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## Glandular Stem Cells A New Source for Myocardial Repair?

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### 1. Introduction

For more than 95% of the patients with end-stage heart failure there is no definitive treatment option up to now (AHA, 2009). This fact is caused by a severe shortage in donor hearts on the one hand and on the other hand by technical and economic limitations of cardiac assist devices and artificial hearts. Thus there is a big need for alternative treatment options. Basically heart failure is due to the inability of adult cardiomyocytes to divide and repair damaged heart muscle. For myocardial regenerative medicine stem cells which show the ability to differentiate into functional cardiomyocytes might be a promising source. Although human embryonic stem cells can differentiate into beating cardiomyocytes (Xu et al, 2002), however the therapeutic use of these cells is not without legal and ethical problems.

Previous studies in animal models have demonstrated the therapeutical potential of intramyocardial injection of adult murine stem cells. In a cell-cell contact dependent manner in mammals, mesenchymal stem cells acquired a cardiomyocyte phenotype (Orlic&Kocher 2001, Kawamoto&Yeh 2003, Wang et. al., 2006). Furthermore spontaneously beating cardiomyocytes were derived from isolated cardiomyogenic cell lines of murine bone marrow stromal cells (Makino et al., 1999) adipose tissue stroma cells (Planat-Bernard et. al., 2004) and from spermatogonial stem cells from the adult mouse testis (Guan et. al., 2006). In human myocardium however, adult stem cells from different origins applied intramyocardially did not show a differentiation into cardiomyocytes (Yoon&Wollert 2005). Thus, the search for an appropriate source of cells being able to differentiate into functional cardiomyocytes in human or to contribute to a contractile myocardial patch is still continuing.

From adult rat pancreatic tissue multipotential stem cells were isolated and differentiated into the endodermal pancreatic and hepatic cells (Zulewski, 2001). Human pancreatic stem cells showed also a differentiation into mesodermal structures, like adipocytes, chondrocytes and osteocytes (Seeberger et. al., 2006). Furthermore rat and human pancreatic stem cells gave rise to cellular aggregates containing cell types of all three germ layers (Kruse et.al, 2004) including ectodermal lineages also shown in further recent publications (Seaberg et. al., 2004; Choi et. al., 2004). But up to now neither in animals nor in humans cardiomyocytes were generated from pancreatic stem cells. Due to the clinical need of cardiomyocytes, we investigated the differentiation of human pancreatic stem cell cultures

into cardiomyocytes, a process potentially promoted by co-culture with human myocardial biopsies.

Myocardial regeneration with artificially applied cardiomyocytes is emerging to a promising issue of significant scientific and clinical impact. Nevertheless the source of cells for human cardiomyocyte differentiation especially from adult tissue is still unclear. We hypothesized that human pancreatic stem cells may differentiate into cardiomyocyte-like cells enhanced when co-cultured with myocardial tissue.

## 2. Harvesting, isolating, culturing

From four patients undergoing surgery after an abdominal injury, pancreatic tissue was obtained with informed consent (ethical accreditation of the Ethics Committees, University Hospital of Lübeck, AZ: 03-065). Stem cells were selected, cultured within fetal calf serum and passaged more than 21 times as described elsewhere (Kruse C et al., 2004). Briefly, the pancreatic tissue was treated with digestion medium containing HEPES-Eagle-medium (pH 7.4), 0.1mM HEPES-buffer (pH 7.6), 70% (v/v) modified Eagle-medium, 0.5% (v/v) Trasylol (Bayer AG, Leverkusen, Germany), 1% (w/v) bovine serum albumin, 2.4 mM CaCl<sub>2</sub> and collagenase (0.63 PZ/mg, Serva, Heidelberg, Germany). After digestion the acini were dissociated, centrifuged and further purified by washing in Dulbecco's modified Eagle's medium (DMEM, Gibco, Germany) supplemented with 20 % fetal calve serum (FCS). The washing procedure was repeated 5 times. The acini were resuspended in DMEM and cultured at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. After 1-2 days of culture spindle-shaped stem cells were observed on the bottom of the cell culture flask. After reaching confluence pancreatic stem cells were subcultured by trypsinization, counted and reseeded with a density of 2-4 x 10<sup>5</sup> cells/cm<sup>2</sup>. This procedure was repeated until sufficient cells were available. For differentiation we used cells from passages 4 and 14.

Pancreatic stem cells as a source of cardio-myocytes can be obtained from patients using minimally invasive procedure during laparoscopy. Due to their retroperitoneal topography this cell harvesting might not be as easy as obtaining cells from blood, bone marrow or fatty tissue. Nevertheless current investigations show that stem cells from salivary glands easier accessible than pancreatic tissue might have a comparable potential generating cardiomyocytes. On the other hand, if these pancreatic stem cells could react as a general donor on the basis of a recently postulated immunological compatibility of adult stem cells (Chiu RCJ, 2005; Puissant et. al, 2005) sophisticated harvesting might not be necessary.

When cells became confluent in culture dishes netlike clusters could be detected. The cell layer was washed with the less nutritive phosphate buffered saline (PBS) and mechanically lifted partially from the culture bottom with a scraper.

Irrespective of whether adult human pancreatic stem cells were cryo-conserved or freshly isolated, their phenotype built netlike cell clusters in different passages. After development of these netlike cell clusters, changing culture conditions and partial mobilization from the bottom, very few cell clusters showed distinct cellular autonomous contractions with about 20 beats per minute. Contracting regions were then video documented in real time and reproduced in five settings.

