Control of lineage specification by the notch pathway in human cardiac precursor cells

Isabelle Plaisance[1], Stephanie Perruchoud[1], Christine Gonzales[1], Patrick Ruchat[2], Andrée Porret[1] and Thierry Pedrazzini[1]

[1]Experimental Cardiology Unit, Department of Medicine, [2]Department of Cardiovascular Surgery, University of Lausanne Medical School, Lausanne, Lausanne, Switzerland

The regenerative potential of the heart relies on a pool of cardiac precursor cells (CPCs). However, the pathways that are required for the mobilization, expansion and commitment of CPCs have not been identified. In the present study, we evaluated whether the Notch pathway was implicated in the commitment and differentiation of human fetal and adult CPCs. Fetal CPCs were obtained from ventricles at 12 to 14 weeks of gestation whereas adult CPCs from right atrial appendages of cardiac patients undergoing surgery. Isolated fetal and adult CPCs demonstrated identical cell surface phenotype, characterized by no expression of hematopoietic markers (CD45) and endothelial stem cell markers (CD34 and CD31), and expression of mesenchymal stem cell markers (CD73, CD90 and CD105). Furthermore, both CPCs expressed early cardiac markers such as Nkx2.5, GATA4 and Mef2c but no late cardiac markers expressed in functional cardiomyocytes. The two populations differ in their cardiogenic potential. In vitro, a large percentage of fetal CPCs differentiate into functional cardiomyocytes and to a lesser extent into smooth muscle cells whereas adult CPCs gave rise essentially to smooth muscle cells. To test their cardiogenic potential in vivo, CPCs were injected into SCID neonates via the temporal vein. Both fetal and adult human cells were detected in the murine heart, and appeared to differentiate into cardiomyocytes. This indicates that, if transferred into the appropriate environment, adult CPCs revealed their cardiogenic potential. The Notch pathway plays crucial roles in the development of cardiovascular system and is still highly activated during the first weeks of age. In order to test whether the Notch pathway could be used to force adult CPCs into the cardiogenic lineage, CPCs were stimulated in vitro using immobilized Notch ligands, i.e. either Jagged 1 or Delta-like-1. In fetal CPCs, both Jagged1 and Delta-like-1-mediated Notch activation increased proliferation and early cardiac gene expression (NKx2.5, GATA4 and Mef2C). In adult CPCs, activation of the Notch pathway using Jagged1 increased the level of Nkx2.5 expression (but not GATA4 or Mef2c) without stimulating proliferation. In contrast, Delta-like1 stimulated proliferation as well as GATA4 and Mef2c expression (but not Nkx2.5). To determine whether the increase in cardiac transcription factor expression reflected a concomitant increase in the number of CPCs, cells were switched to a differentiation medium following stimulation by either Jagged1 or Delta-Like1. Indeed, data indicated that the net production of differentiated cardiomyocytes was increased after transient Notch activation. Altogether, these results indicate that the Notch pathway could be used to reactivate the cardiogenic potential of human adult CPCs.

Sustained and highly tunable delivery of engineered VEGF164 from optimized fibrin matrices ensures normal, stable and functional angiogenesis

Sacchi Veronica, Mittermayr Rainer, Hartinger Joachim, Martino Mikael, Morton Tatjana, Hofmann Anna, Largo Remo, Marshall Jeff, Groppa Elena, Gianni-Barrera Roberto, Ehrbar Martin, Hubbell Jeffrey, Redl Heinz, Banfi Andrea

University Hospital of Basel, Dept. of Biomedicine and Surgery, Hebelstrasse 20, 4031 Basel, Switzerland

Therapeutic angiogenesis is an attractive strategy for treating diseases caused from insufficient vascular supply, e.g. ischemia and wound healing. Vascular endothelial growth factor (VEGF) is the master regulator of physiological and pathological angiogenesis. However, the induction of safe, stable and therapeutically effective angiogenesis requires sustained VEGF delivery for at least 4 weeks at homogeneous microenviromental doses (Ozawa et al, JCI 2004; Von Degenfeld et al, FASEB J 2006). Continuous release of matrix-bound growth factors is a convenient and fully customizable approach for clinical translation of this biological concept. Therefore, here we aim to determine the requirements to induce normal and stable angiogenesis by controlled release of transglutaminase (TG)-bound VEGF from a fibrin gel.

