in vitro disease modeling will help to investigate neuronal dysfunctions associated with specific genetic variations.

**Keywords: neural stem cell; rosette; neural differentiation; cortical neuron; cerebral organoid**
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# Acquisition of fetal features in mesenchymal stem cells derived from iPS cells from aged individuals

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Short life span in culture and restricted differentiation potential are the limiting factors of the in vitro expansion and application potential of human bone marrow derived mesenchymal stem cells (hBM-MSCs) from aged donors. The generation of hMSCs from induced pluripotent stem cells (iMSCs) has been reported as a possible solution. However it is not fully enlightened if there is a reversion of the age-associated phenotype into a younger phenotype upon derivation of iMSCs. To investigate new aspects of potential age influences in deriving iMSCs from iPSCs pluripotency was induced in hBM-MSCs from fetal femur (55 days post conception) and aged donors (60–70 years) and the cells were subsequently differentiated into iMSCs. First of all normal karyotypes were detected in both groups. HBM-MSCs from aged donors showed higher levels of ROS, phosphorylated H2AX as well as slower proliferation rates. Retroviral or episomal reprogramming of fetal hBM-MSCs was more efficient and faster compared to hBM-MSCs from aged donors. IMSCs irrespective of source showed the typical MSC specific immunophenotype and tri-lineage differentiation potential. IMSCs generated from aged hBM-MSCs showed similar morphologies, senescence phenotypes and transcriptomes as fetal hBM-MSCs and their corresponding iMSCs. Secretome analysis revealed a similar profile for parental hBM-MSCs and iMSCs (PDGF-AA, MCP-1, MIF, Serpin E1). All these similarities were also observed in iMSCs originated from human dermal fibroblasts and the ES line H1. In summary we have shown that (a) donor age influences the efficacy of reprogramming of hBM-MSCs into a pluripotent state. (b) The transcriptomes of iPS cells derived from fetal and aged BM-MSCs are more similar to that of hESCs than to that of the parental cells. (c) iMSCs irrespective of donor age and source acquire typical features of BM-MSCs. In conclusion, generation of iMSCs by-passes the shortfalls associated with the expansion of native MSCs and thus makes it possible to maximize their tremendous potential in regenerative medicine.

**Keywords: MSCs; iPS Cells; iMSCs**
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# Different FGF ligands and concentrations with Wnt signalling, induce distinct PSM states

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Presomitic mesoderm (PSM) is the origin of somites that flank both sides of the neural tube and plays a central role in subsequent body patterning. Wnt and FGF signalling control the sequential formation of somites, the origin of musculoskeletal system, and show graded distribution within the PSM of vertebrate embryos. To decipher the role of Wnt and Fgf signalling during PSM specification, we employed ESCs carrying reporters for T, Tbx6 or Msgn1 to differentiate from naïve state to EpiSCs and further to PSM cells. Here we show that by varying the FGF ligand or FGF concentration, the state of PSM cells formed can be altered. When combined with Wnt activation (CHIR99021 (CH)), high FGF concentration supports posterior PSM, whereas low FGF supports an anterior/differentiated PSM state. Our results show that Activin/Nodal signaling causes EpiSCs to be refractory to CH-mediated PSM induction, without affecting T-expression. Activin/Nodal inhibition enhances PSM induction by high FGF/high Wnt signaling. Taken together, this study shows that high FGF, combined with high Wnt signaling induces posterior PSM cells, and that T, Tbx6 and Msgn1 are differently controlled, depending on the type and concentration of the FGF ligand.

**Keywords: embryonic stem cells; epiblast stem cells; presomitic mesoderm; paraxial mesoderm; Wnt; differentiation; FGF; Activin/Nodal; T; Tbx6; Msgn1; Brachyury; Mesogenin1; somitogenesis; somites; FGF2; FGF8**
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# Ectopic HOXB4 enforces hematopoietic development during transition from the hemangioblast to the hemogenic endothelium during pluripotent stem cell differentiation

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Generation of hematopoietic stem cells (HSCs) from pluripotent stem cells, in vitro, holds great promise for future somatic gene and cell therapy. So far, HSCs capable of long-term multilineage reconstitution in mice have only been obtained when the homeodomain transcription factor HOXB4 was ectopically expressed during pluripotent stem cell differentiation. However, the primary "target" cell of HOXB4 during hematopoietic development, in vitro, is not yet known. Its identification is a prerequisite for unambiguously identifying the molecular circuits driving HSC development, at least in vitro. To pin down this cell, we retrovirally expressed HOXB4 or a Tamoxifen-inducible HOXB4-ERT2 fusion protein in different reporter and knock-out mouse embryonic stem cell (ESC) lines. For these experiments, ESCs were differentiated for 6 days as embryoid bodies (EBs), dissociated and subsequently cocultured on OP9 stroma cells for further 3 days. Use of a Runx1(-/-) ESC-line containing a doxycycline-inducible Runx1 coding sequence ("iRunx1"; kindly provided by G. Lacaud, Manchester) uncovered that HOXB4 acts during formation of the hemogenic endothelium (HE) from which HSCs arise. Without Runx1 induction, which arrests hematopoietic development at the HE-stage, ectopic HOXB4 expression mediated an approximately 30-fold increase in the number of circular endothelial HE-sheets being Flk1+VE-Cadherin+Tie2+ and expressing Sox17 and Lmo2. Limiting dilution experiments uncovered that this was due to a significant increase in the number of progenitors generating hemogenic endothelial cells. After additional Runx1 induction, the endothelial cells morphologically underwent an Endothelial-to-Hematopoietic Transition (EHT) as verified at the single cell level by time-lapse microscopy. Concomitantly, they upregulated transcription of Gfi1, Gfi1b and Pu.1, initiated surface expression of the pan-hematopoietic marker CD45 and generated hematopoietic colony forming cells, thus proving their identity as real hemogenic endothelial cells. So far, our results strongly suggest that HOXB4 acts by supporting the entry into the hematopoietic program during mouse pluripotent stem cell differentiation by increasing the number of HE-progenitors, most likely by biasing differentiation of the bipotent progenitor, the hemangioblast, towards hematopoiesis

**Keywords: ESCs; hematopoietic differentiation; HOXB4; hemogenic endothelium; endothelial-to-hematopoietic-transition**
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# In real-time monitoring the fate of transplanted stem cells in the brain

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Introduction: Stroke is one of the most common causes of death in the world. Despite the high prevalence of stroke, there are only limited therapy options, none of which are effective for restoring lost neurological function. Most recently, the use of exogenous stem cells as therapy approach has arisen of high value in restoring neurological function. Here, we describe a completely new strategy to monitor in vivo the fate of transplanted stem cells in real-time. For this purpose, human neural stem cells (H9 hNSC) were stably transduced to express imaging reporters under the control of the early neuronal promoter doublecortin (DCX) or the mature neuron promoter synapsin (Syn) and the cells were monitored with bioluminescence imaging following grafting into mouse brain.

