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1. Introduction

1.1. Cardiovascular Diseases

Cardiovascular diseases (CVDs) had become the United States' number one cause of deaths by the late 1940s (The Framingham Heart Study, 2009) and more people die annually from CVDs worldwide than from any other cause (WHO, 2009). CVDs are caused by disorders of the heart and blood vessels and include coronary heart disease (CHD, heart attacks), raised blood pressure (hypertension), rheumatic heart disease, congenital heart disease and heart failure. In 2004, an estimated 7.2 million people worldwide died from coronary heart disease and 5.7 million died due to stroke, which accounted for the largest percentage of the total of 17.1 million deaths caused by CVDs. There are some major risk factors that can be controlled, treated or modified in order to avoid CVDs such as tobacco use, physical inactivity, overweight and high blood pressure (American Heart Association, 2005). People in low- and middle-income countries are more exposed to risk factors leading to CVDs and are less exposed to prevention efforts than people in high-income countries causing a disproportional affection of these countries: 82% of CVD deaths take place in low- and middle-income countries (WHO, 2009).

CHD and other chronic cardiomyopathies lead to segmental loss of cardiac tissue and scarring of a large portion of the ventricular wall (Beltrami et al., 1994; Joggerst and Hatzopoulos, 2009). Adult hearts have a very limited regenerative potential and the loss of myocytes and coronary vessels after injury is mostly irreversible. Replacement of small areas of dead tissue located within the scarred myocardium has only been occasionally seen in humans (Urbanek et al., 2005). Therefore, the only possible cure for patients with CVDs is transplantation, which is hampered by a low number of suitable donors.

A relatively new approach against CVDs is stem cell therapy. Stem cell populations possess cardiogenic potential and animal models indicate, that the function of ventricular muscle after ischaemic injury is improved after transplantation of mesenchymal stem cells, bone-marrow-derived haematopoietic stem cells, skeletal myoblasts, or embryonic stem cells (Joggerst and Hatzopoulos, 2009). However, the generation of new cardiac tissue is low and the benefits for cell therapy are modest

(Joggerst and Hatzopoulos, 2009). The identification of an optimal cell type which derives from a renewable source and produces sufficient quantities to drive clinically relevant levels of cardiomyogenesis (CMG) is a central challenge for cell-based therapy (Domian et al., 2009).

1.2. Murine Heart Development

The heart is the first functional organ that forms in the developing embryo, starting around day E7. It is composed of a variety of different cell types, indicating the complexity of its morphology. It consists of diverse muscle and non-muscle cell lineages, atrial and ventricular cardiac myocytes, conduction system cells, smooth muscle and endothelial cells of the coronary arteries and veins, endocardial cells, valvular components and connective tissue (Laugwitz et al., 2008). Heart formation therefore needs to be a precisely coordinated process of cellular differentiation and morphogenesis (Akazawa and Komuro, 2005). Cardiogenic mesoderm, the cardiac neural crest and the proepicardial organ have been identified as the three major sources of heart precursors which give rise to the distinct cardiac compartments (Laugwitz et al., 2008). Development of those progenitors is spatially and temporally segregated in the developing embryo (Fig. 1).

The cardiogenic mesoderm itself consists of two different lineages of cardiac precursors. It is distinguished between progenitors giving rise to cells of the first heart

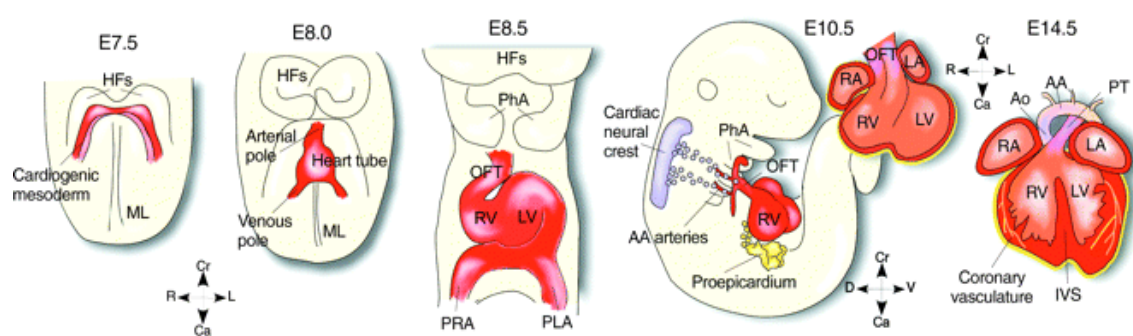


Fig. 1 Murine heart development and origin of heart structures. The cardiogenic mesoderm (red), the cardiac neural crest (purple), and the proepicardium (yellow) are the three major progenitors in murine heart development and contribute to different heart compartments. Cardiogenic mesoderm cells form the linear heart tube and the four chambers of the heart. Cardiac neural crest progenitors engulf the aortic arch arteries after looping occurred on day 8.5 and they furthermore differentiate to vascular smooth muscle cells of the outflow tract. The epicardial mantle and coronary vasculature origins from cells of the proepicardium. AA, aortic arch; IVS, interventricular septum; LA, left atrium; LV, left ventricle; PhA, pharyngeal arches; PLA, primitive left atrium; PRA, primitive right atrium; RA, right atrium; RV, right ventricle (Laugwitz et al., 2008).

field and the second heart field (Buckingham et al., 2005). The first heart field

comprises the cardiac crescent, the linear heart tube, parts of the left and right atria and the left ventricular region. The outflow tract and the right ventricular region make up the second heart field (Buckingham et al., 2005). Highly differentiated cardiomyocytes after birth lose their ability to proliferate. They only grow in size to adapt to a demand for an increased workload (Akazawa and Komuro, 2005).

1.3. Stem Cells

Stem cells are cells with a capacity to self-renew and to generate daughter cells that can differentiate into several lineages to form all of the cell types that are found in the mature tissue. Self renewal means that a progenitor gives rise to daughter progenitors of equivalent developmental potential. Stem cells can either divide symmetrically to two identical cells of the same developmental potential to expand the stem-cell compartment. They can also generate by asymmetric cell division one cell identical to itself and one cell that is distinct. The third option is division to two daughter cells with less potential than the stem cell. The distinct cells go through a series of cell divisions and differentiative steps, forming intermediates or progenitor cells to finally develop to the terminally differentiated cell populations (Smalley and Ashworth, 2003). Stem cells are classified in regard to the different cell types they are able to give rise to.

Totipotent stem cells have the ability to differentiate to all cell types of the body as well as to all cell types that make up the extraembryonic tissue during embryogenesis. The only totipotent cells are the zygote and the developing cells after rapid division of the zygote until the 16-cell developmental stage.

Pluripotent stem cells are able to give rise to cells of all three germ layers of the embryo. However, they cannot form extraembryonic tissue.

Multipotent stem cell have the ability to develop into more than one cell type of the body and unipotent stem cells are restricted to one cell type to which they differentiate (NIH, 2001).

Stem cells reside in a three-dimensional structure, a so called stem cell niche. These stem cell niches consist of a variety of heterologous cells. They must have both anatomic and functional dimensions, specifically enabling stem cells to reproduce or self-renew (Scadden, 2006). A stem cell niche should consist of extracellular matrix,

the stem cells, and signalling cells affecting the stem cells (Lin, 2002). Various elements of the local environment regulate the stem cell developmental status by providing signals for self-renewal or differentiation (Fig. 2) (Scadden, 2006). Adult or somatic stem cells have limited function without the niche, cardiac stem cells cannot exist in the absence of supporting cells which anchor the stem cells to the niche and modulate growth signals (Urbanek et al., 2006). It is the niche that provides the modulation in stem-cell function needed under conditions of physiological challenge (Scadden, 2006). Obtaining signals for activation, cell division occurs and cells migrate out of the niches to sites of cell replacement where they differentiate and acquire the adult phenotype. Niche homeostasis is regulated by division of stem cells which preserves the ideal proportion of primitive and committed cells within the organ (Fuchs et al., 2004).

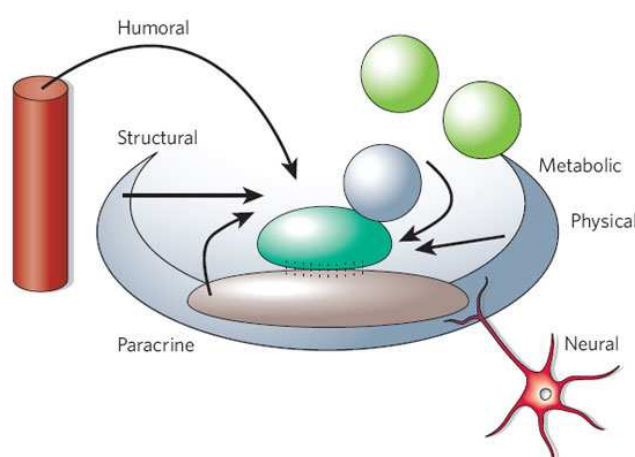


Fig. 2 *Different elements of niche influence on stem cell state.* The local environment of stem cells provides signals to adapt stem cells under conditions of physiologic challenge. Humoral, structural, metabolic, physical, neuronal and paracrine signals influence the differentiation mode of the stem cells (Scadden, 2006).

1.4. Embryonic Stem Cells

The fusion of sperm and egg gametes results in the formation of a zygote and initiates a series of cell divisions. On days 3 – 4, a blastocyst develops which is characterized by the presence of a blastocyst cavity, an inner and outer cell mass (O'Connor and Crystal, 2006). By the time of implantation on days 4 – 5, three distinct cell types are apparent in the mouse blastocyst which give rise to separate cell lineages in later development. The inner cell mass develops to the Epiblast (primitive ectoderm) which gives rise to the entire fetus as well as extraembryonic mesoderm, and to the Hypoblast (primitive endoderm) which forms the extraembryonic endoderm layers of the visceral and parietal yolk sacs. The outer cell mass or trophoctoderm gives rise to all the

trophoblast cell types that make up the majority of the fetal part of the placenta (Chazaud et al., 2006).

Embryonic stem cells (ESCs) are pluripotent cells that are isolated from the inner cell mass of embryos at the blastocyst stage (Fig. 3). The second broad category of stem cells is derived from various fetal and post-natal organs, the so called somatic stem cells. Those stem cells generally have been thought of as tissue specific, able to only give rise to progeny cells found in the tissue of their origin (O'Connor and Crystal, 2006; Wagers and Weissman, 2004).

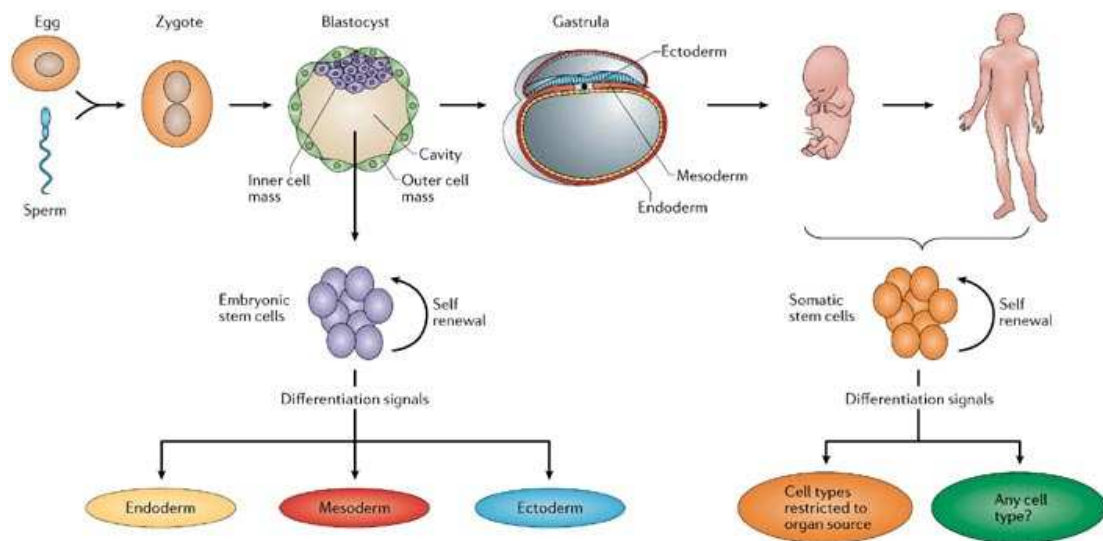


Fig. 3 *Derivation of embryonic and somatic stem cells.* Embryonic stem cells are isolated of inner cell mass cells in the blastocyst stage of embryonic development. They can be cultured, kept in self-renewal mode and upon differentiation signals, they have the ability to form all cell types of the three germ layers. Somatic stem cells are present in many fetal and post-natal tissues. Somatic stem cells are also capable of self-renewal and, with appropriate signals, differentiate into various cell types of the organ from which they are derived (O'Connor and Crystal, 2006).

The advantages of ESCs compared to somatic stem cells are, that they are easier to identify, isolate and to maintain as an established stem cell line (Boiani and Scholer, 2005). As pluripotent cells, they have the ability to divide symmetrically in culture and give rise to daughter cells that are exact copies of the stem cells from which they were derived. Therefore, cells can be expanded in culture before differentiation to all cell types of the embryo can be initiated. They undergo differentiation steps resembling the in vivo developmental programs and are therefore a good model system to study differentiation and lineage commitment.

1.5. Embryoid Bodies

ESCs can be maintained in a pluripotent self renewal state when they are grown on mitotically inactivated, Leukemia Inhibitory Factor (LIF) secreting fibroblast feeder cells. Transfer from feeder cells to gelled plates results in the differentiation of these cells. The formation of embryoid bodies (EBs) is a very well established and reproducible method for mimicking the early in vivo development of the embryo. Embryoid bodies are ESCs which form aggregates without any contact to artificial material. The first cells that occur are primitive endoderm cells which form a single layer surrounding the inner cell mass, which becomes the primitive ectoderm (Ikeda et al., 1999). Therefore differentiating EBs resemble a murine blastocyst in many aspects. At day 4.5 EBs are transferred to gelled plates. Attachment to the matrix induces the differentiation of primitive endoderm to parietal endoderm and migration away from the center of the EB which mimics the migration of parietal endoderm along the inner surface of the trophectoderm in implanting blastocysts (Fig. 4) (Bader et al., 2001).

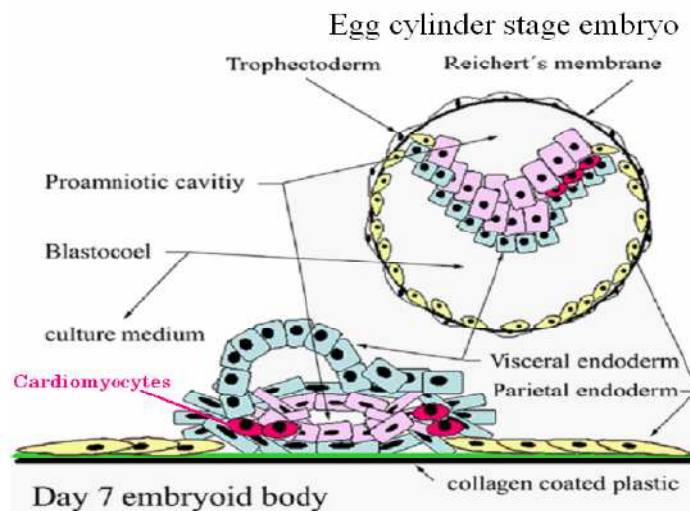


Fig. 4 *Embryoid body development mimics early steps of embryogenesis.* Comparison of an in vitro development of embryoid bodies on day 7 with the in vivo embryogenesis at the egg cylinder stage (Weitzer, 2006).

Further differentiation of EBs results in the formation of cells of all three germ layers such as cardiomyocytes, hematopoietic progenitors, yolk sac, skeletal myocytes, smooth muscle cells, adipocytes, hepatocytes, chondrocytes, endothelial cells, melanocytes, neurons, glia, pancreatic islet cells, and primitive endoderm (Itskovitz-Eldor et al., 2000; Keller, 1995).

1.6. Maintaining Stem Cell Pluripotency

Naive pluripotency of early epiblast and embryonic stem cells is maintained by the activity of three transcriptional organizers, octamer binding transcription factor 4 (Oct4), sex determining region Y box 2 (Sox2), and Nanog. They are described as the trinity of nuclear regulators, building a self-assembling and self-sustaining network to govern pluripotency in vivo and in vitro by continuous suppression of lineage specific factors (Chambers and Smith, 2004; Niwa, 2007). Oct4 and Sox2 also direct the expression of fibroblast growth factor 4 (FGF4), a differentiation inducing protein. FGF4 activates the mitogen activated protein kinase (MAPK) pathway (Kunath et al., 2007) which destabilizes the self-renewal state of stem cells and promotes lineage specification by rendering the cells responsive to further inductive signals (Silva and Smith, 2008) (Fig. 5). This effect of the two stemness factors might appear controversial. However, in development epiblast cells are meant to differentiate. Therefore, the system is made to eliminate ESCs. To keep ESCs in a naive, undifferentiated state requires overcoming normal developmental progression. Conventionally, extrinsic stimulation of Signal transducer and activator of transcription 3 (Stat3) by cytokine leukemia inhibitory factor (LIF) maintains murine ESCs. Furthermore, either bone morphogenetic protein (BMP) or serum have to be added to induce inhibitors of differentiation (ID) proteins (Ying et al., 2003).

FGF4 acts by activation of the mitogen activated protein kinase (MAPK) pathway. FGF4 mutants have minimal requirement for LIF and serum/BMP, however neither LIF, nor serum/BMP block the activation of extracellular signal regulated kinase (ERK). Stat3 and ID proteins may directly antagonize effectors downstream of ERK (Silva and Smith, 2008; Ying et al., 2008).

Therefore, LIF/Stat3 and BMP/Smad/ID signalling do not instruct self-renewal but shield the pluripotent state from induced MAPK signalling (Ying et al., 2008). LIF also promotes ESC growth and viability (Silva and Smith, 2008).

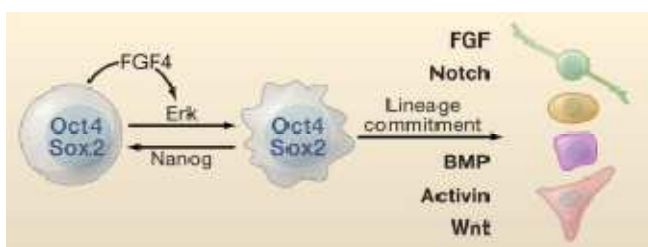


Fig. 5 *Maintaining pluripotency.* Sox2 and Oct4 activate expression of FGF4 which activates the MAPK pathway, resulting in lineage commitment and differentiation (Silva and Smith, 2008).

Maintaining pluripotency does not seem to be predetermined in epigenetics, epigenetic patterns are rather changed due to the differentiation controlling stimuli (Silva and Smith, 2008).

Elimination of differentiation-inducing FGF4 signalling results in a general reduction of differentiation and self renewal is enabled (Ying et al., 2008). However, in the presence of a very potent MEK inhibitor, PD0325901, ESCs degenerated. Culture with a combination of two inhibitors, the MEK inhibitor PD0325901 and the glycogen synthase kinase (GSK3) inhibitor CHIR99021, gives rise to ESCs with clonal propagation, they are able to give somatic chimeras and germline transmission. Shielding from ERK seems to enable self-renewal and this is stabilized by the inhibition of GSK3 (Ying et al., 2008). The two inhibitors liberate ESCs from requirements for exogenous factors without compromise to developmental potency (Ying et al., 2008). Still, the optimal condition of growing ESCs is always with LIF, culture of LIF + the two inhibitors promotes self renewal better than either inhibitor in combination with LIF or the two inhibitors alone. And those combinations work better than LIF + serum/BMP. It was also possible to isolate ESC lines from any mouse strain and from rats with the two inhibitors and LIF (Smith, unpublished data).

Very recently, two other transcription factors, Kruppel like factors 2 and 4 (Klf2 and Klf4) were identified as being important in maintaining pluripotency (Guo et al., 2009; Smith, unpublished data). *Klf2* and *klf4* genes are predominantly downregulated during differentiation of ESCs into Epiblast stem cells (EpiSCs), they are expressed in ESCs but not in EpiSCs. EpiSCs are derived from post-implantation epithelialized epiblasts. They are in a similar ground state as ES cells with an autonomous capacity for survival and replication (Nichols et al., 2009). They are capable of multilineage differentiation in vitro, express the core pluripotency genes Oct4, Sox2, and Nanog but are unable to repopulate a host blastocyst (Brons et al., 2007; Guo et al., 2009; Tesar et al., 2007). Introduction of either *klf2* or *klf4* genes into the genome of EpiSCs followed by culture together with LIF and the two inhibitors resulted in the interconversion of EpiSCs into ESCs, the naive ground state was regenerated (Guo et al., 2009; Smith, unpublished data).

1.7. Cardiac Progenitor Cells

Multicellular organisms require the existence and precise control of stem cells that maintain tissue homeostasis (Boiani and Scholer, 2005). Slowly dividing stem cells give rise to proliferating, lineage restricted progenitor cells which develop to terminally differentiated cells replacing aged or injured cells of the various tissues (Flickinger, 1999; Kajstura et al., 2008; Quesenberry et al., 2005). Although culture and differentiation techniques of ESCs have improved dramatically and ESCs are able to differentiate into cardiomyocytes (CMCs) in vitro, the efficiency of this process is very low and it remains challenging to obtain cardiomyocyte-enriched cultures (Smits et al., 2009).

Recent results suggest that multipotent, cardiac stem cells (CSCs) exist in the heart and constantly give rise to a hierarchy of downstream cellular intermediates and myocyte progeny (Laugwitz et al., 2008). They have been identified in the adult heart and reside in cardiac niches, which contain CSCs and lineage-committed cells. Connexin and cadherins form gap and adherens junctions and connect CSCs to supporting cells such as myocytes and fibroblasts (Urbanek et al., 2006). Different groups already succeeded in the isolation of somatic stem cells of the heart (Beltrami et al., 2003; Oh et al., 2003; Oyama et al., 2007; Pfister et al., 2005). Laugwitz et al. (2008) identified a master heart progenitor cell type that ultimately gives rise to the heart's three major cell types: cardiac muscle, smooth muscle and endothelium. Recent results suggest that a defective CSC compartment is a common denominator of heart failure of different etiologies (Kajstura et al., 2008). Therefore gaining knowledge about the progenitor identity and pathways influencing their differentiation is essential to gain better insights into CMG and for regenerative cardiovascular medicine. For this purpose, it is important to generate an in vitro model that can achieve differentiation of a homogenous population of cardiomyocytes to a reproducible degree (Smits et al., 2009). Wiedner and Weber (Wiedner, 2008) succeeded in the isolation of stable murine cardiovascular progenitor cell (CVPC) lines. CVPCs express the stemness factors Oct4, Nanog, and Sox2 in comparable amounts to ESCs as well as cardiac transcription factors such as *nkx2.5* and *myocyte enhancer factor 2C (mef2c)* already in the undifferentiated state. The cells seem to be somewhere downstream of ESCs, they have a predisposition to the cardiac lineage but still have stem cell character. Similar cells, expressing stem cell specific genes such as *c-kit* as well as the cardiac genes

nkx2.5, *mef2c* and GATA binding protein 4 (*gata4*), were found and isolated from the human heart (Smits et al., 2009).

1.8. Cardiac Transcription Factors

Cardiac transcription factors are essential gene activators that are expressed in the developing heart tissues. The production of structural or regulatory proteins characteristic for CMG is regulated by the expression of cardiac-specific genes. They therefore have an influence on processes that impact the morphogenesis of the developing heart (Bruneau, 2002). Different studies have identified a core group of transcription factors that are required for cardiac development, including members of the NK class of homeodomain proteins, GATA zinc-finger transcription factors, the MADS domain transcription factor Mef2C, Forkhead transcription factors as well as the Hand class of basic helix-loop-helix factors (Black, 2007). *Nkx2.5* is a critical transcription factor in the first and second heart field lineages, *Isl1*, along with *Foxh1* and GATA factors is the key transcriptional regulator of the second heart progenitor field (Laugwitz et al., 2008). *Mef2C* plays an essential role in the transcriptional program for second heart field development whereas it is only weakly expressed in first heart field precursors and its derivatives (Black, 2007). The GATA family of zinc finger-containing transcription factors appears to be potentially critical in regard to progressive differentiation of precardiac cells (Bruneau, 2002).

The function of transcription factors largely depends on the target genes they regulate, therefore identification of such genes is essential to understand the molecular mechanisms in cardiac development (Nakashima et al., 2009). Early cardiac marker genes are *nkx2.5*, *T-box 5 (tbx5)*, *gata4* and *mesoderm posterior 1 (mesp1)*. Late cardiac markers are *myosin heavy chain 6 and 7 (myh6 and myh7)*, *myosin light chain 2 and 2v (mlc2a and mlc2v)* (Kwon et al., 2007).

1.8.1. Nkx2.5

Genetic explorations for cardiac development have started only about a decade ago. The first crucial step in studying transcription factors in heart development was the discovery of the *Drosophila melanogaster* gene *tinman*. It encodes a NK-class homeodomain-containing transcription factor which is required for development of cardial and visceral lineages (Azpiazu and Frasch, 1993; Bodmer, 1993). Homologues

to *tinman*, the NK homeobox genes, were found in vertebrates. *Nkx2.5* is the fifth vertebrate gene identified in the NK-2 homeobox gene family (Lints, 1993).

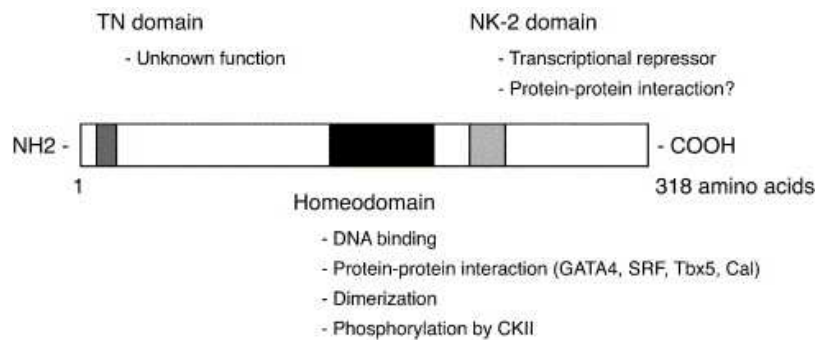


Fig. 6 Domains of the *Nkx2.5* protein. *Nkx2.5* consists of 318 amino acids and is composed of three domains, the TN domain (dark grey), the homeodomain (black) and the NK-2 domain (light grey) (Akazawa and Komuro, 2005).

Nkx2.5 (also called *Csx*) consists of a N-terminal TN domain, a homeodomain which is required for DNA binding, protein interaction, and dimerisation, and a NK-2 specific domain located C-terminal to the homeodomain (Fig. 6) (Harvey, 1996).

Nkx2.5 is a myocardial regulatory protein in both heart fields (Biben and Harvey, 1997) and plays a key role for cardiac transcription in the intricate program of heart development (Bruneau, 2002). In mice, *nkx2.5* expression begins in the cardiogenic mesoderm at embryonic day 7.5 and it is expressed throughout the course of development in the heart primordium, as well as in CMCs (Lints et al., 1993). Its expression pattern is ubiquitous in the heart tube (Christoffels et al., 2000). Its role in cardiac differentiation is crucial for the normal growth of the embryonic myocardium. This becomes apparent in the poorly developed myocardium of mice lacking *nkx2.5* and in the inability to form the primitive cardiac structures beyond the earliest stages of heart looping (Lyons et al., 1995; Tanaka et al., 1999). During the late stage of cardiogenesis, *nkx2.5* plays an important role in lineage specification and maturation of ventricular cardiomyocytes (Akazawa and Komuro, 2005).

Posttranslational modifications, acting either positively or negatively, are likely to account for an important component of the regulation of cardiac transcription factors (Bruneau, 2002). *Nkx2.5* has at least five phosphorylation sites. It was found that cytoplasmic *Nkx2.5* is unphosphorylated. In contrast, the nuclear *Nkx2.5* is

phosphorylated. Casein kinase II (CKII) was identified to phosphorylate serine within the Nkx2.5 homeodomain, increasing DNA binding (Kasahara and Izumo, 1999).

Nkx2.5 physically interacts with other transcription factors such as GATA4, they synergistically activate transcription via unmasking the activation domain of DNA-bound Nkx2.5 as well as recruitment of GATA4 by Nkx2.5 into a transcriptional complex (Bruneau, 2002; Charron and Nemer, 1999).

Nkx2.5 regulates the expression of several cardiac-specific genes, gene expression of *mlc2v*, *atrial natriuretic peptide (anp)*, *cardiac ankyrin repeat protein (carp)*, *mef2C*, *eHAND/HAND1*, *iroquois subclass of homeobox 4 (irx4)* and *hop-sterila (hop)* was reduced in *nkx2.5* deficient embryos (Biben and Harvey, 1997; Bruneau, 2002; Lyons et al., 1995; Tanaka et al., 1999). Working with ESC lines expressing *nkx2.5* at different levels allowed Nakashima et al. (2009) to identify *r-spondin 3 (rspo3)* and *transforming growth factor beta receptor 3 (tgfbr3)* as target genes for *nkx2.5*. *Rspo3* is one of the ligands for Frizzled homolog 8 (Fzd8) and Low-density lipoprotein receptor-related protein 6 (LRP6) and activates the canonical Wnt pathway (Nam et al., 2006). Furthermore, Nkx2.5 modulates transforming growth factor beta (TGF- β) signalling by enhancing the expression of *tgfbr3*, which is a TGF β co-receptor and a critical gene for cardiogenesis (Nakashima et al., 2009). Furthermore, β -*catenin* and *GATA4* were found to contain Nkx2.5 binding sites in their promoter regions and it was detected, that physical interaction between those sequences and Nkx2.5 occurs (Riazi et al., 2009).

1.8.2. Isl1

Isl1 is the key transcriptional regulator of the second heart field lineage during embryogenesis. Its expression is required for survival, proliferation and migration of second heart field progenitor cells. Mutation of Isl1 results in a severe cardiac phenotype in the second heart field. It is expressed in cells that colonize the outflow tract, right ventricle, part of the atria and the inner curvature of the left ventricle (Cai et al., 2003) and its transcription is turned off as the precursor cells differentiate (Laugwitz et al., 2008).

1.9. Desmin

Murine Desmin is a 53 kDa protein which is a member of the type III intermediate filament (IF) group. Desmin IFs occur exclusively in muscle and endothelial cells. It is

the first muscle specific structural protein and is one of the more persistent in several differentiation conditions (Costa et al., 2004). It has two conserved central rod domains (H1 and H2), four linker domains and two globular end domains. The central rod domains are responsible for the polymerization by lateral association (Herrmann and Aebi, 2000). Desmin occurs at the periphery of the Z disk and may act to keep adjacent myofibrils in lateral alignment. Phosphorylation of Desmin regulates the polymerisation of IFs (Huang et al., 2002).

