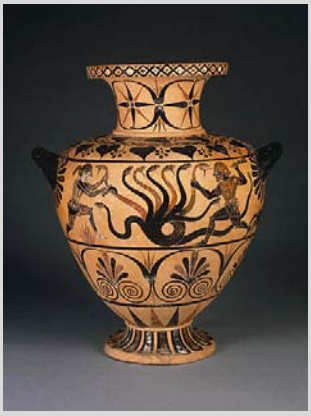
Leopoldina
Nationale Akademie
der Wissenschaften

Symposium
„Regenerative Medicine“



Including “Young Researchers Seminar”

Tübingen,

July 24th - 26th, 2011

EBERHARD KARLS
UNIVERSITÄT
TÜBINGEN

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Title: Caeretan Hydria, Water Jar
Date: about 525 B.C.
Medium: Terracotta

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Organization

Scientific Directors:
Prof. Dr. med. Hans-Peter Zenner, ML
Prof. Dr. Dr. Johannes Schubert, ML
Prof. Dr. med. Eberhard Zrenner, ML

Local Organizers:
Priv.-Doz. Dr. med. Hubert Löwenheim
Prof. Dr. med. Hans-Peter Zenner, ML
Prof. Dr. med. Eberhart Zrenner, ML
Prof. Dr. Marlies Knipper, ML
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Sunday, 24th July

13.00-14:00	**Registration „Symposium Regenerative Medicine“**

YOUNG RESEARCHERS SEMINAR – SEMINAR ROOM BEKESY FOYER
Chair: E. Zrenner

14:00-14:10	Opening **Prof. Dr. E. Zrenner** Tübingen, Germany
14:10-14:50	Regenerative Biology and Regenerative Medicine: “Evolution of new field in research and clinical application” **Priv.-Doz. Dr. H. Löwenheim** Tübingen, Germany
14:50-15:10	Neurodegeneration and Protection in the Retina – Perspective for Regeneration? **Dr. F. Paquet-Durand** Tübingen, Germany
15:10-15:30	Tissue engineering of bone **Dr. D. Alexander** Tübingen, Germany
15:30-15:45	Coffee Break
15:45-16:15	Perspectives for Young Researchers **Prof. Dr. E. Zrenner, ML** Tübingen, Germany
16:15-16:45	Career pathways for Young Researchers in academia versus industry **Dr. Dr. S. Biskup** Cegat GmbH, Tübingen, Germany
16:45-17:30	**Laboratory Tour and “Meet the Principle Investigator"**

Sunday, 24th July

REGISTRATION AND RECEPTION

18.00–20:00 **Registration and Reception**
Symposium Regenerative Medicine
Weingalerie in der Kelter
Schmiedtorstr. 17
72070 Tübingen

Monday, 25th July

OPENING OF THE SYMPOSIUM – LECTURE HALL BEKESY FOYER

08:30-08:40 Welcome
Prof. Dr. H.P. Zenner, ML
Board of Directors, National Academy of Sciences Leopoldina, Halle

08:40-08:50 Opening remarks
Prof. Dr. I.B. Autenrieth
Dean of the Faculty of Medicine, Eberhard Karls University, Tübingen

08:50-09:00 Introduction to the topics of the symposium
Priv.-Doz. Dr. H. Löwenheim
Tübingen
Principle Meeting Organizer

PRINCIPLES OF REGENERATION – Chair: H. Löwenheim

09:00-9:45 Principles of cellular regeneration
Prof. Dr. A. Nordheim
Tübingen, Germany

09:45-10:15 Coffee Break

Principles of regeneration in model organisms

10:15-11:00 Hydra - Cell death as a driving force for regeneration
Prof. Dr. B. Galliot
Geneva, Switzerland

11:00-11:45 Urodels - Principles of regeneration
Prof. Dr. P.A. Tsonis
Dayton, USA

11:45-13:00 Lunch and **Poster Session**

REGENERATION IN HEARING IMPAIRMENT – Chair: M. Ueffing

13:00-13:05 Introduction
H. Löwenheim, Tübingen

13:05-13:50 Defining clinical needs for regenerative medicine in hearing
Prof. Dr. T. Nakagawa
Kyoto, Japan

13:50-14:35 Mechanisms of hair cell regeneration in the vertebrate inner ear
Prof. Dr. M. Warchol
St. Louis, USA

14:35-15:00 Coffee Break

15:00-15:45 Aspects of regeneration in the human inner ear
Prof. Dr. H. Rask-Andersen
Uppsala, Sweden

15:45-16:30 Generation and use of stem cells for the inner ear
Dr. P. Senn
Bern, Switzerland

16:30-17:15 Drug development for regeneration of hair cells
Priv.-Doz. Dr. H. Löwenheim
Tübingen, Germany

17:15-18:30 Coffee Break and **Poster Session**

19:30 Gala Dinner at the Ludwig's (near Hotel Krone)

Tuesday, 26th July

REGENERATION IN MESENCHYMAL TISSUES – Chair: J. Schubert

Vascularization and differentiation

08:30-09:15 The role of vascularization
Prof. Dr. Dr. J. Kleinheinz
Münster, Germany

09:15-10:00 Osteogenic differentiation of stem cells
Priv. Doz. Dr. Dr. J. Handschel
Düsseldorf, Germany

10:00-10:15 Coffee Break

Clinical application

10:15-11:00 Implantation of ex vivo cultured cells and tissues
Prof. Dr. Dr. G. Lauer
Wien, Austria

11:00-11:45 Regenerated cartilage - Regeneration in Head and Neck: is there a future?
Prof. Dr. W. Puelacher
Innsbruck, Austria

11:45-12:30 Bone tissue engineering in reconstructive surgery – basic research and clinical application
Prof. Dr. Dr. S. Reinert
Tübingen, Germany

12:30-13:15 Lunch and **Poster Session**

Tuesday, 26th July

REGENERATION IN VISUAL IMPAIRMENT – Chair: E. Zrenner

13:15-14:00	Optogenetic approaches for restoring photosensitivity in retinitis pigmentosa **Dr. T. Münch** Tübingen, Germany
14:00-14:45	Retinal stem cells **Prof. Dr. D. van der Kooy** Toronto, Canada
14:45-15:00	Coffee Break
15:00-15:45	Neuronal regeneration at eye level **Dr. M.O. Karl** Dresden, Germany
15:45-16:30	Regeneration of vision in inherited retinal dystrophies **Prof. Dr. M. Seeliger** Tübingen, Germany
16:30-17:15	Müller glia as a potential source of stem cells for human therapies to treat retinal degenerative disease **Dr. A. Limb** London, Great Britain
17:15-17:30	Coffee Break
17:30-18:30	**ROUND TABLE – Chair: H.P. Zenner** Interdisciplinary discussion
18:30	**End of the Leopoldina Symposium**

Principles of Regeneration in Urodeles

Panagiotis A. Tsonis

Department of Biology and Center for Tissue Regeneration and Engineering at Dayton, Dayton, OH, 45469-2320, USA

Urodeles, such as newts and salamanders have impressive regenerative capabilities of tissues, organs or body parts, such as limbs, tails, hair cells and eyes. Because of these unparalleled regenerative deeds newts could provide much coveted answers that regenerative medicine is presently seeking. Consequently, these animals have been favored models of regeneration research for over the 200 years since discovery of these activities by Spallanzani in 1768. Significantly, regeneration in newts is mediated primarily by dedifferentiation or transdifferentiation of terminally differentiated cells. In our laboratory we study these events by utilizing as a model the process of lens regeneration. After whole removal of the lens, the pigment epithelial cells (PECs) of the dorsal iris transdifferentiate to produce the new lens. Several issues will be addressed: Re-entry to the cell cycle. Why the same cells from the ventral iris never produce a regenerated lens? Can the ventral iris be induced to transdifferentiate to lens? The process of dedifferentiation. Stemness of newt cells. How robust is regeneration with age and repetition of the insult? What can the newt contribute to issues of regenerative biology and medicine? I will also elaborate on efforts to create resources for research with the newt.

Defining clinical needs for regenerative medicine in hearing.