Methods: The imaging reporters ferritin (Fer) – for magnetic resonance imaging (MRI), firefly luciferase (Luc2) – for bioluminescence imaging (BLI) and GFP – for fluorescence imaging (FI) were cloned in a lentiviral vector system. To ensure that the signal is restricted to neuronal cells the imaging reporters were expressed under the control of either the DCX promoter or the Syn promoter. The H9 hNSCs were transduced by lentiviral gene transfer. A number of 3x105 undifferentiated H9 DCX-Fer-Luc2-GFP cells or H9 Syn-Fer-Luc2-GFP or as control H9 EF1α-Luc2-GFP were implanted in the cortex of nude mice. BLI was recorded twice per week over 6 weeks, or 3 months, respectively. Finally, brains were either examined by electrophysiology in vivo or processed for histology.

Results: The time profile of neuronal maturation upon transplantation of human stem cells into nude mouse brain was well traceable by BLI. At day 0 no reliable signal-to-noise ratio was observed in animals transplanted with cells expressing Luc2 under control of a cell specific promoter. However, at day 4 the DCX-Luc2 signal was clearly detectable and the signal intensity increased continuously over time with a peak at 4 weeks post transplantation. Quantitative analysis of the BL signal (normalized to day 0) revealed that the DCX-Luc2 signal increase is significant (40-fold). The Syn-Luc2 signal was detected after 4 weeks post transplantation and the signal increased over 3 months. In contrast, the EF1α-Luc2 signal in the control group remained stable during the experiment indicating long term graft vitality. We validated the in vivo imaging results by electrophysiology and histology analysis of the engrafted cells. The results confirmed the finding that the transplanted stem cells differentiated spontaneously to early functional neurons.

Conclusions: Here, we describe a new and highly sensitive protocol for functional cell fate mapping to monitor in vivo neuronal differentiation of transplanted hNSC with noninvasive imaging techniques. This novel approach of cell-specific imaging is of particular interest as it provides the key tool to track differentiation, vitality, location, and functionality of transplanted stem cells in a longitudinal study.

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**Keywords: Human neural stem cells; neuronal differentiation; doublecortin promoter; synapsin I promoter; in vivo bioluminescence imaging**
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# Minocycline mitigates the gliogenic effects of pro-inflammatory cytokines on neural stem cells

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Mobilizing endogenous neural stem cells (NSC) in the adult brain has emerged as an experimental concept, aimed at enhancing the brain's regenerative capacity after cerebral lesions such as stroke. However, cerebral ischemia elicits multifaceted neuroinflammatory processes affecting NSC in multiple ways that are only marginally understood to date. The tetracycline minocycline inhibits microglia activation as a hallmark of post-ischemic neuroinflammation and has already been evaluated in clinical trials, but its effects on NSC – in particular on their differentiation potential – are unknown to date. We here investigated the direct effects of minocycline and of pro-inflammatory cytokines on the differentiation potential of NSC in vitro and in vivo. To assess the differentiation potential of NSC in vitro, primary monolayer-cultures of cortical fetal rat NSC were treated with minocycline plus a combination of the pro-inflammatory cytokines TNF-alpha, IL-1β, and IL-6. Differentiation fate of NSC was assessed immunocytochemically. To investigate the effects of minocycline and inflammation in vivo, minocycline or LPS were injected intraperitoneally into adult rats, with subsequent immunohistochemical analyses. Minocycline alone did not affect the differentiation potential or differentiation speed of NSC in vivo and in vitro. Pro-inflammatory cytokines, on the other hand, accelerated the differentiation of NSC, promoting an astrocytic fate while inhibiting neurogenesis in vitro and in vivo. Most interestingly, minocycline counteracted this cytokine-induced rapid glial differentiation and thus restored the neurogenic potential of NSC. Minocycline antagonizes the rapid glial differentiation induced by pro-inflammatory cytokines following cerebral ischemia, without having an own direct effect on the differentiation potential of NSC. Thus, minocycline constitutes a promising drug in stroke research, counteracting the detrimental effects of post-ischemic neuroinflammation in multiple ways.

**Keywords: endogenous neural stem cells; neuroinflammation; minocycline; stroke**
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# Stem Cell Epigenetics

# Epigenetic-senescence-signature reflects heterogeneity of cellular aging within mesenchymal stromal cells

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Mesenchymal stromal cells (MSCs) comprise various different subpopulations. This is reflected by variation in phenotype, proliferation, and differentiation potential of subsets within these cell preparations. On the other hand, standardization of MSCs is a prerequisite for therapeutic applications in regenerative medicine. Therefore a better understanding of cellular heterogeneity is important. Here, we follow the hypothesis that the epigenetic state of cellular aging varies within MSC-preparations as well. To compare single cell derived MSC clones we utilized our Replicative-Senescence-Signature – a biomarker which is based on DNA-methylation levels at six specific CpGs to estimate the state of cellular aging. So far, this analysis was based on DNA-methylation levels that were determined by pyrosequencing of bisulfite converted DNA. This method was now further developed into deep sequencing of barcoded bisulfite amplicons to facilitate simultaneous comparison of multiple clones. The deep sequencing results correlated with pyrosequencing data of the Epigenetic-Senescence-Signature. Furthermore, predictions for cumulative population doublings (cPDs) and passage numbers were in line with real cPDs and passages in the conventional, bulk cultured MSCs. However, there was a huge variability of senescence-predictions for single cell derived colonies and they were hardly associated with real passage numbers. Notably, the DNA-methylation levels at neighboring CpG sites revealed stark changes (ranging from 0 % to 100 %) in single cell derived clones whereas the DNA-methylation levels hardly reflected variance in these genomic regions in bulk MSCs. Significant correlation of Epigenetic-Senescence-Signatures and osteogenic differentiation potential was not observed in single cell derived clones. Our results indicate that the epigenetic state of cellular aging is very heterogeneous within MSCs and this heterogeneity seems to contribute to the phenotypic variability within MSC populations.