Constitutive expression and increased synthesis during mesoderm formation of Desmin cause an up-regulation of the cardiac marker gene *nkx2.5*. At the same time this accelerates early cardiomyogenesis and the cells differentiate into synchronously beating cardiomyocytes. The *desmin* null mutants showed the opposite phenotype, fewer contracting heart cells could be detected when differentiated in EBs (Hofner et al., 2007). The amino-terminal domain of Desmin is important for its function in heart muscle cell development, its deletion decreases in vitro cardiomyogenesis and downregulates *nkx2.5* expression (Hollrigel et al., 2007).

1.10. Wnt Signalling

Wnt proteins are a large family of secreted signalling molecules that regulate crucial aspects of development (Cohen et al., 2008). They are glycoproteins that bind to the seven-transmembrane-spanning receptors frizzled, triggering a signalling cascade which results in altered target gene expression (Fig. 7). In the absence of Wnt, the level of free intracellular β -catenin, the transcriptional mediator of Wnt, is minimized by its phosphorylation by glycogen synthase kinase 3 β (GSK3 β). Free cytoplasmic β -catenin is recruited to a “destruction complex” containing APC, Axin, and GSK3 β . Phosphorylation of β -catenin results in its degradation by the proteasome (Fodde et al., 2001; Hlsken and Behrens, 2000). Binding of Wnt to frizzled activates the receptor leading to the phosphorylation of Dishevelled, which in turn inhibits GSK3 β . Consequently, β -catenin cannot be phosphorylated, and in its unphosphorylated state it translocates to the nucleus where it acts as a transcriptional co-activator that interacts with T cell factor and lymphoid enhancer factor (TCF/LEF) transcription factors to activate Wnt targets (Bejsovec, 2005). Wnt signalling has a crucial role in controlling proliferation and differentiation. It is the key pathway in the regenerative process in many tissues, including the heart (Cohen et al., 2008). In flies, wingless, the founding member of the canonical Wnt family, is required for cardiac lineage determination

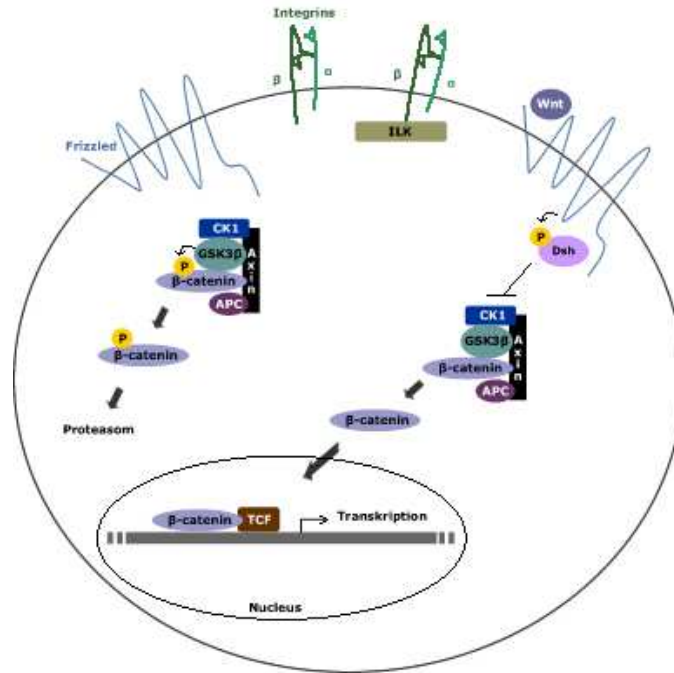


Fig. 7 The canonical Wnt pathway. In the absence of Wnt, β -catenin is phosphorylated by GSK3 β in complex with APC and Axin. Binding of Wnt to its receptor frizzled results in the phosphorylation of Dishevelled (Dsh) which indirectly inhibits GSK3 β kinase activity. β -catenin is free in the cytoplasm and can translocate to the nucleus, activating gene expression of TCF/LEF sites controlled genes (Weiss, 2009).

(Wu et al., 1995). Overexpression of canonical Wnts in dorsal and anterior mesoderm of frogs and chicks inhibits CMC commitment or differentiation. In the cardiac crescent, Wnt signalling downregulates the expression of early cardiac genes *nkx2.5* and *gata4* (Marvin et al., 2001; Schneider and Mercola, 2001). Decrease in the transcriptional activation potential of β -catenin in zebrafish caused hyperproliferation of CMCs, indicating that β -catenin negatively regulates CMG (Rottbauer et al., 2002). In mammals, activation of β -catenin resulted in the expansion of *isl1* expressing CSCs, and in early developmental stages in mouse embryos, β -catenin directly regulates *isl1* expression, suggesting an important role for development of the second heart field (Cohen et al., 2007; Lin et al., 2007). Inhibiting canonical Wnt signalling with Dickkopf1 (*Dkk1*) in embryoid bodies (EBs) between day 4 and 6 resulted in a complete absence of beating EBs, implicating that canonical Wnt is required for CMC formation in the EB system. In addition to those findings, expression levels of *nkx2.5* and *tbx5* were upregulated by exposure to Wnt3a (Kwon et al., 2007). β -catenin expression itself was repressed in cardiac myocytes which is mediated by *nkx2.5* (Riazi et al., 2009). Taken together these data, Wnt signalling seems to have a biphasic

role where β -catenin is necessary at earlier stages of CMG and has an inhibitory effect at later stages of heart development (Lin et al., 2007; Riazi et al., 2009).

1.11. SPARC

Secreted protein acidic and rich in cysteine (SPARC), also termed BMP-40 or osteonectin, is a 43 kDa matricellular glycoprotein which is produced by several cell types, in especially high amounts by the parietal endoderm. SPARC consists of three domains, a N-terminal acidic region, a cysteine rich follistatin-like domain, and an extracellular Ca^{2+} domain (Fig. 8) (Brekken and Sage, 2000). It is expressed during development and in the fetal heart, however not in the adult heart. Its expression is limited to areas with high rates of turnover as well as repair and tissue remodelling (Sage et al., 1989). Very high expression was detected under condition of stress, in response to endotoxin stimulation, heat shock, cellular aging and wound repair (Brekken and Sage, 2000). SPARC was detected extracellularly as well as intracellularly in the nucleus and associated with the nuclear matrix in some cell types (Gooden et al., 1999). It was furthermore detected as a secretion product. Extracellular SPARC binds to growth factors, cytokines and components of the extracellular matrix (ECM), which results in remodelling and explains the role of SPARC in development and wound healing (Brekken and Sage, 2000).

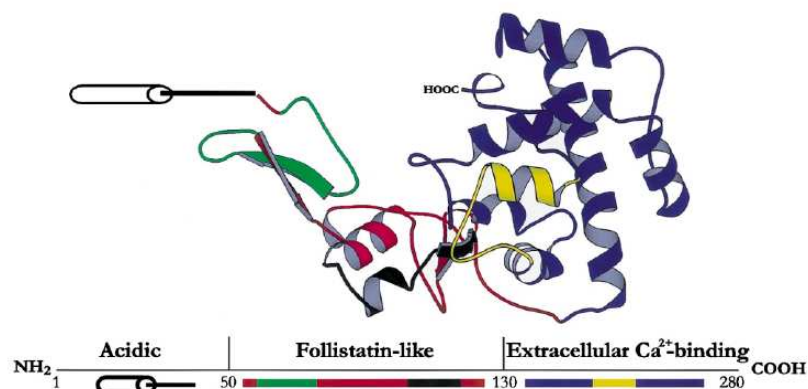


Fig. 8 Structure of SPARC (*Secreted Protein Acidic and Rich in Cysteine*). SPARC consists of three domains, the N-terminal, acidic domain, the follistatin-like, and the extracellular Ca^{2+} -binding C-terminal domain (Brekken and Sage, 2000).

Adenoviral overexpression of SPARC in wild type mice improved the collagen maturation and prevented cardiac dilation and dysfunction after myocardial infarction and therefore indicated that the local production of SPARC is essential for

maintenance of the integrity of cardiac ECM after myocardial infarction (Schellings et al., 2009).

SPARC was found to modulate the kinase activity of Integrin-linked-kinase 1 (ILK1) by binding to the transmembrane protein integrin- β 1 (Barker et al., 2005; Weaver et al., 2008). During adipogenesis, SPARC induced the accumulation and nuclear translocation of β -catenin and therefore its activity as a transcriptional activator, which resulted in an inhibition of adipogenic transcription factors and adipogenesis (Nie and Sage, 2009). Therefore, activation of ILK by SPARC might influence the activity of GSK3, resulting in an alteration of the level of free β -catenin transcription factor in the nucleus (Fig. 9).

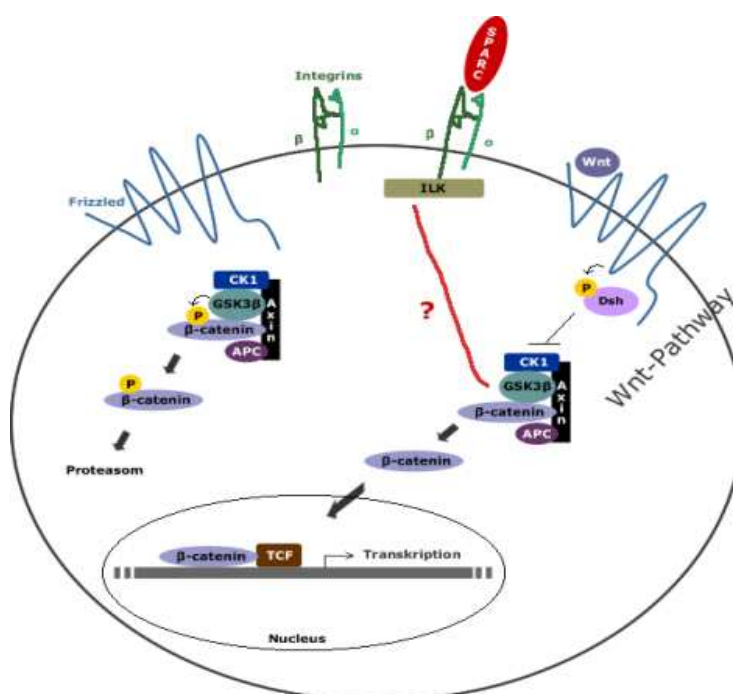


Fig. 9 Possible transduction pathway of SPARC signalling. SPARC binds to integrin β 1, activating ILK1. ILK1 might influence the phosphorylation status of GSK3, altering the concentration of free cytoplasmatic β -catenin which translocates to the nucleus and activates target gene expression (Weiss, 2009).

In human dental pulp cells, SPARC was found to act via the G-protein coupled receptor integrin α β 3, which regulates extracellular signal regulated kinase (ERK) and consequently induced dental pulp cell migration (Pavasant and Yongchaitrakul, 2008).

In respect of cardiomyogenesis, recombinant SPARC was found to enhance early cardiomyocyte formation in EBs when added on days 7 – 10. Upregulation of *nkx2.5* was further detected upon SPARC addition (Stary et al., 2005). Histochemical analysis

revealed, that addition of SPARC to EBs gave rise to an expansion of the peripheral endoderm, with thicker layers of ECM material (Hrabchak et al., 2008).

Here, we wanted to investigate the influence of the two proteins Desmin and SPARC on cardiomyogenesis. Our aim was to determine the mechanism through which Desmin promotes cardiomyogenesis by establishing a link between Desmin and the early cardiac transcription factor Nkx2.5. We furthermore tried to detect the effect of SPARC on cardiomyocyte formation in isolated cardiovascular progenitor cells, whose differentiation potential is restricted to the cardiac lineage. The determination of signalling pathways through which SPARC acts and the detection of effector genes altered in their expression upon SPARC addition would help elucidating the mechanism of its influence.

2. Abbreviations

ANP	Atrial Natriuretic Peptide
APC	Adenomatosis Polyposis Coli
BMP	Bone Morphogenetic Protein
CARP	Cardiac Ankyrin Repeat Protein
CB	Cardiac Body
cDNA	Complementary Deoxyribonucleic Acid
CHD	Coronary Heart Disease
ChIP	Chromatin Immunoprecipitation
c-kit	Kit Oncogene
CK	Casein Kinase
CMC	Cardiomyocyte
CMG	Cardiomyogenesis
CSC	Cardiac Stem Cell
cTNT	Cardiac Troponin T
CVD	Cardiovascular Disease
CVPC	Cardiovascular Progenitor Cell
Cx43	Connexin 43
DAPI	4'-6-Diamidino-2-phenylindole
Dkk-1	Dickkopf-1
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxid
dNTP	Deoxy Nucleotide Triphosphate
DTT	Dithiothreitol
Dsh	Dishevelled
EB	Embryoid Body
EDTA	Ethylenediaminetetraacetic Acid
EGFP	Enhanced Green Fluorescence Protein
EpiSC	Epiblast Stem Cell
ERK	Mitogen Activated Protein Kinase 1
ESC	Embryonic Stem Cell
ETC	Endothelium-like Cell
FACS	Fluorescence Activated Cell Sorting
FGF	Fibroblast Growth Factor
FOX	Forkhead Box
Fzd	Frizzled

GATA4	GATA-binding Protein 4
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFP	Green Fluorescence Protein
GSK3	Glycogen Synthase Kinase 3
HDAC	Histone Deacetylase
ID	Inhibitor of Differentiation
IF	Intermediate Filament
ILK	Integrin Linked Kinase
IRX4	Iroquois Subclass of Homeobox 4
Isl 1	Islet 1
Klf	Kruppel-like Factor
LIF	Leukemia Inhibitory Factor
LRP6	Low Density Lipoprotein Receptor Related Protein 6
MAPK	Mitogen Activated Protein Kinase
MEF2C	Myocyte Enhancer Factor 2C
Mesp1	Mesoderm Posterior 1
MHCα	Myosin Heavy Chain α
MLC	Myosin Light Chain
mRNA	Messenger Ribonucleic Acid
MYH	Myosin Heavy Chain
Nkx2.5	NK2 Transcription Factor Related, locus 5
Oct3/4	Octamer-binding Transcription Factor 3/4
pCMC	Primary Cardiomyocytes
PCR	Polymerase Chain Reaction
Rspo3	R-spondin 3
SC	Stem Cell
siRNA	Short Interfering Ribonucleic Acid
SMC	Smooth Muscle Cell
Sox2	SRY – related Box 2
SPARC	Secreted Protein Acidic and Rich in Cystein
Stat3	Signal Transducer and Activator of Transcription 3
TBX	T Box Family
TCF	T-cell specific Transcription Factor
TGF-β	Transforming Growth Factor β
TGFBR3	Transforming Growth Factor β Receptor 3
Wnt	Wingless-related MMTV Integration Site

3. Results

3.1. Influence of Desmin on Cardiomyogenesis

3.1.1. Interaction of Desmin with *nkx2.5* Promoter DNA

Desmin overexpression in embryonic stem cells (ESCs) results in accelerated cardiomyogenesis and in the development of large, highly interconnected, and synchronously beating clusters of cardiomyocytes (Hofner et al., 2007). A marker for early heart development is *Nkx2.5* (Ching-Ling, L. et al., 1999). Hofner et al. (2007) showed that constitutive expression of *desmin* led to an increase in *nkx2.5* expression. However, the mechanism how this activation works is not known yet. The aim of this experiment was to establish a link between those two proteins. Desmin might directly influence *nkx2.5* expression and we wanted to analyse whether there is a physical interaction between the protein Desmin and the *nkx2.5* promoter region. Furthermore, the time frame in which Desmin binds to the DNA region shall be determined in cells of different genotypes.

Physical DNA-Protein interactions are detected with Chromatin-Immunoprecipitation (ChIP). The experiment was performed with embryoid bodies of different *desmin* alleles and in different developmental stages, with undifferentiated and differentiated cardiovascular progenitor cells, and with primary cardiomyocytes.

3.1.1.1. Interaction in Embryonic Stem Cells

Embryoid bodies were made from AB2.2 wild type ESCs and from DC6 ESCs (*des*^{+/+} *des*^{ect}), which overexpress Desmin. Furthermore 1B44 ESCs (*des*^{Δ1-48/Δ1-48}), whose N-terminus of the *desmin* gene is deleted, and *des*^{-/-} ESCs, which have the whole *desmin* gene deleted, were tested. Samples for the ChIP experiment of all genotypes were taken from days 5 – 9, 1B44 and *des*^{-/-} EBs were also tested on day 10. ChIP was performed with antibodies directed against Desmin and PCRs were made with primers recognising sequences directly upstream the *nkx2.5* start codon (Fig. 10, P1). PCR products were loaded to a 1.5% agarose gel and the samples before (IN) and after (IP) immunoprecipitation were compared.

Nkx2.5 Promoter Region with Primer

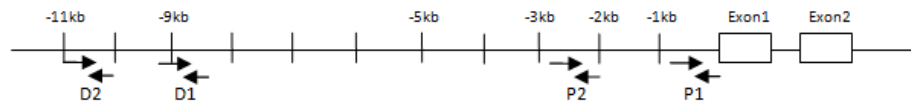


Fig. 10 *Nkx2.5 Promoter Region*. Different primer pairs in the *nkx2.5* upstream region recognising sequences in the *nkx2.5* promoter, P1 and P2, and in the *nkx2.5* enhancer sequence, D1 and D2 (Ching-Ling, et al., 1999).

The wild type EBs ChIP PCR shows, that in the original input samples (IN) there is a clear DNA band at all days. This means that the samples contained *nkx2.5* promoter DNA and therefore the original material had the necessary basic conditions for the immunoprecipitation. At day 5 after immunoprecipitation (IP), no PCR band could be detected indicating that no Desmin protein was bound to the *nkx2.5* promoter DNA at very early developmental stages in wild type embryoid bodies. At day 6, there is a band visible, which suggests a physical interaction of Desmin and *nkx2.5* promoter DNA at this time point. The same result could be seen at day 8. First beating activity in wt EBs was observed on days 6.5 – 7.5. A peculiar observation is, that on day 7 Desmin – *nkx2.5* promoter interaction could not be detected. This is in agreement with unpublished data by Sonja Gawlas. At day 9, no co-immunoprecipitation of Desmin and *nkx2.5* promoter DNA could be observed (Fig. 11).

In the DC6 (*des*^{+/+} *des*^{ect}) EBs Desmin – *nkx2.5* interaction could be detected on day 5, day 6 and day 7, which was one day earlier compared to wt EBs. Desmin was overexpressed in the DC6 cells and this overexpression resulted in the earlier binding of Desmin to the cardiac development marker gene *nkx2.5*. As in the wt cells, two days after the first detected interaction the signal disappeared (Fig. 11) and one day before first beating cardiomyocytes were observed, which was on day 6 in DC6 EBs, the interaction occurred. This suggests, that the developmental progression is identical in the two genotypes, but time-shifted.

In 1B44 (*des*^{Δ1-48/Δ1-48}) EBs no binding of Desmin to the *nkx2.5* promoter DNA could be detected at any tested day (Fig. 11). The missing amino-terminus is thought to be the DNA-binding component of the protein and these results provide another hint that this might be true. *Des*^{-/-} EBs served as a negative control, no Desmin was produced and no PCR band appeared after immunoprecipitation (Fig. 11).

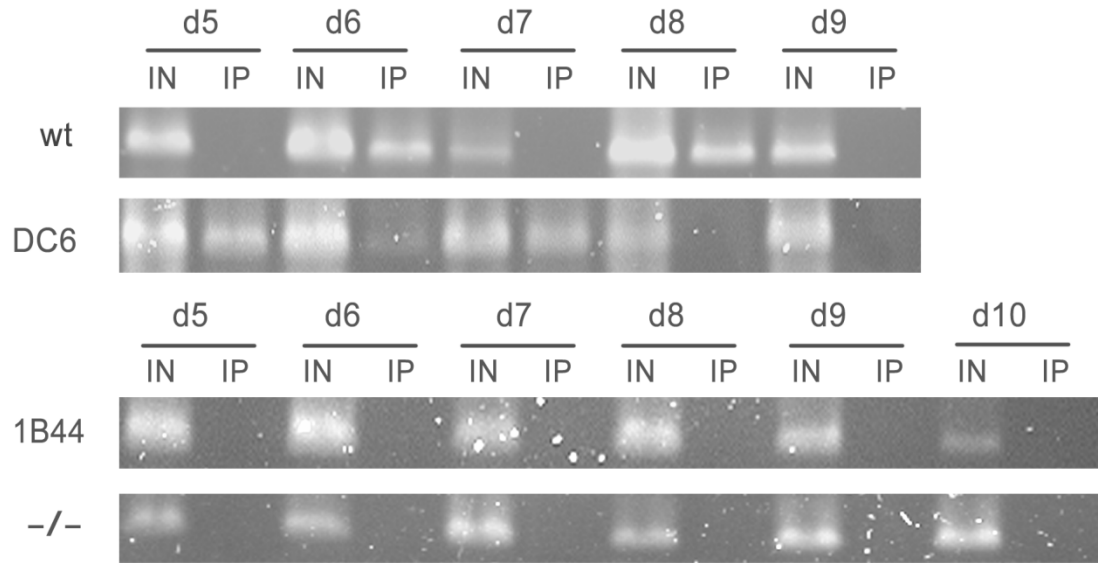


Fig. 11 Chromatin Immunoprecipitation (ChIP) of wt, DC6 (*des*^{+/+} *des*^{ect}), 1B44 (*des*^{A1-48/A1-48}) and -/- (*des*^{-/-}) embryoid bodies. ChIP was performed of wt, DC6, 1B44 and *des*^{-/-} embryoid bodies at different developmental stages. For the Immunoprecipitation Desmin antibody was used and PCRs with *nkx2.5* promoter primers showed whether Desmin physically binds to the *nkx2.5* promoter region at different days of development. The input samples (IN) are the PCR results of the whole chromatin before immunoprecipitation was performed; the immunoprecipitation samples (IP) reflect the PCR results of the immunoprecipitated DNA fragments. The ChIP was done once. The wt and DC6 PCR displays results of three out of seven PCRs. The 1B44 and -/- PCR was made five times, showing the same result.

3.1.1.2. Interaction in Cardiovascular Progenitor Cells and primary Cardiomyocytes

The same ChIP experiment was performed with A5 cardiovascular progenitor cells (CVPCs) and primary cardiomyocytes (pCMCs). Samples of A5 cells were taken in an undifferentiated state as well as on day 17 of cardiac bodies development, where 100% of all cardiac bodies contained differentiated cardiomyocytes. pCMCs were isolated from new born mice (d 0) and immediately used for ChIP. Undifferentiated A5 cells did not have Desmin bound to the *nkx2.5* promoter region. However in differentiated A5 cells, which have already formed beating heart cells in cardiac bodies, a clear interaction between Desmin and the *nkx2.5* promoter region could be detected. Desmin bound to the *nkx2.5* promoter region was still detectable in differentiated heart cells as seen in the primary cardiomyocytes ChIP PCR (Fig. 12).

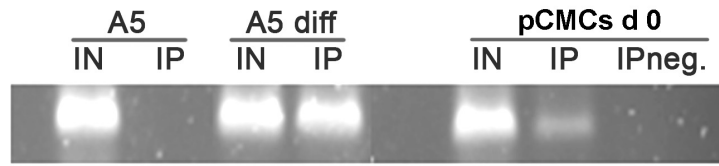


Fig. 12 Chromatin Immunoprecipitation (ChIP) of CVPCs and pCMCs. ChIP was performed of undifferentiated A5 CVPCs, differentiated day 17 A5 CBs and pCMCs isolated from day 0 wild type mice. For the immunoprecipitation Desmin antibody was used and PCRs with *nkx2.5* promoter primers showed whether Desmin physically binds to the *nkx2.5* promoter region. The input samples (IN) reflect the PCR results of the whole chromatin before immunoprecipitation was performed; the immunoprecipitation samples (IP) are the PCR results of the immunoprecipitated DNA fragments. For the IPnegative sample, no antibody was used. The ChIP was made once, displayed A5 results represent two out of five PCR results, pCMC PCRs were made three times, two showed demonstrated results.

3.1.2. Influence of Desmin on the *nkx2.5* Promoter

3.1.2.1. Influence of Desmin on the *nkx2.5* Promoter in C2C12 Myoblasts

The previous ChIP experiment showed that Desmin physically binds to the *nkx2.5* promoter. This binding was detected in embryoid bodies derived from wild type and DC6 embryonic stem cells, in differentiated A5 CVPCs and in pCMCs. The physical binding suggests that Desmin could influence the expression of *nkx2.5*.

In this experiment we wanted to investigate in which way Desmin influences the expression of *nkx2.5*. It should be tested whether it has a positive effect and therefore activates gene expression or whether it inhibits expression by binding to the promoter region.

For that purpose, we made a Luciferase assay with cells transfected with different plasmids. We used a control plasmid containing a *luciferase* reporter gene without a promoter, a vector expressing a *luciferase* gene regulated by the *nkx2.5* promoter (nkx-luc), and a vector expressing a luciferase reporter gene regulated by the *nkx2.5* promoter and enhancer sequences (nkx-enhancer-luc). To test the effect of Desmin overexpression on Luciferase activity, a *desmin* expressing plasmid was used (Des). To downregulate the Desmin level in the cells, we furthermore transfected cells with a plasmid containing a sequence for a siRNA targeted against *desmin* mRNA (Des-siRNA). As an internal control we used a Renilla plasmid, carrying a Renilla *luciferase* gene, which should be expressed in each cell and therefore reflect the cell count and transfection efficiency. A plasmid without any reporter gene (pUC plasmid) was used to keep the amount of DNA constant in the different samples. An in vitro expression

system was established and Luciferase assays were made to determine the expression level of the reporter gene after the transfection.

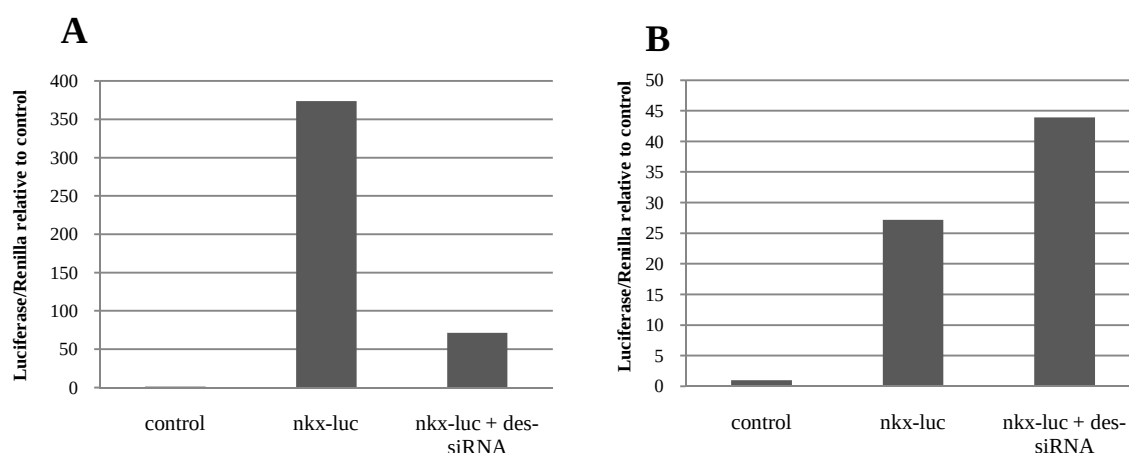


Fig. 13 Effect of desmin knock down on *nkx2.5* expression. C2C12 myoblasts were transfected by calcium phosphate transfection with a control plasmid, containing a promoterless *luciferase* gene, with the *nkx-luc* plasmid that expresses a *luciferase* reporter gene under control of the the *nkx2.5* promoter region. The des-siRNA plasmid was co-transfected with the *nkx-luc* vector in order to test the effect of desmin knock down by the *desmin* siRNA on the *nkx2.5* promoter activity. All cells were furthermore transfected with a Renilla *luciferase* expressing plasmid, which should be constantly expressed in all transfected cells. 48 hours after transfection, the cells were lysed and Luciferase activity was measured. The Luciferase signals were divided by the Renilla signals in order to eliminate fluctuations because of different cell counts or different transfection efficiencies. (A) and (B) display the results of two identical experiments.

In a first experiment 1.2×10^5 C2C12 myoblasts were transfected by Calcium Phosphate Transfection with a control plasmid, the *nkx-luc* plasmid and we furthermore co-transfected *nkx-luc* with a plasmid expressing a siRNA directed against *desmin* mRNA. In addition to those vectors, all cells were transfected with the Renilla plasmid. Renilla *luciferase* should be constantly expressed from this plasmid by all cells and therefore served as an internal control. 48 hours after transfection, the cells were lysed and Luciferase activity was measured. The Luciferase signals were divided by the Renilla signals in order to eliminate fluctuations caused by varying cell counts or different transfection efficiencies. The cells transfected with the *nkx-luc* plasmid produced Luciferase to a greater extent than the control cells (Fig. 13 A). Thus, it could be concluded, that the *nkx2.5* promoter is active in C2C12 myoblasts. The co-transfection with the desmin-siRNA plasmid resulted in a 7 fold reduction of the Luciferase signal compared to *nkx-luc* (Fig. 13 A).

We then repeated the experiment using the same method and plasmids and saw opposite results. The *nkx2.5* promoter again is active in the cells as seen on the

increase in Luciferase activity in the *nkx-luc* transfected cells compared to the control cells. However, the *desmin* siRNA did not cause a decrease in reporter gene expression, in contrast it even enhanced the *nkx2.5* promoter activity and would therefore suggest a negative effect of Desmin on the expression of *nkx2.5* (Fig. 13 B).

However, looking at the Renilla values in both experiments by which the Luciferase activities were divided to avoid fluctuations caused by varying cell counts or different transfection efficiencies we found that there are high fluctuations in the different samples (up to 200 fold) although the same cell count was seeded and the same amount of Renilla plasmid was transfected. Furthermore the transfection efficiency was very low, when detecting the GFP-positive cells (those cells which have taken up a *des*-siRNA plasmid) under the fluorescence microscope (data not shown). Because of these technical problems, a different transfection method, transfection with Lipofectamine 2000, was used for the following experiments.

For transfection with Lipofectamine 2000, the optimal ratio and quantity of transfection agent and DNA had to be determined. Therefore, C2C12 cells were transfected with a GFP expressing plasmid using different Lipofectamine – DNA ratios and quantities. An increase of DNA amount from 5 µg to 8 µg per transfection also increased the transfection efficiency (Fig. 14). Adding higher Lipofectamine concentrations however did not result in a higher amount of transfected cells. No difference could be detected using 8 µg DNA with 10 µl Lipofectamine or 8 µg DNA with 20 µl Lipofectamine. The DNA-Lipofectamine ratio of 1:1.25 using 8 µg DNA with 10 µl Lipofectamine 2000 seemed to be the most efficient for transfection of C2C12 myoblasts and was therefore used for further transfection experiments.

