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1. Introduction

1.1 The cardiovascular disease

Cardiovascular diseases (CVDs) are the leading death cause worldwide as more people die annually from CVDs than from any other cause. With an estimated death toll of 17.3 million people in 2008 CVDs represent 30% of all global deaths (WHO, 2012). About 79 million Americans suffer from CVDs with an estimated 870000 people dying from them each year, making up 36% of all deaths in the United States (AHA, 2007). In general, CVDs are a group of disorders of the heart and blood vessels that include coronary heart disease, cerebrovascular disease, congenital heart disease, rheumatic heart disease, peripheral arterial disease or thrombosis and pulmonary embolism (WHO, 2012). These diseases are usually accompanied or caused by atherosclerosis, the thickening of the inner walls of blood vessels due to accumulation of fatty deposits resulting in the blockage of blood flowing (ischemia) to the heart or brain, and hypertension (raised blood pressure).

The most important behavioral risk factors of CVDs are unhealthy or high-fat diet, physical inactivity (Hansel et al., 2012), tobacco use and harmful use of alcohol. These risk factors are responsible for about 80% of coronary heart disease and cerebrovascular disease cases. The effects of unhealthy diet and physical inactivity may lead to raised blood pressure, raised blood glucose, raised blood lipids, overweight or obesity. These are referred to as intermediate or metabolic risk factors. In addition there are also a number of underlying determinants for CVDs, such as poverty, stress and hereditary factors (WHO, 2012). People with type 2 diabetes mellitus are at a higher risk for CVDs (Hillis et al., 2012), also recent bacterial or viral infections, particularly respiratory tract infections, have been shown to serve as temporarily acting, independent trigger factors to ischemic stroke (Grau et al., 2010). Recently, nutrigenomic analyses revealed that robust genetic associations for CVDs, such as the chromosome 9p21 region, are affected by healthy diet in a way to decrease the risk of myocardial infarction (Do et al., 2011). Of all underlying determinants poverty is still the major cause as over 80% of CVD deaths take place in low- and middle-income countries (WHO, 2012).

Of all deaths related to CVDs, an estimated 7.3 million were due to coronary heart disease (CHD) and 6.2 million were due to stroke (WHO, 2012). Strokes are usually acute events and are mainly caused by either ischemia or hemorrhage, a leakage of blood. The sudden

induction of ischemia by occlusion of a major branch of a coronary artery leads to a series of events that eventually ends with severe irreversible damage or loss (Beltrami et al., 1994; Jennings et al., 1990) of cardiac tissue accompanied by scar formation (Joggerst and Hatzopoulos, 2009). Scar formation is an essential aspect of rapid wound healing, especially in the injured myocardium, which is under constant wall stress and therefore requires a rapid mechanical barrier (Mutsaers et al., 1997). However, scar tissue is largely acellular and lacks the biochemical properties of the host cells. This leads to electrical uncoupling, mechanical dysfunction and loss of structural integrity, ultimately resulting in a dilated cardiomyopathy.

As the adult heart was believed to be a post-mitotic organ the permanent loss of cardiac tissue was seen as an irreversible event (Anversa et al., 2005) that was mostly treated by the use of tissue transplants - harbouring the risk of graft rejection and shortage of donors -, pharmacotherapy, cardiovascular implantable electronic devices (CIEDs) (Gandhi et al., 2012) - which bear the risk of infection - and, secondarily, by lifestyle changes concerning diet, physical activity or alcohol and tobacco use (Kaski and Fernandez-Berges, 2008). Despite significant improvements in therapy, thanks to newer treatment modalities and risk-reduction strategies (Hunink et al., 1997), CVDs remain a continuous health problem, which has prompted research into new therapeutic approaches (Joggerst and Hatzopoulos, 2009).

With the notion that the heart has the capability of self-renewal regulated by a compartment of multipotent cardiac stem cells (CSCs) capable of regenerating myocytes and coronary vessels throughout life, the old paradigm describing the adult heart as a post-mitotic organ has been heavily questioned (Anversa et al., 2005). The discovery of cardiac stem cell populations and newly gained insights into isolation and expansion of these cells *in vitro* makes restorative stem cell therapy a promising approach for treatment of CVDs. Although much knowledge has been gained through more than a decade of research, numerous barriers to successful cardiac regeneration remain. Therefore, a better understanding of processes that lead to both damage and repair is needed before stem cell therapy can be applied in a safe and useful way (Joggerst and Hatzopoulos, 2009).

1.2 Stem cells

1.2.1 Self-renewal and potency of differentiation

Multicellular organisms require the existence and precise control of stem cells that maintain tissue homeostasis by replacement of terminally differentiated, aged or injured cells (Boiani and Scholer, 2005). In 1981 two groups independently discovered the possibility to isolate and cultivate murine embryonic stem cells (ESCs) from the inner cell mass (ICM) of pre-implantation embryos (Evans and Kaufman, 1981; Martin, 1981). Since the first isolation of human ESCs (hESCs) from blastocysts in 1998 (Thomson et al., 1998) the field of stem cell biology went on to further enhance common knowledge on early developmental processes with the future aim of therapeutic use of stem cells or stem cell-derived tissues in regenerative medicine and/or drug discovery.

In general, there are two characteristics that distinguish stem cells from somatic cells: their capacity of self-renewal and their potency of differentiation. Depending on the presence of respective intrinsic and extrinsic stimuli stem cells either proliferate (theoretically) indefinitely giving rise to identical daughter cells without depletion of the stem cell pool, loss of stemness and potency or onset of senescence, or give rise to at least one, or sometimes many, specialized somatic cell types, a process called differentiation that involves both symmetric and asymmetric cell division (Fig.1) (Donovan and Gearhart, 2001; Niwa, 2007; Silva and Smith, 2008). An inherent feature of self-renewal capacity is the ability of stem cells to theoretically survive and proliferate as a single cell to maintain the stem cell pool, which is referred to as clonality, although it is very likely that, in vivo, surrounding helper cells facilitate this process.

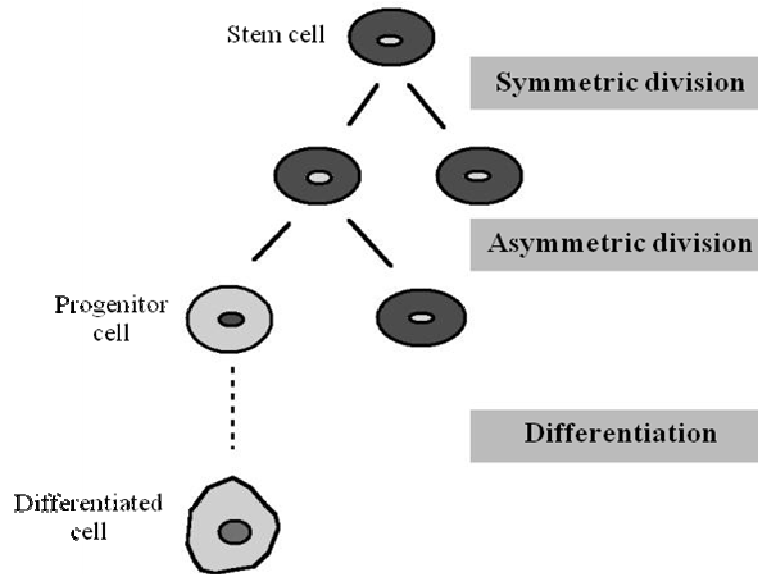


Fig.1: Schematic representation of stem cell division and differentiation. Upon every cell division a stem cell makes the choice between symmetric (two identical daughter cells) and asymmetric cell division (two different daughter cells), eventually yielding a progenitor cell which then differentiates to a specialized cell type (Fagoonee et al., 2009).

There are several classes of potency: totipotency (omnipotency), pluripotency, multipotency (oligopotency) and unipotency (monopotency). This classification basically refers to the developmental potential of a stem cell, the amount of different somatic cell types this cell can give rise to upon differentiation (Fig.2).

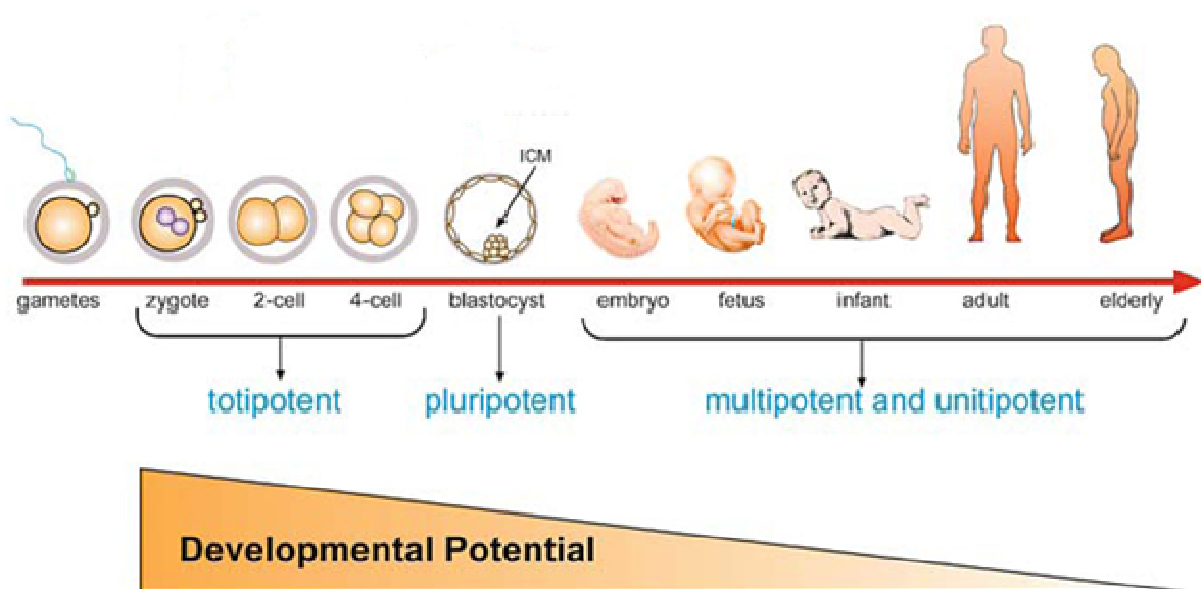


Fig.2: Developmental potential of stem cells over the time-course of a lifetime. Ontogeny begins from a single cell, the zygote. The zygote and all blastomeres of the early embryo (to at least the 4-cell stage) are considered totipotent and therefore capable of autonomously building the whole organism. As development proceeds, the developmental potential (potency of differentiation) of individual blastomeres gradually declines, resulting subsequently in pluripotent, multipotent, unipotent and, finally, terminally differentiated cells (Mitalipov and Wolf, 2009).

Totipotency describes the ability of a single stem cell to give rise to all somatic cell types required for a viable organism from all three germ-layers - ectoderm, mesoderm and endoderm – including extra-embryonic tissue like trophoctoderm. The fertilized oocyte (zygote) and all blastomeres of the embryo to at least the 4-cell stage are considered totipotent (Mitalipov and Wolf, 2009; Western, 2009).

Pluripotency refers to the capacity of a single cell to form cells from all three germ-layers, but not extra-embryonic tissue (Boiani and Scholer, 2005; Donovan and Gearhart, 2001; Ulloa-Montoya et al., 2005). ESCs derived of the ICM of pre-implantation embryos are considered pluripotent, but nevertheless they cannot produce a viable organism on their own because they lack the potential to organize the embryo.

Stem cells that give rise to a limited number of cell types are termed multipotent and often referred to as somatic or adult stem cells. These tissue specific stem cells have less differentiation potential than pluripotent stem cells as they are restricted to give rise to either derivatives of a single germ layer – for example, mesenchymal stem cells (MSCs) are only capable of differentiation into fibroblasts, adipocytes, chondrocytes, muscle cells and so on – or a specific sublineage (Mitalipov and Wolf, 2009; Solter, 2006). Hematopoietic stem cells (HSCs), one of the best studied adult stem cell types, are a prominent example of multipotent stem cells and differentiate along the hematopoietic lineage into at least 8 different cell types, like erythrocytes, leucocytes and lymphocytes (Alenzi et al., 2009).

Unipotent cells produce only one specialized cell-type. Spermatogonial stem cells in the testis are an example for unipotency as they can only differentiate into one cell-type, the spermazoon (Donovan and Gearhart, 2001).

1.2.2 Pluripotent stem cell lines

Three distinct types of mammalian pluripotent stem cell lines have been isolated so far: embryonic carcinoma cells (ECCs), embryonic germ cells (EGCs) and embryonic stem cells (ESCs) (Fig.3) (Donovan and Gearhart, 2001).

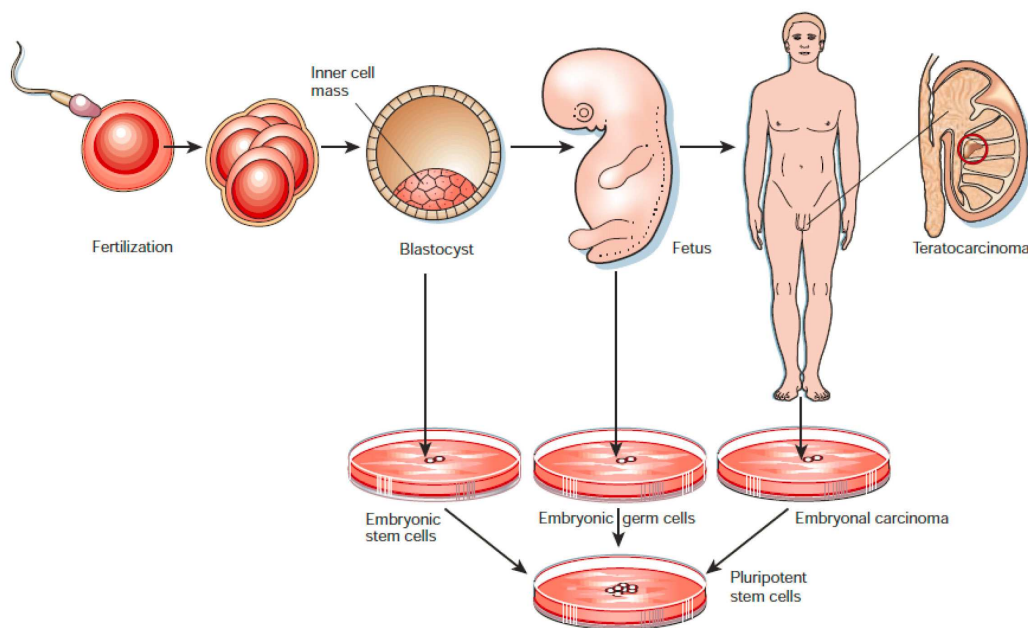


Fig.3: *Origins of pluripotent stem cells in humans.* Different types of pluripotent stem cells can be isolated or derived from different tissues at different times throughout development. EGCs are derived from PGCs isolated from the embryonic gonad. ECCs originate from PGCs as well but were first detected as components of testicular tumors in the adult organism, while ESCs can be isolated from the ICM of the pre-implantation blastocyst-stage embryo (Donovan and Gearhart, 2001).

The field of pluripotent stem cells began in the 1950s with the study of teratocarcinomas which are malignant germ cell tumors comprising both undifferentiated ECCs and differentiated cells from all three germ layers (Yu and Thomson, 2008). ECCs themselves are derived from PGCs, the embryonic precursors of the gametes (Stevens, 1967), and give rise to all differentiated cell types of the tumor (Kleinsmith and Pierce, 1964). First murine ECC lines that could be stably propagated in vitro were established in the 1970s by isolation of ECCs from testicular germ cell tumors (Kahan and Ephrussi, 1970; Rosenthal et al., 1970).

Mammalian EGCs, like ECCs, are derived from cultured mammalian PGCs which can be isolated directly from the embryonic gonad (Matsui et al., 1992; Resnick et al., 1992). When cultivated on feeder layers in the presence of serum and certain growth factors PGCs will form colonies that are morphologically indistinguishable from ECCs and ESCs (Donovan and Gearhart, 2001). The derivation of human EGCs has also been reported (Shamblott et al., 1998). Nevertheless, so far it has not been possible to generate a stable, clonally derived cell line as human EGCs seem to lose their proliferative potential in long-term culture (Turnpenny et al., 2003; Yu and Thomson, 2008).

ESCs are derived from the ICM of the pre-implantation, blastocyst-stage embryo (Evans and Kaufman, 1981; Martin, 1981). Unlike ECCs, murine ESCs have a stable karyotype and the

potential to contribute to a variety of tissues in chimeras, including germ cells, which makes them a practical tool for introducing genetic modifications to the mouse germline (Bradley et al., 1984).

Pluripotent stem cells can also be generated by reprogramming adult somatic cells. The obtained cells are referred to as induced pluripotent stem cells (iPSCs) and, especially in mouse models, share a lot of features with ESCs. Basically, there are three strategies for reprogramming somatic cells (Fig.4).

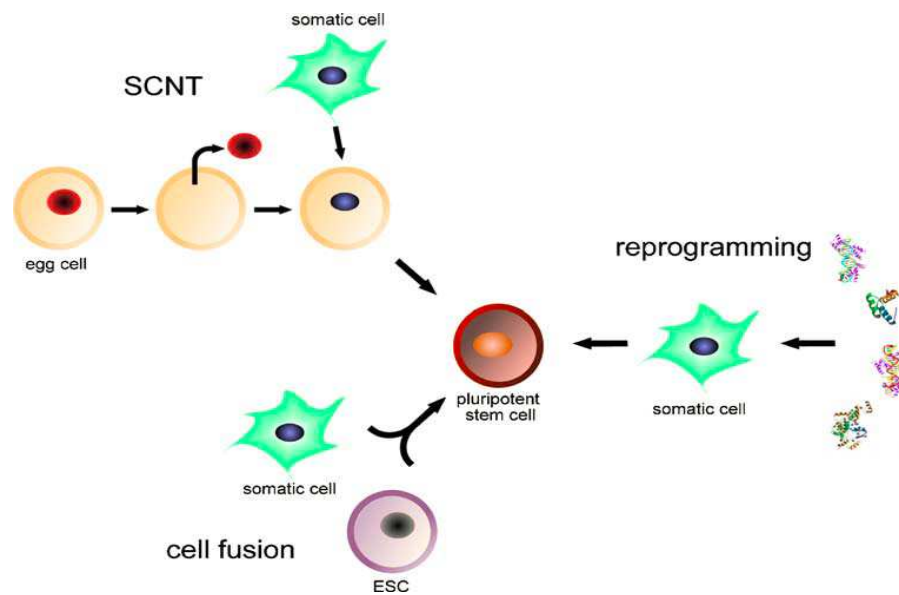


Fig.4: Strategies to generate induced pluripotent stem cells (iPSCs). The generation of iPSCs can be achieved by SCNT (cloning), where a somatic donor nucleus is introduced into an enucleated oocyte. Fusion of a somatic cell with an ESC yields pluripotent tetraploid hybrid cells containing both the somatic and the ESC chromosomes, which limits their use in therapeutic applications. A novel strategy is the direct reprogramming of somatic cells by viral-mediated transduction of defined transcription factors that turn on stemness genes and re-establish pluripotency (Leeb et al., 2010).

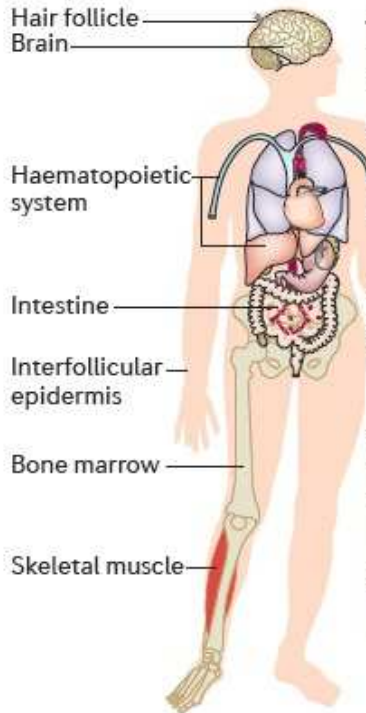
Somatic cell nuclear transfer (SCNT) can be used to generate an iPSC-derived cloned animal by introduction of a nucleus from a somatic donor cell into an enucleated oocyte. This leads to an epigenetic reset of the donor DNA, allowing the epigenetic state of the somatic cell to be reprogrammed to an embryonic state that enables the iPSC to form a blastocyst capable of directing development of a new organism (Jaenisch and Young, 2008). The generation of iPSCs by SCNT that were able to develop into viable organisms was first accomplished in 1997 when Dolly the sheep was cloned (Wilmut et al., 1997). Despite ethical concerns and its low efficiency with human cells SCNT might serve as a powerful tool to create patient-specific iPSCs for therapeutic use (Leeb et al., 2010; Yu and Thomson, 2008).

Another way of generating iPSC is the fusion of somatic cells with ESCs, resulting in tetraploid pluripotent hybrid cells that show morphology, growth rate and antigen expression patterns similar to ESCs (Cowan et al., 2005). Due to the fact that both the somatic and the ESC chromosomes are present these cells cannot be used for therapeutic applications. Recently, a method was established by which selected chromosomes can be removed from ESC-derived hybrid cells (Matsumura et al., 2007), but the question remains if the cells remain pluripotent after removal of all ESC chromosomes (Leeb et al., 2010).

Induction of pluripotency can also be achieved by directly reprogramming somatic cells to iPSCs by introduction of defined transcription factors that reactivate stemness genes. In 2006, mouse embryonic fibroblasts (MEFs) and adult fibroblasts were successfully reprogrammed after viral-mediated transduction of the four transcription factors OCT4, SOX2, KLF4 and c-MYC (Takahashi and Yamanaka, 2006). The obtained iPSCs showed similar morphology and growth properties of ESCs, expressed ESC marker genes, were able to form teratomas containing tissues from all three germ layers and partly contributed to mouse embryonic development when injected into blastocysts. Direct reprogramming is a very promising approach because it circumvents the use of human eggs or embryonic tissue and may be used for the generation of patient-specific iPSCs in therapeutic applications (Jaenisch and Young, 2008; Leeb et al., 2010; Yu and Thomson, 2008).

1.2.3 Adult stem cell lines

Development of a multicellular organism requires a series of events that include cellular proliferation, lineage commitment and lineage progression. The sequential progression of ESCs through these events results in the formation of differentiated cells, tissues and organs that make up the individual organism. However, not all future tissue specific cells progress through this sequence. In various tissues a small number of cells evade terminal differentiation and function as reserve precursor cells which are involved in the maintenance and repair (homeostasis) of the respective tissue throughout the life span of the organism (Young and Black, 2004). These adult stem cells (ASCs) replenish themselves through self-renewal and, upon diverse external stimuli like injury and/or inflammation, differentiate into one (unipotent) or several (multipotent) downstream cell lineages. ASCs are found in many tissues of the human body and can be categorized into two groups: depending on the requirements of the respective tissue ASCs with high or low turnover exist (Fig.5) (Hsu and Fuchs, 2012).



Tissue type	Stem cell location
<i>Tissues with constant turnover</i>	
Haematopoietic system	Bone marrow
Intestine	Fast-cycling: base of crypt Slow-cycling: '+4 position'
Interfollicular epidermis	Basal layer of epidermis
Hair follicle	Bulge
<i>Tissues with low or no turnover</i>	
Brain	Subventricular zone, subgranular zone
Skeletal muscle	Between the basement membrane and the muscle fibres

Fig.5: Locations of adult stem cells in different tissue types. Small numbers of undifferentiated ASCs reside in various tissues in the human body and ensure tissue homeostasis. In tissues with constant or high turnover rates - such as the hematopoietic system, the intestine, the interfollicular epidermis or the hair follicle - they are mainly responsible for tissue maintenance and repair after injury. They also reside in tissues with low or almost no turnover - such as the brain or skeletal muscle. In skeletal muscle ASCs are mainly reserved for tissue repair, while in the brain they are required for the generation of new neurons, either for maintenance of the olfactory bulb (subventricular zone) or after learning stimulations (subgranular zone) (Hsu and Fuchs, 2012).

Epidermal, blood or intestinal cells undergo constant turnover, which necessitates constant activity of the respective stem cells. In the hair follicle, stem cells are only needed periodically for hair growth (Cotsarelis et al., 1990; Morris and Potten, 1999; Tumber et al., 2004), while in relatively dormant tissues, like brain (Doetsch et al., 1999) or skeletal muscle (Collins et al., 2005; Gros et al., 2005), ASCs undergo limited or no cell division but can respond effectively to stimuli, such as learning activities (Villeda et al., 2011; Zhang et al., 2008) in the brain or injury in the skeletal muscle (Conboy and Rando, 2002; Hsu and Fuchs, 2012; Slack, 2008).

A major challenge in the isolation and characterization of diverse ASC types is to prove whether tissue specific ASCs are in fact stem cells or rather represent a multipotent progenitor or transit amplifying cell that is still capable of self-renewal and differentiation into several mature lineages. The difference between progenitors and genuine ASCs can be shown in genetic lineage tracing experiments (Kretzschmar and Watt, 2012), however, due to the lack

of suitable highly specific markers and feasible *in vivo* assays the precise identity of various ASC types has been described only fairly recently (Grompe, 2012). In addition, recent studies have shown that there are two categories of progenitor cells that reside within organs and tissues of postnatal animals: lineage-committed (multi-, tri-, bi- and unipotent) progenitor cells and lineage-uncommitted (epiblastic-like; ecto-, meso- and endodermal) stem cells (Young and Black, 2004). The latter are believed to represent some sort of pluripotent ASC populations which are able to circulate around the body and turn into multiple cell types depending on the local environment in a process called transdifferentiation (Blau et al., 2001; Kucia et al., 2005; Slack, 2008). Altogether, these findings add even more complexity to the challenging task of characterizing the cellular identity of tissue-specific ASCs.

1.2.3.1 Cardiovascular progenitor cells

For decades, the heart was believed to be a post-mitotic organ without any regenerative potential as most cardiomyocytes (CMCs) quit the cell cycle during the perinatal period. The major response to myocardial damage was thought to be hypertrophy of the residual CMCs (Barile et al., 2007; Soonpaa and Field, 1998). With the recent notion that adult mammalian hearts may harbor stem cells with replicative and regenerative potential, this dogmatic view has been heavily challenged. In 2001, a study in patients with myocardial infarction showed an increased number of immature CMCs, situated in the border zone, that were capable of mitosis and may have originated from an endogenous cardiac stem cell pool or from circulating stem cells from non-cardiac tissues (Beltrami et al., 2001; Wu et al., 2008). Since then, the isolation and characterization of putative cardiovascular progenitor cells (CVPCs) has been an emerging field in cardiovascular research.

Numerous research groups have succeeded in the *in situ* identification of these cells in human (Bearzi et al., 2007; Beltrami et al., 2003; Beltrami et al., 2001; Laugwitz et al., 2008; Messina et al., 2004) or murine (Messina et al., 2004; Tallini et al., 2009; Tateishi et al., 2007) hearts. CVPCs have been characterized by expression of the stem cell factor receptor (cKit) (Bearzi et al., 2007; Beltrami et al., 2003; Tallini et al., 2009), stem cell antigen 1 (SCA1) (Matsuura et al., 2004; Oh et al., 2003; Smits et al., 2009), Islet-1 (ISL1) (Laugwitz et al., 2005; Moretti et al., 2006) or members of the ATP-binding cassette (ABC) transporter/multidrug resistance (MDR) protein family (Martin et al., 2004). *In vitro* assays have shown that these cells are clonogenic, capable of self-renewal and have the potential to differentiate into CMCs, endothelial cells (ETCs), smooth muscle cells (SMCs) (Bearzi et al.,

2007; Oyama et al., 2007; Smits et al., 2009; Wu et al., 2008) and, potentially, cardiac fibroblasts (Zeisberg and Kalluri, 2010). This may corroborate the hypothesis that the main cardiac cell types - CMCs, SMCs and ETCs - are derived from a common quasi-primordial CVPC (Moretti et al., 2006). Injection of human or murine CVPCs (*referred to as cardiac stem cells, CSCs, in cited literature*) into ischaemic hearts of mice or rats led to at least partial reconstitution of the myocardium, vessel formation and yielded CMCs with the characteristics of young cells in the heart ventricle (Bearzi et al., 2007; Beltrami et al., 2003). However, their functional contribution to the heart has not unambiguously been demonstrated so far (Taubenschmid and Weitzer, 2012).

The existence of endogenous CVPCs is heavily challenged by the presence of multipotent precursor cells in the peripheral human blood which could be able to transmigrate through vessel walls and populate the heart, acquiring the identity of the resident cells (Cesselli et al., 2009). Also, reprogramming (Efe et al., 2011; Ieda et al., 2010) and transdifferentiation (Takeuchi and Bruneau, 2009) events, as well as the plasticity of somatic cells (Raff, 2003; Zipori, 2004) contradict the proposed CVPC hypothesis. However, even if isolation and culture techniques still have to be further improved, recent advances in the molecular understanding of the developmental aspects and stem cell biology of the cardiovascular system will bring cardiac cell therapy one step closer to clinical application (Taubenschmid and Weitzer, 2012; Wu et al., 2008).

1.2.4 The stem cell niche

Many tissues of the adult organism maintain a population of (adult) stem cells, referred to as regenerative stem cell pool, that govern tissue homeostasis and respond to injurious stimuli (Walker et al., 2009). To sustain this function throughout the entire life of an organism the delicate balance between self-renewal and differentiation must be under tight spatiotemporal control (Li and Xie, 2005). Therefore, these cells reside in highly regulated microenvironments, called niches, which are maintained by a constant dialogue between the stem cells and the surrounding niche cells. The niche protects the stem cells from differentiation or apoptotic stimuli (or any other stimuli that might challenge the stem cell pool) and also prevents them from overproduction, which would eventually lead to cancer (Mitsiadis et al., 2007; Moore and Lemischka, 2006).

In 1978, the niche hypothesis was proposed for the first time (Schofield, 1978) and different niche systems in diverse model organisms have been extensively investigated ever since,

initially in the gonadal tissue from *D. melanogaster* and *C. elegans* (Crittenden et al., 2002; Ellis and Kimble, 1994; Kimble and White, 1981; Xie and Spradling, 2000). In invertebrates the hematopoietic (Adams and Scadden, 2006; Schofield, 1978) and hair follicle (Cotsarelis et al., 1990) stem cell niches are among the better characterized niche models, along with other niche systems that have been investigated lately (Morrison and Spradling, 2008).

In general, the niche contains all cellular and non-cellular components needed for control of the respective stem cell. The interaction of these components with the stem cell is either established by physical contact to a niche cell via tight, adherence or gap junctions, or by diffusible factors secreted from niche cells that modulate the stem cell's transcription via signaling cascades (Fig.6) (Walker et al., 2009).

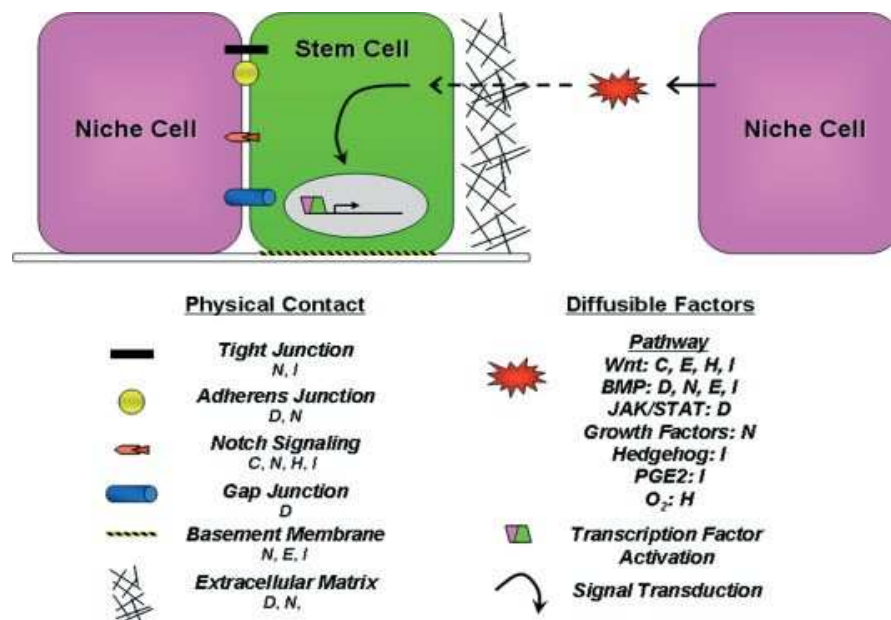


Fig.6: The adult stem cell niche model. The two mechanisms that regulate the adult stem cell are illustrated above. Regulation by physical contact of a niche cell with the stem cell includes tight, adherens and gap junctions, the basement membrane, extracellular matrix proteins and notch signaling. Diffusible factors like signaling ligands, growth factors and prostaglandin E2 (PGE2) are secreted by niche cells and modulate the stem cell's gene expression via diverse signaling pathways, with Wnt, BMP, JAK/STAT and hedgehog signaling among the most prominent ones. Each interaction is shown with a list of single letters designating the niche utilizing it: (C) *C. elegans* germline; (D) *D. melanogaster* germline; (N) neural stem cell (NSC) niche; (E) epidermal stem cell niche; (H) HSC niche; (I) intestinal stem cell (ISC) niche (Walker et al., 2009).

In order to facilitate cell-fate decisions in a proper spatiotemporal manner the niche has to be a highly dynamic and interactive structural unit, as key signaling and molecular cross-talk events have to be patterned to happen in the right place at the right time. As a result, many niche systems share a common feature: anatomical organization ensures stem cell function in space and time, as regulation is established by positive, negative and intercellular signaling

cascades (Moore and Lemischka, 2006). In some niche systems the basement membrane and extracellular matrix (ECM) have been shown to directly regulate adjacent stem cells, also metabolic products, such as calcium, influence stem cell behavior, enabling the cells to respond to varying conditions of tissue state (Scadden, 2006). Recently it was shown that even stem cell progeny within the niche can provide important and diversified feedback mechanisms to regulate their stem cell parents and to ensure control of tissue homeostasis (Hsu and Fuchs, 2012; Mondal et al., 2011).

1.2.5 The pluripotency network as a regulator of stem cell fate decisions

Stem cells are considered to be the most promising source for clinical cell therapy applications. Therefore, elucidating the precise mechanisms that regulate self-renewal, pluripotency and differentiation is of major interest and countless *in vivo* and *in vitro* studies have revealed regulators that play important roles in these processes. The growth of ESCs as a pluripotent population requires a delicate balance between survival, proliferation, self-renewal and differentiation, which is regulated by several intrinsic and extrinsic factors including signaling molecules, transcription factors, cell-cycle regulators, microRNAs and epigenetic modifications (Liu et al., 2007).