## 3. Co-culture with myocardium

To promote self-differentiation into cardiomyocytes, human pancreatic stem cells were co-cultured with biopsies of human myocardium. This myocardial stimulation practically was

achieved by co-culture of the primary cells with each 5 pieces of myocardium (4x4x4mm) for 2 days. The tissue (left ventricular wall, mitral papillary muscle) was received during heart surgery (ethical accreditation of the Ethics Committees, University hospital of Lübeck, AZ 05-206). Heart muscle pieces were adhered to the bottom of the culture dishes for 3 hours until primary pancreatic stem cells ( $1 \times 10^6$ ) were applied. After 48h heart muscle pieces were removed and the stem cells were further cultured as described above. Cells were passaged every time after reaching confluence. Immunocytochemical analyses were performed directly 48 hours after treatment. To investigate the long term effects of differentiation, cells were collected 17 days after treatment for PCR- analyses.

When cells became confluent in culture dishes netlike clusters could be detected. The cell layer was washed with the less nutritive phosphate buffered saline (PBS) and mechanically lifted partially from the culture bottom with a scraper. Contracting regions were then video documented in real time and reproduced in five settings.

To test whether cardio-myocytes grow out of biopsies from heart tissues, we cultured them as described above but without pancreatic stem cells. After two days no outgrowing cells could be detected, thus cell fusion of cardio-myocytes from biopsies with pancreatic stem cells seems very unlikely.

After having been in contact with human myocardium for 48 hours and growing for further 14-40 days in co-culture, cells were partially mobilized from the bottom of the culture flask and treated with a less nutritive culture medium. Many contracting areas (4-6 fold) were found in comparison to the unstimulated cells. However, the structure of a contracting area itself was comparable with that already observed before having been developed spontaneously without contact to myocardium. These autonomous contractions with about 20 beats per minute as well, were found in all five co-cultures investigated.

The number of contracting areas was enhanced by co-cultured human myocardial biopsies.

The influence from cardio-myocytes on glandular stem cells could become already demonstrated by an immunocytochemical visualization of sarcomeres (red) like shown in figure1 seeing a clearly detectable gradient from M, where the myocardium was located, to the periphery. This cellular stimulation could also be documented as described as follows.

These findings of the stem cell transformation in contact with myocardium in-vitro may become repeated in-vivo after an intra-myocardial injection,

#### **4. Analysis of co-cultured glandular stem cells**

After co-culturing and breeding, the influence of the added myocardium to the glandular stem cells was examined by immunocytochemistry of sarcomere-related myosin, immunocytochemistry of cardiac specific troponin, at the electrone-microscopic level and phenotyped with respect to RNA, protein and cardiomyocyte specificity.

##### **4.1 Immunocytochemistry of sarcomere-related myosin**

Both, the stimulated as well as the non stimulated stem cells were seeded on chamber slides and cultured for at least two days before they were fixed with methanol:acetone (7:3) containing 1 g/ml DAPI (Roche, Switzerland) and washed 3 times in PBS. After incubation in 10% normal goat serum at room temperature for 15 min the specimens were incubated with the primary antibody overnight at 4°C in a humid chamber. Primary monoclonal antibody was directed against sarcomere myosin MF 20 (DSHB, USA). After rinsing 3 times with PBS, slides were incubated for 45 minutes at 37°C with Cy3-labelled anti-mouse IgG,

diluted 1:200. Slides were washed 3 times in PBS, covered in Vectashield mounting medium (Vector, USA) and analyzed with a fluorescence microscope (Axioskop Zeiss, Germany). Excluding these detected sarcomeres were released from the biopsy and attached to the stem cells, controls with fibroblasts and endothelial cells were cocultured with myocardium. Within these controls, tested cells were negative for sarcomeres by immunocytochemistry.