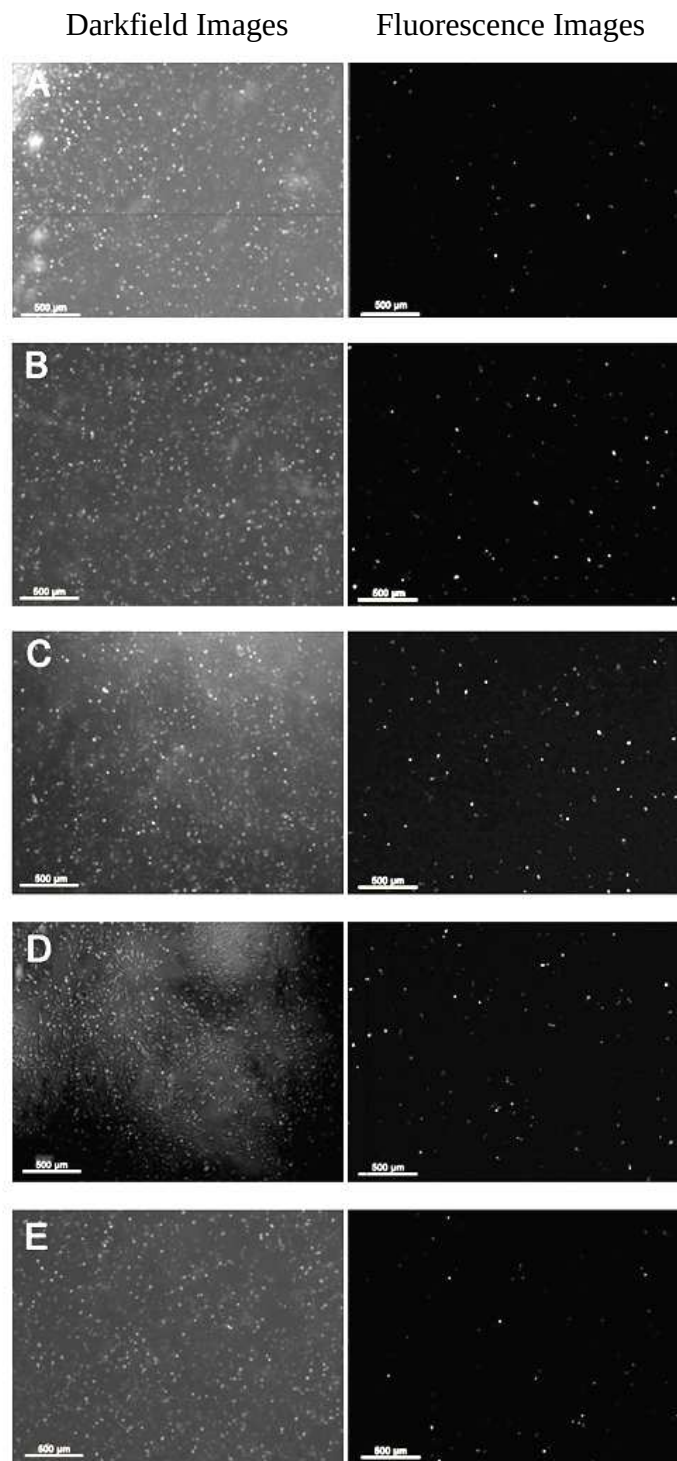


Fig. 14 Transfection efficiency of different Lipofectamine - DNA quantities and ratios. Darkfield and fluorescence images of C2C12 myoblasts transfected with a GFP expressing plasmid using 4 µg DNA + 10 µl Lipofectamine (A), 5 µg DNA + 10 µl Lipofectamine (B), 8 µg DNA + 10 µl Lipofectamine (C), 6 µg DNA + 15 µl Lipofectamine (D), 8 µg DNA + 20 µl Lipofectamine (E).

The following experiment tested the Lipofectamine transfection method with regard to Renilla values. Different cell counts were seeded in duplicates on six well plates and

the same Renilla plasmid concentration was added to the cells. The Renilla activity was measured 48 hours after transfection.

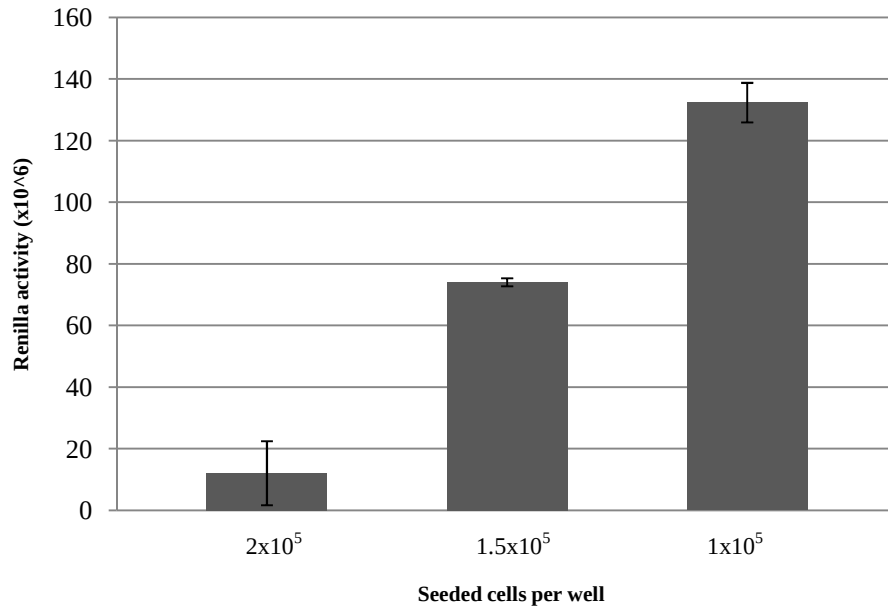


Fig. 15 *Renilla activity in different cell counts.* Different C2C12 cell counts were seeded in duplicates and were transfected with the same Renilla plasmid concentration. After 48 hours, Renilla activity was measured. Data are means of two experiments.

The experiment was made twice and the standard derivation was very low (Fig. 15) which favours this transfection method compared to calcium phosphate transfection. The Renilla activity depended on the seeded cell count. Interestingly, the lower the cell count, the higher the activity (Fig. 15). The cell count was measured when the cells were seeded, 24 hours before the transfection and 72 hours before the Luciferase assay. One possible way of explaining this fact could be that the cells that were not seeded very densely have space to grow and proliferate, are in a better state at the time of transfection and are able to express the plasmid more efficiently whereas the 2×10^5 seeded cells are not in an optimal growing phase and have a lower level of expression. 1.5×10^5 cells per well were used for the following experiments.

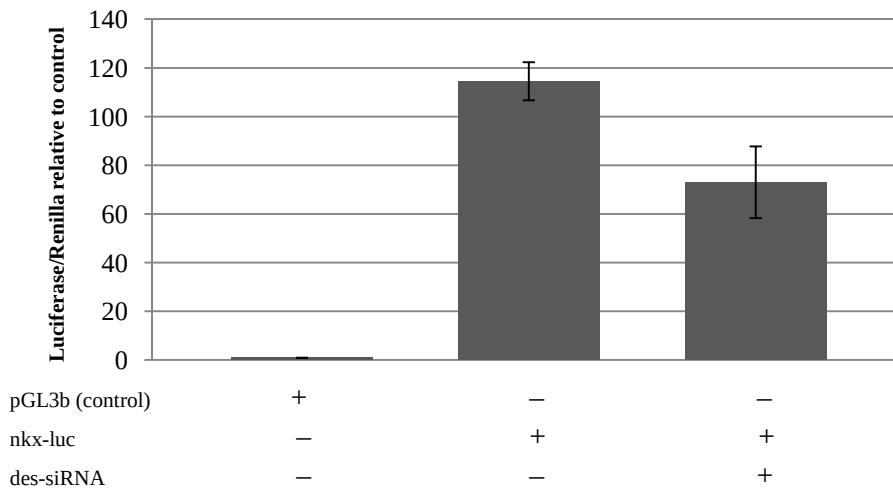


Fig. 16 Effect of desmin knock down on *nkx2.5* expression.. 1.5×10^5 C2C12 myoblast cells were transfected by Lipofectamine transfection with a control plasmid, containing a promoterless *luciferase* gene, with the *nkx-luc* plasmid that expresses a *luciferase* reporter gene under control of the the *nkx2.5* promoter region. The des-siRNA plasmid was co-transfected with the *nkx-luc* vector in order to test the effect of desmin knock down on *nkx2.5* promoter activity. All cells were furthermore transfected with a Renilla *luciferase* expressing plasmid, which is constantly expressed in all transfected cells. 48 hours after transfection, the cells were lysed and Luciferase activity was measured. The Luciferase signals were divided by the Renilla signals in order to eliminate fluctuations because of different cell counts or different transfection efficiencies. Data are means of two experiments.

We then used Lipofectamine transfection with the optimised protocol for the next experiments. 1.5×10^5 C2C12 myoblasts were transfected with a control plasmid, a *nkx-luc* plasmid, and we furthermore co-transfected *nkx-luc* with a desmin knock-down plasmid. siRNA against *desmin* caused a significant decrease in *nkx2.5* promoter controlled Luciferase expression (Fig. 16). This suggests, that downregulation of Desmin inhibits *nkx2.5* expression.

We then wanted to test the effect of Desmin on the *nkx2.5* enhancer region which is located further upstream the *nkx2.5* promoter. For that purpose, we transfected cells with a plasmid carrying a *luciferase* reporter gene regulated by the *nkx2.5* promoter and enhancer sequence (*nkx-enhancer-luc*). We furthermore co-transfected this vector with a plasmid expressing a desmin siRNA. The promoter and enhancer sequences are active in the cells since Luciferase is produced in the *nkx-enhancer-luc* transfected cells compared to the control cells. However, expression could not be inhibited by co-transfection of the *desmin*-siRNA plasmid (Fig. 17).

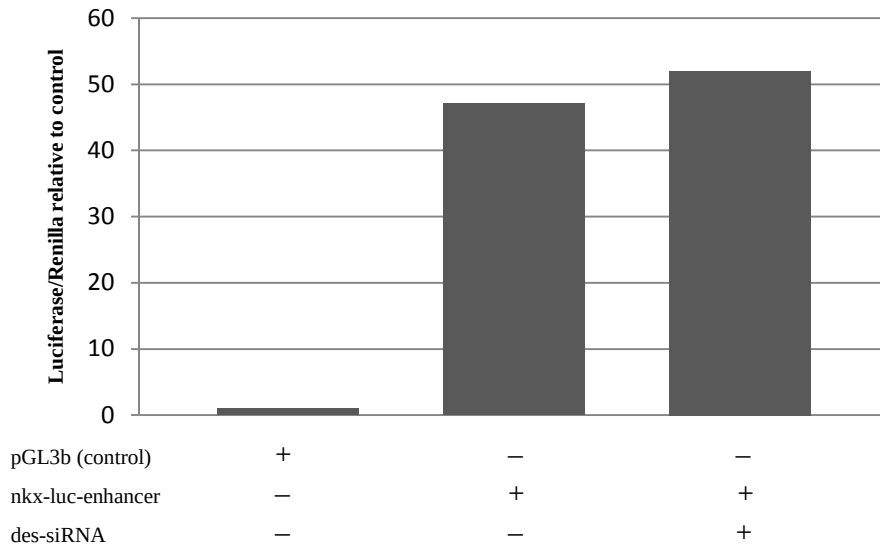


Fig. 17 Effect of desmin knock down on *nkx2.5* expression. 1.5×10^5 C2C12 myoblast cells were transfected by Lipofectamine transfection with a control plasmid, containing a promoterless *luciferase* gene, with the *nkx*-enhancer-luc plasmid that expresses a *luciferase* reporter gene under control of the the *nkx2.5* promoter and enhancer region. The des-siRNA plasmid was co-transfected with the *nkx*-enhancer-luc vector in order to test the effect of desmin knock down by the *desmin* siRNA on the *nkx2.5* promoter and enhancer activity. All cells were furthermore transfected with a Renilla luciferase expressing plasmid, which is constantly expressed in all transfected cells. 48 hours after transfection, the cells were lysed and Luciferase activity was measured. The Luciferase signals were divided by the Renilla signals in order to eliminate fluctuations because of different cell counts or different transfection efficiencies. The experiment was made once.

To test the effect of additional transfected Desmin, another Lipofectamine transfection was made with C2C12 myoblasts. Cells were transfected with a control plasmid, the *nkx*-luc plasmid and with a plasmid expressing *desmin* (Des). All cells were furthermore transfected with Renilla. 48 hours after the transfection the cells were lysed and the Luciferase signal was measured and evaluated in the same way as the other experiments. The co-transfection of the Desmin plasmid caused increased *desmin* expression in the cells which was affirmed by isolating mRNA of transfected cells followed by Desmin PCRs (Fig. 18 B). However, this additional Desmin could not activate the *nkx2.5* promoter. Even less reporter gene was expressed than without the co-transfected Desmin plasmid (Fig. 18 A).

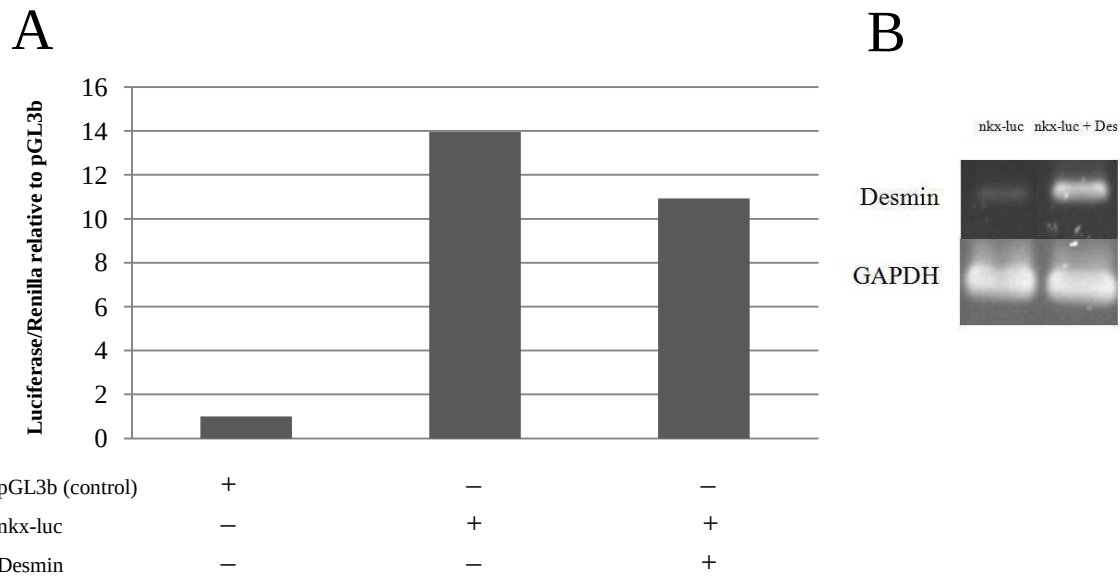


Fig. 18 Effect of ectopic *Desmin* expression on the *nkx2.5* promoter. 1.5×10^5 C2C12 myoblasts were transfected with the nkx-luc plasmid that contains the *nkx2.5* promoter region with a *luciferase* reporter gene. The *Desmin* plasmid contains the *desmin* cDNA and therefore produces a *desmin* mRNA that can be translated to the protein. (A) 48 hours after transfection, the cells were lysed and Luciferase activity was measured. The Luciferase signals were divided by the Renilla signals in order to eliminate fluctuations because of different cell counts or different transfection efficiencies. The experiment was made once. (B) 48 hours after transfection, RNA was isolated, transcribed into cDNA and a PCR with primers for *desmin* was made to confirm the overexpression of *desmin* in the transfected cells. A GAPDH PCR was made to ensure that the same quantities of cDNA was used in the PCR.

3.1.2.2. Influence of *Desmin* on the *nkx2.5* Promoter in primary Cardiomyocytes

Heart cells of five days old mice were isolated and transfected with a control plasmid, the nkx-luc plasmid and we furthermore co-transfected nkx-luc with a plasmid expressing a siRNA directed against *desmin* mRNA. 48 hours after transfection, the cells were lysed and Luciferase activity was measured. We found that the co-transfection with the knock down plasmid did not change the Luciferase activity in pCMCs (Fig. 19). In earlier ChIP experiments it was detected, that *Desmin* is bound to the *nkx2.5* promoter in day 0 pCMCs. In day 5 pCMCs, *Desmin* seems to have no effect on the expression of *nkx2.5*.

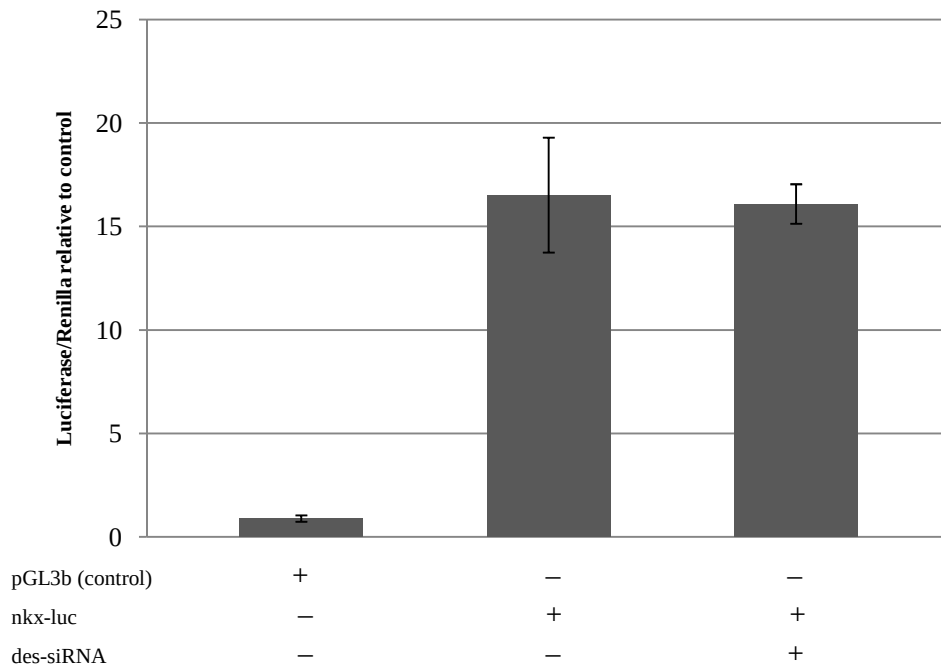


Fig. 19 Effect of desmin knock down on *nkx2.5* expression in primary cardiomyocytes. Primary cardiomyocytes were transfected by Lipofectamine transfection with a control plasmid, containing a promoterless *luciferase* gene, with the *nkx-luc* plasmid that expresses a *luciferase* reporter gene under control of the the *nkx2.5* promoter region. The *des-siRNA* plasmid was co-transfected with the *nkx-luc* vector in order to test the effect of desmin knock down by the *desmin* siRNA on the *nkx2.5* promoter activity. All cells were futhermore transfected with a *Renilla luciferase* expressing plasmid, which is constantly expressed in all transfected cells. 48 hours after transfection, the cells were lysed and *Luciferase* activity was measured. The *Luciferase* signals were divided by the *Renilla* signals in order to eliminate fluctuations because of different cell counts or different transfection efficiencies. The experiment was made once in duplicates.

3.2. Characterisation of Cardiovascular Progenitor Cells

Different groups already succeeded in the isolation of somatic stem cells of the heart (Beltrami et al., 2003; Oh et al., 2003; Oyama et al., 2007; Pfister et al., 2005), however they have never been able to maintain the cells in culture for more than a few passages. Wiedner and Weber (Wiedner, 2008) succeeded in the isolation of stable cardiovascular progenitor cell (CVPC) lines, which express stemness factors as well as myocardial genes in the undifferentiated state. Therefore it can be assumed that they do not have the same potential as ESCs. They seem to be already committed to the cardiac lineage but still have stem cell character. CVPCs of passage 67 still form CBs comparable to CBs of early passages (Fig. 20) and they still differentiate to beating cardiomyocytes, although only at an extent of 50% compared to the early passages. They were already passaged for over 80 passages under continuous cryo-preservation.

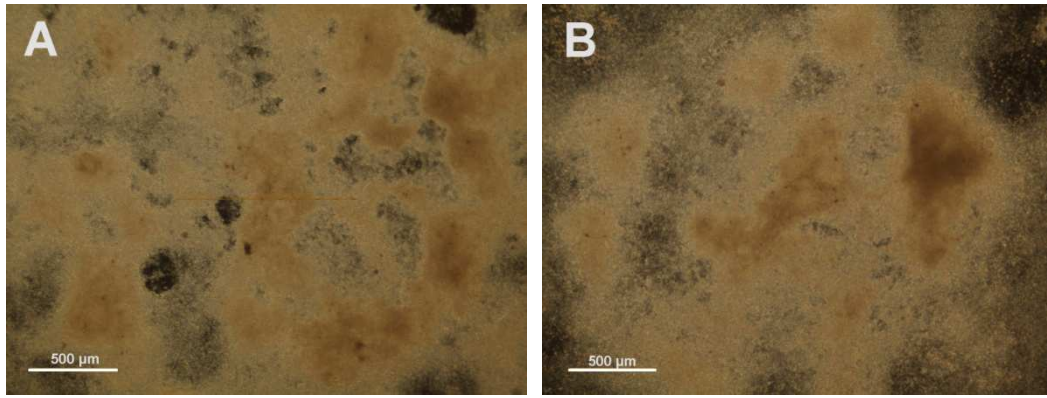


Fig. 20 *Cardiac Bodies of passage 16 and passage 67 CVPCs.* Cardiovascular progenitor cells of an early (p 16, A) and late (p 67, B) passage were differentiated in cardiac bodies. Pictures were taken on day 16 of development.

3.2.1. Differentiation Potential of CVPCs

When grown on feeder cells, CVPCs act like stem cells. They stay in a self-renewal mode and proliferate without differentiating to any other cell type. In the absence of feeder cells CVPCs start differentiating. The differentiation potential of ESCs was examined by growing the aggregates in hanging drop cultures to form embryoid bodies (EBs). This well established method also works for CVPCs. They form cardiac bodies (CBs) and this procedure is the best way of determining and examining the differentiation state of the cells. It could be seen, that CVPCs grown in aggregates without feeder cells exclusively differentiate to rhythmically contracting cardiomyocytes (CMCs), slowly contracting smooth muscle cells (SMCs), and endothelium-like cells (ETCs), the three major cell types of the adult heart. We never observed any nerve-like structures, skeletal muscle cells or erythrocytes even at very late developmental stages. Day 26 A5 CBs were stained with the cardiac marker gene *cardiac Troponin T* and DAPI (Fig. 21 A). Cardiac Troponin T is part of the Troponin complex in heart muscle cells, it links Tropomyosin to Troponin C. Cardiomyocytes build a network of contracting cells and have very tight cell-cell interactions which could be shown by staining the cells with Connexin 43, a gap junction protein (Fig. 21 B).

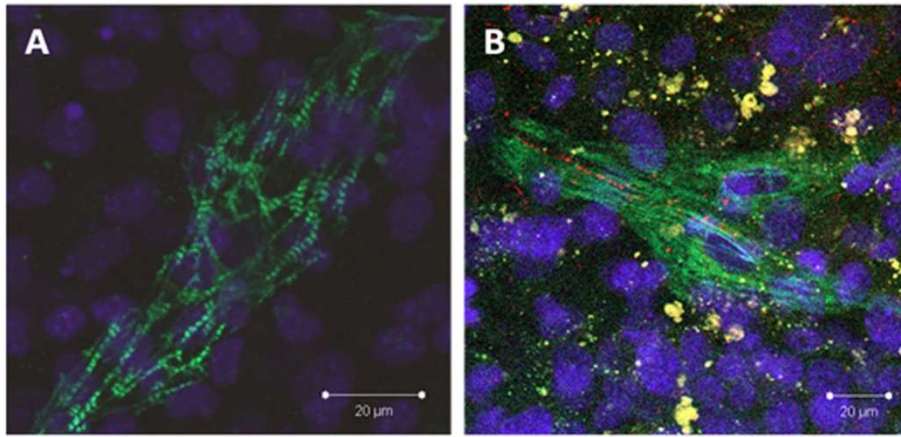


Fig. 21 *Immunofluorescence of differentiated CVPCs.* Cardiomyocytes in day 26 cardiac bodies were stained with cardiac TroponinT (green, A and B) and connexin 43 (red, B).

3.2.2. Comparison of CVPC to ESC Differentiation

When EBs were compared to CBs in regard to morphology, it was detected, that EBs formed a house-shoe like structure at early developmental stages, which has never been observed in CBs. CBs were larger and they showed faster cell spreading (Fig. 22). EBs usually started beating in a distinct area next to the axis (Fig. 22 A, arrow) and then cardiomyocyte formation spreaded at the edge of the EB. In contrast to EBs, first cardiomyocytes started appearing on various areas in CBs at the same time and then started spreading inwards.

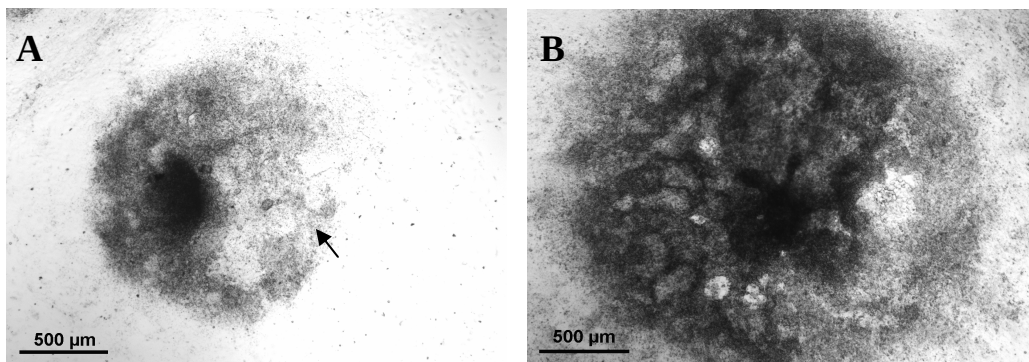


Fig. 22 *Comparison of day 9 EBs and CBs.* Bright field images of AB2.2 EBs (A) and A5 CBs (B) on day 9 of development. Arrow indicates area with first beating cardiomyocytes in EBs.

To observe the beating potential of ESCs and CVPCs, aggregates were made of the two cell types. The number of beating bodies was counted every day for 34 days. First cardiomyocytes started beating in EBs on day 8 of development, whereas in CBs beating cells could only be detected from day 11 on (Fig. 23). Cardiomyogenesis is delayed in CBs by approximately four days compared to EBs. However CMCs in CBs

stayed active over a longer period of time. From day 28 on, no beating cells in EBs could be observed anymore whereas a high percentage of the counted CBs still had rhythmically contracting CMCs.

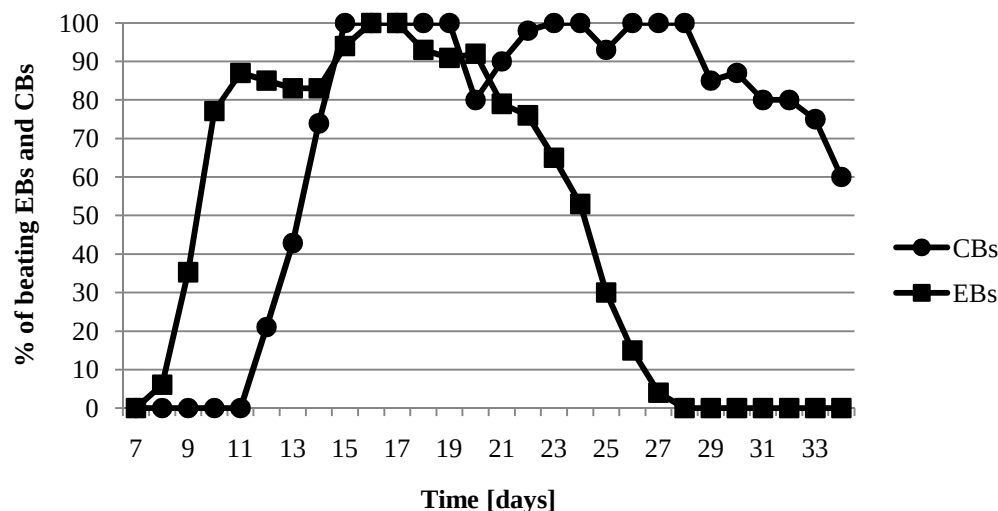


Fig. 23 Development of CMCs in CBs and EBs. A5 CBs and AB2.2 EBs were made and the number of beating bodies was counted every day for 34 days. Cardiomyogenesis in aggregated CVPCs is delayed compared to aggregated ESCs by 4 – 6 days but CMCs stay active over a longer period of time. Data are means of three counted wells, approximately 15 aggregates per well.

3.2.3. Paracrine Influence of EBs on CBs

It might be possible, that other cells in EBs produce cardiomyogenesis accelerating factors that are not provided to the same amount in CBs and therefore cause the earlier onset of CMC development in EBs compared to CBs. To test this hypothesis, and to detect whether those factors are secreted by EBs, CBs were cultured in the presence of EBs. Therefore, CBs and EBs were made and on day 4.7 CBs were transferred to 6 well plates and EBs were placed onto sieve inserts which were put into the wells of the 6 well plate. As controls, CBs were rinsed onto sieve inserts as well and cultured above CBs. Therefore, both, cardiac and embryoid bodies had the same medium and factors secreted by embryoid bodies could influence CMC development in CBs. There was no influence on cardiomyogenesis detectable when CBs were co-cultured with EBs (Fig. 24). This suggests, that secreted factors are not the main reason for the earlier onset of cardiomyogenesis in EBs.

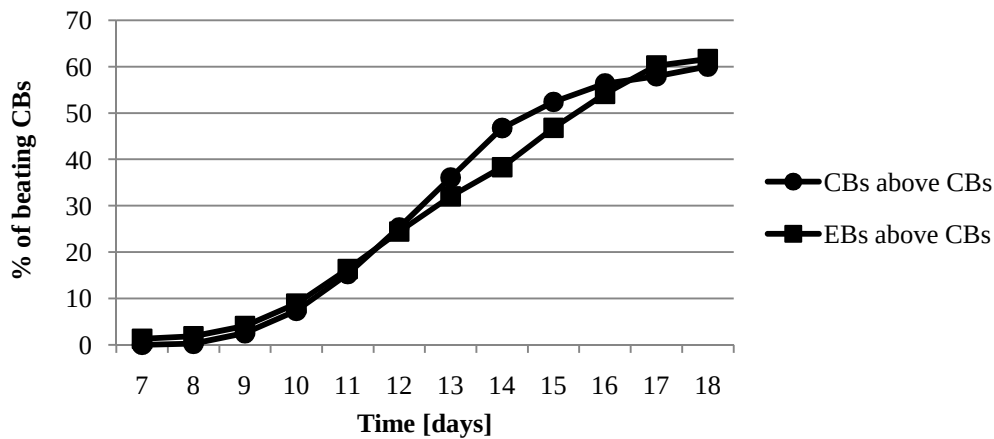


Fig. 24 *Paracrine influence of EBs on CBs.* EBs were cultured in sieve inserts in the same wells as CBs and the possible paracrine effect of factors secreted by EBs was tested. As a control, CBs were as well cultured in sieve inserts above CBs. Data are means of three counted wells, approximately 15 aggregates per well.