**Keywords: epigenetics; MSC; single cell clones; heterogeneity; DNA-methylation**
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# DNA-methylation patterns for tissue-type and aging are erased in mesenchymal stromal cells derived from human induced pluripotent stem cells

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Human mesenchymal stromal cells (MSCs) are heterogeneous cell preparations that lose differentiation potential during culture expansion. Standardization is hampered by variation in starting material and cell culture methods which renders comparison between studies difficult. In contrast, induced pluripotent stem cells (iPSCs) assimilate towards a ground-state. Therefore, generation of MSCs from iPSCs holds the perspective to provide more standardized and homogeneous MSC preparations. We have recently generated iPSCs from bone marrow MSCs. Here, we describe that these MSC-derived iPSCs can be re-differentiated into cells which closely resemble MSCs on functional level. The protocol involves the same culture conditions as used for initial MSC culture using human platelet lysate (hPL) as serum supplement. Within two weeks, iPSC-derived MSCs (iPS-MSCs) down-regulated pluripotency markers and displayed a typical fibroblast-like morphology. The immunophenotype of iPS-MSCs was the same as in primary MSCs (CD14–, CD29+, CD31–, CD34–, CD45–, CD73+, and CD90+) – only CD105 was slightly less expressed in iPS-MSCs. Furthermore, iPS-MSCs were induced towards adipogenic, osteogenic, and chondrogenic lineages. Their differentiation potential was similar to primary MSCs, albeit adipogenic differentiation was less pronounced. Genome wide gene expression profiles supported the notion that iPS-MSCs closely resemble primary MSCs: marker genes for mesodermal lineage and MSCs were expressed at similar levels in iPS-MSCs and primary MSCs. However, DNA methylation (DNAm) profiles – analyzed by Infinium HumanMethylation450 BeadChip technology – revealed marked differences between iPS-MSCs and primary MSCs. Donor-specific differences in DNAm profiles were maintained throughout reprogramming and re-differentiation. However, characteristic DNAm patterns for bone marrow derived MSCs as compared to adipose tissue derived MSCs were not recapitulated in iPS-MSCs. Furthermore, age-related DNAm was reset in iPSCs and not reacquired in iPS-MSCs, indicating that the cells remain epigenetically rejuvenated. Senescence-associated DNAm changes – which accumulate during in vitro culture expansion of primary cells – were also reset by reprogramming into iPSCs but these changes were continuously adopted according to the number of population doublings upon loss of pluripotency. Overall, iPS-MSCs and MSCs are similar in function but iPS-MSCs reveal incomplete re-acquisition of MSC-specific DNAm patterns – particularly of DNAm patterns associated with tissue type and aging. Our results indicate that iPS-MSCs provide an attractive, unlimited, and more standardized source of MSCs.

**Keywords: induced pluripotent stem cells; mesenchymal stem cells; DNA methylation; epigenetic memory; aging**
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# Epigenetic biomarker to support classification into pluripotent and non-pluripotent cells

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Several methods can be used as surrogate assay to determine pluripotency of induced pluripotent stem cells (iPSCs) – e. g. analysis of iPSC-colony morphology, surface marker expression, analysis of individual genes or gene expression profiles, multilineage differentiation potential in vitro, or teratoma formation. There is a clear trade-off between cost and labor-intensive methods on the one hand and reliability on the other. Differentiation potential of iPSCs is also reflected by a unique epigenetic makeup in DNA methylation (DNAm) profiles. Here we describe a simple method to estimate pluripotency which is based on the DNAm level at only three CpG sites. Two of these CpG sites were selected based on their discriminatory power in 258 DNAm profiles (63 pluripotent, 195 non-pluripotent; 450k Illumina BeadChips, www.ncbi.nlm.nih.gov/geo/). They become either methylated or demethylated in iPSCs and their combination is referred to as "Epi-Pluri-Score". In addition, a third CpG located in the pluripotency-associated gene POU5F1 (OCT4) was considered. This epigenetic signature was validated on independent DNAm datasets (264 pluripotent and 1951 non-pluripotent samples; 27k Illumina BeadChips) with 99.9 % specificity and 98.9 % sensitivity. Notably, the method could also discriminate partially or improperly reprogrammed cells. Subsequently, we established pyrosequencing assays to specifically analyze DNAm at the CpGs of the Epi-Pluri-Score. The results allowed reliable classification of 18 pluripotent cell lines and 31 non-pluripotent cell lines. DNAm changes at these three CpGs were subsequently analyzed in the course of differentiation of iPSCs towards mesenchymal stromal cells demonstrating that particularly the CpG site in POU5F1 demarcates early differentiation events. Taken together, DNAm level of three specific CpG sites provides a simple and robust biomarker for analysis of pluripotency with high sensitivity and specificity.

**Keywords: iPSC epigenetics pluripotency biomarker**
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# Epigenetic changes during in vitro activation of hepatic stellate cells

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Hepatic stellate cells (HSC) were identified recently as liver-resident mesenchymal stem cells. In the healthy liver HSCs are characterized by high vitamin A content and they reside in a quiescent state in the space of Disse. Upon liver injury they are activated to myofibroblast-like cells that contribute to liver regeneration and fibrosis. This process is accompanied by changes in morphology and gene expression. It is already known that single genes exhibit an increased DNA methylation during HSC activation, but genome-wide DNA methylation studies are still missing. Therefore, we analyzed global, genome-wide and gene-specific DNA methylation during HSC activation to gain insight into the general epigenetic control of this process. On the global level, HSCs revealed a decrease in DNA methylation of about 60 % during activation. In contrast to this, the genome-wide detection of this modification with Methyl-Mini Seq Epiquest sequencing displayed both, regions of hyper- and hypomethylation. Changes in DNA methylation occurred in promoter regions as well as intragenic CpG-islands. GO-term analysis showed that differentially methylated genes are involved in various cellular processes like the regulation of cell activation and signaling. Several differentially methylated genes were identified that showed a good correlation between DNA methylation and gene expression changes. Finally, L-mimosine treatment and a bromodeoxyuridine assay demonstrated that the observed DNA demethylation is based on an active mechanism independent of DNA synthesis. Taken together, the data show remarkable changes in DNA methylation which may control gene expression during HSC activation. Furthermore our data reveal that initial DNA demethylation during HSC activation depends on an active mechanism. Such epigenetic mechanisms may be essential processes controlling quiesence and activation of adult stem cells.

