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1 Introduction

1.1 Cardiovascular disease

Cardiovascular diseases (CVDs) are the number one cause of death worldwide, with an estimated 17.1 million deaths in 2004. 82% of CVD deaths are in low- and middle-income countries (WHO, 2009). CVDs are the leading cause of death in the Austria accounting for 45% of overall deaths. Although the mortality rate has decreased by 25% since 1990, the CVD related death rate is still 17% higher than the Western European average (WHO, 2004). In the United States CVDs accounted for 35.3% of total deaths in 2005, with an estimated total cost of \$475.3 billion in 2009. Within CVDs coronary heart disease accounts for 52% of overall deaths (American Heart Association, 2009).

A decrease in the number of reported coronary heart diseases (CHDs) as well as the higher survival rate following myocardial infarction (MI) led to a recent decline in overall death rate due to CHDs. Reduction in CVD is largely a result of improved classical risk factor management, such as smoking secession and reduced cholesterol diet. The increased survival rate after acute MI is due to the progress in treatment methods, such as the use of thrombolytic agents (McGovern et al., 1996).

Pharmacological and non-pharmacological therapies have been developed which have significantly enhanced therapy options for a variety of CVDs. However, the loss of cardiomyocytes is irreversible and leads to the development of progressive heart failure. Cardiac transplantation is limited due to a shortage of donor organs, immunosuppression complications, and the possibility of functional failure of the transplanted heart (Hassink et al., 2003).

New therapeutic options for MI patients were made conceivable by the discovery of stem cell populations from somatic tissues as well as embryonic stem cells. The better understanding of the wide differentiation potential of stem cells led to extensive research on tissue renewal. So far, embryonic stem cells, bone marrow mononuclear cells, haematopoietic stem cells, mesenchymal stem cells, endothelial progenitor cells, skeletal myoblasts, and cardiac stem cells are being investigated for treatments after MI (Joggerst and Hatzopoulos, 2009).

Although progress has been made, only modest results have been achieved by increasing tissue renewal of diseased hearts using stem cell therapy (Joggerst and

Hatzopoulos, 2009). For example, skeletal muscle has been investigated as a replacement for cardiac muscle after MI. Skeletal myoblasts have been shown to form grafts of mature muscle after injection into diseased or healthy hearts. These grafts can contract as a reaction to exogenous stimulation. Although the essential formation of electromechanical junctions between cardiac muscle cells and skeletal muscle cells for functional integration into heart tissue was shown in vitro, formation could not be forced in vivo (Reinecke et al., 2000).

During the last three decades the heart was thought to be one of the least regenerative and post-mitotic organs in the human body. Cardiomyocyte response to stress was believed to be limited to hypertrophy and/or cell death (Anversa et al., 2006; Laflamme and Murry, 2005). Lately, it has been proposed that myocardial regeneration after infarction in the human heart is mediated by multipotent cardiac stem cell-like cells (CSCs). These cells are self-renewing but can divide and differentiate into myocytes, smooth muscle cells, and endothelial cells (Beltrami et al., 2003; Urbanek et al., 2005). In 3 months old mouse hearts the presence of senescent and nonsenescent myocytes was observed, indicating that these cells do not have a common origin. These cells are more likely products of a time dependent activation and differentiation of progenitor cells of the heart (Rota et al., 2007). Undifferentiated Y-chromosome-positive cells were found in female hearts after transplantation into male patients. These cells had translocated to the grafted heart and could give rise to coronary arteries, capillaries, and myocytes (Quaini et al., 2002). The heart is now viewed as a self-renewing organ in which myocyte regeneration occurs throughout the lifespan of the organism (Anversa et al., 2006).

CSCs raise hope for new treatment possibilities for MI patients. If in vivo activation of CSCs could be triggered after myocardial infarction, healing processes of affected tissue could be induced (Joggerst and Hatzopoulos, 2009).

1.2 Stem cells

Stem cells can be defined as cells that have the choice between self-renewal and differentiation upon every division, without depleting the stem cell pool. This choice is influenced by signals from the microenvironment or stem cell niche (Alenzi et al., 2009; Ying et al., 2003). Stem cells help to maintain tissue homeostasis by replacing terminally differentiated, aged, and injured cells (Boiani and Scholer, 2005). Stem cells are characterized by self-renewal and potency. Self-renewal means that stem cells can

proliferate indefinitely giving rise to identical daughter cells without the loss of stemness character, potency or going into senescence (Silva and Smith, 2008). Potency describes the ability of stem cells to give rise to at least one, or various somatic cell types (Donovan and Gearhart, 2001). Potency can be classified into totipotency, pluripotency, multipotency, and unipotency.

Totipotency means the ability of a single cell to give rise to all cell types required during embryogenesis, including extra-embryonic tissue, in order to produce a viable animal. The fertilized egg is a totipotent cell (Western, 2009). Recently, it has been shown that normal, fertile animals can be developed from both inner and outer blastomers of the 16-cell embryo, but not the 32-cell embryo (Suwinska et al., 2008).

Pluripotency defines the ability of a single cell to give rise to all cells of an embryo but not extraembryonic trophoctoderm (Boiani and Scholer, 2005; Donovan and Gearhart, 2001).

Multipotency describes the ability of one stem cell to give rise to a number of differentiated cells. Potency is limited compared to pluripotent stem cells. These stem cells are tissue specific and are not isolated from the inner cell mass. Haematopoietic stem cells are an example of multipotent stem cells which can differentiate into at least eight cell types including erythrocytes and white blood cells (Alenzi et al., 2009).

Unipotency means that one stem cell can give rise to only one specialized cell type. Spermatogonial stem cells in the testis are an example of unipotent stem cells. These cells can only differentiate into spermatozoon or remain in self-renewal (Donovan and Gearhart, 2001).

It has been believed that stem cells from a certain tissue can only differentiate into cells native to this tissue. More recently it has been suggested that these cells also have the potential to differentiate to other cell types (Jiang et al., 2002).

Embryogenesis and derivation of stem cells

After fertilization of the mouse oocyte, cell division and compaction take place during mouse embryogenesis (Figure 1). The outer cells of the morula stage form the trophoctoderm (TE), the inner cells form the pluripotent inner cell mass (ICM) (Boiani and Scholer, 2005; Niwa, 2007).

The ICM gives rise to the epiblast and primitive endoderm (PrE). PrE is the precursor of extraembryonic endoderm essential for the nutrition supply of the embryo. The epiblast gives rise to the primitive ectoderm which develops into all three germ layers forming the embryo (Boiani and Scholer, 2005; Niwa, 2007).

The primitive ectoderm is the only cell lineage maintaining pluripotency at the egg cylinder stage and gives rise to all three germ layers (Niwa, 2007). Embryonal carcinoma cells (ECCs) can be derived from primitive ectoderm (Boiani and Scholer, 2005). Primitive ectoderm cannot form TE or PrE (Gardner and Rossant, 1979; Niwa, 2007; Shimosato et al., 2007). The experimental ability to isolate ESCs and ECCs is lost after initiation of gastrulating. Primordial germ cell (PGC) are found in gastrula stage (Boiani and Scholer, 2005).

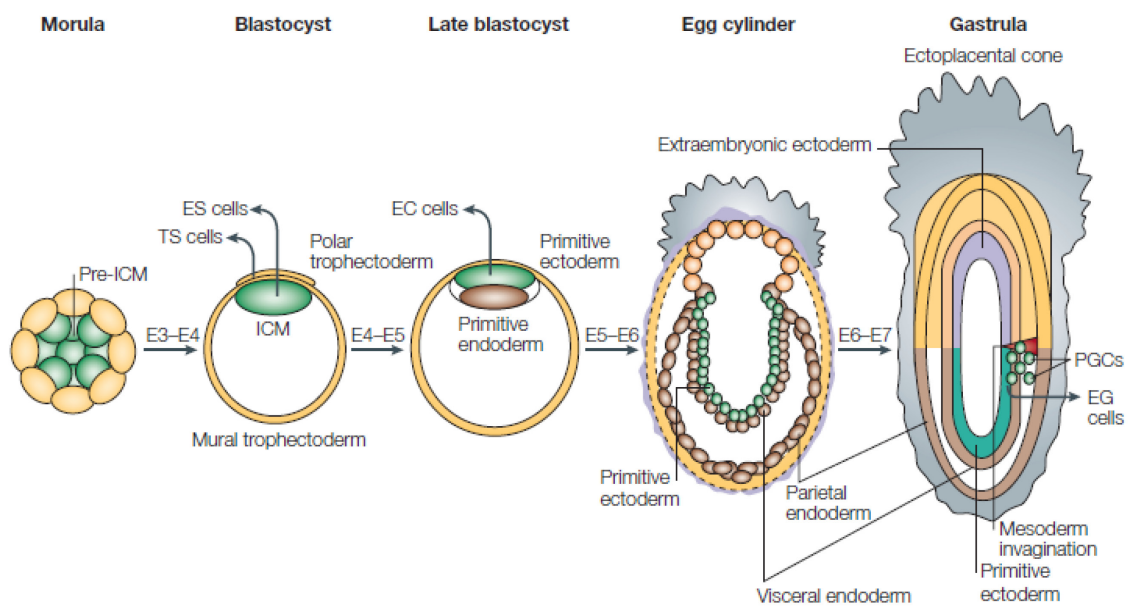


Figure 1 Mouse embryogenesis. The inner cell mass (ICM) gives rise to the epiblast and the primitive endoderm. The epiblast forms the embryo proper while the primitive endoderm gives rise to the extraembryonic endoderm forming extraembryonic tissue. Only the primitive endoderm contains pluripotent stem cells at the egg cylinder stage. ESCs can be derived from blastocysts, ECCs from late blastocysts, and PGCs from gastrula stage (Boiani and Scholer, 2005).

1.3 Pluripotent stem cells

There are three types of pluripotent stem cells: embryonal carcinoma cells (ECCs), embryonic germ cells (EGCs), and embryonic stem cells (ESCs) (Donovan and Gearhart, 2001) (Figure 2). Beyond that, pluripotent stem cells may be generated by reprogramming somatic cells; this is referred to as induced pluripotency.

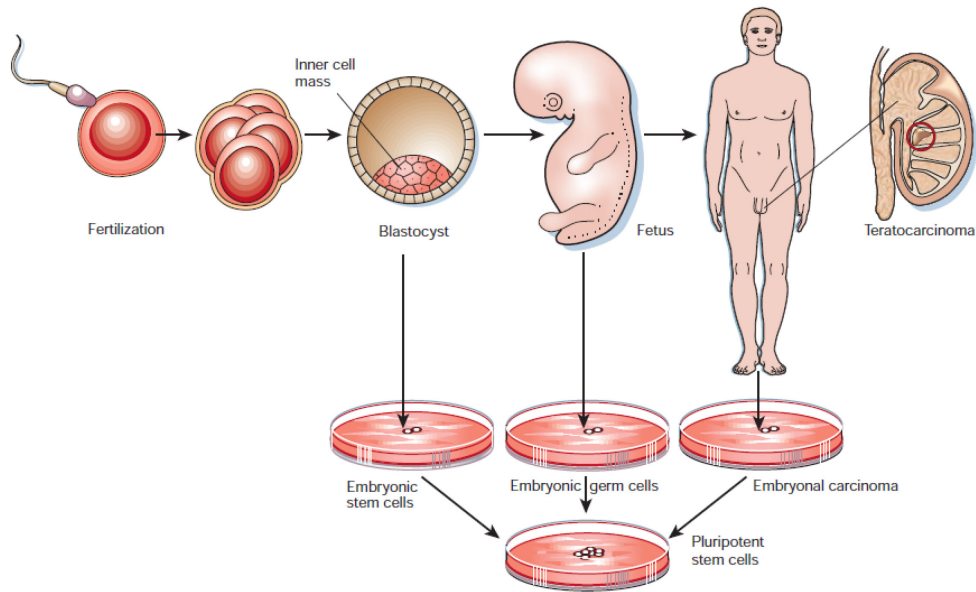


Figure 2 Pluripotent stem cell origins in humans. Pluripotent cells can be derived from the inner cell mass of a blastocyst (embryonic stem cells), from primordial germ cells (embryonic germ cells), and from testicular tumors in adults (embryonal carcinoma cells). (Donovan and Gearhart, 2001)

Embryonal carcinoma cells

Spontaneous testicular germ cell tumors can form during primordial germ cell development. Embryonal carcinoma cells are derived from such germ cell tumors (Stevens, 1967).

Embryonic germ cells

Murine embryonic germ cells originate from in vitro culture of mouse primordial germ cells (PGCs) on feeder layers (Matsui et al., 1992).

Embryonic stem cells

Murine ESCs are derived from the inner cell mass of late mouse blastocysts (Martin, 1981).

Induced pluripotent stem cells

Induced pluripotent stem cells (iPS cells) are generated by reprogramming somatic cells.

- iPS cells can be created by a fusion of ESCs with somatic cells, giving rise to tetraploid cells displaying ESC properties in morphology, growth rate, and antigen expression. Differentiation of these cells can give rise to cells of all three

germ layers. iPS derived from fusion of ESCs and somatic cells still contain the chromosomes of ESC which is a major disadvantage for possible therapeutic use (Cowan et al., 2005).

- Furthermore, iPS cells can be generated from mouse embryonic fibroblasts by introduction of Oct3/4, Sox2, Klf4, and c-Myc. Generated iPS can trigger tumor formation after subcutaneous transplantation in nude mice. Although these cells show morphology and growth properties of ES cells, their gene expression profile is compromised (Takahashi and Yamanaka, 2006).

1.4 Murine embryonic stem cells

ESCs have, along with pluripotency and self-renewal, also the ability to form primary chimaera (Silva and Smith, 2008). Upon transplantation of mESCs into the ICM of E3.5 blastocysts they can completely integrate into the developing embryo giving rise to high rates of chimaerism (Boiani and Scholer, 2005).

For a long time pluripotency was defined as the ability of one cell to differentiate into cells of all three germ layers, without contributing to the trophectoderm lineage (Beddington and Robertson, 1989; Boiani and Scholer, 2005; Donovan and Gearhart, 2001). Recently it was proposed that ICM cells as well as mESCs still possess the ability to form trophectoderm under certain conditions (Niwa et al., 2005; Pierce et al., 1988). Therefore ESCs lack the self-organizing ability to create a whole organism while maintaining the capacity to form all necessary cell types including trophectoderm (Niwa et al., 2005). Upon subcutaneous injection into athymic mice mESC form teratocarcinomas containing derivatives of all three germ layers within 6 weeks (Martin, 1981).

In vitro mESCs can be maintained pluripotent by co-culture of mESC with mitotically inactivated fibroblast feeder cells or in the presence of Leukemia Inhibitory Factor (Martin, 1981; Thompson et al., 1989).

1.5 Embryoid Body formation in mice

When mESCs are cultured in suspension they form round homogenous cell aggregates termed Embryoid Bodies (EBs). EBs consist of an inner core of mESCs coated by a single layer of endoderm-like cells. Their name derives from their similarity to the ICM of mouse embryos (Martin, 1981).

EBs were first made with embryonal carcinoma cells (Martin and Evans, 1975). Cells were cultured in the absence of feeder layers and aggregates of ECCs had formed after 5 days displaying an outer layer of endodermal cells. The presence of feeder layers or gelatin coating of culture plates prevented formation of EBs (Martin and Evans, 1975). Differentiation was observed upon the attachment of EBs on new culture plates. Cells started migrating and formed a halo around cores. Cell lysis and necrosis was observed in core regions of EBs. EBs made from ECCs formed keratinizing epithelium, endodermal cysts, fibroblasts, cartilage, adipose tissue, beating muscles, pigmented cells, and neural cells (Martin and Evans, 1975). Similar organized structures were formed in both EB systems but frequency of these structures was much higher in mESC-derived EBs. EBs derived from mESCs formed more mesoderm-derived structures compared to EBs derived from ECCs (Doetschman et al., 1985)

EBs derived from mESCs form endoderm, mesoderm, ectoderm, and basal lamina between days 3 to 8 of development. By day 5, EB development reaches a state similar to the egg-cylinder stage of a 5-day old embryo. Between day 8 and 10 formation of cystic structures secreting alpha-Fetoprotein and Transferrin takes place, a characteristic of visceral yolk sac endoderm in embryos (Dziadek and Adamson, 1978). These structures are analogous to the visceral yolk sac of the postimplantation embryo (Doetschman et al., 1985). By day 8 of differentiation one third of EBs develop rhythmically contracting areas which continue to beat for over a week (Doetschman et al., 1985). In conclusion, EB formation mimics early embryogenesis to a certain degree.

1.6 Maintaining stem cell pluripotency

In order to potentially use ESCs for therapy it is crucial to understand the complex intrinsic regulatory mechanisms of these cells. Although progress has been made in understanding the function of the three identified stemness factors Oct3/4, Sox2, and Nanog, the extensive mechanisms controlling ESC fate have yet to be determined (Macarthur et al., 2009).

1.6.1 Transcription factor Sox2

Sox2 is a member of the Sox (SRY-related HMG box) gene family and contains one HMG (High Mobility Group)-DNA binding domain (Kamachi et al., 2000). Sox2, like Oct3/4, was shown to be expressed in the ICM, the epiblast, and in germ cells (Avilion et al., 2003; Pesce and Scholer, 2000). Furthermore Sox2 is expressed by multipotent

progenitors in the extraembryonic ectoderm and is a marker for precursor cells within the central nervous system (Avilion et al., 2003).

1.6.2 Transcription factor Oct3/4

Oct3/4 (octamer binding protein) belongs to the POU (Pit-Oct-Unc) transcription factor family and is encoded by the Pou5f1 locus. Presence of Oct3/4 is essential to maintain stem cells in an undifferentiated state. Oct3/4 knockout embryos die at time of implantation due to loss of pluripotency (Avilion et al., 2003; Nichols et al., 1998; Niwa et al., 2000). Repression of Oct3/4 in mESCs leads to differentiation to trophectoderm and consequential loss of pluripotency (Figure 3). Over expression of Oct3/4 in mESCs leads to differentiation of stem cells to primitive endoderm and mesoderm. Therefore the balance of Oct3/4 expression seems crucial in determining the cell fate of mESCs (Niwa et al., 2000).

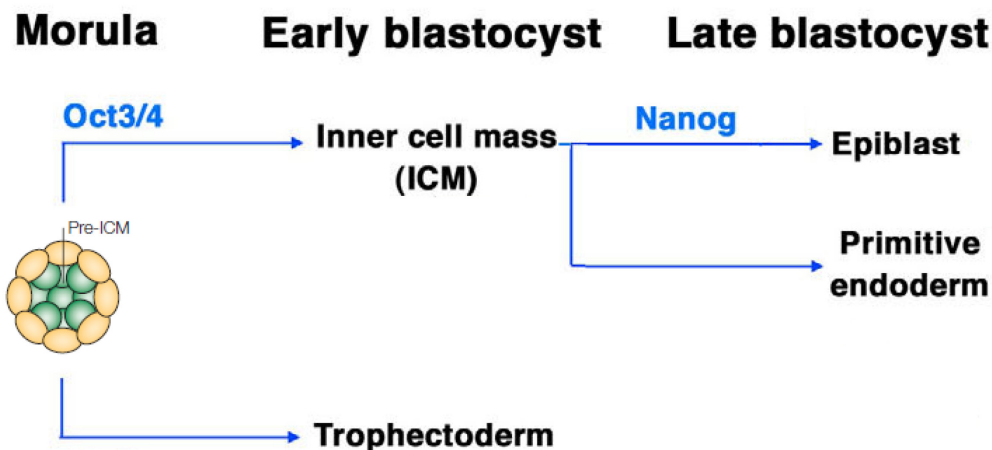


Figure 3 Influence of Oct3/4 and Nanog on embryogenesis. Absence of Oct3/4 expression in morula cells leads to differentiation to trophectoderm. Expression of Nanog determines cell fate between epiblast and primitive endoderm (adapted from Boiani and Scholer, 2005).

1.6.3 Transcription factor Nanog

Nanog is a homeobox protein and plays a fundamental role in the maintenance of pluripotency of the ICM and ESCs (Mitsui et al., 2003). The Nanog expression rate determines cell fate in epiblast cells. Nanog expressing cells remain pluripotent and cells lacking Nanog differentiate into primitive endoderm (Figure 3) and give rise to extraembryonic tissue (Mitsui et al., 2003). Expression of elevated levels of Nanog can prevent differentiation of mESCs in the absence of LIF, while physiological levels of Nanog cannot (Boiani and Scholer, 2005; Mitsui et al., 2003). The Nanog promoter region contains two binding sites for the tumor suppressor p53 and therefore Nanog expression can be directly suppressed by p53 (Lin et al., 2005).

1.6.4 Interaction of stemness factors

Oct3/4 and Sox2 can form a complex and regulate gene transcription. The Oct3/4-Sox2 complex can bind to the FGF4 (fibroblast growth factor 4) enhancer region and thereby promote transcriptional activation of FGF4. FGF4 is expressed in the ICM and epiblast and is essential during embryogenesis (Niswander and Martin, 1992; Rappolee et al., 1994; Yuan et al., 1995). Furthermore the Oct3/4-Sox2 complex can upregulate transcription of Nanog and Oct3/4 (Kuroda et al., 2005; Okumura-Nakanishi et al., 2005; Rodda et al., 2005). Sox2 expression is regulated by POU factor binding sites in the upstream Sox2 enhancer and can therefore be controlled by Oct3/4 (Catena et al., 2004). Oct3/4, Sox2, and Nanog have a positive feedback on their gene expression thereby contributing to self-renewal of mESCs (Boyer et al., 2005) (Figure 4).

In conclusion, stemness factors are regulated by an interwoven network with many feedback loops. Although first insights have been gathered, much more has to be discovered to fully understand the regulation of pluripotency.

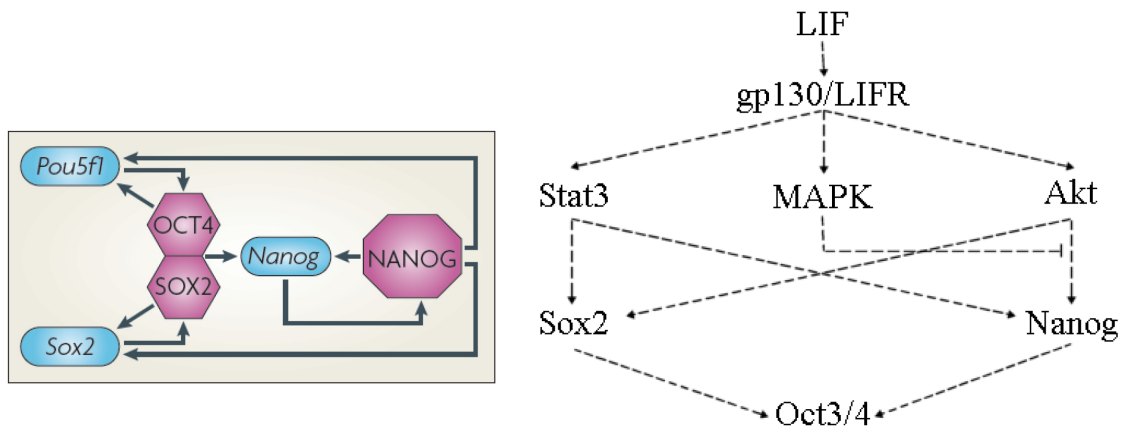


Figure 4 The stemness factor regulatory loop. Nanog influences expression of Oct3/4 and Sox2. Complex of Oct3/4 and Sox2 can regulate Nanog transcription. Auto-regulatory feedback loops are proposed for all stemness genes. (Macarthur et al., 2009). LIF might positively influence stemness factor Sox2 and Nanog transcription via the Stat3, and Akt pathway and inhibit transcription of Nanog via the MAPK pathway. Upregulation of Sox2 and Nanog leads to an activation of Oct3/4 transcription (adapted from Niwa et al., 2009).

1.7 Leukemia Inhibitory Factor

Leukemia Inhibitory Factor (LIF) belongs to the interleukin (IL)-6 cytokine family. The mouse genome encodes for three isoforms of LIF, namely M-LIF (extracellular matrix associated), D-LIF (diffusible), and T-LIF (N-terminal truncated) (Gearing et al., 1987; Haines et al., 1999; Rathjen et al., 1990b). M-LIF and D-LIF are extracellular proteins that are differently expressed during embryogenesis by various embryonic cell types (Rathjen et al., 1990a). T-LIF is retained within the cell (Haines et al., 1999). A soluble form of the mLIF receptor termed LIF-binding protein (LBP) is present at high levels in

serum and even more increased in pregnant mice. LBP most likely prevents systemic effects of locally produced LIF (Layton et al., 1992).

LIF induces heterodimerization of glycoprotein 130 (gp130) receptor and LIF receptor (LIFR) leading to phosphorylation and activation of receptor-associated-Janus Kinases (JAKs). This leads to recruitment and phosphorylation of Stat3 (signal transducer and activator of transcription 3), followed by translocation of pStat3 to the nucleus. Alternatively to the activation of the Stat3 pathway, LIF can activate the mitogen-activated protein kinase (MAPK) pathway (Figure 5) (Davey and Zandstra, 2006; Hirano et al., 1997).

LIF can maintain mESCs in self-renewal and suppress differentiation via the Stat3 pathway (Niwa et al., 1998; Raz et al., 1999; Williams et al., 1988). Furthermore LIF can enhance survival and proliferation in primordial germ cells (De Felici and Dolci, 1991; Matsui et al., 1991). In the absence of LIF mESCs gradually lose responsiveness for LIF prior to loss of stemness factor expression (Oct3/4 and Nanog) and undergo consequential differentiation. Expression of Stat3, gp130, and LIFR are essential for LIF responsiveness and the expression rate correlates with the level of Stat3 activation (Davey et al., 2007). Cells do not differentiate immediately upon LIF deprivation. It is suggested that an autocrine cell density-dependent LIF production can transiently aid maintenance of pluripotency in mESCs in the absence of exocrine LIF (Zandstra et al., 2000). Autocrine expression of (non-LIF) gp130 ligands was also observed in the absence of LIF (Davey and Zandstra, 2006). The endogenous signals supporting Stat3 activation in the absence of LIF cannot fully maintain Stat3, gp130, and LIFR expression. Consequently Stat3 activation decreases over time eventually leading to differentiation, if LIF deprivation persists (Davey et al., 2007).

The cardiac marker Nkx2.5 contains Stat3 DNA binding sites in its promoter region. Therefore a direct influence of Stat3 expression on Nkx2.5 expression and cardiomyogenesis is possible (Foshay et al., 2005).

LIF plays a role in many different cell types and various stages of development. For example LIF has hematopoietic effects such as the enhancement of megakaryocyte and platelet production (Metcalf et al., 1990; Metcalf et al., 1992). LIF can indirectly regulate bone resorption (Martin et al., 1992), act as a survival factor in fetal sensory neurons (Hendry et al., 1992; Thaler et al., 1994), and promote growth and survival of

oligodendrocytes in vitro (Mayer et al., 1994). Uterine expression of LIF is essential for implantation and its absence in LIF^{-/-} mice is embryonic lethal (Stewart et al., 1992). LIFR expression of embryos is not essential for implantation but the disruption of LIFR causes perinatal death (Ware et al., 1995).

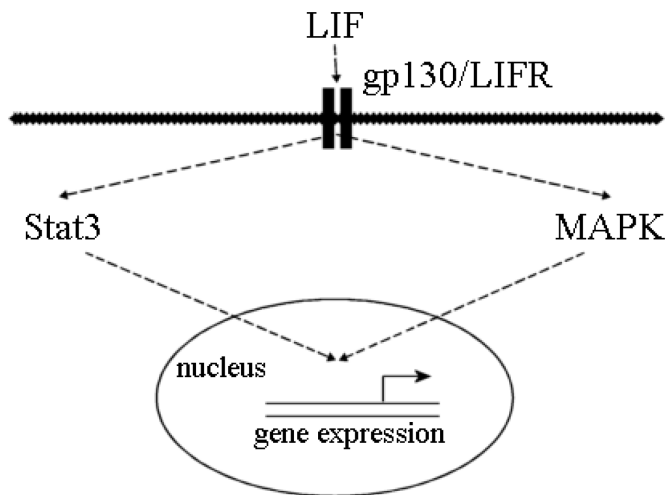


Figure 5 LIF signaling. LIF induces heterodimerization of LIFR and gp130. The Stat3 pathway, or alternatively, the MAPK pathway is activated. pStat3 translocates into the nucleus and positively influences ESC renewal while the MAPK pathway can induce differentiation.

1.8 Bone Morphogenetic Protein 2

Bone Morphogenetic Protein 2 (BMP2) belongs to the transforming growth factors- β (TGF- β) superfamily (Wozney et al., 1988). Mammalian BMP2 shows 75% amino acid identity to Drosophila protein DPP (decapentaplegic) which is responsible for body pattern formation steps during Drosophila development (Capovilla et al., 1994; Ferguson and Anderson, 1992; Zhang and Bradley, 1996).

BMPs signal through heterodimerization of type I and type II receptors. Type I receptors BMPR-IA (ALK3) and BMPR-IB (ALK6) have been identified in mammals, while the expression of BMPR-IB is more restricted. BMPR-II (type II receptor) binds BMP-2 weakly in the absence of type I receptors, while type I receptors bind BMP even in the absence of type II receptors. This stands in contrast to TGF- β receptor systems. Both receptors are serine-threonine kinase receptors and are required for subsequent signal transduction. After type I receptor is activated by type II receptor it mediates signaling through the activation of Smad 1/5/8 by phosphorylation (Figure 6). BAMBI (BMP and Activin Membrane Bound Inhibitor homolog) is a pseudoreceptor for serine-threonine receptors and structurally related to type I receptors. It can stably interact with type I and II receptors and inhibit BMP/TGF induced signaling in a ligand independent

fashion. (Kawabata et al., 1998; Liu et al., 1995; Miyazono et al., 2009; Onichtchouk et al., 1999; Rosenzweig et al., 1995; Sekiya et al., 2004; ten Dijke et al., 1994).

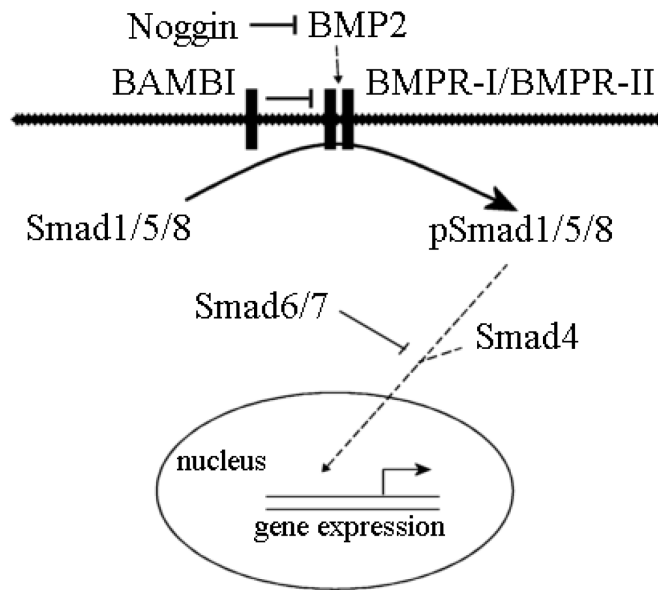


Figure 6 BMP signaling. BMP2 binds BMPR-I and BMPR-II, which leads to phosphorylation and activation of receptor I by receptor II. BMPR-I can now phosphorylate and activate Smad1/5/8. pSmad1/5/8 can associate with Smad 4 and translocate to the nucleus, this can be prevented by Smad6/7. Gene transcription is influenced by interaction with transcriptional co-activators or co-repressors. Extracellular BMP antagonist Noggin can bind BMP ligand and block signaling. Pseudo-receptor BAMBI can inhibit BMP signaling by forming a stable complex with BMP receptors.

BMP2 is essential during early mouse embryonic development. In BMP2 deficient embryos amnion/chorion formation fails and cardiac development is abnormal, thereby showing a role for BMP2 in embryonic and extraembryonic development (Zhang and Bradley, 1996). High expression levels of BMP2 are detected in the myocardial layer of the mouse heart at day E9.5 (Zhang and Bradley, 1996). Treatment of mESCs with BMP2 for 24 h prior to EB formation results in a more than twofold increase of contracting areas and enhances myofibrillogenesis compared to untreated mESCs (Behfar et al., 2002). However, no effect on cardiomyocyte induction by stimulation of mESCs with BMP2/BMP4 was reported by Yuasa et al. 2005.

The BMP antagonist Noggin is expressed on day E7.5 in the cardiac crescent and on day E8.0 in the late crescent, but is barely expressed in the linear heart tube on day E8.5 (Yuasa et al., 2005). Treatment of mESCs with Noggin before or after EB formation does not increase the formation of beating EBs. Only treatment of mESCs with Noggin 3 days prior to EB formation followed by treatment on day 0 results in an increase of beating EBs (Yuasa et al., 2005). These data indicate that BMP signaling is strongly time frame dependent during heart formation. Induction of an acute myocardial

infarction in mice and subsequent injection of mES cells result in a reduction of fibrosis in the affected area. When these mESCs are previously treated with a combination of LIF and BMP2, typical cardiomyocyte morphology and striation patterns are observed 28 days post injection. Untreated cells show more differentiation towards endothelial cells 28 days post injection (Rajasingh et al., 2007).

In chick BMP2 is able to induce expression of myocardial lineage marker Nkx2.5 and GATA4 in ectopic locations. Addition of BMP antagonist Noggin prevents differentiation of precardiac mesoderm into heart tissue (Schultheiss et al., 1997; Zimmerman et al., 1996). BMP2 is insufficient to support viability of precardiac and non-precardiac mesoderm (Lough et al., 1996). Implantation of BMP2 loaded beads in chick can expand Nkx2.5 expression in the anterior paraxial mesoderm specifically. In vitro culture of anterior paraxial mesoderm combined with BMP2 results in formation of robust cardiomyocytes (Schultheiss et al., 1997).

Expression of BMP2/4 overlaps with the expression of inhibitor of differentiation (Id) proteins during embryogenesis. Smad-binding elements are localized in the Id1 promoter region and may also exist in the Id3 promoter region. Expression of Id1 and Id3 is nearly identical during development and might therefore be directly regulated by BMP-induced Smads (Korchynskyi and ten Dijke, 2002; Yanagisawa et al., 2001). Id1 and Id3 double-knockouts are embryonic lethal at E13.5 (Lyden et al., 1999). After loss of Id1 mouse embryonic fibroblasts go into premature senescence (Ruzinova and Benezra, 2003). In tissue culture overexpression of Id1 in endothelial cells can delay senescence but not completely block it (Kiyono et al., 1998; Tang et al., 2002). Upregulation of Id expression is not sufficient for immortalization although elevated levels might be necessary to maintain a highly proliferative state in tumor cells or tissue culture (Ruzinova and Benezra, 2003).

In conclusion BMP2 can both induce and inhibit differentiation via the Smad 1/5/8 signaling pathway. During heart development BMP2 signaling appears to be strongly timeframe-dependent.