Since both VEGF dose and duration are function of degradation rate, we first determined the optimal gel composition that ensures the longest in vivo persistence in skeletal mouse muscle tissue. Then, to determine VEGF therapeutic window, different VEGF concentrations were tested combined with various concentrations of aprotinin, which inhibits plasmin and retards gel degradation. An aprotinin concentration of 56 µg/ml ensured both sufficient persistence and an adequate VEGF release rate. In this condition, after 9 days a VEGF concentration of 25µg/ml triggered the switch from aberrant to predominantly normal angiogenesis. Further decreasing VEGF concentration to 5, 1, 0.1 and 0.01µg/ml consistently led to the formation of only normal capillaries, which were stable and did not regress by 4 weeks, while 100 µg/ml of VEGF yielded large angioma-like vascular structures and pericyte-poor capillaries. Increasing TG-VEGF concentrations starting from 5 µg/ml induced a dose-dependent increase in vessel diameters. On the other hand, increasing VEGF concentrations within the normal range had no effect on the amount of new vessels. Intravascular staining with fluorescent lectin showed that all newly induced vessels were functionally perfused. Finally, we tested the ability of the fibrin TG-VEGF system to induce functional improvement in a rat model of ischemic wound healing. Based on the previous results, a TG-VEGF concentration of 2 µg/ml in the optimized fibrin matrix composition was delivered to wounds produced in ischemic epigastrial skin flaps. Such VEGF treatment significantly improved both flap perfusion and wound healing rate 7 days after ischemia induction and gel application.

In conclusion, in vivo release of TG-VEGF from fibrin hydrogels can be precisely tuned under optimized conditions to efficiently induce dose-dependent, stable and functional angiogenesis.

New role for the Notch-ligand Jagged1 in the adult heart

Melanie Metrich, April Bezdek Pomey, Corinne Berthonneche, Mohamed Nemir, Thierry Pedrazzini.

Experimental Cardiology Unit, Department of Medicine, University of Lausanne Medical School, Lausanne, Switzerland

Background: The Notch ligand Jagged1 is upregulated in the adult damaged heart, and controls the balance between fibrotic and regenerative repair in the adult stressed heart. This effect is partly due to the activation of the Notch1 receptor which releases Notch intracellular domain (N1IC) and which in turn enters the nucleus to upregulate target gene expression (Hes, Hey). However, beside canonical Notch receptor activation, evidence suggests that membrane-bound Notch ligands also release intracellular fragments, which could then activate Notch receptor-independent cellular responses.
Aim: In order to investigate Jagged1 signaling in adult cardiomyocytes and its role in cardiac remodeling, we generated transgenic mice with cardiomyocyte-specific expression of the Jagged1 intracellular domain (J1IC). The transgene was flanked by fused mutated estrogen receptor sequences to confer controlled nuclear translocation by tamoxifen injection.
Methods and results: TG mice treated with tamoxifen were subjected to transverse aortic constriction (TAC) for one week. Cardiomyocyte cross sectional area of TG mice was significantly reduced compared to WT mice after TAC. LV mass was only slightly affected in TG mice since they showed an increase in cardiomyocyte number and a decrease in apoptotic cells that could compensate for cardiomyocyte size reduction. This phenotype was stronger in 14-day-old TG mice which showed a significant reduction in cardiac mass and myocyte size along with an increase in myocyte proliferation, suggesting a delayed maturation of the TG hearts. In addition, N1IC positive cardiomyocytes as well as Notch target gene expression were downregulated in TG mice suggesting that J1IC inhibits Notch signaling in cardiomyocytes and thereby confirming recent data showing that J1IC decreases N1IC binding to its transcription factor RBP-J□.
Conclusion: Altogether, these data present new insights into the role of Notch ligands in the adult heart and in the regulation of the Notch pathway by its ligands. Ongoing experiments aim at determining the mechanism of J1IC effect on cardiac growth and the role of NICD inhibition in this process.