1.2.5.1 Extrinsic regulation via signaling pathways

Several signaling pathways are involved in the maintenance of pluripotency or the induction of differentiation. One of the best characterized extrinsic regulators related to pluripotency in murine ESCs (mESCs) is the leukemia inhibitory factor (LIF), a member of the IL-6 cytokine family (Smith et al., 1988; Williams et al., 1988). LIF directly binds to a heterodimeric receptor (LIFR-gp130 complex) to form a trimeric complex (Zhang et al., 1997) that activates the canonical Janus kinase signal transducer and activator of transcription (JAK/STAT) pathway by phosphorylation of STAT3. Activated STAT3 is then translocated into the nucleus and activates the transcription of genes required for self-renewal.

Additionally, binding of LIF also activates phosphatidylinositol-3-OH-kinase (PI(3)K) signaling which leads to phosphorylation of the serine/threonine kinase Akt. PI(3)K-Akt signaling promotes ESC proliferation (Takahashi et al., 2003) and self-renewal (Paling et al., 2004; Takahashi et al., 2005), but, unlike JAK/STAT signaling, it can be induced by multiple different receptor tyrosine kinases for growth factors, such as the fibroblast growth factor

(FGF) or the insulin-like growth factor (IGF; Fig.8) (Liu et al., 2007; Okita and Yamanaka, 2006).

Alternatively, binding of LIF to the LIFR-gp130 receptor complex can also trigger differentiation by activation of the SRC-Homology-2(SH2)-domain-containing protein tyrosine phosphatase-2 (SHP-2). SHP-2 induces the phosphorylation of extracellular signal-regulated protein kinases ERK1 and ERK2, which increases mitogen-activated protein kinase (MAPK) activity (Fig.7) (Auernhammer et al., 2000; Boeuf et al., 1997; Boiani and Scholer, 2005; Burdon et al., 1999; Niwa et al., 2009).

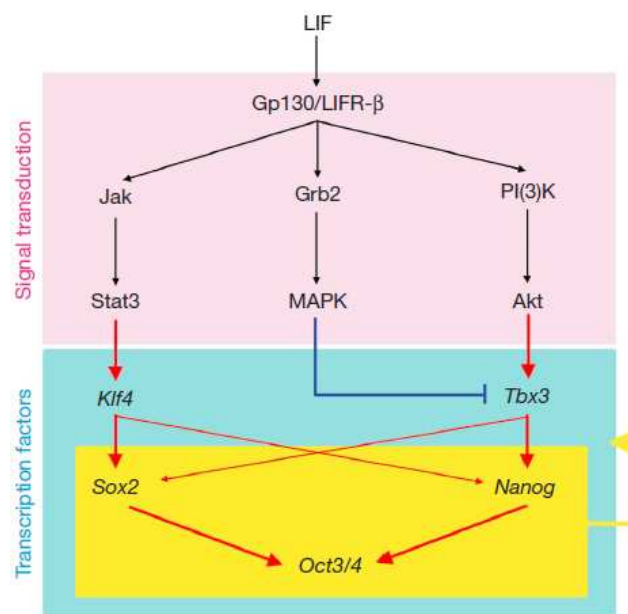


Fig.7: The parallel circuitry of LIF signaling cascades. JAK/STAT signaling activates KLF4, while PI(3)K-Akt signaling stimulates TBX3 transcription. The MAPK pathway plays an antagonistic role by inhibition of TBX3. KLF4 and TBX3 mainly activate the stemness transcription factors SOX2 and NANOG which maintain OCT3/4 expression. SOX2, NANOG and OCT3/4 positively regulate themselves via feedback mechanisms, ensuring stable expression even in the absence of all signals (Niwa et al., 2009).

It has been shown that STAT3 activation is essential to maintain pluripotency in mESCs (Matsuda et al., 1999; Niwa et al., 1998), however, the additional presence of serum also contributes to self-renewal signals, proposing that STAT3 is not strictly sufficient for self-renewal (Ying et al., 2003). Unlike mESCs, LIF/STAT3 signaling does not prevent human ESCs (hESCs) from differentiation (Humphrey et al., 2004; Sumi et al., 2004). LIFR and gp130 are expressed in hESCs, but functional activation of STAT3 by human LIF fails to maintain pluripotency (Daheron et al., 2004). Instead, maintenance of pluripotency in hESCs is achieved by WNT (Sato et al., 2004) and a mutual cooperation of Activin/Nodal and FGF signaling pathways (Vallier et al., 2005).

Along LIF signaling, TGF β /BMP signaling is another crucial regulator governing ESC fate decisions. The transforming growth factor β (TGF β) family of cytokines contains two subfamilies, the TGF β /Activin/Nodal subfamily and the bone morphogenetic protein (BMP)/growth and differentiation factor (GDF) subfamily. Although all diverse TGF β ligands share common sequence and structural features, they elicit quite different cellular responses (Shi and Massague, 2003).

TGF β signaling starts with ligand binding to heterodimeric complexes of either type I or type II serine/threonine kinase receptors. Each TGF β superfamily member binds to a unique combination of type I and type II receptors (Valdimarsdottir and Mummery, 2005). For example, TGF β and activin have high affinity for their type II receptors (Hart et al., 2002), whereas BMPs have higher affinity for their type I receptors than their type II receptors (Kirsch et al., 2000; Shi and Massague, 2003). Binding of TGF β superfamily members triggers complex formation of receptor components and leads to phosphorylation of intracellular signal transduction molecules, called similar to mothers against decapentaplegic (Smad). These are named after their homologues in *Drosophila melanogaster* (MAD; mothers against decapentaplegic) (Sekelsky et al., 1995) and *Caenorhabditis elegans* (Sma) (Derynck et al., 1996; Savage et al., 1996). There are three categories of Smads: receptor-regulated Smads (R-Smads), cooperating Smad (Co-Smad) and inhibitory Smads (I-Smads). Upon ligand binding, only R-Smads are directly phosphorylated (Kretzschmar et al., 1997; Macias-Silva et al., 1996), with Smad2 and 3 responding to signaling by the TGF β subfamily and Smad1, 5 and 8 to signaling by the BMP subfamily (Shi and Massague, 2003). Activated R-Smads form heteromeric complexes with Smad4, the sole Co-Smad known in mammals. These complexes are translocated into the nucleus and function as transcription factors (Fig.8), while I-Smads (Smad6 and 7) suppress TGF β /BMP signaling by inhibiting association between the receptors and R-Smads, competing with R-Smads for binding to the Co-Smad and/or promoting ubiquitin-dependant degradation of receptors and R-Smads (Murakami et al., 2003; Okita and Yamanaka, 2006).

It has been reported that BMP4 or GDF6 cooperate with LIF in the maintenance of pluripotency in mESCs (Ying et al., 2003). Under serum-free culture conditions LIF fails to promote self-renewal, as the cells differentiated along neural lineages. However, addition of BMP4 suppressed neural differentiation and maintained pluripotency, even in the absence of serum. In the presence of LIF, BMP4 contributes to self-renewal by activation of Smad4 which activates members of the Id (inhibitor of differentiation) gene family that functionally

antagonize basic helix-loop-helix (bHLH) transcription factors necessary for neural differentiation (Gerrard et al., 2005; Ruzinova and Benezra, 2003). This effect seems critical as constitutive expression of Id genes circumvents the requirement for BMP4/GDF6 (Ying et al., 2003). In the absence of LIF, BMP4 counteracts the LIF cascade by interaction with different Smads (for example, Smad1, 5 and 8) that have an inhibitory effect on Id gene expression (Boiani and Scholer, 2005; Chambers and Smith, 2004; Liu et al., 2007; Okita and Yamanaka, 2006).

In hESCs, BMP4 induces differentiation into mesoderm and ectoderm, while BMP2 promotes differentiation into extraembryonic endoderm (Schuldiner et al., 2000). Self-renewal in the absence of serum or feeder cells can be established by inhibiting BMP signaling by addition of the BMP antagonist noggin and high doses of bFGF (Okita and Yamanaka, 2006; Xu et al., 2005).

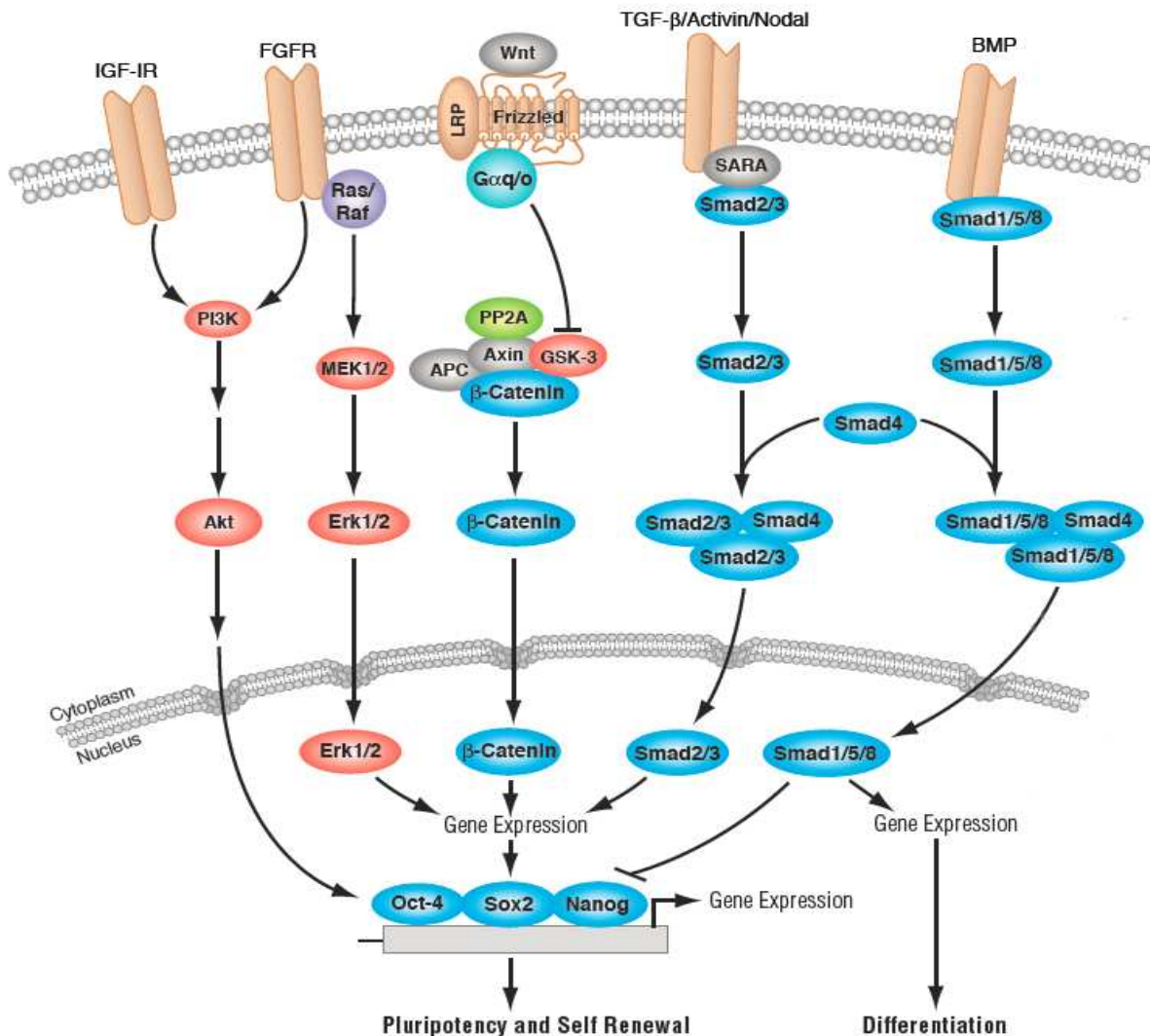


Fig.8: A plethora of signaling pathways contributes to stem cell fate decisions. Several cross-talking signaling pathways govern ESC fate-decisions in murine and human ESCs. While some signals (LIF, WNT and BMP4/TGFβ) mediate self-renewal in mESCs, others function as

inducers of differentiation (BMP2, MEK-ERK). Murine and human ESCs only partially rely on the same signaling pathways to establish pluripotency (WNT), as hESCs additionally require a combination of PI3K-Akt and TGF β /Activin/Nodal signaling for self-renewal (Cell Signaling Technology™, 2012).

One signaling pathway that potentially functions identically in murine and human ESC self-renewal is WNT signaling (Sato et al., 2004). WNT signals are mediated by WNT proteins (Cadigan and Nusse, 1997) and the intracellular messenger β -catenin (Reya and Clevers, 2005), a cytoplasmic protein that also functions in cell-cell adhesion processes. In the absence of WNT signals, β -catenin is phosphorylated by a complex of adenomatous polyposis coli gene (APC), axin and glycogen-synthase kinase-3 β (GSK-3 β). Phosphorylated β -catenin is degraded by ubiquitination, therefore its cytoplasmic level remains low. The canonical WNT pathway is activated upon binding of a WNT protein to its receptor Frizzled, which leads to inhibition of GSK-3 β . As a consequence, β -catenin accumulates in the cytoplasm, travels to the nucleus and triggers expression of target genes (Fig.8) (Boiani and Scholer, 2005; Cadigan and Nusse, 1997; Okita and Yamanaka, 2006; Reya and Clevers, 2005). WNT can also signal independently of β -catenin through so-called non-canonical WNT pathways, which include mechanisms like intracellular calcium signaling, signaling through heterotrimeric G proteins or c-Jun-N-terminal kinase (JNK) signaling (Okita and Yamanaka, 2006; Veeman et al., 2003).

Recent experiments have shown that neural differentiation of mESCs was attenuated upon activation of WNT signaling by overexpression of WNT proteins or inhibition of GSK-3 β (Aubert et al., 2002), which could be partially reversed by addition of the BMP4 antagonist noggin (Haegele et al., 2003). In addition, reversible inhibition of GSK-3 β was shown to maintain pluripotency in both murine and human ESCs by maintenance of stemness gene expression (Sato et al., 2004).

Most of the signaling pathways mentioned above are able to cross-talk with each other. In addition to STAT3 activation, LIF binding can also induce other signaling cascades by JAK-mediated activation of SHP-2, which can bind to both gp130 and LIFR (Schiemann et al., 1997; Stahl et al., 1995). Phosphorylated SHP-2 then either triggers PI(3)K or ERK signaling, with the latter pathway being absolutely required for proper differentiation (Burdon et al., 1999; Qu and Feng, 1998). As another example, LIF and WNT signaling converge on the transcription factor gene c-MYC which is a STAT3 target participating in maintenance of pluripotency on one hand (Cartwright et al., 2005), but is also negatively regulated by GSK-

3 β on the other hand (Sears et al., 2000). Likewise, PI(3)K signaling can be activated by exogenous factors like insulin, but also endogenously by ERas (ES cell-expressed Ras) (Okita and Yamanaka, 2006; Takahashi et al., 2003).

1.2.5.2 Intrinsic regulation via transcription factors

Stemness is established and maintained by activation or repression of genes that function as inducers or regulators of pluripotency. Extrinsic stimuli are conferred to the nucleus via several signaling pathways and activate their target genes which often encode transcription factors. In case of mESCs, three "master" transcription factors related to maintenance of pluripotency (stemness genes) have been characterized: OCT4, SOX2 and NANOG. These "trinity factors" act as key players in the complex transcription factor network that governs ESC fate decisions.

The POU-family transcription factor octamer binding protein (OCT3/4; encoded by the POU5F1 locus) has been shown to be expressed in undifferentiated mESCs, ECCs and EGCs (Boiani and Scholer, 2005; Donovan and de Miguel, 2003; Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1989a; Scholer et al., 1989b). OCT3/4 expression was found in the unfertilized egg and the early embryo before the ICM segregates from the trophectoderm (Pesce and Scholer, 2001). As a consequence, its expression can be detected in the ICM, but is subsequently down-regulated in the trophectoderm. OCT3/4-deficient embryos develop to the blastocyst stage, but die upon implantation because, instead of pluripotent cells, the ICM consists entirely of trophectodermal cells which lack the potential to build a functional epiblast (Nichols et al., 1998; Niwa et al., 2000). This demonstrated the requirement of OCT3/4 for maintenance of pluripotency in mESCs. Furthermore, fate decisions are mediated by different OCT3/4 levels: in mouse embryo models, a less than two-fold increase in expression caused differentiation into primitive endoderm and mesoderm, while repression of OCT3/4 expression led to loss of pluripotency. Therefore, a critical amount of OCT3/4 is needed to ensure stemness, which makes it a master regulator of pluripotency (Chambers and Smith, 2004; Niwa et al., 2000).

SRY-related high-mobility group (HMG)-box protein 2 (SOX2), a member of the HMG-domain DNA-binding-protein family, was shown to be involved in the regulation of transcription and chromatin architecture (Kamachi et al., 2000; Pevny and Lovell-Badge, 1997). Like OCT3/4, SOX2 is expressed in the ICM, the epiblast and in germ cells, however,

unlike OCT3/4, it is also expressed in the extra-embryonic ectoderm and in neural stem cells (Avilion et al., 2003; Zappone et al., 2000). SOX2 was shown to play an essential role in the transcription of several OCT3/4 target genes (Niwa, 2001). A well-known example is FGF4 expression in the ICM, where SOX2 and OCT3 bind to adjacent sites within the FGF4 enhancer and synergistically stimulate transcription (Ambrosetti et al., 2000; Yuan et al., 1995).

The homeodomain-containing transcription factor NANOG (named *Tir nan Og* or *Tir Na Nog* after the mythologic celtic land of the "ever young") was shown to mediate mESC self-renewal and pluripotency in a LIF-independent manner (Chambers et al., 2003; Mitsui et al., 2003; Wang et al., 2003). NANOG can be first detected in the interior cells of compacted morulae and later in the ICM of the blastocyst, where its expression is restricted to the epiblast and excluded from the trophectoderm and primitive endoderm (Pan and Thomson, 2007). NANOG expression is high in ESCs, PGCs, EGCs and teratoma-derived cells and down-regulated during ESC differentiation into adult tissues, correlating with loss of pluripotency (Hart et al., 2004). Mouse embryos lacking NANOG fail to develop a functional epiblast due to differentiation into extra-embryonic endoderm (Mitsui et al., 2003). Most importantly, elevated levels of NANOG promote LIF-independent self-renewal in both mESCs (Chambers et al., 2003; Mitsui et al., 2003) and hESCs (Darr et al., 2006), while physiological levels do not. NANOG overexpressing cells can still spontaneously differentiate upon LIF-withdrawal, while reduced expression results in loss of pluripotency and differentiation, even in the presence of LIF (Hatano et al., 2005). Therefore, NANOG seems to mediate ESC fate-decisions in a dose dependant manner (Boiani and Scholer, 2005; Chambers and Smith, 2004).

Genome-wide analyses of target genes occupied by the "trinity factors" have provided insights into the molecular mechanisms by which they mediate pluripotency and govern cell fate decisions. OCT3/4, SOX2 and NANOG were shown to regulate their own expression, as well as the expression of each other, in a strongly interconnected autoregulatory feedback loop. In addition, they often co-occupy their target genes and collectively target two sets of genes, one that is actively expressed and another one that remains silent until differentiation (Boyer et al., 2005; Jaenisch and Young, 2008; Loh et al., 2006; Niwa, 2007). This autoregulatory circuitry can be seen as a prerequisite for stable gene expression and therefore facilitates maintenance of the pluripotent state (Alon, 2007).

In general, the ground state of ESCs appears to be marked by highly fluctuating gene expression patterns of both pluripotency- and differentiation-affiliated genes. As a consequence, ESC populations are never homogeneous, but rather display variable expression of key factors such as NANOG (Fig.9A) and therefore have different probabilities of self-renewal and even differ in their differentiation potential. The pluripotent state can thus be seen as a constant battle in which the trinity factors dominantly suppress the expression of lineage specification factors (Niwa, 2007; Silva and Smith, 2008; Smith, 2005).

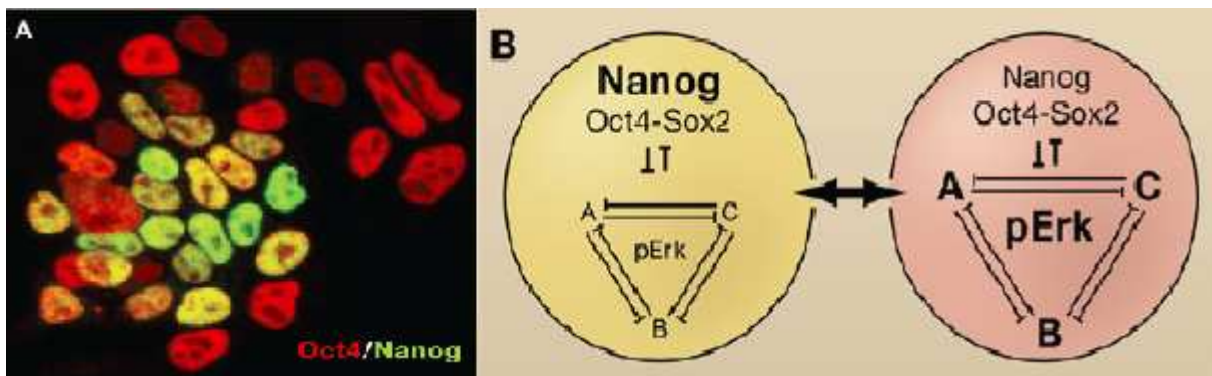


Fig.9: Self-renewal vs. differentiation - a metastable condition. **(A)** ESCs are heterogeneous populations. Cell-to-cell variation creates the possibility for differentiation and is established by highly fluctuating gene expression circuits. **(B)** Autoinductive ERK signaling primes ESCs for lineage specification and differentiation. Lineage-associated transcriptional circuits (A, B and C) are maintained below threshold levels due to suppression by the trinity factors. A destabilized transitional state arises when down-regulation of NANOG coincides with increased activation of ERK. If NANOG expression is restored before commitment is concluded, the actions of pERK are neutralized and the gate is closed (Silva and Smith, 2008).

The fluid transcriptome of ESCs enables them to maintain a metastable pluripotent state, as the transcription factor circuits governing either self-renewal or differentiation act mutually antagonistic. On one hand, OCT4 and SOX2 provide a destabilizing signal by triggering FGF4 (ERK) signaling which drives ESCs towards differentiation. On the other hand, NANOG functions as a pluripotency gatekeeper that antagonizes this autoinductive differentiation signal (Fig.9B). Phosphorylated ERK (pERK) may activate inductive signaling pathways or directly promote differentiation. However, if NANOG levels rise before lineage-decisions are complete the actions of pERK are neutralized and the metastable ground state is restored. Cell-to-cell variation can therefore be seen as a prerequisite for self-renewal, while continually presenting opportunities for lineage commitment (Silva and Smith, 2008).

1.3 Early mammalian development

Embryogenesis begins with the fertilization of an oocyte by a sperm, marked by the fusion of the gametes. The totipotent fertilized oocyte (zygote) has the capacity to give rise to more than 200 different specialized cell types of the adult organism. After fertilization the embryo undergoes a couple of cell divisions until the 8-cell stage is reached (Fig.10), where the cells compact to form a tighter aggregation (morula). This compaction establishes some sort of polarity for the first time in the 16-cell stage morula (E3.0), producing an inside and outside face (Johnson and Ziomek, 1981). Cells on the inside will form the ICM (embryoblast) of the blastocyst, cells on the outside will become the trophectoderm (trophoblast).

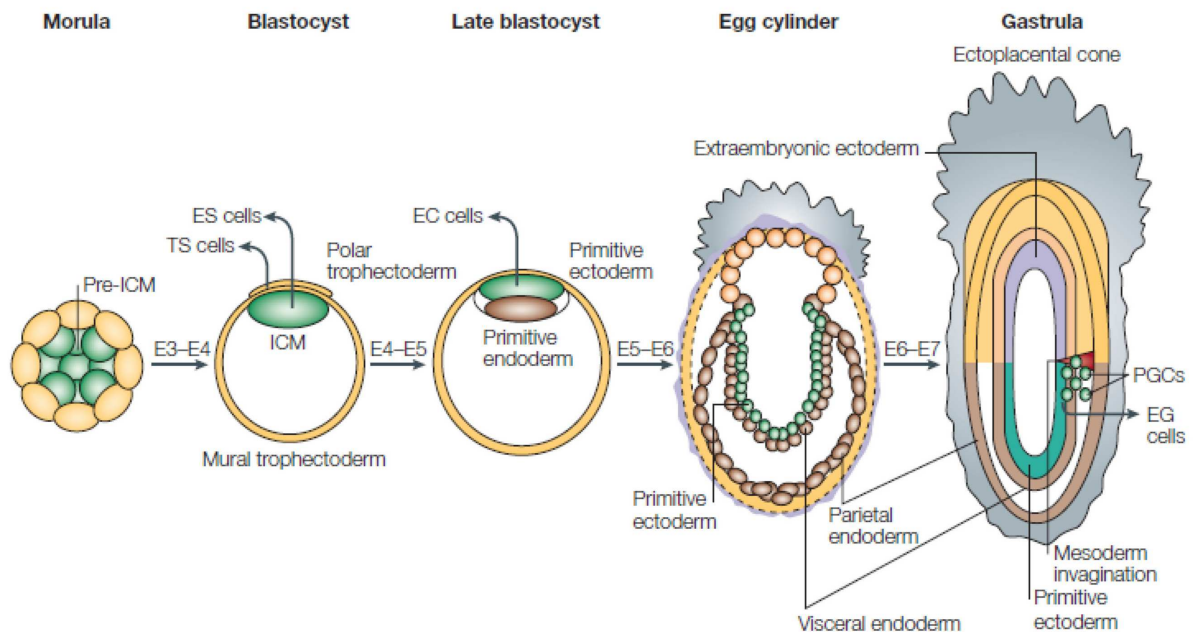


Fig.10: Mammalian embryonic development until gastrula stage. Pluripotent cells of the embryo are tracked in green. The ICM gives rise to the primitive endoderm (hypoblast) and the primitive ectoderm (epiblast). Only the primitive ectoderm contains pluripotent stem cells, while the primitive endoderm gives rise to extra-embryonic structures like the primary yolk sac. ESCs and TSCs can be isolated from the blastocyst, ECCs from the primitive ectoderm in the late blastocyst and EGCs from the PGCs. At E6 and subsequent stages the experimental ability to isolate ESCs, TSCs and ECCs is progressively lost and the embryo starts gastrulating, leading to proper alignment of the three germ layers (Boiani and Scholer, 2005).

In the 32- to 64-cell stage (E3.5) the embryo is referred to as blastocyst and has not increased in mass or volume since fertilization but rather in density. The blastocyst is characterized by the already mentioned ICM, the trophoblast, which will form the placenta, and the blastocyst cavity (blastocoel). At E4.5 the blastocyst hatches from the zona pellucida (ZP) which surrounds it and implants into the uterine wall (nidation). By this time, referred to as egg-cylinder stage, the ICM consists of the epiblast and the hypoblast (primitive endoderm; PrE).

The epiblast will become the primitive ectoderm which will give rise to all three germ layers, the hypoblast will develop into extra-embryonic endoderm necessary for nutrient supply. The next stage, the gastrulation, involves the formation of a mesoderm layer between ectoderm and endoderm and the formation of the primordial germ cells (PGCs) which initially develop in the extra-embryonic surrounding and then start to migrate into the primitive genitals (Boiani and Scholer, 2005; Niwa, 2007).

1.4 Mammalian heart development

In early vertebrate development the heart, the center of the cardiovascular system, is the first organ system to become functional long before other parts in the early embryo are discernable. The development of a fully functional heart requires a set of initial cardiac precursor cells that, driven by underlying highly conserved (Olson, 2006) molecular mechanisms, guide heart development and give rise to distinct cell types which assemble and align in specific compartments to form ventricular chambers, coronary arteries and the conduction system, the major structural and functional components of the mammalian four-chambered heart (Chien et al., 2008; Taubenschmid and Weitzer, 2012). During cardiogenesis, the differentiation of these cardiac precursors is under tight temporal and spatial control (Brand, 2003), yielding a highly diversified set of both muscle and non-muscle cell types, such as atrial/ventricular CMCs, conduction system cells, SMCs, ETCs of the coronary arteries and veins, endocardial cells, valvular components and connective tissue. Three major sources of cardiac precursors have been identified: the cardiogenic mesoderm, originating from the anterior lateral plate mesoderm in all mammals (Zaffran and Frasch, 2002), the cardiac neural crest and the proepicardial organ (Fig.11) (Laugwitz et al., 2008).

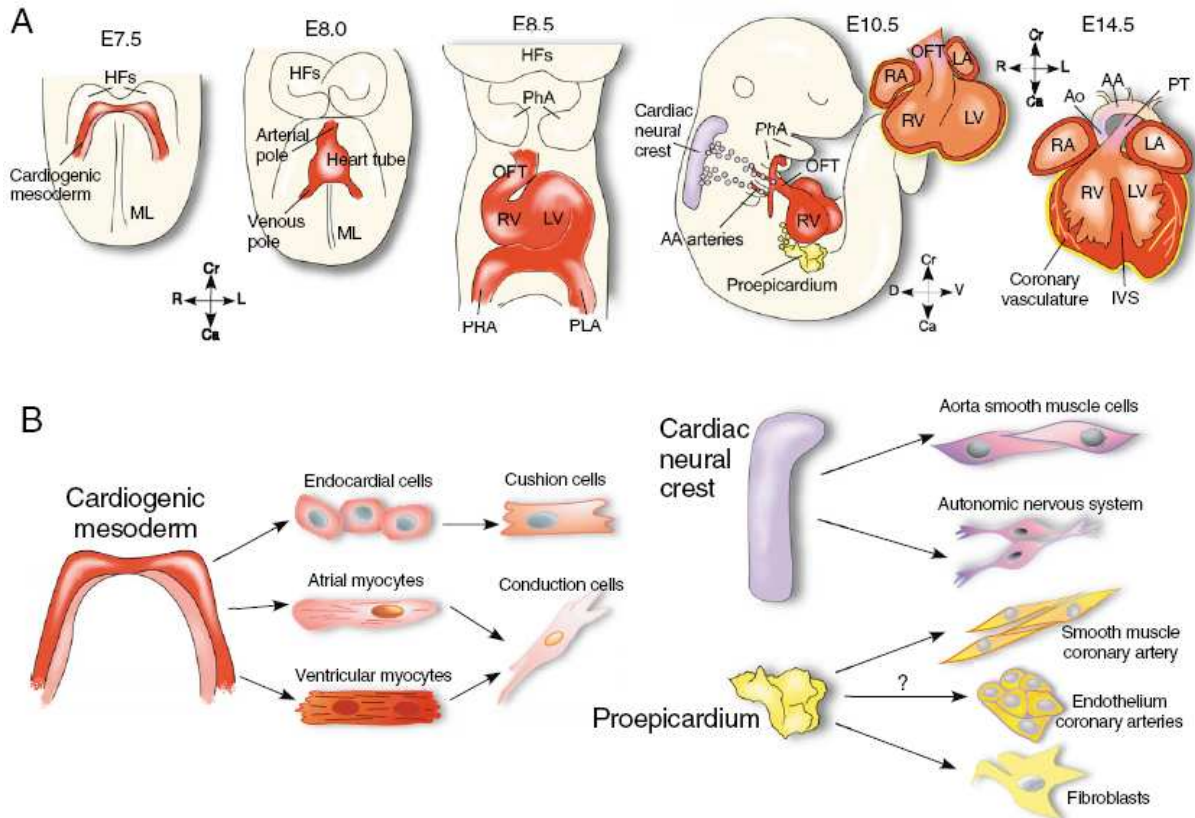


Fig.11: Murine heart development and origin of cardiac cell lineages. (A) Contribution of cardiogenic mesoderm (red), cardiac neural crest (purple) and proepicardial organ (yellow) to different heart compartments during murine heart development. Cardiogenic mesodermal progenitors initially form the linear heart tube (E7.5-E8.0) and ultimately the four heart chambers. After looping of the heart tube cardiac neural crest progenitors start to migrate (E8.5), engulf the aortic arch arteries and contribute to vascular smooth muscle cells of the outflow tract (OFT) around E10.5, while, at the same time, proepicardial organ precursors give rise to the epicardial mantle (yellow) and later to the coronary vasculature. At E14.5 the four heart chambers have already separated by septation and are connected to the pulmonary trunk (PT) and aorta (Ao). Cranial (Cr)-caudal (Ca), right (R)-left (L) and dorsal (D)-ventral (V) axes are indicated. **(B)** Cardiac cell types that arise through lineage diversification of the three embryonic precursor pools in the murine heart. AA, aortic arch; IVS, interventricular septum; LA, left atrium; LV, left ventricle; PhA, pharyngeal arches; PLA, primitive left atrium; PRA, primitive right atrium; RA, right atrium; RV, right ventricle (Laugwitz et al., 2008).

Previous experiments (Kelly et al., 2001; Meilhac et al., 2004; Zaffran et al., 2004) have shown that cardiogenic mesoderm consists of two populations, referred to as heart fields, that contribute to different parts of the heart. The first heart field (FHF), originating from the anterior splanchnic mesoderm, gives rise to the cardiac crescent, later the linear heart tube and ultimately to parts of the atrial chambers and the left ventricular region, while cells from the second heart field (SHF), derived from the pharyngeal mesoderm, are added to the developing heart tube and give rise to the outflow tract, the right ventricular region and main parts of the atrium (Buckingham et al., 2005; Laugwitz et al., 2008).

All diverse cell lineages of both heart fields arise in specific manners from a closely related set of multipotent cardiac progenitors in the early embryonic heart field (Moretti et al., 2006;

Wu et al., 2006). However, so far it has only been possible to isolate and characterize purified populations of cardiac progenitor cells from the SHF, while the lack or unclear expression patterns of unique traceable markers in progenitors of the FHF strongly hampers the possibility of their isolation.

In the past, numerous groups have shown that multipotent cardiac progenitors from the SHF express the LIM-homeodomain transcription factor Islet-1 (insulin gene enhancer protein, ISL1) (Cai et al., 2008; Laugwitz et al., 2005; Moretti et al., 2006). Purified ISL1 positive cardiac progenitors have been shown to be capable of self-renewal and differentiation into SMCs, ETCs and CMCs, the three major cell types in the heart (Bu et al., 2009). Due to the lack of suitable markers purification of cardiac progenitors of the FHF has not been possible so far, nevertheless it has been shown that these cells express cardiac key transcription factors TBX5, HAND1 and NKX2.5 (Taubenschmid and Weitzer, 2012).

1.5 The gene regulatory network in cardiogenesis

The formation of a fully functional heart requires a precise gene regulatory network to ensure tissue patterning and specification of initial cardiac precursor cells towards different types of cardiovascular cell lineages. This evolutionarily highly conserved network is mainly triggered by specific signaling molecules providing up-and downstream signals which are mediated by a plethora of tissue specific transcription factors that activate cardiac specific genes, leading to the formation of terminally differentiated adult heart cells, like CMCs, SMCs, ETCs or cardiac fibroblasts (Srivastava and Olson, 2000).