Takayuki Nakagawa

Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Sensorineural hearing loss (SNHL) is one of the most common disabilities in the world. The majority of SNHL is caused by degeneration of the cochlea. Sound stimuli are transmitted to a cochlea via the middle ear ossicles, and vibrate the basilar membrane, on which the organ of Corti containing the sensory hair cells sits. The hair cells convert sound vibration to neural signals, which are transmitted to the spiral ganglion in the modiolus of a cochlea. Transduction of sound vibration to neural signals is an active process, which required the endocochlear potential, which is generated by the cochlear lateral wall. Thus, the organ of Corti, spiral ganglion and cochlear lateral wall play crucial roles in the auditory function. Degeneration of each cochlear component can cause SNHL, and human temporal bone studies have demonstrated that combined degeneration is sometimes observed in damaged cochleae. Therefore, these cochlear compartments are included in therapeutic targets for treatment of SNHL.

We propose different strategies for hearing restoration according to the stage of cochlear degeneration. SNHL is usually irreversible because of limited capacity of mammalian cochleae for regeneration. In the acute phase of cochlear degeneration, the protection or induction of self-repair may be a pragmatic strategy. For this purpose, pharmaceutical approaches including topical treatment may be preferable. Previous studies have demonstrated several candidates to protect hair cells; however, how to deliver therapeutics to cochlear cells is an obstacle for clinical application. As a resolution for this problem, we have developed drug delivery systems for inner ears using biomaterials. A part of experimental findings in this field has been applied to the clinic.

After disappearance of hair cells, if sufficient numbers of supporting cells still remain in the organ of Corti, transdifferentiation of supporting cells into hair cells is a possible strategy.

The manipulation of notch signaling could be included in candidates for this approach. In case of severe degeneration of not only hair cells but also supporting cells, proliferation of supporting cells is required for restoration of the organ of Corti. Previous studies have demonstrated roles of several cell-cycle inhibitors including p27 in the quiescent status of supporting cells in the organ of Corti.

Recently, the manipulation of such cell-cycle inhibitors has been investigated for induction of cell proliferation of supporting cells. In addition, the existence of stem cell-like cells in the cochlea has also been reported. Therefore, such internal cell populations can be a source of newly generated hair cells.

After disappearance of internal cell sources, a cell transplantation approach may be a candidate of strategies for regeneration of inner ear cells. The capability of several stem cells including iPS cells for differentiation into inner ear cells has been elucidated. The use of development of micro or nano electro mechanical systems could be an alternative for biological regeneration. We have recently generated an artificial cochlear epithelium using piezoelectric materials, which mimics the function of the inner hair cell and basilar membrane.

In near future, the feasibility of such newly developed therapeutic strategies may be estimated. We hope that such efforts will lead to providing SNHL patients with novel therapeutic options for the treatment.

Mechanisms of Sensory Regeneration in the Vertebrate Inner Ear

Mark E. Warchol
Department of Otolaryngology, Washington University School of Medicine, St. Louis MO, USA

Sensory function in the inner ear is mediated by hair cells, which detect mechanical stimuli caused by head motions and sound vibrations. In birds and mammals, a full complement of sensory hair cells is produced during embryogenesis. Those cells can be lost later in life as a result of noise exposure, treatment with ototoxic medications, or as part of normal aging. The ears of mature mammals are unable to replace hair cells, so their loss usually results in permanent deficits in hearing and/or balance. In contrast, all nonmammalian vertebrates appear to be capable of regenerating hair cells after injury. Much current research is aimed at understanding the biological basis of sensory regeneration in the ears of nonmammals and identifying the limitations on regeneration in the ears of mammals. The sensory epithelia of all vertebrate ears are comprised of two general cell phenotypes: hair cells and supporting cells. Regeneration (when it occurs) is mediated by the proliferation or transdifferentiation of supporting cells. In my talk, I will provide an overview of our recent studies aimed at the identification of genes and signaling pathways that regulate the division of supporting cells and the differentiation of new hair cells in the mature avian inner ear. Our current data suggest critical roles for Wnt, FGF and Notch signaling in different phases of this regenerative process.

Aspects on Regeneration in the Human Inner Ear (Cochlea)

Helge Rask-Andersen[1], Jörg Waldhaus[2], Daniel P. Lindholm[1], Wei Liu[1], Kristian Pfaller[3], Rudolf Glueckert[4], Anneliese Schrott-Fischer[4], Marcus Müller[2], Hubert Löwenheim[2]

[1]) Dept. of Surgical Sciences, Section of Otolaryngology, Uppsala University Hospital, Uppsala, Sweden
[2]) Hearing Research Center, Department of Otorhinolaryngology – Head & Neck Surgery, University of Tübingen Medical Center, Tübingen, Germany.
[3]) Dept. of Histology and Molecular Cell Biology, Institute of Anatomy and Histology, Medical University of Innsbruck, Innsbruck, Austria.
[4]) Dept. of Otolaryngology, Innsbruck University Hospital, Innsbruck, Austria

Human cochlea is the most difficult organ to process and analyse due to its location and surrounding bone of exceptional solidity. Despite these obstacles the Swedish anatomist Gustav Retzius already in 1884 presented outstanding microscopic images of its structure including its sensory elements. It was followed more recently by ultrastructural descriptions including immunohistochemistry. The studies were performed on specimens fixed shortly after demise. Here, we present results from analyses based on surgical material obtained at removal of large life-threatening petroclival meningioma with brain stem compression where the ear had to be sacrificed. Ethical consent and patient approval were obtained*. Tissue is directly fixed either with 3% glutaraldehyde or 4% para-formaldehyde followed by decalcification. It allows ultrastructural studies using TEM and SEM and protein/gene analyses including IHC with laser scanning confocal microscopy. Swift removal also permits cell culture and time lapse video recordings (TLVM). The two different types of auditory receptors; the inner (IHCs; 3400) and outer (OHCs; 12500) hair cells is further represented in their innervations; IHCs being innervated by type I neurons (30.000) and OHCs by type II neurons (in man named small ganglion cells and expressing the 57-kDa neuron-specific intermediate filament protein peripherin). The satellite glial cell system surrounding perikarya lack myelin (and MBP) and molecular expression suggests that SG function differs in man

compared to animals. The inner ear has generally been regarded as an area with low cell turnover as well as being immune-privileged. Inherent healing mechanisms exist to induce cell proliferation to close membranous ruptures in endolymphatic hydropic and acoustic overstimulation. Clinical experience and current experimental evidence from other mammals have led to the general conviction that the human organ of Corti and spiral ganglion lack any potential for self-renewal and hence regenerative capability. In the absence of any direct experimental evidence for this opinion we may have underestimated this potential. Human organ of Corti was found to contain supernumerary hair cells and in the organ of Corti 1.8% of all supporting cells were Ki67 labeled with Ki67/p27Kip1 double positive supporting cells. Both findings suggest a low-grade cellular turnover. It supports the intend to develop regenerative strategies to treat human otopathologic entities. Lately, evidence was presented suggesting that even spiral ganglion neurons may self-renew and regenerate, even in man. This could infer that there are possibilities to induce regeneration of neurosensory components and may anticipate future replacement therapy. Results are also presented on cell culture experiments to differentiate and expand human embryonic stem cells and neural progenitors into sequential neurogenin1 - glutamate - TrkB expressing bipolar neurons aimed for cell transplantation.

*informed consent and approval by ethics committee of Uppsala University Hospital
(no. 99398, 22/9 1999, 29/12 2003) conforming to the Declaration of Helsinki

Generation and use of stem cells for the inner ear

[1,2]Pascal Senn, [1]Amir Mina, [2]Stefan Volkenstein, [2]Veronika Starlinger, [2]Kazuo Oshima, [3]Stefano di Santo, [3]Hans Rudolf Widmer and [2]Stefan Heller

[1]University Department of Otorhinolaryngology, Head & Neck Surgery, Inselspital, Bern, Switzerland
[2]Departments of Otolaryngology – Head & Neck Surgery and Molecular & Cellular Physiology, Stanford University, Palo Alto, CA 94305-5793 USA
[3]University Department of Neurosurgery, Inselspital, Bern, Switzerland

The loss of inner ear hair cells leads to incurable balance and hearing disorders because these sensory cells do not effectively regenerate in humans. A potential starting point for therapy would be the stimulation of quiescent progenitor cells within the damaged inner ear. Inner ear progenitor/stem cells, which have been described in rodent inner ears, would be primary candidates for such an approach. Despite the identification of progenitor cell populations in the adult human spiral ganglion, no proliferative cell populations with the capacity to generate hair cells have been reported in vestibular and cochlear tissues of adult humans.
The present study aimed at filling this gap by isolating sphere-forming progenitor cells from surgery-derived and autopsy-derived adult human temporal bones in order to generate inner ear cell types *in vitro*. Sphere-forming and mitogen-responding progenitor cells were isolated from vestibular and cochlear tissues. Clonal spheres grown from adult human utricle and cochlear duct were propagated for a limited number of generations. When differentiated in absence of mitogens, the utricle-derived spheres robustly gave rise to hair cell-like cells, as well as to cells expressing supporting cell-, neuron-, and glial markers, indicating that the adult human utricle harbors multipotent progenitor cells.
Spheres derived from the adult human cochlear duct did not give rise to hair cell-like cell types, which is an indication that human cochlear cells have limited proliferative potential, but lack the ability to differentiate into major inner ear cell

types. However, in additional experiments with donated fetal abortion inner ear tissues, cochlear hair cells were successfully generated, indicating that technology used is principally capable of generating cochlear hair cells in vitro from inner ear stem/progenitor cells but is not yet sufficiently developed to overcome the locks induced by cochlear maturation in adult humans. The generation of human inner ear cell types in culture provides a novel platform for their depth *in vitro* characterization.