Controlled VEGF expression by transduced and FACS-purified mesenchymal progenitors ensures safe angiogenesis and functional improvement in a model of myocardial infarction

Melly L1,2, Marsano A1, Helmrich U1, Heberer M1, Eckstein F2, Carrel T3, Cook S4, Giraud MN4, Tevaearai H3, Banfi A1

1 Cell and Gene Therapy and 2 Cardiac Surgery, Basel University Hospital
3 Department of Cardiovascular Surgery, Inselspital, Bern University Hospital
4 Cardiology, University of Fribourg, Switzerland

Introduction. VEGF can induce normal or aberrant angiogenesis depending exclusively on the amount secreted in the microenvironment around each cell. To make this concept clinically applicable, we developed a FACS-based technique to rapidly purify transduced progenitors that homogeneously express a specific VEGF level from a heterogeneous primary population. Here we aim at inducing safe and efficient angiogenesis in the heart by cell-based expression of controlled VEGF levels.
Methods and Results. Human adipose-tissue stem cells (ASC) were transduced with retroviral vectors expressing either rat VEGF linked to the FACS-quantifiable surface marker CD8, or CD8 alone (CD8) as control. VEGF-expressing cells were then FACS-purified to generate populations producing either a specific (SPEC) or heterogeneous (ALL) VEGF levels.
To determine whether this approach could induce controlled angiogenesis in the heart, 107 cells of each treatment group (CD8, SPEC, ALL) were injected into the normal myocardium of nude rats. After 4 weeks, vessel density was increased 2-3 fold by both VEGF-producing groups. However, ALL cells caused the development of numerous aberrant angioma-like structures, while SPEC cells induced only normal and mature microvascular networks.
To determine the safety and functional efficacy in cardiac ischemia, 70 nude rats underwent myocardial infarction by coronary artery ligation. Two weeks later, animals received at the infarction border either 107 cells of one of the 3 treatment groups or PBS. Four weeks post-treatment, the ejection fraction was significantly worsened by treatment with either ALL VEGF (-13.2%) or control (CD8) cells (-6.4%) as well as the PBS group (-8.1%) compared to SPEC VEGF cells (+1.1%). Similar trends in cardiac function were confirmed by Pressure Volume loop analysis performed at the time of sacrifice. Histological analysis confirmed the induction of aberrant structures in the ALL group, which were completely prevented by SPEC cells similarly to the results in non-ischemic tissue. A positive remodeling effect was observed in the SPEC group, with significantly reduced fibrosis in the infarcted area compared to all other groups (CD8, ALL, PBS).
Conclusions. Controlled VEGF delivery by FACS-purified transduced ASC is effective to reliably induce only normal vascular growth in the myocardium and is a promising novel strategy to achieve safe and therapeutic angiogenesis to treat chronic cardiac ischemia.

Thermo-Reversible Hydrogel / Mesenchymal Stem Cell Injection Approach for Nucleus Pulposus Replacement: Feasibility under Static Loading in a Papain-induced Disc Degeneration Model

Cherry Malonzo1, Samantha Chan1, David Eglin2, Sibylle Grad2, Harald M Bonél3, Lorin M Benneker4, Benjamin Gantenbein-Ritter1

1Tissue & Organ Mechanobiology, Institute for Surgical Technology and Biomechanics, University of Bern, Bern, CH
2AO Research Institute, Davos, CH
3Institute for Diagnostic, Pediatric and Interventional Radiology, Inselspital, University of Bern,CH
4Orthopaedic Department, Inselspital, University of Bern, CH