What is today known to be the core regulatory network governing heart development is the result of numerous evolutionary cycles of gene duplication, modification and selection and can be downsized to a core set of 5 transcription factor gene families: NK-2, MEF2, GATA, TBX and HAND (Fig.12A) (Olson, 2006; Taubenschmid and Weitzer, 2012).

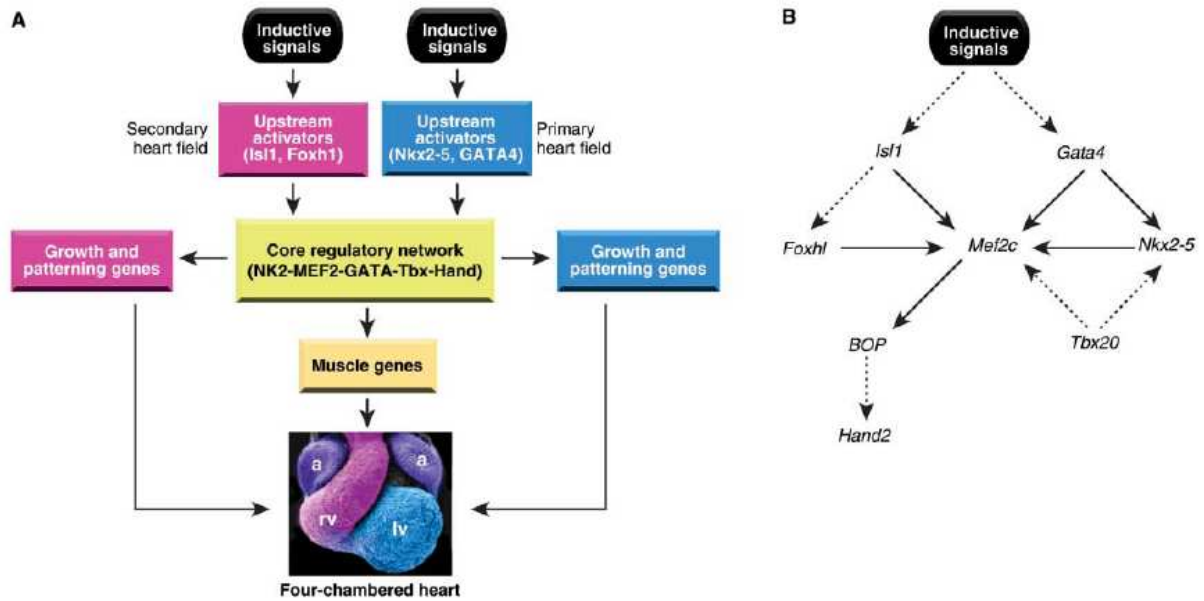


Fig.12: Simplified scheme of the regulatory transcriptional network involved in mammalian heart development. **(A)** Upstream regulatory transcription factor genes of the FHF (blue) and SHF (purple) are activated by inductive signals and their gene products activate genes of the core cardiac network. The core network genes cross- and auto-regulate their expression and function as the central regulatory network for the activation of muscle-specific genes and genes involved in growth and patterning of derivatives of the FHF and SHF. **(B)** The regulatory transcription factor network in the SHF. Direct transcriptional connections are indicated by solid lines, dotted lines indicate connections not yet shown to be direct (Olson, 2006).

During heart development the first specified cardiac progenitors have been shown to originate from mesodermal tissue which is derived from the primitive streak that forms during gastrulation (Robb and Tam, 2004). Initially, the specification of cardiac mesoderm is marked by expression of the T-box (TBX) transcription factor Brachyury (T) (David et al., 2011) and Eomesodermin, another T-box transcription factor, which is essential for epithelial to mesenchymal transition (EMT) in early embryonic development and also directly activates the bHLH transcription factor mesoderm posterior homologue 1 (MESP1) (Costello et al., 2011; Saga et al., 2000). Along with expression of MESP2 (Kitajima et al., 2000), the presence of inductive extrinsic signals like BMP2/4 or FGF (Alsan and Schultheiss, 2002; Schultheiss et al., 1995), which are secreted in the anterior endoderm, is another crucial factor for cardiac specification. Altogether, these upstream signals activate the expression of other cardiac key transcription factors.

Among these factors the two most prominent are the homeodomain transcription factor NK2 transcription factor related, locus 5 (NKX2.5; also referred to as cardiac-specific homeobox gene, Csx (Komuro and Izumo, 1993)), the vertebrate homologue of *tinman* in *D. melanogaster* (Harvey, 1996), and the zinc finger transcription factor GATA-binding protein

4 (GATA4). The GATA family can be divided into two groups: GATA1, 2 and 3 are crucial regulators of hematopoietic stem cell differentiation while GATA4, 5 and 6 are expressed in cardiac mesoderm (Molkentin, 2000) and the developing heart (Charron and Nemer, 1999; Taubenschmid and Weitzer, 2012). Both NKX2.5, the earliest molecular marker of the cardiac lineage (Harvey, 1996), and GATA4 are believed to be the key players in cardiac specification and differentiation. Nevertheless, in contrast to *tinman* in *D. melanogaster*, neither NKX2.5 nor GATA4 can initiate cardiogenesis in vertebrates on their own, but need each other as mutual covalent co-factors, which was shown in transcription and binding assays with the cardiac atrial natriuretic factor (ANF; the major secretory product of embryonic and postnatal CMCs) promoter, a well understood target of NKX2.5 (Durocher et al., 1997). In addition, NKX2.5 and GATA4 regulate each other's expression by mutually reinforcing positive feedback loops (Schwartz and Olson, 1999).

Members of the T-box gene family are other crucial transcription factors in cardiogenesis, playing important roles in both the specification of early mesodermal cells to the cardiac lineage and heart maturation into fully functional trabeculated chambers (Bruneau, 2002). TBX5, which is expressed in the cardiac mesoderm and the myocardium of the fetal and adult heart (Horb and Thomsen, 1999), was shown to associate with NKX2.5 and thus synergistically promotes CMC differentiation (Hiroi et al., 2001). In addition, GATA4 expression was shown to be down-regulated in mice lacking TBX5 and up-regulated in cells overexpressing TBX5, suggesting a possible interaction of TBX5 with GATA4 as well (Bruneau et al., 2001; Heicklen-Klein and Evans, 2004; Plageman and Yutzey, 2005). TBX5, along with TBX20, which was shown to directly interact with NKX2.5, GATA4 and GATA5 (Stennard et al., 2003), also mediates septation of the atria and ventricles from one another and between their left and right sides, while TBX2 and TBX3 act as repressors of the heart chamber gene program in the early heart tube (Bruneau, 2002; Singh and Kispert, 2010). The widespread transcriptional targets and interaction partners of T-box transcription factors along with their response to a variety of upstream signals enable them to promote diverse developmental fate decisions, not just those required for the formation of the heart (Hatcher and Basson, 2001; Stennard and Harvey, 2005).

The MADS-box transcription factor myocyte enhancer factor 2c (MEF2C) is a well-characterized key factor for the proper formation of the outflow tract and the right ventricle and generally serves as a nodal point in the development of the anterior (secondary) heart field (Black, 2007). Its expression is activated by at least two separate mechanisms: on one

hand MEF2C has been shown to be a direct transcriptional target of GATA4 and ISL1 (Dodou et al., 2004), on the other hand it is a target of the forkhead transcription factor FOXH1, which physically and functionally interacts with NKX2.5 (von Both et al., 2004). Furthermore, MEF2C physically interacts with TBX5, making it indispensable for early heart development and CMC differentiation (Ghosh et al., 2009). The putative histone methyltransferase BOP (also known as SMYD1), another regulator of right ventricular heart development, is a direct transcriptional target of MEF2C (Phan et al., 2005), serving as an effective downstream mediator of the actions of MEF2C through activation of the bHLH transcription factor heart- and neural crest derivatives - expressed protein 2 (HAND2; dHAND) (Gottlieb et al., 2002). As most of the early heart specific transcription factors act directly on MEF2C, its activation can be seen as a prerequisite for proper heart development (Fig.12B) (Black, 2007; Potthoff and Olson, 2007; Taubenschmid and Weitzer, 2012).

In the past, numerous experiments with null-mutant mouse-models revealed that mutations in genes encoding transcription factors necessary for heart development are responsible for CHDs. Mutations in or complete lack of any of the transcription factor genes mentioned above cause death in early embryonic development or severe heart malformations and defects (Black, 2007; Clark et al., 2006).

1.6 The embryoid body model system

When cultivated in suspension in the absence of signals promoting self-renewal, ESCs differentiate spontaneously and form 3-dimensional multi-cellular aggregates called embryoid bodies (EBs) (Doetschman et al., 1985; Keller et al., 1993; Wobus et al., 1984; Wobus et al., 1991). These aggregates recapitulate many differentiation steps in early embryonic development and therefore serve as a versatile and reproducible *in vitro* model system in stem cell research. EBs give rise to cells of all three germ layers (Desbaillets et al., 2000; Itskovitz-Eldor et al., 2000) and can be used for differentiation of ESCs into a variety of cell types or *in vitro* knock-out studies (Keller, 1995; Kurosawa, 2007). In addition, the EB model system provides an easy way to identify the potential of stem cells and, above all, allows for investigation of molecular and cellular processes during embryogenesis which cannot be determined experimentally *in vivo*.

Three basic methods for EB formation are commonly used. Removal of ESCs from contact with feeder cells or LIF/FGF and subsequent cultivation in methyl cellulose containing media

or in bacterial grade petri dishes leads to differentiation and EB formation, because the ESCs are unable to adhere to the dish. Secondly, ESCs can be directly cultivated and differentiated on stromal cells, which is a modification of the method described above and often used for studies on hematopoietic development, as the stromal cells provide a supportive environment for the developing EB. The third way to generate EBs is to closely associate ESCs in (methyl cellulose-free) suspension culture, which promotes EB formation due to limited space. This is referred to as "hanging drop" method (Fig.13A). Once assembled, the EBs can be exposed to standard liquid culture conditions where they complete their development (Hopfl et al., 2004; Keller, 1995; Kurosawa, 2007).

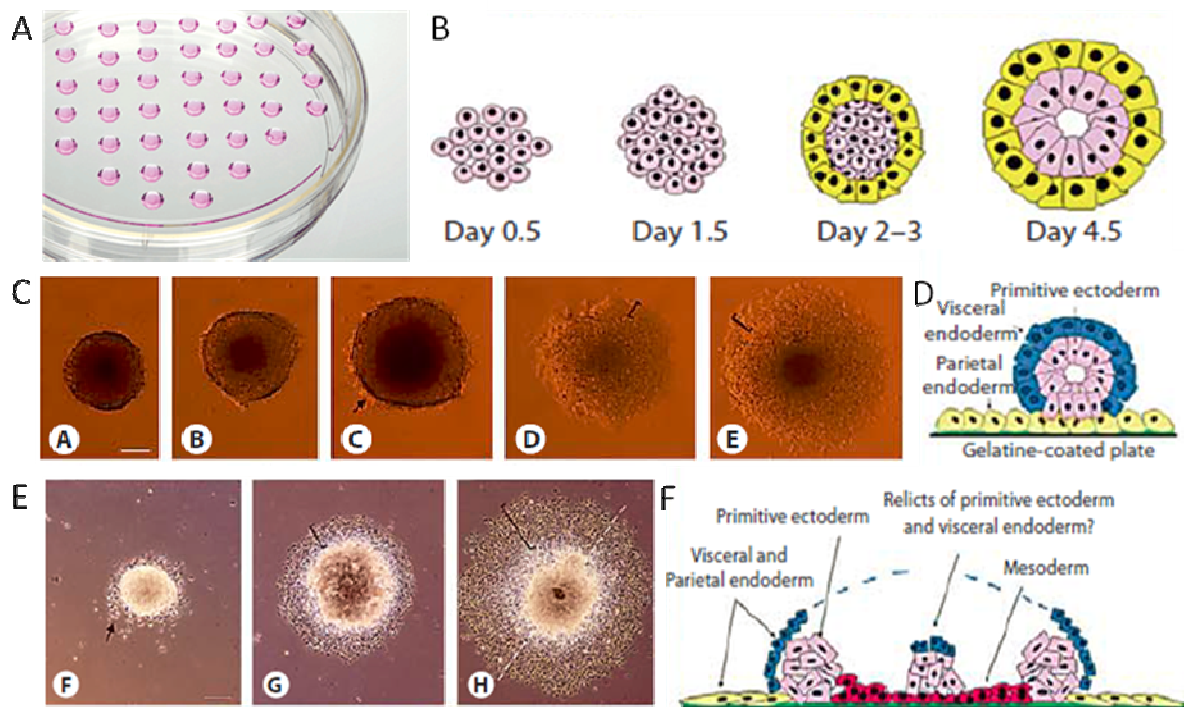


Fig.13: Murine EB formation and cultivation using the "hanging drop" method. (A) Hanging drops on a cell culture dish. (B) Early development of cell types in EBs. Pink: ESCs/primitive ectoderm; yellow: primitive endoderm. (C) Attachment of EBs to a surface breaks the radial symmetry and leads to a bilateral symmetrical aggregate. Attachment of EB to a gelatine-coated culture dish between day 4.5 and 6 (A-E): arrow indicates first attaching primitive endodermal cells (C), brackets show proliferating primitive endoderm (D,E). (D) Cross-section of an attached parietal EB at day 5-6: inner primitive ectoderm (pink) is surrounded by primitive endoderm (blue) which, upon attachment, differentiates into parietal (yellow) and visceral endoderm (on top of the primitive endoderm). (E) Development of attached EB between day 5 and 6: arrow shows migrating primitive endodermal cells (F), brackets show parietal endodermal cells undergoing EMT. (F) Cross-section through the center of an attached EB around day 8 after EMT with ingression of mesoderm. Bars: 100 μ m (Fuchs et al., 2012).

In suspension, EBs consist of an outer cell layer of primitive endoderm (Murray and Edgar, 2001; Rula et al., 2007), resembling the hypoblast of the implanting blastocyst, while the inner cells acquire ectodermal characteristics and form a columnar epithelium (Ikeda et al.,

1999; Komura et al., 2008), resembling the epiblast and later the early egg cylinder stage (E6.5) in murine embryogenesis (Fig.13B). Maintenance of EBs in suspension leads to formation of various cell types from all three germ layers (Fuchs et al., 2012).

Attachment of EBs onto coated culture dishes is mediated by cells of the primitive endoderm which start to migrate radially from the dense center of the EB (Fig.13C and D) while differentiating into the extra-embryonic visceral and parietal endoderm. The primitive ectoderm undergoes EMT which results in development and ingression of mesoderm (Fig.13E and F) (Behr et al., 2005; Denker et al., 2007; Maranca-Huwel and Denker, 2010). The mesodermal precursors give rise to spontaneously and rhythmically contracting CMCs and later SMCs. Also, attachment of the EB as an implantation-like process enables breaking of the radial symmetry in suspension and development of a bilateral symmetry marked by mesoderm formation and axis specification (Fuchs et al., 2012).

1.7 Desmin, a modulator of cardiomyogenesis

The cytoskeleton of eukaryotes is composed of three different filamentous networks: actin filaments, microtubules and intermediate filaments (IFs). In contrast to actin filaments and microtubules, IFs display very restricted tissue specific and developmentally regulated expression patterns (Weitzer et al., 1995).

Desmin, a type III IF protein, is expressed specifically in cardiac, skeletal and smooth muscle tissue (Lazarides et al., 1982; Lazarides and Hubbard, 1976; Weitzer et al., 1995) where it is mainly located at the Z-disks (cardiac and skeletal muscle) and is known to be one of the earliest known myogenic markers in both heart and somites (Herrmann et al., 1989; Kaufman and Foster, 1988; Lin et al., 1994; Schaart et al., 1989). During development, desmin is expressed prior to other muscle-specific structural genes and myogenic bHLH transcription factors like myoD, myogenin or myogenic regulatory factor 4 (MRF4) (Buckingham et al., 1992; Li et al., 1993; Ontell et al., 1993; Sassoon, 1993), indicating that it may modulate myogenic commitment and differentiation (Weitzer et al., 1995). Desmin was shown to be dispensable for cardiomyogenesis *in vitro* (Weitzer et al., 1995) and *in vivo* (Li et al., 1996; Milner et al., 1996), but not for life-long homeostasis of the heart. Desmin null-mutations are not lethal but cause severe heart defects and malformations (myopathies) due to disorganized muscular architecture. In addition, the *in vitro* development of smooth and skeletal muscle cells in EBs is completely blocked in the absence of desmin (Weitzer et al., 1995).

Desmin overexpression was shown to enhance differentiation of CMCs *in vitro* via temporal up-regulation of Brachyury and NKX2.5, while complete lack of desmin only delays cardiomyogenesis (CMG), most likely due to compensation by other type III IFs (Hofner et al., 2007). The fact that aberrant protein forms of desmin cause delayed and decreased CMG in terms of CMC differentiation provides first evidence that desmin takes part in regulating the differentiation of mesoderm into CMCs at the onset of CMG (Hollrigl et al., 2007).

1.8 Previous findings and aim of the study

CVDs, a group of disorders of the heart and blood vessels, are the leading death cause worldwide. Acute MI results in an irreversible loss of CMCs, ultimately leading to progressive heart failure. Recent findings proposing that adult mammalian hearts harbor stem cells with replicative and regenerative potential have heavily questioned the dogmatic view of the heart as a post-mitotic organ. Recently, various groups have succeeded in the isolation of CVPCs from different mammalian species, but failed to establish culture conditions allowing for *in vitro* maintenance of self-renewing and phenotypically stable clonal CVPC lines.

In the past, our group has found a simple, cost-efficient and feasible strategy for the isolation of CVPCs from neonatal and adult murine hearts. This strategy is based on a co-culture system consisting of cells from murine heart preparations, mESCs and LIF secreting feeder cells with selectable markers. The idea was to mimic a surrogate cardiac stem cell niche to foster CVPC survival until a critical mass of CVPCs is reached and clonal autonomously self-renewing CVPC lines can be established (Weber, 2006). Previous characterization of several obtained CVPC clones has demonstrated that our strategy yields euploid cells which are capable of LIF-dependant self-renewal and differentiate exclusively into CMCs, SMCs and ETCs upon LIF deprivation in the CB model system (Gottschamel, 2010; Höbaus, 2009; Walder, 2011). In addition, simultaneous expression of both stemness factors and early mesodermal and myocardial marker genes in undifferentiated CVPCs has revealed a subtle commitment of these cells to the cardiac lineage, which is in sharp contrast to mESC expression patterns (Höbaus, 2009). First attempts to elucidate regulatory processes involved in CMC differentiation have identified several signaling pathways as possible regulators of cardiac cell fate decisions: Inhibition of MAPK or activation of WNT signaling were shown to delay the onset of CMG in EBs/CBs in a spatiotemporal manner (Gottschamel, 2010),

while activation of BMP signaling generally seems to enhance CMC differentiation in a time-dependent manner (Höbaus, 2009).

As most of the preliminary experiments of this study were made with transgenic CVPC lines, the aim of this project was to reproduce previous findings by a thorough characterization of isolated wild-type CVPC lines from hearts of different age. Since our isolation strategy involves short-term co-culture of murine heart cells with mESCs and feeder cells, we particularly wanted to rule out the possibility that obtained CVPCs were not *isolated* but rather *generated*. Therefore, our goal was to prove the cardiac origin of CVPCs to make sure that these cells do not represent *in vitro* artifacts that may arise from cell fusion, transdifferentiation or reprogramming of somatic heart cells. In addition, we wanted to identify the differentiation potential and marker expression patterns of wild-type CVPCs in comparison to transgenic CVPCs and mESCs.

Since CVPCs and the CB model system provide a powerful *in vitro* tool to simulate early cardiac development, we sought to analyze the response of CVPCs to biochemical induction of CMC differentiation by treatment with cardiogenic small molecules. In future therapeutic applications, cardiogenic small molecules may be useful by directly supporting heart regeneration through reactivation of endogenous dormant CVPCs on one side, but also by increasing the yield of functional CMCs during *in vitro* differentiation of potential tissue grafts on the other side.

2. Abbreviations

ABC	ATP-binding cassette
ANF	Atrial natriuretic factor
Ang-2	Angiotensin-2
Ao	Aorta
APC	Adenomatous polyposis coli gene
ASC	Adult stem cell
β-ME	β-Mercaptoethanol
BMP	Bone morphogenetic protein
BMP2/4	Bone morphogenetic protein 2/4
BSA	Bovine serum albumin
CB	Cardiac body
cDNA	Complementary DNA
CgC	Cardiogenol-C
CHD	Congenital heart disease
ChIP	Chromatin immunoprecipitation
CIED	Cardiovascular implantable electronic device
cKit	Stem cell factor receptor
CMC	Cardiomyocyte
CMG	Cardiomyogenesis
CSC	Cardiac stem cell
CSC	Cardiac stem cells
cTnT	Cardiac troponin T
CVD	Cardiovascular disease
CVPC	Cardiovascular progenitor cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxid
EB/CB	Embryoid body/cardiac body
ECC	Embryonic carcinoma cell
ECM	Extracellular matrix
EGC	Embryonic germ cell
EMT	Epithelial to mesenchymal transition
ERas	ES cell-expressed Ras
(p)ERK	(phosphorylated) Extracellular signal-regulated protein kinase
ESC	Embryonic stem cell
ETC	Endothelial cell
FACS	Fluorescence activated cell sorting
FGF	Fibroblast growth factor
FHF	First heart field
FOXH1	Forkhead transcription factor H1
GanC	Gancyclovir
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATA1-6	GATA-binding protein 1-6
GDF	Growth and differentiation factor
gDNA	genomic DNA
GPS	Glutamine/Penicillin/Streptomycin
GSK-3 β	Glycogen-synthase kinase-3β

HAND2	Heart- and neural crest derivatives-expressed protein 2
HBPC	Hair bulge progenitor cell
HDac1	Histone deacetylase 1
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell
HSV-1	(human) Herpes simplex virus type 1
ICM	Inner cell mass
Id	Inhibitor of differentiation
IF	Intermediate filament
IGF	Insulin-like growth factor
iPSC	Induced pluripotent stem cell
ISC	Intestinal stem cell
ISL1	LIM-homeodomain transcription factor Islet 1
JAK	Janus kinase
JNK	c-Jun-N-terminal kinase
LIF	Leukemia inhibitory factor
LIFR	Leukemia inhibitory factor receptor
MAD	Mothers against decapentaplegic
MAPK	Mitogen-activated protein kinase
MDR	Multidrug resistance protein
MEF	Mouse embryonic fibroblast
MEF2C	Myocyte enhancer factor 2C
MESP1/2	Mesoderm posterior homologue 1/2
MHC-α	Myosin heavy chain α
MRF4	Myogenic regulatory factor 4
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
Neo	Neomycin
NKX2.5	NK2 transcription factor related, locus 5
NSC	Neural stem cell
OCT3/4	Octamer binding protein 3/4
OFT	Outflow tract
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGC	Primordial germ cell
PGE2	Prostaglandin E2
PGK	Phosphoglycerate kinase
PI(3)K	Phosphatidylinositol-3-OH-kinase
PrE	Primitive endoderm
PT	Pulmonary trunk
RAS	Renin-angiotensin system
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
SCA1	Stem cell antigen 1
SCNT	Somatic cell nuclear transfer
SDS-PAGE	Sodium dodecylsulfate - polyacrylamide gel electrophoresis
SHF	Secondary heart field
SHP-2	SRC-Homology-2(SH2)-domain-containing protein tyrosine phosphatase-2

SMAD	Similar to mothers against decapentaplegic
SMA-α	Smooth muscle actin α
SMC	Smooth muscle Cell
SOX2	SRY-related high-mobility group (HMG)-box protein 2
SPARC	Secreted protein acidic and rich in cystein
SRY	Sex-determining region Y
STAT(3)	Signal transducer and activator of transcription (3)
TBX5/20	T-box 5/20 transcription factor
TCA	Trichloroacetic acid
TE	Trophectoderm
TGF β	Transforming growth factor β
TK	Thymidine kinase
TM-α	Tropomyosin α
TSC	Trophoblast stem cell
WHO	World Health Association
ZP	Zone pellucida

3. Materials

3.1 Chemicals for molecular biology

Chemical	Company
Acetic acid	Merck, GER
Acryl amide	BioRad, USA
Agarose LE	Biozym, AUT
β -Mercaptoethanol	Loba Feinchemie, AUT
Bromphenolblue	Sigma Aldrich, USA
BSA	Roth, GER
Coomassie Brilliant Blue R250	Merck, GER
Dabco	Sigma Aldrich, USA
dNTPs	Fermentas, LT / NEB, USA
Dimethylsulfoxid (DMSO)	Acros Organics, BEL
Dithiothreitol (DTT)	Acros Organics, BEL
EDTA	Acros Organics, BEL
EGTA	Sigma Aldrich, USA
Ethanol	Merck, GER
Ethidiumbromide	Fluka, CH
Formaldehyde	Merck, Ger
Gelatine	Difco, USA
Giemsa stain	Sigma Aldrich, USA
Glycerin	Merck, GER
Glycerophosphate	Merck, GER
Hydrochloric acid	Acros Organics, BEL
Loading dye	Fermentas, LT / NEB, USA
Methanol	Merck, GER
Magnesiumchloride	Fermentas, LT
Magnesiumsulfate	Fluka, CH
Milk powder (skim milk)	Sucofin, GER
Mowiol	Hoechst, GER
Nalgene Filter	Thermo Fisher Scientific, USA
PCR-Buffer (without $MgCl_2$)	Fermentas, LT / NEB, USA
Ponceau-S	Sigma Aldrich, USA
Protease Inhibitor	Roche, AUT
Reverse Transcriptase Buffer	Fermentas LT
SDS (sodium dodecyl sulfate)	BioRad, USA
Sodiumbicarbonate	Sigma Aldrich, USA
Sodiumchloride	Salinen Austria, AUT
Sodiumhydrogencarbonate	LifeTechnologies, USA
Sodiumhydrogenphosphate	Roth, GER
Sodiumhydroxide	Merck, GER
Trichloroacetic acid	Merck, GER
Tris Base	Life Technologies, USA
Triton X-100	Sigma Aldrich, USA
Tween-20	Sigma Aldrich, USA

3.2 Chemicals for cell culture

Chemical	Company
Amphotericin B	Invitrogen, USA
Angiotensin-2	Sigma Aldrich, USA
D-Glucose	Acros, BEL
DMEM (powder)	Gibco, USA
DMSO (Dimethylsulfoxid)	Sigma Aldrich, USA
Fetal Bovine Serum (FBS)	HyClone, USA
Fetal Bovine Serum (FBS)	Sigma Aldrich, USA
Fetal Bovine Serum (FBS)	Gibco, USA
G418	Gibco, USA
Gancyclovir	Gibco, USA
Gelatin	Difco, USA
Glycine	Applichem, GER
L-(+)-Glutamin	Gibco, USA
Losartan	Sigma Aldrich, USA
Mitomycin C	Acros, BEL
Penicillin G (Kaliumsalz)	Merck, GER
Penicillin/Streptomycin (P/S)	Gibco, CH
Potassium chloride	Sigma Aldrich, USA
Potassiumhydrogensulfate	Fluka, CH
Sodiumhydrogencarbonate	LifeTechnologies, USA
β-Mercaptoethanol	Loba Feinchemie, AUT
Streptomycin	Sigma Aldrich, USA
Colchicine	

3.3 Enzymes

Enzyme	Company
Collagenase II	Worthington, USA
DNase I (RNase free)	Fermentas, LT
Pancreatin	Gibco, USA
Proteinase K	Fluka, CH
RevertAid™ M-MuLV Reverse Transcriptase	Fermentas, LT
RiboLock RNase Inhibitor	Fermentas, LT
Taq DNA Polymerase	Fermentas, LT/NEB, USA
Trypsin	LifeTechnologies, USA

3.4 Kits

Kit	Company
EndoFree Plasmid Maxi Kit	Qiagen, GER
First Strand cDNA Synthesis Kit	Fermentas, LT
peqGOLD Total RNA Kit	PeqLab, GER
RNeasy Mini Kit	Qiagen, GER
SV Total RNA Isolation Kit	Promega, USA

3.5 Primers

Target Gene		Primer Sequence (5' to 3')	T _a (°C)	Cycles (n)	Product Size (bp)
Brachyury (T)	fwd	GAACCTCGGATTACATCGTGAGA	60	36	cDNA: 214
	rev	ATCAAGGAAGGCTTTAGCAAATGGG			genomic: 891
Desmin	fwd	TGAGCCAGGCCTACTCGT	60	30	
	rev	GAGCCTCTGCAGGTCTCTA			genomic: 566
Desmin KO1	fwd	TGAATGAACTGCAGGACGAG	60	30	
	rev	ATAGAAGGGGCCAGGAAAGA			genomic: 945
Desmin KO2	fwd	TCGCCTTCTTGACGAGTTCT	60	30	
	rev	ATAGAAGGGGCCAGGAAAGA			genomic: 322
Eomesodermin	fwd	TGTTTTCTGTTGAAGTGGTTCTGGC	64	29	cDNA: 323
	rev	AGGTCTGAGTCTTGAAGGTTTCATTC			genomic:
GATA4	fwd	GCCTGTATGTAATGCCTGCG	53	31	cDNA: 500
	rev	CCGAGCAGGAATTTGAAGAGG			genomic: 3364
GAPDH	fwd	CGTCTTCACCACCATGGAGA	55	30	cDNA: 300
	rev	CGGCCATCACGCCACAGTTT			genomic: 300
HPRT	fwd	TTGCTGGTGAAAAGGACCTC	60	30	
	rev	TGGCAACATCAACAGGACTC			genomic: 1060
HPRT KO	fwd	TCGCCTTCTTGACGAGTTCT	60	30	
	rev	TGGCAACATCAACAGGACTC			genomic: 846
ISL1	fwd	ATGGGAGACATGGGCGATCC	56	32	cDNA: 368
	rev	CGCAGGGCCAATTCGTCTCC			
MEF2C	fwd	GGCCATGGTACACCGAGTACAACGAGC	66	34	cDNA: 395
	rev	GGGGATCCCTGTGTTACCTGCACTTGG			
MESP1	fwd	AGAAACAGCATCCCAGGAAA	52	32	cDNA: 346
	rev	GTGCCTGCTTCATCTTTA			genomic: 619
MESP1	fwd	CATCGTTCCTGTACGCAGAA	60	30	
	rev	AGCTTGTGCCTGCTTCATCT			cDNA: 367
MHC- α	fwd	GGAAGAGTGAGCGGCGCATCAAGG	58	32	cDNA: 302
	rev	CTGCTGGAGAGGTTATTCCTCG			
NANOG	fwd	AGGGTCTGCTACTGAGATGCTCTG	60	28	cDNA: 364
	rev	CAACCACTGGTTTTCTGCCACCG			genomic: 4793
NKX2.5	fwd	CACCCACGCCTTTCTCAGTC	57	35	cDNA: 513
	rev	TGGACGTGAGCTTCAGCA			
Oct3/4	fwd	CCACCTTCCCCATGGCTGGACA	50	37	cDNA: 1082
	rev	AGGGCTGGTGCCTCAGTTTG			genomic: 4475
p53	fwd	CGCTGCTCCGATGGTGATGG	60	30	cDNA: 343
	rev	TGTCCCGTCCCAGAAGGTTCC			
SOX2	fwd	AACCCCAAGATGCACAACCTC	50	33	cDNA: 202
	rev	CTCCGGGAAGCGTGTACTTA			
SRY	fwd	TTCCAGGAGGCACAGAGATT	60	30	
	rev	TTGGAGTACAGGTGTGCAGC			genomic: 215
Tropomyosin- α	fwd	CAAGCGGAGGCTGATAAGAAGG	55	30	cDNA: 310
	rev	TGCCTCTCTCACTCTCATCTGC			

3.6 Antibodies

Antibody	Dilution	kDa	Company (Catalog number)
goat α -mouse Alexa488	IF 1:1000	-	Molecular Probes, USA (A-11001)
hBMP2/4	WB 1:800	26	R&D Systems, USA (MAB3552)

rabbit α-Desmin	IF 1:80	-	Abcam, USA (ab8592)
mouse α-Desmin	IF 1:50	-	Sigma Aldrich, USA (D1033)
mouse α-Emerin	IF 1:50	-	Novocastra, UK (NCL-Emerin)
GAPDH	WB 1:50000	37	Millipore, USA (MAB374)
mouse α-Lamin A/C	IF 1:500	-	n.a. (Egon Ogris Lab, VBC, AUT)
rabbit α-NKX2.5	IF 1:50	-	Santa Cruz, USA (sc-14033)
donkey α-rabbit Rhodamine	IF 1:400	-	Jackson IR, USA (711-295-152)
rabbit α-SPARC	WB 1:200	43	Santa Cruz, USA (sc-25574)
mouse α-Smooth Muscle Actin	IF 1:50	-	Sigma Aldrich, USA (A2547)
mouse α-cardiac Troponin T	IF 1:50	-	Santa Cruz, USA (sc-20025)

3.7 Cell lines

3.7.1 SNL76/7 fibroblasts (feeder cells)

Mitotically inactivated STO mouse embryonic fibroblasts were stably transfected with both a Neomycin (Neo) resistance and a LIF expression vector (McMahon and Bradley, 1990; Soriano et al., 1991).

3.7.2 mESC lines

The mESC line Ab2.2 was isolated from wild-type 129Sv mice (Soriano et al., 1991). Transgenic mESC line Tag3 represents Ab2.2 ESCs containing two Thymidine kinase (TK) suicide genes from human Herpes simplex virus type 1 (HSV-1) and a Neo resistance cassette (Hollrigel et al., 2001).

The mESC line DC6 was generated by transfection of Ab2.2 ESCs with a desmin expression vector (Hofner et al., 2007), mESC line $des^{-/-}$ by targeted gene disruption as previously described (Hollrigel et al., 2002; Weitzer et al., 1995).

3.7.3 CVPC lines

Transgenic CVPC lines were isolated by positive selection from hearts of two day old out-bred C57BL/6Jx129Sv mice carrying a Neo resistance cassette inserted into one allele of the histone deacetylase 1 (Hdac1) locus (Lagger et al., 2002). After enrichment using a modified 3T3 protocol and clonal propagation under constant Neo selection, 11 independent clonal CVPC lines were obtained (A3, A5, B3, B5, C3, D3, D5, E3, F3, G3 and H3).

Wild-type CVPC lines were isolated by negative selection from hearts of two (mixed female and male), 22 (male) and 180 (female) day old wild-type Balb/C mice. Four clonal CVPC lines from hearts of two day old mice (He2), two clonal CVPC lines from hearts of 22 day old

mice (He22) and one clonal CVPC line from hearts of 180 day old (adult) mice (HeA) were obtained.