1.9 Secreted Protein Acidic and Rich in Cystein

Secreted Protein Acidic and Rich in Cystein (SPARC), also termed Osteonectin or BM-40, is a 32 kDa Ca^{2+} binding glycoprotein belonging to the matricellular family of proteins. The secreted form of SPARC migrates at 43kDa due to the addition of

carbohydrate (Sage et al., 1984). SPARC is produced by many cell types and can modulate growth factor activity, inhibit the cell-cycle, and can function as a counteradhesive protein (Brekken and Sage, 2000).

SPARC is a highly conserved protein. Human and mouse SPARC are 92% identical (Brekken and Sage, 2000). The murine SPARC is encoded by 10 exons localized on chromosome 11 over a 26.5kb span. Exon 1 is untranslated. SPARC consists of an extracellular Ca^{2+} binding module, a Follistatin-like module, and an acidic module (Figure 7).

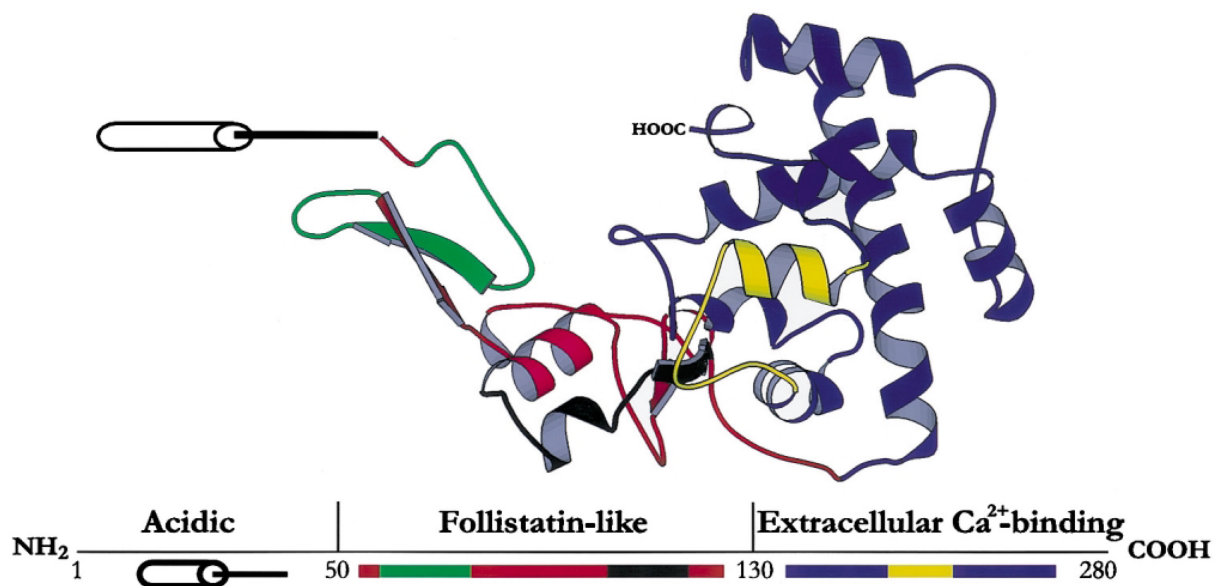


Figure 7 Structure of human SPARC. Module 1 is highly acidic and function is unknown. Module 2 contains structures homologue to repeated domains in follistatin. It might have a function in recruitment to cell surface receptors. Module 3 executes the Ca^{2+} binding activity (Brekken and Sage, 2000).

Expression of SPARC is partially and temporally regulated during development. In the adult it is mainly found in tissues undergoing repair or remodeling (Brekken and Sage, 2000; Yan and Sage, 1999). During mouse embryogenesis SPARC expression is detected on day E9 in the heart primordia (Brekken and Sage, 2000).

SPARC can be found in cell culture media, structures outlining the Golgi apparatus, in nuclei of dividing cells, and in the cytoplasm during metaphase and anaphase (Brekken and Sage, 2000; Gooden et al., 1999).

SPARC protein levels were found to be strongly increased in the left ventricle of the murine heart 7 days after MI (Schellings et al., 2009). SPARC null mice have a much higher risk of dying from cardiac rupture after myocardial infarction (41%) compared to

wildtype mice (9%). While overexpression of SPARC does not affect infarct size, it does prevent cardiac dysfunction and dilatation. Increased SPARC levels have a positive effect on the healing process after MI. In SPARC null mice the cardiac rupture after MI can be prevented by treatment with TGF- β , indicating SPARC mediated TGF- β signaling (Schellings et al., 2009).

Extracellular SPARC binds to integrins- β 1 in lens epithelial cells. Integrin- β 1 can interact with integrin-linked kinase (ILK) via its cytoplasmic tail and thereby modify its activity (Weaver et al., 2008). In adipocyte differentiation SPARC induces β -catenin signaling through ILK. In the presence of siRNA against ILK this effect is no longer observed. Therefore SPARC can modulate Wnt pathway and β -catenin through ILK signaling (Nie and Sage, 2009). In myoblasts, SPARC inhibits phosphorylation of the FGFR1 induced by FGFR1 ligand FGF-2, without affecting interaction between receptor and ligand. The inhibition of FGF signaling induces differentiation to myocytes. SPARC inhibits ERK activation by FGFR1 and thereby influencing the MAPK pathway. SPARC does not affect Akt (thymoma viral proto-oncogene 1) activation (Motamed et al., 2003). In murine mesangial cells SPARC induces expression of TGF β -1 on both mRNA and protein level (Francki et al., 1999).

In the EB system SPARC induces the expression of cardiac markers Nkx2.5 and α -MHC (α -Myosin Heavy Chain) in cardiomyocytes. Furthermore, within 4 h BMP2 expression can be induced by SPARC, both in CBs and in fetal cardiomyocytes. Only BMP2 in combination with SPARC could induce Nkx2.5 expression and promote cardiomyogenesis (Stary et al., 2005).

1.10 Desmin

Desmin is a subunit of the muscle-specific type III intermediate filaments and is expressed in the murine precardiac mesoderm starting day E7.5 (Kuisk et al., 1996). Desmin was shown to directly bind to DNA via its amino-terminus (Li et al., 2003; Tolstonog et al., 2005). Deletions in the amino-terminal of Desmin (amino-acids 1-48) delayed and hampered cardiomyogenesis and inhibited smooth muscle formation in the EB system. Desmin Δ 1-48 inhibited expression of Nkx2.5 and MEF2c in differentiating cardiomyocytes on day 6 and seemed to interfere with onset of cardiomyogenesis. Constitutive overexpression of Desmin promoted differentiation of mESCs and transiently upregulated Nkx2.5. Although Desmin seems to play a supporting role during cardiomyogenesis, it is not sufficient to drive mESCs into the cardiomyocyte

lineage (Hofner et al., 2007; Hollrigl et al., 2002). Gene targeting of Desmin leads to extensive myocyte cell death and to heart failure in mice (Milner et al., 1999; Milner et al., 1996).

2 Rationale

Cardiovascular Diseases account for almost 1 out of 2 deaths in Austria. Loss of cardiomyocytes after acute myocardial infarction is currently irreversible. Recently, cardiac progenitor cells have been isolated by various groups. Reactivation of dormant cardiovascular progenitor cells in the heart would increase tissue renewal after acute MI and could thereby prevent progressive heart failure.

The mechanisms underlying self-renewal of ESCs are only partly understood and even less is known about somatic progenitor cells. Therefore it is important to determine the differences and similarities of mESCs and progenitor cells of the heart. mESCs are isolated from the inner cell mass and are in some sense an in vitro tissue culture artifact. So far it is unclear if the self-renewal of cardiac progenitor cells can be triggered by the same factors as in mESCs or whether they require a specialized microenvironment entirely different from mESCs. The better understanding of the maintenance of multipotency of cardiac progenitor cells will aid in vitro culture. Indefinite culture of cardiac progenitor cells will significantly promote research potential compared to repeatedly isolating new cardiac progenitor cells. The establishment of an in vitro system to mimic cardiomyogenesis will further benefit research.

Investigation of the limitations and the potential of cardiovascular progenitor cells will contribute to the understanding of the regenerative potential of the heart. Furthermore, cardiovascular progenitor cells provide a system to study the signaling mechanisms initiating cardiomyocyte differentiation in the adult heart opposed to heart development during embryogenesis.

3 Abbreviations

AcNPV	<i>Autographa californica</i> nuclear polyhedrosis virus
Akt	thymoma viral prot-oncogene 1
Alk3	bone morphogenetic protein receptor, type IA
Alk4	activin A receptor, type 1B
BAMBI	BMP and activin membrane bound inhibitor homolog
BMP	bone morphogenetic protein
CB	cardiac body
cDNA	complementary deoxyribonucleic acid
CHD	coronary heart disease
CMV	cytomegalovirus
CSC	cardiac stem cell-like cell
cTNT	cardiac troponin T
CVD	cardiovascular disease
CVPC	cardiovascular progenitor cell
D-LIF	diffusible leukemia inhibitory factor
DMEM	dulbecco's modified eagle medium
DMSO	dimethylsulfoxid
dNTP	deoxy nucleotide triphosphate
DTT	dithiothreitol
EB	embryoid body
ECC	embryonal carcinoma cell
EDTA	ethylenediaminetetraacetic acid
ERK	mitogen-activated protein kinase 1
ESC	embryonic stem cell
FGF4	fibroblast growth factor 4
FGFR1	fibroblast growth factor receptor 1
GATA4	GATA-binding protein 4
GFP	green fluorescence protein
gp130	glycoprotein 130
GSKα/β	glycogen synthase kinase alpha/beta
HDAC	histone deacetylase
HMG	high mobility group
HRP	horseradish peroxidase
ICM	inner cell mass
IL6	interleukin 6
ILK	integrin linked kinase
iPS cell	induced pluripotent stem cell
JAK	janus associated kinase
LBP	leukemia inhibitory factor binding protein

MAPK	mitogen activated protein kinase
MEF2C	myocyte enhancer factor 2c
Mesp1	mesoderm posterior 1
MI	myocardial infarction
M-LIF	matrix-associated leukemia inhibitory factor
Nkx2.5	NK2 transcription factor related, locus 5
Oct3/4	octamer-binding transcription factor 3/4
OSMR	oncostatin M receptor
PCR	polymerase chain reaction
PGC	primordial germ cell
Pou5f1	POU domain, class 5, transcription factor 1
PrE	primitive endoderm
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SF	<i>Spodoptera frugiperda</i>
siRNA	short interfering ribonucleic acid
SMA	smooth muscle actin
Smad	mothers against decapentaplegic homolog
Sox2	SRY-related box 2
SPARC	secreted protein acidic and rich in cystein
Stat3	signal transducer and activator of transcription 3
T	brachyury
TE	trophectoderm
TGF-β	transforming growth factor- β
T-LIF	truncated leukemia inhibitory factor
Wnt	wingless-related MMTV integration site
α-MHC	α -myosin heavy chain

4 Results

4.1.1 HDAC1 expression and its correlation with HDAC2 and Nkx2.5 expression

Cardiovascular Progenitor Cells (CVPCs) are derived from neonatal hearts of N1-2 neo^R mice. These mice carry a disruption of one allele of the histone deacetylase 1 (HDAC1) locus by a neomycin resistance cassette. An over expression of HDAC1 was shown to negatively affect Nkx2.5 expression and cardiomyogenesis, whereas an RNAi knockdown of HDAC1 promoted both Nkx2.5 expression and cardiomyogenesis (Liu et al., 2009).

We therefore wanted to investigate if HDAC1 expression was downregulated in HDAC1 heterozygous CVPC clone A5. We compared HDAC1 mRNA expression level of CVPCs to murine embryonic stem cells (mESCs) homozygous and heterozygous for HDAC1. If HDAC1 expression proved to be downregulated in CVPCs, we wanted to see if HDAC2 is upregulated in response.

mESCs 663 are homozygous for the HDAC1 wildtype allele. mESC line 662 are derived N1-2 neo^R mice and carry a heterozygous disruption of the HDAC1 allele. As seen in Figure 8A HDAC1 expression on mRNA level is more abundant in mESC 663 (HDAC1 +/+) and reduced in mESCs 662 (HDAC1 +/-). HDAC2 is upregulated in mESC 662 (HDAC1 +/-) compared to wildtype. No reduction of HDAC1 expression was found in HDAC1 heterozygous CVPC clone A5 compared to mESCs 663 (HDAC1 +/+) (Figure 8B).

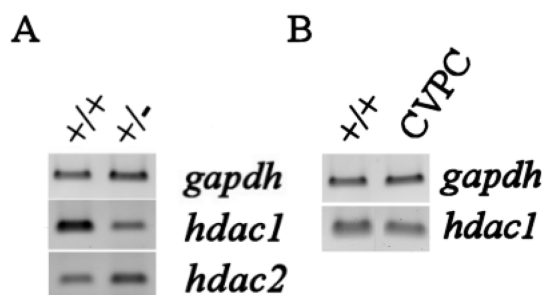


Figure 8 mRNA expression of HDAC1 and HDAC2. GAPDH was used to balance cDNA in RT-PCR analysis. Expression of HDAC1 and HDAC2 mRNA level of mESCs 663 (HDAC1 +/+) and 662 (HDAC1 +/-) is shown in (A). Expression of HDAC1 mRNA level in CVPCs and mESC 663 is shown in (B). HDAC1 is down regulated in HDAC1 heterozygous mESCs, while HDAC2 is upregulated.

Next we wanted to see how HDAC2 is expressed in CVPC clone A5 compared to mESC 662 (HDAC1 +/-) and 663 (HDAC1 +/+). Figure 9 shows that HDAC2 is not upregulated in CVPC clone A5 compared to wildtype mESCs 663. HDAC2 was

upregulated in mESCs 662 heterozygous for HDAC1. Level of HDAC2 expression in CVPCs was reduced compared to mESCs 662 (HDAC1 +/-).

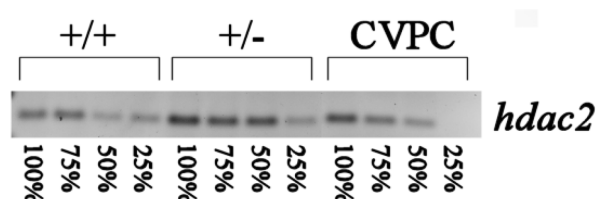


Figure 9 HDAC2 Expression of mESCs and CVPCs. mESCs 663 (HDAC1 +/+), 662 (HDAC1 +/-), and CVPC clone A5 (HDAC1 +/-) were screened for HDAC2 expression on mRNA level. cDNA amount used was balanced by GAPDH expression. cDNA was diluted 1:1; 1:1.33; 1:2; and 1:4. HDAC2 expression is upregulated in HDAC1 +/- cells.

Furthermore, we wanted to investigate a possible connection between levels of HDAC1 and Nkx2.5 in our CVPC cell lines as described by Liu et al. (2009). Unfortunately, Nkx2.5 levels were too low to achieve satisfying results. Nkx2.5 levels of wildtype mESCs 663 were not significantly lower than the Nkx2.5 mRNA level of the heterozygous cell line mESC 662 (data not shown). Expression of Nkx2.5 on mRNA level was lower in CVPCs than in HDAC1 heterozygous mESC line 662 (Figure 10). It was surprising that mESCs 662 and 663 expressed Nkx2.5 in the undifferentiated state, since Nkx2.5 expression was never observed in mESC line AB2.2.

Expression rate of HDAC1, HDAC2, and Nkx2.5 of CVPC clone A5 was approximately the same as the expression rate of these genes in the HDAC +/+ cell line mESC 663.

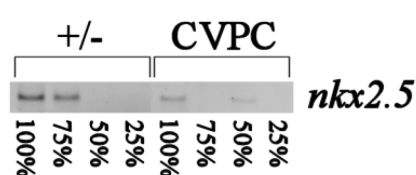


Figure 10 Nkx2.5 expression of mESC and CVPCs. Nkx2.5 mRNA levels were compared in CVPC (HDAC +/-) and mESCs 662 (HDAC +/-). Nkx2.5 levels were very low, therefore template amount had to be doubled.

We could show that HDAC1 heterozygous CVPC clone A5 expressed HDAC1 at levels comparable to homozygous mESC line 663. Expression of HDAC2 and Nkx2.5 were not upregulated. Since CVPCs behaved like wildtype cells in these aspects, we could not show a connection between expression of Nkx2.5 and HDAC1 in CVPCs.

4.1.2 Karyotyping

CVPC isolation procedure included a co-culture of CVPCs with mESCs AB2.2. We wanted to ensure that our cell lines were not fusion products of somatic cells of the

heart and the mESC line AB2.2. If they were fusion products each cell should contain 80 chromosomes. All CVPC lines were clonally derived, therefore, the vast majority of cells was expected to be euploid. In case of fusion cells, the majority should have been aneuploid.

Spindle formation by colchicine treatment was not prevented before karyotyping analysis of all eleven CVPC clones. Therefore cells in all cell cycle steps were present in our samples. Table 1 shows the total number of counted nuclei and the percentage of euploid and aneuploid cells observed. Since in most cases the number of euploid cells was higher than 80%, we reasoned that these cells were indeed euploid (Table 1).

Clone A5 showed 88% euploidy in our original assay. Upon colchicine treatment of clone A5, less than 3% aneuploid cells could be detected (over 50 nuclei analyzed). Since A5 proved to be 97% and H3 95% euploid we selected these clones for all our following experiments.

CVPC clone	% euploid (≤ 47 chromosomes)	% aneuploid (≥ 48 chromosomes)	total counts
A3	66.6%	33.3%	18
A5	88.0%	12.0%	25
B3	90.0%	10.0%	10
B5	90.0%	10.0%	20
C3	60.0%	40.0%	10
D3	90.9%	9.1%	11
D5	61.1%	38.8%	18
E3	82.9%	17.1%	35
F3	75.0%	25.0%	8
G3	86.0%	14.0%	43
H3	95.0%	5.0%	40

Table 1 Chromosome number of CVPC clones. Karotyping was performed without colchicin treatment. Majority of aneuploid cells are likely to represent cells undergoing cell division.

4.1.3 Ground state gene expression on mRNA level

4.1.3.1 Stemness factors

In order to verify that CVPCs can indeed have stem cell character we needed to show that the three stemness factors – Nanog, Oct3/4, Sox2 - were expressed. mRNA analysis showed that all three stemness genes were transcribed in all tested clones on slightly varying levels. mRNA expression levels correlated with expression levels seen in mESC line AB2.2 (Figure 11).

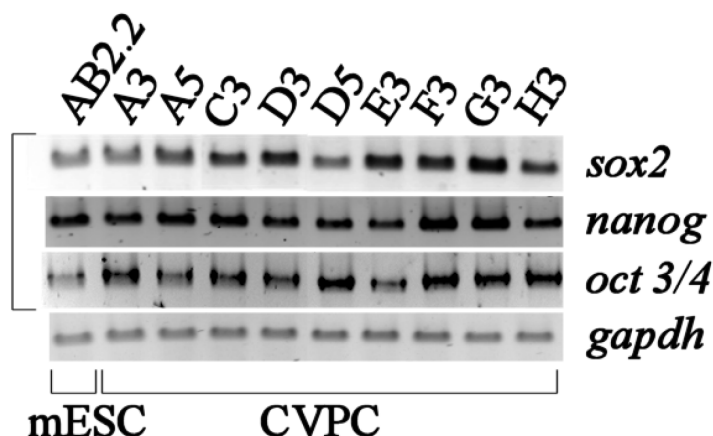


Figure 11 mRNA Expression of stemness factors. mRNA Expression level of stemness factors Sox2, Nanog, and Oct3/4 of 9 CVPC clones and mESC line AB2.2 were tested by RT-PCR. GAPDH was used to balance cDNA amount. All clones express stemness factors at equal level to ESCs.

4.1.3.2 Receptor expression

We wanted to test if CVPCs are potent to react to different external signals. Therefore, we tested for the receptor expression of the signaling pathways we were interested in, namely the Stat3 and BMP2 pathway.

OSMR (Oncostatin M receptor) can bind IL6 family member Oncostatin M followed by heterodimerization with gp130 and subsequent Stat3 signaling. Interestingly, expression of OSMR varied stronger compared to all other tested receptors and proteins. OSMR expression was found to be the highest in CVPC clones A3, F3, and G3 (B3 and B5 not tested) (Figure 12). The clones A3, E3, and G3 produced the least amount of cardiomyocytes in a later experiment (Figure 29). mESC cell line AB2.2 showed weak expression of OSMR. High levels of OSMR expression might therefore have a negative effect on differentiation in the monolayer system.

BAMBI (pseudo-receptor BMP and Activin membrane-bound inhibitor homolog) is a TGF- β -family type 1 receptor but lacks an intracellular kinase domain. BAMBI inhibits TGF- β , BMP, and Activin signaling by preventing formation of receptor complexes in a ligand independent manner (Onichtchouk et al., 1999). BAMBI might also play a role in determining the onset of cardiomyogenesis. It can enhance the expression of c-Myc and cyclinD1 and upregulate Wnt/beta-catenin signaling and therefore result in enhanced cell proliferation (Lin et al., 2008). BAMBI was expressed by all tested clones as well as by mESC AB2.2, meaning TGF- β , BMP, and Activin signaling can be antagonized in CVPCs (Figure 12).

ALK4 (Activin receptor-like kinase 4, also referred to as ActRIB) mediates Activin signaling via heterodimerization with type II receptors ActRIIA or ActRIIB (ten Dijke et al., 1993). ALK4 functions in epiblast and extraembryonic cells to mediate signaling required for egg cylinder organization and gastrulation (Gu et al., 1998). All tested CVPC clones expressed ALK4 receptor on mRNA level showing that CVPC can also respond to Activin signaling (Figure 12).

Since we were interested in the influence of BMP on cardiomyogenesis we tested for expression of receptors responsible for mediating BMP signaling. **ALK3** (BMPRI-IA) is a type I receptor which binds BMP and activates Smad1/5/8 signaling (Kawabata et al., 1998; ten Dijke et al., 1994). ALK3 was expressed in all cell clones at comparable concentrations to AB2.2, meaning CVPCs can respond to BMP2 via the ALK3 receptor (Figure 12).

It is known that LIF plays an essential role in self-renewal of mESCs (Niwa et al., 1998; Raz et al., 1999; Williams et al., 1988). In order to see if CVPCs have the potential to react to LIF in a likewise fashion as mESCs we tested for the expression of the LIF receptor (**LIFR**). As expected, mESC line AB2.2 expressed LIFR on mRNA level. Figure 12 shows that all tested CVPC clones express LIFR on a comparable mRNA levels to mESCs AB2.2. Therefore CVPCs have the potential to react to external LIF (Figure 12).

In conclusion, mRNA expression levels of CVPC clones do not vary significantly, with the notable exception of OSMR expression levels. CVPCs express the necessary receptors to respond to BMP, Activin, and LIF signaling. BAMBI is expressed by CVPCs and can antagonize TGF- β , BMP, and Activin signaling.

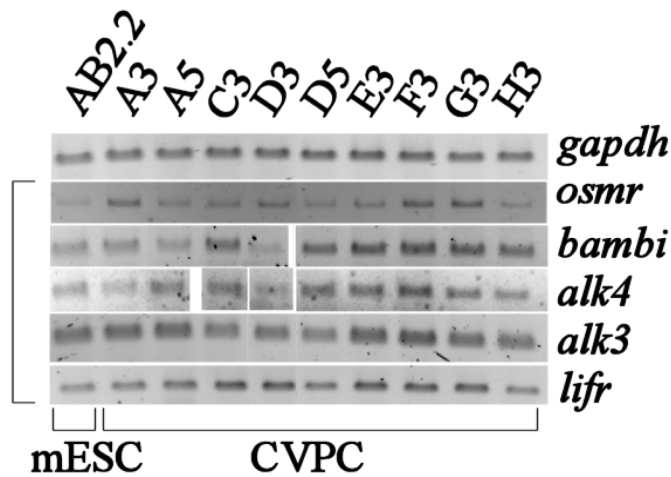


Figure 12 Receptor expression. RT-PCR mRNA expression level of OSMR, BAMBI, ALK4, ALK3 and LIFR in mESCs and CVPCs. GAPDH was used to balance cDNA amount. While LIFR is expressed approximately at a constant level in all cell types, expression of OSMR receptor varies strongly.

4.1.4 mRNA expression shift upon differentiation

We showed mRNA expression levels in the ground state and compared the various CVPC clones. Next, we wanted to investigate the change in mRNA expression level upon differentiation. We selected clone A5 and H3 for further analysis

CVPC were left to differentiate in the monolayer culture and in Cardiac Body (CB) culture. mRNA isolation took place on day 24 of monolayer culture and on day 17 of CB differentiation. EBs were made from mESC AB2.2 and mRNA was isolated on day 17. Furthermore mRNA of AB2.2, A5, and H3 was isolated on day 0.

4.1.4.1 Stem cell specific genes

The expression of stem cell specific genes was tested in CVPC clones A5 and H3 as well as in mESC line AB2.2 to monitor differentiation from stem cells to differentiated cells. As seen in Figure 13, stemness factor Nanog was expressed in all three cell lines in the undifferentiated state (d0) but was depleted upon differentiation (d17, d24). Like Nanog, stemness factor Sox2 was highly expressed in the undifferentiated cells. Downregulation of Sox2 and Nanog expression in monolayer and CB differentiation suggests that CVPCs started differentiating and number of multipotent cells decreased. Lowest expression rate of Nanog and Sox2 was observed in differentiated EBs (Figure 13).

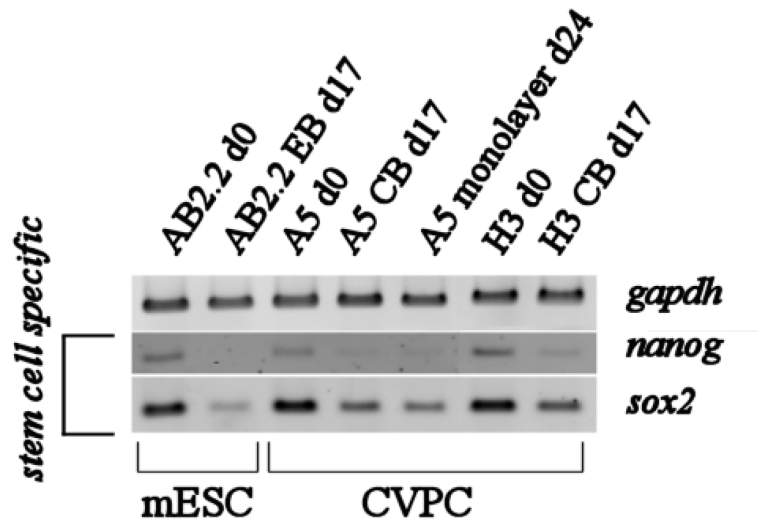


Figure 13 Stemness factor expression of differentiated and undifferentiated CVPCs and mESCs. RT-PCR mRNA expression levels of stemness factors Nanog and Sox2 is shown. GPADH was used to balance cDNA amount. Expression decreases upon initiation of differentiation.

4.1.4.2 Myocardial genes

Cardiac stem cell-like cells (CSCs) were also isolated by Oh et al. (2003) and Oyama et al. (2007). Oh et al. (2003) reported an expression of MEF2c and GATA4 but not Nkx2.5 or α -MHC in the undifferentiated state. Oyama et al. (2007) reported no expression of these markers in the undifferentiated state of their isolated CSCs. Here we wanted to test if CVPCs express cardiac markers in the self-renewing, undifferentiated state. Expression of these markers would indicate a restriction of CVPCs to the cardiac lineage.

The myosin heavy chain (MHC) is the major contractile protein in the heart. Out of all isoforms only α -MHC and β -MHC are expressed in cardiac muscle cells. Cardiac α -MHC is a target for GATA4 (Molkentin et al., 1994). We found an upregulation of α -MHC in CVPCs which is an indicator of cardiac muscle cell formation (Figure 14).

Nkx2.5 is an early marker of cardiac differentiation and one of the first transcription factors expressed in myocardial progenitor cells. Mice carrying a Nkx2.5 mutation in the sequence-specific DNA binding homedomain show abnormal heart formation at E8.5 and die between E8.5 and 10.5. Heart looping does not take place but cardiomyogenic commitment and myofibrogenesis is not inhibited (Lyons et al., 1995; Schultheiss et al., 1995). Nkx2.5 is expressed at low levels in the undifferentiated state of CVPCs but not in the undifferentiated state of mESCs. Upon differentiation Nkx2.5 expression was upregulated and decreased again in late stages of cardiac differentiation (Figure 14, data not shown).

MEF2c (Myocyte Enhancer Factor 2c) belongs to the family of MADS-box containing transcription factors. MEF2c is the oldest known myogenic transcription factor and is highly conserved. It is the only transcription factor associated with differentiation of all muscle types (Black and Olson, 1998). We found that by day 17 of CB differentiation MEF2c expression was downregulated as well. Like Nkx2.5, MEF2c was already expressed in the undifferentiated state of CVPCs along with the stemness factors Nanog, Oct3/4, and Sox2. We interpreted this as a sign of commitment to the cardiac lineage (Figure 14).

Tropomyosin regulates the interaction of Actin and Myosin. Tropomyosin α/β (α/β - TM) are essential during murine cardiac development. α -TM is expressed at higher levels. α -TM null mice are embryonic lethal between E8 to E11.5 (Rethinasamy et al., 1998). In undifferentiated CVPCs α -TM was expressed at low levels and was upregulated upon differentiation (Figure 14).

GATA4 is expressed in the developing heart. GATA4^{-/-} mESCs have reduced potential to undergo cardiac differentiation (Grepin et al., 1995). In contrast, overexpression of GATA4 enhances cardiomyogenesis (Grepin et al., 1997). The GATA4 promoter region shows Nkx2.5 binding elements and expression of GATA4 can therefore be influenced by Nkx2.5. Like the cardiac markers Nkx2.5 and α -TM, GATA4 is upregulated in CVPCs upon differentiation and only expressed at low levels in the undifferentiated state (Figure 14).

We could show a strong increase in expressions of cardiac markers Nkx2.5, α -MHC, α -TM, and GATA4 upon initiation of differentiation. CVPC clone A5 and H3 already expressed these markers at low levels in the undifferentiated state, though expression was higher in clone H3 than in A5. Therefore expression levels of myocardial markers seem to vary between CVPC clones. Expression of myocardial markers in the undifferentiated state is an indicator for the commitment of CVPCs to the cardiac lineage.

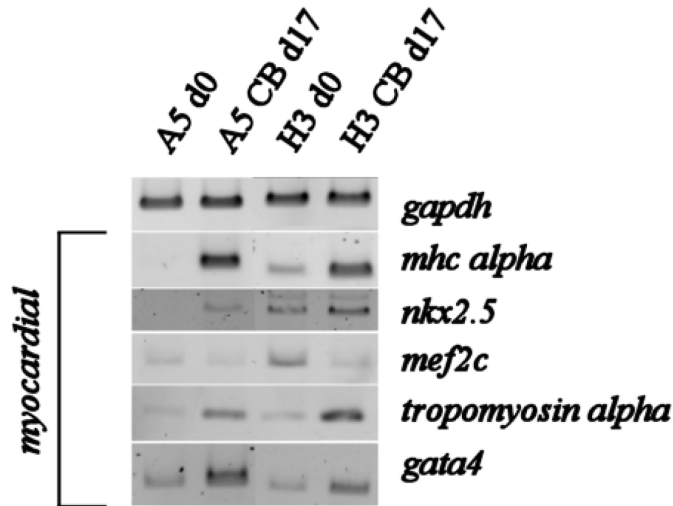


Figure 14 Expression of myocardial genes in differentiated (d17) and undifferentiated (d0) CVPCs. RT-PCR analysis. GAPDH was used to balance cDNA amount. Myocardial markers are already expressed in the undifferentiated state of CVPCs. Differences between CVPC clones can be observed, clone H3 expresses myocardial markers at higher levels than clone A5.

4.1.4.3 Mesodermal genes

The heart is developed from mesoderm. Therefore we tested for an early marker for all mesoderm types (Brachyury) and marker for heart precursor cells (Mesp1)

Mesp1 (mesoderm posterior 1) is expressed in the early mesoderm which gives rise to the extraembryonic and cranial-cardiac mesoderm. It is the earliest marker expressed in the heart precursor cells. Mesp1 heterozygous mice show abnormal heart morphology. Mesp1^{-/-} show a defect in migratory activity of mesodermal cells (Saga, 1998; Saga et al., 1996; Saga et al., 1999). Mesp1 is upregulated upon loss of multipotency and differentiation of CVPCs (Figure 15).

Brachyury (T) is a DNA binding transcription factor. It is one of the key transcription factors in the differentiation and determination of mesoderm in vertebrates (Technau, 2001). In *Xenopus* T is involved in differentiation of all mesoderm cell types, while in mouse mesoderm can form in the absence of T. In Brachyury null mice, posterior mesodermal cells fail to migrate from the tailbud to the primitive streak leading to a failure of the axis to elongate. T/T mutant mESCs are less efficient in leaving the streak than wildtype mESCs after injection into wildtype host blastocysts (Wilson and Beddington, 1997; Wilson et al., 1995). Brachyury is expressed in the undifferentiated state and is downregulated upon initiation of differentiation.

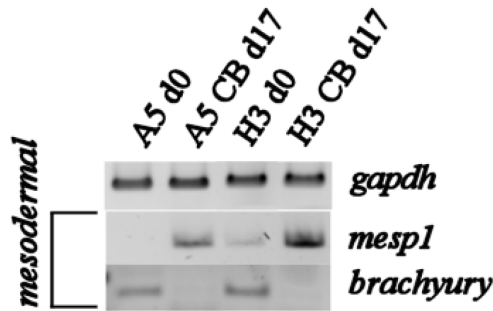


Figure 15 mRNA expression of mesodermal genes during CVPC differentiation. RT-PCR analysis. GAPDH was used to balance cDNA amount. Mesoderm marker Brachyury is expressed in undifferentiated state of CVPCs but is absent in differentiating CVPCs. MEF2c, the earliest marker of heart precursors cells is strongly upregulated after induction of differentiation and is only weakly expressed in undifferentiated state.

4.1.4.4 Receptor genes

We already showed that CVPCs express certain receptors in the undifferentiated state. This suggested that CPVCS are sensitive to Activin, LIF, BMP, and TGF- β signaling. We wanted to test if this competence is lost after differentiation by downregulation of receptor expression or, in contrary, if receptor expression is upregulated. The latter might indicate an importance of that signaling cascade during development.

Expression of Activin receptor ALK4 was not altered during differentiation of CVPCs. However, it is notable that the basic expression seems to vary between CVPC clones (Figure 16). BMP2 receptor ALK3 is strongly upregulated in differentiating CVPCs. This might indicate an important role of BMP signaling during cardiomyogenesis.

ILK (integrin linked kinase) is important for SPARC mediated signaling. ILK is not a transmembrane receptor but is predominantly located on the cytoplasmic side of the plasma membrane and can interact with specific integrins. ILK can for example phosphorylate GSK3 β and thereby influence the Wnt Pathway (Weaver et al., 2008). In CVPCs ILK expression was strongly upregulated upon differentiation (Figure 16).