**Keywords: hepatic stellate cell; stem cell activation; DNA methylation**
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# Deep bisulfite sequencing analysis of germline imprint stability in Angelman syndrome iPSCs

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Genomic imprinting is an epigenetic phenomenon resulting in parent-of-origin dependent gene expression. The hallmark of imprinted genes is a differentially methylated region (DMR) which acquires DNA methylation in one of the parental germlines. This differential DNA methylation is stably maintained throughout development and thought to be stable during reprogramming. Angelman syndrome is an imprinting disorder associated with severe intellectual disability, absence of speech, ataxia and other specific symptoms. It is caused by the disruption of the maternal copy of the UBE3A gene, resulting in absence of funtional UBE3A protein in the brain. Imprinted expression of UBE3A is controlled by the differential methylation at PWS-SRO, which locates to the promotor and exon 1 of SNURF-SNRPN. This methylation is set in the female germline. On the paternal chromosome, the unmethylated PWS-SRO serves as a neuron-specific promoter for a non-coding RNA which overlaps the UBE3A gene in antisense direction, resulting in silencing of the intact paternal UBE3A gene. We generated and characterised iPSCs of a patient with Angelman syndrome and a healthy control person. Several iPSC clones were positively tested for expression of various pluripotency genes using alkaline phosphatase staining, immunohistochemistry, FACS analysis and qRT-PCR. Quality testing included Southern blotting to assess the number of vector integration sites, karyotyping and testing for cellular identity. Analysis of methylation imprint stability was performed using next generation amplicon bisulfite sequencing on the Roche 454 GS Junior system. We investigated the germline DMRs of the six best studied imprinted gene loci: PWS-SRO of the PWS/AS region on chromosome 15q11q13, ICR2 (KCNQ1OT1) and ICR1 on chromosome 11p15.5, CpG85 of RB1 on chromosome 13q14.2, NESP-AS wihin the GNAS locus on chromosome 20q13.2 and IG-DMR between DLK1 and MEG3 on chromosome 14q32. We observed a stable level of differential methylation at PWS-SRO, ICR2 and ICR1. As has previously been described in literature, IG-DMR showed a consistent higher level of methylation in our clones. Methylation levels of CpG85 generally vary depending on the analysed tissue, so an approximate 50 % level of methylation is usually only detected in blood. In all our iPSC clones we observed levels of methylation higher than 90 % at CpG85. NESP-AS showed levels of methylation ranging from 2–64 %. Our data suggest that the methylation imprint is not stable at all imprinted loci during reprogramming and can vary between clones.

**Keywords: iPSCs; UBE3A; Angelman syndrome; methylation; imprinting**
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# Stem Cells in Development

# Endodermal progenitor cells derived from integration-free iPSCs as an in vitro model for dissecting endodermal cell fate decisions

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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. We have generated episomal-derived and integration-free iPSCs (E-iPSCs) from human fetal foreskin fibroblast cells (HFF1). 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 derived endodermal progenitor (EP). These LGR5 positive cells grow as organoids and express HNF4A, PDX1 and CDX2 which are key transcription factors specifying liver, pancreas and intestine. This expression pattern implies that these EPs may be multipotent. 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 bi-potential 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: iPSC; endodermal progenitors; EP**
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# Integration-free iPSCs as a tool for modeling hepatogenesis in vitro

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Induced pluripotent stem cells (iPSCs) are embryonic-like cells and can be generated from somatic cells by the over-expression of OCT4 and SOX2 in combination with either KLF4 and c-MYC or NANOG and LIN28. Like ES cells iPSCs self-renew and are pluripotent, thus making them an idea source for studying human gastrulation in vitro. We have generated episomal-derived and integration-free iPSCs (E-iPSCs) from human fetal foreskin fibroblast cells (HFF1). E-iPSCs were fully characterized and their transcriptomes are more similar to that of hESCs (R2 = 0.9363) in comparison to viral-derived HFF-iPSCs (R2 = 0.8176). We used the E-iPSC line to model hepatogenesis in vitro. The differentiation of hepatocyte-like cells (HLCs) involves a three-step process, from the undifferentiated E-iPSC to definitive endoderm (DE), to hepatic endoderm (HE) and ultimately HLCs. The HLCs were fully characterized biochemically, i.e. glycogen storage, ICG uptake and release, UREA production, and CYP3A4 activity. Ultra-structure analysis by electron microscopy revealed the presence of lipid and glycogen storage, tight junctions and bile canaliculi- all typical features of hepatocytes. Furthermore, the transcriptome of undifferentiated E-iPSC, DE, HE and HLCs were compared to that of fetal liver and primary human hepatocytes (PHH). K-means clustering identified 100 clusters which include developmental stage-specific groups of genes, e.g. OCT4 expression at the undifferentiated stage, SOX17 marking DE stage, AFP and ALB at the HLC stage. The NOTCH and HIPPO pathways were over represented in HLC, fetal liver and PHH. In summary, we have generated episomal-derived iPSCs (E-iPSCs) and demonstrated that they are pluripotent both in vitro and in vivo. These E-iPSCs are able to mimic hepatogenesis and represent a tool for studying human gastrulation at the molecular and cellular levels in vitro.