3.8 Bacterial strains

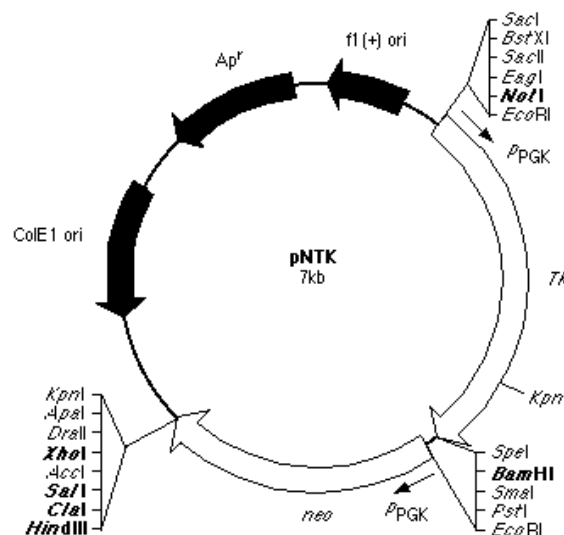
3.8.1. XL1-Blue

The competent *Escherichia coli* strain XL1-Blue (Stratagene, USA) was used for plasmid amplification and preparation.

3.9 Plasmids

3.9.1 pNTK

The pNTK vector contains both the Neo and TK genes, driven by a phosphoglycerate kinase (PGK) promoter, and was used as positive control for genotyping PCR experiments.



3.10 Media and solutions for cell culture

3.10.1 Culture media for ESCs/CVPCs

Dulbecco's Modified Eagle Medium (DMEM)

4.5 l autoclaved MilliQ H₂O are poured into a 5 l Erlenmeyer flask and 66.9 g of DMEM powder are slowly added under constant stirring followed by 18.5 g NaHCO₃. The solution is brought to pH 7.4 with HCl conc., filled up to 5 l with autoclaved MilliQ H₂O and aliquoted through a sterile filter (Nalgene filter, 0.22 µm pore width) into sterile 500 ml medium bottles. Media can be stored at 4°C. An aliquot (3 ml) of each bottle is transferred into a 6-well cell

culture plate, incubated at 37°C, 5% CO₂ and checked daily under the light microscope for contaminations.

M15Hy medium for ESC/CVPC culture

15% HyClone FBS
1% β -Mercaptoethanol (β -ME)
1% GPS
83% DMEM

M15Gi medium for ESC/CVPC culture

15% Gibco FBS
1% β -ME
1% GPS
83% DMEM

M15Si medium for ESC/CVPC differentiation

15% Sigma FBS
1% β -ME
1% GPS
83% DMEM

M10Gi medium for SNL76/7 and feeder cell culture

10% Gibco FBS
1% GPS
89% DMEM

Freezing medium

20% HyClone FBS
20% Dimethylsulfoxid (DMSO)
60% DMEM

3.10.2 Solutions

10x PBS (Phosphate buffered saline)

1.37 M	NaCl
14.7 mM	KCl
78.1 mM	Na ₂ HPO ₄ ·7H ₂ O
26.8 mM	KH ₂ PO ₄
pH 7.2	

Salts are dissolved in 800 ml autoclaved MilliQ H₂O and pH is adjusted to 7.2 with Na₂HPO₄. The solution is filled up to 1 l with autoclaved MilliQ H₂O and sterile filtrated. 10x PBS can be stored at room temperature.

1x PBS

10x PBS is diluted 1:10 in autoclaved MilliQ H₂O.

100x GPS

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

Ingredients are dissolved in 500 ml autoclaved MilliQ H₂O and the solution is sterile filtrated. Aliquots are stored at -20°C. 100x GPS has to be thawed and checked for possible salt precipitation prior to use. In case of precipitation the solution is heated and mixed until it appears clear. Can be stored at 4°C for short-term use.

100x β-ME (10⁻²M)

200 ml	1x PBS
144 μl	β-ME

β-ME and 1x PBS are mixed well and sterile filtrated. Aliquots are stored at -20°C or 4°C when in use.

Trypsin

3.5 g	NaCl
0.5 g	D-Glucose
0.09 g	Na ₂ HPO ₄ ·7H ₂ O
0.185 g	KCl
0.12 g	KH ₂ PO ₄
0.2 g	EDTA
1.25 g	Trypsin
1.5 g	Tris Base
pH 7.6	

Ingredients are dissolved in 500 mL autoclaved MilliQ H₂O and the solution is adjusted to pH 7.6 with HCl conc. After sterile filtration 50 ml aliquots are made and stored at -20°C or 4°C when in use.

1% gelatin stock solution

10 g	gelatin
1 l	MilliQ H ₂ O

10 g gelatin is dissolved in 1 l autoclaved MilliQ and autoclaved. Can be stored at room temperature.

0.1% gelatin working solution

1% gelatin stock solution is diluted 1:10 in autoclaved MilliQ H₂O. Can be stored at room temperature.

4. Methods

4.1 Maintenance and recycling of cell culture consumables

4.1.1 Washing of cell culture glass ware

Used cell culture bottles are filled with hypochlorite and tap water. After 15-30 minutes the bottles are rinsed at least five times with tap water, filled with MilliQ H₂O 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. All other reusable cell culture consumables are also washed with hypochlorite and MilliQ H₂O prior to use and should solely be used for cell culture and never come in contact with detergents.

4.1.2 Washing of glass pipettes

After use, the cotton plug is removed and glass pipettes are put into vessels containing a water/hypochlorite mixture. After several hours the pipettes have to be rinsed with fresh tap water for at least four hours and can then be left in MilliQ H₂O overnight. Pipettes are dried at 80°C for at least 4 hours, placed in pipette boxes and baked at 180°C for 8 hours.

4.2 Culture of SNL76/7 fibroblasts

4.2.1 Gelatin coating of cell culture plates

To facilitate cell adherence cell culture plates can be coated with gelatin. Therefore, the plates are fully covered with a 0.1% gelatin solution and incubated for at least one hour at room temperature or 37°C, 5% CO₂ prior to use. After aspiration of the gelatin solution the plates are ready to use or can be left at 37°C, 5% CO₂ for short-term storage.

4.2.2 Thawing of SNL76/7

10 cm culture plates must be coated with gelatin prior to thawing. SNL76/7 fibroblasts are stored in cryotubes in liquid nitrogen. The cryotube is carefully taken out of the liquid nitrogen tank and thawed in a 37°C bath until just a small icicle is left in the tube. The cryotube is then sprayed with 70% EtOH, dried, transferred into the laminar and flamed before and after opening. The content (1-2 ml) is transferred into a 15 ml Falcon tube and 10 ml M10Gi medium are slowly added drop wise while carefully pivoting the tube. If the medium is added too fast the cells can die because of osmotic shock. The 15 ml Falcon tube containing the cell suspension is centrifuged at 1000 rpm for 7 minutes at room temperature

and the pellet is resuspended in 1 ml of M10Gi medium. Another 3 ml of M10Gi medium are added to the tube and the content is seeded onto a gelatin-coated 10cm cell culture plate. The Falcon tube is washed with additional 4 ml of fresh M10Gi medium to take up any residual cells and the suspension is added to the 10 cm culture plate. Cell viability needs to be checked under the light microscope the next day and the medium has to be changed if necessary.

4.2.3 Cultivation of SNL76/7 fibroblasts

SNL76/7 fibroblasts are incubated at 37°C, 5% CO₂. The medium needs to be replaced regularly with fresh M10Gi medium. At 80-90% confluency the cells are split 1:6-1:8.

4.2.4 Splitting of SNL76/7 fibroblasts

When confluent, cells must be split onto new gelatin-coated 10 cm cell culture plates. The plates are washed with 4 ml 1x PBS (1x PBS is applied to remove traces of M10Gi medium which would inhibit the function of trypsin). 1 ml trypsin is added and the cells are incubated at 37°C, 5% CO₂ for 5-10 minutes or until all cells have detached from the plate surface (detachment can be checked under light microscope). 3 ml fresh M10Gi medium are added to the trypsinized cells and the cells are resuspended well by gentle trituration. For splitting 1:8, 500 µl cell suspension are transferred onto a new gelatin-coated 10 cm cell culture plate containing 7 ml fresh M10Gi medium.

4.2.5 Freezing of SNL76/7 fibroblasts

Confluent cells are washed with 4 ml 1x PBS and, after removal of 1x PBS, 1 ml trypsin is added to the plate. After incubation at 37°C, 5% CO₂ for 5-10 minutes the trypsinized cells are resuspended in 2 ml M10Gi medium and transferred into a 15 ml Falcon tube. 3 ml of freezing medium are slowly added drop wise to the suspension. The content (6 ml) is mixed thoroughly and aliquoted into 3 cryotubes which are stored in a styrofoam box at -80°C. After two days the cryotubes can then be transferred into liquid nitrogen for long-term storage.

4.2.6 Preparation of feeder cells

All cell culture plates used must be gelatin-coated in advance. All but 4 ml of the medium is removed from several confluent SNL76/7 cell culture plates and 80 µl Mitomycin C are added. After a maximum of 4 hours at 37°C, 5% CO₂ the supernatant is aspirated and the plates are washed twice with 1x PBS. 1 ml trypsin is added to each plate and the cells are

incubated at 37°, 5% CO₂ for 5-10 minutes or until all cells have detached from the plate surface (detachment can be checked under light microscope). The cells are resuspended in 5 ml of M10Gi medium (several plates can be pooled) and the suspension is transferred into a 50 ml Falcon tube. Optionally, plates can be washed with additional M10Gi medium for as quantitative as possible cell transfer. The Falcon tube is centrifuged at 1000 rpm for 7 minutes and the supernatant is discarded. The pellet is resuspended in 10 ml of M10Gi medium and the cell number is determined by measurement with a CASY cell counter (10 µm-30 µm) or using a counter chamber. Cells are diluted with M10Gi medium to a viable cell number of 3.5×10^5 cells/ml. For 24-well cell culture plates 0.5 ml suspension/well, for 6-well cell culture plates 2 ml suspension/well, and for 6 cm cell culture plates 4 ml suspension are used. Feeder cell plates are stable at 37°C, 5% CO₂ for up to two weeks and the medium has to be exchanged at least once a week with fresh M10Gi medium.

4.3 Isolation of CVPCs

4.3.1 Isolation using positive selection

Hearts were obtained from 2 day old out-bred C57BL/6Jx129Sv mice with a Neo resistance cassette inserted into one allele of the Hdac1 locus (Lagger et al., 2002). Hearts were placed in ice cold 1x PBS, rinsed thoroughly with 1x PBS, and cut into very small pieces. Residual blood cells were removed by centrifugation at 1000 rpm at room temperature for 1 min and the pelleted tissue was suspended in 1 ml of 1x PBS containing 0.6 mg Pancreatin/0.5 mg Collagenase II and incubated at 37°C on a shaker at 800 rpm for 15 minutes twice. The supernatant of the first incubation period was stored on ice and combined with the second one. After centrifugation cells were suspended in M15Hy medium. 5×10^5 cardiac cells were mixed with 5×10^5 Ab2.2 mESCs (Soriano et al., 1991) and plated into one well of a 24-well plate coated with mitotically inactivated SNL76/7 fibroblasts as feeder cells (McMahon and Bradley, 1990; Soriano et al., 1991). Putative stem cells were enriched by a modified 3T3 protocol for 10 passages, comprising of 3 days culture with daily change of 2 ml M15Hy medium and splitting of the cells 1:3 every third day onto new feeder cells. After 10 passages the cell population was subjected to selection with 180 g/ml G418 for 10 days. C57BL/6Jx129Sv clones surviving G418 selection were expanded and seeded at clonal density on feeder cells under continuing G418 selection. Individual colonies were expanded to clonal cell lines which were frozen at increasing passages.

4.3.2 Isolation using negative selection

The same procedure as in 3.3.1 was applied but instead of C57BL/6Jx129Sv Neo resistant mice hearts from 2, 22, and 180 day old wild-type Balb/C mice were used. Balb/C heart cells were mixed with the transgenic mESC line Tag3 containing 2 TK suicide genes from human HSV-1 and a Neo resistance cassette (Hollrigl et al., 2001). After enrichment using the modified 3T3 protocol for 10 passages the cells were selected twice with 2 mmol/l Gancyclovir (GanC) for 4 days each and individual clones were counter selected for G418 sensitivity. Balb/C wild-type CVPC clones were seeded at clonal density on feeder cells under continuing Gancyclovir selection. Individual colonies were expanded to clonal cell lines which were frozen at increasing passages.

4.4 Culture of mESCs/CVPCs

Murine ESCs and putative CVPCs require the presence of LIF for self-renewal. Therefore, they always have to be cultivated on LIF secreting feeder cell (SNL76/7) layers.

4.4.1 Thawing of mESCs/CVPCs

The feeder cells must be fed with 2 ml fresh M15Hy medium at least 2 hours prior to thawing of ESCs/CVPCs. The cryotubes containing the frozen ESCs/CVPCs are taken out of the liquid nitrogen tank, thawed in a water bath (37°C) until just a small icicle is left, sprayed with 70% EtOH, placed in the laminar and flamed. The content is transferred into a 15 ml Falcon tube and 12 ml M15Hy medium are slowly added dropwise (the tube is gently pivoted after each drop). The cells are centrifuged at 1000 rpm for 6 minutes and the supernatant is discarded. The pellet is resuspended in 1 ml of pre-fed feeder cell supernatant and the cell suspension is transferred back onto the feeder cells. The cryotube may be washed with additional 500 µl of M15Hy medium.

4.4.2 Cultivation of mESCs/CVPCs

Murine ESCs/CVPCs are incubated at 37°C, 5% CO₂. The medium needs to be replaced daily with fresh M15Hy medium. At 80-90% confluency the cells are split 1:2 or 1:3.

4.4.3 Splitting of mESCs/CVPCs

Both feeder cells and mESCs/CVPCs must be fed with fresh M15Hy medium at least 2 hours prior to splitting (2 ml for feeder cells, 1 ml for mESCs/CVPCs). The mESCs/CVPCs are

washed once with 1x PBS and then 200 μ l trypsin (for 24-well plate) are added. The cells are incubated for 15 minutes at 37°C, 5% CO₂, 1 ml of M15Hy medium from previously fed feeder cells is transferred onto the mESC/CVPC suspension and the cells are resuspended well. For splitting 1:2 600 μ l of the ,ESC/CVPC suspension are transferred back onto the feeder cells (for 1:3, 400 μ l).

4.4.4 Freezing of mESCs/CVPCs

The mESCs/CVPCs are fed with 1 ml M15Hy medium at least 2 hours prior to freezing. The cells are washed once with 1x PBS, trypsinized (200 μ l for 24-well plate) and incubated at 37°C, 5% CO₂ for 15 minutes. 1 ml fresh M15Hy medium is added, the cells are resuspended well and transferred into a 15 ml Falcon tube. 1.2 ml freezing medium are added drop wise and the tube is pivoted carefully after each drop to ensure homogenous distribution. After the addition of the freezing medium the cell suspension is divided into two (1.2 ml each) or three (800 μ l each) labelled cryotubes which are stored in a styrofoam box at -80°C for at least two days before they can be transferred into liquid nitrogen.

4.5 EBs and Cardiac bodies (CBs)

4.5.1 Preparation of EBs/CBs

Murine ESCs/CVPCs must be split 1:2 one day prior to EB/CB preparation to assure that the cells are in the log phase. The cells are pre-fed with 1 ml M15Si medium two to four hours prior to EB/CB preparation and 6 well plates are coated with 0.1% gelatin for pre-adsorption of feeder cells.

After two hours the cells are washed with 1x PBS, 200 μ l trypsin are added (for 24-well plate) and the cells are incubated at 37°C, 5% CO₂ for 15 minutes. 800 μ l M15Si medium are added, the cells are resuspended well and transferred onto the gelatin-coated 6-well plate to separate the ESCs/CVPCs from the feeder cells (feeder cells attach to the plate faster than mESCs/CVPCs). After incubation of the 6-well plate for 45 to 60 minutes at 37°C, 5% CO₂ the suspension is transferred into a 15 ml Falcon tube and filled up to 10 ml with M15Si medium. The cell number is measured using a counter chamber (10 μ l suspension used) and adjusted by dilution with M15Si medium (in case the concentration is too high) or centrifugation (1000 rpm, 6 minutes) followed by resuspension in a smaller volume of medium (in case the concentration is too low) to a concentration of 4.5×10^4 cells/ml (equals 900 cells in 20 μ l).

Sterilin coated 10 cm cell culture plates are filled with MilliQ H₂O (the whole plate must be covered) and a 500 µl Eppendorf repeating pipet is set to 20 µl dispensation. The cell suspension is poured into a sterile 10 cm cell culture plate for better handling and 60-100 cell suspension drops are dropped onto the inside of the Sterilin cell culture plate lid (the first and the last drop are always discarded). The drops are placed as close as possible without running risk of merging. Also the drops should not be placed too close to the edge of the dish lid to avoid contamination. The lid is flipped over, placed on the top of the MilliQ filled bottom half and incubated at 37°C, 5% CO₂ (→ day 0).

At day 4.7 the hanging drops have to be transferred onto gelatin-coated 10 cm cell culture plates to allow attachment of the EBs/CBs to the plate surface. Therefore the hanging drop cultures are flamed shortly at the edge of the plate lid and rinsed from the lid onto the bottom of the gelatin-coated dish with 8 ml M15Si medium. The EBs/CBs are stored at 37°C, 5% CO₂ in the incubator.

4.5.2 EB/CB cultivation

CBs must be fed every three days with fresh M15Si medium, but the medium is only partially replaced (day 7 to 10: 3 ml of old medium +8 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 the medium at low concentrations.

4.6 Monolayer differentiation of mESCs/CVPCs

Cells are grown to confluency on 6-well plates and pre-fed with 2 ml M15Si medium two hours prior to trypsinization. 300 µl trypsin are added and the cells are incubated at 37°C, 5% CO₂ for 15 min. Trypsinization is stopped by addition of 1.7 ml M15Si medium and after thorough suspension the cell suspension is transferred onto a gelatin-coated 6-well or 6 cm cell culture plate for pre-adsorption of feeder cells (incubation for 45 -60 minutes at 37°C, 5% CO₂; several wells may be pooled). The cells are transferred into a 15 ml Falcon tube and the cell number is adjusted by dilution with M15Si medium to a concentration of 1.5×10^6 cells/ml (cell suspension may have to be diluted for cell count).

1 ml of the cell suspension is transferred onto a gelatin-coated 6 cm plate and 3 ml M15Si medium are added. Medium is changed every day (4 ml fresh M15Si medium - or less - depending on the condition of the old medium).

4.7 Isolation of genomic DNA

4.7.1 Phenol/Chloroform extraction

The cells are washed with 1x PBS, trypsinized (200 µl for 24-well plate) and incubated at 37°C, 5% CO₂ for 15 minutes. 1 ml fresh M15Hy medium is added, the cells are resuspended well and transferred onto a gelatin-coated 6-well cell culture plate for pre-adsorption of feeder cells. After 45-60 minutes the suspension is transferred onto a new gelatin-coated 6-well cell culture plate and the cells are incubated at 37°C, 5% CO₂ for two more days with daily exchange of medium.

For cell lysis, the cells are washed twice with 1x PBS and 1 ml Lysis Buffer is added to each 6-well, followed by incubation at 60°C for 24 hours. For gDNA extraction, the suspension is transferred into a 15 ml Falcon tube (optionally, cells can be scraped off the surface) and the same volume phenol is added, followed by vigorous shaking (vortex) and centrifugation at 1400 rpm for 8 minutes. The upper aqueous phase containing the gDNA is transferred into a new 15 ml Falcon tube and the same volume phenol/chloroform (1:1) is added. The solution is vortexed thoroughly and centrifuged at 1400 rpm for 7 minutes. The upper clear phase is transferred into a new 15 ml Falcon tube and the same volume chloroform is added. The solution is vortexed thoroughly and centrifuged at 1400 rpm for 5 minutes. The aqueous upper phase is mixed with 1/2 volume ammonium acetate solution and 3 volumes ice-cold absolute EtOH and incubated at -20°C overnight after gentle shaking. After centrifugation at 4000 rpm for 10 minutes the DNA pellet is washed twice with ice-cold 70% EtOH (centrifugation at 4000 rpm for 5 min for each wash step) and resuspended in 100-500 µl TE Buffer or ddH₂O (volume depending on the size of the pellet).

Lysis Buffer

10 mM	Tris-HCl
10 mM	EDTA
300 mM	NaCl
0.4%	SDS
200 ng /ml	Proteinase K
pH 7.4	

TE Buffer

10 mM Tris-HCl
1 mM EDTA
pH 8.0

4.7.2 Wizard® Genomic DNA Purification Kit

The cells are harvested, transferred into a 1,5 ml Eppendorf tube and centrifuged at 13000 rpm for 10 seconds to pellet the cells. The supernatant is removed and the cells are washed with 200 µl 1x PBS (centrifugation at 13000 rpm for 10 seconds for washing). 600 µl Nuclei Lysis Solution are added and the suspension is pipetted to mix for cell lysis until no visible clumps remain. 200 µl Protein Precipitation Solution are added and the solution is vortexed vigorously for 20 seconds. After incubation on ice for 5 minutes the solution is centrifuged at 13000 rpm for 4 minutes to precipitate total protein. The supernatant containing the gDNA is carefully removed and transferred into a new 1,5 ml Eppendorf tube containing 600 µl isopropanol. The solution is gently mixed by inversion and centrifuged at 13000 rpm for 1 minute to pellet the gDNA. The supernatant is discarded and 600 µl 70% EtOH are added. The pellet is washed by gently inverting the tube a couple of times, followed by centrifugation at 13000 rpm for 1 minute. The 70% EtOH is carefully aspirated without disturbing the pellet, the tube is inverted on clean absorbent paper and air-dried for 10-15 minutes. 100-300 µl DNA Rehydration Solution are added (volume depending on the size of the pellet) and the gDNA is rehydrated at 65°C for 1 hour. The solution needs to be mixed periodically by tapping the tube. Genomic DNA can be stored at 4°C.

4.8 Isolation of mRNA

RNA was isolated using the QIAGEN RNeasy Mini Kit. Prior to RNA isolation, the working surface and all used pipettes are carefully cleaned with soap and 70% EtOH or RNase Away decontaminant. In general, one set of pipettes and pipette tips is used exclusively for work with RNA.

The cells are washed with 1x PBS, then 1 ml 1x PBS (for 24-well cell culture plate) is added and the cells are detached from the well surface using a cell scraper. The suspension is transferred into a 15 ml Falcon tube and centrifuged at 1000 rpm for 5 minutes. The cell pellet is washed twice with 1x PBS (centrifugation at 1000 rpm for 5 minutes for each wash step). After washing, the cell pellet is resuspended in 1 ml 1x PBS, the suspension is transferred into

a 1,5 ml Eppendorf tube and centrifuged at 14000 rpm, 4°C for 5 minutes. The supernatant is discarded and the pellet is resuspended in 606 µl RLT Buffer/β-ME (100:1). The suspension is loaded onto a Shredder Column (purple) and centrifuged at 13000 rpm for 2 minutes. The column is discarded, 606 µl RNase-free 70% EtOH are added to the flow-through and the solution is pipetted to mix. For RNA-binding, 600 µl of the solution are loaded onto the RNeasy column (pink) and centrifuged at 14000 rpm for 15 seconds. The flow-through is discarded and the centrifugation step is repeated with the residual 600 µl of the solution. 700 µl RW Buffer are added and the column is centrifuged at 14000 rpm for 15 seconds. The flow-through is discarded, 500 µl RPE Buffer are added and the column is centrifuged at 14000 rpm for 15 seconds. The flow-through is discarded, 500 µl RPE Buffer are added and the column is centrifuged at 14000 rpm for 1 minute to allow EtOH evaporation. The column is transferred into a new 1,5 ml Eppendorf tube, 30 µl RNase-free H₂O are added (for highest yield, H₂O needs to be evenly distributed over the whole column surface) and the column is incubated at room temperature for 1-3 minutes. RNA is eluted by centrifugation at 14000 rpm for 1 minute.

For DNA digestion, 3.75 µl DNase I Buffer (+MgCl₂) and 3.75 µl DNase I are added to 30 µl eluted RNA, followed by incubation at 37°C for 30 minutes. To stop the reaction, 3.75 µl EDTA (25mM) are added and the solution is incubated at 65°C for 10 min while shaking. DNA digestion can be verified running a PCR that specifically amplifies a genomic fragment of GAPDH and subsequent gel electrophoresis.

4.9 Reverse transcription (RT) of mRNA into cDNA

Prior to cDNA synthesis RNA concentration is determined using a NanoDrop UV-Vis Spectrophotometer and the concentration is adjusted to 500 ng/µl. 29.5 µl RNA are mixed with 1 µl d(T) and incubated at 70°C for 10 minutes. After incubation on ice for 3 minutes the sample is centrifuged at 13000 rpm for 30 seconds. 18.5 µl RT-mix are added and the sample is incubated at 42°C for 2 minutes. 1 µl Reverse Transcriptase is added (total volume = 50 µl) following incubation at 42°C for 50 minutes. Next, the sample is incubated at 70°C for 15 minutes and put one ice for 5 minutes afterwards. After centrifugation at 13000 rpm for 2 minutes the cDNA solution can be stored at -20°C.

In case the RNA concentration is lower than 500 ng/µl the whole sample volume after DNA digestion (41.25 µl) is used for RT, resulting in a 1.4-fold increase of the total reaction volume (70 µl instead of 50 µl). Therefore the volumes of all added reagents have to be

multiplied with this factor (RT-mix "1.4-fold"). The same protocol as described above can be used.

RT-mix ($C_{\text{RNA}} \geq 500 \text{ ng/}\mu\text{l}$):

10 μl	5x RT buffer
5 μl	0.1 M DTT
1.5 μl	Riboblock RNase Inhibitor
2 μl	10 mM dNTPs
18.5 μl	

RT-mix "1.4-fold" ($C_{\text{RNA}} < 500 \text{ ng/}\mu\text{l}$):

14 μl	5x RT Buffer
7 μl	0.1 M DTT
2.1 μl	Ribolock RNase Inhibitor
2.8 μl	10 mM dNTPs
25.9 μl	

4.10 Polymerase chain reaction (PCR)

All PCRs were performed with a Biometra T-Personal PCR Cyclor and analyzed by gel electrophoresis on 1.5% agarose gels. A standard PCR reaction mix and cycling program is shown below. Depending on targets and primers, different cycle numbers and annealing temperatures were used.

ddH ₂ O	39.75 - x μl
10x buffer	5 μl
25 mM MgCl ₂	3 μl
10 mM dNTPs	1 μl
Primer forward	0.5 μl
Primer reverse	0.5 μl
mRNA/cDNA/gDNA	x μl (0.1 - 1 μg)
Taq Polymerase	0.25 μl
Total	50 μl

Standard PCR Program:

95°C	1 min
95°C	45 sec
x°C	45 sec
72°C	1 min
72°C	10 min

4.11 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

4.11.1 Protein Isolation for SDS-PAGE

The cells are washed with 37°C 1x PBS and ice-cold, fresh Kinexus Lysis Buffer (100 µl/48-well, 150 µl/24-well, 300 µl/6-well) is added immediately. The cells are scraped off the plate, transferred into a 1,5 ml Eppendorf tube and placed on ice. If necessary, the wells can be washed with additional 50 µl of Kinexus Lysis Buffer.

Kinexus Lysis Buffer

20 mM	Tris-HCl
2 mM	EGTA
2 mM	EDTA
30 mM	Sodiumfluorid
40 mM	Glycerophosphate
10 mM	Sodium Pyrophosphate
2 mM	Sodium Orthovandate
10 µM	Leupeptin
5 µM	Pepstatin A
0.5%	Triton X-100
Proteinase Inhibitor (1 tablet per 100 ml)	
pH 7.0	

4.11.2 Trichloroacetic acid (TCA)/Acetone protein precipitation

Sufficient 100% TCA solution is added to the sample to reach a final TCA concentration of 10% (the sample should turn milky white if proteins are present). The sample is vortexed well and incubated on ice for 30-60 minutes. The sample is centrifuged at 13000 rpm, 4°C for 5 minutes (or until the supernatant is clear and a white protein pellet is present at the bottom of the tube) and the supernatant is discarded carefully. For TCA removal, 800 µl of -20°C acetone are added and the pellet is resuspended by vortexing (the proteins will not dissolve in acetone, they will only be suspended). The protein-acetone suspension is incubated at -20°C for 1 hour and centrifuged at 13000 rpm, 4°C for 5 minutes. The supernatant is discarded and 800 µl -20°C acetone are added to the pellet. For complete resuspension the solution is vortexed well and incubated at -20°C for 10 minutes. The sample is centrifuged at 13000 rpm,

4°C for 5 minutes, the supernatant discarded and washing is repeated at least once more. After the final washing step, the acetone is discarded and the pellet is left to dry completely. The pellet is dissolved in ddH₂O and 5x Sample Buffer can be added right away. After incubation at 95°C for 5-10 minutes, the protein sample can be readily used for SDS-PAGE.

100% TCA solution (1 ml)

1g TCA

0.7 ml ddH₂O

4.11.3 SDS-PAGE

5 ml separation gel are pipetted into the PAGE-chamber and coated with isopropanol for even gel distribution. Once the gel is fully polymerized the isopropanol is removed and the stacking gel is pipetted on top of the separation gel. The SDS-PAGE gels are transferred into the gel chamber filled with SDS-PAGE Running Buffer. 3x Sample Buffer is added to the samples and both samples and marker are incubated at 95°C for 5-10 min before being loaded onto the gel. SDS-PAGE is run between 100V and 150V until the loading dye has almost leaked out at the bottom end.

Separation gel 12.5% (for 2 gels)

5 ml	Polyacrylamide (30%)
3 ml	1.5M Tris/HCl pH8.8, 0.4% SDS
4 ml	ddH ₂ O
120 µl	10% APS
10 µl	TEMED

Stacking gel 5% (for 2 gels)

0.5 ml	Polyacrylamide (30%)
0.75 ml	0.5M Tris/HCl pH6.8, 0.4% SDS
1.75 ml	ddH ₂ O
30 µl	10% APS
2.5 µl	TEMED

10x SDS-PAGE Running Buffer

1.92 M	Glycin
250 mM	Tris-Cl
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	ddH ₂ O
6 mg	Bromphenolblue

Aliquots are made and 150 µl β-ME are added before use.

4.11.4 Western blot

4 Whatman papers are soaked in 1x Blotting Buffer and placed into the semi-dry Western Blot apparatus (Trans Blot SD, Biorad). A nitrocellulose membrane (Whatman, Protran) soaked in 1x Blotting Buffer is placed on top. The SDS-PAGE gel is placed on top of the membrane and topped with 4 more Whatman papers soaked in 1x Blotting Buffer. The device is closed and run at 400mA for approximately one hour. When using a non pre-stained protein ladder, the membrane is incubated in Ponceau S solution for 5 min to visualize the ladder and bands. The bands of the protein ladder are marked on the nitrocellulose membrane before de-staining with ddH₂O (when using a pre-stained protein ladder this step may be skipped, nevertheless, Ponceau S staining confirms protein transfer after blotting and should be carried out as a control).

10x Blotting Buffer

0.48 M	Tris-HCl
0.4 M	Glycin
pH 9.1	

Ponceau-S

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

4.11.5 Antibody incubation and detection

The nitrocellulose membrane is blocked with 5% milk in TBS-T for at least 1 hour at 4°C. The primary antibody is diluted in 5% Milk TBS-T and incubated with the membrane at 4°C for at least 1 hour (or overnight). Incubation is followed by three wash steps with TBS-T for 5-10 min each. The secondary (detection) antibody (linked to horseradish peroxidase; HRP) is diluted in 5% milk TBS-T and incubated with the blot for at least one hour.

For antibody detection, the nitrocellulose membrane is washed thrice with TBS for 5-10 min. ECL Detection Solution 1 and Detection Solution 2 (Amersham) are mixed at a ratio of 1:1 and brought to 1x concentration by dilution with ddH₂O. The nitrocellulose membrane is incubated with 1.5 ml (for 5 x 8 cm membrane) solution for 1-60 minutes, depending on the amount of protein on the membrane. The bands are detected with CL-Xposure Film (Thermo Scientific) in a dark room using an Agfa Curix 60 developer.

5% milk in TBS-T

5%	Skim Milk Powder
0.1%	Tween-20

10x TBS

0.5 M	Tris-HCl
1.5 M	NaCl
pH 7.6	

4.11.6 Stripping of Western blots

For stripping of the primary antibody, the nitrocellulose membrane is washed thrice with 1x TBS for 5 min and transferred into a 50 ml Falcon Tube containing 45 ml Stripping Buffer A. The Falcon Tube is incubated in a 70°C water bath for 30 minutes under constant shaking. The membrane is repeatedly washed with 1x TBS until the β -ME smell has vanished.

For stripping of the secondary antibody, the 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.

Stripping Buffer A

2%	SDS
100 mM	β -ME
62.5 M	Tris-HCl

pH 6.8

4.12 Karyotyping (Giemsa stain)

The cells can be treated with 10 μ l colchicine (1 μ g/ μ l) for 3 hours prior to trypsinization in order to inhibit spindle formation.

Confluent cells (24-well plate) are washed with 1 ml of 1x PBS and 200 μ l trypsin are added. After 15-20 minutes at 37°C, 5% CO₂, 800 μ l M15Hy medium are added and the suspension is transferred into a 15 ml Falcon tube. This step is followed by centrifugation at 1000 rpm for 5 minutes at room temperature. All but 0.5 ml of the supernatant are discarded and the pellet is resuspended in the remaining 0.5 ml. 4 ml 75 mM KCl solution (room temperature) are added drop-wise and the solution is incubated at room temperature for 6 min. The suspension is centrifuged at room temperature at 1000 rpm for 5 minutes. The supernatant is removed and 4 ml of a 3:1 methanol:glacial acetic acid mixture are added with a Pasteur pipette (resuspension). The centrifugation step is repeated, the supernatant is discarded and the pellet is resuspended in 3 ml of a 3:1 methanol:glacial acetic acid mixture. This step is repeated until a satisfying pellet is achieved. The centrifugation step is repeated once more and 0.5 ml of the methanol:glacial acetic acid solution are added (more can be added if the dilution of cells is not high enough).

Before further use, the suspension should be stored at 4°C for at least 3 hours. Glass slides are washed in 70% EtOH and let dry before use. The slides are placed at a 30-60° angle and a Pasteur pipette is used to create individual drops of cells. These drops will run down the slide and thereby spread the cells over the entire slide (for better results the Pasteur pipette can be placed between 3-6 cm above the slide). The suspension can be kept at 4°C over months. For chromosome visualization, the slides are stained with Giemsa stain (Sigma Aldrich, USA) for 5-10 minutes.