These results suggest an importance of BMP signaling mediated by receptor ALK3 during CVPC differentiation. Furthermore signaling trough ILK seems to play an important role and might directly affect Wnt signaling.

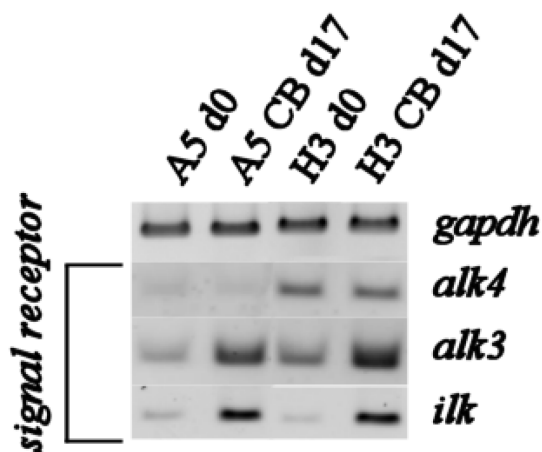


Figure 16 mRNA expression of signal receptors in CVPCs. CVPC clones A5 and H3 were tested for mRNA expression levels of receptors with RT-PCR. Expression of Activin receptors ALK4 is not altered during differentiation, while ALK3 and ILK are both upregulated during differentiation.

4.2 Characterization of Cardiovascular Progenitor Cells

We wanted to characterize murine CVPCs and investigate the similarities and differences between CVPCs and mESCs. We used mESC clone AB2.2 as the basis for comparison.

4.2.1 Murine embryonic stem cells AB2.2

4.2.1.1 Growth on SNL 76/7 feeder layers

Murine ESC strain AB2.2 was cultured on SNL76/7 feeder layers secreting Leukemia Inhibitory Factor (LIF). LIF is known to be sufficient to maintain self-renewal and proliferation of mESCs (Niwa et al., 1998; Raz et al., 1999; Williams et al., 1988).

mESCs AB2.2 showed tight cell-cell interactions and dense colony formation. Colonies had smooth borders. Differentiation to endothelial cells at the rim of these colonies did not take place (Figure 17). Cell clusters were easily distinguishable one day after splitting but started blending in with the feeder layers upon an increase in cell number.

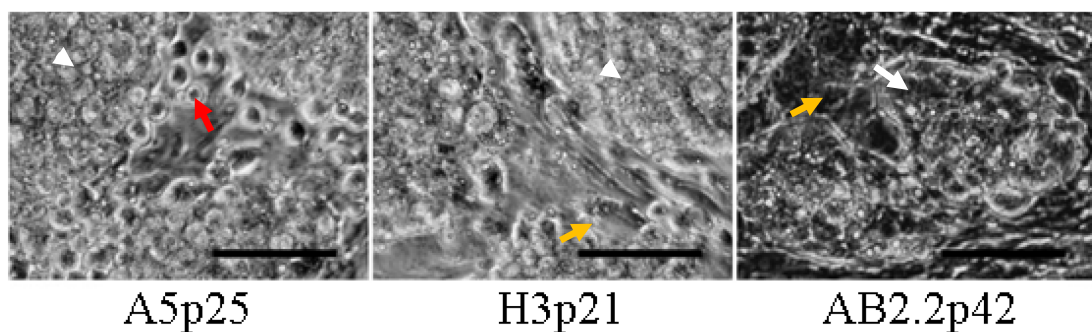


Figure 17 CVPC and mESC morphology. CVPC clones A5 and H3 and mESC clone AB2.2 were grown on feeder layers. White arrowheads indicate undifferentiated CVPCs. White arrow marks undifferentiated ESCs. CVPC colonies are surrounded by differentiated endothelial cells (red arrow). Orange arrows represent feeder cells. mESC AB2.2 form tighter colonies, individual cells cannot be distinguished (bar=100µm).

4.2.1.2 Growth on gelatin coated plates

In order to induce differentiation, murine ESCs AB2.2 were replated from feeder layers onto gelatin coated 24 well-culture dishes at very low cell density ($2-3 \times 10^4$ cells/well).

mESCs AB2.2 tended to aggregate upon transfer onto gelatin coated plates. Cell contacts within colonies were as tight as on feeder layers. Single mESCs AB2.2 were able to form new colonies within three days. Enlarged, differentiated cells started to appear at rims of cell colonies. By day 13 of differentiation colonies branched out forming tree like structures (Figure 18).

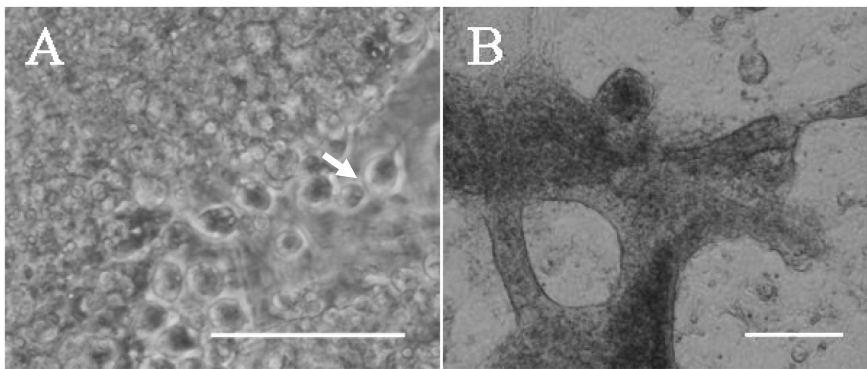


Figure 18 mESC differentiation on gelatin coated plates. mESC AB2.2 on day 4 (A) and day 13 (B) of differentiation. Colony formation is seen in (A), enlarged cells formed in gaps between colonies (white arrow). After 13 days former individual colonies were interconnected by branch like structures (bar=100 μ m (A), bar=300 μ m (B)).

4.2.2 Murine Cardiovascular Progenitor Cells

Eleven clones of Cardiovascular Progenitor cells were isolated from N1-2 neo^R mice. The clones were termed A3, A5, B3, B5, C3, D3, D5, E3, F3, G3, and H3 (Wiedner, 2008).

Since co-culture of mESCs AB2.2 with LIF-secreting feeder layers is essential for the maintenance of self-renewal, we hoped the same held true for murine CVPCs. So far, we were able to culture CVPCs up to passage 68 on SNL76/7 feeder cell layers without a detectable loss of multipotency.

4.2.2.1 Growth on SNL76/7 feeder layers

CVPCs grown on feeder layers appeared increased in size compared to mESCs AB2.2. CVPCs within a colony were surrounded by a halo when observed under a light microscope, while mESCs were not. Therefore, individual CVPCs within a cell colony could be distinguished (Figure 17).

Enlarged CVPCs were found in the gaps between colonies the day after splitting and the number increased thereafter. To monitor the number of enlarged cells we chose CVPC clone A5.

Within 2 passages, consistent with a 6 day time period, the number of enlarged cells formed by CVPC clone A5 did not increase significantly (from 4.6% to 4.8%). Although a percentage of these enlarged cells was transferred upon replating on new feeder layers, these cells did not seem to be able to adhere as well as undifferentiated CVPCs and often settled on top of CVPC colonies. As enlarged cells appeared in gaps between CVPC colonies without the requirement of other enlarged cells being present, we assumed that the newly formed enlarged endothelial cells originated from undifferentiated CVPCs rather than through cell division of replated enlarged cells. The number of differentiated cells developing under a 3T3 protocol remained stable (Table 2). This indicated that these cells had either a lower survival rate, could not attach to feeder layers, or had a significantly decreased growth rate. It took approximately 2 days before the number of enlarged cells started increasing. This means that a 2T2 protocol reduced the formation of enlarged cells but could not inhibit it entirely. The 2T2 protocol potentiated the selection towards fast replicating CVPCs.

We reasoned that 5% differentiation was less influential on further experiments than a constant selection towards fast replicating cells and therefore we set the 3T3 protocol as standard for CVPC culture.

Number of enlarged cells/visual field		Total number of cells/visual field		Percentage of enlarged cells/visual field	
A5p11	A5p13	A5p11	A5p13	A5p11	A5p13
6	2	151	116	3.8%	1.7%
12	8	197	169	6.1%	4.7%
3	14	111	173	2.7%	8.1%
	15		263		5.7%
	5		189		2.6%
21	44	459	910	4.6%	4.8%

Table 2 Differentiation of CVPC clone A5 under regular culture conditions. CVPCs were split 1:3 and sedimentation was monitored with a Olympus CK2 Phase Contrast microscope. The total cell number per visual field and the number of enlarged cells per visual field were determined. The mean percentage of enlarged cells was calculated.

4.2.2.2 *Growth on gelatin coated plates*

CVPCs were transferred from feeder layers onto gelatin coated plates and left to differentiate in the absence of LIF. The cell density was chosen so that almost all cells

adhered individually. Cells did not form tight colonies as observed when grown on feeder layers (Figure 19A) but formed loose cell-cell contacts (Figure 19B). Individual cells could be distinguished. Cell morphology changed from a round shape, when cultured on feeder layers, to an angular shape (Figure 19A+B).

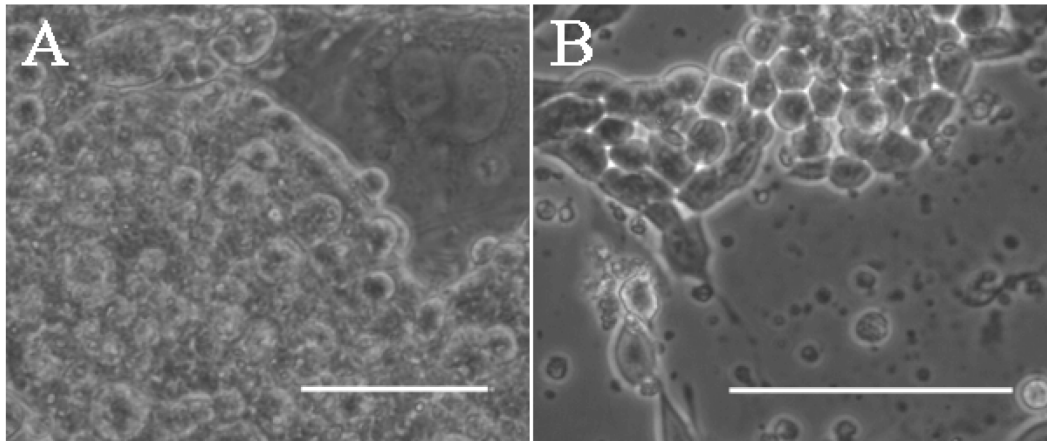


Figure 19 Growth on feeder layers (A) and gelatin (B). CVPCs show tight colony formation upon growth on feeder layers but very loose cell-cell interaction when grown on gelatin coated dishes. Individual cells are much easier to distinguish when cultured on gelatin (B). Cells exhibit round cell morphology on feeder layers (A) and angular cell morphology on gelatin (B) (bar=100μm).

4.2.3 Growth rate

4.2.3.1 Growth rate on feeder layers

We wanted to investigate whether the growth rate of CVPCs differed from the growth rate of mESC line AB2.2 when grown on LIF secreting SNL 76/7 feeder layers.

CVPC clone A5 was grown on SNL76/7 feeder layers. 150 000 cells were seeded and the increase in cell number was monitored on a three day interval. After counting, 150 000 cells were replated on new feeder layers. This procedure was continued over a 22 day time period. Natural logarithm of cumulative cell number was calculated. The doubling time of mESCs AB2.2 on feeder layers was reported to be 17.3h±6.4h under similar culture conditions (Lauss et al., 2005).

When grown on SNL76/7 feeder layers, the growth rate of CVPC clone A5 remained constant over the entire 22 day time period. The doubling time of clone A5 under these conditions was 18.7h±1.2h (Figure 20, Table 3).

These results revealed that the doubling time of CVPC clone A5 was comparable to the doubling time of mESCs AB2.2.

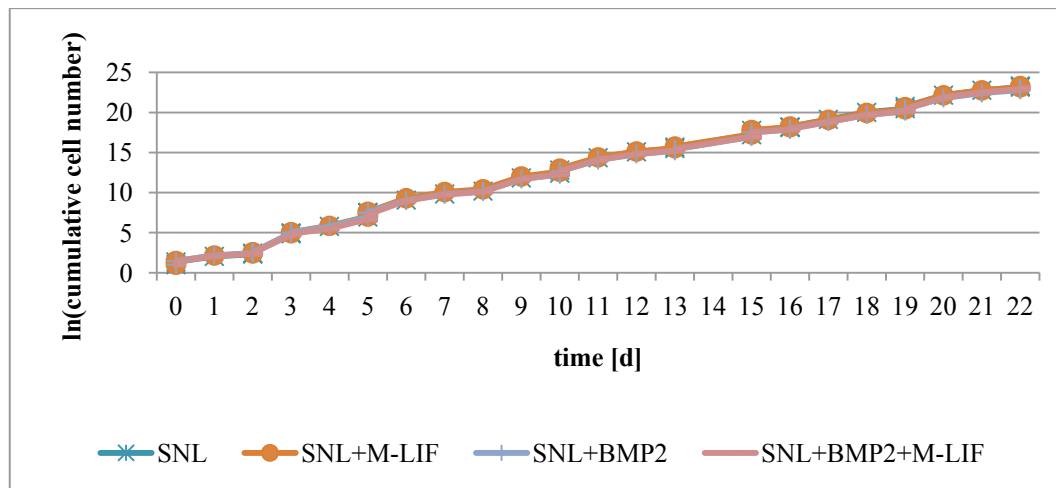


Figure 20 Growth curve of CVPC clone A5. Cells were grown on feeder layers expressing LIF (termed SNL). BMP2 and M-LIF were added at a concentration of 100ng/ml upon feeding every three days. 150 000 cells were seeded per 24 well and every three days cells were counted and the cell number was reduced to 150 000 cells. Natural logarithm of cumulative cell number was calculated.

treatment	Mean doubling time (h)	Standard deviation
mESC line AB2.2	17.3	6.4
CVPC clone A5	18.7	1.2

Table 3 Growth rate of CVPCs and mESCs. CVPCs were seeded at a density of 150 000 cells, after 3 days cell number was determined and 150 000 cells were replated on new feeder layers. Doubling time is mean of 5 consecutive measurements over a 15 day time period. Doubling time and standard deviation of AB2.2 is according to Lauss et al. (2005).

LIF produced by SNL76/7 feeder cells is essential for self-renewal of mESCs. We were interested if elevated levels of LIF could further enhance growth conditions of CVPCs compared to CVPCs grown on feeder layers (SNL). Growth rate was not influenced by the addition of M-LIF but stayed stable around 19 h per cell cycle. Furthermore, addition of Bone Morphogenetic Protein 2 (BMP2) alone or in combination did not affect growth rather of CVPCs on feeder layers (Table 4, Figure 20).

BMP2 is known to induce the *inhibitor of differentiation* genes and we wanted to investigate if addition of BMP2 can prevent formation of enlarged cells in the presence of feeder layers. Cells supplied with BMP2 did not show a decrease in differentiation to endothelial cells (Figure 21).

Increased amounts of BMP2 (100ng/ml) and M-LIF (100ng/ml) did not affect formation and morphology of CVPC colonies compared to untreated cells grown on feeder layers.

CVPC treatment	Mean doubling time (h)	Standard deviation
SNL	18.7	1.3
SNL+M-LIF	19.0	1.2
SNL+BMP2	19.1	0.9
SNL+BMP2+M-LIF	18.9	1.1

Table 4 Doubling time of CVPC clone A5. CVPCs were grown on SNL76/7 LIF-secreting feeder layers (SNL). 150 000 cells were seeded and treated as indicated (100ng/ml BMP2; 100ng/ml M-LIF). After three days cells were counted and 150 000 were transferred onto new feeder layers. Doubling time was calculated. Values are mean of five consecutive measurements over a 15 day time period.

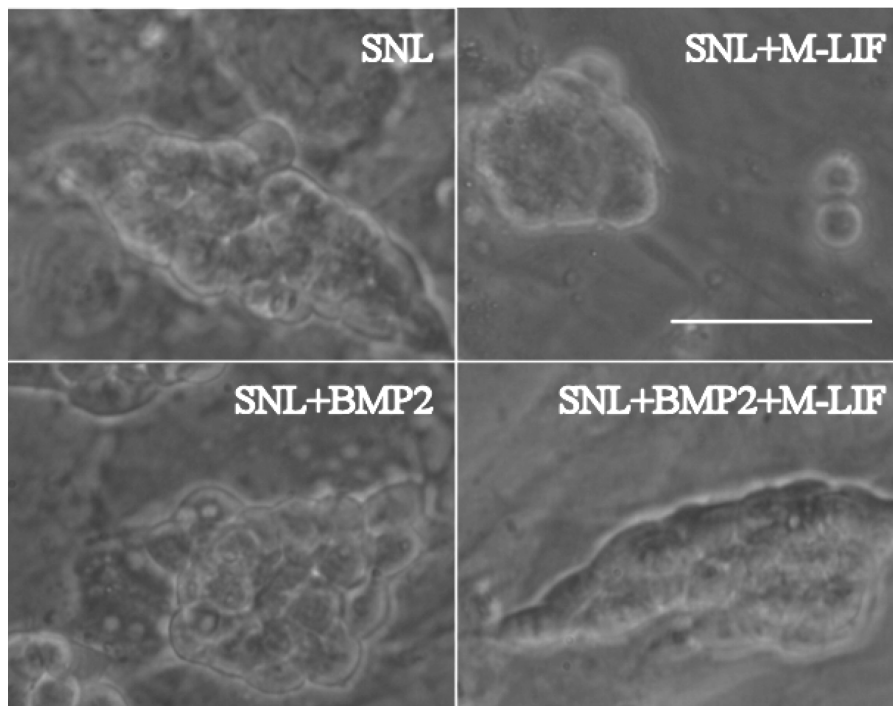


Figure 21 Influence of BMP2 and M-LIF on colony formation. 150 000 A5 CVPCs were seeded onto SNL76/7 feeder layers. BMP2 (100ng/ml) and M-LIF (100ng/ml) were added as indicated. Addition of either of these factors did not effect cell morphology. CVPCs still exhibited colony formation and round cell shape. Addition of BMP2 could not inhibit differentiation to endothelial cells in the gaps between colonies (bar=25µm)

4.2.3.2 Growth rate on gelatin

In the absence of LIF-secreting feeder layers, CVPCs started differentiating and formed cardiomyocytes, smooth muscle cells, and endothelial cells. This differentiation took place without the addition of external factors. The differentiation medium did not vary from growth medium. We wanted to test whether LIF alone is sufficient to maintain self-renewal and growth rate, or if other factors secreted by the SNL 76/7 feeder were essential.

Doubling time of CVPCs grown on gelatin was increased from $19.4\text{h} \pm 1.0$ when treated with M-LIF to $26.2\text{h} \pm 3.8$ in the absence of LIF (Table 4, Table 5). Addition of BMP2 alone could not maintain growth rate and doubling time was increased to $26.0\text{h} \pm 2.6$. In

comparison, addition of insect cell derived M-LIF could sufficiently keep doubling time (19.4 ± 1.0) at nearly the same level as of cells grown on feeder layers (18.7 ± 1.3). Combined addition of BMP2 and M-LIF showed the same effect as M-LIF alone (Figure 22). Since BMP2 alone could not maintain growth rate, M-LIF appeared to be the critical factor. Therefore, M-LIF seems sufficient for maintenance of self-renewal of CVPCs in the absence of feeder layers (Table 4, Table 5).

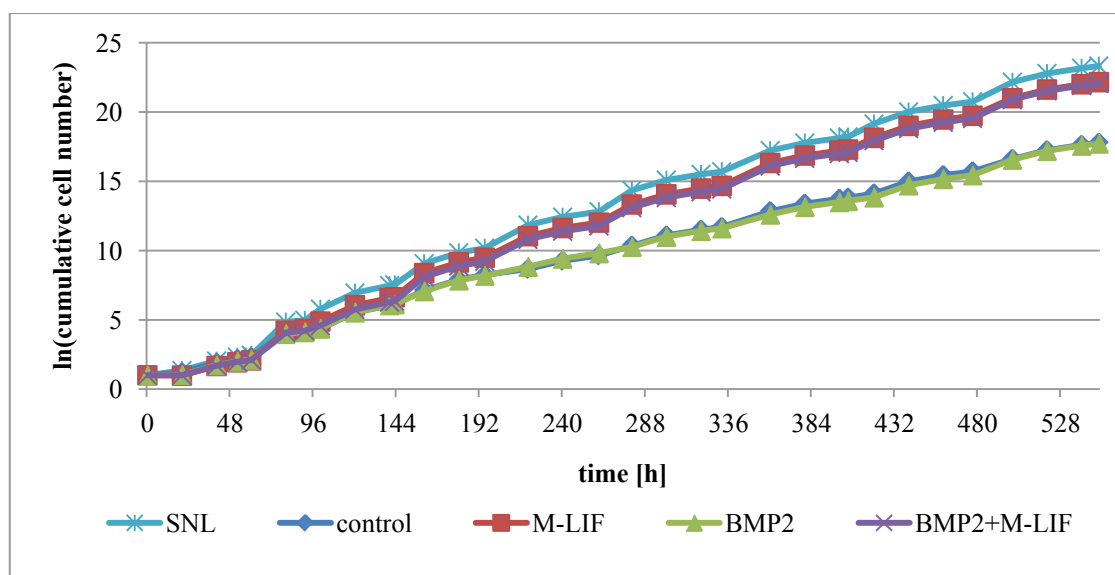


Figure 22 Growth curve of CVPC clone A5. CVPCs termed SNL were cultured on feeder layers. Cells termed control (untreated), M-LIF, BMP2, and BMP2+M-LIF were cultured on gelatin coated plates. 150 000 cells were seeded and counted every three days. After counting, the cell number was reduced to 150 000 and BMP2 (100ng/ml), M-LIF (100ng/ml) were added to cells as indicated. In the absence of LIF cell numbers steadily decreased.

CVPC treatment	Mean doubling time (h)	Standard deviation
control	26.2	3.8
M-LIF	19.4	1.0
BMP2	26	2.6
BMP2+M-LIF	19.1	0.9

Table 5 Doubling time of CVPC clone A5. CVPCs were grown on gelatin coated plates in the absence of feeder layers. 150 000 cells were seeded and treated as indicated (100ng/ml BMP2; 100ng/ml M-LIF). After three days cells were counted and 150 000 were replated on new feeder layers. Values are mean of five consecutive measurements.

Cell morphology of untreated cells did not differ from M-LIF treated cells with the notable exception that cell fragments were frequently observed in the absence of M-LIF (Figure 23). Therefore, the decrease in growth rate shown in Table 5 might not be solely due to a decelerated cell cycle or a decreased self-renewal. Cells died in the absence of M-LIF. After more than 30 days of culture on gelatin coated plates in the absence of LIF, the cell number was significantly decreased and confluence could no longer be reached on a 3T3 protocol (<20% cell density, data not shown). As seen in Table 6,

after 7 days the cell number in the M-LIF treated well (5.0×10^6) was more than 60fold the cell number in the untreated well (8.1×10^4). Viability of the LIF treated cells was higher than 90%, whereas the viability of control was 68%. Viability of BMP2 treated cells (82.8%) was increased compared to untreated cells. Therefore, BMP2 could save cells from cell death in the absence of LIF to a certain degree. Although frequent cell death was observed in BMP2 treated wells cell number was 28.4 fold higher than in untreated wells but 2.2 fold lower than in LIF treated wells after 7 days (Figure 23).

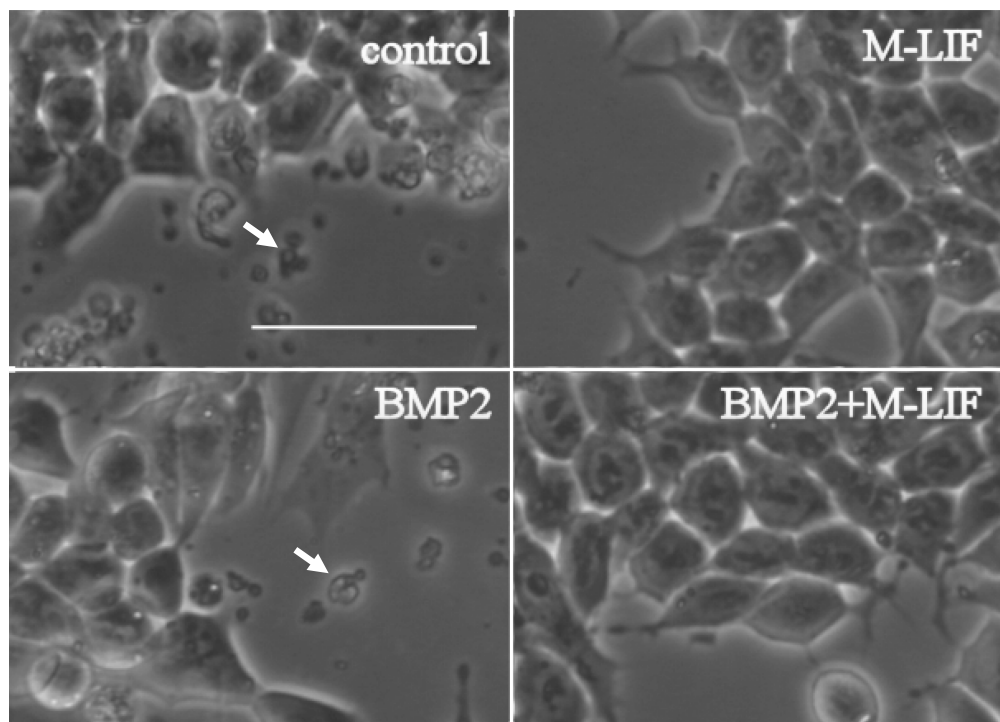


Figure 23 Growth of CVPC clone A5 on gelatin. CVPCs were treated with 100ng/ml BMP2 and/or 100ng/ml M-LIF in the absence of feeder layers as indicated. Both in the control well and the BMP2 treated well dead cells were detected frequently (white arrows) while fragments of dead cells were almost absent in wells supplied with M-LIF (bar=50μm).

CVPC treatment	Cell number/well		Viability (%)
	viable	total	
Control	5.5×10^4	8.1×10^4	67.9
+M-LIF	4.6×10^6	5.0×10^6	91.9
+BMP2	1.9×10^6	2.3×10^6	82.8
+BMP2+M-LIF	2.1×10^6	2.5×10^6	86.5

Table 6 Viability of CVPCs grown on gelatin. Equal cell numbers were seeded (150 000) and cells were cultured for 7 days under M-LIF, BMP2, M-LIF+BMP2 (100ng/ml) treatment of left untreated. Cell number was counted with a CASY counter.

4.2.3.3 Transfer from gelatin coated plates to feeder layers

Morphology of CVPCs instantly changed from round to angular shape upon transfer from feeder layers onto gelatin coated plates. This morphology change was not

influenced by the treatment with BMP2 or M-LIF. We were interested if this morphology change was reversible.

CVPC A5p12 grown on gelatin appeared angular and wide spread, independent of treatment with BMP2 and/or M-LIF (Figure 23). While not forming tight colonies, cells did aggregate. After two passages (5 days) on gelatin 4.5×10^5 cells were replated on feeder layers and treatment with M-LIF and/or BMP2 was stopped. Cells immediately adapted round cell shape similar to the cell shape they displayed from passage 1-11. Cells formed dense colonies, however, individual cells could be distinguished (Figure 24). Cell shape was round and no longer angular. Therefore, cell shape shift was reversible and cell shape adapted depending on matrix. Overall cell size appeared slightly increased. Single enlarged cells were visible (Figure 24). No difference in cell shape or size was detected between cells previously treated with BMP2 and/or M-LIF. Untreated cells showed a slightly increased number of enlarged cells.

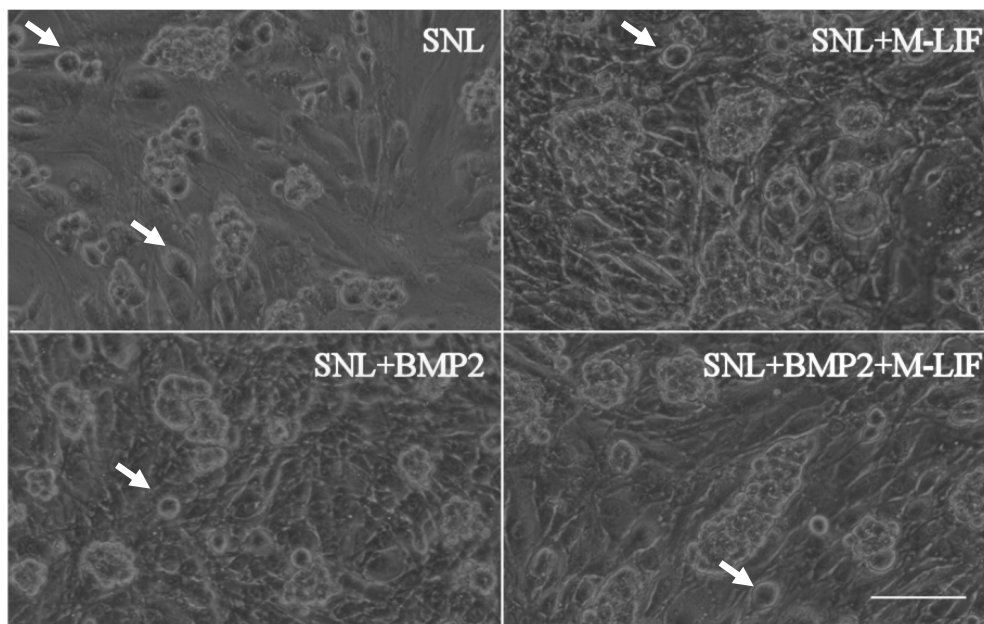


Figure 24 CVPC clone A5 previously cultured on gelatin coated plates. CVPCs were cultured on gelatin coated plates for 5 days, splitting took place on day 2 of culture. CVPCs were treated with 100ng/ml BMP2 or 100ng/ml M-LIF in the absence of feeder layers, as indicated. After 5 days 450 000 cells were transferred back onto feeder layers and pictures were taken the following day. White arrows mark enlarged cells. Untreated cells grown on feeder layers are termed SNL (bar=100µm).

4.2.4 SPARC secretion

Parietal endoderm-secreted SPARC was found to positively influence cardiomyogenesis in mESCs (Stary et al., 2005). Our primary goal was the enhancement of cardiomyogenesis in CVPCs. Therefore, we wanted to test the effect of recombinant SPARC on CVPCs. We performed Dot Blot analysis in order to determine whether CVPCs express and secrete SPARC.

The supernatant of CVPC clone A5 was taken 2 h and 24 h after medium change in order to see if SPARC secretion was a fast or slow process. Figure 25 shows that SPARC was already secreted at a high level 2 h post medium change and was only slightly more enriched in the supernatant during the next 22h.

Parietal Endoderm (PE) is known to secrete SPARC at high levels. Therefore, we compared PE supernatant to CVPC supernatant. SPARC levels were approximately equal. Using known quantities of recombinant SPARC as reference, the SPARC secretion of CVPC clone A5 was determined to be approximately 2 $\mu\text{g/ml}$ under regular culture conditions in 70% confluent wells (Figure 25).

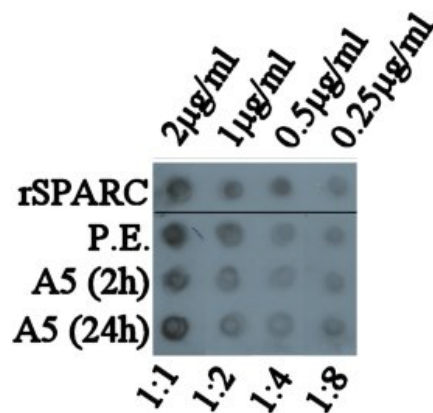


Figure 25 Dot Blot analysis of supernatant. Recombinant SPARC (2 $\mu\text{g/ml}$, 1 $\mu\text{g/ml}$, 0.5 $\mu\text{g/ml}$, and 0.25 $\mu\text{g/ml}$), supernatant of CVPC clone A5 2 hours and 24 h after feeding and supernatant of parietal endoderm were applied on nitrocellulose membrane, respectively. Supernatants were diluted 1:1, 1:2, 1:4, and 1:8 in M15Hy. 1st antibody used was anti-SPARC (1:300) and 2nd antibody was anti-rabbit HRP (1:20 000).

Dot Blot analysis showed secretion of SPARC by CVPCs. Further, we visualized intracellular SPARC in CVPC clone A5 by immunofluorescence. Therefore, in CVPCs SPARC protein can be found both in the intracellular and the secreted form. This stands in contrast to mESC cell line AB2.2 which was found to express SPARC on mRNA level but does not secrete SPARC protein (Stary et al., 2005)

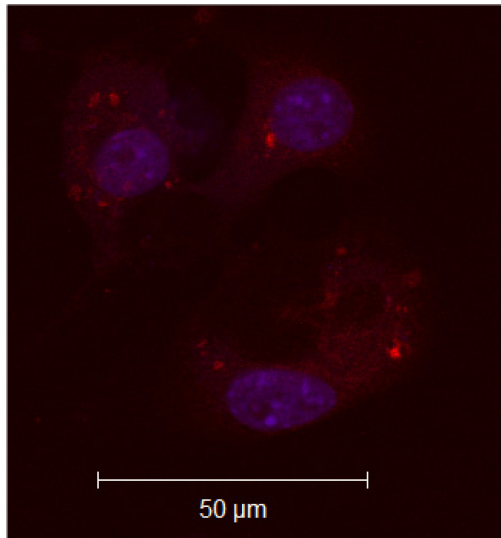


Figure 26 Immunofluorescence of SPARC. SPARC is distributed throughout the cytoplasm of CVPC clone A5. SPARC was detected by anti-SPARC antibodies and with Texas Red-labeled secondary antibodies.

4.3 Differentiation

4.3.1 Monolayer model system

Transfer of mESCs AB2.2 to gelatin coated plates resulted in differentiation, but beating cardiomyocytes were never observed. Upon transfer of CVPCs onto gelatin coated plates they started differentiating into the three cell types of the murine heart, namely cardiomyocytes, smooth muscle cells, and endothelial cells (Figure 27).