3.2.4. Extent of CMC Development in CBs and EBs

Cardiomyogenesis is delayed in CBs compared to EBs (Fig. 23). However, the magnitude of CMC formation is potentiated in CBs. In EBs, small areas on the edge of the body started beating and this beating spreaded on the boarder of the EB. Contraction in CBs started approximately four days later, however contracting cardiomyocytes developed all over the CB and under the light microscope it seemed as if the entire aggregate contracted. This difference in the extent of CMC formation was visualized by indirect immunofluorescence staining of EBs and CBs for the cardiac marker gene *cTNT*. EBs and CBs were fixed on 10 cm plates on days where maximum beating activity was observed (day 11 and day 15 respectively). Bright field and fluorescence images were made (Fig. 25). Only few fluorescent cells were seen in the EB (Fig. 25 B). Those cells reflect the amount of differentiated cardiomyocytes in EBs. Since CBs were taken on day 15, only the central area of the CB is shown (Fig. 25 C). The fluorescent image of the CB (Fig. 25 D) shows that the whole CB was pervaded by *cTNT* positive cells, cardiomyocytes built a network spanning the whole CB.

This was also tested by Fluorescence Activated Cell Sorting (FACS). Therefore, AB2.2 EBs and A5 CBs were made and used on day 11 and day 15 respectively. EBs and CBs were trypsinised and incubated with an antibody against the cardiac marker gene *cTNT* and stained cells were measured in the FACS machine. The amount of stained cells was four times higher in CBs than in EBs (Fig. 26). The observation

under the fluorescent microscope as well as the FACS experiment both show that CVPCs differentiate to highly cardiomyocytes-enriched bodies compared to ESCs.

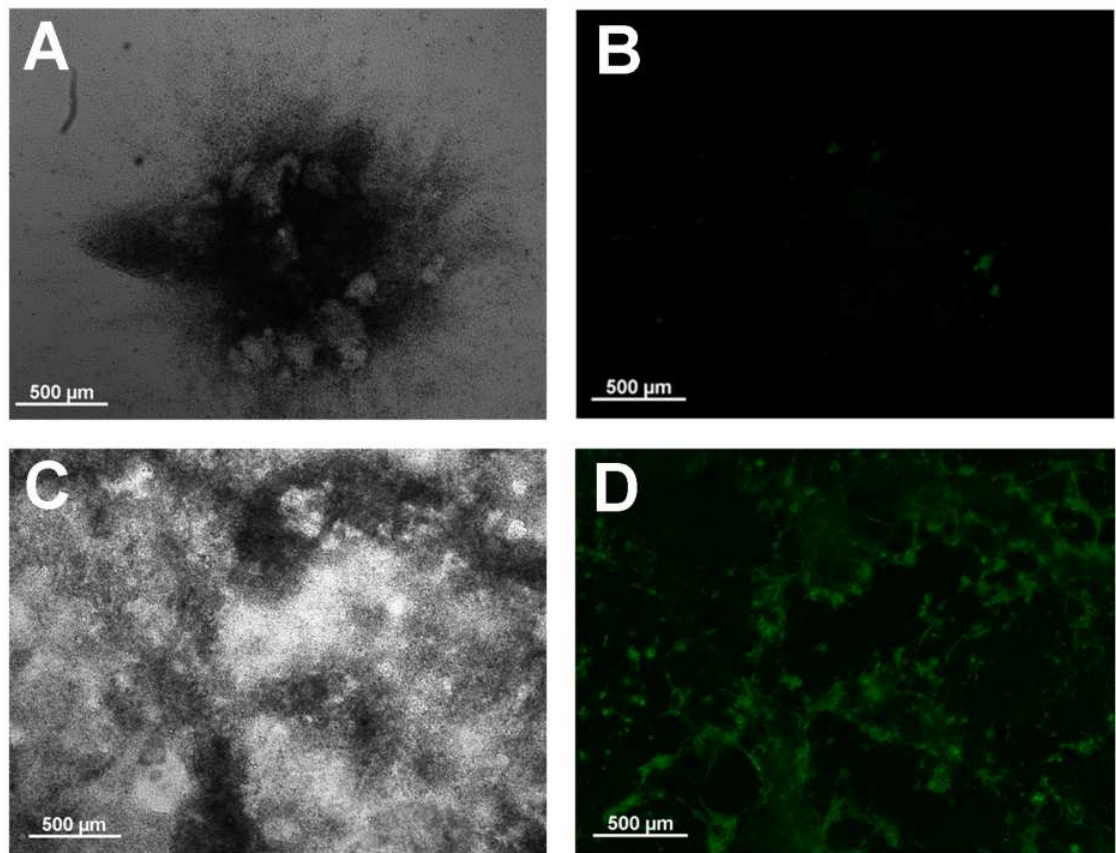


Fig. 25 *Extent of CMC development in CBs and EBs.* Aggregated ESCs and CVPCs were fixed on days of maximum beating activity (day 11 and day 15 respectively). CMCs were stained by indirect immunofluorescence staining with cardiac Troponin T (cTNT) and secondary FITC conjugated antibodies. Bright field image of whole EB (A) and fluorescence image of cTNT positive cells (B) are shown. (C) Bright field image of central area of day 15 CB. (D) Fluorescence image of day 15 CB.

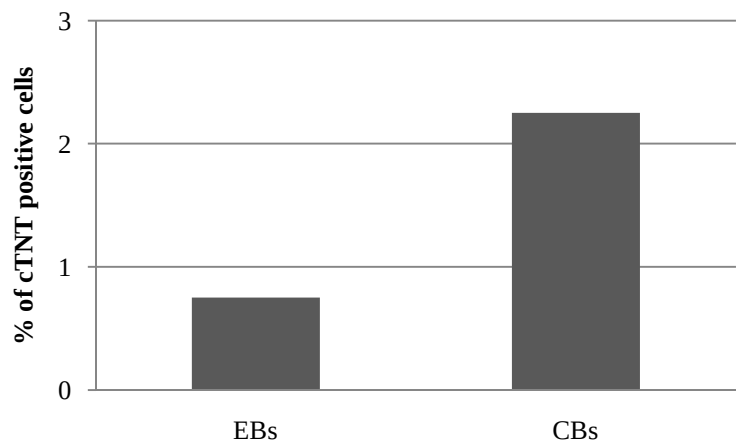


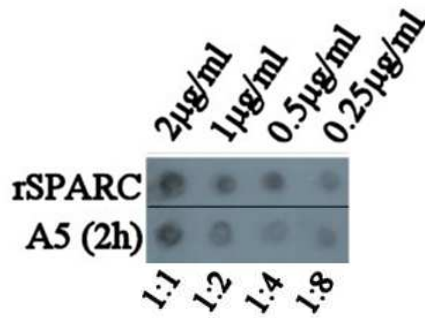
Fig. 26 *Fluorescence Activated Cell Sorting of cTNT stained AB2.2 EBs and A5 CBs.* EBs and CBs were made of AB2.2 and A5 cells respectively. On days where maximum beating activity was observed (day 11 and day 15), EBs and CBs were trypsinised, stained with cTNT and fluorescent cells were measured. The experiment was made three times, data are results of the third, optimized experiment.

3.3. Effect of SPARC on CVPCs

Secreted protein acidic and rich in cysteine (SPARC) is a matricellular glycoprotein which is highly expressed and plays an important role during fetal development. It is also crucial in remodelling and tissue repair (Brekken et al., 1999). SPARC has a positive influence on ESC differentiation. It accelerates and amplifies cardiomyogenesis when added on days 7 – 10 of EB development (Sary et al., 2005). Neutralising α -SPARC antibodies show the opposite effect, they cause a decrease of cardiomyogenesis. Furthermore SPARC enhances the expression of the cardiac marker gene *nkx2.5* in ESCs (Sary et al., 2005). The aim of the following experiments was to test the effect of SPARC on cardiomyogenesis in CVPCs.

3.3.1. Secretion of SPARC during CB Differentiation

We first had to determine which minimum concentration of SPARC had to be added to the cells in order to evoke a cellular response. Sary et al. (2005) used 3 $\mu\text{g/ml}$ of recombinant SPARC. However, CVPCs already express SPARC in an undifferentiated state (Fig. 27 A) (Hoebaus, 2009) as well as upon differentiation. Therefore, the SPARC concentration in the medium of the differentiating cells had to be determined. This was done by DotBlot analysis. A5 CBs were made and supernatant of the CBs was taken every day from day 6 till day 14. The supernatant was directly blotted in a dilution series (1:1, 1:2, 1:4, 1:8) to a nitrocellulose membrane and recombinant SPARC protein of different known concentrations was blotted to the membrane as well. The membrane was stained with α -SPARC antibodies and detected. A5 cells produced the highest concentration of SPARC at the beginning of differentiation. The intensities of the dots were compared to those of recombinant SPARC of known concentrations. On day 6, a SPARC level of about 0.3 $\mu\text{g/ml}$ was detected and afterwards the SPARC concentration decreased in the course of differentiation (Fig. 27 B). Therefore, SPARC seems to be most important in early stages of differentiation and the concentration used in previous experiments with ESCs of 3 $\mu\text{g/ml}$ is sufficient to induce an effect in CVPCs as well.

A**B**

A5 CBs
supernatant

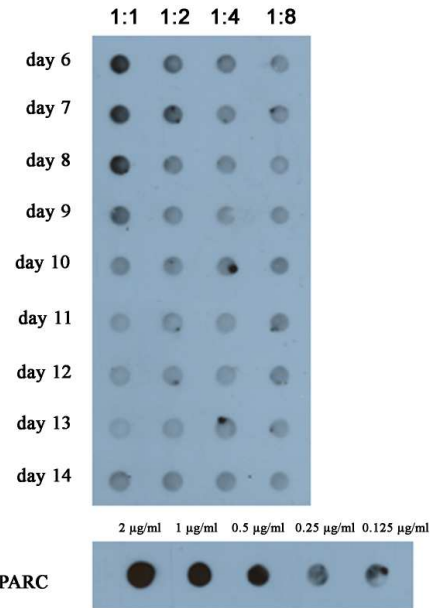


Fig. 27 Concentration of SPARC in A5 supernatant. Supernatant of undifferentiated A5 cells 2 hours after medium change was taken (A) (Hoebaus, 2009) and supernatants of A5 CBs were taken from day 6 till day 14 (B). They were blotted on a membrane in a dilution series (1:1, 1:2, 1:4, 1:8) and known concentrations of recombinant SPARC was blotted to the membrane as well. Detection was made using α -SPARC antibodies.

3.3.2. The Influence of SPARC Addition on CB Development at different Time Points

In EBs, SPARC addition had the most prominent effect when added on days 7 – 10 of differentiation (Stary et al., 2005). However, differentiation of ESCs differs from that of CVPCs and therefore SPARC might influence cardiomyogenesis at different time points in the two cell types. To test the time frame when SPARC influences cardiomyogenesis in CVPCs to the highest degree, neutralising α -SPARC antibody was added to A5 CBs at different time points and beating activity of the CBs was observed (Fig. 28).

SPARC inhibition at all tested time points delayed the onset of cardiomyogenesis. When α -SPARC was added to CBs on days 7 – 10 and 10 – 13 first CMCs were formed 1 and 1.5 days later than in the untreated CBs. However, the extent of beating CBs was similar to A5 control CBs, 90 – 100% of the CBs formed beating CMCs. When α -SPARC was added earlier in development on days 4.7 – 7, cardiomyogenesis was not only delayed but also reduced. Only about 60% of the counted CBs contained contracting cardiomyocytes (Fig. 28). In the DotBlot experiment we detected, that the cells themselves produce higher concentrations of SPARC at early stages of

development and here we show, that early inhibition of SPARC has the most severe phenotype. We chose the time frame of days 4.7-7 for SPARC addition in further experiments.

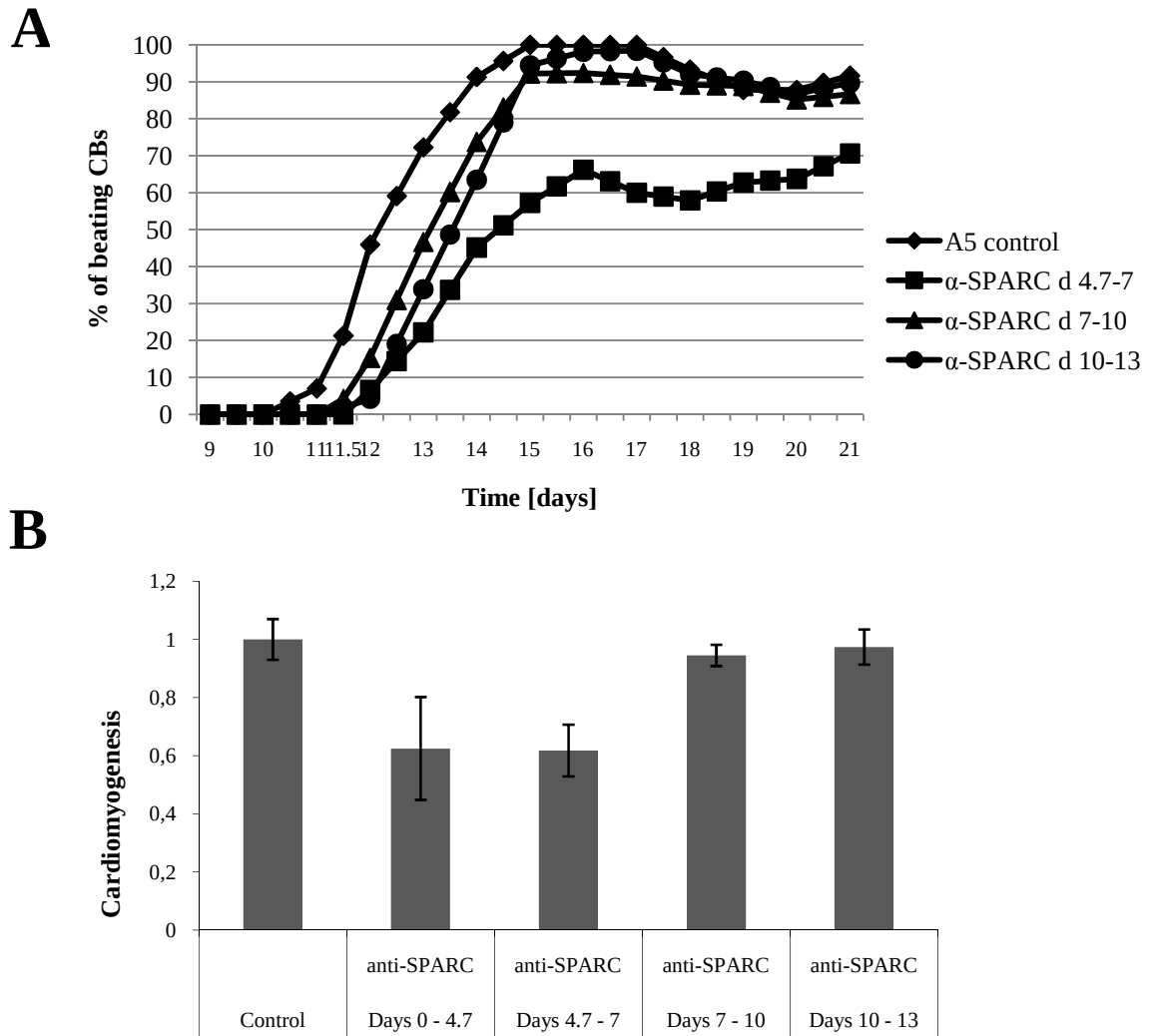


Fig. 28 Effect of α -SPARC on CB differentiation. A5 CBs were made and neutralising α -SPARC antibody was added on days 0-4.7, 4.7-7, days 7-10 and days 10-13. Results are displayed in a graph, values are means of 3 wells, 15 counted CBs per well (A) and bar diagram (B, Auner, H.).

3.3.3. Effect of SPARC and α -SPARC on CB Differentiation

In the following experiment, the influence of SPARC and α -SPARC on days 4.7 – 7 on the differentiation potential of CBs was investigated. A5 CBs were made, rinsed on day 4.7 and either 3 μ g/ml recombinant SPARC or neutralising α -SPARC antibodies (1:100) were added. The medium was changed on day 7. They were imaged under the

light microscope on day 8 (Fig. 29) and the number of contracting CBs was counted every 24 hours until day 20 of development (Fig. 30). CBs treated with SPARC looked similar to the control A5 CBs in regard to size and shape. The α -SPARC CBs however were smaller, they seemed to be hampered in growth and development (Fig. 29). This result suggested, that α -SPARC has a negative influence on CB growth and development.

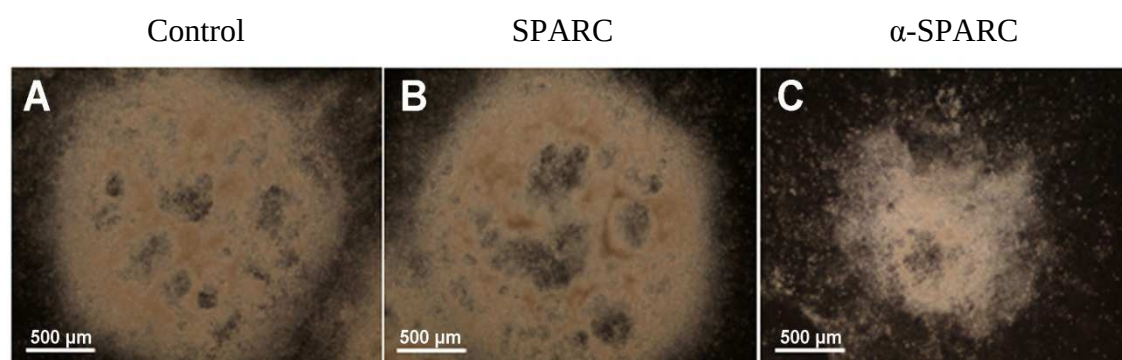


Fig. 29 Darkfield images of control, SPARC and α -SPARC treated A5 CBs. A5 CBs were made and treated with 0.3 μ g/ml SPARC and with neutralising α -SPARC antibodies (1:100) on days 4.7 – 7. Bright field images were made on day 8 of A5 control CBs (A), SPARC (B) and α -SPARC (C) treated CBs.

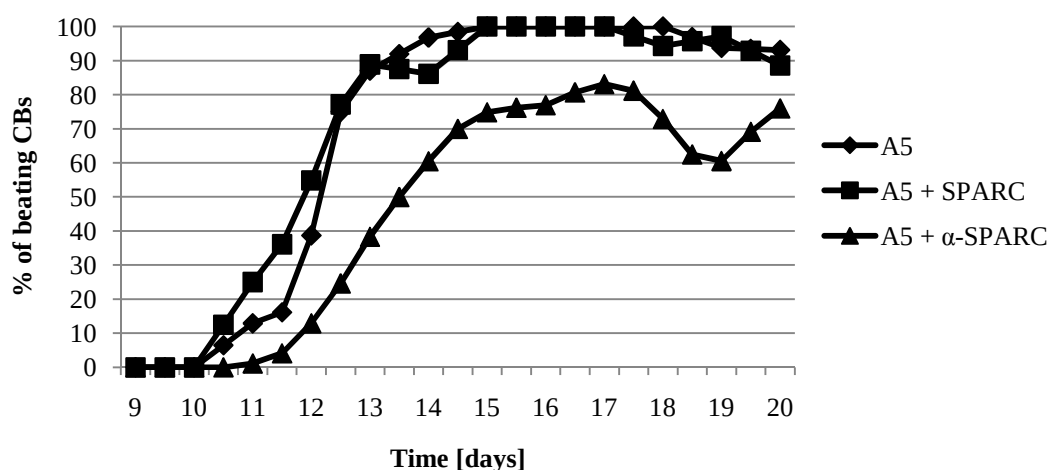


Fig. 30 Influence of SPARC and α -SPARC on CB differentiation. A5 CBs were made and rinsed on day 4.7 onto gelatine coated plates. At the same time, 3 μ g/ml SPARC and neutralising α -SPARC antibodies (1:100) respectively were added to the wells. They were incubated at 37°C. Medium was changed on day 7 and the number of beating CBs was counted every day until day 20 of development. Data are means of two wells, 15 counted CBs per well.

The addition of α -SPARC antibody indeed delayed and reduced the formation of contracting cardiomyocytes (Fig. 30). First beating was observed one day later than in the control and SPARC treated CBs. Cardiomyogenesis was not only delayed but also reduced, 30% of the counted CBs never built any beating cardiomyocytes. Addition of

SPARC caused the opposite effect, it enhanced cardiomyogenesis. First beating activity was observed on the same day as the control CBs, but a higher percentage of CBs developed contracting CMCs at the very beginning of differentiation (Fig. 30). From day 15, 100% of both, untreated and SPARC treated CBs developed beating activity.

The bar diagram in Figure 31 shows the mean of the percentage of beating CBs from days 11 – 13. Addition of SPARC resulted in the enhancement of cardiomyogenesis at the beginning of differentiation. Addition of α -SPARC caused a significant downregulation of contracting CBs. On days 11 – 13, only one third of beating CBs was observed compared to the control and SPARC CBs.

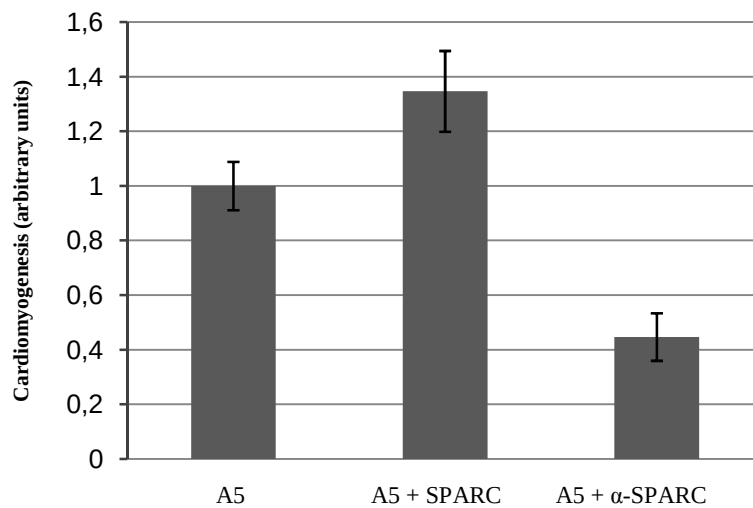


Fig. 31 Influence of SPARC and α -SPARC on CB differentiation. A5 CBs were made and were rinsed on day 4.7 onto gelatine coated plates. At the same time, 3 μ g/ml SPARC and neutralising α -SPARC antibodies (1:100) respectively were added to the wells. They were incubated at 37°C. Medium was changed on day 7 and the number of beating CBs was counted daily. The mean of the percentage of beating CBs on days 11 – 13 was calculated. Data are means of two wells, 15 counted CBs per well.

3.3.4. Effect of SPARC on Wnt Signalling

The previous experiment showed, that SPARC promotes cardiomyogenesis in CVPCs. However, the way in which SPARC influences differentiation is not known yet. We wanted to determine a signalling pathway through which SPARC influences cardiomyogenesis. Integrin α/β was found to be a candidate receptor for SPARC (Nie et al., 2008) and ILK activity is modulated by SPARC (Barker et al., 2005). ILK might act through modulating GSK3 activity, therefore interfering with Wnt signalling.

We wanted to investigate whether SPARC signalling influences the Wnt pathway and modulates activity of β -catenin. For this approach we used the TCF/LEF-luc reporter plasmid, which contains a *luciferase* gene under control of a TCF/LEF binding sites regulated promoter. TCF/LEF sites are sequences recognised and bound by β -catenin. This binding results in an activation of the promoter containing TCF/LEF sites. Therefore, the reporter gene expression reflects the level of active β -catenin in the cell.

The level of β -catenin in the cell is regulated by the Glycogen Synthase Kinase 3 α/β (GSK3 α/β). The active kinase phosphorylates β -catenin which results in its degradation. Unphosphorylated β -catenin can translocate to the nucleus and act as a transcription factor.

Firstly, we detected whether the Wnt signalling can be activated in A5 CVPCs. Therefore we inactivated GSK3 α/β with its inhibitor CHIR99021. CHIR99021 inhibits kinase activity of GSK3 α/β and β -catenin cannot be phosphorylated and degraded. A5 CVPCs were transfected with the TCF/LEF-luc vector and as an internal control with the Renilla plasmid. Four hours after transfection the medium was changed and 3 μ M CHIR99021 was added. The cells were incubated at 37°C and 48 hours after transfection Luciferase activity was measured. GSK3 α/β inhibition by CHIR99021 caused a 14-fold increase of Luciferase activity (Fig. 32). Therefore, Wnt signalling can be activated in CVPCs by inhibition of GSK3 α/β by CHIR99021.

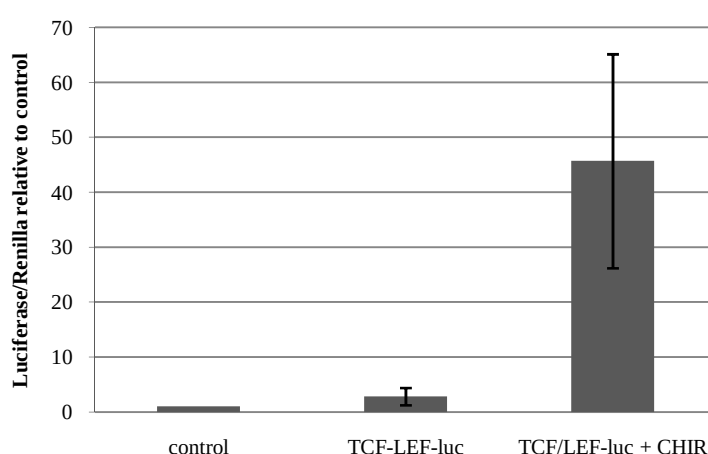


Fig. 32 Effect of GSK3 α/β inhibition on β -catenin activity. A5 CVPCs were transfected with TCF/LEF-luc plasmid, containing a *luciferase* gene under control of a TCF/LEF binding sites promoter. Transfected cells were treated with 3 μ M CHIR99021, an inhibitor of GSK3 α/β and 48 hours after transfection Luciferase activity was measured. Data are means of four experiments.

We then tested the effect of SPARC on Luciferase activity to get an indication about the signalling pathway through which SPARC influences cardiomyogenesis. Therefore, CVPCs were transfected with TCF/LEF-luc and Renilla plasmids and four hours after transfection the medium was changed and 3 μ g/ml SPARC, 3 μ g/ml SPARC together with α -SPARC (1:100) and α -SPARC alone were added, respectively. 48 hours after transfection, the cells were lysed and Luciferase activity was measured. Addition of recombinant SPARC to the transfected cells caused a reduction of Luciferase activity (Fig. 33). β -catenin could not activate the TCF/LEF regulated promoter to the same extent as in the control cells. This leads to the assumption, that SPARC reduced the level of β -catenin that can translocate to the

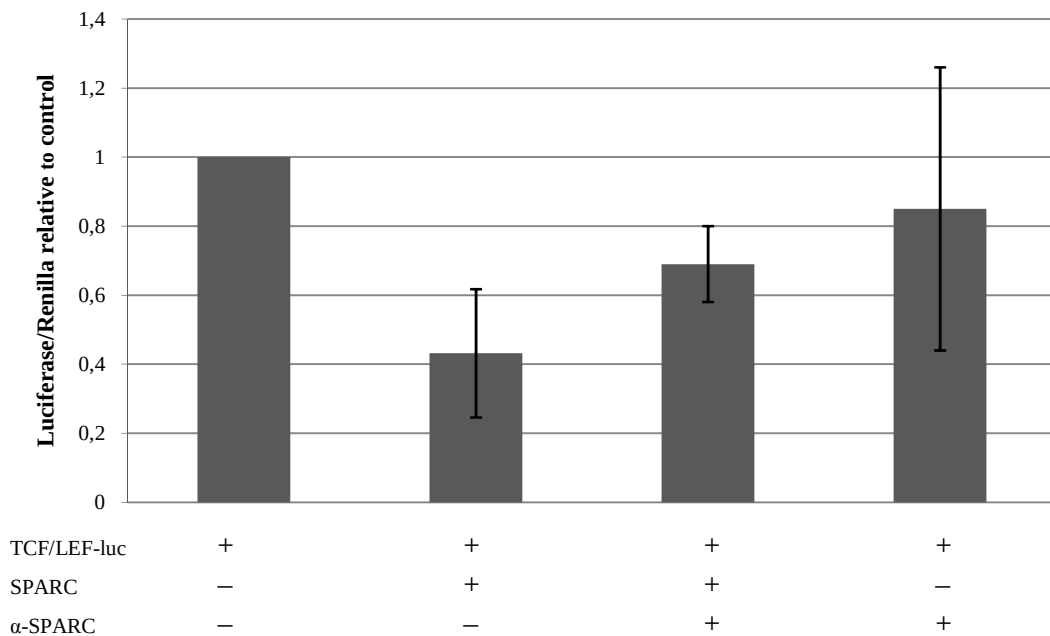


Fig. 33 Effect of SPARC and α -SPARC on β -catenin activity. A5 CVPCs were transfected with the TCF/LEF-luc plasmid, containing a *luciferase* gene under control of a TCF/LEF binding sites promoter. Transfected cells were treated with 3 μ g/ml SPARC, 3 μ g/ml SPARC + α -SPARC antibody (1:100) and with α -SPARC antibody (1:100). 48 hours after transfection Luciferase activity was measured. Data are means of three experiments.

nucleus and act as a transcription factor. This effect was compensated by the addition of α -SPARC to the SPARC induced wells. α -SPARC inhibited SPARC and more Luciferase was expressed than in the SPARC treated samples (Fig. 33). Therefore it can be said, that the inhibitory effect was indeed caused by SPARC addition. When α -SPARC was added to the cells neither an increase, nor a significant decrease was detected. Thus, addition of recombinant SPARC had a negative effect on the level of

free β -catenin in the cells, but SPARC inhibition by α -SPARC did not enhance β -catenin signalling.

3.3.5. Effect of SPARC on Gene Expression

In the previous experiments it was detected, that SPARC has a positive influence on CB differentiation and that it might act through inhibition of β -catenin signalling. We then wanted to test which target genes are influenced by SPARC and how they change their expression after addition of SPARC.