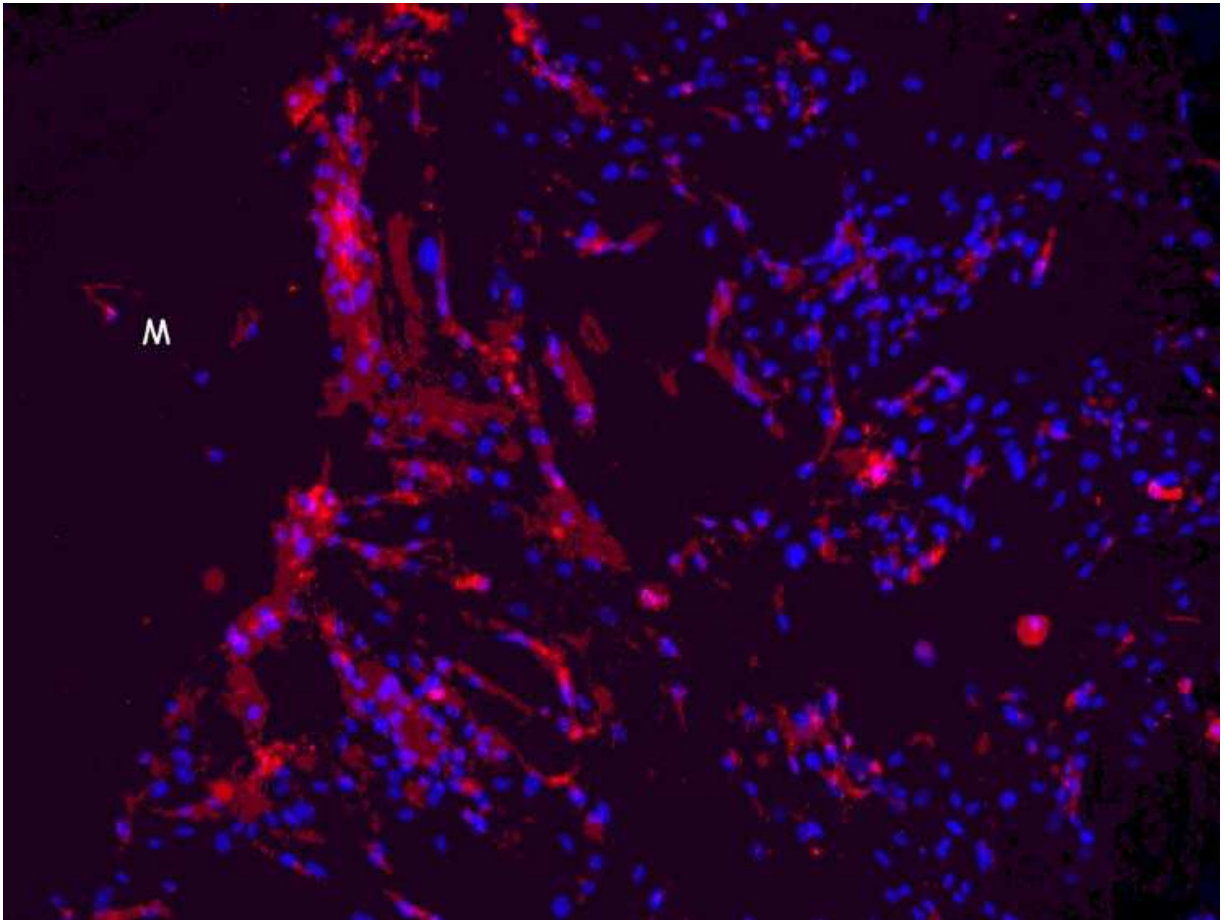


Fig. 1. Immunocytochemical visualization of sarcomeres (red) in transformed adult pancreatic stem cells (blue nuclei) which had been co-cultured with human myocardium (M) for two days. They were clearly positive for the tested antibody compared to the untreated pancreatic stem cells. A decreasing gradient of myosin containing cells from M to the periphery is to be seen.

#### 4.2 Immunocytochemistry of cardiac specific troponin I

Stem cells were co-cultured with myocardial biopsies for 48 hours and bred for further 2 to 4 days after removing the myocardium. Thereafter probes were rinsed 2 times with PBS and dried for 24 hours on air by RT and then fixated by pure acetone for 10 minutes and  $-20^{\circ}\text{C}$ , rinsed again for 2X5 minutes with tris-buffered-saline (TBS) and preincubated with RPMI 1640 (Sigma, USA) with 10% AB serum (Biochrom AG, Germany). Monoclonal anti-troponin I antibody (Clone 2d5, Biozal 1:25) was used as the primary antibody for 60 minutes. Secondary antibody administration (rabbit anti-mouse; DAKO, Denmark; 1:25 for 30 minutes) followed by alkaline phosphatase anti-alkaline phosphatase complex incubation (DAKO, Denmark; 1:50, 30 minutes) was repeated for a total of times each. Finally, substrate

incubation (naphthole/neofuchsin) and counterstaining with hemalaun were performed before microscopic evaluation. Additionally an isotype-control was carried out with mouse IgG 1 (DAKO, Denmark) and for further negative control skeletal muscle was stained. Myocardium was used for a positive control. An isotype-control with mouse IgG 1 (DAKO, Denmark) was negative as well. Additional controls applying on skeletal muscles were negative too. As expected, a control on human myocardium showed positive results (data not shown).

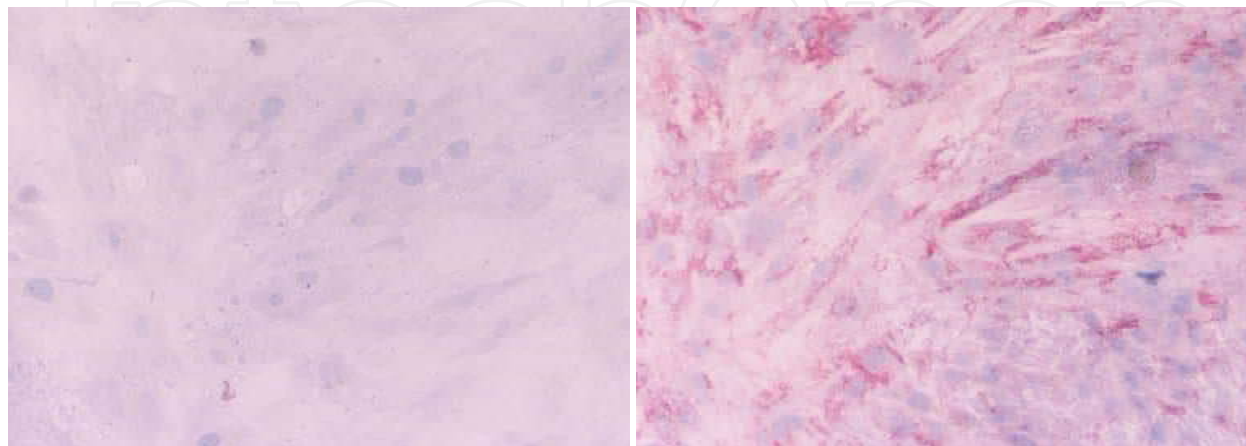


Fig. 2. Human pancreatic adult stem cells with immunocytochemical staining of cardiospecific troponin I (left) and after a two day co-culture with human myocardium (right). The existence of cardiospecific troponin I in transformed cells is clearly demonstrated

#### 4.3 Electron microscopic evaluation

Cells grown on coverslips were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h. Postfixation was performed with 1% OsO<sub>4</sub> in 0.1 M cacodylate buffer for 2 h; samples were dehydrated with ethanol and embedded in araldite (Fluka, Switzerland). Ultrathin sections were stained with uranyl acetate and lead citrate (Ultrastainer Carlsberg System, LKB, Sweden) and were examined with a Philips electron microscope EM 400 at 60 kV (Philips, The Netherlands).