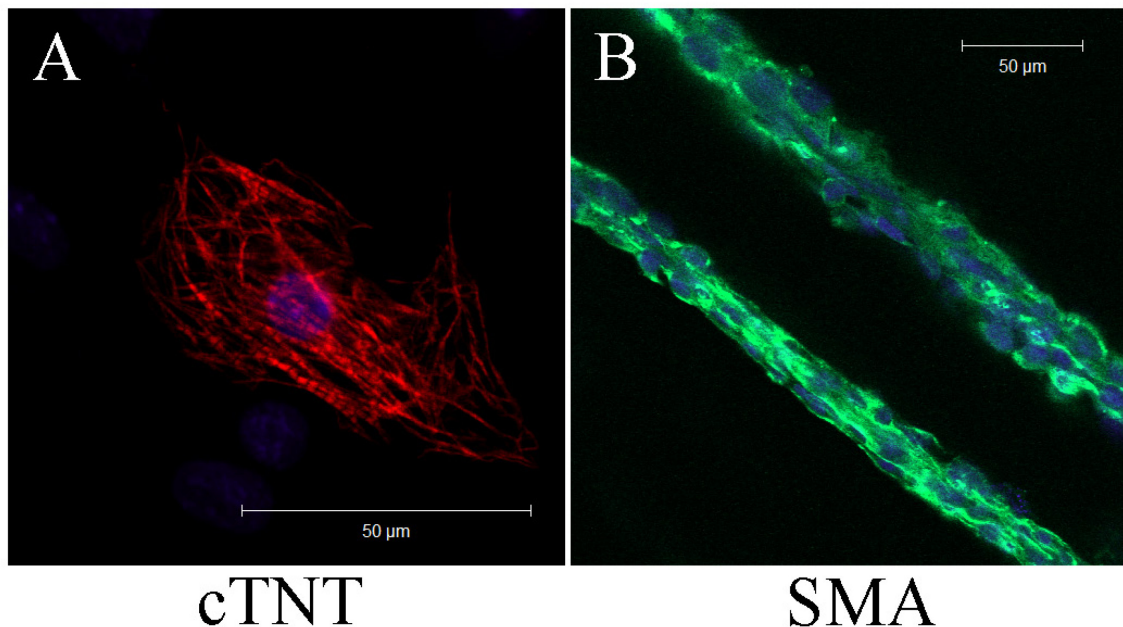


Figure 27 Immunofluorescence labeling of CVPC clone A5. Differentiated CVPCs were separated by trypsinization and fixated on slides. Cardiomyocytes were visualized with anti-cardiac Troponin T antibodies (A). CVPCs were left to differentiate and once smooth muscle contraction was observed, stained with anti-smooth muscle actin antibodies (B).

Cardiomyocyte formation of CVPC was chaotic and the number varied highly between experiments. Smooth muscle cells formed in close proximity to cardiomyocytes and eventually displaced them. Smooth muscle cell formation was very limited and not observed at all times. Since we were not further interested in smooth muscle cell formation but in enhancement of cardiomyogenesis, we set our primary goal in the refinement of differentiation conditions in order to achieve a higher yield of cardiomyocytes.

4.3.1.1 Optimization of differentiation conditions

4.3.1.1.1 Choice of culture plates

We first performed differentiation experiments in 6 well plates at a cell density of 110 000 or 150 000, respectively. As seen in Figure 28, only small areas of the 6 well plates showed signs of differentiation, most of the beating clusters were found around the edges of the culture dishes. Beating cardiomyocyte clusters were rarely observed in the center of the culture dishes. Endothelium formation was predominant. The number of beating clusters was extremely low under the applied differentiation conditions (Figure 29). Since clone A5 seemed the most promising in terms of cardiomyocyte formation, we selected it for further analysis along with clone H3 and G3 which showed intermediate and very low number of cardiomyocytes, respectively. Even though cardiomyocyte formation was not abundant, all eleven clones proved positive for it, whilst mESC cell line AB2.2 did not form cardiomyocytes in this or any of the following experiments under monolayer differentiation conditions.

Since we saw most cardiomyocyte formation at the rim of the culture dishes we switched to 24 wells, hoping we could control conditions better in smaller areas.

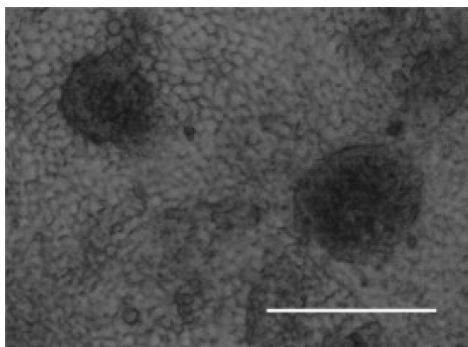


Figure 28 CVPC monolayer differentiation. CVPC clone A5p24 is shown on day 11 after seeding. Dark areas represent areas likely to form cardiomyocytes, areas in between are covered in endothelium (bar=300µm).

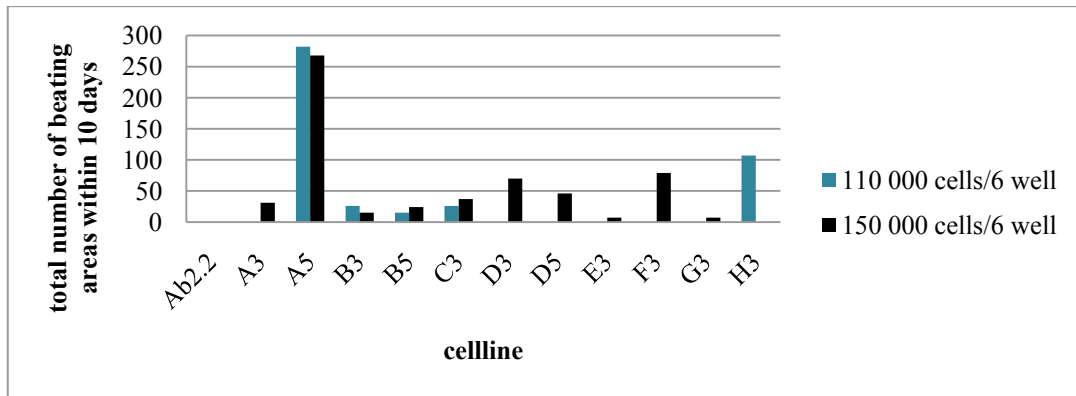


Figure 29 Monolayer differentiation. Total number of beating cardiomyocyte clusters observed over a ten day time period are shown. 300 000 cells were cultured on gelatin coated 24 well for four days prior to seeding onto 6 well plates. 110 000 and 150 000 cells were seeded onto gelatin coated 6 well plates, respectively, and fed every other day, as needed. mESC cell line AB2.2 did not show cardiomyocyte formation.

4.3.1.1.2 Cell number

Seeding cell number was a critical factor as cell-cell contact between CVPCs influenced their differentiation behavior. When the cell number was too high, plates grew over-confluent and cells remained mainly undifferentiated; when the cell number was chosen too low the plates never grew confluent and differentiation hardly took place (Figure 30).

Cells were seeded onto gelatin coated 24 well plates on day zero and clusters of beating cardiomyocytes were observed by day 18 (Figure 30). The number of beating clusters was significantly enhanced compared to our preliminary experiments performed on 6 well plates. In the 6 well system we detected less cardiomyocytes within 10 days than in the 24 well system on 1 day (Figure 29, Figure 30). Beating cardiomyocyte clusters were no longer restricted to the edges of culture wells but were evenly distributed. Cell numbers between 16 000 and 30 000 cells turned out to be the most promising and were adapted for further experiments.

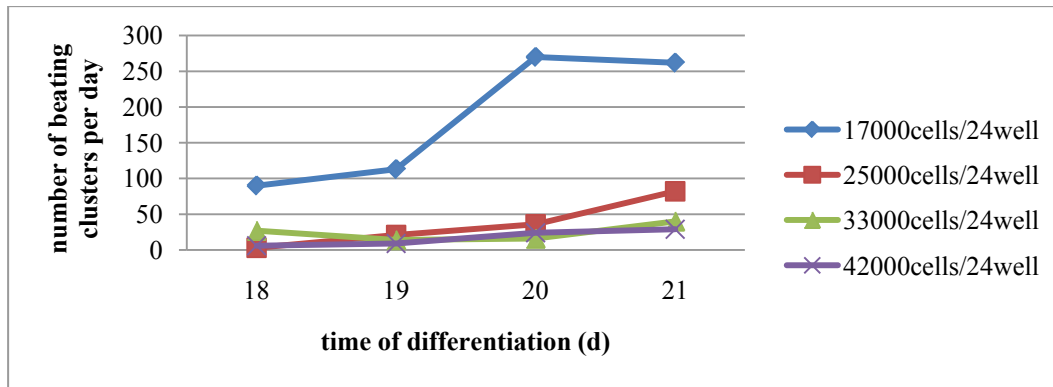


Figure 30 Monolayer differentiation. Indicated cell numbers of CVPC clone A5p15 were seeded onto gelatin coated 24 wells and left to differentiate. Partial (50%) medium replacement took place daily starting day 10. First beating cardiomyocyte clusters were observed on day 18 of differentiation. Wells with the lowest starting number developed the highest number of beating cardiomyocytes.

4.3.1.1.3 Feeding protocol

Higher rates of beating cardiomyocytes were achieved when the medium was only changed partially (data not shown). In order to supply cells with enough nutrients we decided on replacing approximately half of the growth medium every 24h. The first day of medium change depended on the number of seeded cells but was usually between day 3 and 6.

Figure 31 shows the influence of medium change on differentiation. Feeding started on day 5 instead of day 10 (Figure 30, Figure 31), therefore enough nutrients were supplied at all times. A tenfold increase in cardiomyocyte formation was observed when feeding protocol was altered. Partial medium change was continued from then on and percentage of changed growth medium increased every other day as needed.

While CVPC clone A5 needed a higher starting number of around 30 000 cells per 24 well in order to form a maximum of cardiomyocyte clusters (>6000), the same cell number produced much fewer cardiomyocyte clusters (<1000) in clone H3. CVPC clone H3 tended to stay undifferentiated when cell numbers over 20 000cells /24 wells were used. When H3 was seeded at a density of 30 000 cells even after 43 days of differentiation these cells still appeared almost entirely undifferentiated (Figure 32). Therefore, the two CVPC clones A5 and H3 required a different seeding cell number in order to produce a maximum of cardiomyocytes.

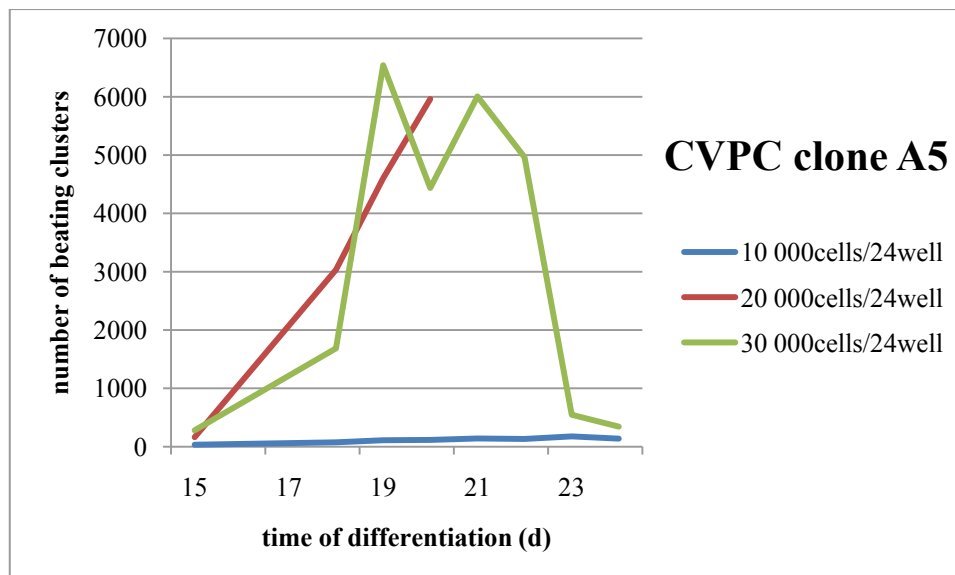
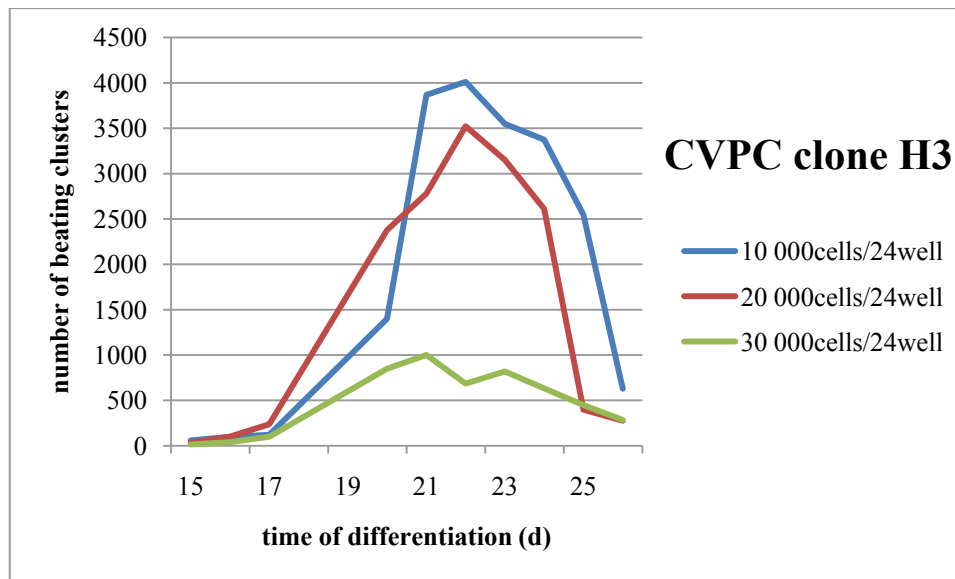


Figure 31 Monolayer Differentiation of CVPC clones A5 and H3. Cells were seeded at 10 000, 20 000, and 30 000 cells per 24 well in 1ml of growth medium, 1 ml of growth medium was added to 20 000 and 30 000 wells on day 5 and to 10 000 on day 7. 1 ml of growth medium was changed daily thereafter. CVPC clone H3 required lower cells numbers to form a maximum of cardiomyocytes, while clone A5 required higher numbers.

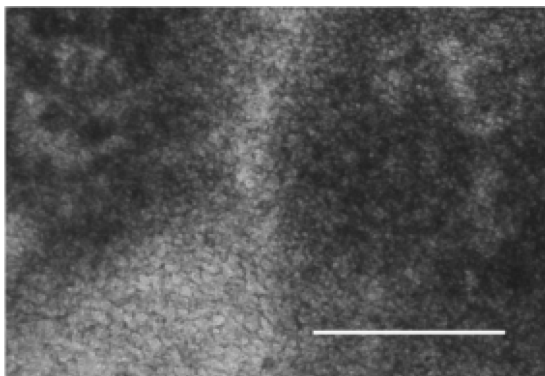


Figure 32 Monolayer Differentiation of CVPC clone H3. CVPC clone H3 was seeded at a density of 30 000 cells/24 well and left to differentiate, medium change took place daily after day 6, on day 5 one ml of growth medium was added. After 43 days of differentiation cells still appear undifferentiated and only low number of cardiomyocytes had formed (bar=300μm).

4.3.1.2 Enhancing cardiomyogenesis

Our main goal was to find ways to direct differentiation towards cardiomyocyte formation and away from smooth muscle and endothelium formation.

Since we already determined that M-LIF was sufficient for self-renewal we decided to culture CVPC clone A5 on gelatin coated plates in the presence of M-LIF. We reasoned that this limits influence of other substances secreted by the feeder cells on CVPCs. Further, we treated A5 with BMP2 and BMP2 combined with M-LIF, respectively. As control we continued to grow CVPC clone A5 on feeder layers. After 30 days of culture under the above described conditions on a 3T3 protocol we let cells differentiate.

Cells treated with BMP2 showed decreased cell survival during the 30 day treatment period (Figure 22). Upon differentiation BMP2 treated cells showed delayed formation and drastically reduced number of cardiomyocyte clusters (Figure 33). Only few and very small areas showed beating cardiomyocytes.

Cells that were treated with M-LIF and not exposed to any other substances secreted by feeder cells, formed twice the number of cardiomyocytes compared to CVPCs cultured on feeder layers. Onset of cardiomyogenesis was enhanced by 2 days. M-LIF treated A5 formed cardiomyocytes to an extent we did not observe before in the monolayer system (Figure 33).

Pre-conditioning CVPCs with a combined treatment of BMP2 and M-LIF for 30 days before differentiation resulted in an onset of cardiomyogenesis and a number of cardiomyocytes comparable to control level.

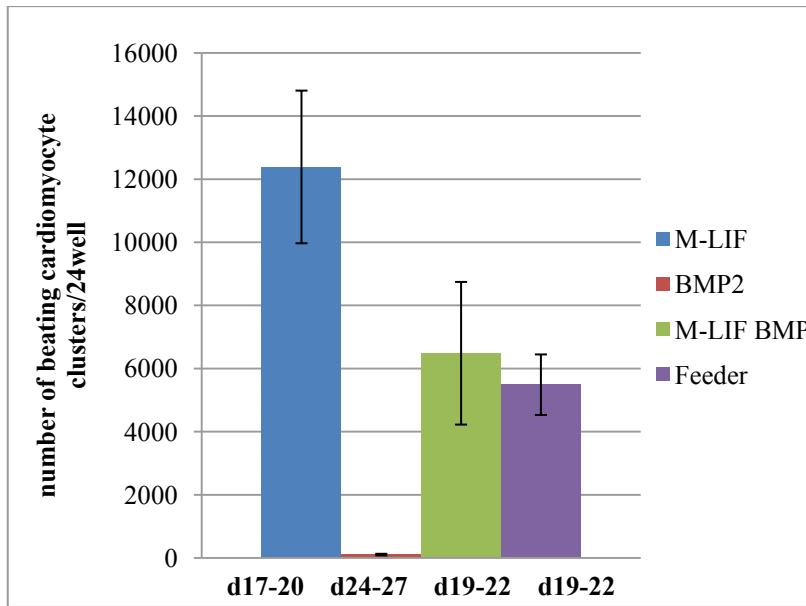


Figure 33 Maximum beating cardiomyocyte clusters of CVPC clone A5. A5 cells were seeded on gelatin coated 24 wells and treated with 0.1 $\mu\text{g/ml}$ M-LIF, 0.1 $\mu\text{g/ml}$ BMP2, or both BMP2 and M-LIF, respectively. Splitting occurred once cells were at least 70% confluent. After 31 days of treatment, 30 000 cells were seeded on gelatin coated 24 wells and partial medium change took place whenever necessary. Cells termed “Feeder” were cultured on feeder layers previous to seeding (30 000/gelatin-coated 24 well). Number of beating clusters was counted daily. Average of the four consecutive days with the highest number of beating clusters was calculated. Time period of maximum cardiomyocyte development is indicated on x-axis. 30 days of BMP2 treatment prior to seeding delayed peak of beating clusters by 5 days and led to 52-fold decrease of beating cluster development compared to untreated cells (Feeder). LIF treatment resulted in a more than 2-fold increase of beating clusters and accelerated development by 2 days.

4.3.2 Cardiac Bodies

We could not sufficiently control monolayer differentiation and the degree of cardiomyogenesis varied between experiments. From mESC research we were familiar with the Embryoid Body (EB) system. Even though we did not expect CVPCs to be able to mimic embryogenic development in a way EBs do, we reasoned that aggregation of CVPCs without attachment to a culture dish might resemble in vivo conditions better.

Cardiac Bodies (CBs) were generated with CVPC clone A5. CBs were not fed daily but according to our standard protocol for EBs. CBs are larger in size and therefore more nutrients were required than supplied by the standard protocol. Beating intensity slowed down when nutrients were too low. Therefore, counted values of feeding days were not incorporated into the statistics but are shown in the appendix.

Figure 34 shows the percentage of CBs with beating cardiomyocytes on a daily basis. First beating cardiomyocytes were observed on day 11 and percentage of CBs with beating cardiomyocytes increased rapidly thereafter. By day 17 all CBs had formed beating cardiomyocytes. Onset of cardiomyogenesis in the monolayer system occurred on day 17 (Figure 31), 6 days later than in CBs.

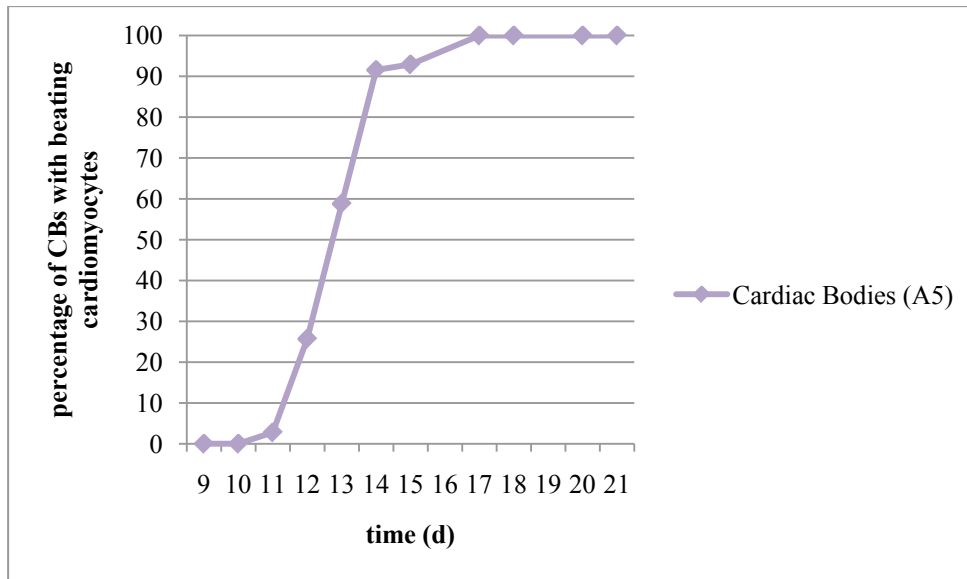


Figure 34 Cardiomyogenesis in CBs. A5p47 (4.4×10^4 cells/ml). Data is mean of 6 control wells. First beating cardiomyocytes were observed on day 11 of differentiation. By day 17 100% of CBs had formed beating cardiomyocytes. Values of feeding days were not included in figure but are given in the appendix (Table 11).

From preliminary experiments we knew that EBs form a horseshoe like structure shortly after attachment to culture plates and right before first cardiomyocytes appear. This structure was mostly absent in CBs. Sometimes horseshoe like structures could be detected but developed most likely by chance. CBs started spreading out after attachment (Figure 35) and increased in size faster than EBs (not shown).

CBs did not form first cardiomyocytes in one specific location as EBs do, in most cases several small beating clusters appeared at once. Around 5 days after first clusters could be observed, cardiomyocytes were spread out all over the CBs, often representing more than 50% of the CB area. Large areas of the CB contracted rhythmically, either in the same rhythm or a chain reaction. Cardiomyocytes of EBs are usually limited to the outskirts of EBs.

Smooth muscle cells were not observed in all CBs. Smooth muscle formation was triggered by unknown factors just like in the monolayer system. Within one culture dish, containing more than 50 CBs, only some CBs formed contracting smooth muscle cells while others did not. This is surprising since these CBs were exposed to the same growth medium. Percentage of CBs forming smooth muscle cells was always very low (<15%). Since smooth muscle formation was not our primary interest, we did not investigate this any further.

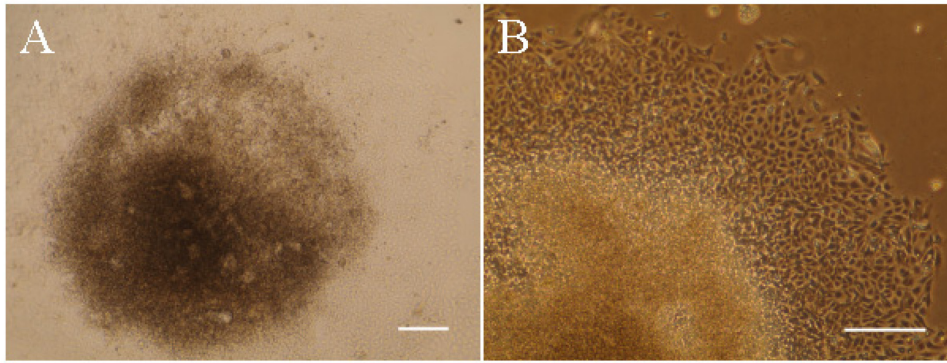


Figure 35 CB on day 7 of differentiation. CB 2 days after adhesion to culture plates (A). Cells around the edges of the CB start spreading out (B) (bar=300µm).

4.3.2.1 Influence of BMP2 and M-LIF on cardiomyogenesis in CBs

Behfar et al. (2002) showed that treatment of mESCs with BMP2 for 24 h prior to EB formation resulted in a more than twofold increase of contracting areas compared to EBs from untreated mESCs.

Rajasingh et al., 2007 reported that mESCs treated with a combination of LIF and BMP2 before injection into mice after an induced myocardial infarction showed typical cardiomyocyte morphology and striation pattern after 28 days

CBs were generated from CVPC clone A5. CBs were treated with M-LIF (100 ng/ml), BMP2 (100 ng/ml), anti-BMP2/4 antibodies (500 µg/ml), anti-LIF antibodies (1 µg/ml), and BMP2 and M-LIF combined, respectively. Substances were added once at beginning of each treatment period, no medium change took place during the treatment periods. Treatment periods were day 0-4.7, day 4.7-7, day 7-10, and day 10-13, respectively. Six control plates were left untreated.

4.3.2.1.1 Leukemia Inhibitory factor

Addition of M-LIF on day 0 resulted in the formation of CBs that were decreased in size and, upon attachment, cell spreading was severely hampered (Figure 36). First beating cardiomyocytes were observed on day 17 with a six day delay compared to control. By day 21, percentage of CBs with beating cardiomyocytes had still not reached 100% (Figure 37A).

M-LIF did not affect time of cardiomyocyte development or number of formed cardiomyocytes when added on day 4.7 (Figure 37). When supplied with M-LIF on day 7 or day 10 cardiomyocyte formation was delayed by 2 days (Figure 37C+ D) and, as seen in Figure 38, number of cardiomyocytes per CB was reduced.

Anit-LIF antibodies enhanced cardiomyogenesis when added on day 0 (Figure 37) and strongly increased the number of beating cardiomyocytes per CB when added on day 0, 4.7 or 10, respectively (Figure 38). Together, addition of anti-LIF antibodies on day 0 enhanced cardiomyogenesis, while addition of LIF delayed it.

When M-LIF and BMP2 were combined, effect of M-LIF was dominant (Figure 38, data not shown).

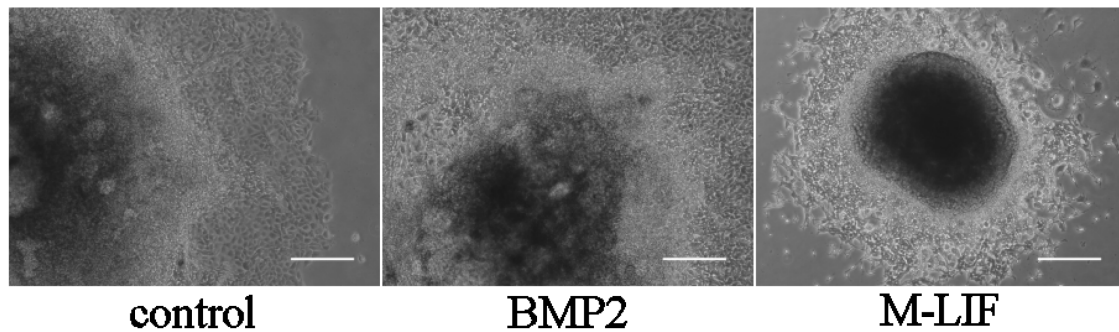


Figure 36 CBs on day 4.7 of development. CBs were treated with BMP2 (100ng/ml) or M-LIF (100ng/ml) between day 0 and day 4.7. M-LIF treated CBs showed decreased cell spreading and appeared undifferentiated in the center. Cardiomyogenesis was delayed. Morphology of BMP2 treated CBs cannot be distinguished from control (bar=300 μ m).

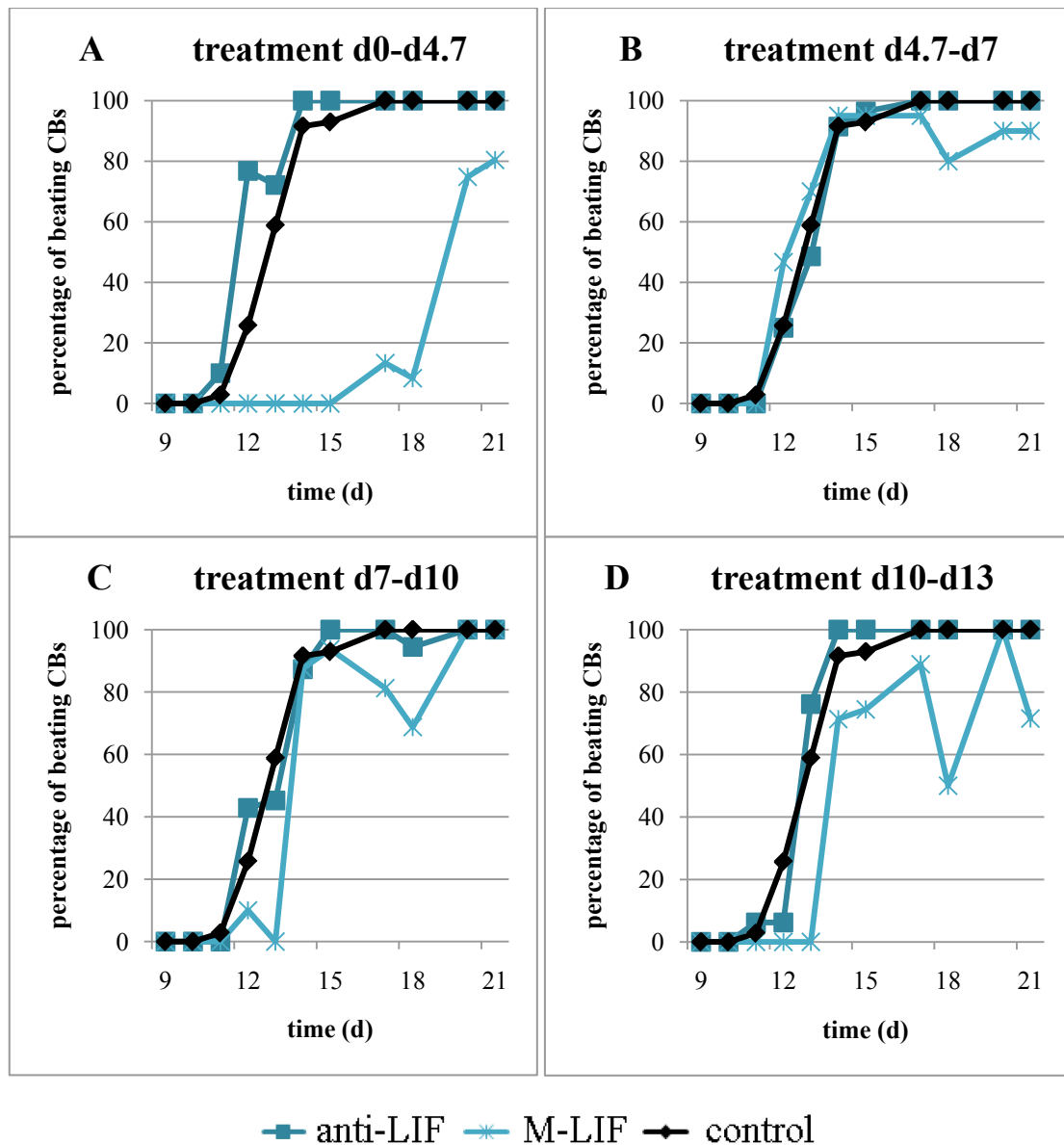


Figure 37 Influence of LIF on CB Development. Addition of M-LIF (100ng/ml) on day 0 (A) resulted in CBs severely reduced in size and delayed cardiomyogenesis, while addition on day 4.7 (B) showed no effect. Addition on day 7 (C) and 10 (D) cardiomyogenesis was delayed by 2 days. Addition of anti-LIF antibodies (1 μ g/ml) had a slightly positive effect on day 0 suggesting that an inhibition of LIF signaling is beneficial during the early stages of cardiomyogenesis.

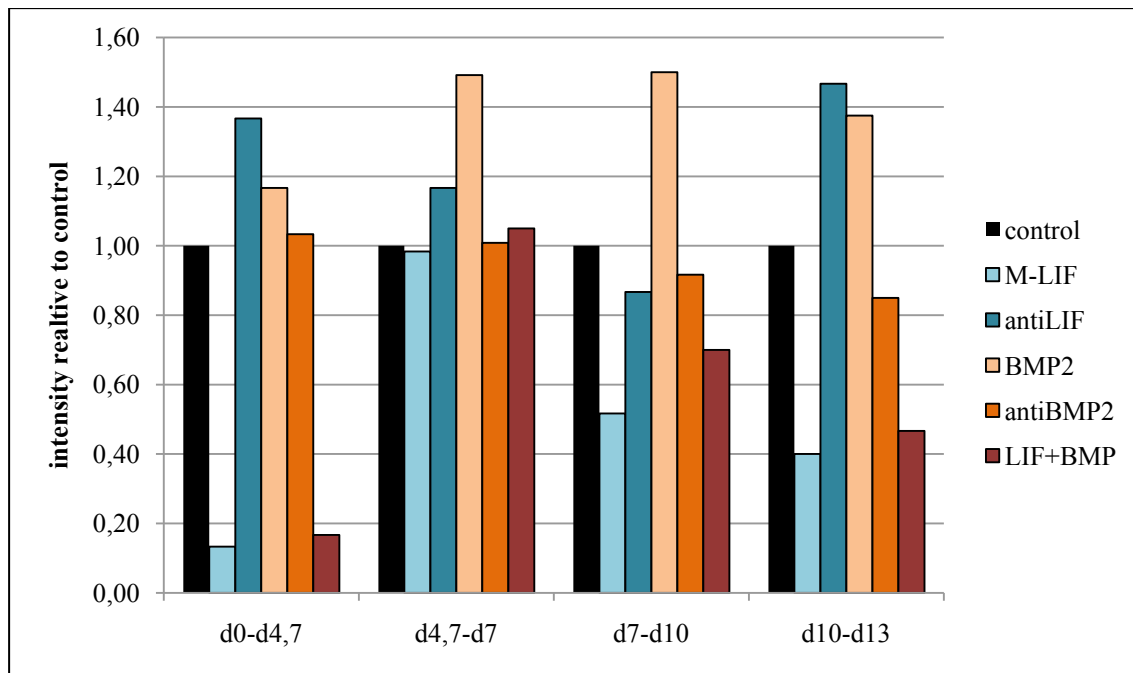


Figure 38 Beating intensity of CBs. Areas of beating cardiomyocytes per CB were quantified daily on a scale from 0 (no activity) to 6 (>70% of the area contracting). Values of day 0-20 were added and set relative to control. Data can be seen in appendix (Table 12). BMP2 treatment enhanced formation of cardiomyocytes per CBs strongly. Addition of anti-BMP2 antibodies slightly reduced number of cardiomyocyte clusters per CB. M-LIF had a negative effect on the number of formed cardiomyocytes. M-LIF and BMP2 combined had an effect similar to M-LIF treatment. Anti M-LIF antibodies increased the number of cardiomyocytes.

4.3.2.1.2 Bone Morphogenetic Protein 2

Addition of BMP2 had a positive effect on cardiomyogenesis at every tested time point of CB development. Not only did it enhance first cardiomyocyte appearance but it also led to a highly increased number of beating cardiomyocytes per CB (Figure 38).