4.13 Immunofluorescence

4.13.1 Pre-extraction and stabilization of the cytoskeleton

Extraction can be performed directly in the 24-well cell culture plate. The medium is discarded and the cells are washed with 37°C 1x PHEM. After washing, the cells are incubated with 2 ml of 37°C Extraction Buffer for 2 minutes at 37°C, 5% CO₂. After pre-extraction, cells can be directly subjected to fixation.

10x PHEM

600 mM	Na-Pipes
250 mM	Na-Hepes
100 mM	Na-EGTA
20 mM	MgCl ₂
pH 6.9	

Extraction Buffer

0.2% (v/v)	Triton X-100
5 µM	Taxol
in 1x PHEM	

4.13.2 Paraformaldehyde (PFA) fixation

For IF, the cells are grown adherent to gelatin-coated glass cover slips inside a 24-well cell culture plate. After washing twice with 1x PBS the cells are fixated by incubation with 4% PFA/1x PBS for 20 minutes at room temperature (alternatively, cells can be fixated in 2% PFA/1x PBS pH7.5 for 25 minutes at room temperature). After removal of PFA/1x PBS the cells are washed with 1x PBS. For permeabilization, the cells incubated with 0.3% Triton X-100/1x PBS for 10 minutes at room temperature (alternatively, cells can be incubated with 0.1% Saponin/1x PBS for 20 minutes at room temperature). The cells are washed twice with 1x PBS and can be stored in 1x PBS at 4°C for weeks.

4.13.3 Methanol fixation

The same protocol as described above is used, but instead of PFA the cells are fixated with ice-cold MeOH for 90 seconds (cells can be incubated with MeOH for up to 5 minutes).

4.13.4 Antibody staining, mounting and detection

After fixation, the cells slides are blocked with 2% bovine serum albumine (BSA)/1x PBS for 90 minutes at 4°C (alternatively, 1% goat serum/1x PBS or 0.1% gelatin/1x PBS can be used). After washing with 1x PBS the primary antibody is added in the appropriate dilution (in the same solution that was used for blocking) and the slides are incubated at 37°C for 45-60 minutes. The cells are washed thrice with 1x PBS and once with 1x PBS-T. The secondary (detection) antibody is added in the appropriate dilution (in the same solution that was used for blocking) and the slides are again incubated at 37°C for 45-60 minutes. The cells are washed thrice with 1x PBS and once with 1x PBS-T. For quenching, the cells are incubated with 0.1M glycine/1x PBS for 5 minutes at room temperature. After washing with 1x PBS the slides are incubated with DAPI/1x PBS (1:5000 - 1:10000) for 10 minutes at room temperature for nuclear staining. The cells are washed with 1x PBS, gently rinsed with ddH₂O and dried. Completely dry cover slips are mounted on glass slides with 55°C Mowiol and let dry overnight in the dark (the edges of the cover slips can be sealed with non fluorescent nail polish). IF-slides need to be stored at 4°C with protection from light. All fluorescent stainings were visualized on a Zeiss LSM-Meta 510 confocal microscope.

Mowiol

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

4.14 Analysis of smooth muscle contraction

CVPCs and mESCs are aggregated and basal SMC contraction rate is monitored over 10 minutes at day 21 and 22. 1 μ M Angiotensin-2 is added to the medium and contraction is again monitored for 10 minutes. After inhibition with 1 μ M Losartan a third measurement is done for 10 minutes. To wash out both Angiotensin-2 and Losartan, the cells are carefully washed twice with 1xPBS and new medium is added. After incubation of 30 min at 37°C, 5% CO₂ the basal contraction rate is monitored again for 10 minutes. A second Angiotensin-2 induction is performed and the contraction rate is monitored for 10 minutes again.

4.15 Plasmid preparation

Plasmid preparation was done with the Endofree Plasmid Purification Maxi Kit from QIAGEN. A Single colony from a freshly streaked selective plate is picked and a starter culture of 2-5 ml LB medium containing the appropriate selective antibiotic is inoculated. The starter culture is incubated overnight at 37°C with 300 rpm shaking. The starter culture is diluted 1/500 or 1/1000 into selective LB medium and grown on a shaker at 37°C with 300 rpm for 12-16 h.

The bacterial cells are harvested by centrifugation at 6000 x g for 15 minutes at 4°C. and the pellet is resuspended in 10 ml Buffer P1 for lysis. 10 ml Buffer P2 are added, the suspension is mixed thoroughly by vigorously inverting the tube 4-6 times and incubated at room temperature for 5 minutes. During the incubation the QIAfilter cartridge is prepared. 10 ml chilled Buffer P3 are added to the lysate which needs to be mixed immediately and thoroughly by vigorously inverting for 4-6 times. The lysate is poured into the barrel of the QIAfilter cartridge and incubated at room temperature for 10 minutes. The cap from the QIAfilter cartridge outlet nozzle is removed and the plunger is gently inserted. The cell lysate is filtered into a 50 ml Falcon tube without applying excessive force. 2.5 ml Buffer ER are added to the filtered lysate which is mixed by inverting the tube approx. 10 times and incubated on ice for 30 minutes. In the meantime, a QIAGEN-tip 500 is equilibrated by applying 10 ml Buffer QBT and allowing the column to empty by gravity flow. For DNA binding the filtered lysate is applied to the QIAGEN-tip 500 and allowed to enter the resin by gravity flow. The QIAGEN-tip 500 is washed twice with 30 ml Buffer QC and the DNA is eluted with 15 ml Buffer QN. The DNA is precipitated by adding 10.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA. The solution is mixed, centrifuged immediately at 15000 x g for 30 minutes at 4°C and the supernatant is discarded carefully. The DNA pellet is washed with 5 ml endotoxin-free room-temperature 70% ethanol and centrifuged at 15000 x g for 10 minutes at 4°C. The pellet is air-dried for 5-10 minutes and the DNA is re-dissolved in a suitable volume of endotoxin-free Buffer TE.

5. Results

5.1 The characterization of putative CVPCs

5.1.1 Validation of the isolation strategy by genotyping

We sought to create a fast and simple *in vitro* strategy which allows clonal isolation and persistent maintenance of CVPC lines from neonatal mouse hearts. The basic idea was to create an artificial stem cell niche mimicking the *in vivo* state as close as possible to foster CVPC survival on one side and to provide LIF in culture to promote CVPC self-renewal on the other side. Therefore, we came up with a three-component-niche-system consisting of Neo resistant SNL76/7 fibroblasts as LIF secreting feeder cells and equal amounts of a cardiac cell population and mESCs (Fig.14).

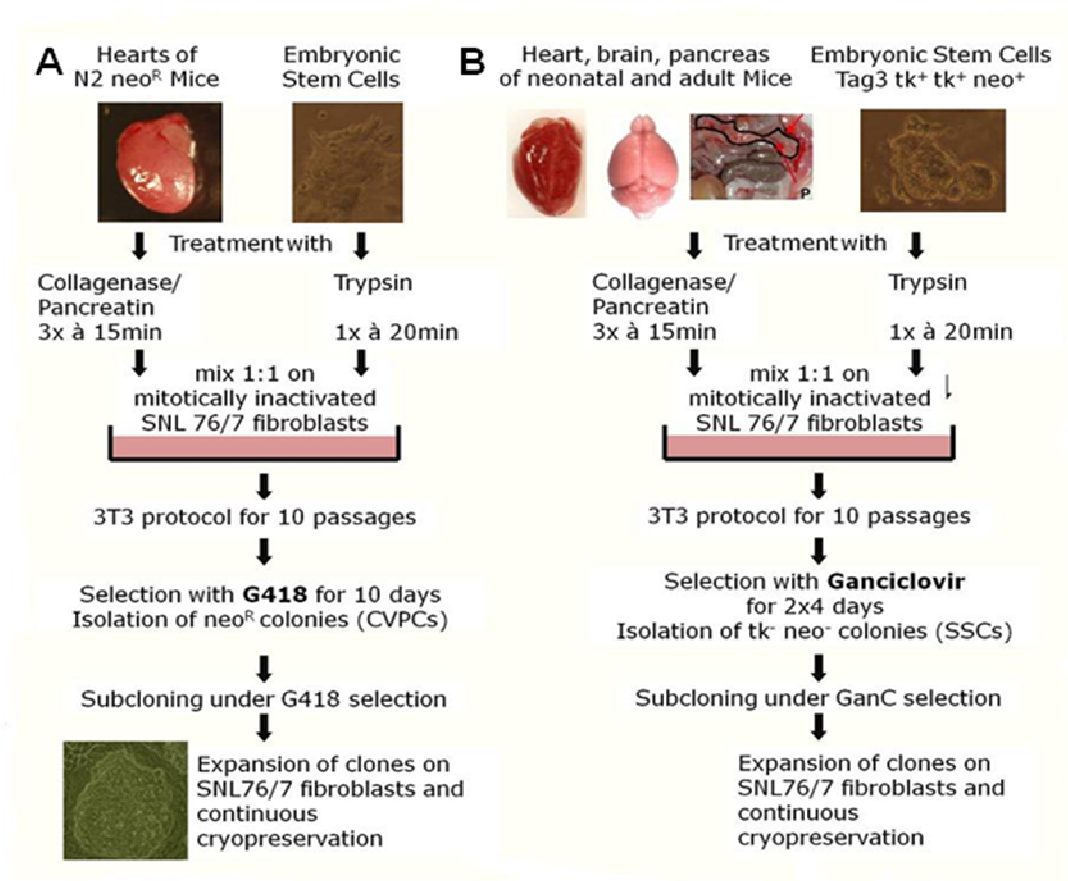


Fig.14: The CVPC isolation strategy. (A) Hearts of 2 day old out-bred C57BL/6Jx129Sv mice with a Neo resistance cassette inserted into one allele of the Hdac1 locus (Lagger et al., 2002) were digested, 5×10^5 heart cells were mixed with an equal amount of wild-type Ab2.2 mESCs (Soriano et al., 1991) and cultivated on LIF secreting SNL76/7 fibroblast feeder layers (McMahon and Bradley, 1990; Soriano et al., 1991). After selection for rapidly cycling stem cells using a modified 3T3-protocol Neo resistant CVPCs were enriched by G418 selection, sub-cloned and expanded. (B) Hearts of 2, 22, and 180 day old wild-type Balb/C mice were used. Balb/C heart cells were mixed with the

transgenic mESC line Tag3 containing 2 TK suicide genes from human HSV-1 and a Neo resistance cassette (Hollrigel et al., 2001). After the modified 3T3 protocol co-culture mESCs were removed by Ganciclovir selection and wild-type CVCPs were sub-cloned and expanded.

The idea of co-cultivating CVPCs with mESCs was based on the notion that mESCs give signals to physically neighboring cells which promote cell survival and self-renewal. We reasoned that, under these culture conditions, it would be possible to enrich a critical mass of putative CVPCs using a modified 3T3 protocol (to select rapidly cycling stem cells) over 10 passages before co-cultivated mESCs were removed from the culture system by drug resistance or suicide gene selection, followed by sub-cloning of putative CVPCs under continuous selection. Somatic heart cells should disappear over a few passages due to low doubling times compared to CVPCs/mESCs and once a critical number of CVPCs is enriched, co-culture with mESCs would no longer be needed due to autonomous CVPC proliferation. Overall, this isolation procedure yielded 11 independent clonal Neo resistant CVPC lines (A3, A5, B3, B5, C3, D3, D5, E3, F3, G3 and H3) and 7 clonal wild-type CVPC lines (4x He2, 2x He22, HeA).

To show that the obtained CVPC lines were in fact isolated from the heart and not results of cell fusion during co-culture with mESCs or reprogramming of feeder cells, we performed genotyping PCRs targeting the respective genetic loci for each cell line in the isolation culture system (Fig.15A and B).

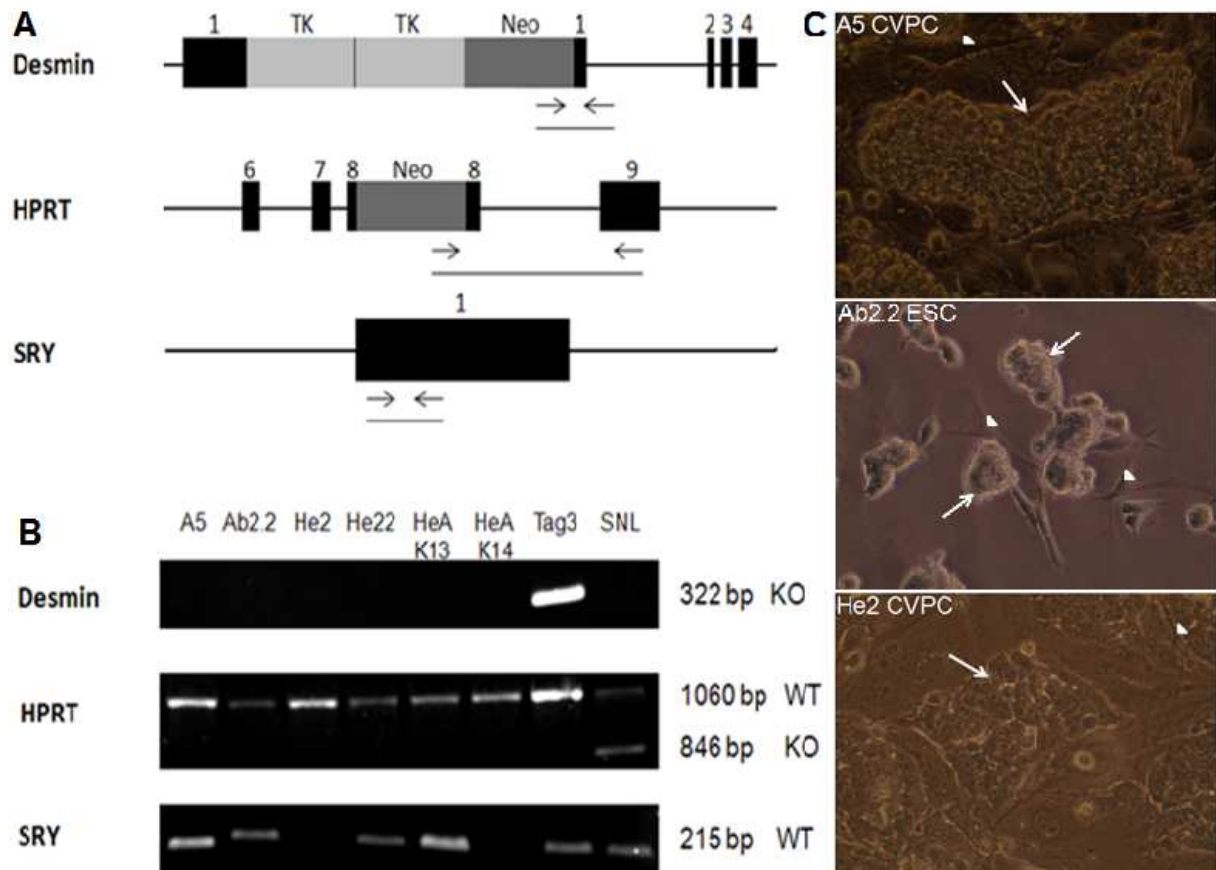


Fig.15: Genotyping targets and PCR results. (A) Targeted genetic loci of Tag3 mESCs (TKTKNeo-cassette in exon 1 of the desmin gene), SNL76/7 fibroblasts (Neo in exon 8 of the HPRT gene) and SRY gene for sex determination. Arrows indicate primer pairs, lines indicate PCR products. (B) Respective PCR results for each targeted locus. Confirmation of desmin-KO in Tag3 mESCs and heterozygous HPRT-KO in SNL76/7 shows that wild-type CVPCs (He2, He22 and two clones of HeA line) are of cardiac origin. This is additionally confirmed by the result that two CVPC lines (He2 and HeAK14) were obtained from female hearts while all other cell lines in the culture system were of male origin. (C) CVPCs (A5, He2) and mESCs (Ab2.2) differ in their morphology. CVPCs appear to have a more globular shape and form looser cell-to-cell contacts in colonies compared to mESCs. Arrows indicate stem cell colonies, arrowheads indicate feeder cells (Olympus CK2 microscope, phase contrast illumination).

Concerning the negative selection method (Fig.14B), we could prove the cardiac origin of CVPC lines isolated from hearts of 2 day old (He2), 22 day old (He22) and 180 day old (HeA clones 13 and 14) wild-type mice. During the isolation procedure these cells were co-cultivated with Tag3 mESCs, which have a TKTKNeo-cassette integrated into exon 1 of the desmin gene (Hollrigl et al., 2001), and SNL76/7 fibroblasts, which have a Neo resistance cassette disrupting exon 8 of one allele of the HPRT (hypoxanthine-guanine phosphoribosyltransferase) gene (heterozygous). Genotyping PCRs with KO-specific primers revealed that He2, He22 and HeAK13/HeAK14 CVPC lines (wild-type desmin and HPRT loci) were neither derived from Tag3 mESCs (desmin-KO) nor from SNL76/7 fibroblasts (HPRT-KO). Hearts from male and female mice had been used for CVPC isolation, therefore SRY (sex-determining region Y) genotyping was used as an additional way to distinguish

CVPC from co-culture cell lines. Thus, we also demonstrated that two CVPC lines (He2 and HeAK14) were isolated from hearts of female mice (no presence of SRY), while Tag3 mESCs and SLN76/7 were derived from male animals.

Likewise, for the positive selection method (Fig.14A) cardiac derivation of CVPC line A5 was demonstrated (data not shown). This transgenic CVPC line was isolated by positive selection from hearts of two day old out-bred C57BL/6Jx129Sv mice carrying a Neo resistance cassette inserted into one allele of the Hdac1 locus (exon 5-7) (Lagger et al., 2002). PCR with KO-specific primers proved the presence of the Hdac1-Neo transgene exclusively in CVPC line A5, but not in co-culture lines Ab2.2 (wild-type mESCs) and SNL76/7 fibroblasts (data not shown). Also, absence of the mutant HPRT locus in A5 CVPCs proved that they were neither derived from SNL76/7 fibroblasts, nor a product of fusion of somatic heart cells with SNL76/7 fibroblasts (Fig.15B).

In addition, undifferentiated CVPCs differ from mESCs in their morphology, as they have a more globular shape and do not form as tightly aggregated colonies in culture. CVPCs isolated by either positive (A5) or negative selection (He2) show similar morphology in contrast to mESCs (Ab2.2) (Fig.15C).

5.1.2 CVPCs display a stable karyotype and do not arise from cell fusion

Even though genotyping results strongly indicated that isolated CVPC lines were neither derived from other cell types in the culture system nor from cell fusion with feeder cells or ESCs, we performed karyotyping analyses to confirm that isolated CVPC lines were diploid. After pre-treating the cells with colchicine to inhibit mitosis the chromosomes were stained and the percentage of diploid, aneuploid and tetraploid cells was determined at different passages (Fig.16).

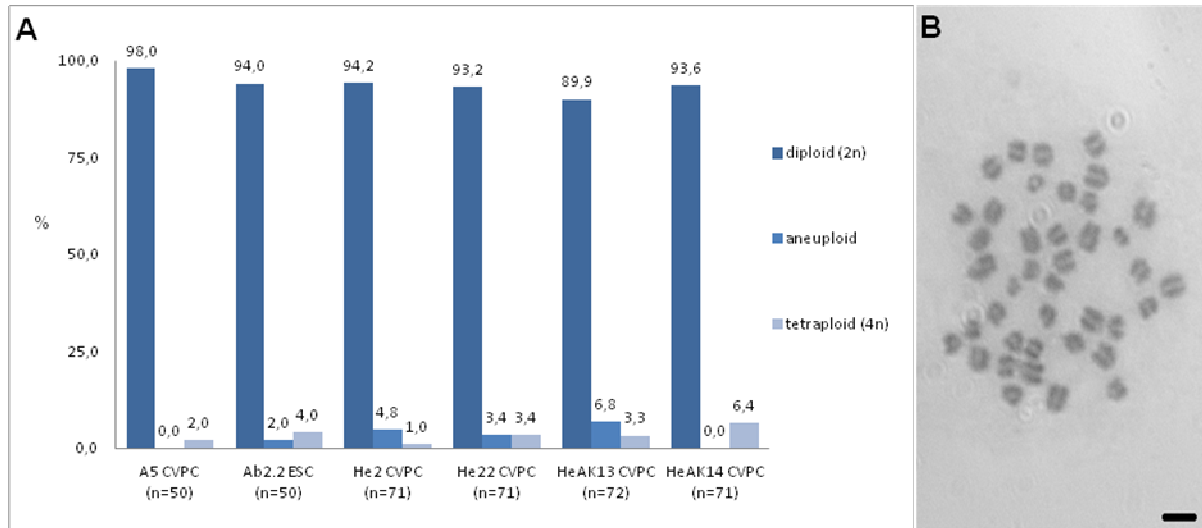


Fig.16: CVPCs display a stable diploid karyotype. **(A)** CVPC lines A5, He2, He22, HeAK13, HeAK14 and mESC line Ab2.2 were subjected to karyotyping analysis at different passages. All cell lines were dominantly diploid with only few aneuploid or tetraploid cells, even after long-term cultivation (A5 CVPC, p124). Percentages are shown above according bars. **(B)** A bright field microscopy picture of a chromosome spread of CVPC clone A5 with 40 discernible chromosomes. Bar = 10 μ M.

Every examined CVPC line displayed a stable diploid phenotype, with levels comparable to those of the mESC positive control. Even after long-term culture for over 100 passages CVPC clone A5 demonstrated diploidy to an extent greater than 90%. Additionally, the few aneuploid or tetraploid cells may also arise from cell culture conditions.

From these results we reasoned that the isolated CVPC lines were "naturally" diploid, even though diploidy theoretically could also be a result of reductive mitosis of tetraploid cells. However, this seems rather unlikely for several reasons: all isolated CVPC lines underwent a 3T3 protocol selecting fast replicating cells. Reductive mitosis requires much more time and therefore only a few pseudo-diploid cells can arise under these culture conditions and would be rapidly diluted out of the culture. Secondly, reductive mitosis is a rather ineffective process, therefore the majority of fusion cells would stay tetraploid, which can neither be stated from the karyotyping results nor from the phenotypic appearance of undifferentiated CVPCs (Fig.15C). Most importantly, it was shown that reductive mitosis can only proceed in the absence of the tumor suppressor gene p53, a crucial cell cycle regulator (Vitale et al., 2010), which is readily expressed in all examined undifferentiated CVPC lines (Fig.17).

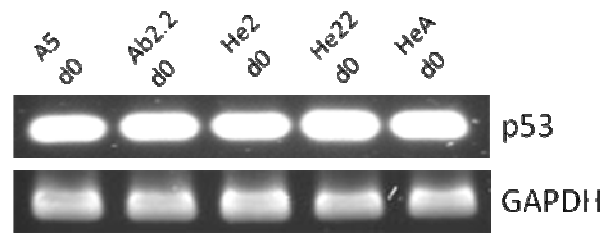


Fig.17: CVPCs express p53 in an undifferentiated state. Reverse transcription PCR (RT-PCR) with cDNA of undifferentiated (d0) CVPC lines demonstrates expression of p53 (GAPDH was used to balance cDNA concentration for RT-PCR). Expression of p53 rules out the possibility of CVPC lines arising from tetraploid fusion cells or pseudo-diploid cells after reductive mitosis.

5.1.3 CVPCs spontaneously differentiate into the three main cardiac cell types upon LIF deprivation

ESCs have the potential to give rise to all somatic cell lineages. In EBs, mESCs differentiate into cells of all three germ layers (Desbaillets et al., 2000; Itskovitz-Eldor et al., 2000), including extra-embryonic endoderm, which has been shown to support CMG (Bader et al., 2001). To test whether differentiating CVPCs are restricted to the cardiac lineage we performed *in vitro* differentiation assays using the EB hanging drop model system. Like mESCs in EB formation, CVPCs were aggregated to cardiac bodies (CBs) after removal of LIF by adsorption of feeder cells. 900 cells per hanging drop were cultivated for 4.7 days before transfer onto a gelatin-coated cell culture dish. After EBs/CBs had attached to the surface, differentiation was monitored daily over 25-30 days by examining the development of CMCs, SMCs and ETCs, the three main cell types of the heart (Fig.18).

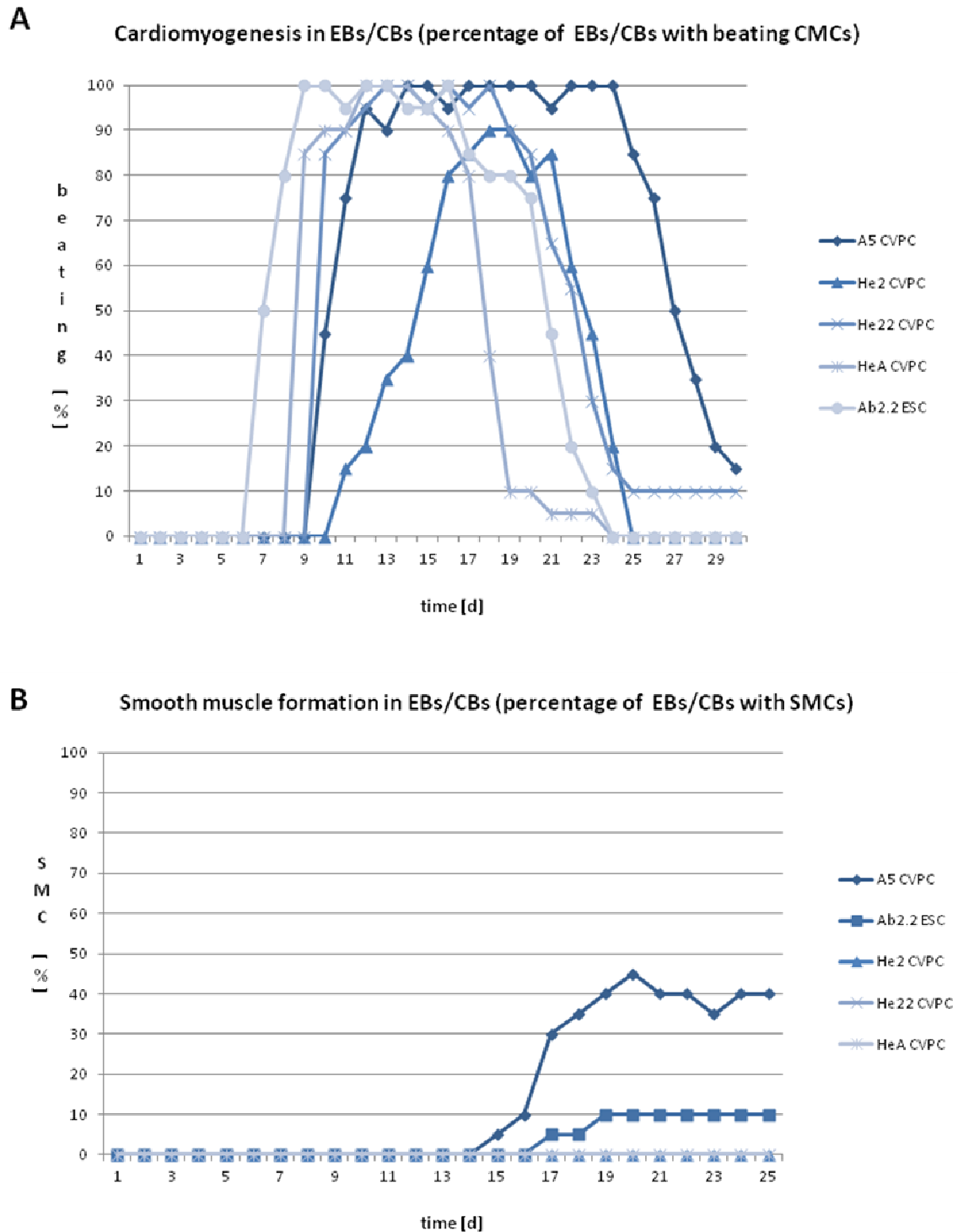


Fig.18: *In vitro* differentiation of CVPCs and mESCs using the EB/CB hanging drop model. Cells were aggregated to EBs/CBs and differentiation was monitored daily over 25-30 days. **(A)** Percentages of EBs/CBs with contracting CMCs (n=20) over 30 days are shown. EBs display beating CMCs earlier than CBs, but, for most CVPC lines, CMC contraction is prolonged compared to EBs. Every CVPC line reached a maximum percentage of CBs with beating CMCs of at least 90%. **(B)** A5 CVPC derived CBs display significantly more SMCs than Ab2.2 mESC derived EBs, indicating their probable restriction to the cardiomyogenic lineage (figure represents data from two independent experiments performed in duplicates).

First visible spontaneously contracting CMCs in differentiating EBs (Ab2.2 mESCs; Fig.19A) could be observed at day 6.5 ± 0.5 . At first only a small fraction of developing CMCs shows rhythmic contraction, at later stages (after 10-12 days) several CMC clusters in one single EB are discernible, visible as large contracting areas (Fig.19B). CMG in EBs proceeded very fast - at day 9, 100% of EBs (n=20, duplicates) displayed beating CMCs - and lasted at least until day 23 (Fig.18A). First spontaneously contracting, parallel aligned SMCs could be observed around d17 (Fig.18B) and were still present to an extent of 10% at the end of cultivation (day 25).

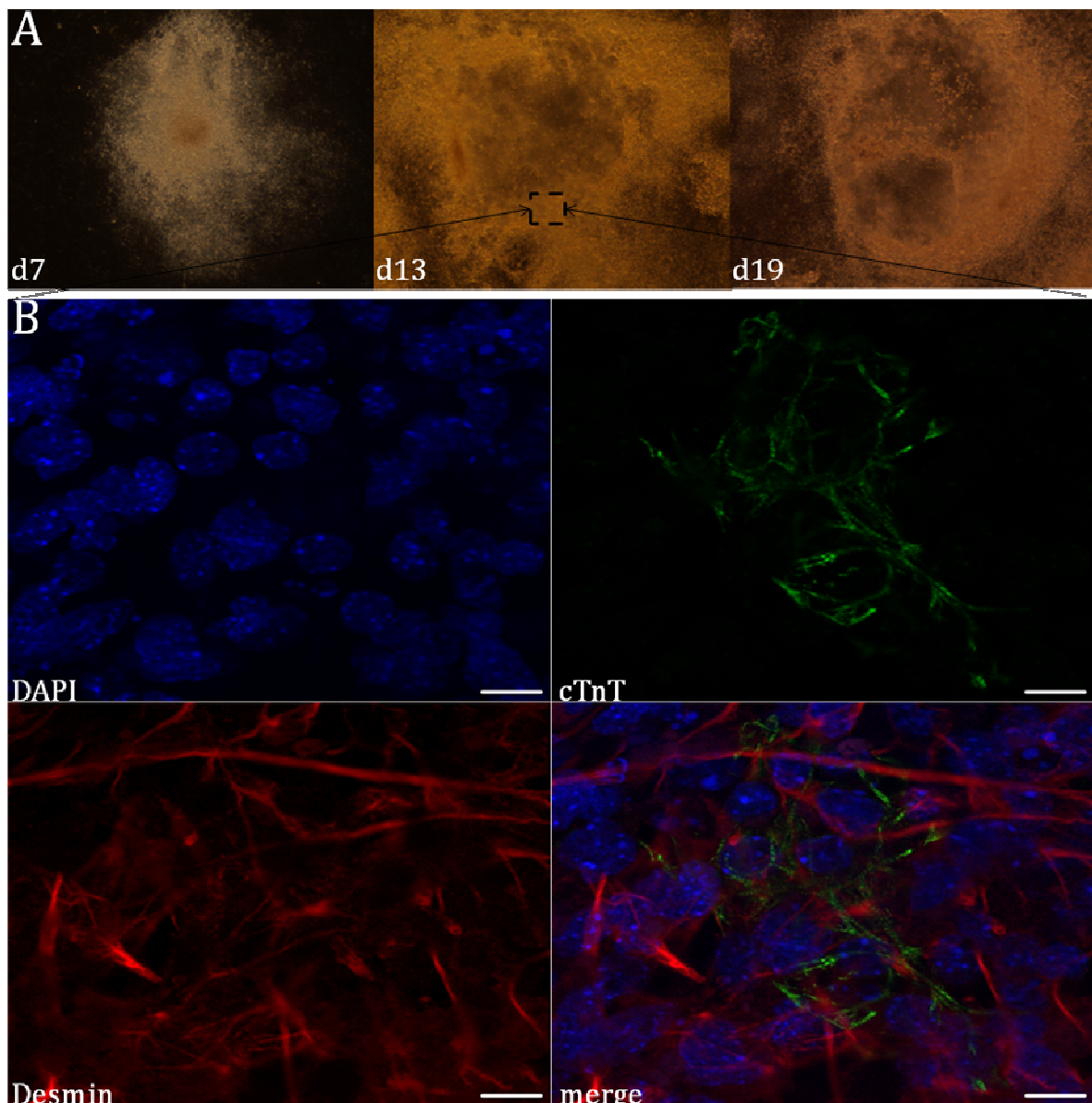


Fig.19: Development of rhythmically contracting CMC patches during EB differentiation. **(A)** Phenotypic appearance of a differentiating EB (Ab2.2 mESC) over time. First spontaneously contracting CMCs develop by day 7, later (between day 11 and 13) large clusters of rhythmically contracting CMCs ("CMC patches") become visible. **(B)** Immunofluorescence staining of a CMC patch in differentiating EBs (day 13) performed with antibodies against the cardiogenic markers cTnT and desmin (Zeiss LSM 510 Meta). Bars = 10 μ m.

In contrast to EBs, development of CMCs derived from CBs required more time, as first spontaneously contracting CMCs were observed at day 8.5 ± 0.5 (He22, HeA) and around day 10 (A5, He2 CVPCs), respectively (Fig.18A). Nevertheless, except for He22 CVPCs, all CVPC lines displayed beating CMC clusters for a longer period than mESCs. 15% of CBs derived from CVPC line A5 showed beating CMCs at day 30, while beating CMCs in EBs derived from mESC line Ab2.2 were present for no longer than day 23. Also, the amount of SMCs in differentiating CBs (A5 CVPC) exceeded SMC formation in EBs (Ab2.2 mESC) by up to 35% (Fig.18B). CBs derived from wild-type CVPC lines He2, He22 and HeA rarely displayed spontaneously contracting SMCs. Smooth muscle-like structures could be observed, nevertheless, in most cases SMC contraction did not occur or was not visible under the light microscope (Fig.20). Also, ETC clusters were visible from day 7 on and, as differentiation was ongoing, large endothelial sheets evolved. Occasionally, vessel-like structures were observed, in rare cases surrounded by parallel SMC bundles.