Therefore, A5 cells were split onto 24 well plates (1:3 ratio) and on the next day, 3 μ g/ml SPARC were added to the wells. RNA was isolated immediately, 2 hours and 3 hours after addition of SPARC. RNA was reversely transcribed into cDNA and PCRs were made using primers recognising different genes of interest (Fig. 34). *Nkx2.5* and *MHC α* are myocardial genes and it could be seen, that addition of SPARC caused an upregulation of their expression after 2 hours. The expression of the myocardial gene *isl1* however was not influenced by recombinant SPARC. *Mesp1* as a cardiovascular marker gene was slightly upregulated. Desmin was found to bind to the *nkx2.5* promoter in ESCs and differentiated CVPCs and overexpression of *desmin* accelerates heart cell formation in EBs (Hofner et al., 2007). Therefore it was interesting whether SPARC influences *desmin* expression and therefore enhances cardiomyogenesis. It was detected, that in the unstimulated A5 cells, no *desmin* mRNA was produced, but the addition of SPARC caused an increase in the *desmin* mRNA level in the cell. The next gene we were interested in was the mesodermal marker gene *brachyury*. Brachyury was upregulated after two and especially three hours of SPARC addition. *Goosecoid* is a gene involved in organising the embryo during early development and indicates differentiation. The undifferentiated control cells did not express this differentiation marker gene. After the addition of SPARC, the cells started producing *goosecoid* mRNA, the level increased between two and three hours after addition. Furthermore, we detected an increase in the mRNA level of Oct3/4 two and three hours after SPARC addition.

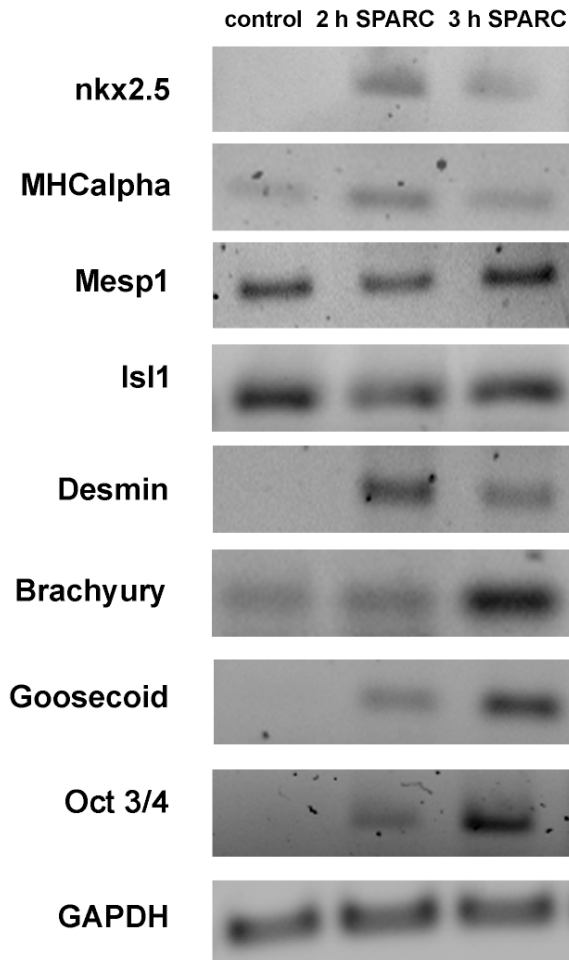


Fig. 34 Influence of SPARC on gene expression. 3 µg/ml recombinant SPARC was added to A5 CVPCs grown for one day on gelatine in monolayers. RNA was isolated of a control well, and 2 and 3 hours after SPARC addition. RNA was reversely transcribed into cDNA and PCRs were made with primers recognising different genes important in differentiation. A GAPDH PCR was made to show that the same amount of cDNA was taken of the three samples.

3.4. GSK3 α/β and MAPK Signalling in Cardiomyogenesis

We have already tested, that SPARC enhances cardiomyogenesis and it influences the level of free β -catenin in the cell. Therefore, the Wnt signalling pathway seems to play an important role in cardiomyogenesis. We then wanted to investigate the influence of the GSK3 α/β and MAPK pathways on cardiomyogenesis.

Therefore, A5 CBs were made, and inhibitors of the two pathways of interest were added on different days of development. The MAPK signalling cascade was inhibited by the MEK inhibitor PD98059 (10 µM), the GSK3 pathway by CHIR99021 (3 µM). The small molecules were added to the CBs on days 0 - 4.7, days 4.7 - 7, days 7 - 10, and days 10 - 13. The number of beating CBs was counted every 24 hours until day 21 of development. Both inhibitors did not influence cardiomyogenesis when added on

days 4.7 – 7 and days 10 – 13, respectively (data not shown). The onset of CMG and percentage of beating CBs was similar to the untreated A5 control cells. However at very early stages of development, PD98059 had a negative influence on cardiomyogenesis (Fig. 35 A). When the MEK inhibitor was added on days 0 – 4.7, the onset of contracting CMC formation was significantly delayed. First contracting cells in the CBs were observed two days later than in the control cells. In contrast, GSK3 α/β inhibition on days 0 – 4.7 barely influenced cardiomyogenesis in CBs. GSK3 α/β activity however seemed to be important at later stages of differentiation. When CHIR99021 was added on days 7 – 10, a drastic delay and reduction of cardiomyogenesis was detected (Fig. 35 B). The onset of CMG was delayed by 2.5 days and 30% of all CBs never contained any cells that differentiated into contracting cardiomyocytes, even at later stages of development. In this time frame, PD98059 addition did not influence CB differentiation.

From these data, it can be reasoned, that at the very beginning of cardiomyogenesis (days 0 – 4.7 of CB development) the MAPK pathway plays an important role whereas GSK3 α/β signaling can be inhibited without any effect. Later in development, on days 7 - 10, MEK inhibition did not influence CMC formation, but GSK3 α/β activity is necessary for proper differentiation of CBs.

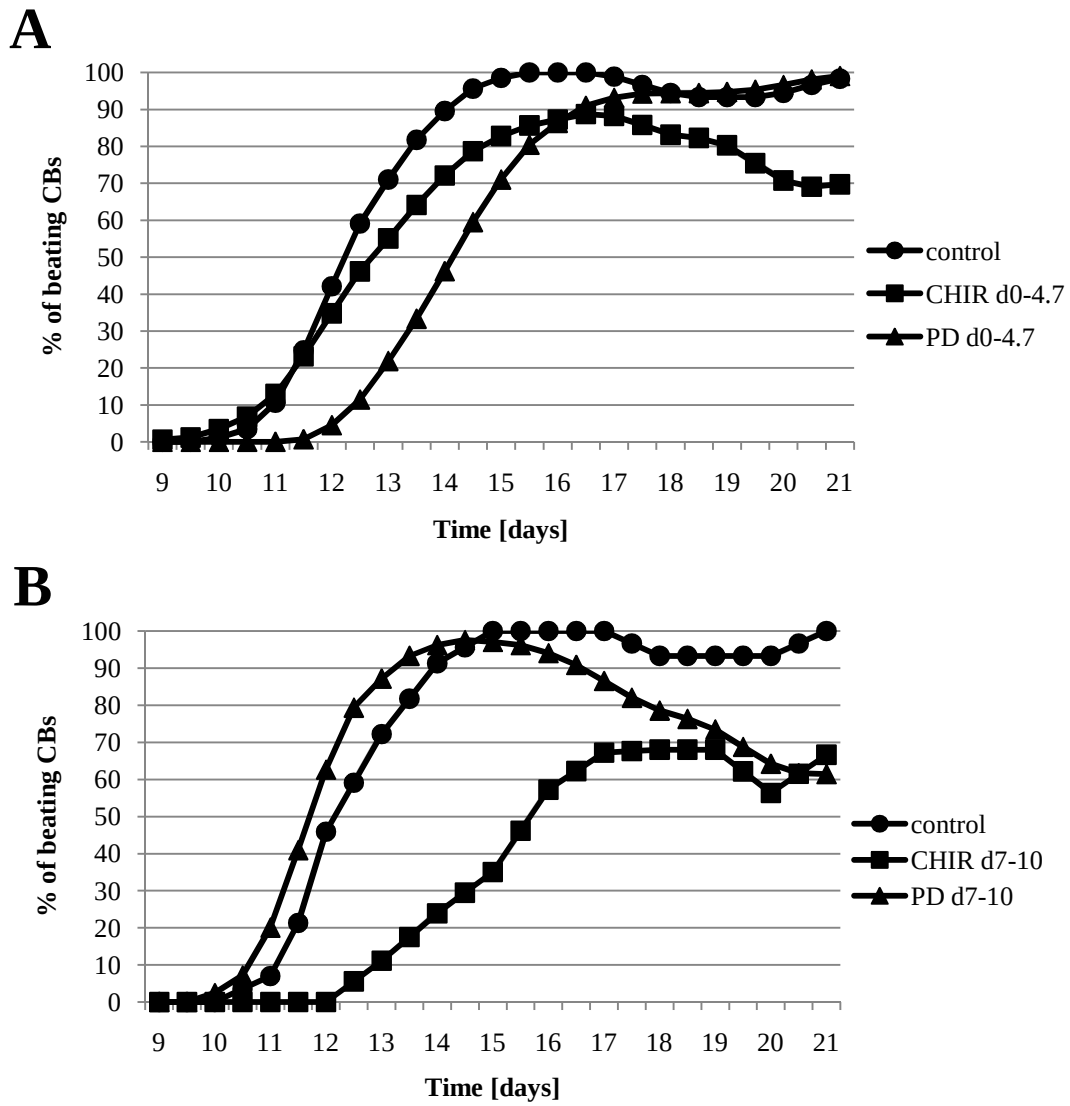


Fig. 35 *Effect of MAPK and GSK3 α/β inhibition on CB differentiation.* A5 CBs were made and were treated with 10 μ M PD98059 (PD), a MEK inhibitor, and 3 μ M CHIR99021 (CHIR), a GSK3 inhibitor. Inhibitor was applied on days 0 – 4.7 (A) and 7 – 10 (B). The CBs were incubated at 37°C and the percentage of beating CBs was calculated every day until day 21 of development. Data are means of three wells, approximately 15 counted CBs per well.

3.5. Generation of a Nkx2.5 – EGFP Reporter Cell Line

Nkx2.5 is a very important gene in heart cell development. During cardiomyogenesis it is one of the earliest heart specific genes and transcription factors that is expressed. It is upregulated in CVPCs upon differentiation and therefore it would act as a good marker gene to monitor development of CVPCs to cardiomyocytes.

The goal was to generate a CVPC line which carries an *EGFP* marker gene under the control of the *nkx2.5* promoter. Therefore, whenever cells express *nkx2.5*, EGFP is produced which can be detected by western blotting, immunofluorescence microscopy and FACS. The effect of the addition of different factors supposed to influence cardiomyogenesis could be investigated with this reporter cell line in an easy way and optimal conditions for differentiation could be determined. A control ESC line carrying an *EGFP* gene knocked into one allele of the *nkx2.5* gene has already been established in our laboratory and the *EGFP* gene activity represents the *nkx2.5* expression in the course of cardiomyogenesis.

3.5.1. Homologue Recombination of A5 Cells

The pCsx-EGFP-PP-DT vector (Hidaka et al., 2003) contains sequences homologue to the murine *nkx2.5* gene but they are interrupted by an *EGFP* and *puromycin* resistance gene. When transfected to cells, the reporter and selection gene flanking sequences homologue to *nkx2.5* can bind to the DNA and if homologue recombination occurs, the *EGFP* and *puromycin* genes are integrated into the genome (Fig. 36).

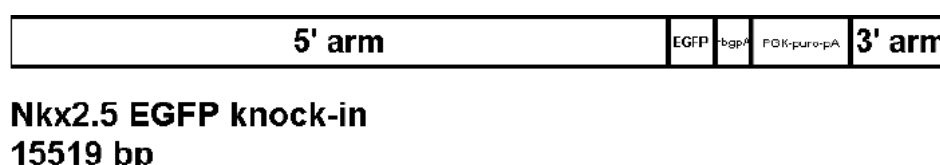


Fig. 36 *Nkx2.5-EGFP knock-in region*. The pCsx-EGFP-PP-DT vector was linearised and brought into A5 cells by electroporation and with homologue recombination, the *EGFP* reporter gene and the *puromycin* resistance gene were knocked into the *nkx2.5* gene (Hidaka et al., 2003).

A5 cells were electroporated with the linearized pCsx-EGFP-PP-DT plasmid. Feeding cells with medium containing puromycin (1 µg/µl) for seven days resulted in a selection for cells which integrated the vector into the genome. Two clones were picked on day 10 after electroporation, on day 16, 8 more colonies were picked. Five of the ten picked clones reached confluence and were named A5-Csx 1 – 5.

3.5.2. Verification and Characterisation of A5-Csx Cell Lines

To confirm that the vector was homologously recombined into the *nkx2.5* sequence and did not insert randomly into the genome, PCR primers were designed. One primer recognized a sequence in the *puromycin* resistance gene within the construct, the other one a sequence 3' of the cassette in the *nkx2.5* genomic region. Only if the construct integrated in the correct location, a PCR product of 1924 bp will occur.

DNA of all five A5-Csx clones was isolated by Phenol/Chloroform extraction. As a positive control, DNA of the already established ESC line carrying one *nkx2.5*-EGFP allele, named MS15, was isolated as well. Furthermore, A5 and AB2.2 DNA served as negative controls. No PCR product was obtained for those two controls. In the MS15 sample, a product of the correct length could be detected. The PCRs of all five clones resulted in a PCR product of the same length (Fig. 37), confirming that all clones carry an *EGFP* gene under control of the *nkx2.5* promoter.

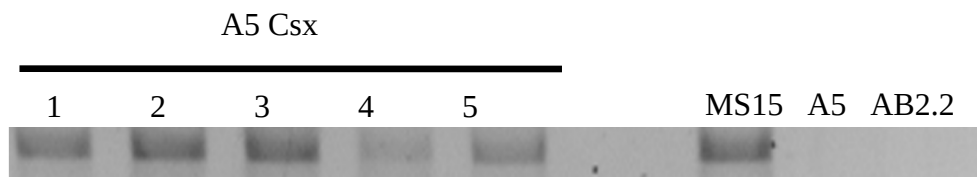


Fig. 37 Detection of successful knock in of the *pCsx-EGFP-PP-DT* vector into the genomic region of the *nkx2.5* gene. PCRs were made with genomic DNA of A5 cells thought to carry an *EGFP* and *puromycin* resistance gene in one allele of the *nkx2.5* locus. Primers were used recognising a sequence inside and outside the homologously recombined *pCsx-EGFP-PP-ST* vector. A PCR product only occurs if the construct integrated into the genome in the desired way.

Since one allele of the *nkx2.5* locus is mutated in the A5-Csx cells, we wanted to differentiate the cells in CBs and compare them to A5 CBs in regard of beating to see whether cells are hampered in cardiomyogenesis due to the mutation in one allele. Furthermore, the expression of *EGFP* on different days of development has been observed by fluorescence microscopy. The same amount of cells was taken to produce A5 and A5-Csx4 CBs and the number of contracting CBs was counted daily. There was no difference in the first appearance of beating CMCs and in the number of rhythmically contracting A5 and A5-Csx4 CBs. First contracting cardiomyocytes were observed on day 10 in both cell types and maximum beating activity was reached at day 14 of development (Fig. 38). The cells heterozygous for *nkx2.5* did not show altered cardiomyogenesis in this setting.

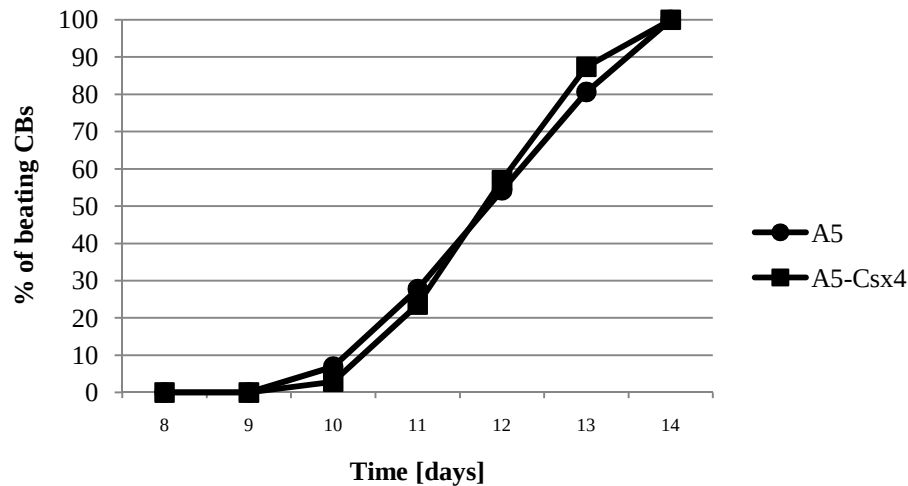


Fig. 38 Development of CMCs in A5 and A5-Csx4 CBs. A5 and A5-Csx4 CVPCs were aggregated to CBs and the percentage of beating bodies was calculated every day for 14 days. Data are means of two wells, 15 CBs per well.

We next wanted to explore, whether A5-Csx cells express *EGFP* and therefore can be observed under the fluorescence microscope. CBs and EBs were made of A5-Csx2, A5, MS15 and AB2.2 cells. The MS15 EBs served as a positive control, AB2.2 and A5 CBs were used as negative controls. After 13 and 14 days of development, EBs and CBs were observed under the fluorescence microscope (Fig. 39). Bright field and fluorescence images of MS15 EBs and AB2.2 EBs were taken. The AB2.2 EBs did not have any fluorescent cells. In MS15 EBs a picture of an area of beating cardiomyocytes was taken and exactly this area contained fluorescent cells. This means, that *EGFP* is expressed in those cells and thus, those cells express *nkx2.5*.

The fluorescence of the A5-Csx2 and A5 CBs was analysed on day 14 by fluorescence microscopy (Fig. 40). Bright field as well as fluorescence images of beating areas of the CBs were taken. Many A5-Csx2 cells were fluorescent. However, having a look at the A5 negative control, a similar picture was seen. It seems that A5 CVPCs had a strong background fluorescence and thus, the additional *EGFP* fluorescence could not be detected. No conclusion could be drawn with this method about the *nkx2.5* expression in CVPC cells.

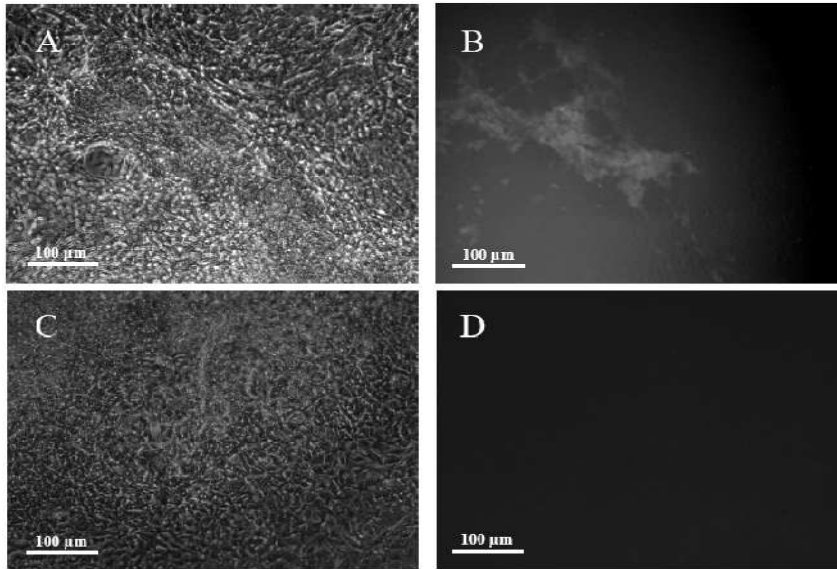


Fig. 39 Expression of *EGFP* in homologously recombined AB2.2 ESCs. EBs were made with MS15 and AB2.2 cells. MS15 cells are ESCs which have an *EGFP* marker gene knocked into one allele of the *nkx2.5* locus. Brightfield image (A) and fluorescence image (B) of MS15 EBs compared to brightfield image (C) and fluorescence image (D) of AB2.2 EBs on day 13 of development.

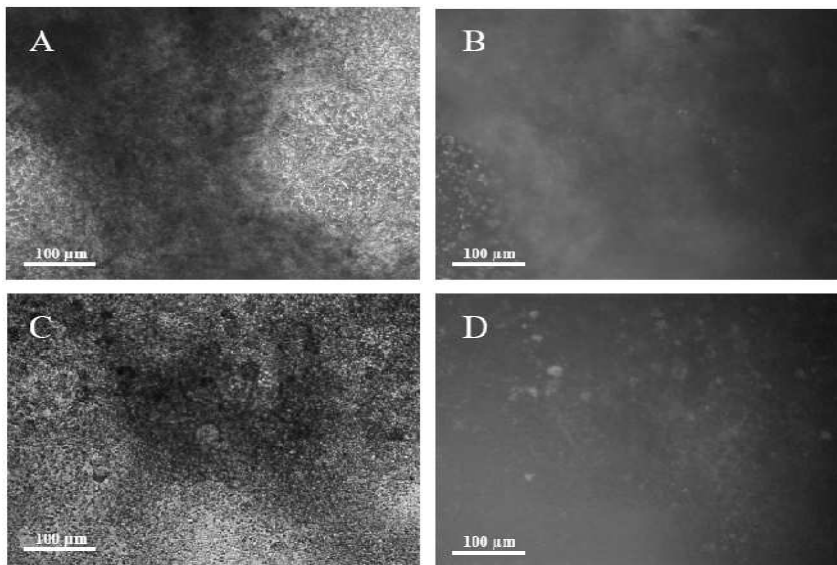


Fig. 40 Expression of *EGFP* in A5 CVPCs. CBs were made with A5-Csx2 and A5 cells. A5-Csx2 cells are CVPCs which have an *EGFP* marker gene knocked into one allele of the *nkx2.5* locus. Brightfield image (A) and fluorescence image (B) of A5-Csx CBs compared to brightfield image (C) and fluorescence image (D) of A5 CBs on day 14 of development.

Therefore, we tried to amplify the EGFP signal by indirect immunofluorescence staining with GFP antibodies and secondary FITC-labelled antibodies. However, even with this amplification, we did not detect any difference between the A5-Csx and A5 cells (data not shown). However, immunofluorescence staining was made on day 14 of development, maybe *nkx2.5* expression and therefore *EGFP* expression was already low at this stage of development and therefore even harder to detect.

We then tried to detect the EGFP expression by Western Blotting. Therefore, A5-Csx4 CBs and A5 CBs were made and proteins were isolated from CBs on days 8 – 14. Proteins were detected with α -GFP antibodies. The A5 samples never showed a GFP signal at any time whereas the A5-Csx4 samples were GFP positive at all measured time points (Fig. 41). These data suggest that *nkx2.5* is expressed in A5-Csx4 cells from days 8 – 14.

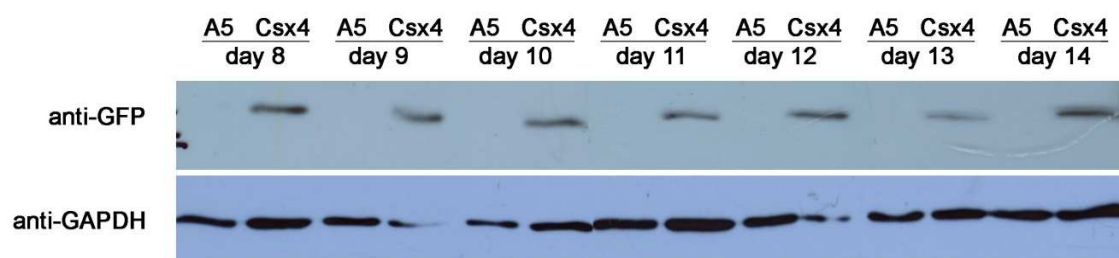


Fig. 41 Western Blot of A5 and A5-Csx4 cells with GFP antibodies. A5 and A5-Csx4 CBs were made and protein was isolated from day 8 till day 14. 35 μ g protein per lane were loaded on a gel, blotted to a nitrocellulose membrane and the proteins were detected with α -GFP antibodies.

Searching for a method suitable to measure quantitative differences in the *EGFP* expression of A5-Csx cells, we tried to make a FACS analysis of A5 and A5-Csx cells. The settings for the FACS analysis could be chosen to disregard autofluorescence and only EGFP fluorescence is taken account of.

A5 and A5-Csx4 CBs were made, on day 9 of differentiation they were trypsinised, resuspended in PBS and the number of fluorescent cells in the two samples was measured. In the A5 sample no GFP positive cells were measured. However 15% of the A5-Csx4 cells expressed GFP (Fig. 42).

This method seems to be the most sensitive and is the most promising approach to investigate differences in the expression of *nkx2.5* in the course of differentiation.

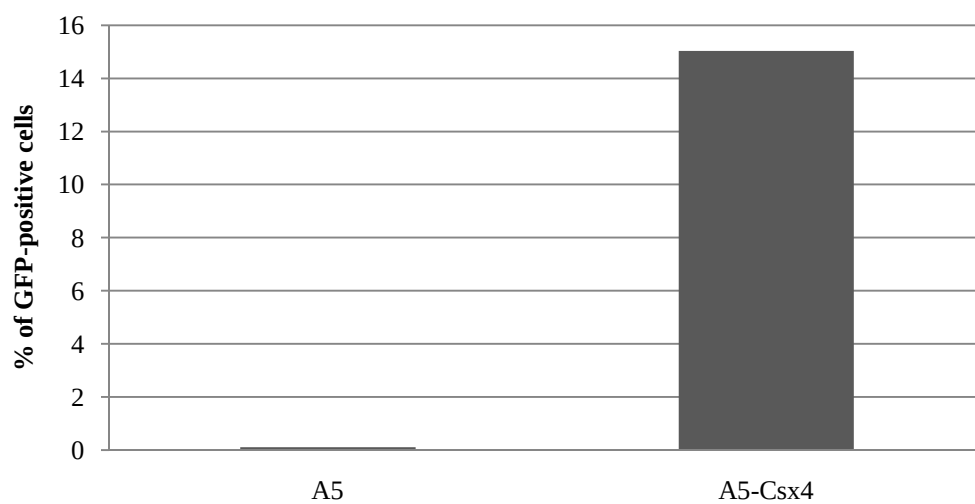


Fig. 42 *FACS of A5 and A5-Csx4 CBs.* CBs were made of A5 and A5-Csx4 CVPCs. On day 9 of development CBs were trypsinised, resuspended in PBS and GFP positive cells were counted by FACS. The experiment was made once.

4. Discussion

The murine heart comprises cardiac stem cells which are supposed to be able to regenerate small areas of dead tissue. However, the regenerative potential of those cells is not sufficient to cure injured hearts. Understanding the mechanisms activating those dormant cells to differentiate and to replace injured cells would be a great achievement for cell based therapy. Here, the isolation of stable cardiovascular progenitor cell (CVPC) lines and the establishment of an in vitro model to determine differentiation conditions and factors influencing cardiomyogenesis is shown. The isolated CVPCs had the potential to differentiate exclusively to cardiomyocytes, smooth muscle, and endothelial cells and therefore are restricted to the cardiac lineage. Adaptation of the embryoid body (EB) formation method from ESCs resulted in a reproducible system for differentiation of CVPCs in cardiac bodies (CBs). A highly increased amount of beating cardiomyocytes was observed in CBs compared to EBs, however the onset of CMG was delayed. We furthermore report the generation of a GFP positive reporter cell line, reflecting the expression level of the early cardiac marker gene *nkx2.5*. The protein Desmin was identified to bind to the *nkx2.5* promoter region in ESCs as well as in differentiated CVPCs and pCMCs. Furthermore, SPARC was shown to have a positive influence on CMC formation by upregulating cardiac specific gene expression. In addition, SPARC regulated Wnt signalling resulting in a decrease of nuclear β -catenin. It was further shown, that Wnt and MAPK signalling pathways play an important role in CMG at different time points of differentiation.

4.1. Isolation, Maintenance, and Differentiation Potential of Stable CVPC Lines

Recently, it was suggested that cardiac stem cells reside in the adult heart and several groups succeeded in their isolation (Beltrami et al., 2003; Laugwitz et al., 2008; Oh et al., 2003; Oyama et al., 2007). However, maintaining them in culture remained challenging. Here, a new approach for the isolation of stable CVPC lines is reported. Murine heart cells were co-cultured with ESCs and feeder cells in order to mimic niche conditions (Wiedner, 2008). This imitation of natural environment kept CVPCs in a condition, allowing self-renewal and maintenance in culture for indefinite time. Therefore, they serve as a good starting material to study differentiation and cardiomyogenesis influencing factors since the cells remain identical for all experiments. CVPCs express the three stemness factors *sox2*, *nanog*, and *oct3/4* at

comparable amounts to murine ESCs. The combined expression of these three transcription factors allows CVPCs to stay in a self-renewal mode when grown on LIF secreting fibroblasts, they can be expanded in culture without losing pluripotency. Simultaneously, the expression of *nkx2.5* and MHC- α was detected in an undifferentiated state (Hoebaus, 2009), showing a predisposition to the cardiac lineage. Upon LIF deprivation, CVPCs start to differentiate, losing their stem cell potential. These cell lines have already been cultured for more than 80 passages under continuous cryo-preservation maintaining their potency to self renew and to form beating CMCs upon differentiation. The adaption of the protocol for the production of EBs (Weitzer et al., 1995) to produce CVPCs derived CBs provided a controllable and highly reproducible system for the study of differentiation to CMCs. CVPCs aggregated in the hanging drop culture without attachment to an artificial surface. In CBs, CVPCs differentiated exclusively to cardiomyocytes, smooth muscle cells and endothelial cells. Cardiomyocytes built networks with tight connexin 43 positive cell – cell interactions. We never observed any nerve-like structures, skeletal muscle cells, adipocytes, or erythrocytes. Compared to EBs, CBs had a less structured and point symmetric development. First beating cells in CBs were observed on day 12, which was approximately four days later than in EBs. The earlier onset of CMG in EBs was not primarily caused by secreting factors produced by the EBs, since co-culture of CBs with EBs did not accelerate CMG in CBs. However, under the same culture conditions CMCs in CBs stayed active over a longer period of time. Contraction in EBs could not be observed after day 28 of differentiation, whereas still a high percentage of CBs consisted of contracting CMCs. We also showed, that the extent of CMC development in CBs was much potentiated compared to EBs. In EBs only small areas with rhythmically contracting cells were observed, whereas in CBs, beating CMCs built a network spanning the entire CB. The continuous self-renewal of CVPCs, their limited differentiation potential, and the high degree of CMC formation makes these cells good candidates for cell therapy.