INTRODUCTION: Nucleus pulposus (NP) replacement by the application of injectable hydrogels seems a straightforward approach for tissue engineering. We investigate a thermoreversible hydrogel (TH-RHG), based on a modified polysaccharide with poly(Nisopropylacrylamide) segments prepared using “click chemistry” and reversible addition fragmentation transfer (RAFT) polymerization. The gel behaves as a liquid at room temperature and hardens at >32°C. In order to inject the hydrogel, a mild papain discdegeneration model (PDDM) is employed that creates a cavity in the intervertebral disc (IVD). Here we investigate the performance of the TH-RHG *in situ* in bovine IVD with or without preconditioned human mesenchymal stem cells (hMSC) with rhGDF-5.
MATERIALS AND METHODS: IVDs were harvested from calf tails, aged 6-9 months, which were received from a local abattoir within 3-5h of slaughter. The bovine IVDs of intermediate size (max. height of 2 cm and diameter of 1.5 cm) were used included the bony endplates and cultured *in vitro* for 16 days. Utilizing our previously established PDDM was necessary to create a cavity in the NP. For the papain injection, a 25G needle was used to inject centrally the enzyme into the bovine IVD. Simultaneously, primary hMSCs expanded from bone marrow obtained from patients undergoing spine surgery (ethically approved) were seeded at 4M cells/mL in 3-D in the TH-RHG and preconditioned with 100ng/mL rhGDF-5 for 7 days.
Afterwards, the TH-RHG was reversed to a fluid and injected into the IVD organ culture and kept under static loading of 0.1MPa for 7 days using custom designed specimen chambers. Experimental design i) fresh d0 control PBS injection ii) PDDM + PBS injection iii) PDDM + TH-RHG + bovine NPCs (Cell control) v) PDDM + TH-RHG + hMSC [4]. Magnetic Resonance Imaging (MRI) was employed to image the hydrogel-filled cavity before and after loading at day 9 and day 16, and RT-PCR comparison of the hMSCs at day 1, 8 and 15 investigated the changes in gene expression. On the same days of cell sampling, cell viability (trypan blue) was determined. Sulphated glycosaminoglycan synthesis (DMMB assay) and DNA content (Picogreen assay) from anterior and posterior annulus fibrosus (AF) segments of each disc at day 16 were performed.
RESULTS: MRI images confirmed a central positioning of the TH-RHG into the PDDM. A considerable drop in volume across all groups in the NP region and consequently of disc height was observed between day 9 and day 16. The RT-PCR results of injected hMSCs showed significant differences between day 8 and day 15 for versican and Sox9 relative to day 1. The results of bovine AF gene expression relative to control AF showed significant differences between groups for ACAN, Col1, Col2, IL-b1, versican, ADAMTS-4, Casp-8, MMP13 and MMP3. Cell viability of injected cells dropped from around ~100% down to ~87% for bovine cells and ~86% for hMSC after 7 days in 3-D culture. It further dropped to ~72% after organ culture. GAG/DNA ratio showed significant difference across groups irrespective of sampled location.
CONCLUSION: MRI imaging of TH-RHG seems a promising protocol for non-invasively observing the gel performance *in vitro.* 10% TH-RHG is a feasible scaffold for *in vitro* 3D preconditioning of hMSCs and can be used at the same time for direct injection into the IVD However, the TH-RHG has been shown to be unable to bare static load leading to a drop of cell viability. Relative gene expression of the hMSC phenotype after “co-culture”, however, points towards “discogenic” differentiation.
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Induced pluripotent stem cells (iPS) attenuate fibrosis in bleomycin injured rat lungs

Gazdahar Amiq, Gard Iwona, Gugger Matthias, Anis Feki, Geiser Thomas

Department of Pulmonary medicine, University Hospital Bern, 3010 Bern

Idiopathic pulmonary fibrosis (IPF) is a progressive and irreversible fibrotic process and a major cause of death. The progressive fibrosis is a result of complex series of interdependent events that occur in response to repeated alveolar epithelial injury. Recently, cell based therapies in regenerative context have been investigated as a novel therapeutic approach, however with limited success. Induced pluripotent stem cells (iPSC) offer a novel and promising potential treatment due to their regenerative properties. As a first proof of concept we evaluated the potential of human iPSC to regenerate the alveolar epithelium in bleomycin injured rat lungs. iPSC derived from human foreskin fibroblasts were instilled intratracheally into bleomycin injured rat lungs. The histological analysis revealed marked improvement in lung architecture with reduction of total collagen content (5319±156.1vs 3404±199.8µg/mg of wet tissue weight) as compared to control animals. In conclusion, iPSC may represent a promising novel therapeutic option for lung injury and fibrosis

A Novel Mouse Model for Ocular Hypertension

Sarode, B1., Arsenijevic, Y2., Schorderet, D3., Larue, L4., Melton, D5., Beermann, F1. and Radtke, F1.

1Swiss Institute for Experimental Cancer Research (ISREC), School of Life Sciences, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland.
2Unit of Gene Therapy and Stem Cell Biology, Jules-Gonin Eye Hospital, University of Lausanne, Lausanne, Switzerland
3IRO − Institute for Research in Ophthalmology, Sion, Switzerland
4Developmental Genetics of Melanocytes, UMR146 CNRS, Institute Curie, Orsay, France.
5Howard Hughes Medical Institute and Department of Molecular and Cellular Biology,
Harvard University, Cambridge, United States.