Drug development for regeneration of hair cells

Hubert Löwenheim[1], Jörg Waldhaus[1], Holger Eickhoff[2], Karl-Heinz Wiesmüller[2], Marcus Müller[1]

Laboratory of Regenerative Medicine, Dept. of Otolaryngology, Hearing Research Center, University of Tübingen, Germany

Sensorineural hearing loss is often caused by damage and subsequent loss of sensory hair cells in the auditory sensory epithelium of the organ of Corti. In humans and other mammals the auditory hair cells are not regenerated when lost. In contrast to the mammalian situation, other vertebrates in particular birds, have retained the ability of spontaneous hair cell regeneration. Several research strategies have been devised to induce hair cell regeneration in mammals. These include manipulation of the cell cycle of supporting cells, direct transdifferentiation of supporting cells into hair cells (i.e. by gene transfer of hair cell transcription factors) or the transplantation of stem cells into the cochlea. In other vertebrate organisms as a predominant principle, hair cell regeneration is achieved by the creation of new cells that arise by proliferation of supporting cells near the site of hair cell loss. At the level of supporting cell devision the functional investigation of cell cycle regulators such as the CKI (Cyclin-dependent Kinase Inhibitor) $p27^{Kip1}$ has revealed their key role in the ability of supporting cells to proliferate. Apart from its biological function $p27^{Kip1}$ also represents a viable molecular drug target that may be manipulated inhibitory mechanisms in order to induce proliferation in inner ear supporting cells. The recent isolation of inner ear progenitor/stem cells form the postnatal rodent organ of Corti indicates a latent endogenous regenerative capacity that is revealed under specific *in vitro* conditions and may be exploited for screening efforts using small molecule libraries. The potential of inner ear progenitor/stem cell propagation and expansion allows for the development of drug screening capabilities in a low-throughput format. Drug candidates from such chemical screens may in turn also generate new molecular targets for therapeutic intervention stimulating endogenous regeneration *in situ*.

Regeneration in mesenchymal tissues – the role of vascularization

Johannes Kleinheinz

Department of Cranio-Maxillofacial Surgery, Research Group Vascular Biology of Oral Structures , University Hospital Muenster, Germany

Living and developing systems need a basic infrastructure to produce good delivery of material and to remove waste. Nature found the solution by developing an archetype of branching to support even smallest structural parts. In humans the highly specified vascular system epitomizes this archetype. This system plays a crucial role in every wound healing and tissue regeneration as well as in functional and structural maintenance of every organ.

There are at least two key processes consisting of an almost similar initial step of development of a mature vascular system and a subsequent organotypic specification of the endothelial cell function. Vessels develop by vasculogenesis (development of new vessels from mesenchymal precursor cells) and angiogenesis (sprouting of new vessels out of pre-existing vessels). This development is controlled and influenced by environmental and molecular mechanisms. Oxygen sensing is the key factor for regulation of vascular responses of mammalian cells. Besides hypoxia, being the strongest natural signal for new vessel formation, a lot of proteins, enzymes, growth factors and transcriptional co-factors were identified and characterized as part of the angiogenic cascade. By external application of growth factors or internal activation of cascades, neoformation, acceleration and securing of the vascular system could already be demonstrated. Based on the experience in jaw bone regeneration, the influence of angiogenesis on osteogenesis and bone regeneration could be shown, especially the osteogenic-angiogenic coupling. A persisting problem seems to be the development of an intrinsic vascular network inside extracorporal tissue-type constructs, which is pivotal for survival. For this reason it is questionable if extracorporally assembled

implantable systems are the best way for tissue regeneration or if the specific activation of internal regenerative capacities are more effective. Future investigations will aim at activation or inhibition of trigger factors such as the oxygen sensors HIF-1α and PCG-1α. In addition, techniques to assess the oxygen tension on the tissue or cellular levels and intravital imaging of tissue-specific vascularization have to be developed or improved.

Osteogenic differentiation of stem cells

Jörg Handschel
Department for Cranio- and Maxillofacial Surgery, Heinrich-Heine-University, Düsseldorf, Germany

Due to trauma, tumor resection, cysts, inflammation or atrophy after loss of function bony defects may occur that may not self-heal and therefore need to be reconstructed to restore functionality and form. In these so called critical-size bone defects, autologous or allogenic cells may be required in addition to compatible biomaterials for the successful defect healing. Recently, various stem cell types including pluripotent stem cells and mesenchymal stem cells (MSC) from different sources were described as potential candidates for bone tissue engineering approaches.

Currently, two types of pluripotent stem cells are being intensely investigated, namely embryonic stem cells and induced pluripotent stem cells (iPS). Advantages and disadvantages of both methods are compared with respect to their applicability in bone tissue engineering.

Furthermore, the osteogenic potentials of MSC from adipose tissue, umbilical cord blood, bone marrow or dental pulp are compared with each other and with the potential of pluripotent stem cells. For the osteogenic differentiation of stem cells, efficient in vitro protocols were developed. Moreover, approaches are presented that increase differentiation efficiency and rapidness. Finally, a cell culture method for the production of osteogenic microspheres is presented that are particularly suited for osteogenic tissue engineering.

Implantation of ex vivo cultured cells and tissues

Günter Lauer

University Hospital for Cranio, Maxillofacial and Oral Surgery, Medical University of Vienna, Vienna, Austria

The transplantation of cultured epithelial cells was pioneered more than 30 years ago in general and plastic surgery for patients that suffered from burns. In oral and maxillofacial surgery the transplantation of epithelial / mucosa cells in patients after tumour resection was introduced as early as 1990. To also be able to use the cultured cells in elective surgical procedures in oral and maxillofacial surgery the culture technique was modified to avoid xenogenous feeder cells and xenogenous serum. Since then, not only cultured epithelial cells and epithelial tissue but also cultured osteoblasts have been used as grafts to improve soft tissue conditions in the oral cavity and to fill osseous defects and deficits in the jaw.

In the oral cavity for example, it is often necessary to create stable soft tissue around dental implants or in the denture-bearing area. Instead of split mucosa grafts from the palate, cultured mucosa cell grafts can be used. Initially these grafts only consisted of gingival keratinocytes. Clinical follow-up revealed that there was considerable shrinkage of the grafted surfaces where these pure epithelial grafts had been used. In comparison to split skin or split mucosa, the pure epithelial grafts lack a submucosal connective tissue with fibroblasts. Therefore, we developed an oral keratinocyte-fibroblast graft, consisting of a collagen sponge seeded with these two types of cells. The application of this type of oral keratinocyte-fibroblast mucosa graft led to less wound shrinkage so a wider rim of keratinized mucosa around the dental implants was maintained. Thus, after healing of the oral keratinocyte-fibroblast graft the quality of the grafted sides had been improved considerably.

In oral and maxillofacial surgery there are several conditions requiring hard tissue replacement such as cysts in the jaw, atrophic alveolar crests post extraction and malformations as in the cleft alveolus in patients suffering from cleft lips and palates. In these clinical situations cultured autogenous osteoblasts have been

applied successfully. The cultured osteoblast grafts - collagen sponges seeded with bone cells from the maxilla as a type of tissue engineered bone – were able to instigate bone growth when implanted in osseous defects or deficits of limited size. The in vitro cultured bone cells have shown to be an alternative to conventional autogenous bone grafts ie: from the iliac crest.