**Keywords: iPSC; HLC; hepatocytes**
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# Freezing of regional identity upon in vitro conversion of NES cells into radial glia-like neural stem cells

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In recent years, adherently growing human neural stem cells with different characteristics and from different sources have been established using growth factor-based protocols. A still open question is to what extent these diverse stem cell populations reflect transitional stem cell states observed across nervous system development. In the developing embryo early neuroepithelial stem (NES) cells with a polarized morphology and responsiveness to regionalizing morphogens give rise to radial glia (RG) cells, which generate region-specific neurons. Recently stable neural cell populations reminiscent of NES cells have been obtained from pluripotent stem cells (It-NES cells) and the fetal human hindbrain (hbNES cells). Here, we explore whether these cell populations, similar to their in vivo counterparts, can give rise to regionally specified RG-like cells. To that end we propagated It-NES and hbNES cells temporarily in differentiating conditions. Upon re-initiation of growth factor treatment, these cells were found to enter a developmental stage reflecting major characteristics of RG cells. These RG-like neural stem (NS) cells could be expanded for multiple passages and express markers typically associated with RG cells, while NES cell markers were down-regulated. Gene ontology analysis of differentially expressed genes revealed enrichment of transcripts associated with embryonic development and cell proliferation in NES cells whereas RG-like NS cells showed pronounced expression of genes associated with glial cells, brain morphology and neuronal physiology. Interestingly, RG-like NS cells derived from NES cells with different regional identity exhibited stable region-specific transcription factor expression with anteriorized and posteriorized cells maintaining their positional identity during multiple passages of in vitro proliferation. Preservation of positional identity was remarkably robust and could not be overcome even by strong regionalizing factors such as retinoid acid. Upon differentiation RG-like NS cells generated functional region-specific neurons appropriate for their positional identity. Importantly, RG-like NS cells obtained from pluripotent stem cell-derived It-NES cells and hbNES cells as well as primary human RG cells showed similar properties, suggesting that conversion of NES cells into RG-like NS cells recapitulates the developmental progression of early NES cells into radial glia cells observed during nervous system development.

**Keywords: neural stem cells; radial glia cells; neural development;**
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# Lineage tracing of hematopoietic stem cells to epidermal Langerhans cells

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Hematopoietic stem cells give rise to all cells in blood and lymphoid tissue, including dendritic cells (DC). DC are professional antigen presenting cells with a pivotal role in immunity and tolerance. A special type of DC are Langerhans cells (LC), DC in skin epidermis that represent a first barrier for invading pathogens. LC originate from embryonic precursors in fetal liver that seed the developing skin from E14.5 of mouse development. Shortly after birth LC precursors differentiate into LC, which is characterized by up-regulation of CD11c, MHC class II (MHC-II) and langerin expression. The LC network is maintained during life by a self-renewing local precursor without contribution from bone marrow. We have shown before that the helix-loop-helix transcription factor Id2 is a critical regulator of LC biology and adult Id2-/- mice lack LC (Hacker et al., Nat Immunol. 2003; Seré et al., Immunity 2012). Here we investigated how Id2 regulates LC precursor development, recruitment to skin and differentiation. We performed kinetics of LC differentiation and expansion in epidermis of neonatal Id2-/- mice. Surprisingly, we detected MHC-II+CD11c+ cells in Id2-/- epidermis at postnatal day 3 (P3) but these cells did not up-regulate langerin during differentiation. Furthermore, the number of MHC-II+ cells at P3 was lower in Id2-/- epidermis compared to Id2+/- littermates and further decreased with time. At P22, virtually all MHC-II+ cells had left the Id2-/- epidermis. Interestingly, we found that Id2-/- cells expressed lower levels of the adhesion molecules EpCAM and E-Cadherin compared to Id2+/- cells. Taken together, this suggests that in the absence of Id2 LC are impaired in anchoring in the epithelial environment. To study the underlying mechanism of LC recruitment to and anchoring in epidermis, we used a lineage tracer model of Id2-CreERT2xRosa26eGFP (Rawlins et al., Development 2009). We show that Id2-eGFP expression is low in embryonic LC precursors and is enhanced shortly after birth, thereby underscoring the requirement of Id2 for LC differentiation and expansion. In summary, our data demonstrate that (i) development of embryonic LC precursors and their recruitment to epidermis are not dependent on the transcription factor Id2 and (ii) anchoring of LC in epidermis requires Id2.

**Keywords: embryonic precursors; dendritic cells; epidermal Langerhans cells; lineage tracing; transcription factor Id2**
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# Transcriptional Regulation & Non-Coding RNAs

# Pre-rosette NSCs for studying the impact of miRNAs on dopaminergic differentiation

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Midbrain dopaminergic (mDA) neurons are progressively lost in patients suffering from Parkinson's disease. Thus, much effort has been made to derive in vitro counterparts of mDA neurons from human stem cells that could be used for drug screening approaches or cell replacement therapies. It is either possible to directly generate mDA neurons from human pluripotent stem cells (hPSC) or to use intermediate populations of self-renewing neural stem cells (NSCs) that can be directed towards dopaminergic differentiation. There are different types of neural stem/precursor cell (NSC/NPC) populations that can be generated from hPSCs. These populations can be discriminated based on their ability to form neural rosettes, their differentiation potential and their responsiveness to morphogens, including dopaminergic patterning factors. Based on experiments using rosette-forming neuroepithelial-like stem cells (lt-NES cells; Koch et al, 2009), we have recently shown that miRNAs can be used to modulate dopaminergic differentiation (Stappert et al, 2013). As a next step we set out to transfer these findings to a NSC population representing an earlier, pre-rosette stage more amenable to dopaminergic patterning cues. For that purpose, we first implemented and adapted a protocol for pre-rosette NSC generation (Reinhardt et al, 2013). Immunofluorescence analysis confirmed the pre-rosette identity of these cells with expression of the neural precursor marker Nestin and a non-polarized expression of membrane proteins such as ZO1 and N-cadherin. As another validation step we assessed the potential of the cells to transit into a rosette-forming stage upon FGF2 treatment. Next, we transduced the cells with a conditional overexpression system coding for several miRNA constructs and selected target genes. In order to analyze the impact of these miRNAs on neuronal differentiation, we also introduced a genetic neuronal reporter construct. This construct contains an EGFP cassette under control of the Doublecortin DCX promoter, which is selectively activated in young neurons. With these tools in hand we expect to explore the role of miRNAs in pre-rosette NSCs with the intention to further optimize the in vitro generation of dopaminergic neurons.