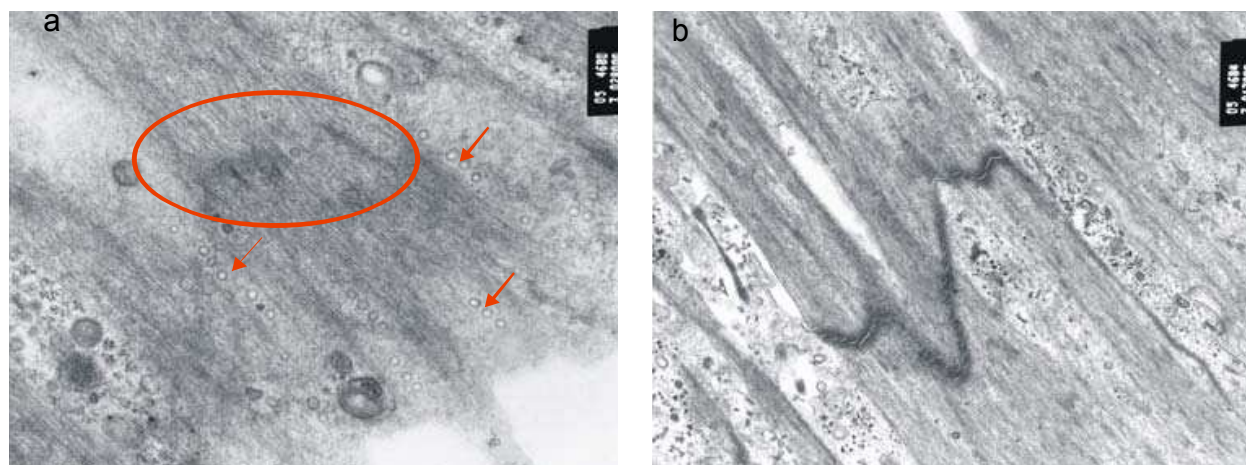


Fig. 3. Electron micrographs (magnification 13 000X) four days after a 48-hour contact with biopsies of human myocardium. Myofilaments and structures of less (left) and complete developed intercalated disc (right) are demonstrated

Electron microscopy (Fig. 3 left) showed differentiated cardiomyocytes containing a number of contractile fibrils. Furthermore, different developmental stages of intercalated discs were observed. While intercalated discs are slightly but clearly recognizable (encircled, Fig. 3 left) in some cells, they are as well differentiated as in mature tissue in others (Fig. 3 right).

#### 4.4 Semiquantative RT-PCR analysis

Total cellular RNA was isolated using NucleoSpin®RNA II-Kit (Macherey-Nagel, Germany). 0.5 µg of total RNA was reverse transcribed into cDNA using Superscript II Reverse Transcriptase RNase H- (RT, Invitrogen, The Netherlands) and oligo dT-Primers (Invitrogen, The Netherlands) according to the manufacturer's instructions. The PCR's were performed in 50 µl reaction volume using Taq DNA Polymerase (MBI Fermentas GmbH, Germany). The reactions were carried out for 38 cycles. Control run of RNA without reverse transcriptions were performed to avoid contamination with genomic DNA and produced no bands. To normalize cDNA concentration in different RT-probes, we measured relative expression of GAPDH as representative for an internal housekeeping gene control.

The expected fragment sizes and optimal PCR annealing temperatures used were as follows: GAPDH , 5':gagtcacggatttggtcgt, 3':ggaagatggtgatgggattt (213bp, 58,8°C), troponin T2, 5':gattctggctgagaggagga, 3':tggagacttctggttatcgttg (197bp, 62,6°C), alpha-actin, 5':gtgtgacgacgaggagacca, 3':cttctgaccataccacca (154bp, 62,6°C), desmin, 5':ctgtccctcccactctgt, 3':agcccctgcttctaagtc (250bp, 62,6°C). A purified human heart RNA (Ambion, USA) and a carcinogenic cell line (HEp-2) served as functional controls for PCR-primer. PCR-analyses were carried out in a total of four settings.

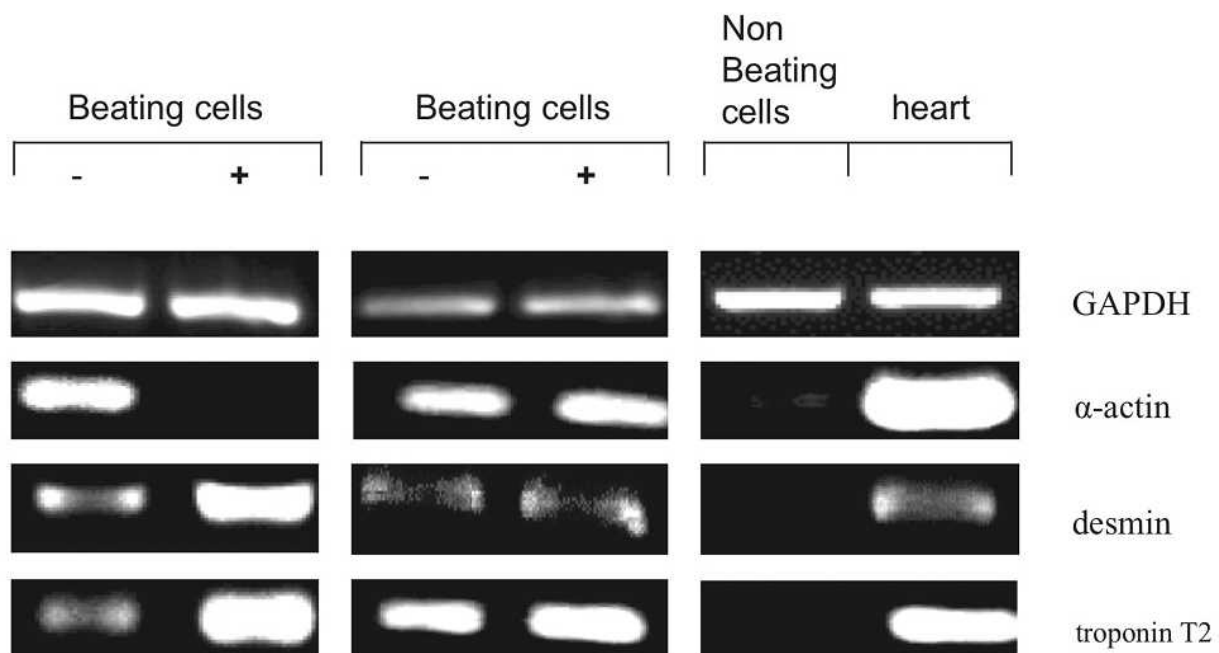


Fig. 4. Gene expression analysis with the specific PCR primers for the target genes  $\alpha$ -actin, desmin and troponin T2 isoform1 demonstrated a greater increase of muscle cell-specific molecules in co-cultured beating cells (+) than in untreated beating cells (-). Non beating carcinogenic cells did not show any muscle specific marker. As a positive control served human heart cDNA (heart)