In summary, up to now implantation of ex vivo cultured cells work as a tissue graft and help to avoid donor side defects and morbidity. However, further research will be necessary particular in respect to tissue vascularisation to create ex vivo tissue that allows for the replacement of larger tissue volumes.

Regenerated cartilage – Regeneration in Head and Neck: is there a future?

Wolfgang C. Puelacher

Laboratory for Tissue Engineering – University Clinic for Craniomaxillofacial Surgery, Leopold Franzens University Clinic, Innsbruck, Austria, EU

The technologies of biomedical tissue engineering and regenerative applications represent rapidly growing fields in basic science and medicine including head and neck surgery. Tissue engineering approach combines the biological properties of living cells and physiochemical properties of individually designed materials in order to enable the foundation of artificial tissues. Two main strategies of cell based tissue engineering are promoted: cell transplantation and cell enhancement technologies. Cartilaginous tissue may be seen as an ideal tissue to generate using tissue engineering: cartilage basically consists of one cell type, the chondrocyte, which shows a limited regenerative capacity clinically. Chondrocytes can be easily isolated in great numbers, show high viability, are readily multiplied in vitro and do not show significant vascularisation. The chondocytes are nourished by diffusion and exhibit a low oxygen requirement compared to other tissues. All these properties make them an ideal candidate for transplantation and survival by diffusion until successful engraftment takes place.

Cartilaginous cell transplantation respectively cell-housing-construct implantation shows different important impacts on clinical outcome including harvesting procedures, cell isolation, cell culture technique, cell screening, cell transplantation devices, implantation procedure, and immunological aspects.

Summarizing, the understanding of fundamental biology associated with cartilaginous tissue regeneration is essential for the development of approaches to enhance cell differentiation, cell function and neomorphogenesis of cartilaginous tissue.

Bone tissue engineering in reconstructive surgery – basic research and clinical applications

Siegmar Reinert, Dorothee Alexander

Dept. of Oral and Maxillofacial Surgery, University Hospital Tuebingen

The main focus of our basic research is to develop biofunctionalized cell-seeded implants for facial reconstructive surgery. For this purpose, we characterize in detail the mesenchymal stem cell type derived from jaw periosteum on the one hand and on the other hand we try to identify the optimal osteoinductive biomaterial for this stem cell type.

Tissue engineering applications require a well-defined stem cell source. Jaw periosteal cells (JPCs) comprise a heterogeneous population and not all isolated JPCs are capable to mineralize in vitro. To identify the osteogenic progenitor cells we screened a series of mesenchymal stem cell markers and identified the CD271 (low affinity nerve growth factor receptor) and the newly defined marker MSCA-1 (mesenchymal stem cell antigen) to be differentially expressed in mineralizing versus non-mineralizing JPCs. By magnetic cell sorting using specific anti-CD271 and anti-MSCA-1 antibodies we isolated the positive and negative cell fractions and analyzed the purity and the osteogenic potential of these cells. We could demonstrate that MSCA-1 but not CD271 designates the osteogenic progenitor cells within the entire JPC population[1,2]..

Otherwise, bone regeneration constructs require the optimal scaffolding material carrying adhesive, proliferative and osteoinductive properties. We analyzed open-cell polylactic acid (OPLA), collagen and calcium phosphate scaffolds for their suitability for human JPC and found a significant increase of proliferation rates in OPLA scaffolds vs. Coll / CaP scaffolds at three time points.

In clinical applications lifting of the sinus floor is a standard procedure for bony augmentation that enables dental implantation. We present the clinical results of

dental implants placed after the insertion of periosteum-derived, tissue-engineered bone grafts in sinus lifts with a follow-up of six years. The cell-polymer constructs were transplanted by sinus lift 8 weeks after harvesting. Up to now we did no see any complications and no implants were lost.

1. Alexander D, Schäfer F, Olbrich M, Friedrich B, Bühring HJ, Hoffmann J, Reinert S.
MSCA-1/TNAP selection of human jaw periosteal cells improves their mineralization capacity.
Cell Physiol Biochem. 2010;26(6):1073-80.
2. Alexander D, Schäfer F, Munz A, Friedrich B, Klein C, Hoffmann J, Bühring HJ, Reinert S.
LNGFR induction during osteogenesis of human jaw periosteum-derived cells.
Cell Physiol Biochem. 2009;24(3-4):283-90.
3. Alexander D, Ardjomandi N, Munz A, Friedrich B, Reinert S.
ECM remodeling components regulated during jaw periosteal cell osteogenesis.
Cell Biol Int. 2011 Apr 28. [Epub ahead of print]
4. Alexander D, Hoffmann J, Munz A, Friedrich B, Geis-Gerstorfer J, Reinert S.
Analysis of OPLA scaffolds for bone engineering constructs using human jaw periosteal cells.
J Mater Sci Mater Med. 2008 Mar;19(3):965-74.

Optogenetic approaches for restoring vision in blindness

Thomas Münch
Retinal Circuits and Optogenetics, Werner Reichardt Centre for Integrative Neuroscience, Tuebingen, Germany

In the last few years, the field of optogenetics has evolved at an amazing pace. One application are is to use light-sensitive proteins in the treatment of blindness. Such optical neuromodulators, for example channelrhodopsin, are expressed in retinal cells, rendering them light sensitive. It has been shown that this treatment can restore vision in mouse models for retinal degeneration.

I will review the recent developments in this field, covering the different treatment strategies that have been used, including the targeting of cell types and the choice of optical neuromodulator. I will also consider new developments in the field of the light-sensitive proteins themselves, and how these new versions of optical neuromodulators might improve the optogenetic treatment of blindness.

Retinal Stem Cells

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Retinal stem cells are present in the ciliary margin of the adult mouse and human human eye. These retinal stem cells can be prospectively isolated, and thus do not arise from a transdifferentiation process come to all peripheral RPE cells. Single adult retinal stem cells can give rise to all retinal cell types, but only a minority of these becomes photoreceptors when differentiated *in vitro*. Modulation of gene expression in retinal stem cells can greatly enrich for photoreceptor progeny, as can differentiation in the presence of taurine and retinoic acid in vitro. Furthermore, when these RSC photorecptor progeny are transplanted into mouse eyes, they show integration and differentiation into the outer retinal layer. Most important, electrophysiological and behavioral tests show that these transplanted cells can promote functional recovery in transducin mutant mice lacking functional rod photoreceptors. More recent work has been directed at delivering these retinal stem cell progeny into the subretinal space within a biodegradable gel that improves the spread of the donor cells across the retina, as well as potentially their survival and integration. Finally, we have begun a search for the factors that limit the proliferation of the endogenous retinal stem cells in vivo in the adult eye.

Neuronal regeneration at eye level

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Our studies in regeneration are motivated by the major question whether or not in neurodegenerative diseases the surviving cells have any capacity that may be utilized to therapeutic benefit. Retinal degenerations lead to loss of vision that is increasingly impacting health of an ageing society. Development of various therapeutic interventions targeting prevention, progression and repair is therefore pressing and will not only contribute to maintain mobility, life quality and independence, but also to reduce the social annual cost. Whereas currently in human's loss of vision due to neuronal cell death is permanent retinal regeneration is well established in non-mammalian vertebrates. After retinal damage in fish this regeneration is complete; in chicken it is limited to some types of retinal neurons. Recent studies in rodents suggest that depending on the experimental approach a limited regenerative program can be stimulated, but the types, numbers and functionality of neurons regenerated are controversial. Here I present a general overview of our results focusing on the major avenues and barriers in retinal regeneration.
Retinal damage in adult mice was caused by intraocular injection of neurotoxins or light damage. Retinas were analyzed using immunostaining, 3D confocal imaging and quantitative PCR. Retinal damage itself induced de-differentiation of Müller glia – for example, upregulation of GFAP and Pax6 expression. Unfortunately, in comparison to fish and chick Müller glia did not spontaneously re-enter cell cycle thereby preventing regeneration in mice. To overcome this limitation we tested several mitogens by intraocular injection two days after neuronal damage. The

combination of FGF1 and insulin was most successful in stimulating regeneration in the adult mouse retina in vivo. This treatment led to regeneration of BrdU+ GABAergic amacrine cells, as defined by NeuN, Calretinin, Pax6, Prox1 and GAD67 expression. Moreover, using a genetic lineage trace I showed that a population of GFAP expressing Müller glia is the source of neuronal regeneration. Interestingly, exposure to bright light leads to photoreceptor damage and subsequent injection of growth factors does not increase the number of regenerated amacrine neurons compared to growth factor injections alone. In contrast, NMDA induced neurotoxic injury with subsequent growth factor stimulation led to a significant higher number of regenerated amacrine neurons. In subsequent experiments none of the treatments tested so far after retinal light or NMDA damage led to regeneration of rod (Nrl-GFP), bipolar (mGluR6-GFP) or retinal ganglion cells (Thy1-CFP) in adult mice in vivo. Thus, Müller glia can be induced to proliferate, dedifferentiate and regenerate retinal neurons in very limited numbers, most likely amacrine cells, in the mouse retina. Although more limited than chicken or fish retina, the adult mouse retina has the potential to regenerate inner retinal neurons after damage. In ongoing studies we are investigating novel ways to overcome barriers of retinal regeneration in mammals.