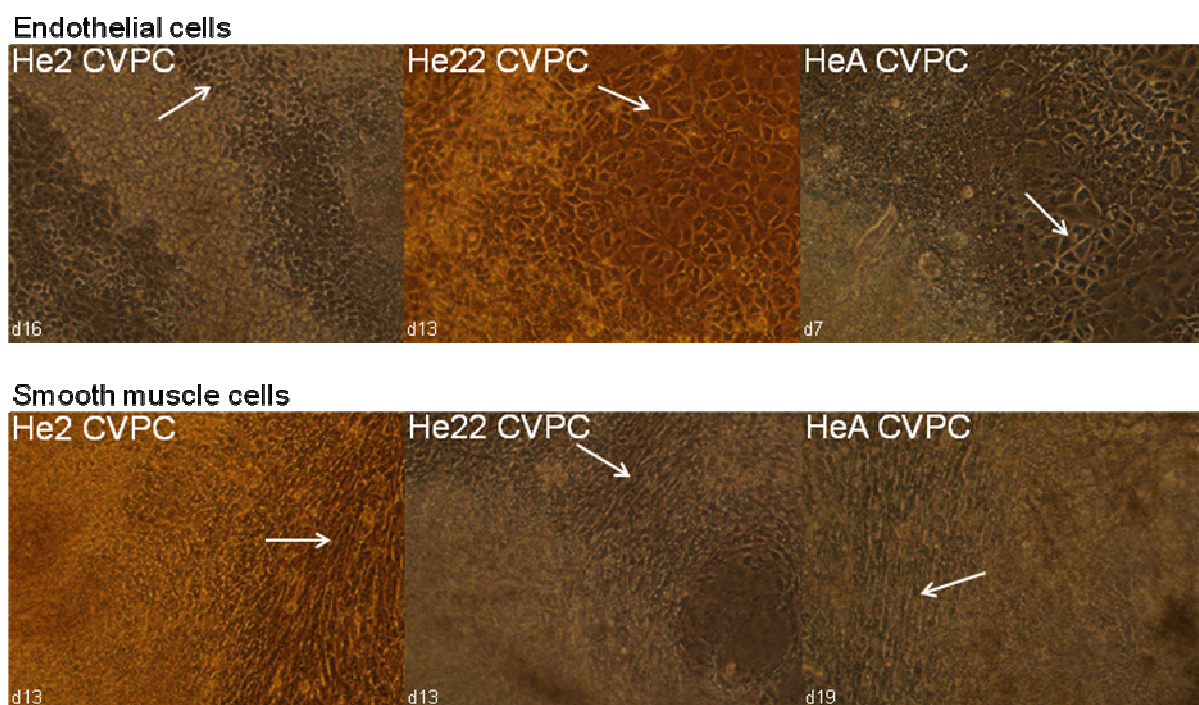
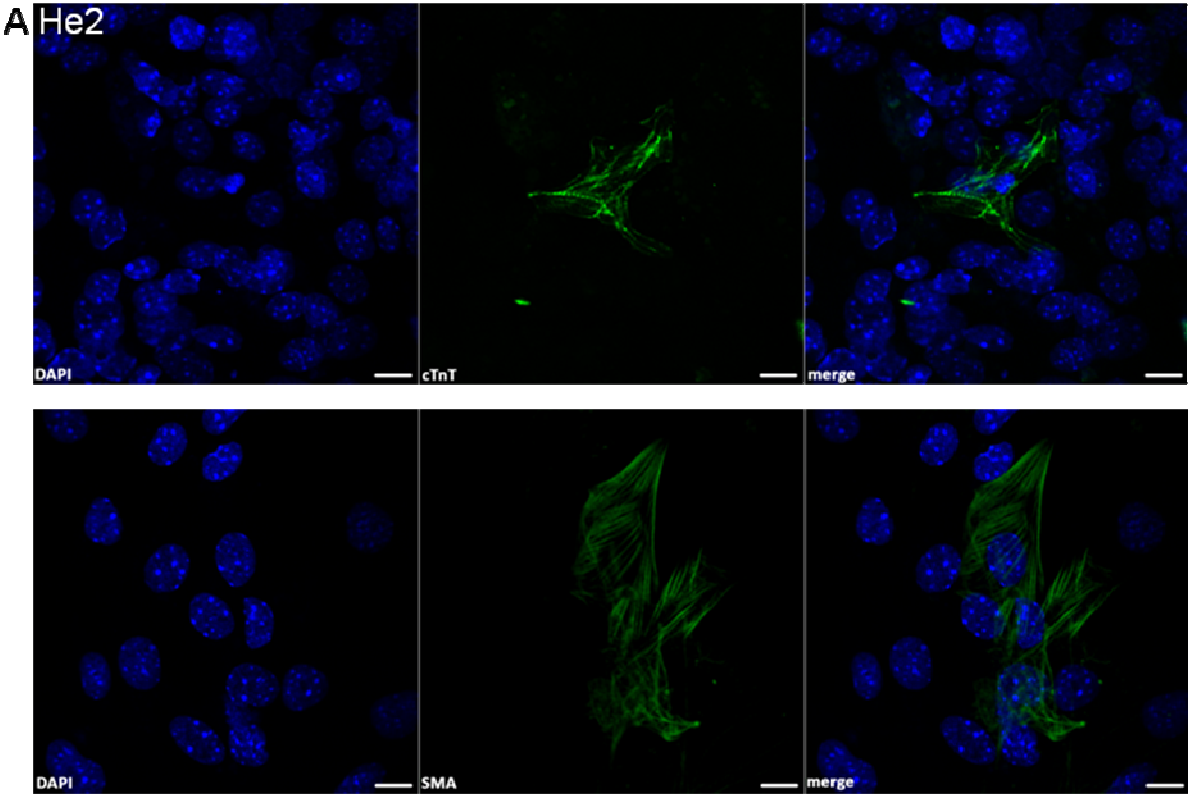


Fig.20: Wild-type CVPCs differentiate into ETCs and SMCs in a CB *in vitro* differentiation system. Wild-type CVPC lines He2, He22 and HeA displayed ETC clusters from day 7 on. At later stages, larger areas of endothelial sheets were observed. By day 13, smooth muscle-like parallel bundles of premature SMCs which rarely show spontaneous contraction are visible (Olympus CK2 microscope, phase contrast illumination).

Since spontaneous contraction of smooth muscle fibers was hardly ever observed under the light microscope during *in vitro* differentiation of CBs derived from wild -type CVPC lines

He2, He22 and HeA, we performed immunofluorescence microscopy to check for the presence of CMC marker cTnT and smooth muscle marker SMA- α . CVPCs were differentiated in CBs for 20 days, dissociated and stained (Fig.21). Like A5 CVPCs and Ab2.2 mESCs (Fig.18, Fig.19), all three wild-type CVPC lines were shown to have the potential to give rise to CMCs and SMCs in CB differentiation assays.



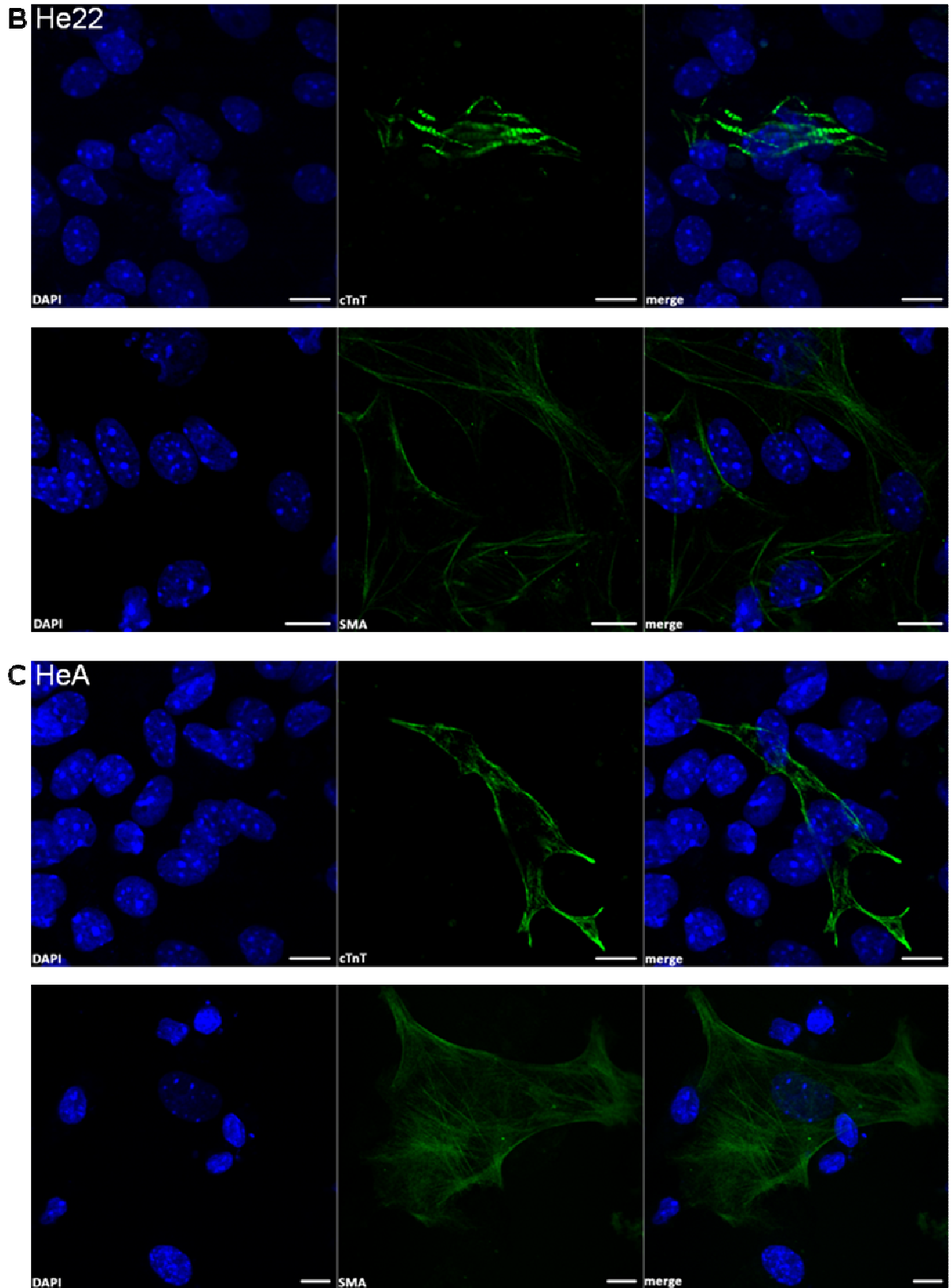


Fig.21: Wild-type CVPC lines He2, He22 and HeA give rise to CMCs and SMCs. After differentiation in CBs for 20 days, He2 (A), He22 (B) and HeA (C) CVPCs were dissociated, fixed and stained with antibodies against cTnT and SMA- α for immunofluorescence microscopy. Nuclei were visualized by DAPI staining (Zeiss LSM 510 Meta). Bars = 10 μ m.

5.1.4 CVPCs lack the ability of self-organization when grown in aggregates (CBs) in contrast to ESCs

Similar to the phenotypic differences we observed between undifferentiated CVPCs and mESCs in culture (Fig.15C), EBs and CBs differed in their morphology as well. Attachment of EBs (Fig22, AB2.2 mESC d7) to the cell culture plate surface after 4.7 days breaks the radial symmetry observed during EB formation in suspension. A horseshoe-shaped ring of cells surrounding the center of the EB develops and first mesodermal cells evolve between this ring and the center (Fig22, AB2.2 mESC d13). This gastrulation-like process leads to establishment of a bilateral symmetry (Fuchs et al., 2012).

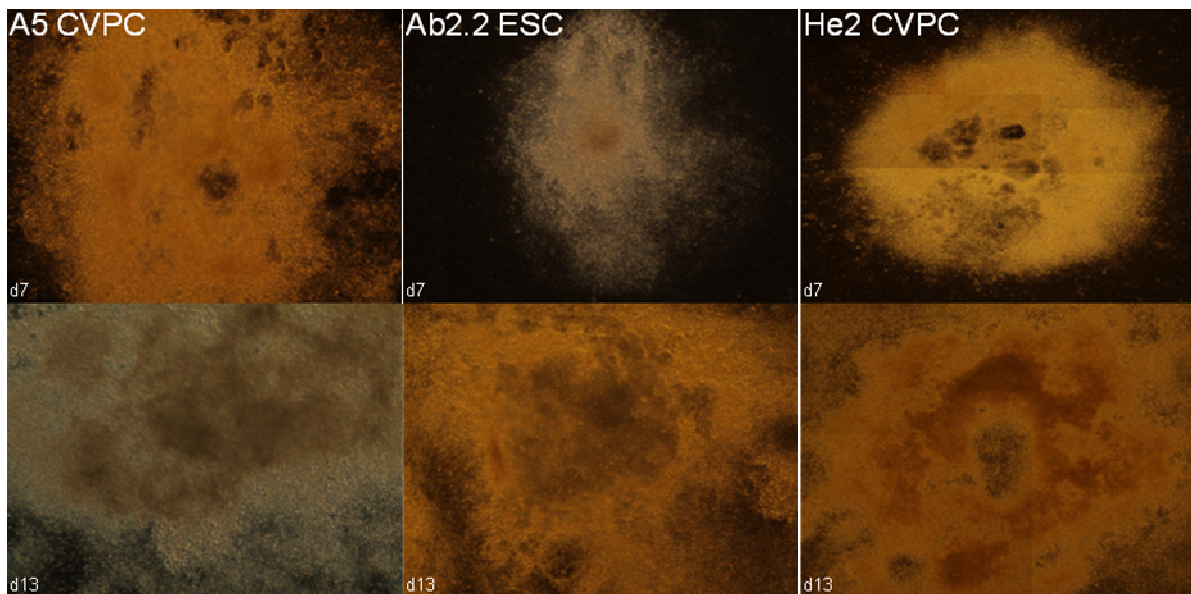


Fig.22: EBs and CBs differ in their morphology. CB (A5, He2) and EB (AB2.2) morphology was monitored over 25-30 days of differentiation. Radial symmetry in EBs diminishes after attachment and bilateral symmetry is established after a gastrulation-like process which leads to mesoderm ingression. As a consequence, CMC development is limited to this mesodermal area surrounding the center of the EB. CBs do not show these organizational features and CMCs evolve in a chaotic manner, encompassing much larger areas of the CB compared to EBs (Olympus CK2 microscope, phase contrast illumination).

In CBs (Fig.22, A5 CVPC and He2 CVPC), none of these processes or structures was observed. CB morphology lacked any symmetry and first beating CMCs developed unpredictably and randomly at several areas. In addition, CBs usually displayed more CMC clusters than EBs at later stages of differentiation and the size of beating CMC patches was bigger than the size observed in EBs. Generally, CB size significantly exceeded EB size, but no structural self-organization was observed whatsoever. This is in accordance with previously published data (Fuchs et al., 2012) demonstrating that CBs lack the potential of

self-organization *in vitro* compared to EBs, which may be due to the loss of this unique ESC-feature as a consequence of CVPCs being already committed to the cardiac lineage.

5.1.5 CVPCs express stemness and early mesodermal markers in an undifferentiated state

To verify that CVPCs are capable of self-renewal like mESCs we analysed the expression of stemness transcription factor genes. Total mRNA of undifferentiated (day 0) CVPC clones (A5, He2, He2 and HeA) and mESCs (AB2.2) was isolated and transcription of SOX2 and NANOG was monitored by RT-PCR (Fig.23). Undifferentiated CVPCs, like ESCs, express stemness markers, indicating their capability of self-renewal. During the early phase (day 0 - day 13) of *in vitro* differentiation in CBs/EBs, SOX2 and NANOG expression was continuously down-regulated while expression of early mesodermal lineage-specific markers was already detectable (data not shown).

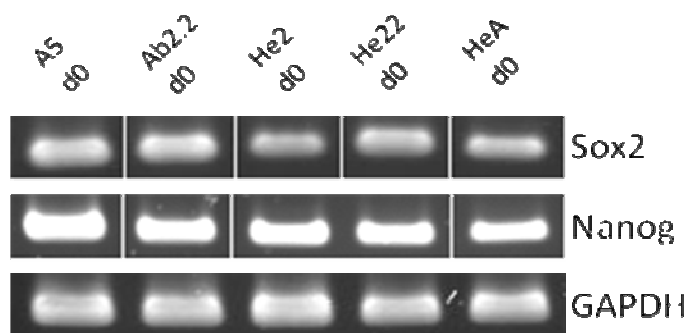


Fig.23: Expression of stemness factors enables autonomous CVPC self-renewal. Like mESC line AB2.2, all tested CVPC clones express stemness transcription factors SOX2 and NANOG at slightly varying levels in an undifferentiated state. GAPDH was used to balance cDNA amounts used for RT-PCR.

Cardiac mesoderm is specified by expression of Brachyury (David et al., 2011) and Eomesodermin, which is essential for epithelial to mesenchymal transition (EMT) and directly activates MESP1 (Costello et al., 2011; Saga et al., 2000). Together, Brachyury and MESP1 govern cardiac specification as upstream regulators of core cardiac transcription factors like NKX2.5, GATA4, ISL1 or MEF2C.

We reasoned that, if they were indeed committed to the cardiac lineage, undifferentiated CVPCs might already express early mesodermal transcription factors in combination with stemness factors. Therefore, total mRNA of undifferentiated CVPC clones (A5, He2, He22 and HeA) and mESCs (AB2.2) and total mRNA of CVPCs/mESCs after differentiation in CBs/EBs for 13 days was isolated and expression of Brachyury and MESP1 was analysed with RT-PCR (Fig.24).

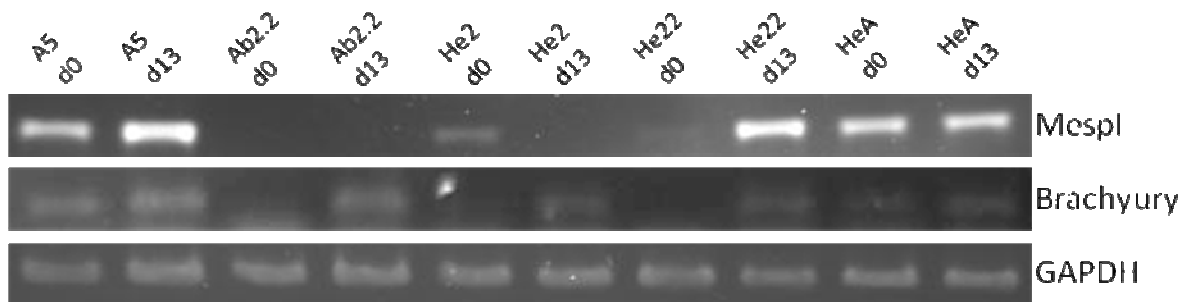


Fig.24: CVPCs express early mesodermal transcription factor genes in an undifferentiated state. CVPCs and mESCs were differentiated in aggregates for 13 days and total mRNA was isolated. Expression levels of Brachyury and MESP1 in undifferentiated (d0) and differentiated (d13) cells was analysed with RT-PCR. CVPC clones (A5, He2, He22 and HeA) express MESP1 in an undifferentiated state at varying levels. A5 and HeA CVPCs also express Brachyury in an undifferentiated state. Throughout early CB/EB differentiation, MESP1 and Brachyury are upregulated. GAPDH was used to balance cDNA amounts used for RT-PCR.

All CVPC clones express the early cardiac mesoderm marker MESP1 in an undifferentiated state at varying levels, which might be due to donor variability, as hearts from different mice at different ages with different genotypes were used for CVPC isolation (Fig.14). Upon differentiation in CBs/EBs for 13 days, MESP1 was up-regulated, resulting in primitive mesoderm specification during early development and, ultimately, development of contracting CMCs. MESP1 expression in undifferentiated mESCs (AB2.2) was never observed. Surprisingly, MESP1 expression was not detectable in differentiated EBs (AB2.2 d13) and He2 CVPC-derived CBs (He2 d13). In EBs this could be a result of completed cardiac mesoderm specification, which is based on the observation that first contracting CMCs develop on average 2-3 days earlier in EBs than in CBs (Fig.18A). Additionally, CMG in terms of visible CMC contraction and SMC formation is prolonged in most CVPC clones (Fig.18) compared to mESCs, making it possible that the time-window for cardiac mesoderm specification by MESP1 expression in mESCs is shorter than in CVPCs. This is in accordance with RT-PCR results, where MESP1 is strongly expressed in differentiating CVPCs (A5 d13, He22 d13 and HeA d13; Fig24). In differentiating CBs derived from CVPC clone He2, MESP1 expression surprisingly was not observed, which could also be due to completed cardiac mesoderm specification, but not because of earlier CMC development like in EBs - which was never observed compared to other CVPC clones - but rather because of the fact that He2 CVPCs never displayed CMC and SMC formation to the same extent as other CVPC clones (Fig.18).

Brachyury expression was up-regulated throughout differentiation in EBs/CBs for 13 days (Fig.24), with comparable expression levels observed in differentiated mESCs (AB2.2) and CVPCs (A5, He2, He22 and HeA). This indicates ongoing mesoderm specification as a

prerequisite for cardiac differentiation. In sharp contrast to undifferentiated mESCs (AB2.2), where Brachyury expression was never observed, some undifferentiated CVPC clones (A5, He2, HeA) already express Brachyury at moderate levels. This, in combination with MESP1 and stemness factor (SOX2, NANOG) expression observed in some undifferentiated CVPC clones, indicates the subtle cardiac commitment of CVPCs.

After assessing the transcriptional levels of stemness and mesodermal marker genes in CVPCs we also wanted to identify the expression of early and late stage-specific myocardial markers throughout differentiation in CBs/EBs. After 13 days of differentiation total mRNA was isolated and expression was monitored with RT-PCR (Fig.25).

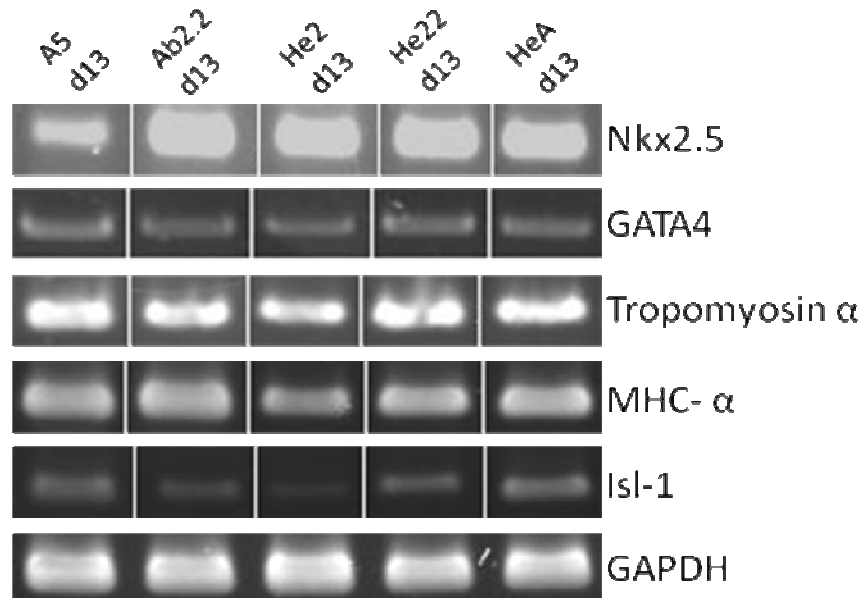


Fig.25: CVPCs express early and late stage-specific cardiac markers upon differentiation. CVPCs and mESCs were differentiated in aggregates for 13 days and total mRNA was isolated. RT-PCR demonstrates expression of both early stage-specific myocardial markers NKX2.5, GATA4 and ISL-1 and late stage-specific myocardial markers TM- α and MHC- α . GAPDH was used to balance cDNA amounts used for RT-PCR.

With the previously observed up-regulation of mesoderm-specifying markers MESP1 and Brachyury upon differentiation (Fig.24), transcriptional levels of myocardial markers were also expected to increase over time. Even though some CVPC clones (A5 CVPC) displayed myocardial marker expression in an undifferentiated state (Fig.31), high expression levels were only observed upon differentiation. Like mESCs, all CVPC lines expressed early stage-specific cardiac markers NKX2.5, GATA4 and ISL-1 upon differentiation, as well as late stage-specific myocardial markers Tropomyosin α (TM- α) and myosin heavy chain α (MHC- α). TM- α is an integral sarcomeric protein involved in calcium-dependent striated muscle

contraction by regulation of actin-myosin interactions and is essential for proper murine cardiac development (Jagatheesan et al., 2010; Rethinasamy et al., 1998). MHC- α , the major structural protein in the adult myocardium, is exclusively expressed in the heart and has been shown to be partially regulated by GATA4 (Molkentin et al., 1994).

Summarizing RT-PCR results, there is a strong indication that CVPCs appear to represent some sort of early cardiac precursor, given their ability to self-renew (Fig.23) while already expressing mesodermal marker genes in an undifferentiated state (Fig.24). None of these features were ever observed in mESCs. The possible cardiac commitment of CVPCs is additionally supported by preliminary experiments showing that undifferentiated CVPCs, not mESCs, secrete secreted protein, acidic, rich in cysteine (SPARC) which promotes *in vitro* CMG (data not shown) (Sary et al., 2005). Upon differentiation, CVPCs exclusively give rise to ETCs, CMCs and SMCs, which is consistent with expression of early and late-stage specific myocardial markers.

5.1.6 SMCs derived from CVPCs respond to Angiotensin-2

Angiotensin-2 (Ang-2), the primary effector molecule of the renin-angiotensin system (RAS), plays a pivotal role in the cardiovascular system by regulating blood vessel constriction (Zablocki and Sadoshima, 2011). Heart failure is related with RAS up-regulation and acute myocardial ischemia-reperfusion increases Ang-2 expression (Oyamada et al., 2010). Mature SMCs respond to Ang-2 with increased contraction rates. Since spontaneous SMC contraction was observed during *in vitro* differentiation of CBs and EBs (Fig.18B), SMC response to Ang-2 and subsequent inhibition with the competitive Ang-2 inhibitor Losartan seemed to be a feasible assay to analyze the functionality of CVPC-derived SMCs. Basal SMC contraction rates in CBs (A5, He2) and EBs (AB2.2) after 21 and 22 days of differentiation and the effects of Ang-2 and subsequent Losartan addition to the medium (both 1 μ M) were monitored under the light microscope (Fig.26).

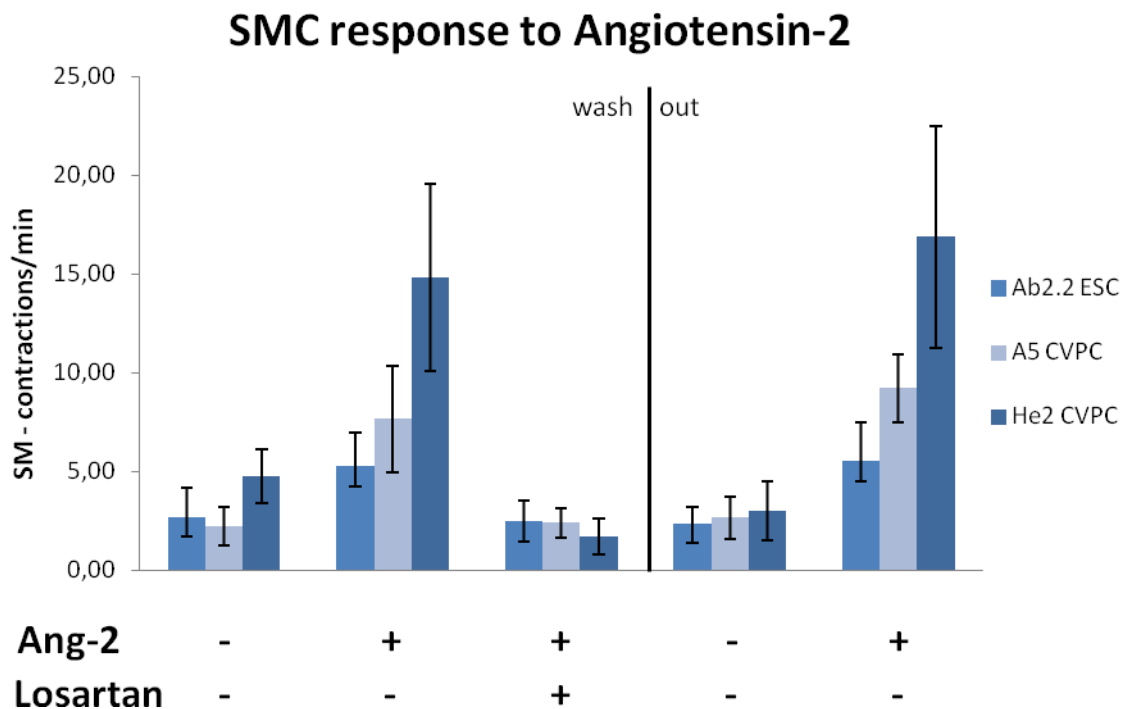


Fig.26: CVPC-derived SMCs respond to Ang-2 with enhanced contraction. Cells were aggregated to CBs/EBs and Ang-2 response was monitored after 21 and 22 days of differentiation. Addition of 1 μ M Ang-2 to the medium resulted in significant increase of the SMC contraction rate, which could be reverted by inhibition with 1 μ M Losartan. After a wash out of both substances the basal level was reconstituted. A second SMC stimulation with Ang-2 led to increased contraction rates to a similar extent compared to the first one, showing that the method was reproducible.

Both CVPC- and mESC-derived SMC displayed basal contraction rates of about 5 contractions/minute. Addition of Ang-2 to the medium resulted in an immediate 2 to 3-fold increase of the contraction rate, which was reversible by addition of the competitive Ang-2 inhibitor Losartan. After a wash-out of both substances and incubation for 30 minutes the basal SMC activity was reestablished. A second induction with Ang-2 led to enhanced contraction rates similar to the first treatment. CVPC-derived SMCs responded to Ang-2 much stronger than mESC-derived SMCs, suggesting that CVPC-derived SMCs are in a more advanced developmental stage.

5.2 The role of MK142 in cardiomyogenesis

In recent years, numerous screens for small molecules that promote cardiac lineage specification and differentiation have been performed by various research groups in order to find a starting point for drugs that promote myocardial repair (Russell et al., 2012; Sadek et al., 2008; Willems et al., 2011; Wu et al., 2004). The common goal is to gain further insights

into mechanisms of stem cell fate decisions, lineage priming and transdifferentiation to enrich enough cardiac cell types for future therapeutic application after MI.

5.2.1 MK142 enhances and prolongs functional CMC formation

MK142 is a novel derivative of Cardiogenol-C (CgC), a small cell-permeable diaminopyrimidine which has been shown to induce mESCs (Wu et al., 2004) and murine hair bulge progenitor cells (mHBPCs) (Yau et al., 2011) to differentiate into CMCs. We wanted to test whether treatment with MK142 exerts the same effects observed with CgC in *in vitro* differentiation assays. We chose the CB/EB hanging drop model as method of choice and performed pilot experiments where the effects of both substances on cardiac differentiation were screened over time (day 0 to day 13) to identify treatment periods that gave the most significant results (data not shown). From preliminary data evaluation we came up with the following treatment groups: MK142 treatment from day 0 to day 4.7 (during CB/EB aggregation), CgC treatment from day 4.7 to day 7 (after CB/EB adherence) and a combination of both, with MK142 treatment from day 0 to day 4.7 followed by CgC treatment from day 4.7 to day 7. Cells treated with DMSO were used as a negative control. CVPCs (A5 and He2) and mESCs (AB2.2) were aggregated and the substances were added over the respective periods throughout differentiation. The percentage of CBs and EBs with spontaneously contracting CMCs was monitored over 25 days (Fig.27).