To test the long-term effect of differentiation PCR-analyses were performed two weeks after co-culture. The target genes for  $\alpha$ -actin, desmin and troponin T were detected to a somewhat greater extent in pancreatic stem cells cocultured with myocardium as compared to untreated cells (Fig. 4).  $\alpha$ -actin could partially not be amplified, whereas desmin and troponin T showed greater amounts in most of the experiments. It remains unclear why  $\alpha$ -actin is suppressed in some cultures. One explanation could be that the differentiation progression of single cell lines follows different roads. We could find a myocardial differentiation within 14 days, but also a shutdown of genes, e.g.  $\alpha$ -actin, in between. Fig.4 demonstrates typical results of these two possibilities.

However, as the differences between co-culture and untreated cells were slightly, the differentiation seemed to abate during time. Probably a permanent stimulus might be necessary to keep the cells in differentiation processes.

## 5. Homing

Applying stem cell therapy in a failing myocardium, the dimension of an intra-myocardial cell homing is significant. Thus a comparison of the homing potential between glandular (GSCs) and mesenchymal stem cells (MSCs) was performed within the myocardium of a big animal model.

In 6 African Bore Goats the intra- myocardial homing of glandular stem cells and MSCs (CD133<sup>+</sup>) was evaluated. Glandular stem cells were characterized by red PKH26 respectively green PKH67 (MSCs) makers. Myocardial samples were taken after one resp. three hours (n=3), others were harvested 6 weeks after injection in additional three goats. Frozen tissue slices were generated and examined for the marked cells.

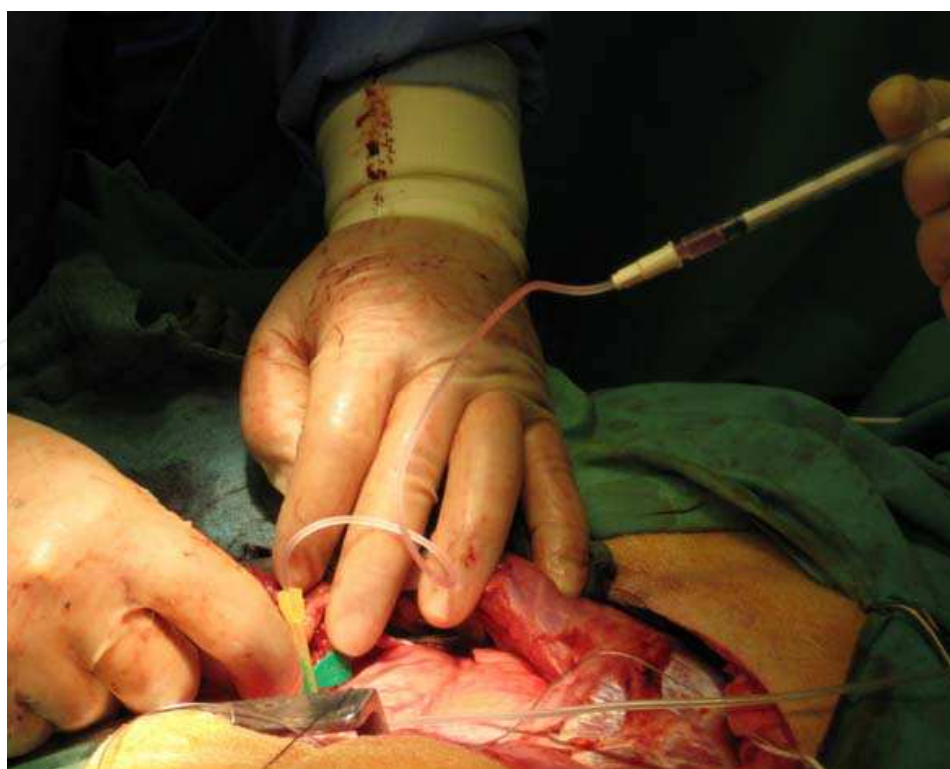


Fig. 5. Through a left lateral thoracotomy and exposure of the left heart a mix of one million of each cell type was injected into three locations of the goat's myocardium of the left ventricle