Regeneration of vision in inherited retinal dystrophies

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Several lines of promising regenerative strategies for inherited retinal dystrophies have been developed in the immediate past years. Whereas substantial advances have been made in a number of areas, none has currently progressed as far as gene therapy, the focus of this presentation. Gene therapy, the main representative of curative approaches, has led to the successful restoration of function using recombinant adeno-associated viral vectors (rAAV) but also lentiviral vectors (LV), and has in the case of RPE65 already culminated in first human clinical trials. Based on proof-of-principle studies in animal models, several other candidates for human gene transfer like X-linked retinoschisis and achromatopsia now come to the fore. In contrast, some symptomatic approaches aim at more central structures of the visual system and/or those that degenerate late in the course of a disease, like the use of channelrhodopsin/halorhodopsin to make retinal cells intrinsically light sensitive. Other attempts try to retard the disease process by slow release of neuroprotective factors. Importantly, the rescue of vision requires an adequate functional and morphological assessment at different sites along the visual pathway. For retinal degenerations, optical coherence tomography (OCT) and electroretinography (ERG) appear best suited for the clinical and experimental follow-up. A particular challenge is that the application of treatment commonly leads to locally limited improvements in functionality, and so novel concepts and/or techniques to take care of the topographic differences may be required to establish reliable and meaningful clinical endpoints.

Human Müller glia as a potential source of stem cells for therapies to treat retinal degenerative diseases

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Müller glia plays a retina regenerative role throughout the zebrafish life. The neural progenicity of these cells has been also identified in newborn chick and rodents, and although a subpopulation of Müller glia with stem cell characteristics is present in the adult human retina, they do not display a regenerative ability in this species. Bundles of Müller glia expressing nestin and the neural stem cell markers SOX2, PAX6, NOTCH1 and CHX10, are observed in the most anterior, non laminated human neural retina. When retinal explants are cultured with EGF, Müller stem cells *in situ* re-enter the cell cycle as judged by expression of the proliferating antigen Ki67. These cells can be isolated from the adult human retina and become spontaneously immortalized *in vitro*. Culture with growth and differentiation factors induces Müller stem cells to differentiate into retinal neurons. This is illustrated by observations that culture with retinoic acid, taurine and IGF1 induces these cells to acquire photoreceptor morphology and to upregulate their expression of photoreceptor markers, such as CRX, Nr2e3, IRBP, rhodopsin and recoverin. In addition, culture with FGF2 and DAPT, a γ-secretase inhibitor that inhibits the transcription factor Notch1, induces Muller stem cells to express markers of retinal ganglion cell (RGC) precursors such as BRN3b, ISL1 and HuD, acquire a characteristic neural morphology, and respond to nicotinic stimulation (characteristic of RGC but not Müller glia) with increased cytosolic calcium levels *in vitro*.
When enriched populations of photoreceptor cells were transplanted into the subretinal space of P23-H rhodopsin mutant rats (a model of photoreceptor degeneration), animals showed a significantly improvement in the A-wave amplitude of the electroretinogram as compared to control animals. Confocal microscopy analysis of immuno-stained retinal sections showed that transplanted

photoreceptors had migrated and integrated into the host outer nuclear layer where they expressed markers of mature photoreceptors including rhodopsin.

Similarly, when enriched populations of cells expressing RGC phenotype were transplanted into the vitreous of rats with NMDA induced RGC depletion, grafted cells migrate into the RGC layer and appeared to extend long neural-like projections along the transplanted region. Most important, they induced a partial restoration of the negative scotopic threshold response of the electroretinogram (ERG), an indicative measure of RGC function, in these animals.

These observations suggest that Müller stem cells isolated from the adult human retina have the ability to differentiate *in vitro* into photoreceptors and RGC precursors, two major types of retinal neurons, with the potential to restore retinal function *in vivo*. Taking into consideration that Müller stem cells can be easily sourced from the adult human eye, these cells may constitute a valuable tool for the design of cell based therapies to treat human retinal degenerative conditions.

(1) Characterization and differentiation of isolated retinal progenitor cells

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Objectives: Regenerative or cellular therapy has emerged in recent years as one of the most promising alternatives in the treatment of many diseases, including retinal degenerations such as retinitis pigmentosa or age related macular degeneration. A strategy to restore visual function in these patients is to replace the cells affected by retinal progenitor cells (RPC) that are predisposed to differentiate into neurons in the retina. On the other hand, obtaining and characterizing neurospheres from stem and progenitor cells of different models of retinal disease can also be a way to study the mechanisms involved in these diseases. With this background, the objective of this work was to characterize and differentiate neurospheres derived from retina of mice.

Material and methods: Mice (C3H genetic background) were used in this study. The animal care and protocols were approved by the Ethics Committee of the institution and adjusted to the Spanish law concerning animal experiments.

Progenitor cells were isolated from retina (Multipotent retinal progenitors express developmental markers, differentiate into retinal neurons, and preserve light-mediated behavior. Klassen HJ, Ng TF, Kurimoto Y, Kirov I, Shatos M, Coffey P, Young MJ. Invest Ophthalmol Vis Sci. 2004 Nov;45(11):4167-73) at postnatal day 1. We proceeded to culture these cells until day 7. The neurospheres formed were characterized with the following markers: nestin, Ki-67, GFAP, MAP2, recoverin, TUJ-1 and NF200. We also proceeded to the differentiation of neurospheres

culturing them in the absence of growth factors to further characterize them with the same markers of differentiation to day 14.

Results: Neurospheres from progenitor cells express markers of retinal neurofilament and proliferation, like GFAP, TUJ1, Ki-67 and nestin. Differentiated cells express neuronal markers like TUJ-1 and photoreceptors markers (i.e. recocerin).

Conclusion: Cells obtained from retinas have progenitor properties and neurogenic potential, providing potential sources of cells for transplantation.

(2) Tissue-resident mesenchymal stem cells of head and neck tissues – characteristics and functions

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Introduction: Multipotent mesenchymal stem cells (MSCs) are present in bone marrow and other tissues such as adipose tissue, muscle, pancreas, liver, tendon, etc. Recent evidence suggests that MSCs interact with different immune cell subsets and thus may be important regulators of local tissue immunity. Until now, most immunological studies refer to bone marrow-derived MSCs (bm-MSCs).

Methods/Results: Here, we report for the first time on the isolation and characterization of multipotent nasal mucosa-derived mesenchymal stem cells (nm-MSCs). Nm-MSCs show a plastic adherent and fibroblast-like morphology and are able to form colonies which can be expanded for at least 14 passages. Following an initial proliferation period, nm-MSCs express the typical bm-MSCs marker antigens CD29, CD44, CD73, CD90 and CD105. Nm-MSCs were able to differentiate along the adipogenic, chondrogenic and osteogenic pathway. Tissue-specific differentiation was confirmed by histochemical and immunofluorescense staining as well as by reverse transcriptase PCR (RT-PCR) for defined marker genes. Nm-MSCs are immunologically active and responsive, produce a set of inflammatory cytokines and express a number of chemokine receptors.

Conclusion: Our study to best of our knowledge is the first description of human tissue-resident MSCs from nasal mucosa. These cells may be an alternative adult strem cell resource for regenerative tissue repair and auto-transplantation in the clinical approaches and may represent important regulators of local mucosal immunity.