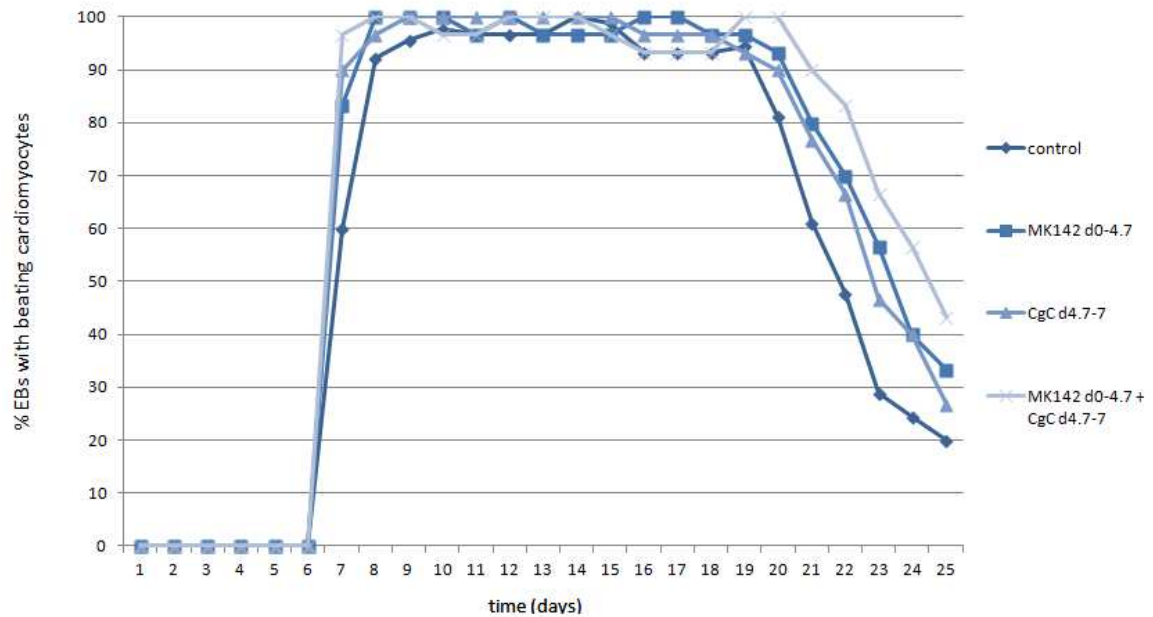
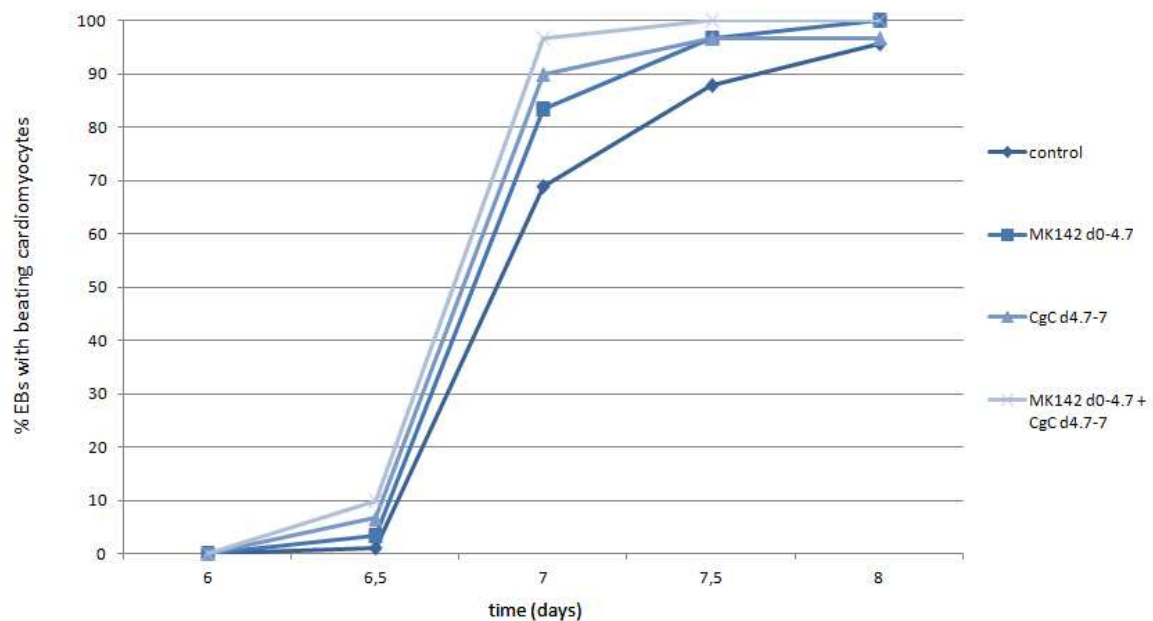
A**Ab2.2 ESC - Influence of MK142 and CgC on CMG****B****Ab2.2 ESC - Influence of MK142 and CgC on CMG (d6-8)**

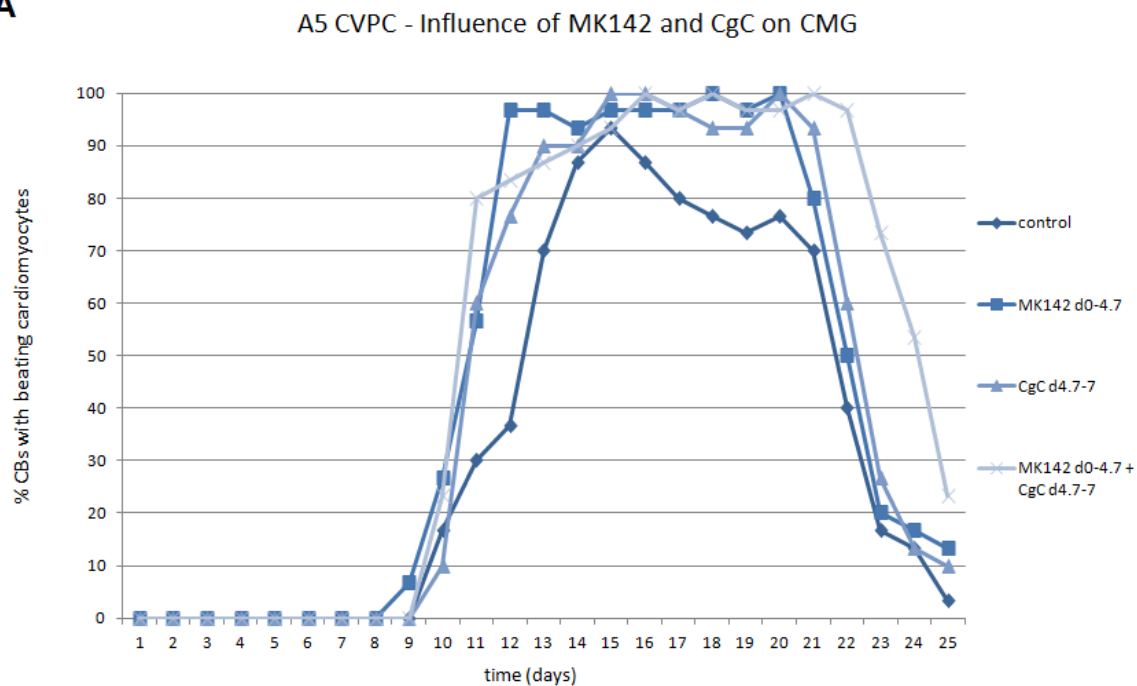
Fig.27: MK142 induces differentiation of mESCs into CMCs. **(A)** mESCs (AB2.2) were differentiated in EBs for 25 days and percentage of EBs with beating CMCs was monitored over 25 days. Like CgC, MK142 promoted CMC differentiation, albeit to a moderate extent. Beating CMCs were observed for significantly longer periods, especially after treatment of mESCs with both substances. **(B)** Treatment of mESCs with MK142 enhanced CMC formation at the onset of CMG (substances were added over indicated treatment periods).

Consistent with published data on the effects of CgC treatment, MK142 treatment led to enhanced CMC differentiation in EBs, even though the effect was rather moderate (Fig.27A). First EBs with spontaneously contracting CMCs were observed from day 6 on and ongoing

CMC formation proceeded rapidly within 2 days, with 95-100% of EBs (n=20, duplicates of each treatment group) displaying beating CMCs at day 8 (Fig.27B). More importantly, more beating CMCs were observed at the end of cultivation after 25 days in both MK142 and CgC treated EBs. This effect was even stronger when both substances were applied.

In differentiating CBs (A5, He2) the same effects were observed, but to in a much stronger manner (Fig.28). Like in EBs, MK142 and CgC induced CMC differentiation (Fig.28A). More significantly, in CBs (A5) treated with MK142 from day 0 to day 4.7 appearance of first spontaneously contracting CMCs preceded all other treatment groups by one day (Fig.28B), suggesting that MK142 and CgC function via different mechanisms. Again, a combinational treatment with MK142 and CgC yielded significantly more beating CMCs at the end of cultivation.

A



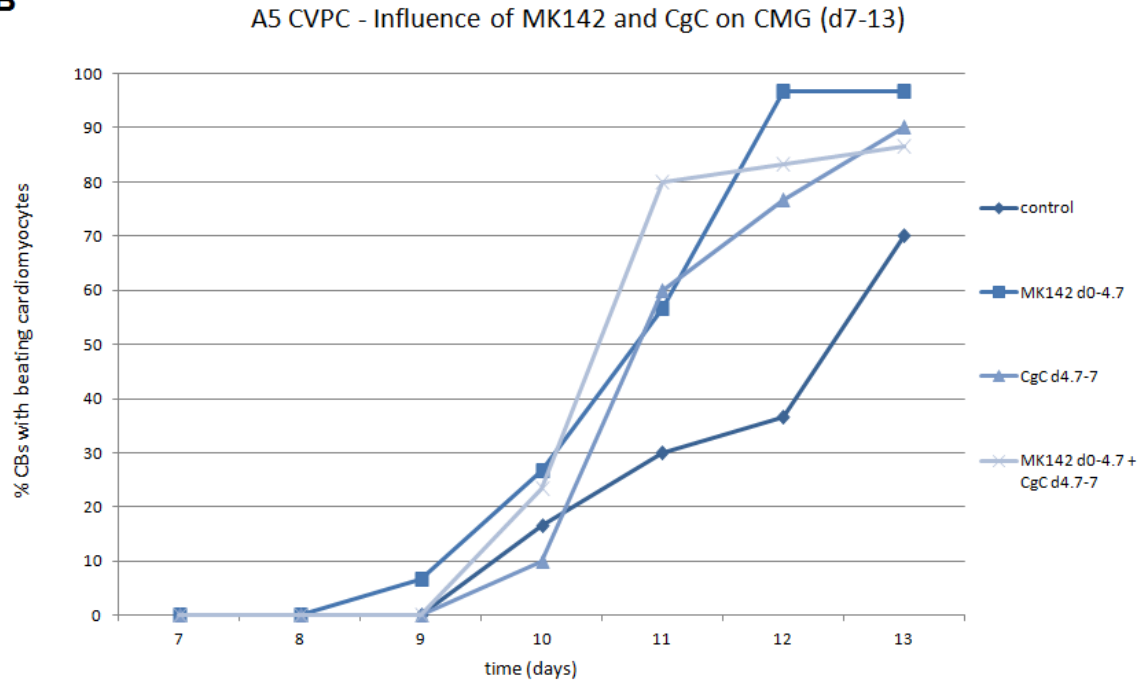
B

Fig.28: MK142 induces differentiation of CVPCs into CMCs. **(A)** CVPCs (A5) were differentiated in CBs for 25 days and percentage of CBs with beating CMCs was monitored over 25 days. Like CgC, MK142 promoted CMC differentiation, but to a much bigger extent than in mESCs. Beating CMCs were observed for significantly longer periods, especially after treatment of CVPCs with both substances. **(B)** Appearance of first spontaneously contracting CMCs in CBs treated with MK142 preceded all other treatment groups by 1 day (substances were added over indicated treatment periods).

Likewise, MK142 enhanced CMC formation in differentiating He2 CBs, but this time no inductive effect of CgC treatment was observed (Fig.29A). Treatment of CBs with Mk142 resulted in the strongest CMC induction observed in all three analyzed cell lines (AB2.2, A5, He2), approximately doubling the percentage of CBs displaying spontaneously contracting CMCs compared to the control group. Again, application of both MK142 and CgC led to prolonged appearance of beating CMCs compared to the control group. Surprisingly, this effect was exceeded by MK142 treatment alone. Also, first appearance of beating CMCs preceded all other treatment groups, including CgC treatment, by 2 days (Fig.28B).

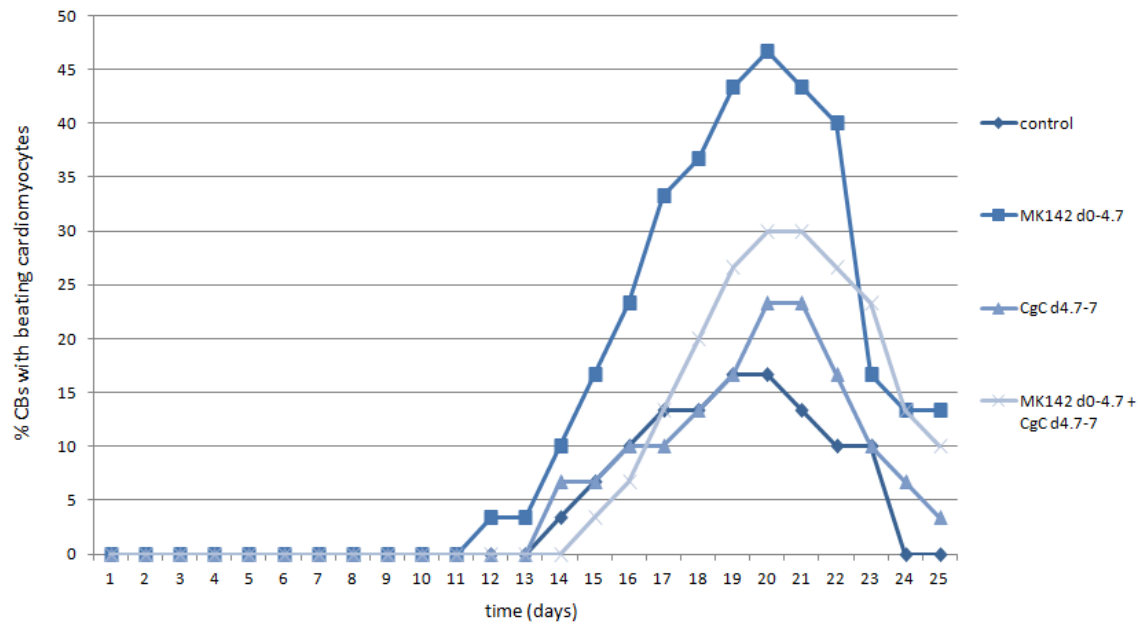
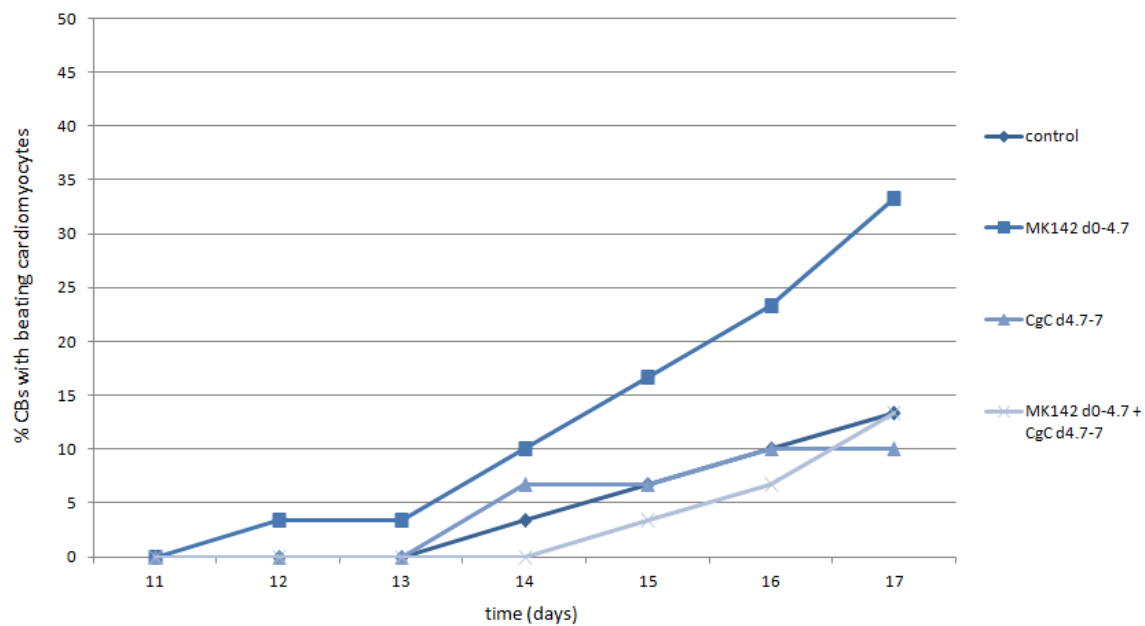
A**He2 CVPC - Influence of MK142 and CgC on CMG****B****He2 CVPC - Influence of MK142 and CgC on CMG (d11-17)**

Fig.29: MK142 induces differentiation of CVPCs into CMCs. (A) CVPCs (He2) were differentiated in CBs for 25 days and percentage of CBs with beating CMCs was monitored over 25 days. MK142 promoted CMC differentiation to a much bigger extent than in mESCs, while the inductive effects of CgC treatment were not significant. Treatment of CVPCs with both substances did not result in prolonged appearance of beating CMCs at the end of cultivation. (B) Appearance of first spontaneously contracting CMCs in CBs treated with MK142 preceded all other treatment groups by 2 days (substances were added over indicated treatment periods).

These results strongly indicate the ability of the CgC derivative MK142 to induce differentiation of mESCs (AB2.2) and both transgenic (A5) and wild-type (He2) CVPCs into

spontaneously contracting CMCs. In contrast to experiments with CgC treated mHBPCs (Yau et al., 2011), CMC contraction was always observed in EBs/CBs treated with MK142, suggesting that MK142 is a more subtle inducer of differentiation into functional CMCs. Additionally, in contrast to CgC, MK142 was shown to significantly enhance CMC formation in all tested cell lines, demonstrating its versatility for future clinical application.

5.2.2 MK142 induces differentiation of mESCs and CVPCs into functional SMCs

After the observation that mESCs and some CVPC lines also give rise to spontaneously contracting SMCs (Fig.18B, Fig.20) upon differentiation in aggregates we also wanted to test whether MK142 can also induce SMC development. Given the fact that wild-type CVPC lines He2, He22 and HeA only occasionally displayed functional SMCs, we only used mESC line AB2.2 and the transgenic CVPC line A5 for the experiment. The cells were differentiated in aggregates and MK142 and CgC treatments were applied over the same periods as in the CMC differentiation assay. The appearance of contracting SMCs in EBs and CBs was monitored over 25 days.

In EBs, treatment with MK142 induced SMC formation compared to untreated cells, albeit at rather moderate levels (Fig.30A). Neither CgC treatment nor a combination of MK142 and CgC were demonstrated to trigger SMC differentiation of mESCs. CgC treated EBs even displayed less contracting SMCs than the control group, while treatment in combination with MK142 resulted in SMC differentiation comparable to untreated cells. Thus, CgC might rather have an inhibitory effect on SMC formation, even reverting MK142 induced SMC differentiation, while favorably inducing differentiation into CMCs.

Opposite results were obtained from *in vitro* differentiation assays of CBs. MK142 significantly induced differentiation of CVPCs into functional SMCs (Fig.30B). At maximum, 83,3% of analyzed CBs displayed spontaneously contracting SMCs compared to 60% in untreated cells. In CBs, CgC treatment and a combination of CgC and MK142 both led to a significant increase of SMC formation as well, but not to the extent observed with MK142 treatment alone. However, whether this is due to the cardiac lineage commitment of CVPCs or to preferred CMC differentiation over SMC differentiation after treatment with both substances remains unclear and needs to be evaluated in additional experiments.

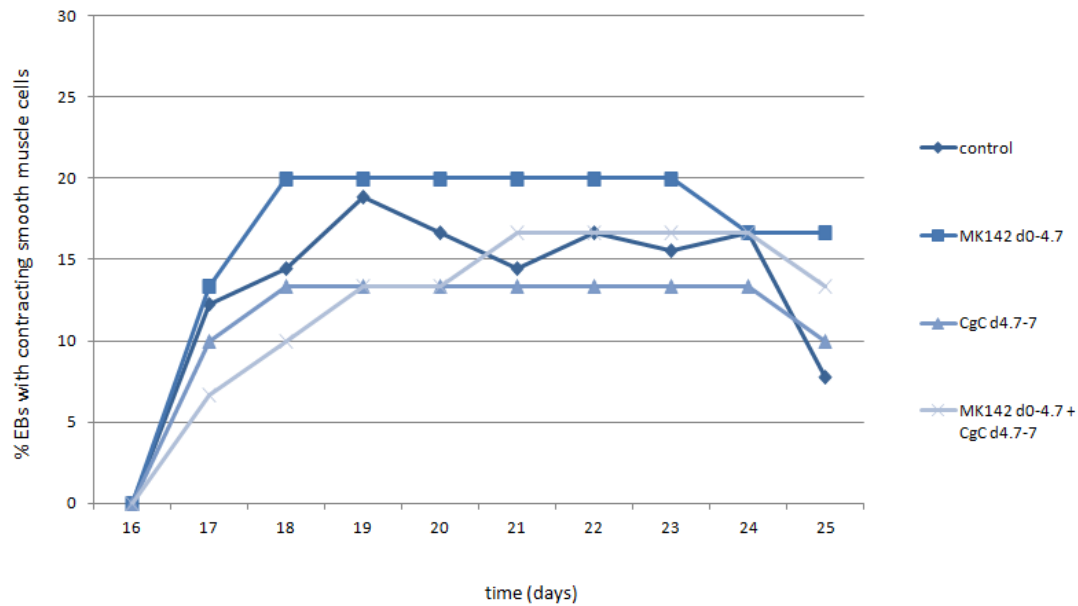
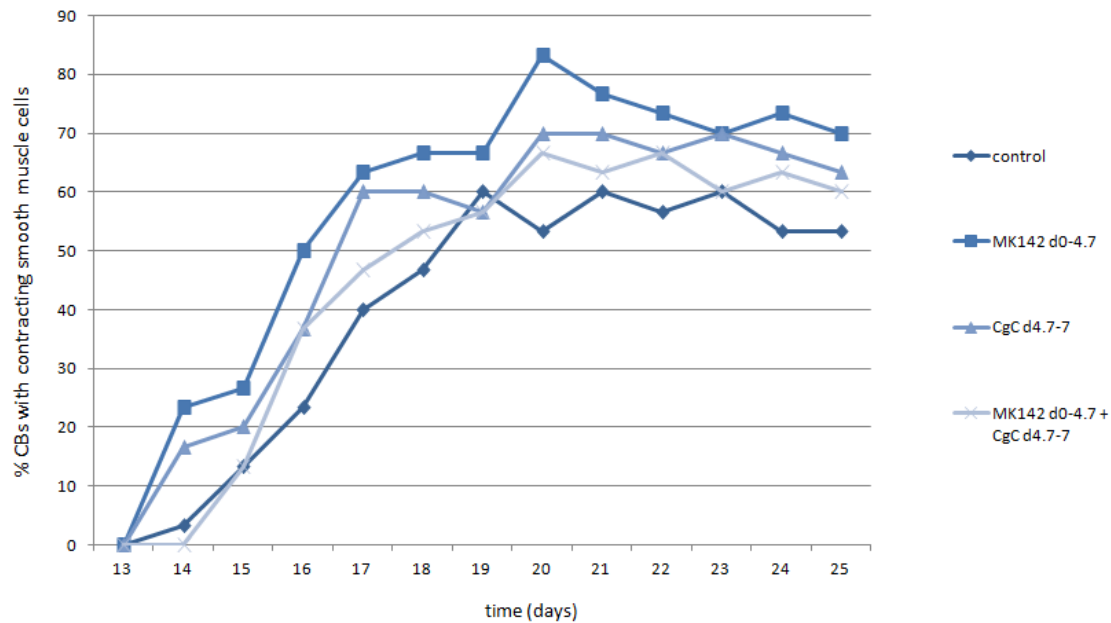
A**Ab2.2 ESC - Influence of MK142 and CgC on SMC formation (d16-25)****B****A5 CVPC - Influence of MK142 and CgC on SMC formation (d13-25)**

Fig.30: MK142 induces SMC formation in EBs and CBs. AB2.2 mESCs and A5 CVPCs were differentiated in EBs/CBs for 25 days and percentage of EBs/CBs with spontaneously contracting SMCs was monitored over 25 days. **(A)** MK142 treatment resulted in moderate induction of mESC differentiation into SMCs, while CgC seems to rather decrease SMC formation. **(B)** In CBs, MK142 led to significantly increased differentiation of CVPCs into functional SMCs. CgC treatment also resulted in increased SMC formation compared to untreated cells (substances were added over indicated treatment periods).

5.2.3 MK142 induces cardiac differentiation via up-regulation of early mesodermal and cardiac regulatory genes:

After the observation that MK142 induces cardiac differentiation we wanted to identify possible mechanisms for this effect. Since wild-type CVPCs (He2) only occasionally gave rise to functional SMCs, we only used the transgenic CVPC line A5 for the experiment. Due to the fact that it is hard to take samples from CBs before day 4.7, we differentiated the cells in monolayer culture to make the culture system accessible for mRNA isolation. A5 CVPCs were treated with MK142 for 5 days and expression levels of mesodermal and myocardial markers were monitored in comparison to a control group by RT-PCR (Fig.31).

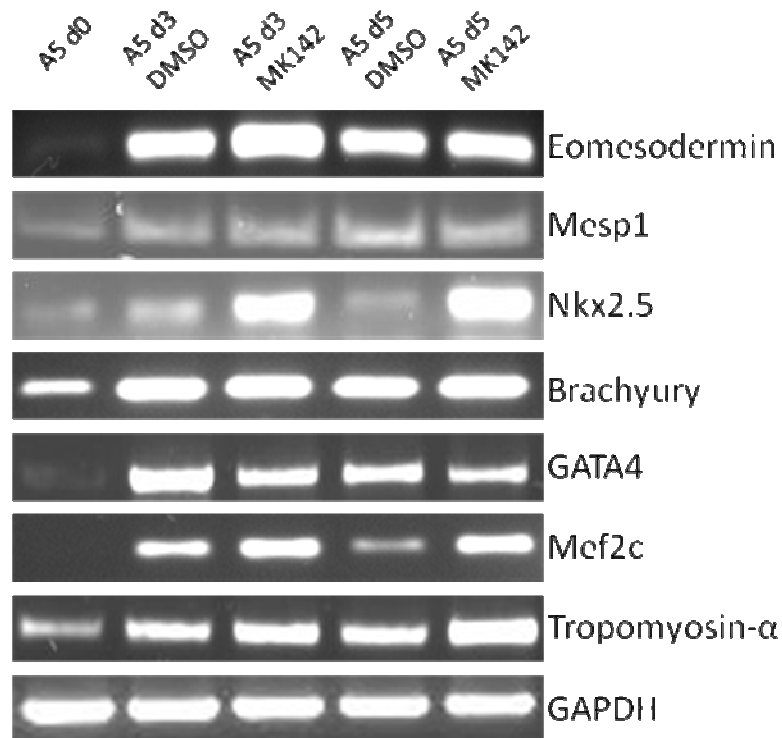


Fig.31: MK142 induces cardiac differentiation by up-regulation of NKX2.5 expression. CVPCs were differentiated in monolayer culture and MK142 was added for 5 days. After 3 and 5 days, mRNA was isolated and transcriptional levels of mesodermal and myocardial markers were compared to a non-treated control. MK142 functions via up-regulation of NKX2.5, which enhances MEF2C expression. Note the expression of mesodermal and early cardiac markers in undifferentiated CVPCs. GAPDH was used to balance the amount of cDNA used for RT-PCR.

Treatment with MK142 resulted in significantly increased expression of the cardiac key transcription factor NKX2.5 within 3 days. In addition, a slight transcriptional up-regulation of eomesodermin, the earliest mesodermal marker along with MESP1, was observed. Since eomesodermin and MESP1 act upstream of NKX2.5, this up-regulation might support the effect of MK142 treatment on NKX2.5 expression. Consequently, expression of MEF2C, a direct downstream target of NKX2.5, was increased. In combination, these events lead to

enhanced mesodermal and myocardial specification, which is consistent with the increased differentiation of mESCs and CVPCs into functional CMCs and SMCs observed in EB and CB *in vitro* differentiation experiments.

5.3 Desmin modulates cardiomyogenesis by temporal nuclear localization

The muscle specific type III IF protein desmin is one of the earliest known myogenic markers in both heart and somites. Desmin expression is first visible in the murine precardiac mesoderm around day 7 (Kuisk et al., 1996), however, desmin mRNA was detected in EBs by day 5 (Weitzer et al., 1995), suggesting a role of desmin in early embryonic development.

5.3.1 Desmin is localized in the nucleus of mESCs and CVPCs at the onset of cardiac differentiation

In previous chromatin immunoprecipitation (ChIP) experiments (Fuchs, 2007; Gawlas, 2011; Gottschamel, 2010), desmin was shown to bind to regulatory sequences of the NKX2.5 gene between day 6 and 8 of *in vitro* cardiogenesis of mESCs (AB2.2). In mESCs that overexpress desmin (DC6), the premature expression of desmin advanced the time of interaction with the NKX2.5 promoter and enhancer by one day (data not shown). To support these findings on a novel cardiac regulatory function of desmin, we wanted to visualize its temporal intra-nuclear localization with IF microscopy.

To make sure that detected desmin fluorescence signals were indeed localized inside the nucleus we performed z-stack microscopy (12 to 16 slices, 0.34 -0.52 μm steps) to screen each cell from bottom to top. Only those signals were counted intra-nuclear that were exclusively present over the middle focal planes of the stack with the highest nuclear staining intensities (Fig.32).

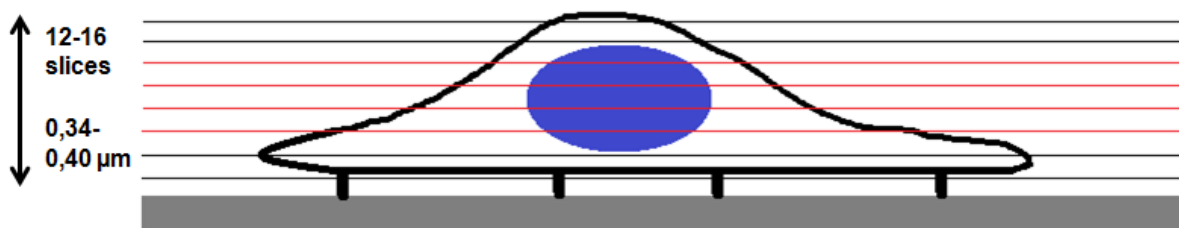


Fig.32: Z-stack confocal microscopy for detection of nuclear localized desmin. Z-stack microscopy was performed in 0.34 - 0.40 μm increments from bottom to top. For fluorescence signal detection of intra-nuclear desmin, only focal planes within the maximum range (red lines) of nuclear staining intensity were used for evaluation.

Three cell lines were used for the experiment, A5 CVPCs ($\text{des}^{+/+}$), DC6 mESCs ($\text{des}^{+/+} \text{des}^{\text{ect}}$) and a desmin knock-out mESC line ($\text{des}^{-/-}$) as negative control. The cells were aggregated for EB/CB *in vitro* differentiation, dissociated at day 7 and subjected to IF z-stack microscopy. To get a more precise idea of the exact nuclear position, DAPI nuclear staining was combined with lamin A/C staining (Fig.33). Lamins belong to the intermediate filament protein family and have been shown to be the major structural component of the nuclear lamina, a fibrous protein meshwork underlying the inner nuclear membrane (Aaronson and Blobel, 1975).

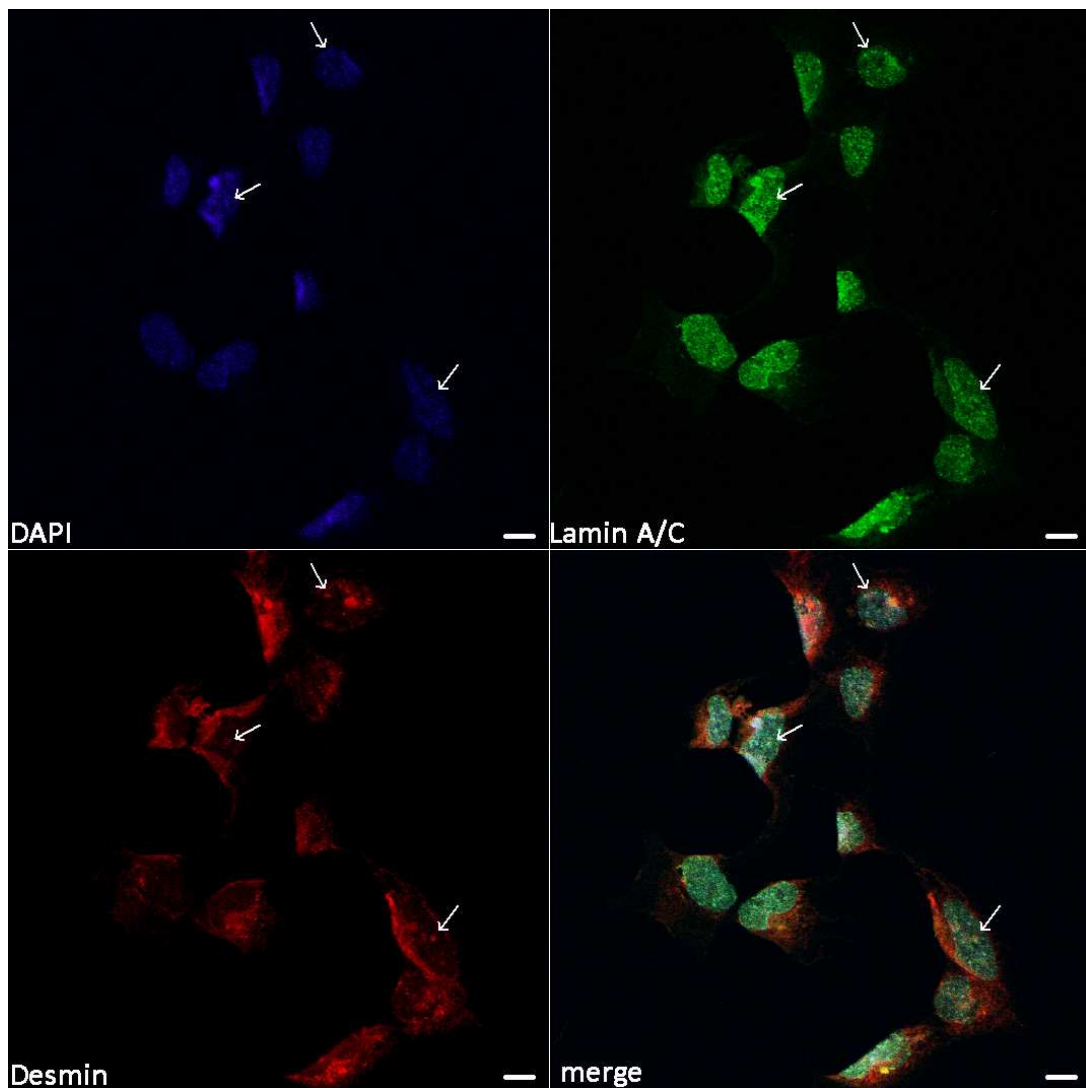


Fig.33: Desmin is located in the nucleus of differentiating CVPCs. CVPCs (A5) were differentiated in aggregates for 7 days, dissociated and stained with antibodies against lamin A/C (green) and desmin (red). Nuclei were stained with DAPI. Arrows indicate positions of intra-nuclear desmin signals which were only present over 1-3 optical sections ($12 \times 0.34 \mu\text{m}$ z-stack, Zeiss LSM 510 Meta). Bars = $10 \mu\text{m}$.

In differentiating A5 CVPCs (day 7), desmin was localized in the nucleus of 10,4% of analyzed cells (Fig.33, Fig.38A). Lamins are also located in the nuclear interior in a diffuse pattern (Stuurman et al., 1998), which explains the background observed inside the nucleus.

Thus, lamin A/C staining was replaced by staining with antibodies against the inner nuclear membrane protein emerin which is located exclusively at the nuclear rim (Manilal et al., 1996; Nagano et al., 1996). Consequently, in focal planes going through the nucleus, emerin staining results in a ring-like structure, significantly reducing the number of false positive desmin signals (Fig.34).