**Keywords: miRNA; neural stem cells; dopaminergic neurons; Parkinson's disease; hPS cells**
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# Long non-coding RNAs control lineage programming during early embryonic development

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It is hypothesised that early life forms relied on RNA both for the storage of genetic information and for the catalytic functions that sustained life. Later on during evolution, the coding of genetic information was entitled to chemically more stable DNA and the catalytic function was bestowed to more chemically versatile and flexible proteins. Although a major player in controlling translation and splicing events in higher eukaryotes, RNA was for a long time believed to solely act as a messenger between DNA and proteins. However, large scale genomic studies in the past decade have significantly challenged that notion. Interestingly, neither the number of protein families with specific function nor the number of encoded proteins changed enough across metazoan evolution to explain the glaring differences in developmental complexity in higher order eukaryotes. There is increasing evidence that apart from the use of alternative splicing to expand the functional proteome in more developmentally sophisticated organisms, the answer might be lying in the huge gap between transcribed genetics information and translated genetic information. In fact, ~98 % of the transcribed human genome is not translated. It has been demonstrated that the transcribed, but untranslated part of the genome increases dramatically with increasing developmental complexity. This suggests that these sequences harbour extensive regulatory non-coding information which might dictate gene expression during increasingly complex development and differentiation programs. In order to identify these non-coding elements, we mapped the dynamic transcriptome during human cardiovascular development using a holistic approach combining state-of-the-art pluripotent stem cell-based defined in vitro differentiation systems and systems biology. This lead to the identification of ~2000 novel long non-coding RNAs (lncRNA) that were specifically expressed in defined developmental stages. Seventy-six of these lncRNAs were evolutionarily conserved, indicating a central role in developmental decisions. We then functionally characterized 3 lncRNAs essential for pluripotency, mesoderm commitment and cardiovascular development, respectively. By in vivo studies using developing zebrafish embryos, we could indeed confirm that these lncRNAs are functionally conserved through vertebrate evolution. Taken together, our data indicate that conserved lncRNAs orchestrate lineage decisions during early embryonic development.

**Keywords: long non-coding RNAs; RNA-seq; lineage commitment; embryonic development; cardiovascular biology**
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# Investigation of miRNA depending regulation of different protein targets in hybrid cells derived from breast cancer cells and cells with stem cell properties

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Cell fusion is a process occurring in cancer. Due to this, new cells with new properties could originate that may differ from their parental cells. We obtained our hybrid cells by spontaneous fusion events in vitro between a breast cancer cell line (HS578T Hyg) and a cell line with stem cell properties (M13SV1-EGFP-Neo). From a series of obtained hybrid cell lines we picked two clones (M13HS-2, M13HS-8) for further investigations. Previous studies revealed i. a. a different behavior in cell migration depending on an altered cell signaling compared to each other as well to the parental cells. In the present study we focus on a deeper characterization on mRNA expression, miRNA expression and protein regulation by miRNA. Microarray studies for the tested cell lines for mRNA and miRNA were made. Some interesting mRNAs were analyzed by PCR and their protein expression was investigated by Western Blot. miRNAs of interest were validated by quantitative real time PCR (qPCR) and Luciferase based functional studies. Likewise, an over expression system for miR-222-3p was designed. Microarray analyses for mRNA and miRNA revealed that each tested cell line exhibit a unique mRNA and miRNA expression profile. Comparison of microarray data showed a putative correlation for MMP1 and ERĐ expression levels and miRNA expression levels in the investigated cell lines. For instance, MMP1 was markedly upregulated in both hybrid cell clones, whereas M13SV1 cells lack MMP1 expression, which correlates to high expression of MMP1 regulating miRNAs (miR-133a, miR-221-3p, miR-222-3p, miR-365-3p, miR-373-5p) in this cell line and low expression of those miRNAs in the hybrid cell lines. However, luciferase based functional studies showed a decrease of luciferase activity for miR-222-3p, miR-365-3p and miR-373-5p in all tested cell lines suggesting that these miRNAs were functional active. This is consistent with the regulation of MMP1 in M13SV1, but is in contrast to the expression of MMP1 in the other cell lines. By contrast, M13SV1 cells showed high expression of ERα whereas all other cell lines lack ERα expression. However, miRNAs which suppose to regulate ERα (miR-221-3p, miR-222-3p, miR-373-5p) were higher expressed in M13SV1, whereas all other cells showed lower miRNA expression. Western blot showed that over expression of functional active miR-222-3p had no effect on ERα expression in each tested cell line. This possibly indicates that the tested miRNAs contribute less to ERα regulation. These data show that hybrid cells from cancer cells and cells with stem cell properties differ in their mRNA and miRNA expression profile compare to the parental cells as well to each other.

**Keywords: hybrid cells; miRNAs; miR-222-3p; MMP1; ERĐ;**
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# Development of an inducible cell culture model for transcriptional interference at the Angelman syndrome gene UBE3A

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Transcriptional interference (TI) is the suppression of one transcriptional process by another. In mammals, it is assumed to be involved in the establishment of maternal DNA methylation marks and - in some cases – the regulation of imprinted gene expression. UBE3A is one of the imprinted genes probably silenced by TI. The paternal UBE3A gene is suppressed in the brain by the expression of an overlapping long non-coding antisense RNA. Loss of function of the maternally inherited UBE3A allele leads to Angelman syndrome, which is characterized by intellectual disability, seizures, ataxia and a happy demeanor. Understanding the mechanism of gene silencing of the paternal UBE3A allele is essential for the development of strategies for its reactivation. The aim of our study is to investigate the mechanism of TI in a cell culture model based on human Flp-In T-Rex 293 cells. We constructed a minigene consisting of a test promoter driving EGFP expression. It is positioned downstream of an inducible CMV promoter controlling expression of an overlapping ß-globin transcript. Test promoters under study are UBE3A as example of an imprinted gene and HBA2 and MSH2 as controls. The HBA2 and MSH2 promoters were used because TI was observed as result of microdeletions in patients. These deletions led to overlapping sense-antisense (HBA2) or sense-sense (MSH2) transcription resulting in gene silencing and induction of DNA methylation. For UBE3A and HBA2, the promoters were in an antisense orientation with regard to ß-globin expression, whereas the MSH2 promoter was in a sense orientation. In all three cases we observed upregulation of the ß-globin transcript and downregulation of EGFP expression at the mRNA and protein level. We are currently investigating if the induction of gene silencing is reversible. These studies demonstrate the versatile use of our model for the investigation of TI in the regulation of gene transcription.