(3) Expression of stem cell pluripotency-inducing factors during chick retina regeneration

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The embryonic chicken can regenerate its retina by the reprogramming of the retinal pigmented epithelium (RPE) and by the activation of stem/progenitor cells present in the Ciliary Margin (CM) located in the anterior region of the eye. It has been demonstrated that somatic cells can be reprogrammed *in vitro* to generate induced pluripotent stem cells (iPSC) by expression of Oct4, Sox2, cMyc and Klf4. However, there is limited information concerning the reprogramming during the process of regeneration *in vivo*. Here, we test the hypothesis that reprogramming of the RPE share similarities to the reprogramming of somatic cells that generate iPSC. Therefore, we analyzed the expression of stem cell pluripotency factors during chick retina regeneration. We first collected CM and RPE by Laser Capture Microdissection (LCM) from embryos at day 4-7. Among all factors, only Sox2, cMyc and Klf4 mRNAs were detected by RT-PCR in the CM. Sox2 was clearly detected by immunofluorescence in the CM and central retina but not in the RPE. In contrast, only cMyc mRNA was detected in the RPE. During retina regeneration, Sox2, cMyc and Klf4 remained expressed in the CM. However, Sox2 and Klf4 were induced after 4 hrs post-retinectomy during RPE reprogramming, and their expression was maintained up to day 3 in the presence of FGF. Interestingly, Sox2 was immediately up-regulated in the RPE at 4 hrs post-retinectomy even in the absence of FGF but its expression is completely lost at day 3 post-retinectomy. These results suggest that FGF is necessary to maintain the expression of Sox2 during RPE reprogramming. Oct4 and Nanog were not detected during regeneration indicating that reprogrammed RPE cells do not generate pluripotent cells.

(4) Regulatory miRNAs target genes for cytoprotective peptides of the digestive tract

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The digestive tract with its immensely large surface in contact to the environment outside the body and constantly exposed to various substances, many of them toxic, infectious, or carcinogenic, requires constant protection, and, if it fails, regeneration of cells within the epithelial layer. Among protective molecules the epithelial cells synthesize three secreted peptides can be found. Due to their folded secondary structure motif (3 Cys-Cys bridges) they were termed “trefoil family peptides” (TFF). Deletion of each of the coding genes in mouse models results in either irritation of the mucosal layer, increased susceptibility to inflammation upon bacterial or chemical challenge or even to development of adenomas and adenocarcinomas. The genes contain a variety of regulatory DNA motifs and show variable expression responding to endo- and exogenic stimuli.

To extend our knowledge of cellular response in need to cytoprotection or regenenration of destructed areas we investigated which role is played by a new class of regulatory molecules, the microRNAs (miRNA). Screening for deregulated miRNA in all 3 mouse models detected sets of miRNA that target genes involved in caloric maintenance or in cancer pathways. In the latter, specific miRNAs in mice corresponded exactly to miRNAs noted in human patients with adenocarcinomas. The ongoing study will confirm the comparative study by in-detail analysis of the concerned miRNAs in cell or tissue models.

(5) Cyclic nucleotide-mediated neurite outgrowth and motility of human model neurons and canine glial cells

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Due to the failure of injured axons to regenerate, injury to the adult mammalian central nervous system often results in a permanent loss of sensorimotor function. Cultured neurons and glial cells can be employed as in vitro models to predict the potential of bioactive chemicals affecting neural repair mechanisms. Here, we developed a model of differentiating neurons from human embryonal carcinoma stem cells (NT2). NT2 cells were induced to differentiate into neuronal phenotypes following two weeks of treatment with retinoic acid in aggregate culture. Nestin positive progenitor cells migrated out of NT2 aggregates and differentiated into β-III-tubulin expressing neuronal cells. Culturing the NT2 cells for an additional 7-14 days resulted in increased percentage of β-III-tubulin expressing cells, elaborating a long neurite positively stained for axonal marker (Tau) and pre-synaptic protein (synapsin).

We then asked whether neurite outgrowth from NT2 neurons is modulated by second messenger pathways. Since cyclic nucleotide signalling pathways have been investigated as a regulator of neurite outgrowth/regeneration in several experimental systems, we used chemical activators and inhibitors of cAMP/PKA pathway in our culture. The adenylyl cyclase activator, forskolin, and cell permeable analogue of cAMP, 8-Br-cAMP increased the percentage of β-III-tubulin positive

cells bearing neurites as well as the extension of existing neurites. Application of the protein kinase A inhibitors, H-89 and Rp-cAMP, blocked neurite formation.
These findings implicate the cAMP/PKA signalling pathway as positively regulator for neurite formation.
Since functional recovery through axonal growth is of high clinical significance, we tried to improve neurite regeneration not only by chemical manipulation, but also by creating a permissive cellular environment. Therefore we measured cellular motility of the glial cells in a scratch wound assay under same treatment as the neurons. The data demonstrated that chemical manipulation of cyclic nucleotide signalling mediates neuroglial motility.

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(6) Altered expression of securin (Pttg1) and serpina3n in the auditory system of hearing-impaired *Tff3*-deficient mice

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Tff3 peptide exerts important functions in cytoprotection and restitution of the gastrointestinal (GI) tract epithelia. Moreover, its presence in the rodent inner ear and involvement in the hearing process was demonstrated recently. However, its role in the auditory system still remains elusive. Our previous results showed a deterioration of hearing with age in *Tff3*-deficient animals.

Present detailed analysis of auditory brain stem response (ABR) measurements and immunohistochemical study of selected functional proteins indicated a normal function and phenotype of the cochlea in *Tff3* mutants. However, a microarray-based screening of tissue derived from the auditory central nervous system revealed an alteration of *securin* (*Pttg1*) and *serpina3n* expression between wild-type and *Tff3* knock-out animals. This was confirmed by qRT-PCR, immunostaining and western blots.

We found highly down-regulated *Pttg1* and up-regulated *serpina3n* expression as a consequence of genetically deleting *Tff3* in mice, indicating a potential role of these factors during the development of presbyacusis.

(7) Sphere forming potential of the organ of Corti is related to Sox2 enhance methylation status

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Sphere formation of isolated mouse auditory epithelial cells as observed at postnatal day 4 (P4) indicates the presence of regenerative potential residing in the mammalian inner ear. The capacity to isolate those so called otospheres declines during maturation of the organ and is finally absent in the functional adult organ of Corti at P21 in mice.

The goal of our study was to investigate if the potential to isolate otospheres is related to the epigenetic regulation of Sox2 expression. Transcription factor Sox2 is proposed to control the opposing phenomena of self-renewal and differentiation in otic progenitors. Using qRT-PCR we found constitutive Sox2 expression form embryonic day 13.5 (E13.5) to P21 with a transient maximum in the maturating organ of Corti at P4. This pattern implied a concentration dependant function for Sox2. Consequently we compared Sox2 mRNA expression levels during otic development and in the otosphere model to the DNA methylation profiles of the Sox2 promoter and its otic enhancers NOP1 and NOP2 using Sequenom's MassARRAY platform.

We found that (1) otic progenitors (E13.5) and the otospheres were characterized by moderate methylation of the otic Sox2 enhancer elements NOP1/2 in combination with a basal Sox2 mRNA expression. (2) The high methylation of enhancers NOP1/2 corresponded to a cellular phenotype devoid of self-renewing potential in the organ of Corti at P21 and after otosphere differentiation in vitro. (3) Supplementation of the otospheres with EGF also triggered high methylation of

enhancers NOP1/2 corresponding to an otosphere phenotype devoid of self-renewing potential.

We conclude that the regenerative potential of the organ of Corti is related to Sox2 epigenetic status. We speculate that our observations support the continued exploration of treatment strategies aiming at regeneration of hair cells by dedifferentiation or reprogramming of differentiated cochlear supporting cells.

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(8) Redox modulation of NO signaling by redox-active inorganic complex as a therapeutic approach in □-cell regeneration

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Although nitroxyl (HNO) has been of interest to the chemists for more than a century it is the discovery of nitric oxide (NO) as the endothelium-derived relaxing factor that lead to resurgence of the research of HNO. Both chemical and biological characteristic of HNO makes it unique among NO congeners. Like NO, HNO induces vasodilatation but also targets signaling pathways distinct from NO. More importantly HNO modifies cysteine residues in proteins which can lead to modulation of intracellular signaling. One of the potential targets for HNO could be Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) which regulates expression of antioxidants enzymes and promotes tissue regeneration.