BMP2 had the strongest effect on cardiomyogenesis when added on day 4.7 and day 7, respectively. In both cases 100% of CBs had formed beating cardiomyocyte clusters by day 13, 4 days earlier than in controls (Figure 39). As seen in Figure 38, the number of beating cardiomyocytes per CB was strongly increased in all cases.

Interestingly, anti-BMP2/4 antibodies only had a slight negative effect on the onset of cardiomyogenesis when added on day 0, 7, or 10 but had no effect when added on day 4.7 (Figure 39). The number of cardiomyocytes per CB was not influenced by anti-BMP2/4 antibodies when added on day 0 or 4.7 and was slightly attenuated when added on day 7 or 10 (Figure 38).

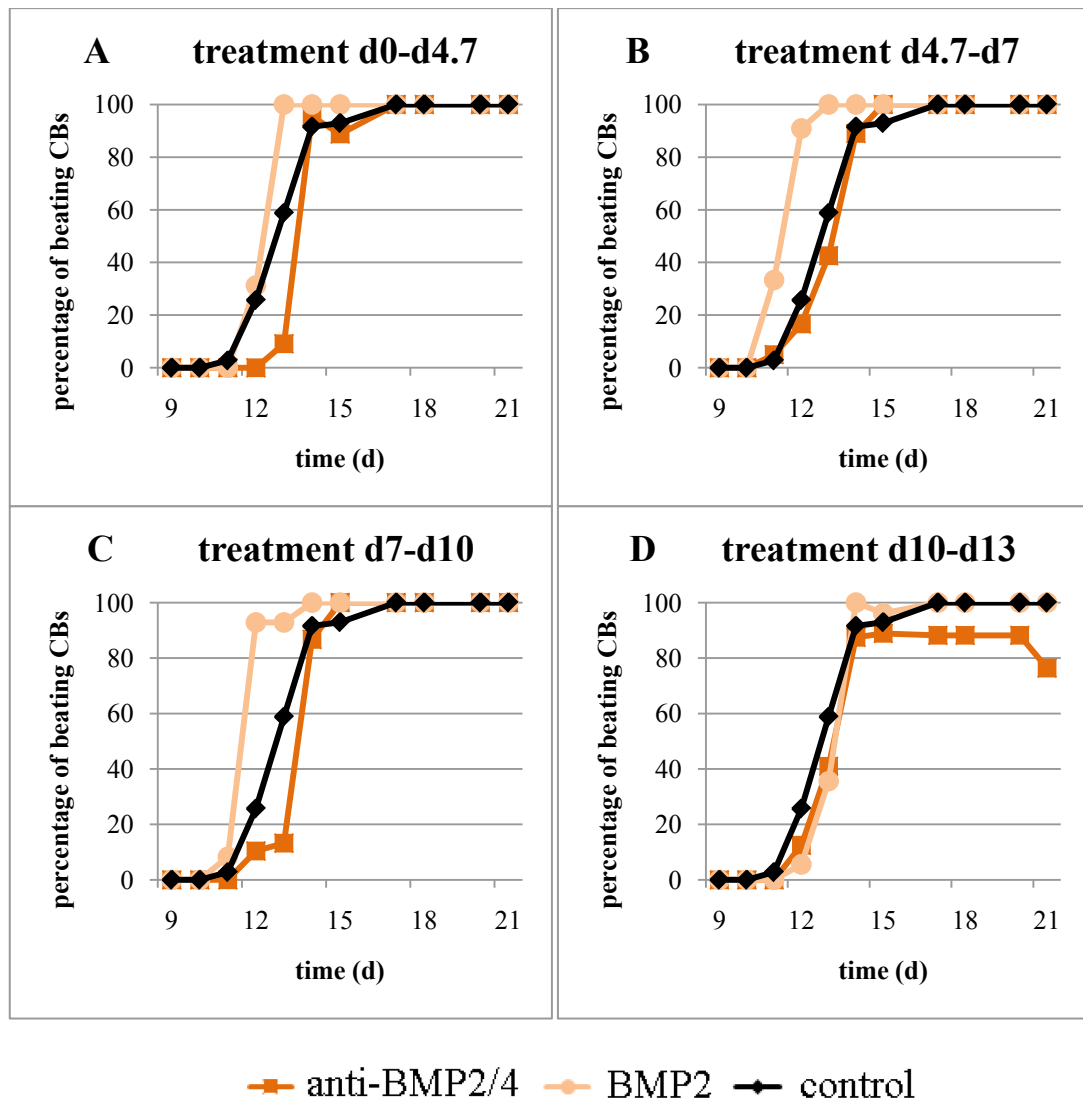


Figure 39 Influence of BMP2 on CB Development. BMP2 (100ng/ml) enhanced cardiomyogenesis when added on day 0 (A), 4.7 (B) or day 7 (C), while addition of BMP2/4 antibodies negatively influences cardiomyogenesis on these days. On day 10 BMP and BMP antibodies had no effect (D).

4.3.2.1.3 Influence of M-LIF and BMP2 on phosphorylation levels in CBs

We wanted to investigate which pathways are upregulated during CB development. Protein Lysats of CBs treated with BMP2, M-LIF, anti-LIF antibodies, and control were prepared. Cell lysis took place at the end of each treatment period (d4.7, d7, d10, d13 respectively). Cell number on day 4.7 was not high enough to balance protein level of Western Blots.

Phosphorylation of Stat3 was highly elevated in all CBs treated with M-LIF relative to overall Stat3 levels. CBs treated with anti-LIF antibodies showed a down regulation of pStat3 during treatment periods of d4.7-7 and d7-10. pStat3 levels were not altered between control and BMP2 treated CBs (Figure 40).

Different treatment of CBs did not influence phosphorylation of Erk1/2. pERK1/2 was upregulated in all CBs by day 13 (Figure 40).

pSmad1/5/8 was upregulated in CBs treated with BMP2 during a time period between d4.7-7, d7-10, and d10-13. No activation was detectable on day 4.7 when CBs were treated between d0-4.7.

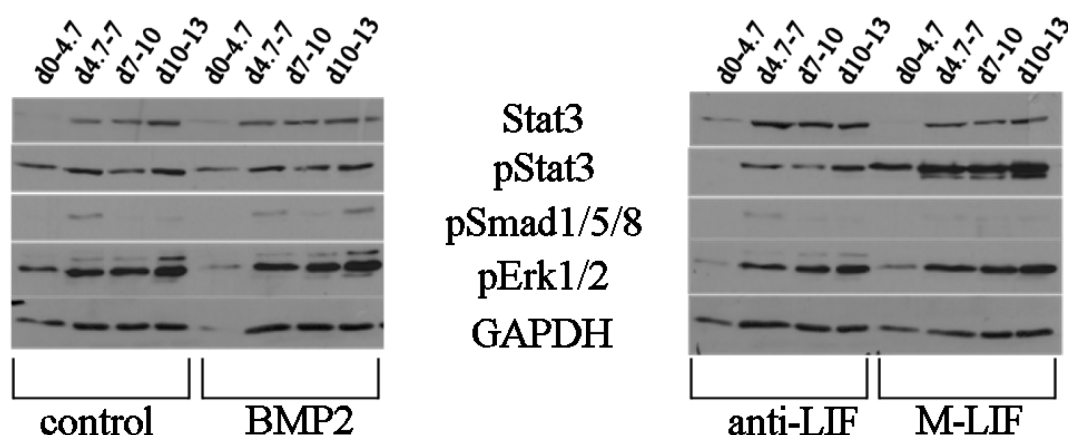


Figure 40 Western Blot of CB Lysats. CBs were treated with BMP2 (100ng/ml), M-LIF (100ng/ml) and anti-M-LIF antibodies (1 μ g/ml) from day 0-4.7, 4.7-7, 7-10, and 10-13, respectively. At the end of each treatment period, proteins were isolated. Western Blot analysis with Stat3, pStat3, pSmad1/5/8, pErk1/2 and GAPDH were performed. pStat3 is increased upon M-LIF treatment. BMP2 induces pSmad1/5/8 signaling. pErk2 is not influenced by treatment.

This suggests a role of BMP and MAPK signaling during CB development. Stat3 signaling seems to remain relatively stable during cardiomyogenesis.

4.3.2.2 *GSK3 inhibitor Chir99021*

In previous experiments we saw a positive effect of SPARC on cardiomyogenesis of CVPC clone H3 (H.Auner, unpublished data). Since SPARC was shown to signal via ILK, which in turn controls GSK3 activity, we had reason to believe that SPARC might signal through the Wnt pathway by affecting GSK3 activity. We therefore wanted to test if Chir99021, an inhibitor of GSK3, can mimic SPARC effect and enhance the number of cardiomyocytes. Since our preliminary experiments with Chir99021 were performed using CVPC clone H3, we continued with the same clone.

Cells were seeded onto gelatin coated plates to induce differentiation and were treated with Chir99021 (1.399 μ g/ml 1:1, 1:2 1:10, 1:50, 1:70, 1:100, 1:150) daily. After 24 days of treatment only dilutions higher than 1:50 had formed beating cardiomyocytes but the number was drastically reduced compared to control levels (data not shown). Since cells looked mainly undifferentiated (Figure 41A), we replated cells treated with 1.399 μ g/ml back onto feeder layers on day 20 of treatment. After three days cell

morphology (Figure 41B) was indistinguishable from untreated control cells grown on LIF-secreting feeder layers. In order to test whether these cells still had the potential to form cardiomyocytes we made CBs. In Figure 41C a CB is shown on day 4.5, morphology is round and shows no noticeable alterations. A CB after attachment on day 7 is shown in Figure 41D. CBs showed first beating cardiomyocytes on day 10 and by day 18 100% of CBs had formed beating cardiomyocytes. This means that the multipotency of CVPC potential was not decreased after 20 days of culture in the presence of GSK3 inhibitor Chir99021. Continuous Chir99021 treatment and consequential inactivation of GSK3 could not induce cardiomyogenesis but rather blocked it. Whether a time restricted inactivation of Chir99021 enhances cardiomyogenesis remains to be determined.

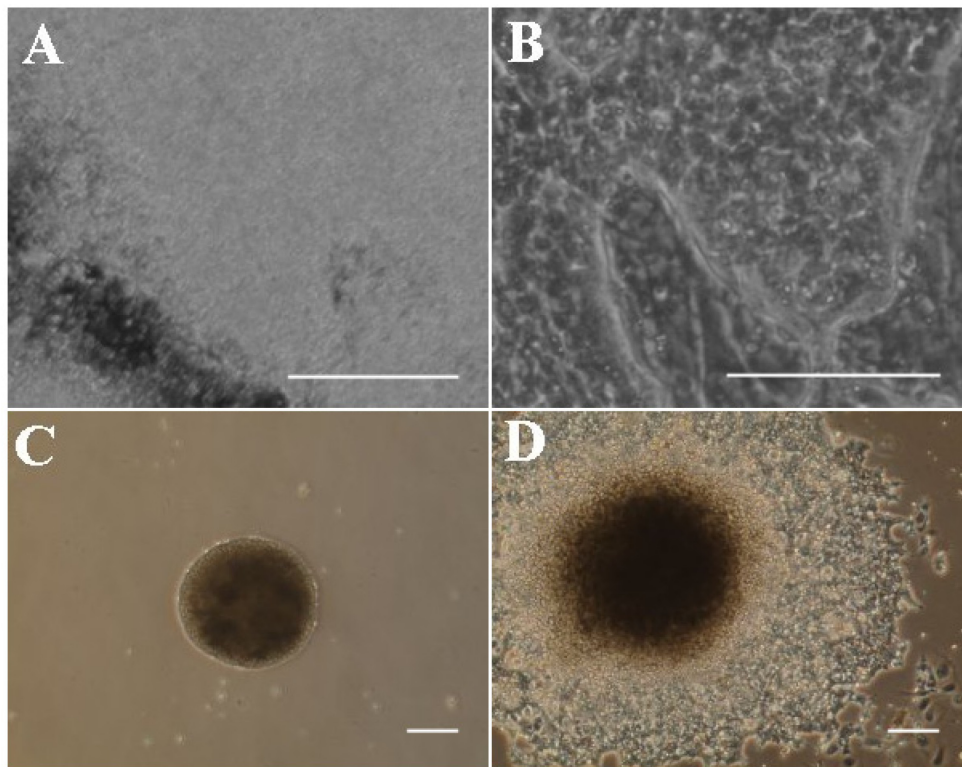


Figure 41 Chir99021 treated CVPCs. CVPC clone H3 after 20 days of treatment with 1.399 $\mu\text{g/ml}$ Chir99021 on gelatin (A). Cells three days post transfer of cells from (A) onto feeder layers are shown in (B). Normal colony formation took place. Cells could not be distinguished from control wells grown on feeder layers. Cells from (B) were used to create CBs. (C) shows CB on day 4.5, (D) on day 7 (bar=200 μm (A), bar=100 μm (B), bar=300 μm (C,D)).

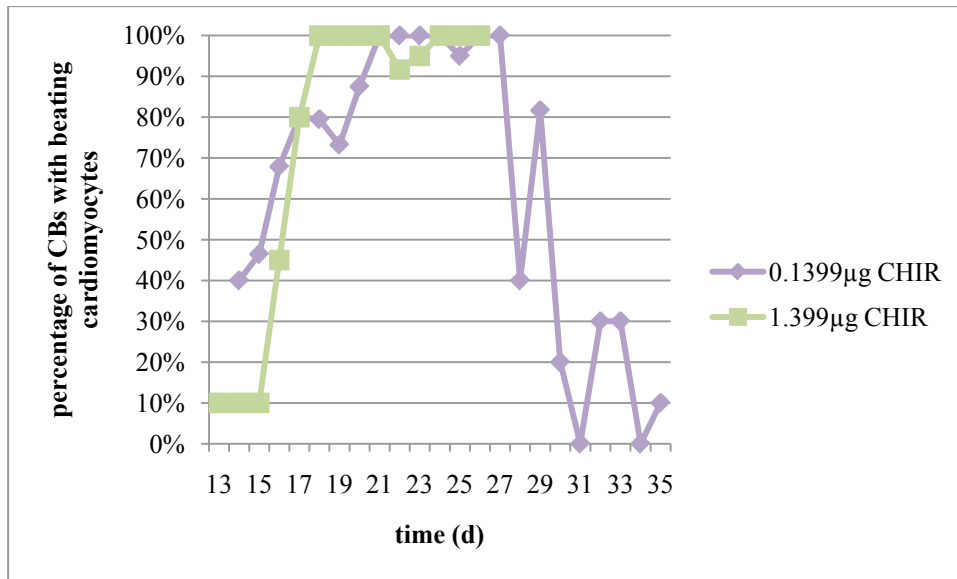


Figure 42 Cardiomyocyte formation of Chir99021 treated cells. CBs were generated from CVPC clone H3. Cells were previously treated with 1.399 µg/ml for 20 days and 0.1399 µg/ml for 11 days, respectively. All CBs were able to form beating cardiomyocytes.

4.4 Transfections

Nkx2.5 is an early marker for cardiomyogenesis. Therefore, we wanted to use Nkx2.5 expression as a reporter system for differentiation towards the cardiac lineage in CVPCs. We used a Plasmid containing a Luciferase reporter gene under the control of the promoter region of the Nkx2.5 locus (NKE24).

4.4.1 Establishment of a transfection protocol

We first had to establish a transfection protocol for CVPCs since we had not performed transfections in these cells before. Therefore, we used the pGM3-EGFP-Vimentin plasmid so we could visually detect GFP expression by a Zeiss Axio Cam MR Plus without performing luciferase assays.

4.4.1.1 Cell density

According to supplier information cells should be between 90-95% confluent on day of transfection with Lipofectamine 2000, and a cell number of $0.5-2 \times 10^5$ was suggested. Since CVPCs are much smaller than fibroblasts we chose 1×10^5 , 5×10^5 , 1×10^6 , and 5×10^6 cells. Only 5×10^6 cells reached 95% cell density by time point of transfection. When 1×10^5 cells were seeded, wells were still not confluent 48h after transfection, while all the other wells were. Although GFP expression was detectable in all test transfections, the strongest GFP expression was detectable when the smallest starting cell number was chosen. GFP expression and detection was impeded when cell number was too high, lower cell numbers made it easier to detect GFP signal (Table 7).

GFP expression was detectable 24 h post stop of Lipofectamine 2000 reaction and increased within the next 24 h. After 48 h, expression reached a maximum and after approximately 72 h, expression started decreasing (Table 7). For all further luciferase experiments $1-1,5 \times 10^5$ cells/24 well plate were chosen as standard cell number and 48 h post transfection-stop was chosen as point of detection.

Cell number	Cell density after 24 h	Transfection efficiency (24 h)	Cell density after 48 h	Transfection efficiency (48 h)
1×10^5	30-40%	+++	60-70%	++++
5×10^5	>60%	+++	>95%	++
1×10^6	>80%	+	>95%	++
5×10^6	>95%	+	>95%	+

Table 7 pGM3-EGFP-Vimentin Lipofectamine 2000 Transfection of CVPC Clone A5. Lipofectamine 2000 to DNA Ratio used was 4:1. Transfection efficiency increased overtime and had a maximum around 48 h post transfection and slowly decreased thereafter. High cell density strongly influenced transfection efficiency. Transfection efficiency was determined by estimating the ratio of GFP positive cells to GFP negative cells.

4.4.1.2 DNA to Lipofectamine 2000 ratio

Once a suitable cell number was established we had to choose an optimal DNA to Lipofectamine 2000 ratio. DNA (μg) to Lipofectamine 2000 (μl) was suggested to be chosen between 1:2 and 1:3, the minimum being 1:0.5 and the maximum being 1:5. We therefore chose 1:3, 1:4, and 1:5 as our test concentrations.

Figure 43 shows fluorescence and phase contrast images of clone A5p14 transfected with pGM3-EGFP-Vimentin. No difference in transfection efficiency between varying Lipofectamine 2000 concentrations could be detected after 28h and 52h. We therefore chose to lower DNA to Lipofectamine 2000 ratio even further. We next tried ratios of 1:0.5, 1:1, 1:2, and 1:3. Within the latter three no significant variance of transfection efficiency could be detected, only ratio of 1:0.5 yielded less GFP positive cells (data not shown). A DNA to Lipofectamine 2000 ratio of 1:1 was set as standard for further transfections.

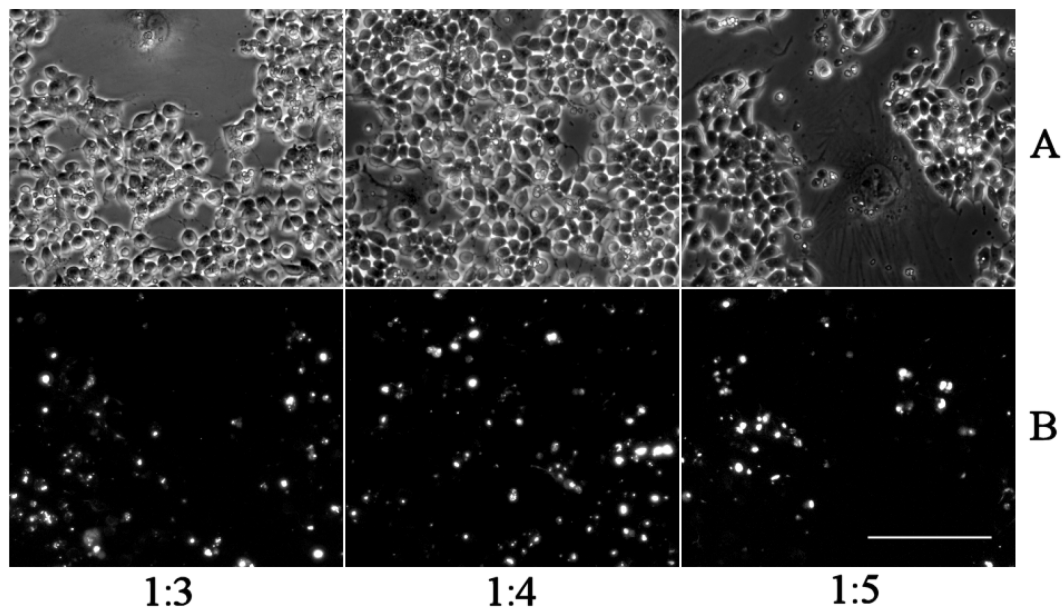


Figure 43 pGM3-EGFP-Vimentin transfection. Phase contrast images (A), GFP fluorescence (B) after 52h. 1.5×10^5 cells were seeded. The DNA to Lipofectamine 2000 ratio is indicated at the bottom. Transfection efficiency did not increase when the ratio was increased but stayed stable (bar=50 μ m).

4.4.1.3 Medium conditions

First we performed transfections with 1 ml medium per 24 well. Since less medium means a higher chance of reagent-cell interaction, the medium volume was lowered step wise to eventually 500 μ l per well. Lipofectamine 2000 can be toxic for cells and reaction should not continue over night. Medium change was suggested to take place between 4 h and 6 h post transfection. We did not detect a significant difference between 3 h and 6 h of incubation and therefore 3 h were chosen as standard protocol (data not shown).

In summary, standard protocol was chosen to contain a DNA to Lipofectamine 2000 ratio of 1:1, transfection time was set to 3 h in 500 μ l medium, and detection was carried out 48 h post transfection stop.

4.4.2 Monitoring Nkx2.5 expression

4.4.2.1 Dilution

We used the sea pansy (*Renilla reniformis*) luciferase to determine transfection efficiency. Renilla values were often too high to be within the linear detection interval and therefore samples had to be diluted. In order to test whether dilution of samples alters measured values we performed a dilution assay. Two wells were transfected with NKE24 and Renilla according to standard protocol. NKE24 carried a luciferase reporter gene under the Nkx2.5 promoter region. Samples were diluted 1:7.5, 1:22.5, and 1:45.

Values are mean of the duplicates. The purple graph shows mean value and standard deviation of all six measurements (Figure 44).

Firefly to renilla ratio is slightly influenced by dilution protocol but standard deviation between all 6 measurements was very low ($\sigma=0.004$). Therefore, we set a dilution of 1:36 as standard.

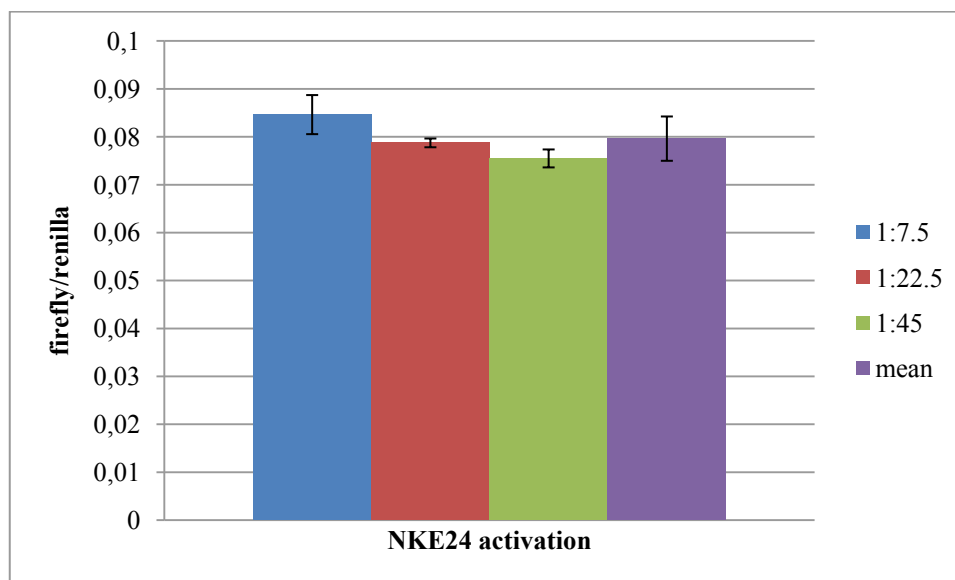


Figure 44 NKE Expression. CVPCs A5p22 were transfected with the NKE24 plasmid. NKE24 to renilla ratio was chosen to be 1:100. Lysats were diluted in Lysis Buffer as indicated ($x=0.08\pm0.004$).

4.4.2.2 Influence of GFP expression on Luciferase assays

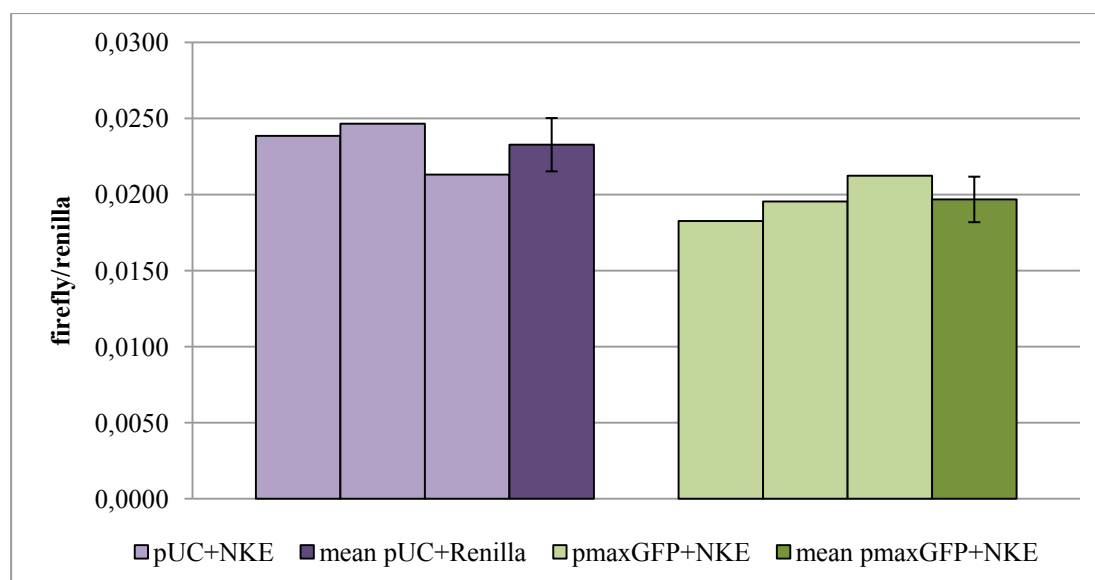
From transfection experiments with other cell lines we had reason to believe that plasmids encoding a green fluorescence protein (GFP) somehow interfered with renilla and firefly detection. We therefore wanted to test whether these assumptions held true in CVPCs. As seen in Table 8 GFP excitation spectra are rather close to firefly and renilla emission spectra. Therefore, we wanted to ensure that GFP does not interfere with the detection method.

To test our theory we used the pmaxGFP plasmid, which encodes GFP under a CMV (Cytomegalovirus) promoter. Cells were transfected with pmaxGFP, NKE24, and pUC, respectively.

	emission	excitation
Monster GFP (Promega)	515nm	480-505nm
pEGFP	507nm	488nm
firefly	560nm	
renilla	475nm	

Table 8 Emission scale. Excitation spectra of GFP are close to emission levels of firefly and renilla. We were concerned that there might be an interference with renilla and firefly emission. Monster GFP is derived from *Montastrea cavernosa* (Great Star Coral).

As seen in Table 9, renilla values were strongly reduced when GFP plasmid was co-transfected, which went hand in hand with previous results from other cell lines. This concerned us since renilla luciferase activity was our indicator of transfection efficiency. Firefly values were also reduced when GFP was co-transfected. Since the ratio of firefly to renilla between pmaxGFP and pUC transfection was not strongly influenced ($\sigma=0.0025$) we decided that we could continue transfection experiments.



NKE	Firefly (F)	Renilla (R)	FF/R	Mean	σ
+pUC	3990	167220	0,0239	0,0233	0,0018
	4440	180040	0,0247		
	5090	238890	0,0213		
+pmaxGFP	2150	117770	0,0183	0,0197	0,0015
	2000	102340	0,0195		
	2210	104050	0,0212		

Table 9 Influence of GFP on luciferase detection. Renilla values indicate transfection efficiency and are used to standardize measurements. Transfection efficiency was much lower when GFP plasmid was co-transfected. Ratio of firefly to renilla stayed approximately the same.

4.4.2.3 *Nkx2.5* expression in CVPCs

We transfected CVPCs with the NKE24 plasmid to monitor the *Nkx2.5* promoter activation by measuring the luciferase reporter gene levels. NKE24 encodes the luciferase gene under the control of the *Nkx2.5* promoter region (NKE). Control cells were transfected with a pGL3b plasmid encoding a luciferase gene without a promoter region (pGL3b).

Cells were transferred onto gelatin coated plates one day prior to transfection. Figure 45B shows NKE luciferase values relative to pGL3b luciferase values. The *Nkx2.5* promoter was indeed activated in CVPCs since luciferase activity of NKE transfected

cells was more than 30fold higher relative to pGL3b luciferase activity. Next we decreased the NKE24 Plasmid-DNA amount to 50% and used the pUC plasmid to compensate DNA decrease. This lead to a reduction of luciferase reporter gene activity by around 50%. This showed that an addition of a second plasmid to the Lipofectamine 2000 reaction had no negative influence on the reporter gene activity. In Figure 45A the firefly values of the same experiment are shown relative to the renilla values.

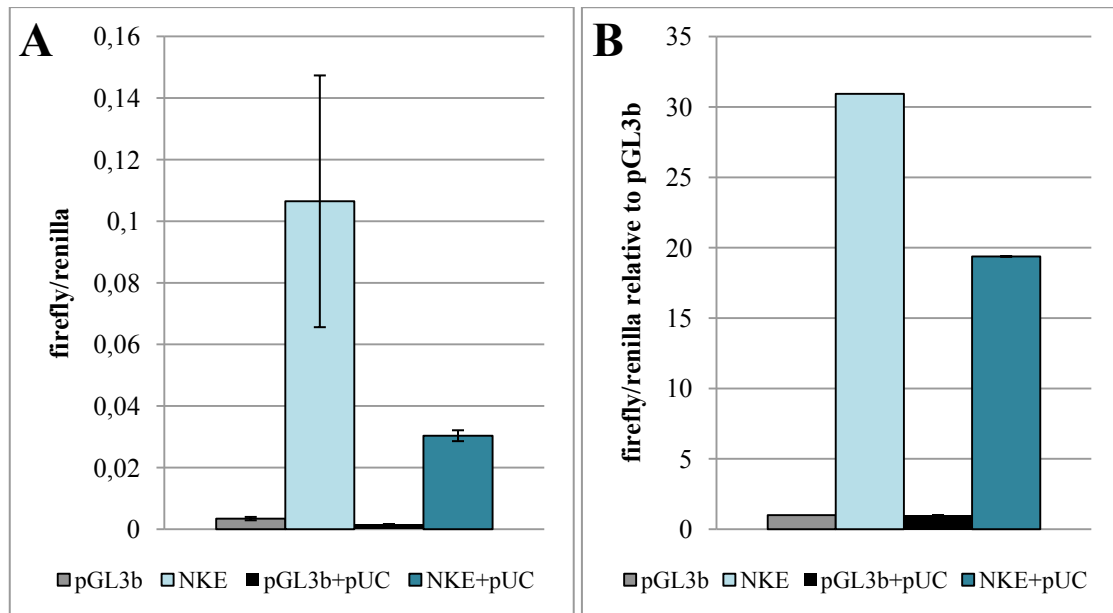


Figure 45 Luciferase gene expression. A5p28 was seeded at 10^5 cells/24 well. The plasmid DNA to renilla ratio was 100:1, the total DNA amount was 1000ng DNA (DNA ratio: pGL3b 100%, NKE 100%, pGL3b+pUC 50%+50%, NKE+pUC 50%+50%). Luciferase was predominantly produced in cell lines transfected with the NKE24 plasmid, cell lines lacking this plasmid showed weak expression (A). The firefly/renilla ratio relative to pGL3b levels is shown in (B). Transfection of half the NKE plasmid amount reduced firefly/renilla values to approximately one half of the original values.

4.4.2.4 Influence of BMP2 and SPARC on Nkx2.5 expression

From preliminary experiments we knew that SPARC had a positive influence on cardiomyogenesis in CVPCs (H.Auner, unpublished results). Earlier we showed that BMP2 positively influences cardiomyogenesis in CBs. We wanted to investigate if we can observe a change in Nkx2.5 promoter activation rate after addition of BMP2 or SPARC.

Standard transfection protocol was used and BMP2 (100 ng/ml) and SPARC (1 μ g/ml) were added right after Lipofectamine 2000 reaction was stopped. Triplicates were made. As seen in Figure 46 no significant differences in luciferase reporter expression were detected within the tested timeframe. This does not exclude that SPARC and BMP2 can influence Nkx2.5 expression but rather shows what within 48 h no significant change in expression rate can be detected.

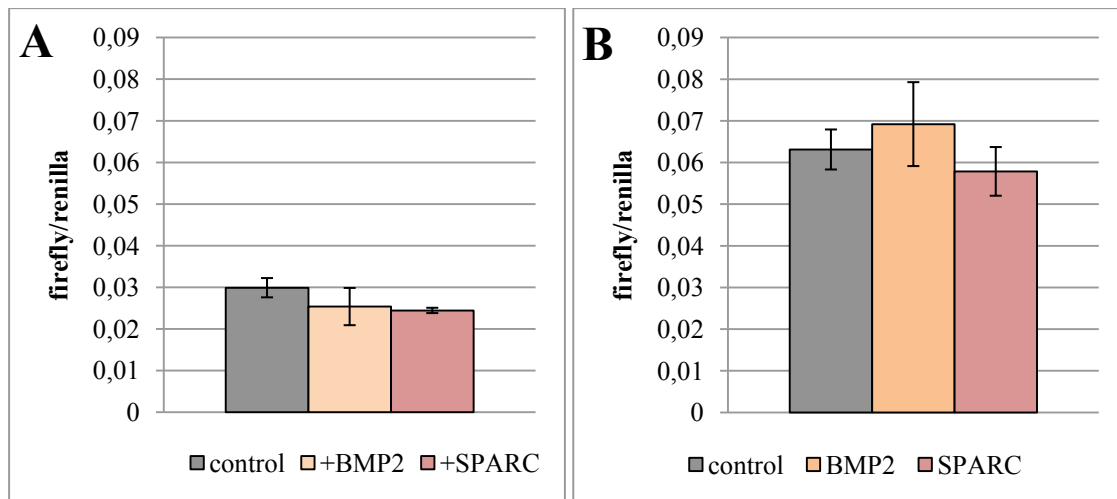


Figure 46 Nkx2.5 activation by BMP2 and SPARC. A5p27 (A) and A5p14 (B) were transfected with the NKE24 plasmid and the renilla plasmid at a ratio of 100:1. BMP2 and SPARC have no influence on Nkx2.5 promoter activation 48h post transfection. Luciferase values are shown in appendix (Table 13).

4.4.2.5 Influence of Desmin on Nkx2.5 expression

We previously found that Desmin can bind to the Nkx2.5 promoter region in mESCs AB2.2 and DC6 (Desmin over-expressing cells) (C.Fuchs, S.Gawlas, unpublished data).

We wanted to see if in CVPCs over-expression or knock-down of Desmin influences Nkx2.5 expression. We therefore transfected CVPC clone A5 with Desmin encoding vector pBK-RSV-Desmin; Desmin siRNA expressing vector knockdown-4 (kd-4); random, non-coding siRNA expressing vector knockdown-control (kd-ct); and NKE24 as a reporter vector, respectively.