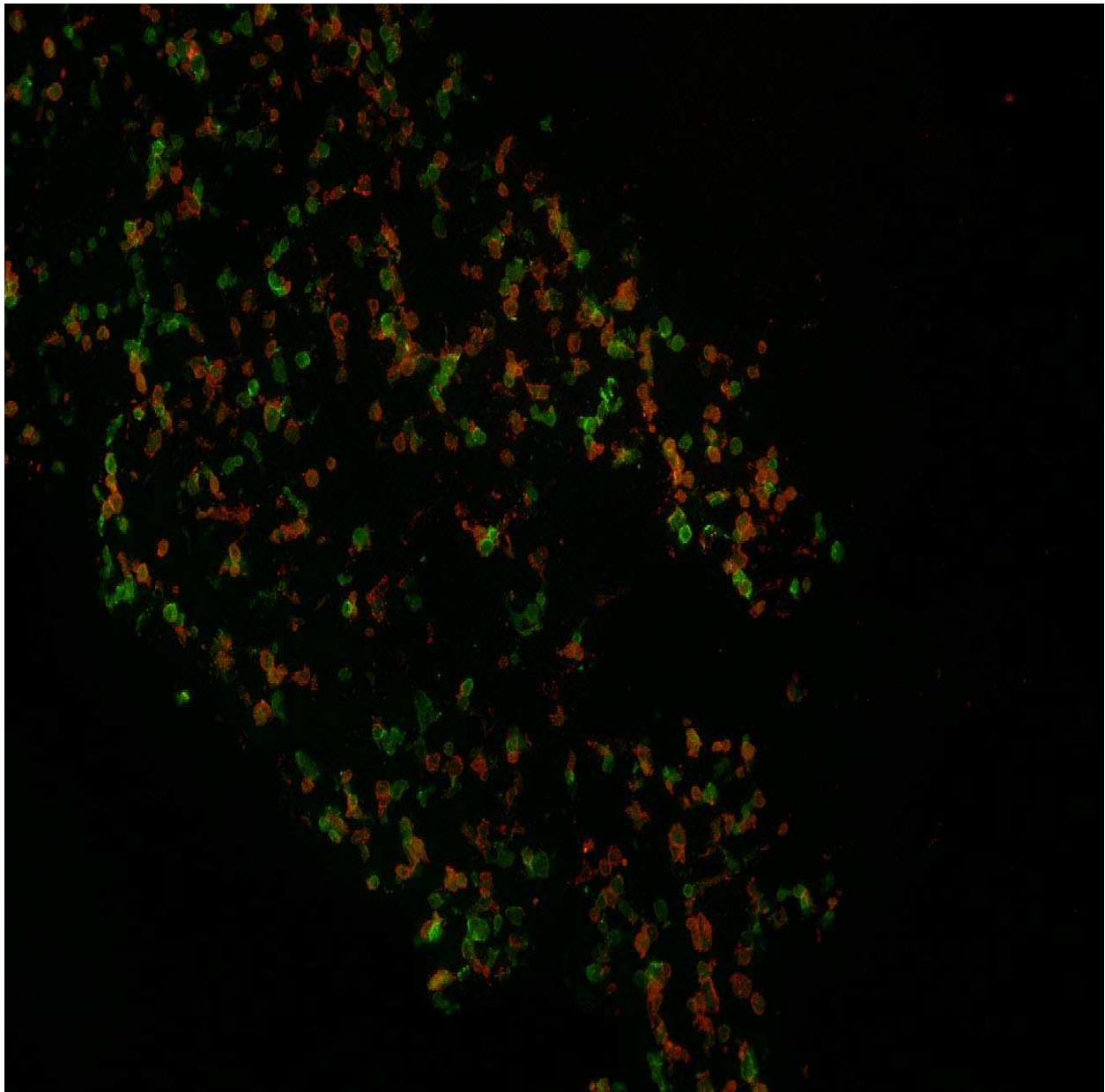


Fig. 6. a A mix of GSCs and MSCs (CD133<sup>+</sup>) are shown before intra- myocardial injection . GSCs are characterized by red PKH26 respectively MSCs by green PKH67 (MSCs) makers. They stay within the cell membrane for about three months. Cell counts were performed after one and three hours and 6 weeks after the intra-myocardial injection

Red PKH26 markers for glandular stem cells (GSCs) respectively green PKH67 markers for mesenchymal stem cells (MSCs) will enable to detect the intramyocardial injected stem cell within a time frame of six months. Within this time frame it should be possible to visualize connexin anti-bodies for the detection of gap junctions.

Using a mix of an intra- myocardial injection of GSCs and MSCs, solely in MSCs (green) a significant cell migration into the surrounding myocardium (n=3) was observed. After 6 weeks nearly all GSCs remained within the myocardium while the MSCs disappeared almost completely. Within the frozen myocardial slices >90% of the marked stem cells were identified as GSCs (red) but <10% as green MSCs.