We have recently demonstrated that in vitro, a patented manganese-based superoxide mimic (MnSODm) that has enetered phase III clinical trials, can also bind NO (beside removing toxic superoxide radical) and reduce it to HNO. Here we show that this also happens *in vivo* on the cellular level. Furthermore, we demonstrate that intracellularly formed HNO then activates translocation of Nrf2 into nucleus. Finally, on the animal model of diabetes we show tremendous □-cell regeneration and restoration of normal insulin level after 7 day treatment with MnSODm

This effect is also marked by overexpression of antioxidant enzymes in the tissue and the translocation of Nrf-2 into nucleus (the same effect being seen even in the healthy, MnSODm-treated rats). Overall, our results represent a new perspective in therapy as they offer modulation of intracellular signaling by small, cheap, redox-active inorganic molecules, as a key to tissue rescuing.

(9) Chemical Modification of the Epigenetic Pattern Improved the Osteogenic and Hepatic Differentiation Potential of aged Human Ad-MSCs

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Objective:
With the increase of donors' age, adipose-derived mesenchymal stem cells (Ad-MSCs) lose their potential to proliferate and differentiate. We previously found age-related differences in the epigenetic status and pluripotency characterization of Ad-MSCs isolated from young and old donors. Consequently, Ad-MSCs from old donors exhibited a lower hepatic and osteogenic differentiation capacity. We have learned from a couple of reports that 5-Azacytidine (AZA) and BIX01294 (BIX) are compounds which could affect the epigenetic patterns of stem cells. Aim of the present study was to find out whether Ad-MSCs from old donors could be epigenetically activated by 5-Azacytidine (AZA) or BIX01294 (BIX) to improve their function as target cells after hepatic or osteogenic differentiation.

Material and methods:
Ad-MSCs were isolated from human abdominal adipose tissue by surgical excision, following the ethical guidelines of the MRI. Donors of Ad-MSCs were divided into young (≤45yrs) and old (>45yrs) groups. Ad-MSCs from each donor were treated with AZA (2.5, 5, 20 µmol/l) and BIX (0.1, 0.2 µmol/l) for 24, 48 and 72 h. DNA was extracted for global methylation measurement and RNA for testing expression of pluripotency markers (Oct4, Nanog, Sox2, c-Myc, KLF4, GAPDH was used as house-keeping gene) by qPCR. After 3 weeks of differentiation, hepatic and osteogenic characteristics were measured. Different CYP 450 enzymatic activities (Phase I/ II), urea and glucose production were measured to evaluate the hepatic characteristics. For the determination of the osteogenic characteristics, alkaline phosphatase (AP) activity assay and staining, as well as Alizarin Red and von Kossa staining were performed. Meanwhile, all measurements were performed on

non-treated Ad-MSCs as negative control, and also on human hepatocytes (hHeps) as positive control.

Results:
As the age of donors of Ad-MSCs is increasing, the global methylation status of their genomic DNA is also rising, which is accompanied with a decrease in expression of the pluripotency markers Oct4, Nanog, Sox2, c-Myc and KLF4. In particular, we found a significant decrease of Nanog and Sox2 expression in Ad-MSCs from old donors, in comparison with those from donors who are less than 45 years old. Treatment with AZA or BIX significantly decreased the genomic DNA methylation of the Ad-MSCs. This effect was most prominent in the cells derived from aged donors (AZA caused a decrease by 30%, BIX by 60%), which was associated with changes in the expression of pluripotency related-genes: Both chemical treatments significantly increased expression of Nanog and Oct4 ($p < 0.05$ vs. untreated) from aged Ad-MSCs, BIX: 3.0- and 5.2-fold, AZA: 2.5- and 1.8-fold, respectively. This pretreatment with AZA or BIX improved the subsequent differentiation into hepatocyte- or osteoblast-like cells. Pretreated hepatocyte-like-cells demonstrated an elevated urea and glucose metabolism as well as improved phase I/II enzyme activities. CYP 1A1, CYP2B6 activity, and HFC Resorufin conjugation were significantly improved in comparison to non-treated cells. In some cases CYP450 activities and urea production of AZA or BIX-treated cells even reached similar levels as primary human hepatocytes. Comparable results were also obtained after osteogenic differentiation. AP activity and staining as well as production of mineralized matrix (Alizarin Red and von Kossa staining) were found significantly improved particularly in Ad-MSC-derived osteoblast-like cells of old donors.

Conclusion:
Our data suggest that the treatment with AZA and BIX seems to be a promising approach to reactivate human Ad-MSCs from old donors, bringing them up to the level of cells from young donors. Consequently, this leads to a comparable differentiation capacity to the cells derived from young donors. This approach may overcome the deficit of aged mesenchymal stem cells to differentiate into target cells without the high risks of using inducible pluripotent stem (iPS) cell technology such as gene integration and tumor genesis. Therefore, this chemical approach might provide an option to modify Ad-MSCs before their application in autologous cell therapy/transplantation in elderly patients.

(10) A transient postnatal niche for neural progenitors in the cochlea nucleus

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The cochlear nucleus (CN) shelters the second echelon of neurons along the auditory pathway. There is a critical period in early postnatal development (before postnatal day 14) of the CN where removal of the afferent input evokes dramatic neuron death, and an enormous gain of plasticity occurs. There are no studies available, which determine if neural stem/progenitor cells are involved in this process. Here we report the occurrence of neural progenitors in the postnatal cochlear nucleus. We show that these cells can be clonally expanded, an indication for self-renewal, a defining feature of stem cells. Furthermore, we determined that Wnt- and/or TGF-beta signalling is involved in the maintenance of these stem cells. TGF-beta- and Wnt signalling play multiple roles in neural development, and Wnt signalling has recently been shown to be essential for clonal expansion of neural stem/progenitor cells.

We demonstrated with a sphere formation assay the postnatal existence of neural progenitors in all three subdivisions of the cochlear nucleus (anteroventral, posteroventral, dorsal). The ability of sphere formation decreases from postnatal day 1 until postnatal day 14. Using an Axin2-LacZ reporter mouse, we identified Wnt-active cells in all CN-derived neurospheres as well as in the central areas of postnatal cochlear nucleus sections. After incubation of cochlear nucleus cells with

a fluorescent substrate of beta-galactosidase, and fluorescence activated cell sorting (FACS), we could isolate a population of Axin2+ cells that is able to form spheres that display Wnt-activation using the Axin2-reporter assay. Axin2- cells are also able to form spheres, but to a lesser degree. When we added Wnt agonists, we detected increase in sphere formation; Wnt-antagonists decrease the number of spheres. Furthermore, addition of BMP4 completely inhibited sphere formation, whereas noggin, a BMP-antagonist, starkly increased the number of spheres. In a gene expression microarray analysis of native CN tissue, CN spheres and CN-sphere derived neuron-like cells, we found a number of genes that are up-/down-regulated in specific patterns. Some of these genes might be involved in progenitor maintenance and differentiation.

In summary, our results suggest that the cochlear nucleus maintains a transient postnatal niche for neural progenitor cells during its critical plastic period. Sphere formation from these stem/progenitor cells can be modulated by activators and inhibitors of Wnt- and TGF-beta-signalling.

(11) Preventing noise-induced hearing loss by blocking phosphodiesterase-5

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Noise-induced hearing loss (NIHL) is a global health hazard with considerable patho-physiological and social consequences, which, until today, has no effective treatment. In the heart, lung, and other organs, cGMP has been described to facilitate protective processes in response to traumatic events. We therefore analyzed NIHL in mice with a genetic deletion of cGMP-dependent protein kinase type I (cGKI) and found significantly increased noise vulnerability. In wild-type animals, in sensory hair cells and neurons of the inner ear, cGKI was expressed

and partly overlapped with the expression profile of cGMP-hydrolyzing phosphodiesterase 5 (PDE5). Treatment with PDE5 inhibitor vardenafil almost completely prevented NIHL and caused a cGKI-dependent upregulation of poly (ADP-ribose) in hair cells and the spiral ganglion, suggesting an endogenous protective cGMP-cGKI signaling pathway culminating in activation of poly (ADP-ribose) polymerase. These data point to the high potential of vardenafil or related drugs for the therapy of NIHL.

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(12) Auditory brainstem response changes in tinnitus

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Tinnitus is often accompanied by hearing loss, hyperacusis, and aberrant neuronal activity known to lead to changes in neuronal plasticity and central reorganisation. Since human and rat ABR functions are generated by the activity within distinct auditory brainstem-, midbrain-, and interbrain-structures, we focused on the ABR signal waveform to reveal information about the brain areas being involved in tinnitus.