**Keywords: transcriptional interference; UBE3A; imprinting; Angelman syndrome**
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# Computational prediction of stem cell regulation by lncRNAs via DNA-RNA triple helices

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From the ENCODE project we know that up to 90 % of the human genome is potentially expressed in at least one cell type. Only a small percentage of the transcripts are protein coding, the rest are non-coding transcripts. Of particular interest are long (> 200bps) non-coding RNAs (lncRNA). The few functionally characterized lncRNAs have important functions as regulators of gene transcription, chromatin structure and mRNA stability during cell development and diseases. A lncRNA can act as a scaffold promoting the interaction of several proteins, RNA and DNA. One example is FENDRR, which has been shown to interact with the Polycomb complex and to bind to particular DNA regions via RNA-DNA triple helices. This causes the repression of the genes Foxf1a and Pitx2 and the regulation of lateral mesoderm development in mouse. We are interested in the computational prediction of RNA-DNA triple helices. Triple helices are formed by a single stranded RNA/DNA molecule (triplex-forming oligonucleotides), which binds to the major groove of a double helix via Hoogsten hydrogen bonds. Recent computational methods have been proposed for the detection of DNA-RNA binding sites for a given RNA and DNA sequence. We have extended this approach by a statistical framework called Triplex Domain Finder. The tool explores the usage of triple helices as a mechanism for particular lncRNAs that form potential DNA binding domains (DBD). The two main methods, promoter test and region test, are implemented to detect the statistically significant DBDs with the particular promoters or regions. Moreover, the DNA binding sites from the predicted DBDs are likely to be functional and can be used to indicate potential target genes, i. e. genes with high binding site coverage in their promoter. We have applied our tool to the promoter regions of genes differentially expressed after FENDRR knock down experiments. We could accurately predict the same DNA binding domain and DNA binding sites previously experimentally validated. Currently, we study the potential involvement of triple helix forming lncRNAs as a regulation mechanism during mesenchymal stem cell differentiation.

**Keywords: lncRNA;triple helices; cell regulation; prediction**
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# Deciphering molecular details underlying the function of cell identity specifying transcription factors

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Cell identities are specified by transcriptional regulatory networks through which transcription factors establish highly regulated gene expression programs. As a paradigm for cell identity specification, the properties of the pluripotent stem cells are maintained by a core circuitry that depend on OCT4 and SOX2, two transcription factors that bear a DNA major groove binding POU domain and a minor groove binding HMG domain respectively. OCT4 and SOX2 are also critical for the induction of pluripotency in somatic cells. In pluripotent cells OCT4 and SOX2 associate on DNA regulatory regions, thus enhancing or modifying the sequence specificity compared to the individual proteins. Interestingly, in the early stages during induction of pluripotency, it was proposed that OCT4 explores the genome independent of SOX2. Here we report a mechanism by which SOX2 influences the orientation, dynamics, and unbinding profiles of both DNA-binding domains of OCT4. This involves an interplay between protein-protein interactions and DNA-mediated allostery. We consider that such a mechanism enables OCT4 to use its DNA binding domains and the interaction partners available in a certain biological context to access alternative genome exploration routes. Furthermore, we provide the structural basis for the genomic redistribution of OCT4 during stem cell differentiation to primitive endoderm that depends on its interaction with SOX17. Ultimately, we show that in silico design of the cooperative DNA recognition by transcription factors is attainable and can be used to manipulate transcriptional circuitries. This study enhances the understanding of the context specific function of OCT4 and provides a general perspective on how DNA-binding cooperativity is modulated by different types of interactions.

**Keywords: OCT4; cooperative DNA recognition; transcriptional regulation; stem cell pluripotency and differentiation; molecular dynamics and free energy calculations**
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# Reciprocal interaction of bifunctional microRNA-9/9* and the Notch signaling pathway in human neural stem cells

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The balance between self-renewal and differentiation of neural stem cells requires tight regulation to assure proper neural development. In this context, Notch signaling has been shown to be an important promoter of stemness. In contrast, the bifunctional brain-enriched microRNA miR-9/9* has been implicated in promoting neuronal differentiation. We set out to explore a potential interplay between Notch signaling and miR-9/9* using a homogeneous population of human embryonic stem cell-derived neuroepithelial-like stem cells generated in our lab (lt-NES cells, Koch et al., PNAS 2009). Our miR-9/9* gain-of-function analyses indicate that both miRNAs contribute to the shift of lt-NES cells from self-renewal to neuronal differentiation. In line with this effect, miR 9/9* decrease Notch activity by targeting NOTCH2 and HES1, two members of the Notch signaling cascade. Vice versa, expression levels of miR 9/9* were found to depend on the activation status of Notch signaling. While gain of Notch function inhibits differentiation of neural stem cells, we observed that it also induces miR-9/9* expression by direct binding of the NICD/RBPj transcriptional complex to miR-9/9* genomic loci. Taken together, these data point to an intricate feedback interaction between bifunctional miRNA-9/9* and the Notch pathway controlling the delicate balance between self-renewal and differentiation of human neural stem cells.

**Keywords: neural stem cells; Notch; miR-9/9*; proliferation; differentiation**
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# MicroRNA-181a modulates human neural stem cell maintenance and dopaminergic differentiation

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Recent evidence indicates that microRNAs (miRNAs) can be placed in midst of the regulatory mechanisms underlying neural stem cell maintenance, differentiation and fate choice. However, given the large number of miRNA species in existence, our understanding of miRNA-based regulation during neurogenesis remains incomplete, in particular with regard to human neural development. Here, we focused on miR-181a, a ubiquitous regulator of development highly expressed in the neural lineage. Using pluripotent stem cell-derived long-term self-renewing neuroepithelial-like stem cells (lt-NES cells) we set out to investigate the impact of miR-181a on neuronal differentiation. Based on gain- and loss-of-function analyses we found that miR-181a is required for shifting the cells from a self-renewing phenotype towards neuronal differentiation, similar to the pro-neuronal miR-124. With regard to its role as promoter of neuronal differentiation, miR-181a down-regulates several neural stem cell-associated genes such as NOTCH2, Musashi2 as well as LIN28A/B. Targeting of LIN28A/B by miR-181a disrupts the LIN28/let-7 feedback loop leading to an up-regulation of let-7, which itself acts as a promoter of differentiation. In addition to its effect on general neuronal differentiation, miR-181a has a specific effect on neuronal subtype differentiation and promotes the emergence of TH-positive dopamine-like neurons. We further found that miR-181a targets the germ cell nuclear factor GCNF, which has been previously associated with forebrain and midbrain development and was here identified as a negative regulator of dopaminergic differentiation. In addition, miR-181a also enhances Wnt signaling probably by targeting several negative modulators of this pathway, which further promotes the dopaminergic lineage. Finally, we have integrated these findings into a transfection-based miRNA modulation approach that could be combined with the currently used patterning protocols to generate dopamine neurons. Taken together, this study uncovers a mechanistic role of miR-181a in regulating human neural stem cell maintenance and is the first report on a miRNA promoting the generation of dopamine neurons, which are of high interest for translational stem cell research, particularly in the context of Parkinson's disease.