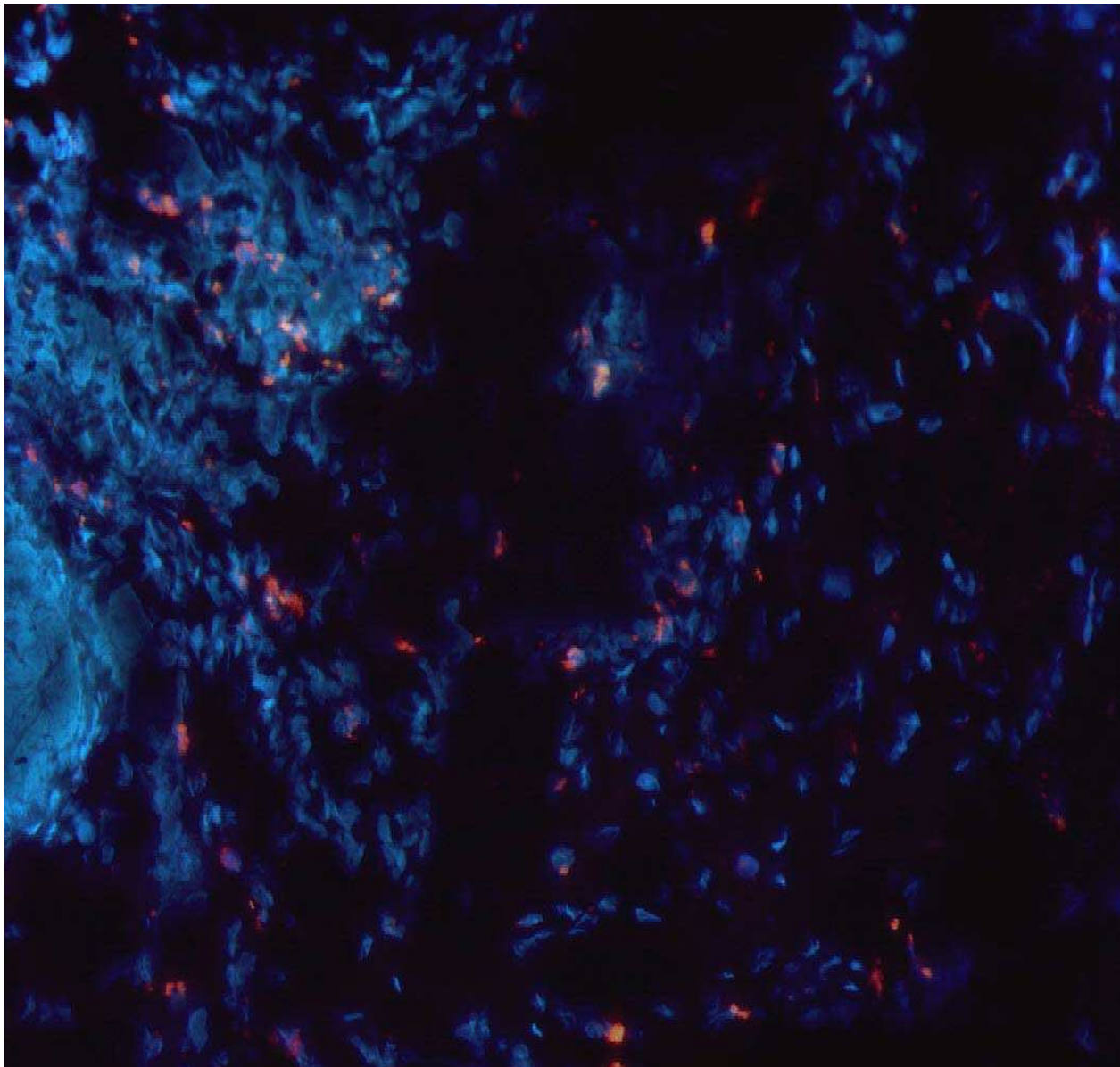


Fig. 6. b Mainly glandular stem cells (GSCs) stained by red PKH26 were found six weeks after intramyocardial injection in the myocardium (blue) of the Bore Goats (n=3). Due to a >90% homing of GSCs combined with the ability developing cardio- myocyte like cells, glandular stem cells might become a very promising treatment option in the therapy of a failing myocardium

## 6. Clinical application

Adult bone marrow derived adult stem cells i. e. mesenchymal stem cells (MSCs) were successfully applied clinically to restore the myocardium solely and in combination with trans-myocardial laser (channels) for myocardial revascularization because MSCs mainly transform into capillaries ) but not significantly into cardio-myocytes. (Stamm C et al.,2003 and Steinhoff G et al., 2006).

Adult glandular stem cells (pancreatic, parotic and submandibular) however are able to form tissue with the distinct ability to generate cell types of all three germ layers (Kruse C et

al., 2004). We showed that mesenchymal cells from this tissue differentiate into cardiomyocytes promoted in co-cultures with human myocardial biopsies (Guldner et al., 2006).