We detected an activation of the Nkx2.5 promoter region in all NKE24 transfected clones, luciferase signal was minimal when transfected with pGL3b alone. Overexpression of Desmin by the pBK-RSV-Desmin vector did not result in the expected activation of the Nkx2.5 promoter region and the consequential expression of the luciferase reporter gene (Figure 47). Co-transfection of NKE24 with any other plasmid than pUC resulted in a strong downregulation of luciferase reporter gene activity. Interestingly, transfection efficiency relative to pGL3b varied strongly between the experiments but within the individual experiments it remained proportional (Figure 47).

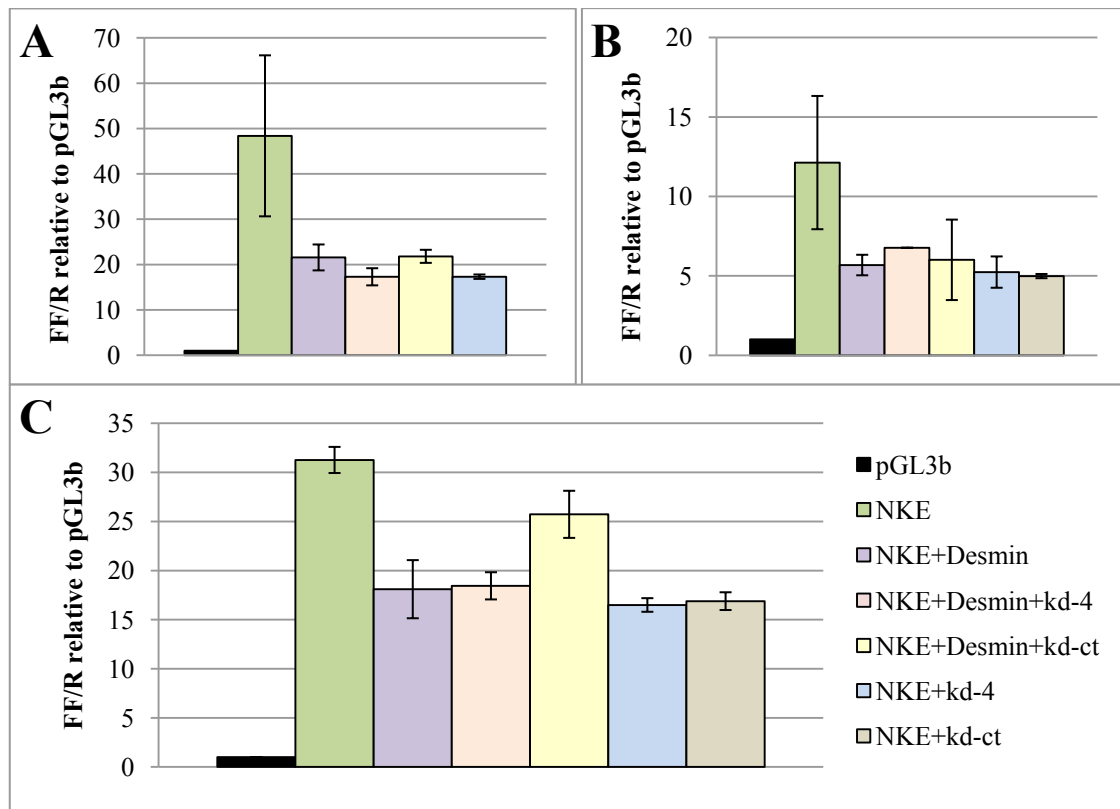


Figure 47 Luciferase Assay of CVPC clone A5. Cells were transfected at a plasmid to renilla ratio of 100:1. DNA level was kept constant at 1000ng, 330ng of each plasmid was used (Renilla 10ng), vector pUC was used to compensate DNA amount. (A), (B), and (C) show firefly/renilla values relative to pGL3b values of three individual experiments. Although transfection protocol was not altered efficiency varied strongly. Average firefly to renilla ratio is about 4 times higher in (A) than in (B). Co-transfection of a second plasmid together with the NKE24 plasmid led to a strong downregulation of luciferase reporter gene activity. Luciferase values are shown in appendix (Table 14, Table 15, Table 16).

As seen in Table 10 viability of cells was influenced depending on transfected plasmids. Survival of cells containing a Desmin over-expressing or a Desmin knock-down vector was decreased. NKE and kd-ct did not influence viability of cells.

transfected plasmids	viable cells per transfected well after 24h		
NKE	+++	+++	+++
NKE+Desmin	+	++	++
NKE+Desmin+kd-4	++	++	+
NKE+Desmin+kd-ct	++	++	++
NKE+kd-4	+	++	++
NKE+kd-ct	+++	+++	+++

Table 10 Viability of transfected cells. 100 000 A5p11 were seeded. Over expression and knockdown of Desmin influenced viability of cells within 24h. NKE and kd-ct plasmids did not decrease viability.

In order to test whether the pBK-RSV-Desmin plasmid was functional we isolated protein lysats and performed Western Blot analysis with α -Desmin antibodies. Lane 2 and 4 show an upregulation of Desmin protein level proofing that transfection of the pBK-RSV-Desmin plasmid resulted in an overexpression of Desmin (Figure 48). Signal is reduced in lane 2 due to lower protein loading amount compared to other lanes (see

GSK3 expression rate), therefore Desmin levels are expected to be even higher than shown in lane 2. Lane 3 shows downregulation of Desmin protein level in presence of vector kd-4, proofing that vector kd-4 was indeed functional. According to lane 6, knockdown control (kd-ct) had no influence on internal Desmin protein levels, while Figure 47 shows a reduction of luciferase reporter gene activity compared to transfection of NKE by itself.

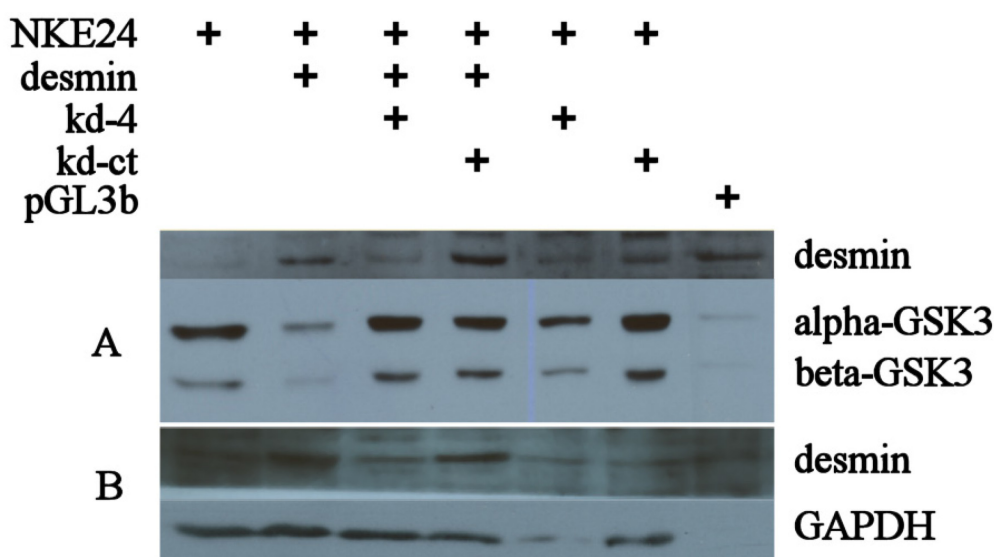


Figure 48 Western Blot analysis of Desmin protein expression. CVPCs A5p11 were transfected with indicated plasmids, pUC was used to balance total DNA amount. GSK3 and GAPDH were used to determine loaded protein amount per lane. Desmin is upregulated in lane 2 and 4. Western Blots of two individual experiments are shown in (A) and (B).

4.5 SPARC signaling

We previously showed a positive influence of SPARC on cardiomyogenesis (H.Auner, unpublished data). Therefore, we wanted to investigate the SPARC signaling pathway.

4.5.1 Proof of method

Leukemia Inhibitory Factor (LIF) signals via the Stat3 and MAPK pathway. Since an effect of LIF on Stat phosphorylation was reported to take place within 30 min protein lysats were prepared at time point zero, 4 min, 10 min, 30 min and 60 min (Rajasingh et al., 2007). Medium was changed at time point zero and M-LIF (100ng/ml) and BMP2 (2 µg/ml) were added to samples termed 4 min, 10 min, 30 min and 60 min. Proteins of sample 0 min were isolated without a medium change.

Upon M-LIF addition, phosphorylation of Stat3 was induced after 4 min relative to overall Stat3 level. Phosphorylation led to an activation of the Stat3 pathway. pErk levels were not increased under these conditions in two independent experiments

(Figure 49). Therefore MAPK signaling was not induced within 1 h after M-LIF addition.

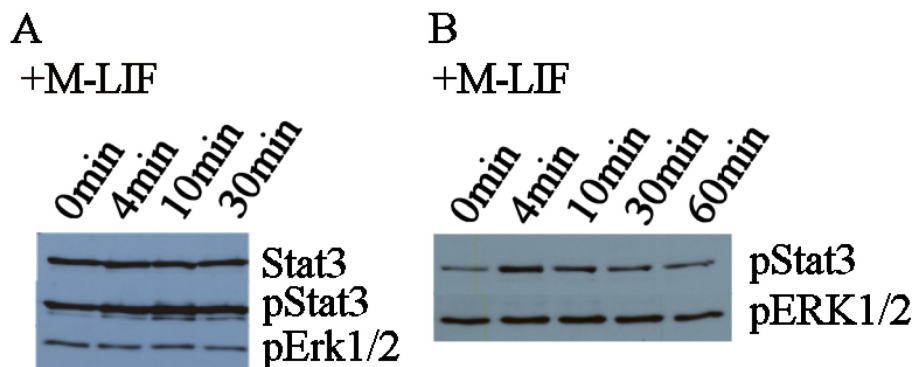


Figure 49 Western Blot M-LIF Induction. Cells were treated with 100ng/ml M-LIF at timepoint zero. Proteins were isolated at 0, 4, 10, 30, and 60 minutes after induction. Western Blots with Stat3, pStat3 and pERK1/2 antibodies were made. (A) shows upregulation of pStat3 10 min after M-LIF induction relative to total amount of Stat3. pErk1/2 levels are not influenced under these conditions. (B) shows activation of Stat3 at 4 min and 10 min compared to pErk1/2 levels.

To further proof our method we induced cells with BMP2 and tested for activation of Smad1/5/8 by phosphorylation. An increase of pSmad1/5/8 was detected after 10 minutes and further increased after 30 min and 1 h (Figure 50). No increase of pSmad1/5/8 was seen in controls (not shown). BMP2 had no influence on phosphorylation of Stat3.

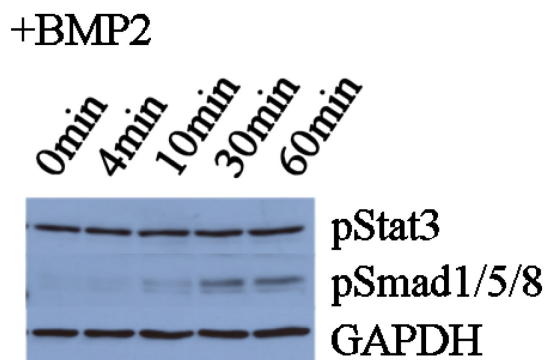


Figure 50 Western Blot BMP2 Induction. Cells were treated with 2 μ g/ml BMP2 at timepoint 0 min. Proteins were isolated at 0, 4, 10, 30, and 60 minutes after induction. Western Blots with pStat3, pSmad1/5/8, and GAPDH antibodies were made. Addition of BMP2 did not influence phosphorylation of Stat3 but lead to an increase of phosphorylation of Smad1/5/8.

Since we could both induce phosphorylation of Smad1/5/8 by BMP2 addition as well as phosphorylation of Stat3 after LIF addition we concluded that our method was functional.

4.5.2 SPARC signaling

SPARC has been reported to bind to β -Integrin 1, which is linked to ILK1. ILK1 influences phosphorylation of GSK3 and therefore the Wnt Pathway (Weaver et al., 2008).

Phosphorylation of GSK3 was shown to take place within 5-30 min after signal induction (Markou et al., 2008). If SPARC signaled through GSK3 inactivation - thereby influencing the Wnt Pathway - we should have seen an increase in GSK3 phosphorylation.

We chose timepoints 0, 4, 10, 30, and 60 minutes after SPARC addition for protein isolation. At timepoint 0 medium was changed to eliminate SPARC secreted by CVPCs. Recombinant SPARC was added to growth medium. Medium of sample 0 min was not changed, since isolation took place immediately.

At first we detected a noticeable decrease of Glycogen Synthase Kinase 3 $\alpha+\beta$ (GSK3) phosphorylation of Serine 9 and Serine 21 after 4 min and 10 min, meaning an activation of GSK3. GSK3 phosphorylation levels were back to initial levels 30 min post SPARC induction (T. Gottschamel, unpublished results). 4min after SPARC induction we could show a decrease in phosphorylation of Akt (also referred to PKB or Rac), arguing that Akt was rendered inactivate (Figure 51). One of the essential functions of Akt is the negative regulation of GSK3 activity by phosphorylation. Inactivation of Akt leads to an activation of GSK3 which goes hand in hand with the reported activation of GSK3. pStat3 levels were not influenced by addition of SPARC to growth medium (Figure 51).

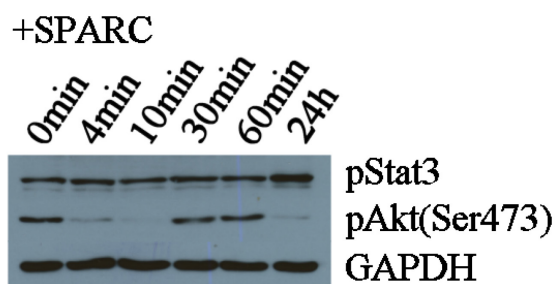


Figure 51 SPARC Signaling. Proteins were isolated for sample 0 min and growth medium of the remaining wells was changed, point of medium change was termed timepoint 0. SPARC (0.3 μ g/ml) was added at timepoint 0 min. A strong decrease in Akt phosphorylation could be detected, level of Stat3 phosphorylation was not influenced.

Since we wanted to test through which signaling pathway SPARC can regulate gene transcription we tested the influence of MAPK pathway inhibitor PD98059 and GSK3 inhibitor Chir99021 in combination with SPARC.

PD98059 was added 45min before reaction start therefore medium change took place 45min before timepoint 0. SPARC was added at timepoint 0. Influence of SPARC on pAkt could no longer be detected and pGSK3 levels were increased (Figure 52A). This was surprising since block of MAPKK1 activation should not interfere with the activation of Akt and also not with the deactivation of GSK3. Controls showed no elevation in pGSK3 level induced by PD98059 after 45min of treatment (Figure 52B). Unfortunately, we showed that the medium change at timepoint 0 min severely interfered with phosphorylation of GSK3 and Akt (Figure 53). Since protein isolation protocol used for experiments proved to be unsuitable no further Western Blot data is shown.

+SPARC+Pd98059

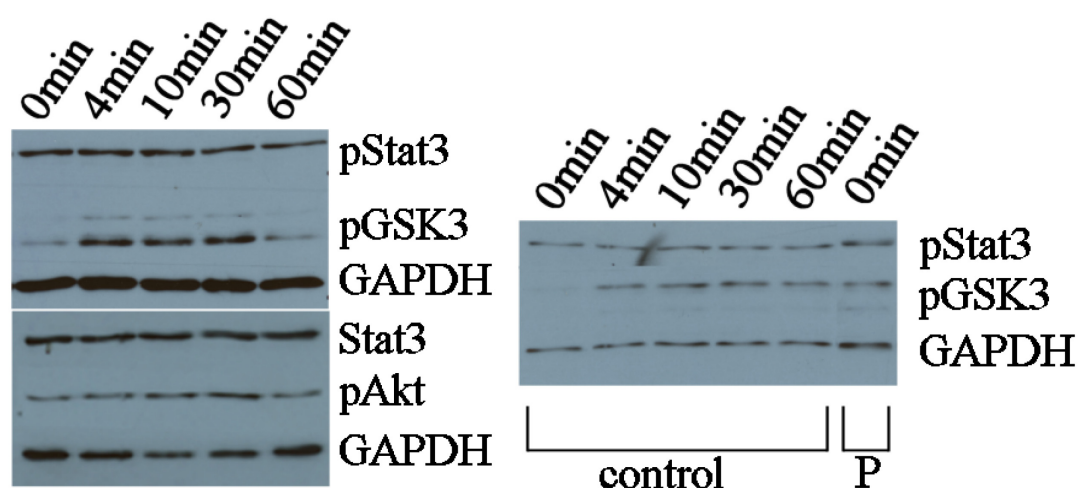


Figure 52 SPARC and inhibitor of MAPK induction. (A) MAPK pathway inhibitor PD98059 (10⁻⁵M) was added to wells 45 min prior to SPARC (0.3 μg/ml) (induction at timepoint zero. Timeperiod of SPARC induction is indicated. GSK3 was inactivated after 4 min, pAkt and pStat3 levels were not influenced. (B) Growth medium was changed 45 min prior to protein isolation. PD98059 (=P) was added at a concentration of 10⁻⁵M upon medium change. After 45 min of PD98059 treatment no influence on pGSK3 or Stat3 level could be observed.

4.5.2.1 Change of growth medium influences GSK3 phosphorylation

We proved that protein isolation protocol worked for the Stat3 and the Smad pathway. But protocol seemed to influence phosphorylation of GSK3 and Akt.

We tested whether feeding protocol influenced pGSK3 and pAkt levels. Medium was changed 2 h prior to protein isolation for control 1 and 2. Proteins of control 1 and timepoint 0 min of control 2 were isolated without a second medium change. Growth medium of remaining wells of control 2 were changed at timepoint 0.

Figure 53 reveals that medium change at timepoint zero was responsible for downregulation of pGSK3 after 4 min while pStat3 and pErk1/2 levels were not

influenced. Since all other Western Blot experiments were performed under these conditions we could not draw any conclusions from them and no further Western Blot data is shown.

Since CVPCs express high levels of SPARC within 2 h and therefore we could not adapt our protein isolation protocol. According to Figure 53 the medium change would have to take place at least 30 min prior to SPARC induction in order to avoid an influence on pGSK and pAkt levels.

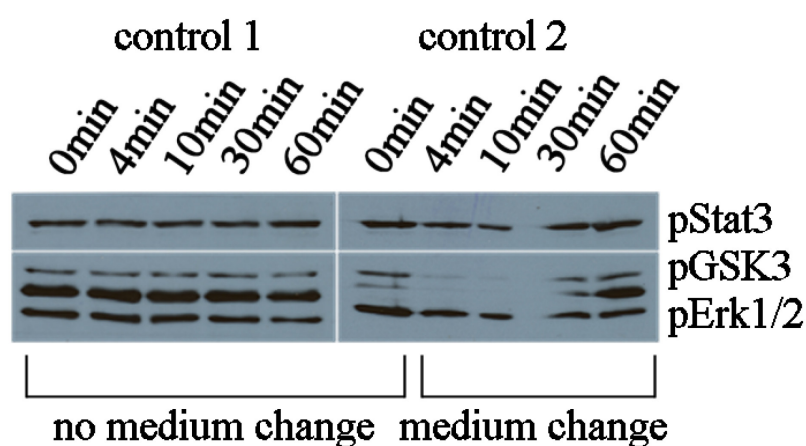


Figure 53 Change of growth medium influences pGSK3 level. Growth medium of samples was changed at time point zero as indicated. This medium change resulted in a deactivation of GSK3 by phosphorylation. Erk2 and Stat3 phosphorylation levels were not influenced.

5 Discussion

Recently, cardiac stem cell-like cells (CSCs) have been discovered in the adult heart. To better understand the self-renewal capacity of the murine heart, it is necessary to establish a model system in which molecular and cellular mechanisms of the progenitor cell reactivation and the underlying cytokine influences can be studied. Here we report the isolation of a stable Cardiovascular Progenitor Cell (CVPC) line from the murine heart which exclusively differentiates to cardiomyocytes, smooth muscle cells, and endothelial cells. The potential of these cells is limited and we reason that these cells might therefore provide new opportunities for the regeneration of injured heart tissue in the future. Restricted potency of these cells also implicates lower risks than ESC therapy. We propose that the CB system is a valid system for the study of CVPC differentiation. It allows better investigation of the pathways thriving and inhibiting differentiation of these cells. Furthermore the most important differences and similarities between CVPCs and the most commonly studied mESCs were determined. We show that LIF is sufficient to maintain CVPC self-renewal and negatively effects differentiation. We demonstrate that inhibition of LIF signaling is essential to induce cardiomyogenesis and LIF is dominant over the positive influence of BMP2 on cardiomyogenesis. BMP and Stat3 signaling play a time dependent role during cardiomyogenesis.

5.1 Isolation of a stable cardiovascular progenitor cell line

The heart was thought to be one of the least regenerative organs in the human body (Laflamme and Murry, 2005). Cardiomyocytes were believed to terminally differentiate and withdraw from the cell cycle after birth. Therefore myocardial injury would result in permanent myocardial loss and to cardiac dysfunction (Oyama et al., 2007; Towbin and Bowles, 2002). Recently, CSCs have been isolated by various groups suggesting that the heart maintains self-renewal capacity after birth (Beltrami et al., 2003; Oh et al., 2003; Oyama et al., 2007; Pfister et al., 2005).

Here we report the isolation of the first stable cardiovascular progenitor cell (CVPC) line from neonatal mice (Weber, 2006; Wiedner, 2008). CVPC isolation protocol included a co-culture step with mESC line AB2.2. Here we show that CVPCs are euploid and not aneuploid fusion products of mESCs and somatic cells. We have successfully cultured CVPCs up to passage 68 without a detectable loss in multipotency

or self-renewal capacity. Thereby we provide further proof that the murine heart maintains a regenerative capacity after birth.

5.2 CVPCs simultaneously express stemness markers and cardiac lineage markers

CVPCs express at least three stemness factors, namely Sox2, Nanog, and Oct3/4 at a level comparable to murine ESCs. Expression of these three transcription factors give CVPCs the potential to remain in the undifferentiated stem cell state. CVPCs express cardiac markers such as Nkx2.5 and α -MHC simultaneously with stemness factors. Upon LIF deprivation and consequential differentiation, the expression of the stemness factors is downregulated and lost eventually, leading to terminal loss of multipotency. Oh et al. (2003) reported the isolation of cardiac progenitors from the adult murine heart. These cells did not express cardiac markers Nkx2.5 and α -MHC, but did express cardiac markers such as MEF2c and GATA4 on mRNA level in the undifferentiated state. Oyama et al. (2007) reported no expression of cardiac markers in the undifferentiated state of their isolated CSCs. In contrast, CVPCs expressed MEF2c, GATA4, as well as Nkx2.5 and α -MHC on mRNA level in the undifferentiated state. Therefore we reason that our CVPC clones are further downstream of these more primitive progenitor cells and possibly further committed to the cardiac lineage. Expression of stemness genes proves that CVPCs have stem cell character and can remain in the undifferentiated state. Co-expression of cardiac lineage markers shows that CVPCs are not pluripotent as mESCs but that differentiation potential is limited to cell types of the heart. The difference in expression pattern of CVPCs distinguishes them from CSCs isolated by other groups. The fact that CVPCs appear to be a so far unique cell line might also explain why we were able to culture CVPCs up to passage 68 while others could not.

5.3 CVPCs differentiate to cardiomyocytes in the absence of inductive signals

So far differentiation of CSCs to cardiomyocytes was reported to be induced in response to 5'-azacytidine (Oh et al., 2003), in response to oxytocin and trichostatin (Oyama et al., 2007), or by co-culture with adult cardiomyocytes (Pfister et al., 2005). Differentiation of mESCs AB2.2 in the monolayer system never yielded beating cardiomyocytes. Here we show differentiation of CVPCs to cardiomyocytes, smooth muscle cells, and endothelial cells without the addition of differentiation inducing substances or co-culture with adult cardiomyocytes. In our hands, deprivation of LIF

was sufficient to induce CVPC differentiation in regular growth medium. Differentiation potential of CVPCs remained limited to smooth muscle, cardiomyocyte, and endothelium formation and could not be increased by addition of LIF, BMP2, GSK3 inhibitors, or MAPK inhibitors. The fact, that differentiation to non-cardiac lineage cells could not be forced further supports that future use of CVPCs for treatment of cardiac injuries limits risk compared to mESCs since CVPCs are strongly committed to the cardiac lineage. Taken together this means that CVPCs are already pre-destined to differentiate into cells of the cardiac lineage and that this differentiation process is initiated once self-renewal is no longer aided.

5.4 The Cardiac Body model system

CVPCs started differentiating in the absence of LIF. We wanted to establish a more reproducible system to study CVPC differentiation than the monolayer system. Embryoid Bodies (EBs) are used as an in vitro model system to investigate early steps of embryogenesis (Doetschman et al., 1985; Martin and Evans, 1975). We reasoned that 3D-aggregation of CVPCs in the absence of contact to artificial surfaces might mimic in vivo heart formation better than cell surface attachment in the monolayer culture system. We adapted our standard EB protocol for the formation and named CVPC-derived structures Cardiac Bodies (CBs) (Hofner et al., 2007; Weitzer et al., 1995). In comparison to EBs, CBs only gave rise to endothelial cells, smooth muscle cells, and cardiomyocytes. First beating cardiomyocytes were observed on day 11. This is 6 days earlier than in the monolayer system. The onset and degree of cardiomyogenesis was reproducible in the CB system, while these factors varied between experiments in the monolayer system. The CB system was not as dependent on starting cell number and feeding protocol as the monolayer system. Taken together, we reason that the CB system is a reliable system to study early stages of cardiomyogenesis and provides a microenvironment that is beneficial for cardiomyogenesis compared to the monolayer system. The number of cardiomyocytes per overall cell number was increased, the onset of cardiomyogenesis was enhanced and the timeframe in which first cardiomyocytes were observed was narrowed. Therefore, the CB system allowed us to investigate the influence of external factors on cardiomyogenesis in a more controllable approach than the monolayer system.

The formation of smooth muscle cells depended on unknown factors and was only observed sporadically. Within one culture plate, containing between 10 and 90 CBs,

only few developed contracting smooth muscle cells although CBs were exposed to the same growth medium. Therefore, smooth muscle formation appeared dependent on locally restricted secreted factors or entirely independent of secreted factors. So far we do not understand the mechanisms driving smooth muscle development but we could show that neither BMP2 nor M-LIF had a beneficial effect on smooth muscle formation.

5.5 CVPCs differ from mESC in cell morphology and differentiation potential

Morphology of CVPCS differs from mESCs while doubling time of CVPCs is comparable to doubling time of mESCs (Lauss et al., 2005). Furthermore CVPCs have a limited differentiation potential compared to pluripotent mESCs. The risk of differentiation to unwanted cell types upon injection into the murine heart would therefore be lower than the risk of mESC injection. If we understand the mechanisms initiating differentiation to cardiomyocytes, a reactivation of dormant progenitor cells could be induced after myocardial infarction in the future.

5.6 CVPCs express SPARC

Recombinant SPARC was shown to increase cardiomyogenesis in EBs, while addition of anti-SPARC antibodies attenuated the effect. mESCs AB2.2 express SPARC on mRNA level but do not secrete SPARC protein (Stary et al., 2005). Here we report a cell autonomous expression of SPARC by CVPCs. We could demonstrate both intracellular and extracellular localization of SPARC in CVPCs. This further distinguishes CVPCs from mESC, which do not secrete SPARC. Autocrine SPARC signaling might therefore play a role in cardiomyogenesis of CVPCs. Due to the high levels of cell autonomous SPARC secretion we were not able to determine the pathways through which SPARC signals in CVPCs. However, we could show an upregulation of ILK on mRNA expression level during CB development. SPARC was shown to influence Wnt signaling through ILK (Nie and Sage, 2009). It remains to be determined if ILK upregulation in CVPCs is linked to SPARC signaling.

5.7 LIF is sufficient for CVPC self-renewal

The isolation procedure of CVPCs included culture on SNL76/7 LIF-producing feeder cells. LIF is known to maintain mESC in an undifferentiated state (Niwa et al., 1998; Raz et al., 1999; Williams et al., 1988). Zandstra et al. (2000) showed that addition of LIF to mESCs potentiates the choice towards self-renewal and away from differentiation in cells undergoing cell division. Here we show that recombinant LIF or

feeder cell-secreted LIF are sufficient to maintain self-renewal and inhibit differentiation of CVPCs. Furthermore, we demonstrate a positive effect of M-LIF on growth rate of CVPC. However, Zandstra et al. (2000) reported no immediate impact of LIF on proliferation or survival of mESCs. Raz et al. (1999) showed a correlation between LIF concentration and proliferation for one out of two tested mESC lines. Together, this means that LIF-associated cell proliferation varies within mESCs. Here we show that CVPC cell proliferation is indeed LIF-associated and that a LIF deprivation results in a decrease of growth rate.

In mESCs an autocrine LIF production or an autocrine non-LIF gp130 ligand production was suggested to temporarily maintain mESCs in self-renewal after LIF deprivation. This effect was shown to be cell number dependent (Davey and Zandstra, 2006; Zandstra et al., 2000). In case this holds true for CVPCs, it would explain why successful differentiation in the monolayer system was highly cell number dependent. We observed that high amounts of cardiomyocytes only developed when the seeded cell number was low. When the cell number was chosen too high, cardiomyogenesis did not take place and cells appeared undifferentiated. Whether an autocrine production of LIF or a non-LIF gp130 ligand takes place in CVPCs will be tested in the future. Addition of anti-LIF antibodies, anti-LIFR-antibodies, or anti-gp130 antibodies, respectively, should show if CVPCs are able to form beating cardiomyocytes when seeded at higher cell numbers. If so, CVPC differentiation is inhibited by an autocrine LIF/gp130-ligand production at high cell density. The addition of M-LIF antibodies to the CB system resulted in an earlier onset of cardiomyogenesis as well as an increased number of cardiomyocytes per CB. This strongly indicates that an autocrine LIF production does indeed take place in CVPCs.

5.8 LIF negatively affects cardiomyogenesis in the CB system

Here we showed a negative effect of LIF on CB development. CBs treated with LIF during day 0-4.7 showed severely hampered CB development. CB size was decreased, cell spreading was impeded, and cardiomyocyte formation was delayed by 6 days. In contrast, addition of anti-LIF antibodies during that time period resulted in an increase of cardiomyocytes per CB. Cardiomyocyte formation in 100% of CBs was reached 4 days earlier in anti-LIF treated CBs than in untreated CBs. Although addition of LIF at all time points of CB development resulted in drastically decreased cardiomyogenesis per CB, its influence was the most critical during the first 5 days. We showed that LIF is

a strong inducer of self-renewal and inhibits differentiation of CVPCs. Therefore, inhibition of LIF signaling appears to be important during cardiomyogenesis in a time dependent manner. These results are in line with the findings that LIF maintains self-renewal and suppresses differentiation in mESCs (Niwa et al., 1998; Raz et al., 1999; Williams et al., 1988).

In contrast, we saw a positive effect of LIF on cardiomyogenesis in the monolayer system. It is notable, that in the monolayer system we treated CVPCs with LIF for a time period prior to onset of differentiation while in the CB system we treated CVPCs during differentiation. We reason that the increase in cardiomyogenesis of M-LIF treated cells compared to cells grown on feeder layers is mainly due to the absence of other factors secreted by feeder cells that might negatively affect differentiation or aid self-renewal. This might also explain the immediate onset of cardiomyogenic program upon LIF deprivation. In conclusion we do not think the monolayer system represents a good model for the investigation of the events underlying cardiomyogenesis. Slight changes in the feeding protocol, minimal cell number changes, and other unknown factors had significant influences on cardiomyogenesis, problems that were not observed in the CB model. The most significant finding from the monolayer system was that CVPCs can form cardiomyocytes, smooth muscle cells, and endothelial cells in the absence of LIF without the need of inductive signals.

5.9 BMP2 enhances cardiomyogenesis in the CB system

mESCs treated with BMP2 for 24 h prior to EB formation resulted in a more than twofold increase of contracting areas and an enhanced myofibrillogenesis by day 9, compared to EBs from untreated mESCs (Behfar et al., 2002). Here we report a positive influence of BMP2 on cardiomyogenesis in CBs. Addition of BMP2 on day 4.7 or day 7 resulted in a 1.5fold increase in cardiomyocyte formation per CB. Furthermore, cardiomyocyte formation in 100% of BMP2 treated CBs was reached up to 4 days earlier than in control. Addition of anti-BMP2/4 antibodies delayed onset of cardiomyogenesis by a maximum of 2 days but had only low influence on the number of cardiomyocytes per CB. The morphology of BMP2 and anti-BMP2/4 treated CBs was indistinguishable from untreated CBs. Taken together, BMP2 has a strong positive effect on cardiomyogenesis, which is further supported by the upregulation of pSmad1/5/8 signaling during CB development. We showed that pSmad1/5/8 signaling

was upregulated in CBs on day 7 and day 13, independent of treatment with BMP2. Inhibition of BMP2 does not prohibit cardiomyogenesis but impedes it.

In comparison, from the monolayer system we learned that BMP2 is insufficient to maintain CVPCs in self-renewal. Cell number was constantly declining and cell death was frequently observed in the absence of LIF. After 31 days of culture of CVPCs under continuous BMP2 treatment in the absence of LIF the potential of CVPCs to form beating cardiomyocytes was severely diminished. Survival was severely influenced and a lot of these cells most likely lost stem cell character during the 30 days of LIF deprivation. The fact that some cells still maintained the potency to form cardiomyocytes showed that a low number of CVPCs survived an entire month of LIF deprivation. From these results we can conclude that BMP2 is insufficient to maintain self-renewal of CVPCs but cannot draw conclusions about the influence of BMP2 on cardiomyogenesis.

When LIF was added in combination with BMP2 the number of formed cardiomyocytes correlated with the number of cardiomyocytes formed by CVPCs grown on feeder cells. One explanation for this is that BMP2 aids self-renewal and further inhibits differentiation in the presence of LIF by inducing the inhibitor of differentiation genes but is not sufficient to maintain self-renewal in the absence of LIF.

5.10 Inhibition of GSK3 does not affect CVPC multipotency

During EB development Wnt signaling is required for the commitment of mesodermal cells into a cardiomyocyte lineage (Naito et al., 2006). Addition of Wnt3a or GSK3- β inhibitor BIO between day 0 and 3 resulted in an increase of beating EBs, while in late phases of cardiac development Wnt signaling inhibits proliferation and/or maturation of committed cardiomyocytes. Here we show that continuous GSK3 inhibition by Chir99021 negatively influences cardiomyogenesis in the monolayer system. We demonstrated that even after 20 days of LIF deprivation and GSK3 inhibition, a pool of multipotent CVPCs survived and could give rise to CBs with normal cardiomyocyte development. Whether a time restricted inhibition of GSK3 has beneficial effects on cardiomyogenesis cannot be concluded from these results.