**Keywords: human neural stem cells; dopamine neurons; microRNA; miR-181a; neuronal differentiation**
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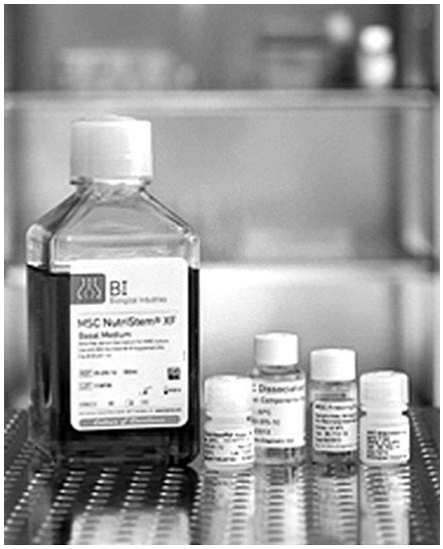


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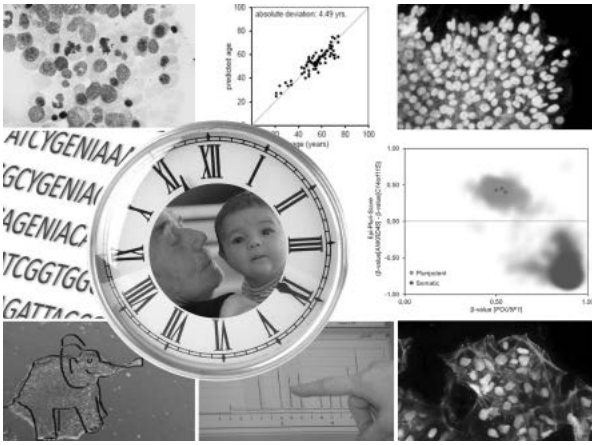

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# TRINOVA Biochem

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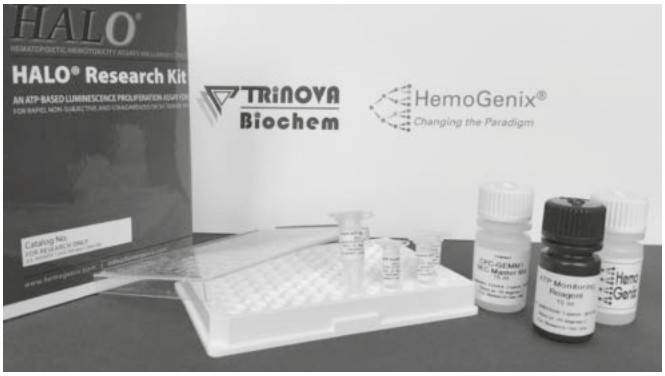


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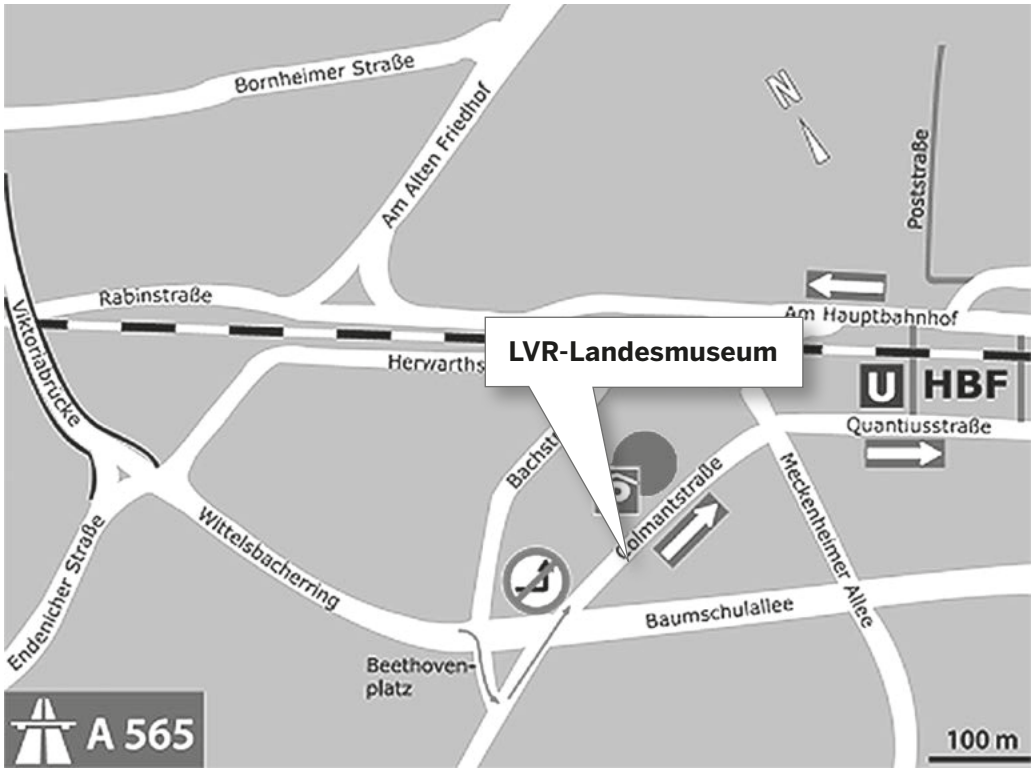
# _Contact & Disclaimer

**Networking Reception at LVR-Landesmuseum April 21st, 7:30 pm, Colmantstr. 14–16, 53115 Bonn**

The museum is very easy to reach with public transportation. It is only a few minutes' walk away from Bonn Central Train Station. From there you take the underground passageway behind the train station, exiting right onto Quantiusstrasse, and then cross Quantiusstrasse to Colmantstrasse. The museum is located on the right-hand side.

**Arrival by public transportation from the World Conference Center, Bonn:**
From station *Heussalle/Museumsmeile* you can take trams 61/16 (direction of Tannenbusch Mitte/ Niehl Sebastianstr.) or 66 (direction of Siegburg Bf) to Bonn main station.



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