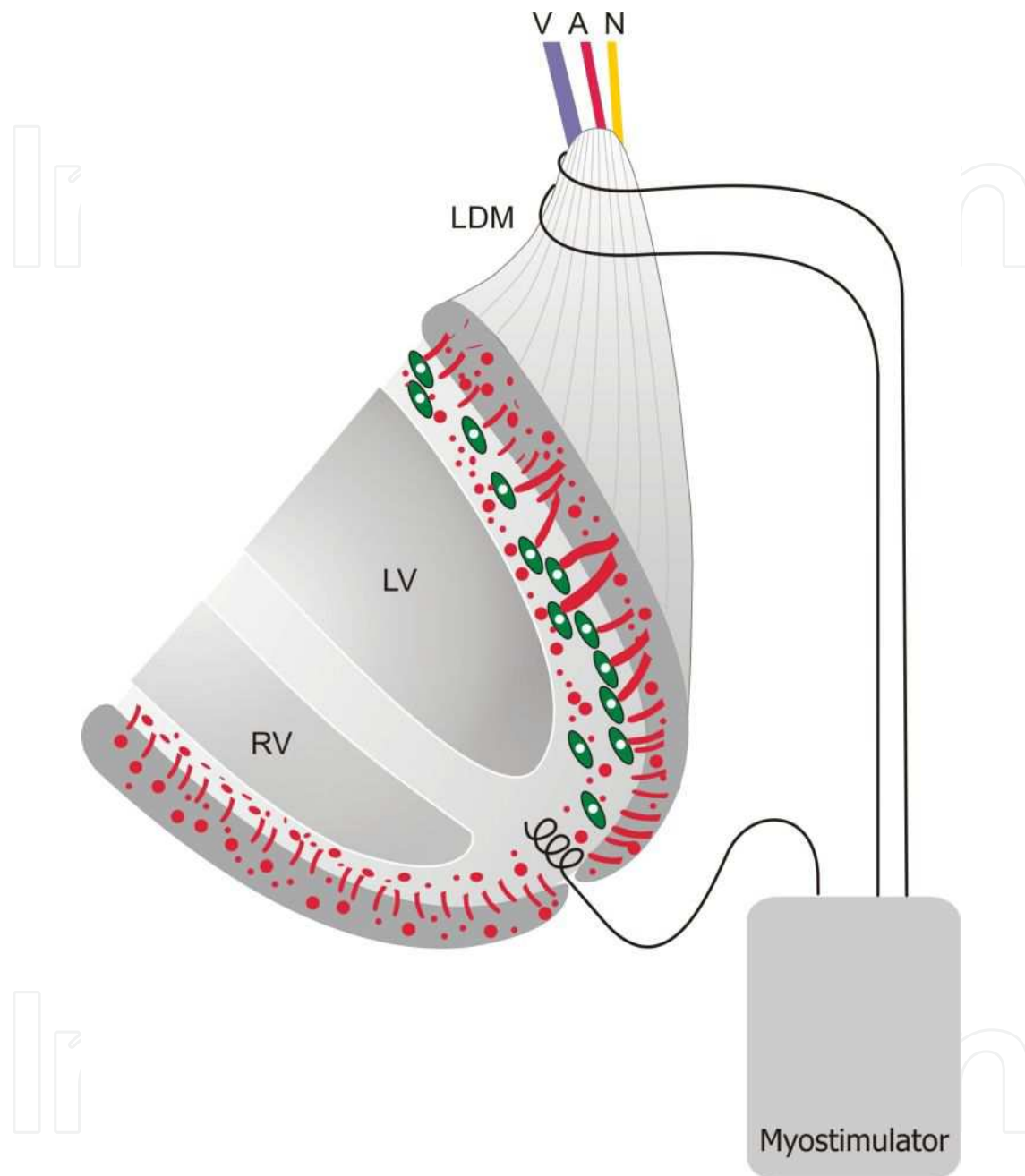


Fig. 7. The concept of a glandular stem cell cardiomyoplasty is designed as a treatment option for end-stage heart failure. It combines an expected regenerative potential of transformed adult glandular stem cells into cardiomyocytes within the myocardium or onto the myocardium and the potential of a hypercapillarized latissimus dorsi muscle (LDM) wrapped around the heart for stem cell nutrition and girdling. Muscle transformation from glandular stem cells into cardiomyocytes is documented. A hypercapillarization (capillary to fiber ratio) of 36% within the LDM was found after an intermittent electrical stimulation over 14 days by an implanted myostimulator delivering a controlled stimulation pattern by a new designed devise (Microstim myostimulator, Germany)

Harvesting pancreatic stem cells may cause severe complications due to pancreatic fistulas accompanied with a peritonitis. Biopsies of the glandula parotis may injure the facialis nerve with facialis paresis. The authors assume minor complications are to expect using the submandibular gland by harvesting cells. An amount of more than 100 mio. stem cells are expected from each submandibular gland, which can become harvested in the same operation injecting them into the myocardium.

Glandular stem cells have the potential transforming into cardio myocytes (Guldner et al., 2006). Furthermore GSCs showed a more than 90% homing in comparison with MSCs after 6 weeks injected in a goat's myocardium (n=3). Combined with their ability developing cardio-myocyte like cells, glandular stem cells are expected to be superior to MSCs for myocardial repair.

In goats and humans electrical continuous and intermittent stimulation of the latissimus dorsi muscle (LDM) has been shown to enhance capillary density of skeletal muscle tissue likewise in small animals as shown by Hudlicka O et al., 1984; Dawson JM et al., 1989; Mathieu-Costello et al., 1996; and Skorjanc D et al., 1998. Additionally known are data of capillary density in intermittent stimulated human sized animals, correlated with functional data like blood flow at rest and under exercise which were evaluated elsewhere (Guldner et al., 2008). The recent investigations in goats demonstrated a 36% higher capillary to fiber ratio in comparison with the non stimulated control after 14 days of electrical pacing of the in-situ LDM (Guldner et al., 2008). An electrically stimulated and therefore hyper-capillarized latissimus dorsi muscle (LDM) and wrapped around the heart could serve for GSC's nutrition (Mannion et al., 1992, 1993, 1996 ;Salmons et al., 1998; Guldner et.al.2008).

A stem cell cardio-myoplasty combines a cellular cardio-myoplasty (Stamm et. al, 2003; Steinhoff&Stamm, 2006) with a muscular dynamic cardio-myoplasty (Carpentier& Chachques 1985). The additional elastic girdling from LDM around the heart (Figure 7) reduces left ventricular's wall tension and therefore reduces the oxygen consumption of the myocardium (Hagège A A et al., 1995).

## 7. Conclusion

These are first investigations demonstrating the feasibility of generating autonomously contracting cardio-myocyte-like cells from adult human glandular stem cells and their transformation in contact with myocardium. Injecting them into the myocardium is an in vivo co-culturing. We expect in opposite to the injection of bone marrow derived stem cells, resulting in capillaries, a substantial increase of cardiomyocytes. This mechanism might be helpful because of the inability of adult cardiomyocytes to divide and repair damaged heart muscle.

Harvesting glandular stem cells intra-operatively might become feasible in the same operative procedure as the intramyocardial implantation likewise it has been practiced already with bone-marrow-derived stem cells (Steinhoff&Stamm, 2006). The estimated amount of harvested glandular stem cells from one glandular submandibularis might be many-fold higher than the amount of MSCs applied nowadays clinically.

Regarding a superior intra-myocardial homing of GSCs in comparison to MSCs stem cell therapy with GSCs might become an advanced cellular treatment option in the regenerative medicine for end-stage heart failure.

Glandular stem cells might enable intramyocardial injections solely or in combination with a pre-stimulated cardiomyoplasty, a muscle powered cardiac assist procedure with an increased capillary to fiber ratio, which is to perform by adequate electrical stimulation patterns. This muscular indirect myocard revascularisation may not only increase myocardial's blood supply supporting the implanted GSCs but will additionally decrease the oxygen consumption by a girdling effect. This is due to a decreases wall tension of the myocardium. Thus glandular stem cells should enable a glandular stem cell cardiomyoplasty which combines an expected regenerative potential of transformed adult glandular stem cells into cardiomyocytes within the myocardium with the potential of a hypercapillarized latissimus dorsi muscle (LDM) wrapped around the heart for stem cell nutrition and girdling.

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## **Regenerative Medicine and Tissue Engineering - Cells and Biomaterials**

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Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage for knee-replacement procedures, urinary bladder replacement, urethra substitutes and cellular therapies for the treatment of urinary incontinence. The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues reconstructed from readily available biopsy material induce only minimal or no immunogenicity when reimplanted in the patient. This book is aimed at anyone interested in the application of Tissue Engineering in different organ systems. It offers insights into a wide variety of strategies applying the principles of Tissue Engineering to tissue and organ regeneration.

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