4.2. Generation of a Nkx2.5-EGFP Reporter Cell Line

Nkx2.5 is the most important early cardiac gene in first heart field CMG. Forced expression of *Nkx2.5* is sufficient to enhance cardiogenesis in murine ESCs (David et al., 2009). It is upregulated during differentiation of CVPCs and would therefore serve as a good marker gene to keep track of CVPCs differentiating to CMCs. Here we

report the generation of a stable CVPC line carrying an *EGFP* reporter gene in one allele of the *nkx2.5* locus. Expression of *EGFP* was controlled by the *nkx2.5* promoter and reflected the *nkx2.5* expression in the cells. Two previously found aspects had to be taken into account before differentiating these cells. Firstly, it was shown that heterozygous mutation of *nkx2.5* negatively influenced cardiac morphogenesis and blocked atrioventricular conduction (Biben et al., 2000). Secondly, it was detected, that EGFP binds to myosin. This binding had a negative effect on the contractile function of cells (Agbulut et al., 2007). However, differentiation of the generated *nkx2.5*-EGFP cells did not show differences in their cardiomyogenic potential compared to CVPCs which is in accordance to findings of other groups working with similar cell lines (Hidaka et al., 2003). Since the cardiogenic potential was not influenced, the generation of a *nkx2.5*-EGFP reporter cell line provided a useful in vitro culture system to monitor *nkx2.5* expression during development and the effect of several CMG influencing factors. The GFP signal however was very weak and for in vitro experiments, FACS analysis was shown to be the most promising detection method. Further copies of *nkx2.5* promoter – EGFP integrated into the genome could enable the detection of EGFP in CVPCs. The *nkx2.5*-EGFP cell line could also be used to track the fates of CVPCs and differentiating cardiomyocytes in vivo. Injection of EGFP-positive CVPCs into the murine heart could show the reintegration and regenerative potential of those cells in the adult, injured heart.

4.3. Influence of Desmin on *nkx2.5*

The intermediate filament type III protein Desmin was shown to upregulate the expression of the cardiac marker gene *nkx2.5* during mesoderm formation in EBs (Hofner et al., 2007). However, the mechanism how this activation works is not known yet. Here, we detected a physical interaction of Desmin with the *nkx2.5* promoter on day 6 and day 8 in wild type EBs. First beating in wild type EBs occurred on days 6.5-7.5. In Desmin overexpressing EBs (DC6 ESCs) an interaction was already detected on day 5, and remained on day 6 and day 7. The interaction started and also ended one day earlier than in the wild type cells. The *desmin* overexpressing EBs started beating one day earlier than the wild type EBs, first beating could normally be seen from day 6.5 to day 7. In wild type EBs, as well as in Desmin overexpressing EBs Desmin interaction with the *nkx2.5* promoter could be detected one day before the onset of contraction. It seems as if the development of heart cells is shifted one day in advance

in DC6 cells since the interaction as well as the beating was detected one day earlier than in wild type cells. These results suggest a correlation between binding of Desmin to the *nkx2.5* promoter and cardiomyocyte formation. This interaction might be a necessary step for initiation of cardiomyogenesis. However, in wild type and DC6 EBs no or very little interaction of Desmin and the *nkx2.5* promoter was observed on the day where first beating occurred. Probably, for contraction, a reconstruction of the intermediate filament structure has to occur and for this short time, Desmin is recruited out of the nucleus. At later stages of differentiation no interaction was observed, Desmin's action as a transcription factor might be redundant. The 1B44 EBs whose Desmin N-terminus is deleted did not show any interaction of Desmin with the *nkx2.5* promoter. The Desmin amino-terminus was detected to be important for cardiomyogenesis (Hollrigl et al., 2002). Our results provide evidence, that the N-terminus is important for DNA binding. The lack of DNA – *nkx2.5* promoter interaction might be a reason for the negative effect of the Desmin amino-terminus deletion on cardiomyogenesis.

We then tested whether Desmin - DNA interaction occurs in CVPCs and in pCMCs. ChIP was made of undifferentiated and differentiated CBs (day 17). Interaction of Desmin with the *nkx2.5* promoter was seen in the differentiated CBs which already formed beating CMCs, however no binding occurred in the undifferentiated samples. Therefore it seems that the interaction takes place in the course of development and might be an important step in heart cell formation. In day 17 CBs, already a high percentage of CBs formed rhythmically beating clusters. At this time point, the Desmin – *nkx2.5* promoter interaction already stopped in EBs. Differentiation of CVPCs in CBs results in the production of CMC at a high level, however the onset of CMG is delayed. CMCs in CBs are furthermore active over a much longer period of time compared to CMCs in EBs and the production of further CMCs might be induced at later time points as well. Therefore, it might be reasonable, that the interaction in CBs still occurs at later stages of differentiation. Furthermore, we found Desmin bound to the *nkx2.5* promoter region in differentiated heart cells of new born mice. This provides another evidence for the hypothesis stating that the longer CMCs stay active, the longer Desmin is bound to the *nkx2.5* promoter.

Nkx2.5 is already expressed in undifferentiated CVPCs. However, the degree of its expression is very low as long as cells are obtained with signals for self-renewal. Differentiation inducing signals could result in binding of Desmin to the *nkx2.5*

promoter and thereby increase the level of expressed *nkx2.5* which in turn might lead to commitment and final differentiation.

The effect of the *Desmin-nkx2.5* promoter interaction on gene expression was examined by transfection of C2C12 cells with plasmids containing a *luciferase* reporter gene under the control of the *nkx2.5* promoter. Downregulation of *desmin* by *desmin* siRNA and coherent downregulation of *nkx2.5* expression would proof the *Desmin* – specific effect on *nkx2.5* promoter activity. We showed that the *nkx2.5* promoter was indeed active in C2C12 myoblasts and we could demonstrate a downregulation of reporter gene expression after *desmin* knock down. These data are in line with our ChIP data from which we hypothesised that binding of *Desmin* to the *nkx2.5* promoter enhances its activity resulting in CMC differentiation. They are also in accordance with Hofner et al. (2007) who revealed *Desmin* as a positive regulator of cardiomyogenesis. However, *desmin* overexpression decreased promoter activity. This corresponds to studies suggesting a negative feedback loop of *Desmin* when reaching a certain concentration in the cells (Hoebaus, 2009). In primary cardiomyocytes, *desmin* knock down did not influence promoter activity. In earlier ChIP experiments it was detected, that *Desmin* is bound to the *nkx2.5* promoter in day 0 pCMCs. Here, downregulation of *Desmin* in day 5 pCMCs did not alter *nkx2.5* expression. In the 5 days further developed cells *Desmin* might not have an influence on *nkx2.5* expression anymore.

4.4. Influence of SPARC on CVPCs

Recombinant SPARC was described to enhance CMG in ESCs (Sary et al., 2005). ESCs express SPARC on mRNA level but SPARC could not be found as a secreted protein (Sary et al., 2005). The addition of recombinant SPARC to EBs on days 7 – 10 increased the amount of differentiated CMCs whereas neutralizing α -SPARC antibody showed the opposite effect (Sary et al., 2005). Unlike ESCs, undifferentiated CVPCs secrete SPARC at high levels (Hoebaus, 2009) and in the course of CB development, SPARC secretion decreased. Assuming a positive influence for SPARC on CMG, SPARC might therefore be important in early steps of differentiation. This was affirmed by detecting a time dependent negative influence of neutralizing α -SPARC antibody and a positive effect on CMG upon addition of recombinant SPARC to CBs on days 4.7 – 7. Despite the fact that the onset of CMC formation in CBs is delayed compared to EBs, SPARC influenced CMG at earlier time points in CBs than in EBs.

CVPCs might have to be provided with SPARC earlier in differentiation. The ground state of CVPCs is already progressed in development compared to the ground state of ESCs. ESC differentiation might firstly go through progenitor cell formation and at this stage, SPARC can positively influence CMG. However, despite addition of SPARC to CBs, the onset of CMC formation is delayed compared to EBs, therefore SPARC is not able to replace the microenvironment and possible niche conditions that are mimicked in EBs.

To gain better insights into the mode of SPARC action, the short time effect of SPARC on genes involved in differentiation was tested by detecting their mRNA level two and three hours after addition of recombinant SPARC. Several genes altered in their expression pattern upon SPARC addition could be identified. We detected an increase in expression of the early cardiac marker gene *nkx2.5*. *MHC α* as a late gene in cardiac development was not significantly upregulated, giving another reason for classifying SPARC as a protein involved in early CMG. The myocardial gene *isl1* expression was not changed upon SPARC addition. *Nkx2.5* is expressed in first and second heart field progenitors, whereas *isl1* was identified as the key transcriptional regulator of the second heart field progenitor cell lineage (Laugwitz et al., 2008). This provides evidence that SPARC may promote differentiation of first heart field progenitors or that CVPCs are progenitor cells of the first heart field. In addition to the other cardiac markers, SPARC slightly upregulated the expression of *mesp1*, which is a master regulator sufficient to induce cardiovascularogenesis in ESCs (David et al., 2008). Desmin was found to bind the *nkx2.5* promoter in EBs and differentiated CVPCs and *desmin* overexpression enhanced CMG in EBs (Hofner et al., 2007). Here, an increase of *desmin* mRNA after two and three hours of SPARC addition was detected. A link between two positive regulators of CMG was established. SPARC might increase Desmin protein concentration. Desmin was shown in earlier ChIP experiments to bind to the *nkx2.5* promoter region in ESCs, differentiated CVPCs and pCMCs. It was further identified to positively influence *Nkx2.5* expression in C2C12 myoblasts, suggesting the possibility that Desmin might activate the expression of the cardiac marker as well in ESCs and CVPCs. Therefore, *nkx2.5* gene expression enhancement by SPARC might act via *desmin* upregulation. In addition to cardiac markers, SPARC upregulates the expression of the differentiation inducing factor *goosecoid*. It is expressed in vivo during early development and is involved in organising the embryo, indicating differentiation. Furthermore the mesodermal marker gene *brachyury* was

upregulated as well. A link might be established between the upregulation of *desmin* and *brachyury*, since Desmin was shown to upregulate Brachyury expression during mesoderm formation (Hofner et al., 2007). These results suggest that SPARC promotes myocardial differentiation by upregulating the expression of cardiac marker genes of the first heart field. However, SPARC might as well play an important role earlier in development during differentiation of other cell types by upregulating markers important for organisation of the embryo and for mesoderm formation.

4.5. MAPK and Wnt Signalling in CVPCs

An important step in elucidating mechanisms involved in CMG is the detection of signalling pathways important for proper development. We could determine the contribution of two pathways involved in several developmental mechanisms, the MAPK and the Wnt pathway, to CMG. Inhibitor of MAPK pathway added at the very beginning of differentiation from days 0 – 4.7 delayed the onset of contraction in CBs. Inhibition at later time points did not affect CMG. This is in accordance with previous found results where activated ERK was detected during the first four hours after conjugation of xenopus animal cap (AC) and anterior endoderm (AE) explants. Inhibition of ERK in AC/AE explants during those four hours significantly reduced CMG whereas no effect was seen at later time points (Samuel and Latinkic, 2009). Therefore, CVPCs seem to activate the MAPK pathway at the very beginning of differentiation, which might be a necessary step for proper heart cell formation. The contribution of the second pathway, the Wnt signalling pathway, was tested by inhibition of GSK3. Inhibition of GSK3 resulted in an increase of free β -catenin in CVPCs which translocates to the nucleus. Elevated levels of active β -catenin transcription factors on days 7 – 10 of differentiation delayed the onset of CMG and reduced the number of beating CBs. At earlier stages of development GSK3 inhibition did not alter CBs differentiation. Therefore, Wnt signalling seems to be redundant at early stages of cardiomyocyte development and important later in differentiation. Kwon et al. (2007) reported canonical Wnt signalling as being a regulator for CMG during specific windows of development. Activation of the Wnt/ β -catenin pathway after gastrulation in xenopus laevis embryos prevented cardiac differentiation (Samuel and Latinkic, 2009). Recent studies suggested that active β -catenin is necessary at early stages of CMG whereas it might have an inhibitory effect later in development

(Lin et al., 2007; Riazi et al., 2009). Our results affirm the assumption, that elevated nuclear β -catenin levels in CVPCs reduce CMG at later stages of development.

Since the signalling pathway through which SPARC induces cellular responses is not known yet we tried to detect whether a link between SPARC and one of these two pathways influencing CMG could be established. The addition of recombinant SPARC downregulated the expression a *luciferase* gene under the control of a β -catenin regulated promoter. This negative regulation was reverted by neutralizing α -SPARC antibodies, proving evidence for the selectivity of the downregulation caused by SPARC. Since SPARC might therefore act through the transcriptional mediator β -catenin, the binding of SPARC to integrin- β followed by activation of ILK (Barker et al., 2005; Weaver et al., 2008) could result in a modulation of GSK3 β activity negatively influencing Wnt signalling and downregulating genes regulated by β -catenin controlled promoters. Under the premise that SPARC enhances cardiac differentiation and decreases active β -catenin transcription factor, downregulation of nuclear β -catenin leads to enhanced CMG in CVPCs. These data are in contrast to results showing that β -catenin activity is necessary for specification, migration and expansion of progenitors of the second heart field (Cohen et al., 2007; Kwon et al., 2007). Therefore, different signalling pathways are activated in CVPCs than in progenitor cells of the second heart field, classifying them into a distinct type of progenitor cells and providing another indication for an influence of SPARC on first heart field progenitors. The cardiac transcription factor *Mesp1* was found to inhibit Wnt signalling by binding to the Wnt-inhibitor Dkk-1 (David et al., 2008). SPARC was shown to upregulate *Mesp1* expression, providing another possible explanation for its effect on β -catenin activity.

5. Conclusion

The very limited regenerative potential of the heart makes stem cell therapy a new approach against cardiovascular diseases. The isolation of stable CVPC lines exclusively differentiating to cardiomyocytes, smooth muscle cells and endothelial cells, providing an unlimited, renewable source of identical cells, fulfilled a central challenge in the search for curing methods. CVPCs exclusively differentiate to cells of the adult heart and it is possible to get highly CMC-enriched cultures making them good candidates for cell therapy. The generation of a transgenic CVPC line carrying an *EGFP* gene in one allele of the *nkx2.5* locus provided a good reporter cell line to study alterations of the expression of that early cardiac marker gene upon differentiation and addition of CMG influencing factors. Two proteins, Desmin and SPARC, could be identified as CMG influencing factors. Desmin protein enhances CMG and was found to bind to the *nkx2.5* promoter in ESCs, differentiated CVPCs, and pCMCs. SPARC was detected to be a positive regulator of CMG by decreasing the promoter activity of β -catenin regulated genes in the cells and enhancing the expression of cardiac marker genes of the first heart field. It furthermore upregulates markers important for differentiation and mesoderm formation. MAPK signalling plays an important role immediately after initiation of differentiation whereas Wnt signalling is necessary for proper CMC formation at later stages of development.

Further investigations of CVPCs will lead to a better understanding of early steps in CMG. It remains to be detected whether those cells have the ability to reintegrate into the murine heart. With the help of these cells, an important step towards the ultimate goal, the regeneration of injured or dead tissue by reactivating or reintegrating cardiac stem cells *in vivo*, can be made.

6. Material

6.1. Enzymes

Collagenase	Wothington, USA
DNAse I, RNAse free	Fermentas, Lithuania
Pancreatin	Sigma, USA
Proteinase K	Fluka, CH
RiboLock RNAse Inhibitor	Fermentas, Lithuania
RevertAid™ M-MuLV RT	Fermentas, Lithuania
Restriction Enzymes	New England Biolabs, USA
Taq DNA Polymerase	Fermentas, Lithuania
Trypsin	LifeTechnologies, USA

6.2. Chemicals for Cell Culture

β-Mercaptoethanol	Loba, A
Amphotericin B	Invitrogen, USA
D-Glucose	Acros, B
DMEM powder	Gibco, USA
DMSO (Dimethylsulfoxide)	Sigma, USA
Fetal Bovine Serum (FBS)	HyClone, USA
Fetal Bovine Serum (FBS)	Gibco, USA
Fetal Bovine Serum (FBS)	Sigma, USA
Fetal Horse Serum (FHS)	Gibco, USA
Gelatine	Difco, USA
Glycine	Applichem, D
L-(+)-Glutamin	Acros, B
Mitomycin C	Acros, B
Penicillin G Potassiumsalt	Merck, D
Penicillin/Streptomycin (P/S)	Gibco, USA
Potassium chloride	Sigma, USA
Potassiumhydrogenphosphate	Fluka, CH
Sodiumhydrongencarbonate	LifeTechnologies, USA
Streptomycin	Sigma, USA

6.3. General Chemicals and Material

Acetic acid	Merck, D
Agarose, Biozyme LE	Biozyme, D
APS	Biorad, USA
β-Mercaptoethanol	Loba Feinchemie, AUT
Bromphenolblue	Sigma, USA
BSA	Roth, D
Chloroform	Acros, B
Coomassie Brilliant Blue R250	Merck, D
Dabco	Sigma, USA
dNTPs	Fermentas, Lithuania
Dimethylsulfoxid (DMSO)	Acros, B
Dithiothreitol (DTT)	Acros, B
EDTA	Acros, B
EGTA	Sigma, USA
Ethanol	Merck, D
Ethidiumbromide	Fluka, CH
Formaldehyde	Merck, D
Glycerine	Merck, D
Glycerophosphate	Merck, D
Glycin	Sigma, USA
HeBS	Promega, USA
HEPES	Merck, D
Hydrochloric acid (HCl)	Acros, B
Isoamylalcohol	Merck, D
Leupeptin	Roche, D
Lithiumchloride	Merck, D
Lipofectamine 2000	Invitrogen, USA
Loading Dye	Fermentas, Lithuania
Methanol	Merck, D
Magnesiumchloride	Fermentas, Lithuania
Magnesiumsulfate	Fluka, CH
Milkpowder	Fixmilch instant, AUT
Mowiol 2-88	Hoechst, D
Nalgene Filter	Nalagene Labware, USA
NaDoc 10%	Sigma, USA

NP-40 10%	Sigma, USA
PCR-buffer without MgCl₂	Fermentas, Lithuania
PEG₆₀₀₀ and PEG₈₀₀₀	Calbiochem, D
Pepstatin	Roche, D
Phenylmethanesulfonylfluoride (PMSF)	Fluka, CH
Polyacrylamide	Merck, D
Ponceau-S	Sigma, USA
Proteinase Inhibitor	Roche, D
Protein A SepharoseTM CL-4B	GE Healthcare, S
Pyrophosphate	Fluka, CH
Reverse Transcriptase Buffer	Invitrogen, USA
SDS (Sodium Dodecylsulfate)	BioRad, USA
Sodiumazid	Acros, B
Sodiumbicarbonate	Sigma, USA
Sodiumchloride	Salinen Austria, AUT
Sodiumfluorid	Donauchemie, AUT
Sodiumhydrogencarbonate	LifeTechnologies, USA
Sodiumhydrogenphosphate	Roth, D
Sodiumhydroxide	Merck, D
Sodiumthiosulfate	Merck, D
TEMED	Sigma, USA
Trichloroacetic acid	Merck, D
Tris Base	LifeTechnologies, USA
Triton X100	Sigma, USA
Tween-20	Sigma, USA

6.4. Kits

EndoFree Plasmid Maxi Kit	Qiagen, D
RNeasy Mini Kit	Qiagen, D
Dual Luciferase Reporter System	Promega, USA

6.5. Inhibitors and Recombinant Proteins

Chir99021	Axon, NL
PD98059	Cayman, USA
SPARC	Sigma, USA

6.6. Plasmids

6.6.1. Renilla Plasmid

This pRL-TK vector is an internal control plasmid with a *Renilla luciferase* reporter gene, that is constantly expressed in the cells. *Promega* #E2241.

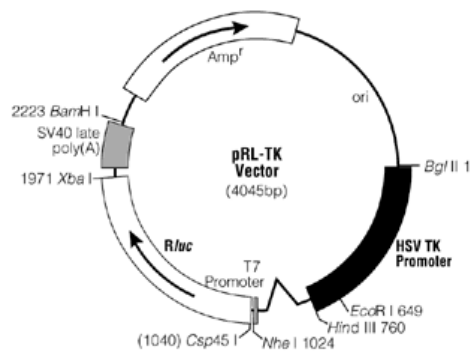


Fig. 43 *Promega Renilla Plasmid*

6.6.2. pGL3-Basic (Control) Plasmid

The pGL3-Basic vector is a control plasmid containing a *luciferase* gene without a promoter. *Promega* #E1751.

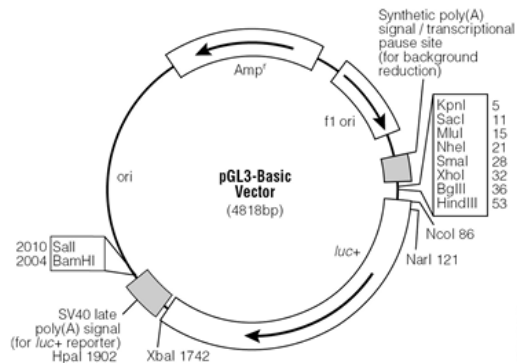


Fig. 44 *Promega pGL3-Basic Plasmid*

6.6.3. pUC Plasmid

This pUC18 vector is a plasmid without any reporter gene, it is added to keep the DNA amount in the different samples constant.

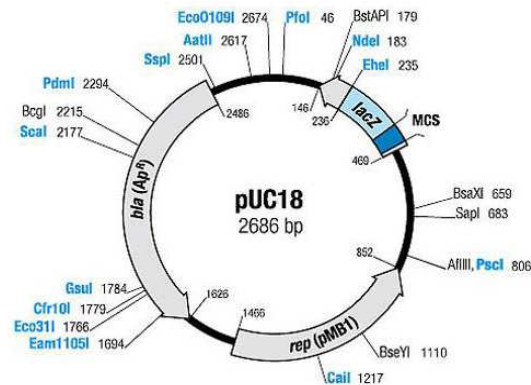


Fig. 45 Promega pUC18 Plasmid

6.6.4. Nkx – luc Plasmid

The Nkx – luc plasmid is a Promega pGL3b plasmid (#E1751) with an insert of the *nkx2.5* promoter region in front of a *luciferase* reporter gene and was obtained by Dr. Katherine Yutzey (USA).

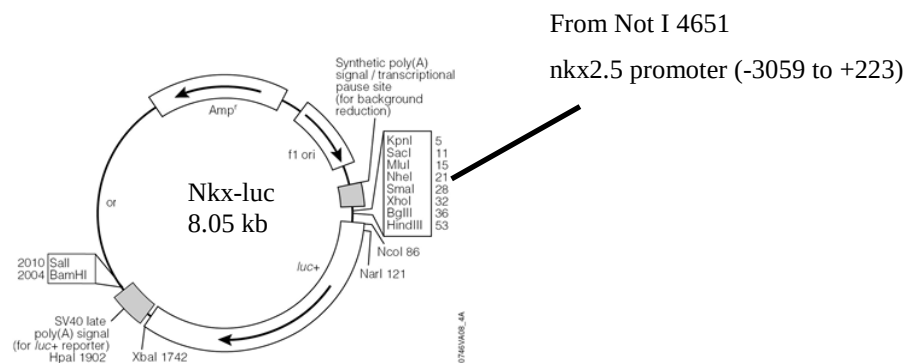


Fig. 46 Nkx – luc plasmid: pGL3-Basic vector with *nkx2.5* insert

6.6.5. Nkx – enhancer – luc Plasmid

The Nkx – enhancer - luc plasmid is made by Sonja Gawlas, the “minimal cardiac enhancer” sequence of the *nkx2.5* promoter (published by Ching-Ling, L. et al., 1999) is cloned into the Nkx – luc plasmid.

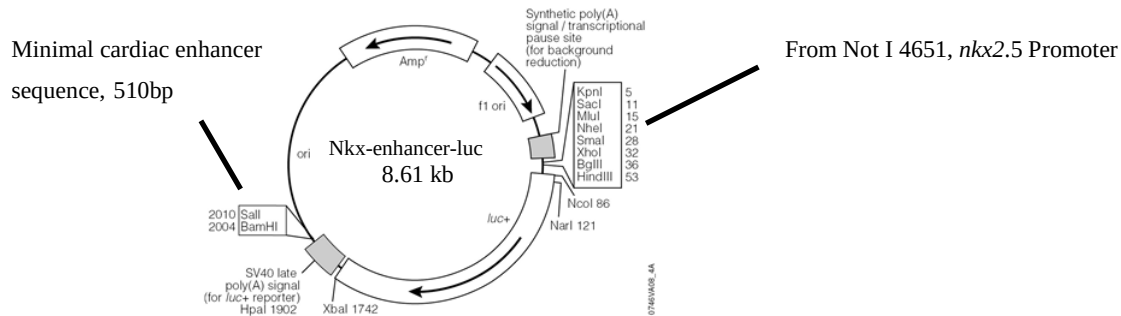


Fig. 47 Nkx – enhancer - luc Plasmid

6.6.6. Des – siRNA Plasmid (Clone 4)

This Desmin – siRNA plasmid contains an antisense sequence for the mouse *desmin* mRNA. The original vector in which the following sequence is clones is the Promega pGeneClip hMGFP Vector (Promega #C8790).

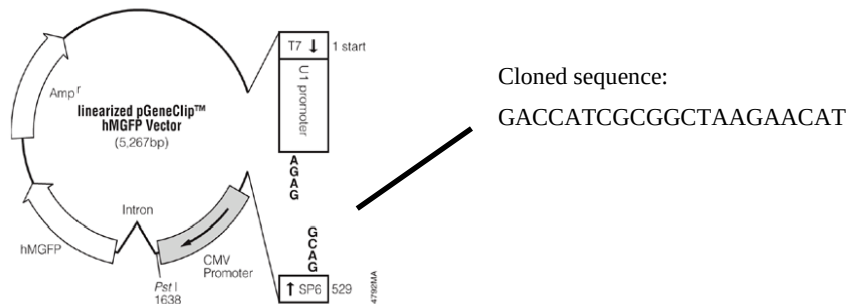


Fig. 48 Desmin – siRNA Plasmid

6.6.7. Knock Down Negative Plasmid

This plasmid is a control plasmid having the same components of the Des – siRNA plasmid except that the insert is a sequence that does not have any similarities with the *desmin* mRNA.

Cloned sequence: GGAATCTCATTCGATGCATAC

6.6.8. Desmin Plasmid

This Plasmid contains the *desmin* cDNA and is used for ectopic expression of Desmin in cells. Stratagene #212209.

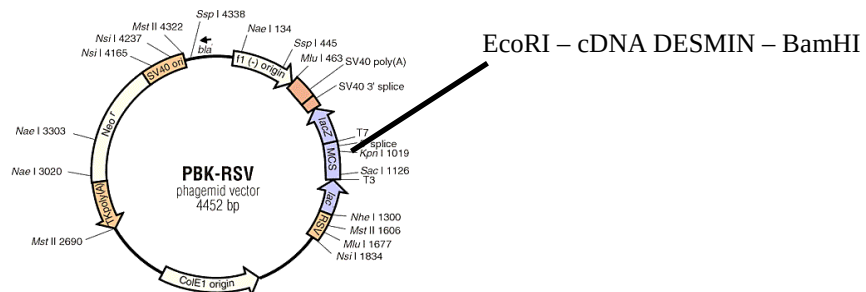
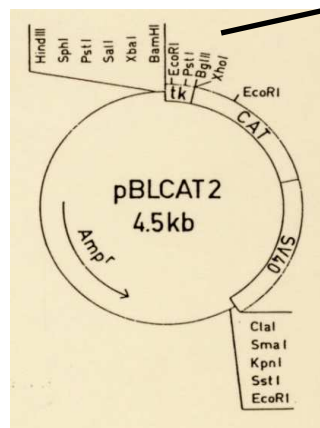


Fig. 49 Stratagene PBK-RSV vector with the Desmin cDNA insert

6.6.9. TOPFlash Plasmid

This plasmid carries a luciferase gene under the control of a TCF/LEF binding sites containing promoter, which is activated by binding of β -catenin (obtained by Hartmund Beug, IMP, Vienna).



TCF binding sites were cloned into the blunt HindIII site, the CAT gene was replaced by the Luciferase gene.

Fig. 50 TOPflash Plasmid: pBLCAT2 plasmid with TCF/LEF binding sites and Luciferase reporter gene.

6.6.10. pmaxEGFP

This pmaxGFP plasmid encodes GFP under the control of a strong CMV promoter (Cytomegalovirus promoter). It was provided by Artaker Matthias (Medical University of Vienna, Amaxa #VSC-1001).

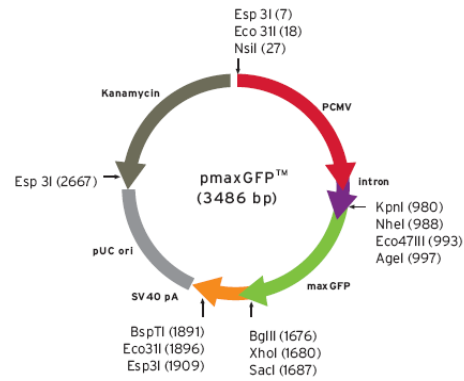


Fig. 51 Amaxa pmaxGFP plasmid

6.6.11. pCsx-EGFP-PP-DT

This vector was obtained from Hidaka (Hidaka et al., 2003). It contains regions homologue to the *nkx2.5* gene interrupted by an EGFP marker and a puromycin resistance selection gene. It can be used for homologue recombination of the *nkx2.5* gene.