5.11 Transfection of CVPCs using Lipofectamine 2000

Here we report the establishment of a transfection protocol for CVPCs using Lipofectamine 2000. We show that GFP encoding plasmids have a negative effect on

Luciferase detection. We could prove an activation of the Nkx2.5 promoter region in CVPCs after 72 h of culture on gelatin. Since Nkx2.5 is an early cardiac marker, this means that the cardiac program is already initiated at this time point. However, we could not show a direct influence of SPARC or BMP2 on Nkx2.5 expression within a 48 h timeframe. If Nkx2.5 expression is positively regulated by BMP2 or SPARC at other time points cannot be deduced from these results. Stary et al. (2005) showed an upregulation of Nkx2.5 expression on mRNA level 4 h after SPARC addition in EBs on day 7 as well as in primary fetal cardiomyocytes. This discrepancy between these results and ours might be explained by the fact that both primary fetal cardiomyocytes as well as EBs on day 7 are already much further progressed in the cardiomyocyte formation than CVPCs are after 72 h. Therefore, SPARC might enhance cardiomyogenesis in a time dependent manner.

5.12 Desmin overexpression and knockdown negatively effects CVPC survival

Constitutive overexpression of Desmin in mESCs was shown to transiently upregulate Nkx2.5 expression in EBs (Hofner et al., 2007). Transfection of CVPCs with Desmin encoding plasmid resulted in a significant increase of intracellular Desmin concentration but did not result in an upregulation of Nkx2.5 expression. Co-transfection with a plasmid encoding a Desmin siRNA (kd-4) diminished Desmin expression, while a non-coding siRNA expressing plasmid (kd-ct) did not affect Desmin levels. Surprisingly, co-transfection of each of these plasmids with the NKE24 plasmid led to a downregulation of Nkx2.5 expression. Hofner, et al. (2007) reported a Nkx2.5 activation by Desmin between day 2 and 4 of EB differentiation. Therefore, the timeframe we chose for Nkx2.5 activation by Desmin might not be optimal, especially since we worked with the monolayer system in which cardiomyogenesis is delayed compared to EB development. We reason that the overexpression of Desmin might result in a negative feedback loop when reaching a certain Desmin threshold but we could not explain why co-transfection with a non-coding siRNA expressing plasmid (kd-ct) had a negative effect on Nkx2.5 expression, although it did not affect intracellular Desmin levels and toxicity of this vector was ruled out. Here we show that both overexpression and downregulation of Desmin have negative effects on CVPC cell survival. Transient transfection of CVPCs did not allow us to measure Luciferase activity more than 96 h post transfection. To further investigate influence of Desmin overexpression and knockdown in CVPCs stable cell lines need to be generated.

5.13 The study of Cardiovascular Progenitor Cells will contribute to the better understanding of the postnatal regenerative potential of the murine heart

The mechanisms underlying the differentiation of cardiac precursor cells to mature cardiomyocytes are not fully known and are predominantly studied during embryogenesis. It remains to be investigated whether the reactivation of stem cells in the mature heart underlies the same signal induction cascades as during early heart formation. The study of cardiovascular progenitor cells will aid the better understanding of the regeneration capacity of the adult heart and might lead to new insights on how to initiate differentiation of CVPCs to adult cardiomyocytes *in vivo*. The greatest benefit would be the reactivation of endogenous CVPCs, thereby omitting necessity of immunosuppression due to transplantation or injection of donor cells. Here we provide further evidence for the existence of CVPCs in the neonatal murine heart. We showed that these cells can self-renew *in vitro* and remain undifferentiated. Their differentiation potential is limited to the three cell types of the heart, namely smooth muscle cells, endothelial cells, and cardiomyocytes. Further, we introduced the Cardiac Body system, a new model to study cardiomyogenesis of CVPCs *in vitro*. Further investigation of the limitations and the potential of murine CVPCs will contribute to a better understanding of the regenerative potential of the murine heart.

6 Material

6.1 Chemicals and material for molecular biology

Chemical	Company
Acetic acid	Merck, D
Acryl amide	BioRad, USA
Agarose, Biozyme LE	Biozyme, D
Aprotinin	Sigma, USA
β -Mercaptoethanol	Loba Feinchemie, A
Bromphenolblue	Sigma, USA
BSA	Roth, D
Cellular membrane	Roth, D
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
Giemsa stain	Sigma, USA
Glycerine	Merck, D
Glycerophosphat	Merck, D
Hydrochloric acid (HCl)	Acros, B
Imidazole	Sigma, USA
Leupeptin	Roche, AUT
Loading Dye	Fermentas, Lithuania
Methanol	Merck, D
Magnesiumchloride	Fermentas, Lithuania
Magnesiumsulfate	Fluka, CH
Milk Powder (Skim Milk)	Sucofin
Mowiol	Hoechst, D
Nalgene Filter	Thermo Scientific, USA
Ni-NTA agarose beads	Quiagen, D
PCR-buffer without $MgCl_2$	Fermentas, USA
Pepstatin A	Roche, AUT
Ponceau-S	Sigma, USA
Proteinase Inhibitor	Roche, AUT
Pyrophosphate	Fluka, CH
Reverse Transcriptase Buffer	Invitrogen, USA
SDS	BioRad, USA
Sodiumbicarbonate	Sigma, USA
Sodiumchloride	Salinen Austria, AUT
Sodiumfluorid	Donauchemie, AUT
Sodiumhydrogencarbonate	LifeTechnologies, USA
Sodiumhydrogenphosphate	Roth, D
Sodiumhydroxide	Merck, D
Sodium Orthovandate	Sigma, AUT

Sodium Pyrophosphate	Fluka, CH
Sodiumthiosulfate	Merck, D
Trichloroacetic acid	Merck, D
Tris Base	LifeTechnologies, USA
Triton X-100	Sigma, USA
Tween-20	Sigma, USA

6.2 Chemicals for cell culture

Chemical	Company
β-Mercaptoethanol	Loba, A
Ampothericin B	Invitrogen, USA
Chir99021	Axon, NL
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
Gelatin	Difco, USA
Glycine	Applichem, D
L-(+)-Glutamin	Gibco, USA
Lipofectamine 2000	Invitrogen, USA
Mitomycin C	Acros, B
PD 98059	Cayman, USA
Penicillin G Kaliumsalz	Merck, D
Penicillin / Streptomycin (P/S)	Gibco, CH
Potassium chloride	Sigma, USA
Potassiumhydrogenphosphate	Fluka, CH
Sf-900 SFM medium	Gibco, USA
Sodiumhydrongencarbonate	LifeTechnologies, USA
Streptomycin	Sigma, USA

6.3 Enzymes

Enzyme	Company
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

6.4 Proteins

Protein	Company
Recombinant SPARC	Sigma, USA
M-LIF	Purified from insect cells
BMP2	R&D Systems

6.5 Kits

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

6.6 Cell Lines

6.6.1 SNL76/7-Fibroblasts

STO Fibroblasts were transfected with a stable neomycin resistance expression vector and a LIF expression vector by Allan Bradley (McMahon and Bradley, 1990)

6.6.2 Bacteria Strain XL1-Blue

Bacteria strain XL-1 Blue (Stratagene, USA) was used for transformation.

6.6.3 Embryonic stem cells

The embryonic stem cell line AB2.2 was isolated by Allan Bradley from 129Sv mice (Soriano et al., 1991). The embryonic stem cell line 662 was isolated from a 129Sv/BL6 N1-2 neo^R mouse (Lagger et al., 2002). The embryonic stem cell line 663 was isolated from 129Sv/BL6 mice (Lagger et al., 2002).

6.6.4 Cardiovascular Progenitor Cells

CVPC cell lines were isolated by Wolfgang Weber from heart tissue of N1-2 neo^R mice. This mouse line encodes a neomycin-resistance gene in one allele of the HDAC1 locus and can therefore survive selection with neomycin or neomycin analogs (e.g. G418). In order to increase the isolation success rate these heart tissue derived cells were co-cultured with murine wild type ESCs. This co-culture was used to supply possible cardiovascular progenitor cells with external factors that are usually supplied by the stem cell niche. Since niche conditions are unknown, the presence of ESCs and the proteins, cytokines, etc. secreted by these, was thought to possibly keep CVPCs in stem cell mode. After 10 passages of co-culture (3T3 protocol) on feeder layers a selection against hypertrophic cells and towards fast replicating cells had already taken place. Addition of the neomycin analog G418 resulted in death of murine wild type stem cells. CVPC clones were gained by seeding one cell per plate and expanding this culture under treatment of G418. 11 clonal cell lines were derived named A3, A5, B3, B5, C3, D3, D5, E3, F3, G3, and H3.

6.7 Primers

Primer name		Primer sequence	Annealing temp	Cycles	PCR product (bp)	
BAMBI	Fwd	5' – TTCCAAGAGCGAAGCCTCA – 3'	58°C	30	cDNA	214
	Rev	5' – CTCATCCTGCAGCCTCTTGT – 3'			Genomic	651
Brachyury (T)	Fwd	5' – GAACCTCGGATTACATCGTGAGA – 3'	60°C	36	cDNA:	159
	Rev	5' – ATCAAGGAAGGCTTTAGCAAATGGG – 3'			Genomic:	891
Desmin	Fwd	5' – TGACAACCTGATAGACGA – 3'	50°C	37	cDNA:	390
	Rev	5' – TTCTTATTGGCTGCCTGA – 3'			Genomic:	1972
Desmin	Fwd	5' – TGATGAGGCAGATGAGGGAG – 3'	50°C	37	cDNA	246
	Rev	5' – TGAGAGCAGAGAAGGTCTGG – 3'				
GATA4	Fwd	5' – GCCTGTATGTAATGCCTGCG – 3'	53°C	31	cDNA:	500
	Rev	5' – CCGAGCAGGAATTTGAAGAGG – 3'			Genomic:	3364
GAPDH	Fwd	5' – CGTCTTCACCACCATGGAGA – 3'	55°C	29	cDNA:	300
	Rev	5' – CGGCCATCACGCCACAGTTT – 3'			Genomic:	300
ILK	Fwd	5' – CTCTGCGGTGGTTGAAAT – 3'	52.5°C	35	cDNA	190
	Rev	5' – GAAACATGCATAGTGAAGTGG – 3'			Genomic	360
LIFR	Fwd	5' – AGGAATGCCACAATCAGAGG – 3'	57°C	30	cDNA	190
	Rev2	5' – TCCAAGTGTCTGGTCCCTTT – 3'			Genomic	499
MEF2c	Fwd	5' – GGCCATGGTACACCGAGTACAACGAGC – 3'	66°C	34	cDNA:	395
	Rev	5' – GGGGATCCCTGTGTTACCTGCACTTGG – 3'				
Mesp1	Fwd	5' – AGAAACAGCATCCCAGGAAA – 3'	52°C	32	cDNA:	346
	Rev	5' – GTGCCTGCTTCATCTTTA – 3'			Genomic:	619
α-MHC	Fwd	5' – GGAAGAGTGAGCGCGCATCAAGG – 3'	57.7°C	32	cDNA:	302
	Rev	5' – CTGCTGGAGAGGTTATTCCTCG – 3'				
Nanog	Fwd	5' – AGGGTCTGCTACTGAGATGCTCTG – 3'	60°C	28	cDNA:	364
	Rev	5' – CAACCACTGGTTTTTCTGCCACCG – 3'			Genomic:	4793
Nkx2.5	Fwd	5' – CACCCACGCCTTTCTCAGTC – 3'	57°C	35	cDNA:	513
	Rev	5' – TGGACGTGAGCTTCAGCA – 3'				
Oct 3/4	Fwd	5' – CCACCTTCCCATGGCTGGACA – 3'	50°C	37	cDNA:	1082
	Rev	5' – AGGGCTGGTGCCTCAGTTTG – 3'			Genomic:	4475
OSMR	Fwd	5' – GGCAAATCACTGAAGGCTCA – 3'	62°C	30	cDNA:	801
	Rev	5' – CACACCAGTCCACAACATAG – 3'				
α-Tropomyosin	Fwd	5' – CAAGCGGAGGCTGATAAGAAGG – 3'	55°C	30	cDNA:	310
	Rev	5' – TGCCTCTCTACTCTCATCTGC – 3'				

6.8 Antibodies

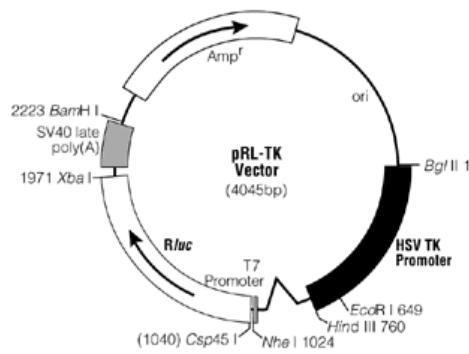
Antibody	Dilution	kDa	Host	Catalog #	Company
Desmin	WB 1:300	50-55	mouse	D1033	Sigma, USA
Desmin	WB 1:200	53	rabbit	8592	Abcam, USA
Erk1/2	WB 1:300	42	rabbit	n.A.	Upstate, USA
GAPDH	WB 1:60 000	37		MAB374	Millipore, USA
GSK3 α/β	IF 1:50	51, 47	mouse	7291	Santa Cruz, USA
Human BMP-2/4				MAB355 2	R&D Systems, USA
Mouse LIF				AF449	R&D Systems, USA
mouse-FITC	IF 1:200		donkey	715-095-151	Jackson Labs, USA
p-Akt (Ser473)	WB 1:1000	60	rabbit	9271	Cell Signaling, USA
p-Akt (Thr308)	WB 1:1000	60	rabbit	9275	Cell Signaling, USA
p-GSK3 α/β (Ser21/9)	WB 1:1.000	46, 51		9331	Cell Signaling, USA
p-GSK3 α/β (Tyr276/216)	IF 1:50	51, 47	goat	11758	Santa Cruz, USA
p-Smad1 (Ser463/465)/ Smad5 (Ser463/465)/ Smad8 (Ser426/428)	WB 1:500	60	rabbit	9511	Cell Signaling, USA
p-Smad2 (ser465/467)	WB 1:1000	60	rabbit	3101	Cell Signaling, USA
p-Stat3	WB 1:1000	79, 86	rabbit	9131	Cell Signaling, USA
rabbit-FITC	IF 1:200		donkey	711-095-152	Jackson Labs, USA
Smooth Muscle Actin	IF 1:800		mouse	A 2547	Sigma, USA
SPARC	IF 1:50, WB 1:200	43	rabbit	25574	Santa Cruz, USA
Stat3	WB 1:1000	79, 86	rabbit	9132	Cell Signaling, USA
Troponin T, Cardiac Isoform Ab-1	IF 1:100-200		mouse	MS-295-P	Thermo Scientific, USA

WB=Western Blot, IF=Immunofluorescence

6.9 Plasmids

6.9.1 Renilla

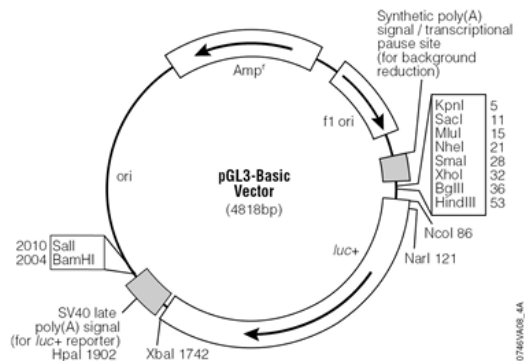
The pRL-TK vector contains a renilla luciferase reporter gene under the control of a T7 promoter. This plasmid was used as an internal control to measure transfection efficiency.



Promega Renilla Plasmid (#E2241)

6.9.2 pGL3b

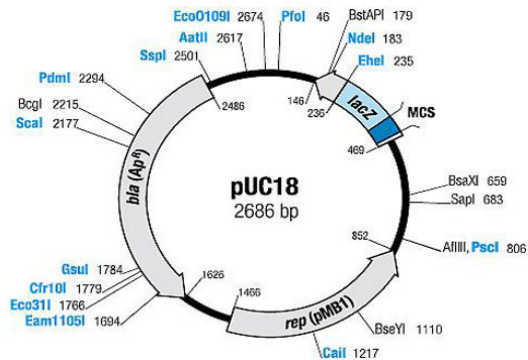
The pGL3-Basic vector encodes a firefly luciferase gene but lacks a promoter.



Promega pGL3-Basic Plasmid (#E1751)

6.9.3 pUC

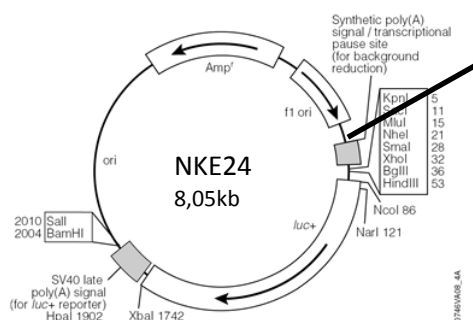
The pUC18 plasmid does not encode for a luciferase reporter gene but is used to compensate plasmid DNA amount in transfections with varying plasmid numbers.



Promega pUC18 Plasmid

6.9.4 NKE24

NKE24 is a pGL3b plasmid with the Nkx2.5 promoter inserted in frame with the luciferase reporter gene. It was obtained from Dr. Katherine Yutzey (USA).

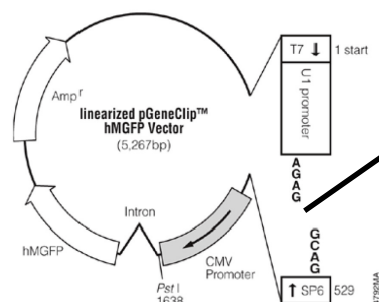


pGL3-Basic vector with Nkx2.5 insert

From Not I 4651
Nkx2.5Promoter (-3059to +223)

6.9.5 Knock down-4

The kd-4 plasmid encodes for antisense RNA to the murine Desmin mRNA inserted into the Promega pGeneClip hMGFP Vector.



Cloned sequence:
GACCATCGCGGCTAAGAAC

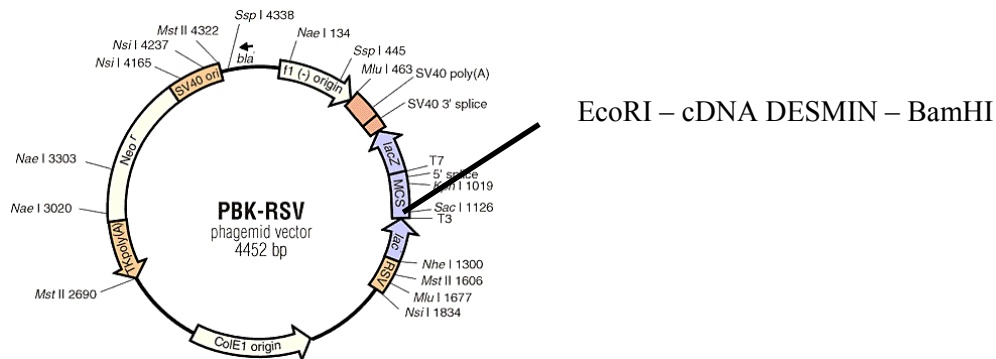
Desmin Knock Down4 Plasmid, Promega (#C8790)

6.9.6 Knock down control

The kd-ct plasmid is identical to the kd-4 plasmid with the exception that it encodes for a non-coding RNA. Cloned sequence: GGAATCTCATTCGATGCATAC

6.9.7 Desmin

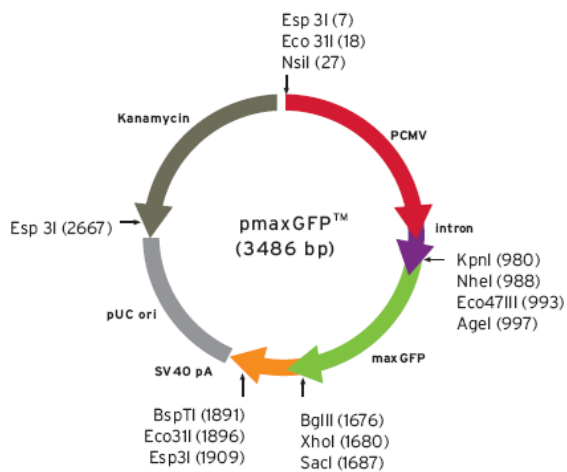
The Desmin plasmid is a PBK-RSV plasmid encoding for the Desmin cDNA under the RSV promoter and is used for ectopic Desmin overexpression.



Stratagene PBK-RSV vector with a Desmin cDNA insert (#212209)

6.9.8 pmaxEGFP

The Amara pmaxGFP control plasmid encodes GFP under the control of a Cytomegalovirus promoter (CMV). This plasmid was kindly provided by M. Artaker.



Amaxa pmaxGFP plasmid

7 Methods

7.1 Cell culture maintenance

7.1.1 Washing of glass pipettes

After use, cotton plug is removed and pipettes are put into vessels containing a mixture of water and hypochlorite. After several hours glass pipettes are rinsed with fresh tap water for at least four hours. The pipettes are left in MilliQ overnight. Pipettes are dried at 80°C for 4 hours, placed in pipette boxes and baked at 180°C for 8 to 10 hours. If pipettes come in touch with soap they can no longer be used for tissue culture work with ESCs or CVPCs.

7.1.2 Washing of cell culture glass ware

Glass ware is filled with hypochlorite and tapwater. After 15-30 minutes bottles are rinsed at least five times with tap water and then filled with MilliQ and left overnight. Bottles can be emptied and left to dry on the next day. Bottles are autoclaved at 120°C at 1.4 bar for 20-30 minutes.

Magnets and other tissue culture appliances are also washed with hypochlorite and MilliQ prior to use and should solely be used for tissue culture and never come in contact with soap.

7.2 Media and solutions

7.2.1 Solutions

10x PBS (Phosphate buffered saline)

1.37 M NaCl

14.7 mM KCl

78.1 mM Na₂HPO₄·7H₂O

26.8 mM KH₂PO₄

pH 7.2

Salts are dissolved in 800 ml of MilliQ and titrated to a pH of 7.2 with Na₂HPO₄·7H₂O solution. Fill up to 1 l mark with MilliQ and sterile filtrate (Nalgene Filter, Nalgene Membrane, 0.22 µm pore width). Solution can be stored at room temperature.

1x PBS

Dilute 50 ml of 10x PBS with 450 ml of autoclaved MilliQ in laminar flow.

100x GPS (Glutamine-Penicillin-Streptomycin)

4.25 g NaCl
1.5 g Penicillin
2.5 g Streptomycin
14.6 g L-(+)-Glutamine

Dissolve ingredients in 500 ml of MilliQ and sterile filtrate. Aliquot and store at -20°C.
Prior to use thaw and resuspend till suspension is clear. Store at 4°C.

100x β-Mercaptoethanol (10⁻²M)

200 ml 1x PBS
144 µl β-Mercaptoethanol

Add β-Mercaptoethanol to PBS and sterile filtrate before aliquoting. Store at -20°C for longer time periods and at 4°C when in use.

Trypsin

3.5 g NaCl
0.5 g D-Glucose
0.09 g Na₂HPO₄·7H₂O
0.185 g KCl
0.12 g KH₂PO₄
0.2 g EDTA
1.25 g Trypsin
1.5 g Tris Base

Add ingredients and fill up to 500 ml with MilliQ and bring to a pH of 7.6 with concentrated HCl. Sterile filtrate the solution and aliquot in 50 ml falcons and store at -20°C. When in use store at 4°C, mix well before use.

1% gelatin stock solution

Dissolve 10 g gelatin in 1 l of MilliQ and autoclave before further use. Store at room temperature.

0.1% gelatin solution

Dilute 50 ml of 1% gelatin stock solution with 450 ml autoclaved MilliQ. Store at room temperature.

Dulbecco's Modified Eagle Medium (DMEM)

Add 4.5 l to a 5 l Erlenmeyer flask and put on top of a magnetic stirrer. Slowly add 66.9 g of D-MEM followed by 18.5 g of Natriumhydrogencarbonat. Bring to pH 7.4 with concentrated HCl. Sterile filtrate and store at 4°C. Pipette 3 ml aliquots of each bottle into 6 wells and culture at 37°C, 5%CO₂. Check daily under the microscope for bacterial growth.

Culture media

M10Gi medium for SNL76/7 and feeder layers

10% Gibco FBS Serum
1% GPS
89% DMEM

M10Gi medium for ESCs

10% Gibco FBS Serum
1% β-Mercaptoethanol
1% GPS
88% DMEM

M15Gi medium for ESCs

15% Gibco FBS Serum
1% β-Mercaptoethanol
1% GPS
83% DMEM

M15Hy medium

15% HyClone FBS Serum
1% β-Mercaptoethanol
1% GPS
83% DMEM

M15Si medium

15% Sigma FBS Serum
1% β -Mercaptoethanol
1% GPS
83% DMEM

Freezing medium

60% DMEM
20% FBS
20% DMSO

Gelatin coating of cell culture plates:

Cell culture plates must be coated with gelatin prior to use in order to provide a matrix that cells can adhere to. Therefore, 0.1% gelatin is applied to all cell culture plates two hours prior to use. Gelatin must be removed before further use of culture dishes. Plates coated with gelatin are stored at 37°C, 5% CO₂.

7.3 Maintenance of SNL67/7**7.3.1 Thawing of SNL76/7**

10 cm culture plates must be coated with gelatin prior to thawing. SNL76/7 are stored in cryotubes in liquid nitrogen. Cryotubes are carefully removed from liquid nitrogen tank and thawed in a 37°C bath until only a thin icicle is left in the tube. Cryotubes are sprayed with 70% EtOH, dried, placed in the laminar flow and flamed before and after opening. Content (1 ml-2 ml) is transferred into a 15 ml falcon tube and 10 ml M10Gi are added drop by drop while pivoting over a period of at least 5 minutes. If M10Gi is added too fast cells will undergo an osmotic shock and burst. 15 ml falcons are centrifuged at 1000 rpm for 7 minutes at room temperature (Heraeus Biofuge, swing out buckets). Pellet is resuspended in 1 ml of M10Gi. Another 3 ml of M10Gi are added to 15 ml falcons and content is transferred onto a gelatin-coated 10cm culture dish. Falcons are washed with 4 ml of fresh M10Gi and solution is added to culture dishes. Check plates the next day and change medium if necessary.

7.3.2 Cultivation of SNL76/7

SNL67/7 cells are cultured at 37°C, 5% CO₂. Medium must be replaced by fresh M10Gi if color indicator switches to orange. Cells must be split when plate is confluent.

7.3.3 Splitting of SNL76/7

When confluent, cells must be split onto new gelatin-coated culture dishes. Plates are washed with 4 ml 1x PBS. PBS is applied to remove traces of M10Gi which would interfere with the function of trypsin. 1 ml of trypsin is added to culture plate and plates are placed into incubator (37°C, 5% CO₂) for 5 minutes or until cell contacts are dissolved. If unsure, cells can be examined under a light microscope. Cells will display a round morphology and are not elongated as in their adhesive state. 3 ml of fresh M10Gi are added to culture plates to stop trypsin reaction. Cells are resuspended and 500 µl are transferred onto gelatin-coated culture plates containing 7 ml of fresh M10Gi (for 1:8 splitting).

7.3.4 Freezing of SNL76/7

When SNL76/7 are confluent, cells can be frozen. Therefore, cells are washed in 4 ml PBS. After removal of PBS 0.7 ml trypsin are added to 10cm plate and placed into incubator (37°C; 5%) for 10 minutes. Trypsinized cells are resuspended in 2 ml of M10Gi and transferred to a 15 ml falcon tube. 2.7 ml of freezing medium are added drop by drop to the suspension. This should be done very slowly. The contents is divided into two cryotubes and placed in a styrofoam box which is then sealed with tape and placed into a -80°C freezer. After two days cryotubes can be moved to a liquid nitrogen tank.

Freezing medium:

60% DMEM

20% Gibco FBS Serum

20% DMSO

7.3.5 Generating feeder cells

All culture dishes used must be gelatin-coated in advance. Confluent SNL76/7 plates are used to make feeder cells. All but 4 ml of the medium is removed and 80 µl Mitomycin C are added to the culture dishes. After a maximum of 4 hours at 37°C, 5% CO₂ the supernatant is removed and plates are washed twice with 1x PBS. 1 ml of trypsin is added to each plate and placed at 37°, 5% CO₂ for 5 minutes or until cells are

floating around. Cells are resuspended in 5 ml of M10Gi, if more than one plate is trypsinized cells can be pooled (maximum of three plates) and solution is transferred into a 50 ml falcon. In order to transfer most cells, plates are washed with an additional 10 ml M10Gi which can be pooled again. The falcon is centrifuged at 1000 rpm for 7 minutes (Heraeus Biofuge, swing out buckets) and supernatant is discarded. Pellet is resuspended in 10 ml of M10Gi. Cell number is measured with CASY cell counter (10 μ m-30 μ m). Cells are diluted till viable cell number per ml reaches 3.5×10^6 . For 24 well plates pipette 0.5 ml/well, for 6 well plates 2 ml/well, and for 6 cm plates use 4 ml of suspension. Feeder cells can be kept at 37°C; 5% CO₂ for up to two weeks and have to be fed at least once a week with M10Gi.

7.4 Maintenance of mouse embryonic stem cells

To keep mouse ESCs undifferentiated they are co-cultured with feeder cells (see above) which express leukemia inhibitory factor.

7.4.1 Thawing of mESCs

Thawing of ESCs is quite similar to thawing of SNL76/7. Feeder cells must be fed with M10Hy at least 2 hours prior to thawing of mESCs. Cryotubes are removed from liquid nitrogen tank, thawed in a 37°C water bath until only a small icicle is left and then sprayed with 70% EtOH, placed into laminar and flamed. Content is transferred to 15 ml falcon tubes and 12 ml of M15Hy are added drop by drop, this should be done slowly and tubes should be pivoted after each drop. Tubes are centrifuged at 1000 rpm for 6 minutes and supernatant is discarded. Cell pellets are resuspended in 1 ml of pre-fed feeder cell supernatant and then transferred back onto feeder cells. Wash tubes with another 500 μ l of fresh M15Hy. Cells most likely do not have to be fed within the next 48 h.

7.4.2 Cultivation of mESCs

ESCs are cultivated at 37°C, 5% CO₂. Medium must be replaced by fresh M15Hy daily. Cells must be split when plate is confluent.

7.4.3 Splitting of mESCs

Feeder cells and ESCs must be fed with fresh M15Hy two hours prior to splitting (feeders 2 ml, ESC 0.5 ml). ESCs are washed once with 1x PBS. After removal of PBS trypsin is applied to plates (200 μ l/24 well; 500 μ l/6 well; 1 ml/10cm) and plates are placed at 37°C, 5% CO₂ for fifteen to twenty minutes. In case of 24 wells 1 ml of

M15Hy medium (for all other wells at least thrice the amount of trypsin used) from previously fed feeder cells is transferred onto ESCs and resuspended well. For splitting 1:2 600 µl of suspension are transferred back onto feeder cells (1:3, 400 µl).

7.4.4 Freezing of mESCs

Cells are fed 2 hours prior to freezing. Cells are washed with PBS and trypsinized (200 µl/24 well) and after 15 minutes at 37°C, 5% CO₂ 800 µl fresh M15Hy are added to 24 wells. Cells are resuspended and transferred into 15 ml falcons. 1.8 ml freezing medium is added drop by drop (slow!) to falcons, after each drop falcons must be shaken slightly to insure homogenous distribution. After addition suspension is divided into two cryotubes, these are labeled and placed into to a styrofoam box, which is then sealed and placed at -80°C for at least two days before transfer of cryotubes into liquid nitrogen tank.

Freezing medium:

60%	DMEM
20%	HyClone FBS Serum
20%	DMSO

7.5 Maintenance of CVPCs

See 7.4 Maintenance of mouse embryonic stem cells (mESCs).

7.6 Embryoid bodies and Cardiac Bodies

7.6.1 Generation of EBs/CBs

mESCs/CVPCs are cultured on feeder cells in 24 wells and must be split at a ratio of 1:2 one day before EB/CB formation in order for cells to be in log phase. Cells are fed 2 hours prior to starting point with M15Si and 6 well plates are gelatin-coated. After 2 hours cells are washed with 1x PBS and 200 µl of trypsin are added to each 24 well. Plates are placed in an incubator at 37°C, 5% CO₂ until cell bonds are disassociated. 800 µl of M15Si are added to stop the reaction and suspension is transferred onto gelatin-coated 6 well plates containing 2 ml of M15Si in order to separate stem cells from feeder cells. Feeder cells adhere faster to the gelatin-coated surface of the 6 well plates while ESCs/CVPCs remain nonattached for a longer period of time. After 50 minutes of incubation at 37°C, 5% CO₂ supernatant is transferred into a 15 ml falcon. Suspension is filled up to 10 ml total volume and cell number is measured using a

CASY counter. Suspension is diluted with M15Si to a cell concentration of 4×10^4 cells/ml for ESCs and $4,5 \times 10^4$ cells/ml for CVPCs. 2 ml of these dilutions are enough for one 10cm dish of hanging drops. Sterilin culture plates are filled with autoclaved MilliQ water (a 4 mm high water level is enough). A repeating pipette is set to 20 μ l dispensation (equals 800 cells per drop). Repeating pipette is held at a 90° angle. Drops are placed as close as possible without running risk of merging. Do not place drops too close to the edge of the bacterial dish lid to avoid contamination. Flip lid over and place on top of water filled bottom half. Place carefully at 37°C, 5% CO₂; this timepoint is referred to as day 0. At day 4.7 the hanging drop cultures have to be transferred onto gelatin-coated 10 cm tissue culture plates to allow attachment of the EBs/CBs to the culture dishes. Hanging drop cultures are flamed shortly at the edge. 8 ml of M15Si are used to rinse EBs/CBs from the lid onto the bottom of a gelatin coated culture plate. Place plates at 37°C, 5% CO₂ and do not move for the following 48 h.

7.6.2 Culture of EBs/CBs

EBs/CBs must be fed every 3 days with M15Si, medium is only partially replaced (day 7 and 10: 3 ml of old medium +5 ml fresh medium; day 13 to 24: 4 ml old medium +10 ml fresh medium, day 25 and thereafter: 5 ml old medium +12 ml fresh medium). Partial medium replacement ensures that secreted factors remain in medium at low concentration.

Beating of EBs/CBs is observed on a daily basis; both number of beating EBs/CBs and intensity of beating areas is quantified.

7.7 Isolation of Proteins for SDS-PAGE

Cells are washed with 37°C 1x PBS and icecold, fresh Kinexus Lysis Buffer (e.g. 100 μ l/48 well, 150 μ l/24 well) is added immediately. Cells are scraped off and transferred into an Eppendorf tube and placed on ice. If necessary, wells can be washed with additional 50 μ l of Kinexus lysis buffer.