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Cellular signaling mediated by Notch receptors results in coordinated regulation of cell growth, survival and differentiation. Melanocyte specific inactivation of both receptors results in dose dependent hair graying, due to loss of melanocytes and melanocyte stem cells but nothing is known about the role of Notch signaling in pigment cells in the eye.
In order to study the consequences of Notch gain of function in pigment cells in the eye we used Mart1::Cre transgenic mice in which Cre is expressed specifically in ciliary body, iris and retinal pigment epithelium (RPE) in the eye. By crossing these mice with Rosa26::NotchIC transgenic mice we were able to induce overexpression of NotchIC (NIC) in pigment cells in the eye. Mart1Cre:ROSA26::NICl/+ mice developed ocular pathology, characterized by ciliary body hyperplasia and buphthalmic eye. Intraocular pressure (IOP) was increased in the mutant mouse which was responsible for buphthalmic eye. We confirmed that the ciliary body hyperplasia was of pigment cell origin by MITF staining. Ciliary body hyperplasia showed Ki67 staining indicating hyperproliferation. We also found that the RPE was maintained in an undifferentiated state as a consequence of Notch1 overexpression, as determined by analysis of RPE65 and Pax6 expression. In addition, we observed retinal detachment in the Notch mutant mice which was further confirmed by *in vivo* imaging of the eye by OCT. ERG of the Notch mutant mice reveals absence of rods and cones response indicating blindness in the mouse. Fluorescein angiography confirms impaired retinal vasculature as a consequence of Notch gain of function in the pigment cells in the eye. Treatment of the mutant eyes with Glaucoma therapy reduces the IOP indicating this mouse can be used as a model for ocular hypertension for screening new hypotensive drugs. Overall, our data reveals that overexpression of Notch1 in pigmented cells has pleiotropic effects and this mouse can be used as model for ocular hypertension.

DICER1 – A CRUCIAL MEDIATOR IN NOTCH-DRIVEN T-ALL

Junker Fabian, Koch Ute, Dubey Christelle, Radtke Freddy

ISREC, EPFL, SV2533, Station 19, Lausanne CH-1015

T cell acute lymphoblastic leukemia (T-ALL) is one of the most common pediatric hematopoietic malignancies. Activating mutations in the human NOTCH1 receptor have been discovered in more than 50% of pediatric T-ALL cases attributing a crucial role for Notch signaling. Although disregulation of the Notch signaling pathway and certain downstream target genes (Hes1, c-myc) are important players in T-ALL, the precise molecular pathways that control disease development are incompletely understood. T-ALL can be reliably mimicked in mouse models either by expressing the Notch intracellular domain (NICD) genetically from the Rosa-26 locus or via retroviral transduction of hematopoietic progenitors. The aim of this study is to determine the role of microRNAs (miRNAs) in Notch1-driven T-ALL.

Emerging evidence suggests that miRNAs contribute to tumorigenesis acting either as tumor promoters or suppressors. In the context of T-ALL, it has recently been suggested that miRNAs may act as oncogenes (oncomiRs), co-operate with Notch1 and accelerate disease progression in a T-ALL mouse model. However, it is not clear whether miRNAs are essential for T-ALL development, tumor cell progression and survival or tumor maintenance.

We addressed these questions in in vivo studies using an inducible loss of function (LOF; Dicerlox/lox) of Dicer, an enzyme essential to miRNA biogenesis, in a retrovirally Notch1-driven T-ALL setting. We showed that miRNAs are required during the early phase of disease onset using CD4-Cre-mediated LOF of Dicer in T-cell progenitors. Dicer-deficient animals did not succumb to T-ALL suggesting that functional Dicer is required during disease initiation. Also, using Mx1-Cre, we showed in a model of late-stage T-ALL that the ablation of Dicer in leukemic cells leads to a drastically improved survival in the respective animals. In addition, we established several transplantable monoclonal T-ALL cell lines from moribund animals, which allow us to study the role of miRNAs in disease maintenance in greater detail both in vitro and in vivo. We thus showed that, when compared to physiological peripheral T-cells, several distinct miRNAs are upregulated in the T-ALL cell lines such as miR-19b and miR-92a. These highly conserved miRNAs have been proposed to target Bim, Pten and Fbw7, all known regulators of apoptosis and cell growth. Consequently, the mRNA levels of these proteins are reduced in the T-ALL cell lines, and conditional deletion of Dicer in vitro leads to an increase in apoptosis. We are now studying thephysiological regulation of these miRNA candidates in vivo, and are developing strategies to ablate them both in vitro and in vivo.