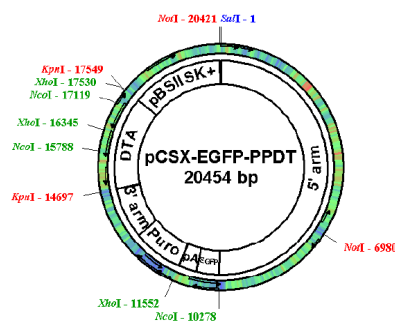


Fig. 52 pCsx-EGFP-PP-DT plasmid

6.7. Primer

Primer Name		Sequence 5' - 3'	Annealing	Cycles	Product (cDNA)
ANF	fwd	CGG TGT CCA ACA CAG ATC TG	52,5°C	36x	187 bp
	rev	TCT CTC AGA GGT GGG TTG AC			
Brachyury	fwd	ATC AAG GAA GGC TTT AGC AAA TGG G	60°C	36x	159 bp
	rev	GAA CCT CGG ATT CAC ATC GTG AGA			
Csx	fwd	CCA TTT GTC ACG TCC TGC ACG ACG	57°C	45X	1924 bp
	rev	GCT TGC ACT TGT AGC GAC GGT TC			
Desmin	fwd	TGA CAA CCT GAT AGA CGA	50°C	37x	390 bp
	rev	TTC TTA TTG GCT GCC TGA			
GAPDH	fwd	CGT CTT CAC CAC CAT GGA GA	55°C	29x	300 bp
	rev	CGG CCA TCA CGC CAC AGT TT			
GATA4	fwd	GCC TGT ATG TAA TGC CTG CG	53°C	31x	500 bp
	rev	CCG AGC AGG AAT TTG AAG AGG			
Goosecoid	fwd	GCA CCA TCT TCA CCG ATG AG	52.7°C	35x	190 bp
	rev	AGG AGG ATC GCT TCT GTC GT			
Isl1	fwd	CGG TGC AAG GAC AAG AAA	49°C	38x	346 bp
	rev	CAA TAG GAC TGG CTA CCA			
MCE _{cis} Element	fwd	CGA CGAG GAA ACT CGG AGC TA	54°C	38x	124 bp
	rev	CTC TGC TGT GTG GCC TTG TA			
Mef2C	fwd	GGC CAT GGT ACA CCG AGT ACA ACG AGC	66°C	34x	395 bp
	rev	GGG GAT CCC TGT GTT ACC TGC ACT TGG			
Mesp1	fwd	AGA AAC AGC ATC CCA GGA AA	52°C	32x	346 bp
	rev	GTG CCT GCT TCA TCT TTA			
MHC α	fwd	GGA AGA GTG AGC GGC GCA TCA AGG	57.7°C	32x	302 bp + 473 bp
	rev	CTG CTG GAG AGG TTA TTC CTC G			
MLC1v	fwd	TCA GGA AGC CCA GGG CAG GC	56°C	38x	86 bp
	rev	GGA GTC CGA ACC ACT CCT TC			
nkx2.5	fwd	CAC CCA CGC CTT TCT CAG TC	57°C	40x	513 bp
	rev	TGG ACG TGA GCT TCA GCA			
nkx2.5 Promoter1	fwd	CTC TGT TTG CTT TCT CGC CA	52.5°C	38x	246 bp
	rev	ATT GGA GAC AGG CAG CGT TA			
Oct 3/4	fwd	GGCAAATCACTGAAGGCTCA	62°C	30x	801 bp
	rev	CACACCAGTCCACAACATAG			
pCSX	fwd	GAT GTG GAA TGT GTG CGA GG	55°C	45x	1700 bp
	rev	TGG ACG TGA GCT TCA GCA C			
T Promoter	fwd	ATA AAT ACA GCC GAG GTG GCC T	55°C	30x	176 bp
	rev	CGA CGC TTT CCT TAC AGG AAG			

6.8. Antibodies

Antibody	Company
cardiac TroponinT	Thermo Scientific #MS-295
Connexin43	Sigma #C6219
Desmin	SigmaAldrich #D1033
Desmin	Abcam #ab8592-500
GAPDH	Millipore, #P04406
GFP	Cell Signalling #2956
SPARC	Santa Cruz, #sc-25574
α-mouse FITC	Dianova #715-095-151
α-mouse-HRP	Pierce, #31430
α-rabbit-HRP	Dianova, #111-035-003
α-rabbit-TR	Dianova #711-075-152

6.9. Cell Lines

6.9.1. Fibroblasts

- SNL 76/7 Fibroblasts

The SNL76/7 cell line was established by Allan Bradley. It derives from the murine STO fibroblast cell line but additionally has a *LIF* gene and a *neomycin*-resistance gene inserted into the genome (McMahon and Bradley, 1990).

- C2C12 Fibroblasts

The C2C12 cell line was produced by Gelen Blau. C2C12 cells are myoblasts committed to produce myotubes upon differentiation.

6.9.2. Embryonic Stem Cells

- AB2.2

The AB2.2 cell line is a murine wild type embryonic stem cell line isolated by Allan Bradley from the mouse strain 129Sv (Soriano et al., 1991).

- DC6

The DC6 cell line is a murine embryonic stem cell line that overexpresses Desmin ($des^{+/+} des^{ect}$). It was produced by Sonja Puz (Puz, 1999).

- Des^{-/-}

The Des^{-/-} cell line is a *desmin* knock out murine embryonic stem cell line produced by Georg Weitzer (Milner et al., 1996).

- Des^{Δ1-48/Δ1-48}

The Des^{Δ1-48/Δ1-48} cell line is a murine embryonic stem cell line with a mutated *desmin* gene. The first 48 amino acids of the N-terminus are deleted (Hollrigel et al., 2002).

- MS15

The MS15 cells line was established by Martina Stary. It is an embryonic stem cell line carrying an *EGFP* reporter gene and a puromycin resistance gene in one allele of the *nkx2.5* locus. The reporter and resistance genes are expressed under the control of the *nkx2.5* promoter.

6.9.3. Cardiovascular Progenitor Cells

Twelve different cardiovascular progenitor cell lines were isolated by Georg Weitzer and Marc Widner from newborn mice carrying a neomycin resistance gene in one allele of the *hdac1* locus. The hearts of nine days old mice were cut out, digested with

a collagenase/pancreatin solution, co-cultured with embryonic stem cells and feeder cells for 10 passages following a 3T3 protocol and selection with G418 resulted in the death of murine wild type embryonic stem cells and the isolation of twelve clonal cardiovascular progenitor cell lines. Here, the cell lines A5 and H3 were used for experiments.

6.10. Bacteria Strains

XL-1 blue

Stratagene, USA

6.11. Material for bacteria culture

6.11.1. LB Medium

- 10 g NaCl
- 10 g Tryptone
- 5 g yeast extract
- 1 l ddH₂O

The three reagents are mixed until the chemicals are dissolved. The LB medium is portioned into 500 ml bottles and autoclaved at 100 – 120°C at 1.4 bar for 20 to 30 minutes.

6.11.2. LB – plates

7.5 g agarose (1.5%) are added to a 500 ml bottle of LB – medium. Then, the bottle is autoclaved and melted for 30 minutes at 180 Watt in the microwave. The liquid is cooled down, antibiotics are added, and the medium is poured into petri dishes. The plates are kept at room temperature to solidify and then stored at 4°C.

7. Methods

7.1. Cell Culture

7.1.1. General Stem Cell Culture Workings

Glass material for stem cell culture should not encounter with washing-up liquid, because already a small amount of it causes death of embryonic stem cells. Therefore it is cleaned in a different way than normal laboratory glass ware.

7.1.1.1. Washing of Glass Bottles for Media and Solutions

Empty bottles are filled with tap water and a few drops Hypochlorid. They are kept at room temperature for about an hour. Then, the bottle is rinsed out with tap water until no Hypochlorid is left and it is then washed with milliQ-H₂O. It is filled with milliQ-H₂O, kept at room temperature for 24 hours, dried and finally autoclaved at 120°C and 1 bar.

7.1.1.2. Washing of Pipettes

After usage, the glass pipettes are stored in Hypochlorid containers. When they are full, the pipettes are transferred to another container and are rinsed with tap water for at least 4 hours. The pipettes are then stored in milliQ-H₂O over night and afterwards dried in a drying oven at 60°C. A piece of cotton is plugged into each pipet, they are put into the pipet boxes and are baked at 180°C for 8 hours.

7.1.2. Buffer and Media for Cell Culture

10x PBS (Phosphate Buffered Saline) stock solution

- 1.37 M NaCl
- 14.7 mM KCl
- 78.1 mM Na₂HPO₄ x 7H₂O
- 26.8 mM KH₂PO₄
- Saturated Na₂HPO₄ is added to a final pH of 7.2.
- The solution is sterile filtered (Nalagene Filter, 0.22 µm pore width).

100x GPS (Glutamine-Penicillin-Streptomycin)

- 4.25 g NaCl
- 1.5 g Penicillin
- 2.5 g Streptomycin

- 14.6 g L-(+)-Glutamine
- MilliQ-H₂O is added to a final volume of 500 ml.
- The solution is stored at -20°C and then kept at 4°C after thawing.

100x β-Mercaptoethanol (10⁻²M)

- 200 ml 1x PBS
- 144 μl β-Mercaptoethanol (14 M)
- The solution is sterile filtered.
- The solution is stored at -20°C and then kept at 4°C after thawing.

Trypsin

- 3.5 g NaCl
- 0.5 g D-Glucose
- 0.09 g Na₂HPO₄ x 7H₂O
- 0.185 g KCl
- 0.12 g KH₂PO₄
- 0.2 g EDTA
- 1.25 g Trypsin (Gibco)
- 1.5 g Tris Base
- MilliQ-H₂O is added to a final volume of 500 ml.
- Concentrated HCl is added to a final pH of 7.6.
- The solution is stored at -20°C and then kept at 4°C after thawing.

1% Gelatine Stock Solution

10 g Gelatine (Difco) are soluted in 1 l milliQ-H₂O. The solution is sterile filtered.

DMEM (Dulbecco's Modified Eagle's Medium)

A 5 l Erlenmeyer Flask is filled with 4.5 l milliQ-H₂O. Half a DMEM can (Gibco, +4500 mg/l Glucose, -NaHCO₃, -Pyruvate #52100-039) is added and dissolved. 18.5 g NaHCO₃ are added. Concentrated HCl added to a final pH of 7.4. MilliQ-H₂O is added to a final volume of 5 l and the medium is sterile filtered into cell culture falsks.

Freezing Medium

- 60% DMEM
- 20% Fetal Bovine Serum (company depends on cell line, use the one they are normally cultured with)
- 20% DMSO (Dimethylsulfoxid)

PBS/Collagenase/Pancreatin Solution

- 10 ml 1x PBS
- 5 mg Collagenase (Worthington)
- 6 mg Pancreatin (Gibco)

7.1.3. Growing Media

Medium for Fibroblasts (M10Gi)

- 89% DMEM
- 10% Fetal Bovine Serum (Gibco)
- 1% GPS (Glutamine-Penicillin-Streptomycin)

Medium for embryonic stem cells and cardiovascular progenitor cells (M15Hi)

- 83 % DMEM
- 15% Fetal Bovine Serum (HyClone)
- 1% GPS (Glutamine-Penicillin-Streptomycin)
- 1% β -Mercaptoethanol

Medium for Embryoid and Cardiac Bodies (M15Si)

- 83% DMEM
- 15% Fetal Bovine Serum (Sigma)
- 1% GPS (Glutamine-Penicillin-Streptomycin)
- 1% β -Mercaptoethanol

Medium for primary Cardiomyocytes (pCMC)

- 59% DMEM
- 20% Fetal Bovine Serum (Gibco)

- 20% Horse Serum (Gibco)
- 1% GPS

7.1.4. Gelation of Tissue Plates

0.1% Gelatin solution is pipetted on the tissue plates so that the bottom is covered with the solution. It is kept at room temperature for two hours and then the solution is aspirated.

7.1.5. Fibroblasts

7.1.5.1. Culture of SNL76/7 Fibroblasts

Embryonic stem cells grow on so called feeder cells, a layer of fibroblast cells which produce LIF and therefore keep the embryonic stem cells in an undifferentiated state. The SNL76/7 cell line derives from the STO fibroblast cell line but additionally has a LIF gene inserted into the genome. The cells are cultured on 0.1% gelled 10 cm cell culture plates at 37°C and 5% CO₂. They are fed with M10Gi as soon as the colour of the medium changes and when they reach confluence, they are split in an up to 1:8 ratio.

7.1.5.2. Culture of C2C12 Fibroblasts

The fibroblasts are incubated at 37°C and 5% CO₂ in 10 cm tissue plates and are split every second or third day in a 1:8 ratio.

7.1.5.3. Thawing of Fibroblasts

The cells are stored in 2 ml Kryotubes in liquid nitrogen at -180°C.

The tubes are taken out of the liquid nitrogen. The cells are thawed in the 37°C water bath and transferred to a 15 ml falcon. M10Gi medium (6 ml medium/1 ml cell suspension) is added drop by drop, after each drop the falcon is gently shaken. The cells are then centrifuged for 7 minutes, 1000 rpm at room temperature. The medium is aspirated and the cells are resuspended in 4 ml fresh M10Gi. The cells are transferred to a (gelled) 10 cm culture plate, the falcon is rewashed with 4 ml M10Gi, which are also transferred to the culture plate. The cells are dispersed by moving the plate in a shape of an eight and are then incubated at 37°C.

7.1.5.4. Splitting of Fibroblasts

The medium in the plate is aspirated and the cells are washed with 1x PBS. The PBS is aspirated and 1 ml Trypsin is pipetted to the cells. The plate is incubated at 37°C for 5 minutes. The cells are resuspended in 7 ml fresh M10Gi medium and 1 ml of this suspension is transferred to a new (gelled) 10 cm plate (1:8 ratio). 8 ml fresh M10Gi are added and the plate is incubated at 37°C.

7.1.5.5. Freezing of Fibroblasts

The medium is aspirated and the cells are washed with 1x PBS. 700 µl Trypsin are added to the cells and the plate is incubated for 10 minutes at 37°C. The cells are resuspended in 2 ml M10Gi and transferred to a 15 ml falcon. 2.7 ml freezing medium are added drop by drop, after each drop the falcon is gently shaken. The cell suspension is apportioned to three Kryotubes, the tubes are put into a Styrofoam box which is placed into the -80°C freezer for at least 24 hours and then the tubes are stored in liquid nitrogen.

7.1.5.6. Production of Feeder Cells

One 10 cm culture plate with confluent SNL76/7 cells is enough for about two 24 well plates. In the course of producing feeder cells, the SNL76/7 cells have to get mitotically inactivated so that they do not divide anymore but still produce all factors needed for self-renewal.

The medium of the 10 cm culture plate is aspirated except 4 ml. 80 µl Mitomycin C are added drop by drop and the plate is incubated at 37°C for 3 – 4 hours. The medium is aspirated and the cells are washed twice with 1x PBS. 1 ml Trypsin is pipetted to the cells and they are incubated at 37°C for 10 minutes. The cells are resuspended in 5 ml M10Gi and transferred to a sterile 15 ml falcon. The plate is rewashed with 10 ml M10Gi, which are also pipetted to the falcon. The falcon is centrifuged for 7 minutes, 1000 rpm at room temperature and the supernatant is aspirated. The pellet is resuspended in 10 ml M10Gi and the cell count is determined. The cells are diluted to a concentration of 3.5×10^5 cells/ml and seeded to gelled 24 well plates, 0.5 ml per well. The plates are incubated at 37°C and can be used on the next day when the cells adhere to the plate.

7.1.6. Embryonic Stem Cells (ESCs) and Cardiovascular Progenitor Cells (CVPCs)

7.1.6.1. Culture of Embryonic Stem Cells and Cardiovascular Progenitor Cells

The cells are incubated at 37°C and 5% CO₂ in 24 well plates, are fed every 24 hours with M15Hi and are split at approximately 90% confluence in a 1:3 ratio.

7.1.6.2. Thawing of Embryonic Stem Cells and Cardiovascular Progenitor Cells

The cells are stored in 2 ml Kryotubes in liquid nitrogen at -180°C.

Two hours before thawing, the feeder cells on which the cells will grow are fed with 2 ml M15Hi. The tubes are taken out of the liquid nitrogen. The cells are thawed in the 37°C water bath and transferred to a 15 ml falcon. M10Gi medium (approximately 6 ml medium/1 ml cell suspension) is added drop by drop, after each drop the falcon is gently shaken. The cells are then centrifuged for 7 minutes, 1000 rpm at room temperature. The medium is aspirated and the cells are resuspended in 1 ml medium of the prefed feeder cells. They are then transferred to the feeder well. The cells are incubated at 37°C.

7.1.6.3. Splitting of Embryonic Stem Cells and Cardiovascular Progenitor Cells

Two hours before splitting, the feeders on which the cells will grow are fed with 2 ml M15Hi and the embryonic stem cells that will be split are fed with 1 ml M15Hi. The medium in the well is aspirated and the cells are washed with 1x PBS. The PBS is aspirated and 200 µl Trypsin are pipetted to the cells. The plate is incubated at 37°C for 15 minutes. The cells are resuspended in 1 ml medium of the prefed feeder cells and 400 µl (if splitting ratio is 1:3) of this suspension are transferred to the feeder well. The plate is incubated at 37°C.

7.1.6.4. Freezing of Embryonic Stem Cells and Cardiovascular Progenitor Cells

Two hours before freezing, the embryonic stem cells are fed with 1 ml M15Hi. The medium is aspirated and the cells are washed with 1x PBS. 200 µl Trypsin are added to the cells and the plate is incubated for 15 minutes at 37°C. The cells are resuspended in 800 µl M15Hi. 1 ml freezing medium is added drop by drop directly to the well, after each drop the plate is gently shaken. The cell suspension is apportioned to two Kryotubes, the tubes are put into a Styrofoam box which is placed into the -80°C freezer for at least 24 hours and then the tubes are put into the liquid nitrogen.

7.1.7. Embryoid Bodies and Cardiac Bodies

7.1.7.1. Production of Embryoid Bodies and Cardiac Bodies

One day before embryoid bodies (EBs) and cardiac bodies (CBs) are made, the embryonic stem cells and cardiovascular progenitor cells that will be used for the EBs and CBs are split in a 1:2 ratio so that the cells are in an optimal condition.

Two hours before starting, the cells are fed with 1 ml M15Hi. The medium is then aspirated and the cells are washed with 1x PBS. 200 µl Trypsin are added to the cells and the plate is incubated for 15 – 20 minutes at 37°C. The cells are resuspended in 800 µl M15Si and pipetted to a gelled 6 well plate, and the original well is rewashed with 1 ml medium. The plate is incubated for 1 hour at 37°C so that the fibroblasts adhere to the gelled plate but the embryonic stem cells do not. The supernatant is pipetted to a 50 ml falcon and is expanded to 10 ml with M15Si. The cell count is determined. The cells are diluted to a concentration of 4×10^4 cells/ml (for EBs) and 4.5×10^4 cells/ml (for CBs) and measured again until the correct cell count is detected in the solution. Sterile bacteria plates are filled with autoclaved milliQ-H₂O. 20 µl drops are pipetted to the lid of the bacteria plates (approximately 100 drops per lid). The lid is put on the plate containing the water and the plate is incubated at 37°C. The day, on which the embryoid and cardiac bodies are made is day 0. On day 4.5 (EBs) and day 4.7 (CBs) the drops in the lid are rinsed with about 8 ml M15Si medium into a gelled 10 cm cell culture plate and the embryoid and cardiac bodies are dispersed by rocking to two different directions. The plate is incubated at 37°C.

7.1.7.2. Culture of Embryoid Bodies and Cardiac Bodies

Embryoid and cardiac bodies are fed every third day, however a part of the old medium is kept and fresh medium is added to the old one.

On day 7 and day 10, 3 ml old medium are kept in the plate and 8 ml fresh M15Si are added.

On day 13, day 16, day 19 and day 22, 4 ml old medium are kept in the plate and 10 ml fresh M15Si are added.

From day 25, 5 ml old medium are kept in the plate and 12 ml fresh M15Si are added.

7.1.7.3. Trypsinisation of Embryoid and Cardiac Bodies

The plates are washed twice with 1x PBS. 2 ml of Trypsin/Collagenase/Pancreatin (for 20 ml: 20 ml Trypsin, 10 mg Collagenase, 12 mg Pancreatin) are added to each plate and they are incubated at 37°C for 20 minutes. The cells are resuspended in 8 ml M15Si and pooled into a 50 ml falcon. The falcon is centrifuged for 10 seconds, the supernatant is collected, the pellet is resuspended in 2 ml Trypsin/Collagenase/Pancreatin and incubated at 37°C for 10 minutes. The cells are resuspended in 8 ml M15Si, transferred to a 50 ml falcon. The falcon is centrifuged for 10 seconds, the supernatant is pooled, the pellet is resuspended in 1 ml Trypsin/Collagenase/Pancreatin and incubated at 37°C for 7 minutes. The cells are resuspended in 5 ml M15Si and pooled with the previous supernatants. After centrifugation for 5 minutes at 1000 rpm, the pellet can be resuspended in the desired solution.

7.2. Isolation of primary Cardiomyocytes

The hearts of newborn mice are cut out and placed into a tube containing 1x PBS (up to three hearts per tube). The PBS is removed and the hearts are cut into small pieces. 1 ml 1x PBS is added and the tube is centrifuged for 2 minutes at 1200 rpm. The PBS is aspirated and 1 ml PBS/Collagenase/Pancreatin solution is added. The solution is incubated at 37°C for 7 minutes. It is then centrifuged for 2 minutes at 1200 rpm. The supernatant is removed. 1 ml PBS/Collagenase/Pancreatin solution is added and incubated at 37°C for 3 minutes. The mixture is centrifuged for 2 minutes at 1200 rpm and the supernatant is removed. The cells are resuspended in pCMC medium and transferred to a 1% gelatine coated 6 cm plate. The cells are incubated at 37°C for 60 minutes. The supernatant is transferred to a new 1% gelatine coated plate which is incubated at 37°C.

7.3. DNA Isolation

DNA is isolated of one confluent 24 well.

The cells are trypsinised, resuspended in 1x PBS and transferred into a Kloesch tube. The tube is centrifuged for 10 minutes at 1200 rpm and 4°C. The supernatant is aspirated, the pellet resuspended in 1 ml 1x PBS and centrifuged for 10 minutes at

1200 rpm and 4°C. The supernatant is aspirated, the pellet is resuspended in 800 µl PK-Buffer and incubated at 50°C and 700 rpm over night. On the next day, the solution is partitioned into two tubes and 400 µl Phenol are added to each tube. The solution is mixed by inverting several times and then centrifuged at 14000 rpm for 10 minutes. The upper phase is transferred into a new Kloesch tube and again 400 µl Phenol are added, inverted and centrifuged for 10 minutes at 14000 rpm. The upper phase is again transferred into a new tube and 580 µl Chloroform + 20 µl Isoamylalcohol are added, the tubes are inverted and then centrifuged for 10 minutes at 14000 rpm. The upper phase is transferred into a new tube. 1 ml 96% EtOH and 50 µl 3M NaOAc are added and the tubes are kept at -80°C for 1.5 hours. They are then centrifuged at 14000 rpm for 15 minutes at 4°C to pellet the precipitated DNA. The supernatant is aspirated and the pellet is washed with 500 µl 70% EtOH. The tube is centrifuged for 10 minutes at 14000 rpm and 4°C. The supernatant is aspirated and the pellet is dried. 40 µl milliQ-H₂O are added and the tubes are incubated at 37°C and 700 rpm for 30 minutes. The content of the two tubes is pooled and the DNA is stored at -20°C.

PK Buffer:

- 0.5 ml 1 M Tris pH 8
- 20 µl 0.5 M EDTA pH 8
- 100 µl 10% SDS
- 200 µl Proteinase K 10 mg/ml
- 9.18 ml dH₂O

7.4. PCR

Master Mix

- 38.75 µl milliH₂O
- 5 µl 10x Taq Buffer (Fermentas #EP0402)
- 3 µl 25 mM MgCl₂ (Fermentas #EP0402)
- 1 µl 10 mM dNTPs (Fermentas #R0192)
- 0.5 µl Primer forward (Vienna BioTec)
- 0.5 µl Primer reverse (Vienna BioTec)
- 0.25 µl Taq Polymerase (Fermentas #E0402)

The master mix is pipetted into PCR tubes (Kloesch) and 1-2 μ l of sample DNA are added. The tubes are placed into a Biometra T-Personal PCR machine.

PCR Conditions

1. 94°C 60 seconds
2. 94°C 45 seconds
3. Annealing 45 seconds (annealing temperature depends on primers)
4. 72°C 60 seconds → back to step 2 for 28-39 cycles
5. 72°C 240 seconds
6. 4°C

7.5. mRNA Isolation and Reverse Transcription

To isolate mRNA from cells, the Quiagen RNeasy Mini Kit is used.

The medium of the cells of interest is aspirated and the cells are washed with 1x PBS. The PBS is aspirated and 1 ml 1x PBS is added. The cells are scraped off, resuspended and transferred to 15 ml falcon tubes. They are centrifuged for 5 minutes, 1000 rpm at room temperature and the pellet is resuspended in 1 ml 1x PBS. The mixture is transferred into a tube, centrifuged for 5 minutes at 14000 rpm and 4°C and the supernatant is aspirated. β -Mercaptoethanol is added to RTL buffer (1:100). 606 μ l of this solution are added to each tube, the pellet is resuspended and pipetted into a Shredder Column. It is centrifuged for 2 minutes, 13000 rpm at room temperature. The column is discarded and 606 μ l of 70% EtOH (RNase free) are pipetted to the solution, mixed and 600 μ l of this mixture are pipetted to a RNeasy column. The column is centrifuged for 15 seconds at full speed. The flow through is discarded and the other 600 μ l of the mixture are pipetted to the RNeasy column. The column is again centrifuged for 15 seconds at full speed and the flow through is discarded. 700 μ l RW1 are added followed by 15 seconds full speed centrifugation and flow through removal. This step is repeated with 500 μ l RPE. Then 500 μ l RPE are added, the column is centrifuged for 1 minute at 13000 rpm, and the column is put into a new 1.5 ml tube. 30 μ l RNase-free water are added and the tubes are centrifuged for 1 minute, 13000 rpm at room temperature. The column is discarded and the DNA is digested by adding 3.75 μ l DNase buffer (Fermentas) and 3.75 μ l DNase I (Fermentas). After incubation for 30 minutes at 37°C, 3.75 μ l EDTA (Fermentas) are added to stop the DNA digestion and the tubes are incubated at 65°C for 30 minutes. The samples are kept on

ice and a PCR with GAPDH Primers is made with the samples as templates to detect whether there is still some genomic DNA left in the samples. During running the PCR the samples are frozen at -80°C. The PCR products are loaded on a 1.5% agarose gel which is run at 130 Volt for about 1 hour. The gel is then put into an Ethidiumbromid bath for about 20 minutes and afterwards the DNA bands are detected under an UV light.

If there is no band visible, the protocol can be continued and the RNA can be reversely transcribed.

1 µl oligo-d(T) is added to each sample and they are incubated at 70°C for 10 minutes. The samples are then kept on ice for 3 minutes and centrifuged for 30 seconds, 13000 rpm at room temperature. 18.5 µl RT (reverse transcription) Mix are added to each sample. The samples are incubated for 2 minutes at 42°C and 1 µl Reverse Transcriptase (Fermentas) is added to each sample. They are incubated at 42°C for 50 minutes. They are further incubated at 70°C for 15 minutes. Then, the samples are kept on ice for 5 minutes and centrifuged for 2 minutes, 13000 rpm. The samples can be stored at -20°C.

The first thing that has to be tested is, if there is the same amount of cDNA in each sample. Therefore a PCR is made with Primers for the house-keeping gene GAPDH. If all GAPDH bands on the gel have the same intensity, PCRs with primers for the sequence of interest can be made.

1x RT Mix

- 10 µl 5x FS Buffer
- 0.5 µl 0.1 M DTT
- 1.5 µl RNase Inhibitor
- 2 µl 10 mM dNTPs

7.6. Chromatin Immunopäzipitation (ChIP)

With this method, it is possible to detect protein-DNA interaction.

Here, the interaction of the Protein Desmin with the *nkx2.5* promoter region should have been detected.

300 μ l Formaldehyd/10 ml medium are added to the 10 cm plates on which cells of interest grow (to X-link proteins and DNA). The plate is incubated for 40 minutes at room temperature. 500 μ l Glycine/10 ml medium are added and the plate is shaken for 5 minutes (to stop X-linking). The supernatant is aspirated and the cells are washed with ice cold 1x PBS. 1 ml ice cold 1x PBS is added and the cells are scraped off and transferred to a falcon tube. They are centrifuged for 5 minutes at 1200 rpm and 4°C. The supernatant is aspirated and the pellet is washed with 5 ml WASH I. Therefore, the pellet is resuspended in 1 ml WASH I, then 4 ml WASH I are added, the falcon is shaken and kept on ice for 10 minutes. This is followed by centrifugation for 5 minutes, 1200 rpm, 4°C. The washing step is repeated with WASH II. The pellet is resuspended in 1 ml Lysis Buffer. The solution is transferred to a 2 ml Kloesch tube and stored at 4°C over night.

The cells are sonicated so that they burst and that the DNA is fractured into pieces of 600 – 800 bp length. The Kloesch tube is kept on ice and the sonication is repeated 16 times with 90% Dot Cycles and 45% Output for 15 seconds. After each sonication the tubes are kept on ice for 35 seconds. The cell fragments are centrifuged for 10 minutes, full speed, 4°C. The supernatant is collected and the OD at 280 nm is measured with a NanoDrop machine. Therefore 2 μ l of sample are soluted in 98 μ l ddH₂O and OD₂₈₀ is measured. The samples are then brought to an equal volume depending on their protein concentration. If OD₂₈₀ is 0.1 $\rightarrow$ 100 μ l sample are used. Lysis Buffer to a final volume of 140 μ l is added, 260 μ l Elution Buffer and 20 μ l 4M NaCl are added. The tubes are incubated at 65°C and 300 rpm over night (in order to reverse the x-link).

The proteins are degraded by adding 8 μ l 0.5 M EDTA, 16 μ l 1 M Tris pH 6.5 and 2 μ l Proteinase K (20 mg/ml). The samples are incubated for 1 hour at 55°C and 400 rpm. Under the flue, 600 μ l Phenol are added to the samples, the lid is sealed with Parafilm, the samples are vortexed and centrifuged for 2 minutes, full speed at room temperature. The upper phase is collected in a new Kloesch tube and the procedure is repeated with Chloroformisopropanol (24:1). The supernatant is collected in a new Kloesch tube and 800 μ l 96% EtOH (-20°C), 12 μ l 3 M NaOAc and 1 μ l Glycogen are added. The samples are kept at -20°C for at least 2.5 hours. This is followed by centrifugation at 4°C for 25 minutes at full speed. 800 μ l 70% EtOH are added to wash the pellet. It is centrifuged for 10 minutes at 4°C and full speed. The supernatant is

aspirated and the pellet is air-dried. 100 μ l ddH₂O and 1 μ l RNase are added and incubated at 37°C for half an hour.

These samples are used as the Inputs (IN). The length of the DNA fragments is checked. Therefore 20 μ l of the samples are loaded on a 0.8% agarose gel.

The samples for the Immunoprecipitation are then made.

The Protein A beads have to be prepared before usage. Therefore, 2-3 spatula tips of Protein A beads are put into a Kloesch tube and 1 ml TE Buffer is added. The beads are incubated for 30 minutes at room temperature. They are then centrifuged for 3 minutes, 1000 rpm at room temperature. Afterwards, they are washed three times with TE. The amount of beads is estimated and the same amount of blocking solution is added. The beads are shaken at 4°C for 30 minutes. Then, the tube is centrifuged for 3 minutes at 1000 rpm. The supernatant is discarded, the pellet is washed with TE, centrifuged and the supernatant is again discarded. The amount of beads is estimated and the same amount TE Buffer is added.