Kinexus lysis buffer

20 mM	Tris pH7.0
2 mM	EGTA
5 mM	EDTA
30 mM	Sodiumfluorid
40 mM	Glycerophosphate

10 mM Sodium Pyrophosphate
2 mM Sodium Orthovanadate
10 μ M Leupeptin
5 μ M Pepstatin A
0.5% Triton X-100
Proteinase-Inhibitor (1 tablet per 100 ml)

7.7.1 Bradford protein concentration measurement

5x Bradford Stock Solution is diluted 1:5 in ddH₂O and 200 μ l of solution are transferred into each 96 well. 4 μ l of sample are added to each well, duplicates are made. BSA (2.5 μ g/ μ l, 1.25 μ g/ml, 0.625 μ g/ μ l, 0.313 μ g/ μ l, 0.156 μ g/ μ l) gradient is used for standard curve. 4 μ l of standard dilutions are added to 200 μ l of 1x Bradford solution in duplicates. Protein concentrations are measured at 595 nm on a Labsystems Multiscan RC with the Labsystems Multiscan Genesis Software. Values should be below 1. Standard curve is calculated, regression should be above 0.996. Protein concentrations of samples are calculated according to standard curve.

5x Bradford stock solution

100 mg Coomassie
50 ml absolute MeOH or EtOH
50 ml 85% H₃PO₄
fill up to 200 μ l with dd H₂O
store in the dark

7.8 SDS-PAGE

5 ml of running gel is poured and coated with isopropanol. Once the gel is hardened isopropanol is removed and stacking gel is poured. SDS-PAGE gels are transferred into running buffer. 3x sample buffer is added to samples at a ratio of 1:3. Samples and marker are boiled at 95°C for 10 min before applying to gel. SDS-PAGE is run between 100V and 150V till the loading dye has leaked at the bottom end.

Running gel 12.5% (makes 2 gels)

5 ml PAA
3 ml 1.5M Tris/HCl pH8.8, 0.4% SDS
4 ml ddH₂O
120 μ l 10% APS

10 μ l TEMED

Stacking gel 5% (makes 2 gels)

0.5 ml PAA

0.75 ml 0.5M Tris/HCl pH6.8, 0.4% SDS

1.75 ml ddH₂O

30 μ l 10% APS

2.5 μ l TEMED

10x Page buffer

1.92 M Glycin

250 mM Tris

1% SDS

3x Sample buffer (Laemmli reducing)

3 ml Glycerin

0.9 g SDS

3.75 ml 1.5M Tris/HCl pH8.8 0.4%SDS

1.75 ml ddH₂O

6 mg Bromphenolblue

Aliquot and add 150 μ l

7.9 Western Blotting

4 Whatmann papers are soaked in 1x blotting buffer and are placed into the semi-dry Western Blot gear (Trans Blot SD, Biorad). Nitrocellulose membrane (Whatman, Protran) soaked in 1x blotting buffer is placed on top. The SDS-PAGE gel is placed on top of all this and topped with 4 more Whatmann papers soaked in 1x blotting buffer. Device is closed and run at 400 mA for one hour. After successful Western Blotting nitrocellulose membrane is transferred into Ponceau S solution for 5 minutes. Ponceau S staining visualizes protein-ladder and protein-bands. Bands of protein ladder are marked on nitrocellulose membrane with a charcoal pencil before de-staining with ddH₂O.

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

7.9.1 Antibody incubation and visualization

Nitrocellulose membrane is blocked in 5% Milk in TBS-T for at least one hour at 4°C. First antibody is diluted in 5% Milk TBS-T and incubated at 4°C for at least one hour. Incubation is followed by three wash steps in TBS-T for 6-10 min each. Second antibody linked to horseradish peroxidase or alkaline phosphatase is diluted in 5% Milk TBS-T and incubated for at least 1h.

5% Milk in TBS-T

5% Skim Milk Powder

0.1% Tween-20

TBS

10x TBS

0.5 M Tris

1.5 M NaCl

pH 7.6

7.9.1.1 Detection of HRP-conjugated antibodies

Nitrocellulose membrane is washed thrice with TBS for 6-10 minutes. Lumigio Reagent A (Cell Signaling) and Peroxide Reagent B (Cell Signaling) are mixed at a ratio of 1:1 and are brought to 1x concentration by dilution with ddH₂O. 1,5 ml of solution is used to coat one nitrocellulose membrane (5x8cm) and incubated for one minute. Bands are detected with CL-XPosure Film (Thermo Scientific) in a dark room using an Agfa Curix 60 developer.

7.9.1.2 Detection of AP-conjugated antibodies

Membrane is washed three times with TBS-T for 6-10 min each and is then incubated with 10 ml AP-Buffer containing 66 µl of NBT (added first) and 33 µl of BCIP (added second). Incubation takes place in the dark, within 5-60 minutes bands start appearing. Reaction is stopped with ddH₂O.

AP-Buffer

100 mM Tris HCl pH9.5

100 mM NaCl

5 mM MgCl 6 H₂O

BCIP

0.5 g BCIP

10 ml 100% Dimethylformamide

store at 4°C

NBT

0.5 g NBT

10 ml 70% Dimethylformamide

store at 4°C

7.9.2 Stripping of Western Blots

7.9.2.1 Stripping of primary and secondary antibody

Membrane is washed thrice for 5 minutes in 1x TBS and is then transferred into a 50 ml falcon containing 45 ml Stripping Buffer A. Falcon is placed in a 70°C water bath for 30 min under constant shaking. Nitrocellulose membrane is repeatedly washed with 1x TBS until β-Mercaptoethanol smell has vanished.

Stripping Buffer A

2% SDS

100 mM β-Mercaptoethanol

62.5 mM Tris-HCl pH 6.8

7.9.2.2 Stripping of secondary antibody

Nitrocellulose membrane is incubated in 0.2 M NaOH for 7 minutes at room temperature followed by four 6 minute wash steps in TBS-T. Secondary Antibody should be removed now. Only use antibodies produced in different donor than the previous one.

7.9.3 Dot Blot

Dot Blot analysis can be used to estimate protein concentrations of a sample compared to known concentrations of the purified protein if a specific antibody is available.

2 µl of sample are spotted onto nitrocellulose membrane, each spot should be comparable in size and should not exceed 3-4 mm diameter. After membrane has dried it is blocked in 5% BSA in TBS-T for one hour at room temperature. Primary antibody is diluted in BSA/TBS-T and incubated for at least 30 minutes or longer. Membrane is washed thrice with TBS-T for 5 minutes each. Secondary antibody (HRP- or AP-conjugated) is diluted in BSA/TBS-T and incubated for at least 30 minutes. For detection protocol see 7.9.1.

TBS

20 mM Tris-HCl

150 mM NaCl

pH7.5

TBS-T

0.05% Tween 20 in TBS

BSA/TBS-T

0.1% BSA in TBS-T

7.10 Isolation of RNA from ESCs and CVPCs

Cells have to be fed two hours prior to RNA isolation. If cells are grown on feeder cells they are trypsinized, resuspended in M15Hy and plated on gelatin-coated wells for 50 minutes at 37°C, 5% CO₂ in order to separate stem cells from feeder cells. Feeder cells adhere faster to the gelatin-coated surface of the 6 well plates while ESCs/CVPCs remain nonattached for a longer period of time. After 50 minutes of incubation at 37°C, 5% CO₂ supernatant is transferred into a 15 ml falcon. If cells are not grown on feeders the preadsorption can be left out and cells are trypsinized, resuspended and directly transferred to 15 ml falcons. Falcons are rotated for 5 min at 1000 rpm at room temperature, and pellet is washed with 4 ml of PBS. Centrifugation step is repeated and pellet is resuspended in 1 ml 1x PBS and transferred to an Eppendorf tube. Tubes are centrifuged at 14 000 rpm for 5 min at 4°C.

RNeasy Mini Kit, Qia-shredder protocol

Pellets are resuspended in 600 µl of RLT buffer-β-Mercaptoethanol (100:1) and loaded onto RNeasy Mini Spin Column (pink) and centrifuged at room temperature for 2 minutes at 13 000 rpm. Column is discarded and 600 µl of 70% EtOH are added to flow-through. 600 µl of this suspension are loaded onto QIAshredder Spin Column

(purple) and centrifuged for 15 seconds at 13 000 rpm at room temperature. Flow-through is discarded and the remaining 600 µl of suspension are loaded onto column and centrifugation step is repeated. Flow-through is discarded and 700 µl RW1 is loaded onto column and centrifugation is repeated as before. After flow through is discarded 500 µl of RPE are loaded and centrifuged for 2 minutes at same conditions. This last step is repeated once more, the column is transferred into a fresh 2 ml collection tube and spun down for 1 minute at 13 000 rpm. Column is transferred into a clean Eppendorf tube and 35 µl RNase-Free Water are added and nucleic acids are eluted by a 1 minute centrifugation step at 13 000 rpm at room temperature.

DNA digestion

3.75 µl DNase 1 buffer and 3.75 µl DNase 1 are added and tube is placed at 37°C for 30 minutes. To stop the digestion 3.75 µl EDTA are added and tube is placed on shaker for 10 minutes at 65°C. After this, the tube is stored at -80°C. Successful digestion of DNA has to be checked by running a GAPDH PCR. If digestion was successful only positive control should show a band at 300bp.

Reverse Transcription

RT-Mix

10 µl	5x Buffer
5 µl	0.1M DTT
1.5 µl	RiboLock RNase Inhibitor
2 µl	10mM dNTPs

1 µl d(T) is added to DNA-free sample and incubated at 70°C for 10 minutes followed by 3 minutes on ice and 15 seconds spinning at 13 000 rpm. 18.5 µl RT-Mix are added to sample and incubated at 42°C for 2 minutes. 1 µl Revert Aid Reverse Transcriptase is added to samples and incubated for 50 minutes at 42°C. This step is followed by 15 minutes at 70°C and 5 min on ice. After 2 minutes of centrifugation at 13 000 rpm samples are stored at -80°C. Concentration of nucleic acids is measured by Nanotrop.

When comparing samples with each other the amount of sample added to RT-PCR master mix must be balanced by GAPDH RT-PCRs first.

7.11 PCR

Mastermix

38,75 µl	dH ₂ O	
5 µl	10x buffer	(Fermentas #EP0402)
3 µl	25 mM MgCl	(Fermentas #EP0402)
1 µl	10 mM dNTPs	(Fermentas #R0192)
0.5 µl	Forward primer	(Vienna BioTec)
0.5 µl	Reverse primer	(Vienna BioTec)
0.25 µl	Taq Polymerase	(Fermentas #EP0402)

A mulitple of the mastermix can be made and aliquots of 49 µl are added to each PCR tubes (Klösch) and 0.9-2 µl of sample DNA or cDNA are added. Tubes are placed in Biometra T-Personal PCR machine.

PCR-Program:

1. 94°C for 60 sec
2. 94°C for 45 sec
3. k°C for 45 sec usually 50°C<k<66°C depending on primers
4. 72°C for 60 sec
5. Repeat step 2-4 for 28-39 cycles, depending on T_M of primers
6. 72°C for 240 sec
7. 4°C

7.12 Immunofluorescence

Cells are adherent to culture dishes and are washed once with 1x PBS followed by addition of 4% Paraformaldehyde. After 20 min at room temperature, cells are washed twice with 1x PBS. 0.1% Saponin in 1x PBS is used to permeabilize cells and after 20 minutes wash step is repeated. Blocking is achieved with 2% BSA in TBS/PBS for 10 minutes. 1st Antibody is diluted in antibody dilution solution and incubated for 1 h at 4°C. The application of parafilm on top of cell layer may help to evenly distribute antibody. Cells are washed thrice with 1x PBS for ten minutes each followed by an incubation with secondary antibody for one hour at 4°C. Cells are washed once with 1x PBS for 10 minutes followed by a 10 minute wash step with 1x PBS containing a 1:1000 dilution of DAPI. After this, cells are washed twice for 10 min with 1x PBS and coated with 55°C Mowiol. Coverslip is applied while Mowiol is still warm and slides

are stored in the dark. Once Mowiol hardend, cover slips are sealed with non-fluorescing nail polish. A LSM-Meta 510 (Zeiss) Microscope is used to visualize staining.

Antibody Dilution Solution

2% BSA in 1x TBS

7.13 Karyotyping

Cells can be treated with 10 µl colchicine (1 µg/ µl) for 3 h prior to trypsinization in order to inhibit spindle formation.

Confluent cells (24 wells) are washed with 1 ml of 1x PBS and 200 µl of trypsin are added. After 15-20 min at 37°C 5% CO₂ 800 µl M15Hy are added and suspension is transferred to a 15 ml falcon. This step is followed by 5 min centrifugation at 1000 rpm at room temperature. All but 0.5 ml of supernatant are discarded and pellet is resuspended in the remaining 0.5 ml. 4 ml of 75 mM KCl buffer (room temperature) are added drop-wise. Let stand at room temperature for 6 minutes. After this, suspension is centrifuged at room temperature for 5 min at 1000 rpm. Supernatant is removed and 4 ml of a 3:1 methanol:glacial acetic acid mixture are added with a Pasteur pipette. Centrifugation step is repeated, supernatant discarded and 3 ml of methanol:glacial acetic acid are added. This step is repeated till a satisfying pellet is achieved. After that centrifugation step is repeated once more and 0.5 ml of methanol:glacial acetic acid are added. More can be added if dilution of cells is not high enough. Before further use suspension should be stored at 4°C for at least 3 h. Wash slides in 70% Ethanol and let them dry before use. Place slides at a 30-60° angle and use a Pasteur pipette to create individual drops of cells. Drops will run down the slide and thereby spread the cells over the entire slide. Place Pasteur pipette between 3-6 cm above slides to achieve better results. Suspension can be kept over months at 4°C.

7.14 Purification of recombinant M-LIF

The SF9 cell line was derived from pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda*. The SF9 cell line is highly susceptible to infection with *Autographa California* nuclear polyhedrosis virus (AcNPV baculovirus), and can be used with all baculovirus expression vectors. SF9 cells are commonly used to isolate and propagate recombinant baculoviral stocks and for the productions of recombinant proteins.

SF9 freezing medium

20%	Gibco FBS serum
20%	DMSO
60%	Sf-900 medium

SF9 culture medium

93%	Sf-900 medium
1%	Streptomycin
2%	Gibco serum

7.14.1 Thawing of SF9 insect cells

SF9 cells are kept in a liquid nitrogen tank in freezing medium containing 20% Gibco FBS Serum. Kryotube is thawed in water bath till only a small icicle remains, sprayed with 70% EtOH and flamed before opening. Contents is transferred into 15 ml flasks and 12 ml of SF9 20% Gi are added drop by drop. Falcon is centrifuged at room temperature for 4 minutes at 1000 rpm. Pellet is resuspended in 1 ml thawing medium and another 5 ml of thawing medium are added and mixed well. Suspension is transferred into 75cm² culture flask containing 5 ml of thawing medium. Culture at 28°C but do not close lid fully so that air can circulate. Replace medium after 24h by fresh thawing medium to remove dead cells. When cells are confluent scrape them off with a Pasteur pipette and transfer them into a falcon and centrifuge for 5 minutes at room temperature (RT) at 1000 rpm. Resuspend pellet in Sf-900 15% Gi serum and culture till confluent. Reduce serum to 10%, then 5% and finally 2% serum every time cells are confluent and split. Sf900 2% Gi is used as regular culture medium.

Thawing medium

3,6 ml	Gibco
16,4 ml	SF9 medium

7.14.2 Maintenance of SF9 insect cells

Cells are kept at 28°C in a SF9 culture medium. Lids of culture flasks should not be fully closed. Medium should be changed about twice a week.

7.14.3 Infection of SF9 insect cells

When density reaches 40x10⁶ cells per culture flask (75cm²) cells are ready to be transfected. 40x10⁶ cells are diluted in 20 ml Sf-900 culture medium and baculovirus is

added according to the desired MOI. After 96 h cells are scraped off and transferred into 50 ml falcons. Falcons are centrifuged at 1000 rpm at room temperature for 5 minutes. Supernatant is transferred into a new falcon and stored at -80°C. Pellet is washed with 5 ml of 1x PBS and centrifuged for 5 minutes at room temperature at 1000 rpm. Supernatant is discarded and pellet can be stored at -80°C.

7.14.4 Protein purification from SF9 insect cells

Cell pellet is thawed on ice and resuspended in 3.5 ml of lysis buffer (use two Eppis). After 15 min on ice, use a Douncer to crush cells by moving up and down approximately 50 times (on ice). Keep suspension on ice for another 10 minutes and centrifuge at 4°C for 20 min at 14 000 rpm. Keep 10 µl of supernatant for analysis. The remaining supernatant is divided into four Eppendorf tubes containing approximately 200 µl of Ni-NTA agarose beads (Quiagen) each. Ni-NTA agarose beads are previously pipetted into Eppendorf tubes and spun down for 2 min at 13 000 rpm at RT, supernatant is removed and beads should come up to 0.1 ml marker on Eppendorf tube. 300 µl of lysis buffer are added followed by centrifugation for 2 min at 13 000 rpm at RT and removal of supernatant before sample can be added. After addition of supernatant to the beads the tubes are rotated on 4°C for at least 2.5 h or overnight. After that Eppendorf tubes are centrifuged at 4°C for 5 minutes at 14 000 rpm, supernatant is discarded and 500 µl of wash buffer are added and mixed well followed by another centrifugation step. Washing is repeated 3 times. 200 µl of elution buffer I are added to the beads and tubes are shaken for 10 min at 800 rpm followed by 5 minutes centrifugation at 4°C at 14 000 rpm. Supernatant is collected and is named eluate 1. This step is repeated to achieve eluate 2. 200 µl of elution buffer 2 are used to accumulate eluate 3 and another 200 µl of elution buffer 2 to get eluate 4. Eluates are stored at 4°C.

Lysis buffer

50 mM NaH₂PO₄

300 mM NaCl

10 mM Imidazole

adjust pH to 8 using 1 M NaOH

add NP-40 at a dilution of 1:100 directly before use

add Aprotinin, Leupeptin, Pepstatin A at a dilution of 1:1000 each right before use

Wash buffer

50 mM NaH_2PO_4

300 mM NaCl

10 mM Imidazole

adjust pH to 8 using 1 M NaOH

add Aprotinin, Leupeptin, Pepstatin A at a dilution of 1:1000 each right before use

Elution buffer I

50 mM NaH_2PO_4

300 mM NaCl

50 mM Imidazole

adjust pH to 8 using 1 M NaOH

add Aprotinin, Leupeptin, Pepstatin A at a dilution of 1:1000 each right before use

Elution buffer II

50 mM NaH_2PO_4

300 mM NaCl

250 mM Imidazole

adjust pH to 8 using 1 M NaOH

add Aprotinin, Leupeptin, Pepstatin A at a dilution of 1:1000 each right before use

7.14.5 Dialysis of purified protein from SF9 insect cells

If the purified protein is used for cell culture work it needs to be purified prior to use. About 2 cm of cellulose tubular membrane (Roth) per Eppendorf tube is needed. Membrane is rinsed with distilled water and then placed in a 50 ml falcon containing 50 ml of solution 1. Falcon is placed in a 60°C water bath for 3 h in order to remove traces of heavy metal ions. Prior to use the membrane is rinsed with distilled water.

The rear end of a Pasteur pipette is heated over a burner and used to burn holes into the lids of sterile Eppendorf tubes. 400 μl of sample are added into each tube and 2 cm of membrane tube is cut off and cut open to create a 2 cm x 2 cm surface. Membrane is placed on top of Eppendorf tubes and lids are closed, fixating the membrane to the tubes. Place tubes upside-down in individual 50 ml falcons and centrifuge for 4 min at 1000 rpm. Tubes should not leak, in case they do, replace membrane and repeat centrifugation step to make sure tubes are sealed well. Tubes are placed upside-down in a beaker and placed in 1x PBS at a volume of 1:1000 in relation to the sample amount.

The following day content is transferred into fresh Eppendorf tubes and protein concentration is measured by Nanodrop.

Western Blot can be performed to ensure presence of M-LIF protein in solution. Previously performed mass spectroscopy (MALDI-TOF) showed that M-LIF makes up about 2% of total protein concentration.

Solution 1

1 mM EDTA
2% Sodiumhydrogencarbonate

7.15 Transfection of CVPCs

Transfection medium

83% DMEM
15% HyClone
1% L-Glutamine (amino acids)
1% β -Mercaptoethanol

24 wells are gelatin-coated two hours prior to use. Confluent cells are trypsinized and counted with a CASY counter. 10^5 cells per well are seeded in 1 ml of transfection medium free of antibiotics. Cells are cultured over night and can be transfected the next morning.

7.15.1 Transfection with pGM3-EGFP Vimentin MIDI

EGFP transfections are used to optimize transfection protocol. DNA to Lipofectamine 2000 ratio of 1:1 are used. 1 μ l of Lipofectamine 2000 is added to 49 μ l of DMEM-1% Glutamine and kept at room temperature for 5 minutes. 1 μ g pGM3-EGFP Vimentin MIDI DNA is added to DMEM-1% Glutamine to a total volume of 50 μ l and mixture is then added to Lipofectamine 2000-DMEM solution. After 20 minutes at room temperature the entire 100 μ l are added to the cells seeded the day before (after reducing the medium to 0.5 ml/well). After three hours of incubation at 37°C, 5% CO₂ the supernatant is replaced by regular M15Hy culture medium. Efficiency of transfection can be visualized in YFP channel on a Zeiss Axio Stereomicroscope after 20h.

7.15.2 Transfection with plasmid DNA and renilla

DNA to Lipofectamine 2000 ratio used is 1:1. Renilla to plasmid DNA ratio used is 1:100.

1 μ l of Lipofectamine 2000 is added to 49 μ l of DMEM-1% Glutamine and kept at room temperature for 5 minutes. 1 μ g plasmid DNA is added to DMEM-1% Glutamine to a total volume of 50 μ l and mixture is then added to Lipofectamine 2000-DMEM solution. After 20 minutes at room temperature the entire 100 μ l are added to the cells seeded the day before (after reducing the medium to 0.5 ml per well). After three hours of incubation at 37°C, 5% CO₂ the supernatant is replaced by regular M15Hy culture medium. To see if SPARC, M-LIF, or other proteins have short time influence they can be added to cells at this time point. After 48 h wells are washed with 1x PBS twice and 500 μ l of 1x Passive lysis buffer are added. With the help of a pipette tip contact surfaces for the lysis buffer are scraped. After 50 min cells are resuspended and transferred into an Eppendorf tube. Wells are washed with 100 μ l of 1x Lysisbuffer. 6 μ l of sample are diluted with 30 μ l of 1x lysis buffer and 20 μ l of the mixture are transferred onto 96 well micro plate. Make sure samples are mixed well before any dilution step. Renilla and firefly values are measured with a LB960 Microplate Luminometer (Berthold Technologies). Renilla values are measured as an internal control to determine transfection efficiency per well. Therefore, firefly values are divided by the renilla values in order to create comparable results.

Passive lysis buffer

Promega Dual-Luciferase Reporter Assay System Kit #E19910

8 Appendix

8.1 Differentiation

control	beating CBs	Total CBs	percentage	intensity
d9	0	38	0	0
d10	0	38	0	0
d11	2	38	5	2
d12	13	54	24	5
d13	36	63	57	10
d14	58	63	92	15
d15	58	62	94	16,5
d16	58	62	94	18
d17	63	63	100	24
d18	63	63	100	21,5
d19	61	63	97	16
d20	63	63	100	20,5
d21	63	63	100	18,5

Table 11 Cardiomyogenesis in CBs (Figure 34). A5p47 (4.4×10^4 cells/ml). Data is total of 6 control wells. Number of beating CBs was determined daily. Intensity of beating was quantified on a scale from 0 (no activity) to 6 (>70% of the area contracting) and represents sum of all 6 control wells.

treatment period (d)	0-4.7	4.7-7	7-10	10-13
M-LIF	0,13	0,98	0,52	0,40
BMP2	1,17	1,49	1,50	1,38
M-LIF+BMP2	0,17	1,05	0,70	0,47
anti-LIF	1,37	1,17	0,87	1,47
anti-BMP2/4	1,03	1,01	0,92	0,85
control	1,00	1,00	1,00	1,00

Table 12 Beating intensity of CBs (Figure 38). CBs were treated with M-LIF (100ng/ml), BMP2 (100ng/ml), anti-BMP2/4 antibodies (500 µg/ml), anti-LIF antibodies (1 µg/ml), and BMP2 and M-LIF combined, respectively. Substances were added on day 1 of treatment period. Treatment periods were day 0-4.7, day 4.7-7, day 7-10, and day 10-13, respectively. 6 control plates were left untreated. Areas of beating cardiomyocytes per CB were quantified daily on a scale from 0 (no activity) to 6 (>70% of the area contracting). Values of day 0-20 were added and set relative to control. Control values can be seen in Table 11.

8.2 Transfections

NKE	Firefly (FF)	Renilla (R)	A		σ
			FF/R	Mean	
blank value	180	650	0.2769		
control	4240	147750	0.0287	0.0299	0.0023
	4070	124990	0.0326		
	4440	156220	0.0284		
BMP2	3480	163870	0.0212	0.0254	0.0045
	4630	153730	0.0301		
	4410	178290	0.0247		
SPARC	4810	191430	0.0251	0.0244	0.0006
	3780	158340	0.0239		
	3640	149770	0.0243		
blank value	180	650	0.2769		
control	2330	35490	0.0657	0.0631	0.0048
	2560	38720	0.0661		
	1680	29180	0.0576		
BMP2	3730	52030	0.0717	0.0692	0.0101
	3070	39460	0.0778		
	2810	48350	0.0581		
SPARC	2340	44790	0.0522	0.0579	0.0058
	2760	48070	0.0574		
	3210	50230	0.0639		

Table 13 Nkx2.5 activation by BMP2 and SPARC (Figure 46). A5p27 (A) and A5p14 (B) were transfected with the NKE24 plasmid and the Renilla plasmid at a ratio of 100:1. BMP2 and SPARC have no influence on Nkx2.5 promoter activation 48h post transfection.

transfected plasmids	FF	R	FF/R	FF/R/[pGL3b]	Mean value	Standard deviation
NKE+pUC	5000	46110	0.1084	60.93	48.38	17.75
	4610	72310	0.0638	35.82		
NKE+Desmin+pUC	4070	96980	0.0420	23.58	21.56	2.86
	4600	132300	0.0348	19.54		
NKE+Desmin+kd 4	1790	54010	0.0331	18.62	17.29	1.89
	1950	68690	0.0284	15.95		
NKE+Desmin+kd-ct	6690	180880	0.0370	20.78	21.80	1.44
	6710	165230	0.0406	22.82		
NKE+kd-4+pUC	2800	89110	0.0314	17.66	17.31	0.49
	2410	79810	0.0302	16.97		
pGL3b+pUC	540	309390	0.0017	1.00		
	490	270150	0.0018			
blank	280	500	0.5600			

Table 14 Luciferase Assay of CVPC clone A5 (Figure 47A). 330 ng of each plasmid was used (Renilla 10 ng), vector pUC was used to compensate to a total of 1000 ng. Firefly values (FF), Renilla values (R), Firefly relative to Renilla (FF/R), FF/R relative to pGL3b (FF/R/[pGL3b]).

transfected plasmids	FF	R	FF/R	FF/R/ [pGL3b F/R]	Mean value	Standard deviation
NKE+pUC	1390	8580	0.16	15.09	12.13	4.19
	1420	14440	0.10	9.16		
NKE+Desmin+pUC	1350	20500	0.07	6.13	5.68	0.65
	1120	19980	0.06	5.22		
NKE+Desmin+kd 4	1060	14600	0.07	6.76	12.12	7.58
	730	3890	0.19	17.48		
NKE+Desmin+kd- ct	1740	20790	0.08	7.80	6.01	2.53
	1920	42430	0.05	4.22		
NKE+kd-4+pUC	940	19300	0.05	4.54	5.23	0.99
	1970	30940	0.06	5.93		
NKE+kd-ct+pUC	3140	59830	0.05	4.89	4.98	0.13
	3570	65510	0.05	5.08		
pGL3b+pUC	380	35400	0.01	1.00		
blank	280	620	0.45			

Table 15 Luciferase Assay of CVPC clone A5 (Figure 47B). 330 ng of each plasmid was used (Renilla 10 ng), vector pUC was used to compensate to a total of 1000 ng. Firefly values (FF), Renilla values (R), Firefly relative to Renilla (FF/R), FF/R relative to pGL3b (FF/R[pGL3b]). The values that are highlighted red were not incorporated in graphic.

transfected plasmids	FF	R	FF/R	FF/R/ [pGL3b F/R]	Mean value	Standard deviation
NKE+pUC	3170	33090	0.0958	30.32	31.26	1.33
	4090	40200	0.1017	32.20		
NKE+Desmin+pUC	2630	37050	0.0710	22.47	18.10	2.96
	2590	48440	0.0535	16.92		
	1830	36430	0.0502	15.90		
	2830	52360	0.0540	17.11		
NKE+Desmin+kd 4	970	15120	0.0642	20.30	18.44	1.38
	1190	20280	0.0587	18.57		
	1240	22040	0.0563	17.81		
	1380	25570	0.0540	17.08		
NKE+Desmin+kd- ct	1470	19320	0.0761	24.08	25.72	2.40
	1640	22300	0.0735	23.28		
	1420	15990	0.0888	28.11		
	1480	17080	0.0867	27.42		
NKE+kd-4+pUC	1410	27890	0.0506	16.00	16.49	0.69
	1850	34480	0.0537	16.98		
NKE+kd-ct+pUC	4200	75870	0.0554	17.52	16.88	0.90
	3580	69760	0.0513	16.24		
pGL3b+pUC	330	110520	0.0030		1.00	
	310	93000	0.0033			
blank	270	571	0.4729			
	250	550	0.4545			

Table 16 Luciferase Assay of CVPC clone A5 (Figure 47C) 330 ng of each plasmid was used (Renilla 10 ng), vector pUC was used to compensate to a total of 1000 ng. Firefly values (FF), Renilla values (R), Firefly relative to Renilla (FF/R), FF/R relative to pGL3b (FF/R[pGL3b]).

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10 Zusammenfassung

Herz-Kreislauf-Erkrankungen sind weltweit die häufigste Todesursache. Allein in Österreich stirbt fast jede zweite Person an Herz-Kreislauf-Erkrankungen. Das adulte Herz wurde lange Zeit für ein post-mitotisches Organ ohne regenerativer Kapazität gehalten. Dadurch wäre ein Herzinfarkt verursachtes Sterben von Kardiomyozyten irreversibel. Innerhalb der letzten Jahre wurde das Vorhandensein von Herzvorläuferzellen von verschiedenen Arbeitsgruppen gezeigt. Eine in-vivo Aktivierung und Differenzierung dieser Zellen würde neue Therapiemöglichkeiten für Herzinfarkt-Patienten eröffnen. Daher ist die Untersuchung der Signaltransduktionskaskaden die der Aktivierung und Differenzierung dieser Vorläuferzellen unterliegen essentiell und würde zu einem besseren Verständnis der Selbsterneuerungskapazität des murinen Herzens beitragen.

In dieser Arbeit berichten wir über die Isolierung einer stabilen, euploiden, murinen Herz-Vorläufer-Zelllinie (cardiovascular progenitor cell (CVPC) line). Wir konnten diese Zellen über 60 Passagen kultivieren ohne einen Verlust der Selbsterneuerungskapazität oder einer Minderung der Potenz festzustellen. CVPCs exprimieren die Stammzellmarker Oct3/4, Nanog und Sox2 gleichzeitig mit den Herzzellmarker Nkx2.5, Mef2c und GATA4. Letzteres steht im Gegensatz zu murinen embryonalen Stammzellen (mESCs). Die Expression dieser Herzzellmarker steht im Einklang mit der Potenz-Limitierung dieser Zellen im Vergleich zu mESCs. CVPCs differenzieren ausschließlich zu Zellen des Herzens, nämlich Kardiomyozyten, glatter Muskelzellen und Endothelzellen. Sowohl die Expression von Herzzellmarkern im undifferenzierten Zustand als auch die Limitierung des Differenzierungspotentials sind Hinweise darauf, dass CVPCs bereits auf die Differenzierung zu Zellen des Herzens eingeschränkt sind. Die Differenzierung von CVPCs wird durch den Entzug von Leukemia Inhibitory Factor (LIF) eingeleitet und ist unabhängig von anderen induzierenden Faktoren. LIF ist nötig und hinreichend um die Selbsterneuerung von CVPCs zu erhalten und hat einen negativen Effekt auf die Differenzierung von CVPCs. Eine zeitlich begrenzte Inhibierung der LIF-Signalgebung unterstützt die Differenzierung von CVPCs und Bone Morphogenic Protein 2 erhöht die Differenzierung zu Kardiomyocyten im Cardiac Body System signifikant.

CVPCS unterscheiden sich deutlich von den häufig untersuchten mESCs. Da ihr Potential limitiert ist und CVPCs bereits auf die Differenzierung zu Zellen des Herzens

eingeschränkt sind, stellen sie verglichen mit mESCs ein niedrigeres Risiko in Stammzelltransplantationen dar. Weitere Untersuchungen von CVPCs werden zu einem besseren Verständnis der Selbsterneuerungskapazität des murinen Herzens führen.

11 Abstract

Cardiovascular Diseases are the leading cause of death globally and account for almost one out of two deaths in Austria. The adult heart was thought to be a post-mitotic organ without a regenerative capacity. Therefore cell death of cardiomyocytes after myocardial infarction (MI) was believed to be irreversible. Recently, the existence of cardiovascular progenitor cells in the adult heart was demonstrated by various groups. The activation and differentiation of these cells *in vivo* would provide a new therapy option for MI patients. Therefore, the study of the cell signaling cascades underlying cardiomyocyte differentiation and cardiovascular progenitor cell activation *in vitro* would benefit the better understanding of the *in vivo* regenerative capacity of the murine heart.

Here we report the isolation of a stable, euploid murine cardiovascular progenitor cell (CVPC) line. We successfully cultured these cells for over 60 passages without a loss of self-renewal capacity or a decrease in potency. CVPCs express stemness factors Oct3/4, Nanog, and Sox2 simultaneously with cardiac markers Nkx2.5, Mef2c, and GATA4, the latter stands in contrast to murine embryonic stem cells (mESCs). The expression of cardiac markers in the undifferentiated state of CVPCs goes hand in hand with the potency-limitations of these cells compared to mESCs. CVPCs are restricted to differentiate to cell types of the cardiac lineage, namely cardiomyocytes, smooth muscle cells, and endothelium. The expression of cardiac markers in the undifferentiated state and the limitation of the differentiation potential indicate that CVPCs are already committed to give rise to cells of the cardiac lineage. The differentiation of CVPCs is induced upon Leukemia Inhibitory Factor (LIF) deprivation and is not dependent on other inductive signals. Furthermore, LIF is necessary and sufficient to maintain self-renewal of CVPCs and has a negative effect on differentiation. Inhibition of LIF signaling aids differentiation of CVPCs and Bone Morphogenetic Protein 2 significantly enhances differentiation to cardiomyocytes in the Cardiac Body system in a time dependent manner.

CVPCs are clearly distinct from the most commonly studied mESCs. Since their potential is limited and CVPCs are committed to the cardiac lineage, they bear lower risks than mESCs in stem cell transplantation approaches. Further investigation of CVPCs will contribute to a better understanding of the self-renewal capacity of the murine heart.

12 Curriculum Vitae

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