In a rat animal model we exposed rats to mildly traumatizing noise, resulting in slight hearing loss but, nevertheless, leading to tinnitus in only a subgroup of animals. Animals were categorized for experiencing tinnitus or no-tinnitus by our behavior model (Rüttiger et al., Knipper, 2003). In addition, the expression of trauma and activity related genes was analysed in the cochlea and in the central auditory system. ABR responses were analysed for amplitude and latency changes in relation to stimulus sound pressure level and hearing threshold by selecting characteristic signal deflections (wave-I to wave V) and correlation measures that delineate the functional loss by noise and tinnitus.

Here, we present a summary of recent analyses correlating expression of different genes with functional and physiological data.

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(13) MOLECULAR ASPECTS OF TINNITUS

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OBJECTIVE To compare different aspects on molecular level between equally hearing impaired animals with tinnitus and without tinnitus induced by acoustic trauma.

METHODS Well-trained adult female Wistar rats that served as a behavior model were exposed to different acoustic trauma paradigms and sacrificed at different time points post-trauma. Then immunohistochemistry for CtBP2, as a measure for inner hair cell ribbon structure and a combined protein and mRNA assay for Arc/Arg3.1 expression in the different regions of the brain were performed.

RESULTS Changes were found for inner hair cell ribbon numbers in tinnitus animals as compared to no-tinnitus animals. These changes could be correlated to Arc/Arg3.1 expression in hippocampal CA1 and the basolateral amygdale.

CONCLUSIONS Arc/Arg3.1 could be used as a tinnitus-specific marker and modulating Arc/Arg3.1 expression may be an attractive approach for tinnitus therapy.

(14) Stimulation of neurite outgrowth of spiral ganglion cells in the organotypic culture model of mouse by neurotrophins and TrkB ligands

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Background: The performance of a cochlear implant is dependent on the number of neurons available for stimulation and by the nerve-electrode interaction. This interaction may be improved by outgrowth of neurites towards and onto the electrode. However, the inhibitory activity of myelin cells, mediated by the low-affinity neurotrophin receptor p75NTR inhibit neurite outgrowth. Effective stimulation mediated via TrkB receptors can be achieved by neurotrophins such as BDNF (brain derived neurotrophic factor). As a paradox, the stimulatory effect of BDNF is opposed by its simultaneous binding to the low-affinity p75NTR receptor in an inhibitory environment.

Methods: An organotypic culture model of the mice spiral ganglion was developed (strain NMRI; established postnatal day 4-6). Coatings with different extracellular matrices (ECM) were compared with respect to their growth stimulating effect. The neurite outgrowth was analyzed and quantified with ImageJ. Stimulation of neurite outgrowth was quantified for BDNF and synthetic, selective TrkB ligands. Inhibition of neurite outgrowth was assessed for MAG-Fc (myelin-associated glycoprotein), PI3K (phosphoinositide-3-kinase) inhibitors and PKA (protein kinase A) inhibitors.

Results: The extracellular matrix coating laminin/poly-D-lysin provided the best environment for neurite outgrowth. Stimulation with BDNF was achieved in a dose and a time dependent manner. Neurite outgrowth was also stimulated by a synthetic TrkB ligand comparable to BDNF stimulation. Selective inhibitors of the TrkB-signaling inhibited these effects.

Conclusion: The organotypic culture model of the spiral ganglion is suitable for the assessment of stimulatory and inhibitory compounds. A synthetic, selective TrkB ligand proves to be a potential candidate for selective stimulation of neurite outgrowth.

Learning outcome: The search for selective TrkB ligands may prove valuable in order to avoid p75NTR receptor mediated effects in an inhibitory environment.

(15) Development of a bioreactor system aiming at the in vitro culture of the functionally mature Organ of Corti

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Due to the difficult anatomical approach and complex cytoarchitecture basic research of the mammalian inner ear is still a challenge. In vitro culture models allow for the investigation of physiology, development, degeneration and regeneration of hearing as well as pharmacological drug screening and ototoxicity studies. In vitro cultures provide defined experimental parameters leading to enhanced reproducibility. Substantial progress has been made in culturing the inner ear in the past, however these techniques still require fragmentation and isolation of the sensory epithelia from its natural environment. We developed a culture technique, using a horizontally rotating culture vessel (Synthecon, USA) that facilitates the organ culture of the entire and intact inner ear within the bony labyrinth. As has been shown for several tissues, creation of simulated microgravity conditions results in low shear stress, enhanced mass transfer and cell-cell-association as well as better paracrine and autocrine cellular communication. The bony part of the basal turn and the apex are removed to facilitate perfusion of the perilymphatic space. Previous studies have shown that inner ears of postnatal day 7 mice can be maintained in culture for up to seven days. We speculated that hypoxic conditions as in the standard in vitro situation (about 140 mmHg pO2) result in hair cell loss in mature ears. We extended our system by introducing an oxygenator to achieve elevated oxygen partial pressures in a continuous flow of media within the rotating culture vessel. Results show, that high level oxygenation results in enhanced survival of outer hair cells in 21 day old mice cultured for 24 hours. Areas of complete hair cell loss were reduced compared to non-oxygenated culture conditions.

(16) Acute noise induced hearing loss is reduced by local application of steroids

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Noise exposure, despite protection measures, often leads to an acute noise trauma. The acute noise trauma quite frequently results in a permanent threshold shift (PTS). Treatments to limit the PTS show that corticosteroids may provide an effective medication. Here we investigated the use of corticosteroids in a preclinical animal model of acute noise trauma. Exposure to impact noise led to a permanent hearing loss in the entire frequency range. Hair cell loss was observed in the middle and apical region of the cochlea. Both the permanent hearing loss and hair cell loss was reduced after 2 week treatment with corticosteroids in a dose-dependent fashion. After noise trauma, local application of high-dose dexamethasone (1 and 4 mg/ml), prednisolone (25 mg/ml) or methylprednisolone (12.5 mg/ml) at the round window of the cochlea showed highest effects. With dexamethasone and prednisolone a statistically significant threshold improvement was observed. Dexamethasone also a clearly improved hair cell preservation compared to the untreated controls. To achieve high drug concentrations, effective to treat acute noise trauma, the application of steroids to the round window of the cochlea was necessary.

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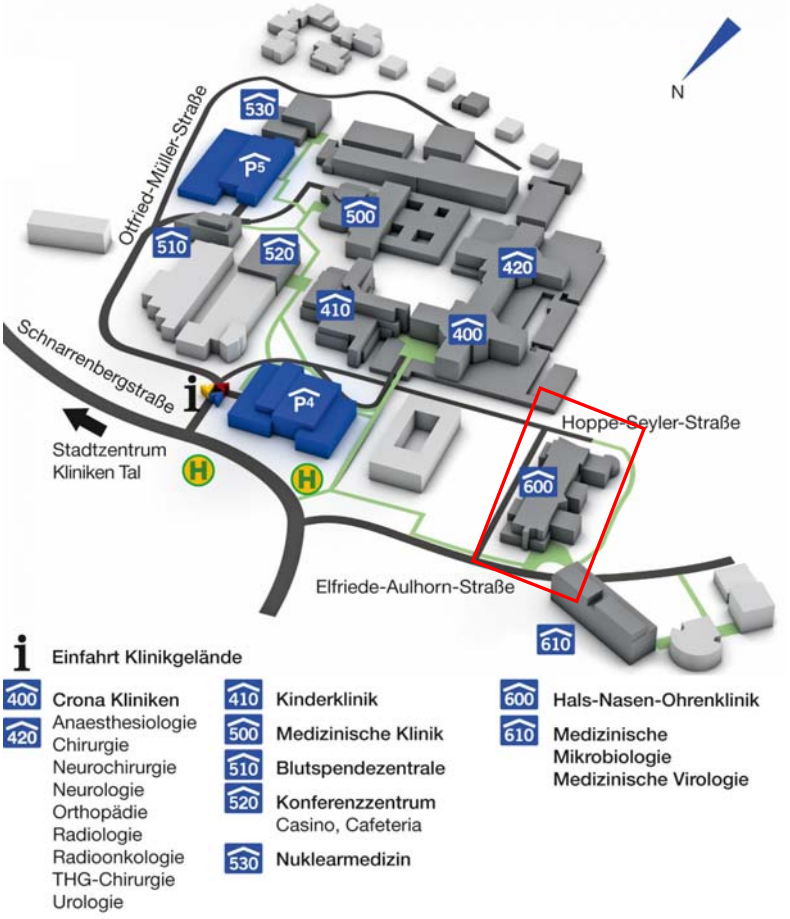

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