The sonicated samples are brought to an equal volume depending on their concentration. If OD₂₈₀ is 0.1 $\rightarrow$ 100 μ l sample are used. Lysis Buffer to a final volume of 140 μ l is added and 860 μ l Dilution Buffer are pipetted to the solution. 25 μ l Protein A beads are added to the samples and the tubes are shaken at 4°C for one hour to pre-clear the chromatin. The pre-cleared chromatin is then centrifuged for 5 minutes, 1200 rpm at 4°C. The supernatant is transferred to a new Kloesch tube, 2.5 μ l α -Desmin antibody are added and the tubes are shaken at 4°C over night. 35 μ l Protein A beads are added and the tubes are shaken at 4°C for 4 hours. The tubes are centrifuged, the supernatant is discarded and 900 μ l of RIPA buffer (to get rid of unspecific binding) is added to the immune-complexes. The tubes are shaken for 10 minutes at 4°C and then centrifuged for 5 minutes, 1200 rpm at 4°C. The washing step is repeated with HI Salt buffer (to get rid of unspecific binding) and with LiCl buffer (to get rid of RNA crosslinked to complexes). The washing step is finally repeated twice with TE Buffer (to neutralise). After removal of the supernatant, 400 μ l Elution Buffer are added to the beads. The complexes are shaken for 5 minutes, 300 rpm at room temperature. 20 μ l 4 M NaCl are added and the tubes are gently shaken at 300 rpm at 65°C over night to reverse the X-link. The proteins are degraded by adding 8 μ l 0.5 M EDTA, 16 μ l 1 M Tris pH 6.5 and 2 μ l Proteinase K (20mg/ml) and incubating them for 1 hour at 55°C

and 400 rpm. Under the flue, 600 µl Phenol are added to the samples, the lid is sealed with Parafilm, the samples are vortexed and centrifuged for 2 minutes, full speed at room temperature. The upper phase is collected in a new Kloesch tube and the procedure is repeated with Chloroformisopropanol (24:1). The supernatant is collected in a new tube and 800 µl 96% EtOH (-20°C), 12 µl 3M NaOAc and 1 µl Glycogen are added. The samples are kept at -20°C for at least 2.5 hours. This is followed by centrifugation at 4°C for 25 minutes at full speed. 800 µl 70% EtOH are added to wash the pellet. It is centrifuged for 10 minutes at 4°C and full speed. The supernatant is aspirated and the pellet is air-dried. 100 µl ddH₂O and 1 µl RNase are added and incubated at 37°C for half an hour.

These samples are used as the Immunoprecipitation (IP) samples.

For the following PCRs, 2 µl of the INPUT samples and 10 µl of the IMMUNOPRECIPITATION samples respectively are used as template DNAs.

PCR Master Mix (1x):

- 37.75 µl milliH₂O
- 5 µl 10x Taq Buffer (Fermentas #EP0402)
- 4 µl 25 mM MgCl₂ (Fermentas #EP0402)
- 1 µl 10 mM dNTPs (Fermentas #R0192)
- 0.5 µl Primer forward (Vienna BioTec)
- 0.5 µl Primer reverse (Vienna BioTec)
- 0.25 µl Taq Polymerase (Fermentas #EP0402)

PCR conditions:

1. 95°C 4 minutes
2. Annealing 90 seconds (Annealing temperature depends on Primer)
3. 72°C 90 seconds
4. 95°C 60 seconds
5. Annealing 30 seconds
6. 72°C 45 seconds → back to step 4
7. 72°C 90 seconds
8. 4°C

The PCR products are loaded on a 1.8% agarose gel (the agarose concentration depends on the length of the produced fragment, the smaller the product, the higher the agarose concentration should be in order to get a good separation), which is run at 130 V for about 1.5 hours. The gel is then put into an Ethidiumbromid bath for about 20 minutes and afterwards the DNA bands are detected under an UV light.

Buffer for Chromatin Immunoprecipitation:

WASH I

- 10 ml 10% Triton x-100
- 8 ml 0.5 M EDTA
- 400 µl 1 M Hepes
- ddH₂O ad 400 ml
- +200 µl/10 ml Proteinase Inhibitor (add immediately before use)
- +10 µl/10 ml PMSF (add immediately before use)

WASH II

- 20 ml 4 M NaCl
- 800 µl 0.5 M EDTA
- 400 µl 0.5 M EGTA
- 4 ml 1 M Hepes
- ddH₂O ad 400 ml
- +200 µl/10 ml Proteinase Inhibitor (add immediately before use)
- +10 µl/10 ml PMSF (add immediately before use)

Lysis Buffer

- 100 µl 10% SDS
- 20 µl 0.5 M EDTA
- 50 µl 1 M Tris pH 8.1
- 20 µl Proteinase Inhibitor
- 1 µl PMSF
- ddH₂O ad 1 ml

Elution Buffer

- 200 µl 10% SDS
- 100 µl 1 M NaHCO₃
- 10 µl 1 M DTT (Dithiothreitol)

- ddH₂O ad 1 ml

TE Buffer

- 4 ml 1 M Tris pH 8
- 800 µl 0.5 M EDTA
- ddH₂O ad 400 ml
- The solution is autoclaved.

Blocking Solution (for 100 µl beads)

- 2 µl Herring Sperm
- 10 µl BSA (10 mg/ml)
- 5 µl NaAcid 2%
- 84 µl TE

Dilution Buffer

- 800 µl 10% SDS
- 44 ml 10% Triton x-100
- 960 µl 0.5 M EDTA
- 6.68 ml 1 M Tris pH 8,1
- 16.7 ml 4 M NaCl
- ddH₂O ad 400 ml

RIPA Buffer

- 15 ml 4 M NaCl
- 20 ml 1 M Tris pH 8
- 4 ml 10% SDS
- 20 ml 10 M NaDoc
- 40 ml 10% NP-40
- ddH₂O ad 400 ml

HI Salt Buffer

- 50 ml 4 M NaCl
- 20 ml 1 M Tris pH 8
- 4 ml 10% SDS
- 40 ml 10% NP-40
- ddH₂O ad 400 ml

LiCl Buffer

- 100 ml 1 M LiCl
- 20 ml 1 M Tris pH 8
- 20 ml 10% NaDoc
- 40 ml 10% NP-40
- ddH₂O ad 400 ml

Elution Buffer

- 1 ml 10% SDS
- 0.5 ml 1 M NaHCO₃
- 50 µl 1 M DTT
- ddH₂O ad 5 ml

7.7. Cloning Systems

7.7.1. Production of competent E.Coli

A colony of E.Coli XL1-blue is picked, transferred to a flask containing 5 ml LB medium, and is incubated over night at 37°C. The bacteria culture is diluted with 45 ml LB medium in a 250 ml flask and is incubated until a density of OD₆₀₀ = 0.3 – 0.6 is reached (about 2 hours). The culture is centrifuged at 4°C for 10 minutes and 1000 g. The supernatant is aspirated and the pellet is resuspended in 5 ml TSB. The solution is kept on ice for 10 minutes and then aliquoted in pre-cooled Kloesch tubes. The bacteria are shock-frozen in liquid nitrogen and then stored at -80°C.

TSB (sterile filtered)

- 10% PEG₆₀₀₀ or PEG₈₀₀₀
- 5% DMSO
- 10 mM MgCl₂ x 6 H₂O
- 10 mM MgSO₄ x 7 H₂O
- LB medium

7.7.2. Transformation of competent E.Coli

2 µl of Plasmid DNA are pipetted to 500 µl of competent E.Coli XL1-blue and are mixed by inverting the tube. The suspension is kept on ice for 30 minutes, transferred to 42°C for 30 seconds and immediately put on ice. 250 µl LB medium are added and

the mixture is shaken (300 rpm) at 37°C for 60 minutes. Antibiotic selective LB-agar plates are prewarmed and 10 µl and 30 µl of the bacteria solution are spread to the plate and incubated over night at 37°C. The next day single colonies can be picked.

7.7.3. Glycerol Stocks of E.Coli

Glycerolstocks are used for long-term storage of bacteria containing a specific plasmid.

A single colonie of competent E.Coli transformed with the plasmid of interest is picked and transferred into a cuvette tube containing 2 – 5 ml antibiotic selective medium. The bacteria are incubated and shaken over night at 37°C. The next day, 850 µl of the overnight culture are mixed with 150 µl sterile 87% Glycerol and are transferred to a cryotube. The bacteria are shock-frozen in liquid nitrogen and then frozen at -80°C.

7.7.4. Plasmid Preparation

To transfect cells with plasmids, they have to be extracted from bacteria that were transformed with the plasmid of interest. For the Plasmid Preparation, the Qiagen MaxiPrep Kit is used.

A piece of a glycerol stock is transferred to an antibiotic-selective LB-agar plate and incubated at 37°C over night. The next day, a single colony is picked and transferred into a tube containing 3 ml antibiotic selective LB medium. The tube is shaken at 37°C for about 4 hours and then the content of the tube is transferred into a 1 l flask containing 150 ml antibiotic selective LB medium. The flask is incubated and shaken at 37°C over night. The overnight culture is centrifuged for 15 minutes at 4°C and 8500 rpm. The pellet is resuspended in 10 ml Buffer P1 and transferred to a 50 ml falcon. 10 ml Buffer P2 are added, the tube is inverted and kept at room temperature for 5 minutes. 10 ml Buffer P3 are added, the tube is inverted 5 – 6 times and kept at room temperature for 10 minutes. It is then centrifuged for 5 minutes at 8000 rpm and the supernatant is transferred to a Qiagen Cartridge. The content is pushed through the cartridge into a new falcon. 2.5 ml ER buffer are added, the tube is inverted about 10 times and kept on ice for 30 minutes. A Qiagen-tip 500 is filled with 10 ml QBT and the column is emptied by gravity flow. Then the filtered lysate is applied to the Quiagen-tip and left until it flew through the tip. The tip is washed two times with 30 ml Buffer QC. Then 15 ml Buffer QN are added to elute the DNA into a new flask. 10.5 ml isopropanol are added, mixed and centrifuged for 30 minutes at 15000 rpm

and 4°C. The supernatant is discarded, 5 ml 70% Ethanol are added and the tube is again centrifuged for 10 minutes at 4°C and 15000 rpm. The supernatant is aspirated, the pellet is dried and then dissolved in 600 µl TE-buffer. The DNA concentration is measured on a NanoDrop machine and the Plasmid DNA is stored at -20°C.

7.8. Transfection

7.8.1. Transfection of Fibroblasts

7.8.1.1. Calcium Phosphate Transfection

1.2×10^5 C2C12 fibroblast cells are seeded to each well of a gelled (0.1% gelatine) 6 well plate. The plate is incubated over night at 37°C. On the next day, the medium is aspirated and 2.5 ml fresh M10Gi medium are added to each well. The plate is incubated for about 3 hours. For each well one 2 ml Kloesch tube is prepared with 300 µl HeBS buffer. For each well one 1.5 ml Kloesch tube is prepared with 12 µg of Plasmid-DNA dissolved in milliQ-H₂O to a total volume of 263 µl and 37 µl 2 M CaCl₂. The HeBS tubes are put on a vortex machine and the 300 µl DNA solution is pipetted to the HeBS buffer. The mixture is vortexed for 30 more seconds and is then kept on room temperature for 30 minutes. The DNA solutions are then pipetted to the wells and the plate is incubated at 37°C. About 24 hours after the transfection the medium is aspirated, the cells are washed twice with 1x PBS and 3 ml fresh M10Gi are added to each well. The plate is further incubated at 37°C. After 48 hours the transfected cells can be tested in further experiment (in this case either with Luciferase Assay or RNA isolation).

7.8.1.2. Lipofectamine Transfection

1.5×10^5 C2C12 fibroblast cells are seeded to each well of a gelled (0.1% gelatine) six-well plate. They are seeded in 2 ml antibiotics-free and serum reduced medium (M5Gi w/o antibiotics). The plate is incubated at 37°C over night. On the next day, for each well one Kloesch tube is prepared with 10 µl Lipofectamine 2000 reagent (Invitrogen) in 240 µl antibiotics- and serum-free medium. The mixture is kept at room temperature for 5 minutes. For each well one Kloesch tube is prepared with 8 µg of Plasmid – DNA dissolved in antibiotics- and serum – free medium to a total volume of 250 µl. The Lipofectamine is then pipetted to the DNA tubes, gently mixed and kept at

room temperature for 20 minutes. The Lipofectamine – DNA complexes are then pipetted to the wells and incubated at 37°C. 6 hours or 24 hours after transfection, the medium is aspirated, the cells are twice washed with 1x PBS and fresh M10Gi medium is added. After 48 hours the transfected cells can be tested in further experiment.

7.8.2. Transfection of CVPCs

7.8.2.1. Lipofectamine Transfection

1×10^5 CVPC cells are seeded on each well of a gelled 24 well plate in M15Hi medium. The plate is incubated at 37°C over night. On the next day, the medium is aspirated, the cells are washed with 1x PBS and 500 μ l antibiotics-free medium (DMEM + 15% HyClone Serum + 1% Glutamine) are added to each well 2 hours before transfection. 1 μ g DNA is mixed in 50 μ l of DMEM + 1% Glutamine, 1 μ l Lipofectamine 2000 is soluted in 50 μ l DMEM + 1% Glutamine. The solution is kept at room temperature for 5 minutes. The DNA is pipetted to the Lipofectamine solution and kept at room temperature for 20 minutes. 100 μ l are pipetted to each well and the plate is incubated at 37°C for 3 hours. Then the medium is aspirated and 2 ml (or less – at least 750 μ l) M15Hi are added. After 48 hours the transfected cells can be tested in further experiment.

7.9. Luciferase Assay

7.9.1. Luciferase Assay of transfected Fibroblasts

For the Luciferase Assay, the Promega Dual-Luciferase Reporter Assay System Kit is used.

48 hours after transfection the medium is aspirated from the 6 well plate and the cells are washed twice with cold 1x PBS. 1 ml 1x Passive Lysis Buffer is added to each well and the plate is incubated at room temperature for 40 minutes. The cells are resuspended and transferred into Kloesch tubes. 50 μ l of the cell suspension are pipetted into a 96 well Luciferase plate. For the Luciferase machine (Berthold LB960) LARII (for Firefly Luciferase values) and Stop&Glo solution (Stop&Glo Buffer:Stop&Glo Reagent 50:1, for Renilla Luciferase values) are prepared. The Firefly and Renilla Luciferase activity are measured (Fig. 53).

7.9.2. Luciferase Assay of transfected Cardiovascular Progenitor Cells

For the Luciferase Assay, the Promega Dual-Luciferase Reporter Assay System Kit is used.

48 hours after the transfection the medium is aspirated, the cells are washed with 1x PBS, and 200 μ l 1x Passive Lysis Buffer are added to each well. The plate is incubated for 60 minutes at 37°C or room temperature. The cells are resuspended and transferred into Kloesch tubes, the wells are washed with 100 μ l 1x Passive Lysis Buffer (1x PLB), and are diluted 1:10 in 1x PLB. For the Luciferase machine (Berthold LB960) LARII (for Firefly Luciferase values) and Stop&Glo solution (Stop&Glo Buffer:Stop&Glo Reagent 50:1, for Renilla Luciferase values) are prepared. (about 100 μ l per sample). 20 μ l of the lysate are pipetted into a Luciferase plate and Luciferase activity is measured (Fig. 53).

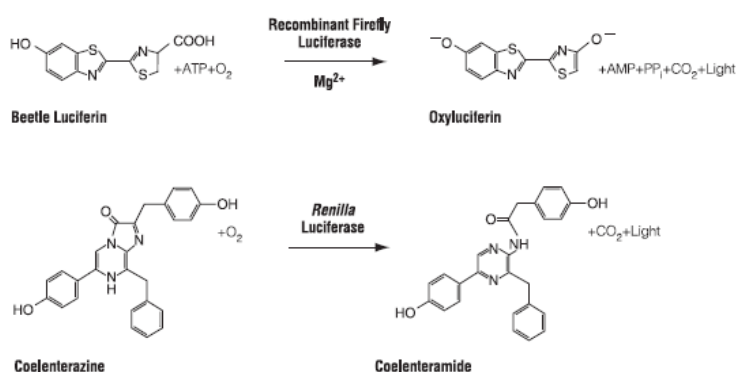


Fig. 53 Bioluminescent reactions catalyzed by firefly and Renilla luciferases (Promega).

7.10. Homologue Recombination of cardiovascular progenitor cells

The pC_{SV}-EGFP-PP-DT plasmid was cut with Sal I enzyme to linearize. Therefore, 3 μ l Sal I (NEB #R0138S) are incubated with 30 μ l NEBuffer 3 (NEB #B7003S) and 3 μ g of Plasmid DNA in a total volume of 300 μ l at 37°C over night. 1.1×10^7 CVPC cells are pipetted into an electroporation cuvette and 260 ng linearized plasmid DNA are added (25 μ l). The cuvette is kept at room temperature for five minutes and afterwards cells are electroporated at 230 V and 500 μ F in a BioRad electroporation machine. The content of the cuvette is put onto a 10 cm feeder plate which is then incubated at 37°C. The cells are fed for 7 days with medium containing puromycin (1 μ g/ μ l) and for 2 days with puromycin – free medium. After this time, small colonies could be observed with clonal cells which should contain the nkx2.5-EGFP knock in construct. As soon, as colonies are seen on the plate, clones are picked. Therefore,

feeder cells in 96 wells are prefed with M15Hi medium, and 20 μ l Trypsin are put into empty 96 well plates. The colonies are scraped off the 10 cm plate, put into the Trypsin wells, incubated for 10 minutes at 37°C and are resuspended with 70 μ l of the prefed feeder cell medium. The cells are transferred to the feeder wells and incubated at 37°C for five days and are then transferred to 48 well feeder plates. They are fed every day and when the clones reach confluence, they are transferred to a 24 well feeder plate. This step is passage 0. The clones are fed every day. After three days when they reach confluence, they are split in a 1:2 ratio, one part is further cultivated, the other one is frozen (passage 1).

7.11. Fluorescence Activated Cell Sorting (FACS)

Here, the extent of heart cell formation in embryoid and cardiac bodies was measured with FACS after staining with the fluorescent heart cell marker cardiac Troponin T. Embryoid and Cardiac Bodies are trypsinised. The pellet is resuspended in 4% Paraformaldehyd in PBS and incubated at room temperature for 20 minutes. The solution is centrifuged for 10 minutes at 1200 rpm and the pellet is resuspended in PBS. The solution is again centrifuged for 10 minutes at 1200 rpm and the pellet is resuspended in PBS. The next centrifugation for 10 minutes at 1200 rpm is followed by dissolving the pellet in 0.15% Saponin in PBS to permeabilise the cells. The solution is centrifuged for 8 minutes at 1200 rpm. The pellet is resuspended, incubated at room temperature for 10 minutes with 2% BSA-PBS to block unspecific binding sites and then partitioned to two tubes. The tubes are then centrifuged for 5 minutes at 1000 rpm, one pellet is dissolved in primary antibody solution (cardiac TroponinT, Thermo Scientific #MS-295,1:200 in 2% BSA-PBS), the other one in 2% BSA-PBS (as a negative control) and the tubes are incubated at 4°C for at least one hour or over night. The suspension is centrifuged for 8 minutes at 1200 rpm, and the pellet is resuspended in PBS. This wash step is repeated twice. After the last step, the cells are partitioned to two tubes each, and one tube each is incubated with secondary antibody solution (anti mouse-FITC, Jackson ImmunoResearch 1:200; #711-095-151) in order to get four samples. The cells are incubated at 4°C for at least one hour or over night. The tubes are then centrifuged for 10 minutes at 1200 rpm and the pellet is resuspended in PBS. This wash step is repeated twice. The pellet is then dissolved in 1 ml PBS and the cells are analyzed on a BD FACSCalibur machine.

7.12. Immunofluorescence

Indirect immunofluorescence microscopy was used to detect the extent of heart cell formation in embryoid and cardiac bodies by staining them with the cardiac marker cardiac Troponin T (cTNT). Furthermore, the cardiac bodies were stained with Connexin 43 to detect whether they form tight cell-cell interactions.

Embryoid and cardiac bodies are grown on 10 cm plates either covered with cover slip, in order to get highly magnified and detailed pictures, or without cover slips for overview screens. 10 ml 4% Formaldehyd in 1x PBS are added to each plate and they are rotated at room temperature for 20 minutes. The plates are washed twice with 1x PBS and 2 ml 0.1% Saponin in 1x PBS are added. The plates are rotated for 20 minutes to permeabilise the cells. The cells are then washed twice with 1x PBS and 5 ml 2% BSA-PBS are added to each plate to block unspecific binding sites. The plates are swayed for 10 minutes. The cells are then incubated with primary antibody in 2% BSA-TBS (cardiac TroponinT, Thermo Scientific #MS-295,1:200; Connexin 43, Sigma #C6219, 1:4000) at 4°C for at least one hour. They are then washed three times with 1x PBS for 10 minutes. Secondary Antibody in 2% BSA-TBS is added (Dianova α -mouse-FITC #715-095-151, 1:200; Dianova α -rabbit-TR #711-075-152) and incubated for one hour at 4°C. The cells are washed 10 minutes with 1x PBS. DAPI (Invitrogen #D1306) is diluted 1:1000 in 1x PBS, added to the plates and swayed for 5 minutes. The plates are washed twice with 1x PBS for 15 minutes and those without cover slips are observed with a Zeiss Axiovert 200M microscope. The cover slips are put on glass slides with 50 μ l of 55°C Mowiol. After drying of the Mowiol, the cover slips are fixed with nail polish. The fluorescence of the cells on the slides is observed with a Zeiss LSM 510 confocal microscope.

Mowiol

- 6 g Glycerin
- 2.6 g Mowiol 2-88
- 5% DABCO
- 6 ml ddH₂O

The components are mixed and incubated at room temperature for 2 hours. 12 ml 0.2 M Tris/HCl pH 8.5 are added and the solution is heated at 50°C for 10 minutes. It is then centrifuged for 15 minutes at 5000 rpm and aliquots are stored at -20°C.

7.13. Western Blot

7.13.1. Protein Isolation

Cells are washed with 1x PBS and Kinexus Lysis Buffer is pipetted to the plate. The cells are scraped off the plate, resuspended, transferred to Kloesch tubes and stored at -20°C.

Kinexus Lysis Buffer

- 20 mM Tris pH7
- 2 mM EGTA
- 5 mM EDTA
- 30 mM Sodiumfluorid
- 40 mM Glycerophosphat
- 10 mM Pyrophosphate
- 2 mM Sodiumorthovanadate
- 10 µM Leupeptin
- 5 µM PepstatinA
- 0.5% Triton X-100
- Proteinaseinhibitor
- ddH₂O

7.13.2. Bradford Protein Concentration Measurement

The concentration of the proteins is measured with Bradford measurement.

The Bradford Dye is diluted 1:4 and 196 µl dilution are pipetted into a 96 well plate. Different BSA concentration samples are made, and 4 µl of each (0 mg/ml; 0.156 mg/ml; 0.313 mg/ml; 0.63 mg/ml; 1.25 mg/ml; 2.5 mg/ml) are pipetted to the wells in duplicates. For the samples, 199 µl Bradford dilution and 1 µl cell lysate are mixed in each well in duplicates. OD₅₉₅ is measured with a Labsystem Multiscan RC machine. The protein concentration is calculated according to the BSA standards.

Bradford Dye

- 0.5 mg/ml Coomassie Blue
- 25% Methanol
- 42.5% H₃PO₄

7.13.3. SDS Separation

For protein separation, 12.5% SDS Polyacrylamid gels are used. Firstly, the separation gel is poured into a gel apparatus and it is covered with isopropanol. As soon as the gel is hardened, isopropanol is removed, the stacking gel is poured and a comb is put into the liquid gel. The hardened gel is then transferred into 1x PAGE buffer. After Bradford measurement, the lysates are diluted with ddH₂O and 3x Sample buffer to 30 µg protein in 24 µl per sample. The samples are boiled for 5 minutes at 95°C, centrifuged for 2 minutes at 13000 rpm and loaded to the gel. The gel is run firstly at 90 V until the samples are collected and build a sharp lane which enters the separation gel, the proteins are then separated at 120 V.

Stacking Gel 5%

- 0.333 ml 30% Polyacrylamide
- 0.25 ml 1 M Tris/HCl pH 6.8; 0.4% SDS
- 1.385 ml ddH₂O
- 10 µl 10% APS
- 2 µl TEMED

Separation Gel 12.5%

- 2.083 ml 30% Polyacrylamide
- 1.875 ml 1 M Tris/HCl pH 8.8; 0.4% SDS
- 0.948 ml ddH₂O
- 40 µl 10% APS
- 4µl TEMED

3x Sample Buffer

- 187.5 mM Tris/HCl pH6.8
- 6% SDS
- 30% Glycerol
- 0.15 M DTT
- 0.03% Bromphenolblau

10x PAGE Buffer

- 1.92 M Glycine
- 250 mM Tris
- 1% SDS

7.13.4. Blotting

The proteins separated on a gel are blotted onto a Nitrocellulose Membrane and then stained with specific primary and secondary antibodies.

The gel is put onto five Whatman paper and one Nitrocellulose membrane and is covered with another five Whatman paper (everything soaked in 1x Blotting Buffer). The proteins are blotted at 400 mA for 60 minutes. The membrane is then stained with Ponceau solution to visualize protein marker bands. It is destained with ddH₂O and blocked in 5% Milk-TBST for one hour at 4°C. Primary antibody solution in 5% Milk-TBST or 5% BSA is added (SPARC, Santa Cruz, #sc-25574, 1:200; GFP, Cell Signalling #2956, 1:666) and the membrane is swayed at 4°C for at least 60 minutes. The blot is washed three times for 5 minutes with 0.1% TBST. HRP-conjugated secondary antibody solution in 5% Milk-TBST or 5% BSA is added (α -rabbit-HRP, Dianova, #111-035-003, 1:15000; α -mouse-HRP Pierce, #31430, 1:15000) and the membrane is rotated at 4°C for at least 60 minutes. The membrane is washed twice for 5 minutes with 0.1% TBST and once with 1x TBS and is detected (Cell Signaling 20x LumiGLO Reagent and 20x Peroxide #7003). The film (Thermo Scientific #34089) is developed in an AGFA Curix60 machine.

10x Blotting Buffer

- 0.48 M Tris
- 0.4 M Glycin
- pH 9.1

Ponceau-S

- 100 mg Ponceau-S
- 100 ml 1% glacial acetic acid

10x TBS

- 0.5 M Tris
- 1.5 M NaCl
- pH 7.6

0.1% TBST

- 1x TBS
- 0.1% Tween

7.14. Dot Blot

Dot Blot Analysis was performed with supernatants from CVPC cultures. The DotBlot apparatus BIORAD-DOT™ 96 wells was used.

A nitrocellulose membrane is wetted with 1x TBS and clamped into the apparatus. 400 µl TBS are pipetted into each well. Different concentrations of supernatant and protein of interest are pipetted to the wells. The apparatus is kept on room temperature for one hour, then the solution is sucked through the membrane so that the proteins are attached to the nitrocellulose membrane. The wells are washed by sucking 200 µl 1x TBS through the membrane. The membrane is put out of the machine and dried for 15 minutes. It is then blocked with 5% Milk-TBST for 30 minutes. Primary Antibody solution in 5% Milk-TBST is added (SPARC, Santa Cruz, #sc-25574, 1:200) and the membrane is incubated at 4°C for 60 minutes. It is washed three times with 0.1% TBST for 5 minutes. HRP-conjugated secondary antibody solution in 5% Milk-TBST (α -rabbit-HRP, Dianova, #111-035-003, 1:15000) is added and the membrane is incubated at 4°C for 60 minutes. The membrane is washed twice for 5 minutes with 0.1% TBST and once with 1x TBS and is detected (Cell Signaling 20x LumiGLO Reagent and 20x Peroxide #7003). The film (Thermo Scientific #34089) is developed in an AGFA Curix60 machine.

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9. Abstract

Cardiovascular diseases account for the highest percentage of deaths worldwide. The heart comprises cardiac stem cells, however its regenerative potential is very limited. Understanding the mechanisms and identifying factors activating those dormant cells and making them potent to differentiate and replace injured cells would be a great achievement for cell based therapy.

Here, two proteins, Desmin and SPARC, were identified as positive regulators of cardiomyogenesis. Desmin accelerates and enhances cardiomyocyte formation in embryonic stem cells by binding to the cardiac transcription factor *nkx2.5* promoter. Embryonic stem cells however differentiate into cardiomyocytes only to a low extent. The successful isolation of a stable cardiovascular progenitor cell line, which exclusively differentiates to cardiomyocytes, smooth muscle cells, and endothelial cells, provides an indefinite source for studying methods and mechanisms to obtain highly cardiomyocytes enriched cultures. Recombinant SPARC promotes cardiomyogenesis by upregulating cardiac specific gene expression such as *nkx2.5*. SPARC was furthermore determined to act via modulation of Wnt signalling. Wnt and MAPK signalling pathways play an important role during cardiomyogenesis at different time points of development. To study the influence of different factors on *Nkx2.5* expression, an *nkx2.5* – EGFP – positive cardiovascular progenitor reporter cell line was established.

10. Zusammenfassung

Herz-Kreislauf-Erkrankungen machen den größten Anteil an weltweiten Todesfällen aus. Das Herz besitzt zwar somatische Herzstammzellen, allerdings hat es ein nur sehr limitiertes regeneratives Potential. Ein großer Schritt für Stammzelltherapie als Heilungsmethode von Erkrankungen des Herzens wäre es, Mechanismen und Faktoren herauszufinden, die Herzstammzellen aktivieren und sie dadurch dazu zu bringen, verletztes und totes Herzgewebe zu ersetzen.

In dieser Arbeit werden zwei Proteine, Desmin und SPARC, als positive Regulatoren der Herzzellentwicklung beschrieben. Desmin beschleunigt und verstärkt die Bildung von Herzzellen in embryonalen Stammzellen indem es an die Promoterregion des frühen Herzzellmarkers *nkx2.5* bindet. Embryonale Stammzellen entwickeln sich allerdings nur zu einem geringen Ausmaß zu Herzzellen, viele andere Zelltypen entstehen während der Differenzierung. Daher brachte die Isolierung von stabilen kardiovaskulären Vorläuferzelllinien eine unerschöpfliche Quelle von immer gleichbleibenden Zellen, die ausschließlich zu Kardiomyocyten, glatten Muskulaturzellen und Endothelzellen differenzieren. Diese machen es möglich, Methoden und Mechanismen herauszufinden, mit denen man Kulturen bekommt, die stark mit Kardiomyocyten angereichert sind. Rekombinantes SPARC fördert die Herzzellentwicklung indem es herzzellspezifische Gene hinaufreguliert. SPARC wirkt indem es in den Wnt Signaltransduktionsweg eingreift. Der Wnt und MAPK Signaltransduktionsweg spielen zu verschiedenen Zeitpunkten in der Herzzellentwicklung eine wichtige Rolle. Um die Auswirkung verschiedener Faktoren auf die *Nkx2.5* Expression zu testen wurde weiters eine *nkx2.5* – EGFP – positive Herzvorfürerzelllinie hergestellt.

11. Curriculum Vitae

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Annual Meeting of the Austrian Association of Molecular Life Sciences and Biotechnology, in Innsbruck, Austria, 21st to 23rd of September, 2009.