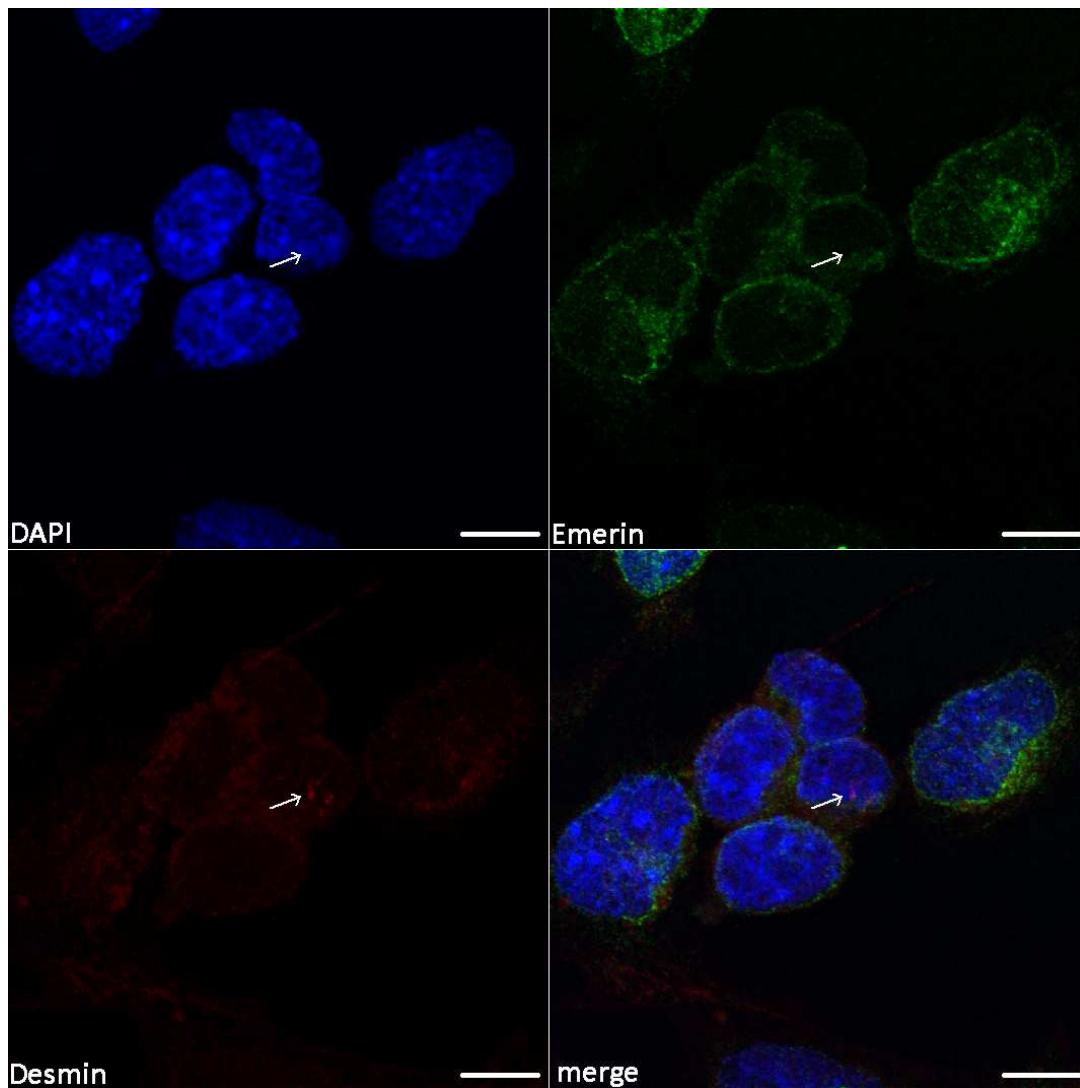


Fig.34: Desmin is located in the nucleus of differentiating mESCs. mESCs (DC6) were differentiated in aggregates for 7 days, dissociated and stained with antibodies against emerin (green) and desmin (red). Nuclei were stained with DAPI. Arrow indicates position of intra-nuclear desmin signals which were only present over 1-3 optical sections (12 x 0.39 μm z-stack, Zeiss LSM 510 Meta). Bars = 10 μm .

In differentiating DC6 mESCs (day 7), desmin was located in the nucleus of 7.5% of analyzed cells (Fig.34). Even though DC6 mESCs overexpress desmin, the amount of cells with intra-nuclear desmin is about 3% less than in CVPCs. This corroborates *in vitro* differentiation data where CVPCs were shown to differentiate into CMCs and SMCs at higher ratios than mESCs. Desmin signals were never observed in $\text{des}^{-/-}$ mESCs. The possible regulatory role of desmin

in early CMG is underscored by the fact that, in both mESCs (data not shown) and CVPCs, desmin was located in the nucleus before any functional CMCs were observed *in vitro*.

5.3.2 Desmin co-localizes with nuclear NKX2.5 protein during early cardiogenesis

After desmin interaction with regulatory sequences of the NKX2.5 gene was demonstrated with ChIP, we wanted to visualize this interaction, as intra-nuclear desmin not necessarily has to be located at the NKX2.5 locus. The NKX2.5 transcription factor binds to its own promoter region via its C-terminus in a negative feedback loop (Li et al., 2007; Tanaka et al., 1999). Therefore, desmin interaction with the NKX2.5 promoter can be demonstrated by co-localization of desmin and the NKX2.5 protein (Fig.35).

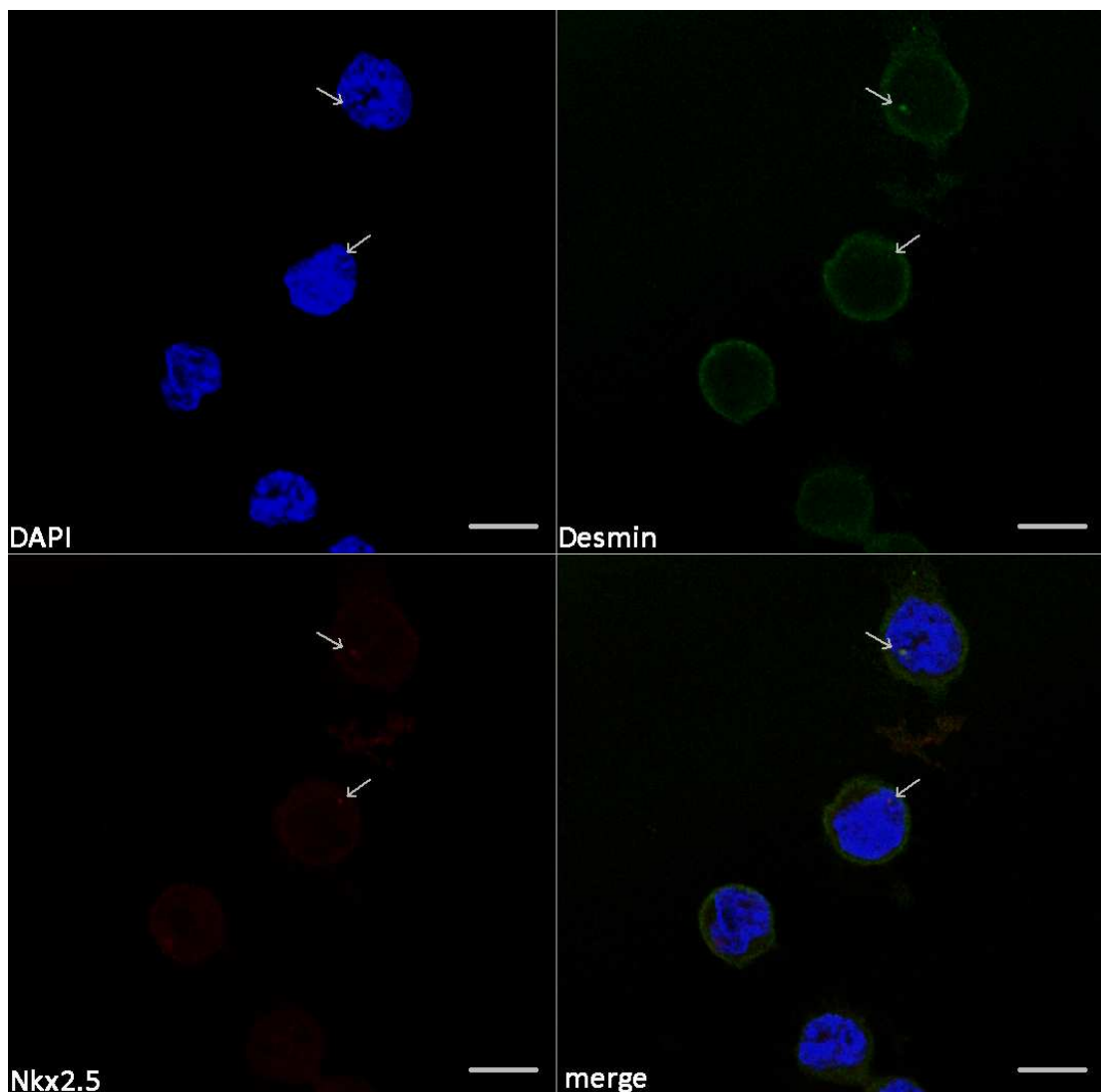


Fig.35: Desmin and NKX2.5 co-localize in the nucleus at the onset of differentiation. mESCs (DC6) were differentiated in aggregates for 7 days, dissociated and stained with antibodies against desmin (green) and NKX2.5 (red). Nuclei were stained with DAPI. Arrows indicate co-localization of desmin and NKX2.5 signals which were only present over the same 3-4 optical sections (12 x 0.40 μm z-stack, Zeiss LSM 510 Meta). Bars = 10 μm .

In some differentiating mESCs (DC6), desmin co-localized with NKX2.5 in the nucleus after 7 days of differentiation (Fig.35). This is consistent with ChIP results where interaction of desmin with regulatory sequences of the NKX2.5 gene in DC6 mESCs was observed between day 5 and 7. To visualize this co-localisation, it was important that desmin and NKX2.5 signals were only present in the same optical sections in the middle of the z-stack (Fig.36).

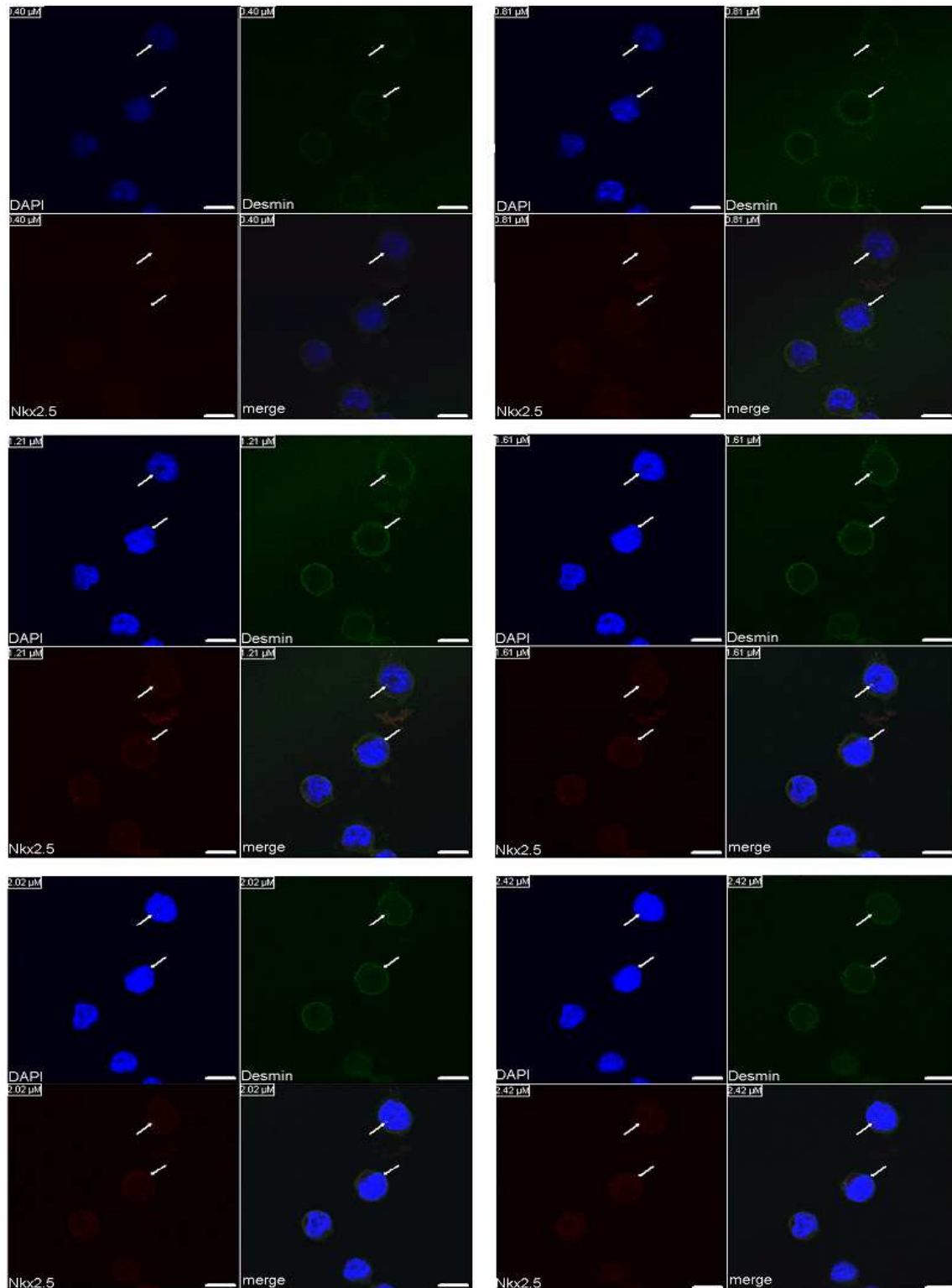


Fig.36: *Confocal z-stack microscopy demonstrates interaction of desmin and NKX2.5. Six optical sections from bottom to top are shown (0.40 μ m - 2.42 μ m, increment 0.40 μ m). Both signals appear and disappear over the same focal range (Zeiss LSM 510 Meta). Bars = 10 μ m.*

5.3.3 Desmin is present in the nucleus of immature CMCs

With the notion that desmin translocates to the nucleus and stimulates cardiac differentiation via temporal co-regulation of NKX2.5, we wanted to examine if desmin is still present in the

nuclei of immature CMCs, where cardiac differentiation has not yet been fully terminated. In previous experiments, desmin was shown to be localized in the nucleus of few primary CMCs but not of terminally differentiated CMCs (data not shown). Confocal z-stack microscopy of CVPCs (A5) and mESCs (DC6, *des*^{-/-} mESCs) after 13 days of differentiation in aggregates demonstrated nuclear localization of desmin in a small portion of immature CMCs (Fig.37).

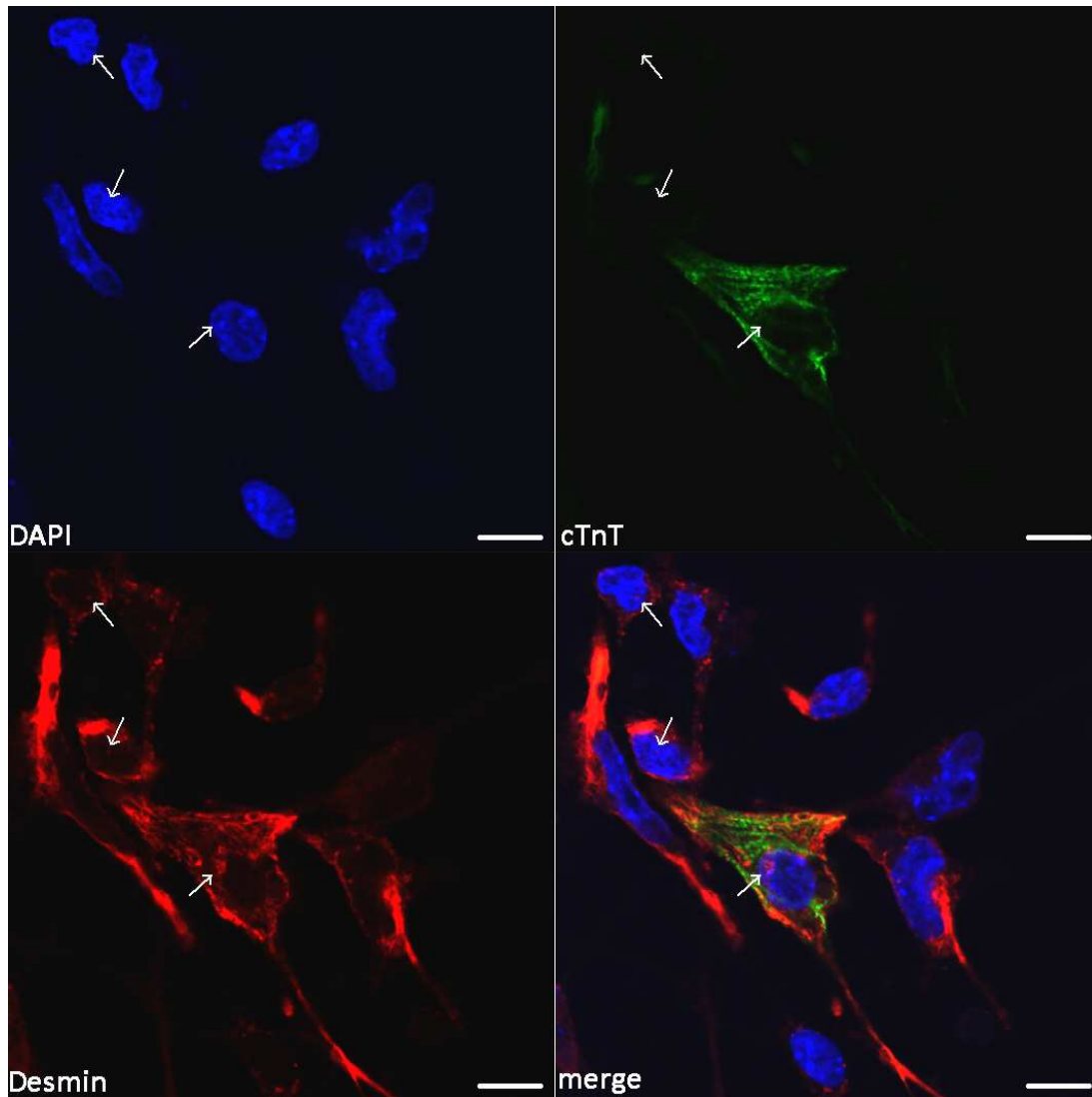


Fig.37: *Desmin is localized in the nucleus of immature CMCs.* CVPCs (A5) were differentiated in aggregates for 13 days, dissociated and stained with antibodies against cTnT (green) and desmin (red). Nuclei were stained with DAPI. Arrows indicate positions of intra-nuclear desmin signals which were only present over 1-3 optical sections (16 x 0.40 μm z-stack, Zeiss LSM 510 Meta). Bars = 10 μm .

In CBs (A5), 12.2% of all analyzed cells had differentiated into cTnT-positive CMCs, compared to 9.0% in DC6 mESCs and 6.1% in *des*^{-/-} mESCs (Fig.38B). In *des*^{-/-} mESCs, appearance of CMCs is reduced, consistent with previous results that demonstrated that a lack of desmin rather delays than prevents cardiogenesis (Hofner et al., 2007). Of all CVPC-

derived CMCs, 5.7% still displayed desmin located in the nucleus, compared to 4.0% in desmin overexpressing DC6 mESCs. Intra-nuclear desmin was neither observed in differentiating $\text{des}^{-/-}$ mESCs (Fig.38A) nor in $\text{des}^{-/-}$ mESC-derived CMCs.

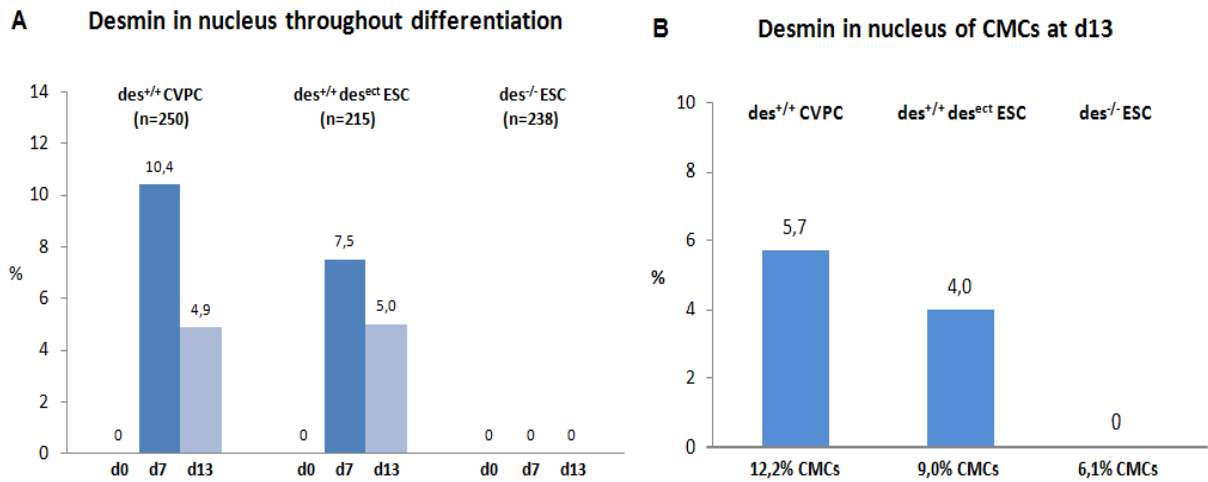


Fig.38: A time-course of nuclear desmin localization levels throughout early cardiogenesis. **(A)** The percentage of undifferentiated (d0) and differentiating cells (d7, d13) with desmin located in the nucleus was monitored with confocal z-stack microscopy. At the onset of cardiogenesis (d7) nuclear desmin levels were higher compared to the time when first immature CMCs had developed (d13), suggesting a regulatory role of desmin in cardiac specification. **(B)** Desmin was still located in the nucleus of few immature CMCs, but not of terminally differentiated CMCs.

Desmin was never located in the nucleus of undifferentiated CVPCs and mESCs, but the observation that intra-nuclear desmin localization levels increased to up to 10.4% during ongoing early cardiac specification and decreased thereafter suggests a subtle involvement of desmin in the regulation of early cardiogenesis. In addition, the amount of desmin localized in the nucleus seems to correlate with the induction of CMC differentiation, as intra-nuclear desmin levels in CVPCs were 3.9% higher than in mESCs, resulting in 3.2% more CMCs by day 13 of differentiation. At day 13, no difference in intra-nuclear desmin levels between differentiating CVPCs and mESCs were observed. Thus, desmin may function in a spatio-temporal manner by inducing NKX2.5 expression at the onset of cardiogenesis.

6. Discussion

In the last decade, various research groups succeeded in the isolation of putative CVPCs (*CSCs in cited literature*) from mammalian hearts. Most of them used preparative FACS (fluorescence activated cell sorting) assays to separate CVPCs from somatic cells in heart preparations. Several stem cell -related surface antigens were used as targets for isolation. C-kit positive CVPCs were the first stem cells indentified in the rat heart (Beltrami et al., 2003) and have been extensively characterized. Some groups indicated that Sca-1 positive progenitors are the predominant cardiac stem cell population (Matsuura et al., 2004; Oh et al., 2003), while others refer to ISL-1 positive cells as "primordial" CVPCs (Laugwitz et al., 2005). Also, a small fraction of MDR-1 positive cardiac side population cells has been shown to contribute to diverse lineages (Martin et al., 2004). Thus, CVPCs seem to be a heterogenic cell population expressing several stem cell antigens. However, there is no unique marker capable of providing absolute identification of stem cells *in vivo* to date (Leri et al., 2005).

6.1 A novel strategy for the isolation of clonal CVPCs

Here we provide a simple and robust strategy to reproducibly isolate CVPCs from both neonatal and adult murine hearts (Höbaus, Heher, Gottschamel et al., 2012). We use a three-component culture system consisting of Neo^R LIF-secreting feeder cells and a 1:1 ratio of mESCs and cells from heart preparations, based on the notion that mESCs secrete factors that foster CVPC survival and self-renewal. Once a critical mass of CVPCs is enriched, other cells in the culture system are removed by drug selection and CVPCs are clonally derived by limiting dilution under further selection. Two isolation strategies were tested (Fig.14): cell preparations of Neo^R (A5 CVPCs) and wild-type (He2, He22, HeA CVPCs) hearts with subsequent positive (Neo) or negative (TK) drug selection. Positive selection yielded 11 Neo^R CVPC clones, negative selection 4 wild-type CVPC clones.

A major concern about the origin of CVPCs is based on the culture system itself which involves co-culture of an unknown initial amount of CVPCs with mESCs and LIF-secreting fibroblasts over several weeks. Thus, it is possible that CVPC clones may arise from cell transdifferentiation or reprogramming of somatic heart or feeder cells *in vitro*. This would mean that CVPCs were not isolated, but rather artifacts generated under the used culture conditions (Peran et al., 2010). Results from genotyping PCR experiments targeting the transgenic and wild-type loci, respectively, of all cell types in the culture system demonstrated

that CVPCs were neither derived from the feeder cells, nor from the respective mESC co-culture line (Fig.15). Also, direct reprogramming or transdifferentiation of somatic cells without forced expression of lineage specific markers or addition of cardiogenic compounds occur at low efficiencies and usually require longer culture periods (Ieda et al., 2010; Takahashi and Yamanaka, 2006), mostly producing cells with a meta-stable cardiac phenotype (Acquistapace et al., 2011; Condorelli et al., 2001). All obtained CVPC clones isolated with our method were capable of continuous LIF-dependent self-renewal *in vitro* and displayed a constant cardiogenic differentiation potential.

Another possible way to generate CVPCs *in vitro* is fusion of somatic heart cells with mESCs in co-culture. Genotyping experiments proving that CVPCs or feeder cells were not derived from mESCs provided a first hint that this is not the case. In addition, karyotyping results demonstrated that CVPCs were diploid (Fig.16). Pseudo-diploidy of CVPCs after cell fusion and subsequent reductive mitosis can be ruled out for several reasons. The modified 3T3 protocol we used selects fast replicating cells. Reductive mitosis is a rather slow process, yielding only a few pseudo-diploid cells that would be rapidly diluted out of the culture. In addition, reductive mitosis is inefficient, thus the majority of fusion cells would stay tetraploid, which was not observed in regard to karyotyping results and the size of undifferentiated CVPCs (Fig.15C). Most importantly, reductive mitosis requires the absence of p53, which was expressed in all undifferentiated CVPC clones (Fig.17), and a majority of pseudo-diploid cells are non-viable (Vitale et al., 2010). Also, p53 expression greatly reduces the efficiency of reprogramming (Hong et al., 2009; Kawamura et al., 2009; Marion et al., 2009; Zhao et al., 2008).

CVPCs may also arise from multipotent progenitor cells present in the peripheral blood (Cesselli et al., 2009), but we never observed hematopoietic cells during *in vitro* differentiation experiments. In addition, CVPCs express several mesodermal and myocardial markers not present in bone marrow-derived stem cells.

6.2 CVPCs simultaneously express stemness and mesodermal markers in an undifferentiated state

Similar to mESC, LIF was sufficient to keep cultivated CVPCs in an undifferentiated state. CVPCs were demonstrated to be capable of LIF-dependent self-renewal by expression of stemness markers NANOG and SOX2 at levels comparable to mESCs (Fig.23). Additionally, some CVPC lines expressed two of the earliest mesodermal markers, MESP1 and brachyury,

at varying levels in an undifferentiated state, which is in sharp contrast to mESCs (Fig.24). CVPC line A5 also displayed expression of early myocardial markers NKX2.5 and GATA4 (Fig.31) in an undifferentiated state. Altogether, these RT-PCR data are in accordance with previously reported expression patterns of CVPCs (*CSCs in cited literature*) isolated with preparative FACS by other groups (Laugwitz et al., 2005; Matsuura et al., 2004; Oh et al., 2003). From the simultaneous expression of stemness and early mesodermal and cardiac markers we suggest that CVPCs are already committed to the cardiac lineage, while still being able to self-renew. CVPCs have been cultivated for up to 149 passages without any visible phenotypic or karyotypic changes and still gave rise to functional CMCs and SMCs upon differentiation in CBs. From reports of isolated cardiac side population cells that did not express any cardiac or mesoderm stage-specific markers (Oyama et al., 2007), we suggest that our isolated CVPCs lie downstream of these more unspecified cells. This cardiac commitment is supported by the observation that CVPCs predominantly give rise to ETCs, CMCs and SMCs.

However, further elucidation of the exact developmental stage of isolated CVPCs is of major importance, as stage specific markers are present in all cells undergoing lineage differentiation which, especially with FACS, complicates recognition of the actual primitive cells in a heterogenic population (Leri et al., 2005).

6.3 CVPCs spontaneously give rise to ETCs, CMCs and SMCs upon LIF deprivation

In contrast to CVPCs isolated by other groups which only differentiated upon chemical induction (Matsuura et al., 2004; Oh et al., 2003; Oyama et al., 2007) or co-cultivation with neonatal (Laugwitz et al., 2005) or mature (Pfister et al., 2005) CMCs, LIF deprivation was sufficient to induce *in vitro* differentiation of CBs into ETCs, CMCs and SMCs (Fig.18, Fig.20, Fig.21), the three major cell types of the heart. In contrast to mESCs, CVPCs also gave rise to spontaneously contracting CMCs in monolayer culture. In addition, CBs lacked self-organization phenomena regularly observed in EBs (Fig.22) (Fuchs et al., 2012).

First endothelial cells in CBs were visible from day 7 on, while differentiation into spontaneously beating cTnT positive CMCs required 8 to 10 days without addition of cardiogenic substances in normal growth medium, delayed by 2 days compared to EBs. Nevertheless, for most CVPC lines, CBs displayed larger functional CMC clusters over time and more persistent CMC differentiation than EBs, with prolonged appearance of beating

CMCs over time. All CVPC lines expressed early and definite myocardial markers at slightly varying levels after differentiation in aggregates for 13 days (Fig.25).

Additionally, all CVPC lines reproducibly gave rise to SMA- α positive SMCs, usually to a higher extent than observed in mESC differentiation. CVPCs isolated from adult murine hearts rarely gave rise to spontaneously contracting SMCs. CVPCs isolated from neonatal hearts regularly differentiated into functional SMCs which, in contrast to other isolated CVPC lines (Beltrami et al., 2003), responded to physiological Ang-2 levels with increased contraction rates (Fig. 26). The observation that CVPCs responded to Ang-2 much stronger than mESCs suggests a more advanced developmental stage of CVPC-derived functional SMCs. The potential of CVPCs to give rise to functional CMCs and SMCs along with the observed expression of both stemness and stage-specific myocardial markers in undifferentiated CVPCs supports the theoretical cardiac commitment of these cells. Since *in vitro* differentiation of CVPCs occurred spontaneously upon withdrawal of LIF, we hypothesize that stimuli for both self-renewal and cardiac differentiation are in constant competition, resulting in CVPCs primed for cardiac cell fate decisions. Elucidating a possible mechanism for this specification will provide further insight into early cardiac development.

6.4 MK142 is a novel cardiogenic small molecule that induces both CMC and SMC differentiation

MK142 is a novel derivative of the small cell-permeable diaminopyrimidine CgC which has been shown to induce *in vitro* CMC differentiation of mESCs (Wu et al., 2004) and murine hair bulge progenitor cells (mHBPCs) (Yau et al., 2011). Using the EB/CB model system to mimic early cardiomyogenesis, we demonstrated that MK142 treatment for 5 days is sufficient to induce differentiation into functional CMCs. Consistent with the previously demonstrated cardiac commitment of CVPCs, this induction was significantly stronger in these cells (Fig.28, Fig.29) compared to the moderate effects observed in mESCs (Fig.27). Nevertheless, compared to previous experiments with CgC in mHBPCs (Yau et al., 2011), MK142 and CgC treatment always resulted in spontaneously contracting CMCs, but functional CMC levels were higher in MK142 treated than in CgC treated CVPCs. Moreover, a combination of both substances led to prolonged appearance of spontaneously contracting CMCs in mESCs and CVPCs, suggesting partly independent induction mechanisms for MK142 and CgC.

In contrast to CgC, MK142 also induces functional SMC differentiation, likewise at moderate levels in mESCs compared to the significant increase observed in CVPCs (Fig.30). Surprisingly, CgC reverted MK142-induced SMC differentiation to control levels, which may be a consequence of the CgC-mediated induction of CMC differentiation at the expense of SMC differentiation.

CgC was reported to induce CMC differentiation via up-regulation of cardiac markers NKX2.5, GATA4, MEF2C, MHC or TBX5 (Wu et al., 2004; Yau et al., 2011). Preliminary RT-PCR results demonstrated that MK142 may function via up-regulation of NKX2.5 and consequential MEF2C up-regulation which ultimately leads to increased expression of the definite cardiac marker Tropomyosin- α . This transcriptional cascade might be initiated by slight up-regulation of eomesodermin, the earliest mesodermal marker along with MESP1, which together trigger NKX2.5 expression (Fig.31).

The use of cardiogenic small molecules is a promising approach to circumvent the risks of exogenous stem cell therapy of the injured heart. Screening of chemical libraries has identified compounds that were shown to enhance the endogenous regenerative potential of the myocardium by inducing cardiac differentiation (Sadek et al., 2008). Novel combinational strategies involving pre-delivery treatment of stem or progenitor cells with small molecules like drugs or growth factors followed by injection of these cells has been successful in clinical trials of peripheral vascular disease (Dimmeler et al., 2001; Sasaki et al., 2006). Here we demonstrated that MK142 is a versatile and strongly inductive cardiogenic compound which increases both functional CMC and SMC differentiation in a variety of cell types.

6.5 Desmin plays a modulatory role in early cardiogenesis

Desmin, a type III intermediate filament, is one of the earliest expressed proteins in mesodermal cells committed to the myocardial lineage (Kuisk et al., 1996; Li and Capetanaki, 1994) and has previously been demonstrated to induce differentiation of mESCs into CMCs via up-regulation of brachyury and NKX2.5, while deletion of its aminoterminal region significantly reduced myocardial differentiation (Hofner et al., 2007; Holtrigl et al., 2007; Holtrigl et al., 2002). Supported by data showing that type III intermediate filaments interact with DNA (Tolstonog et al., 2005; Traub and Shoeman, 1994), these findings suggest a modulatory role of desmin in early cardiogenesis.

ChIP experiments demonstrated binding of desmin to regulatory sequences of the NKX2.5 gene at the onset of myocardial differentiation in mESCs and CVPCs (Fuchs, 2007; Gawlas,

2011; Gottschamel, 2010). Supporting these data, the nuclear localization of desmin in differentiating mESCs and CVPCs was demonstrated with confocal z-stack IF microscopy. In contrast to undifferentiated cells where desmin was never localized in the nucleus, mESCs and CVPCs displayed intra-nuclear desmin at the onset of cardiac differentiation (Fig.33, Fig.34). Moreover, desmin and NKX2.5 co-localized in the nucleus of a subset of cells (Fig.36). Reduced but still detectable levels of intra-nuclear desmin were observed in immature CMCs (Fig.37), while terminally differentiated CMCs never displayed desmin in the nucleus, indicating that desmin is involved in the specification of mesodermal progenitors towards the myocardial lineage in a spatiotemporal manner. In this respect, desmin might act as a scaffold, stabilizing the transcriptional complex by bringing regulatory sequences of the NKX2.5 gene into close proximity.

7. Conclusion and Outlook

Here we provide a feasible, robust and cost-efficient strategy for the isolation of CVPCs from both neonatal and adult murine hearts. The simplicity of the method, which does not require surface antigen expression analysis with preparative FACS, allows for adaption to other organs or species. We demonstrate that isolated CVPCs are capable of self-renewal while displaying a subtle commitment to the myocardial lineage. This limited potential is of major importance for clinical application, significantly reducing the risk of uncontrolled cell growth *in situ*, as CVPCs only give rise to the main cell types of the heart upon differentiation. In addition, the possibility to obtain CVPCs from adult hearts may facilitate isolation of human CVPCs from biopsies, which can be used for drug discovery, cardiotoxicity screenings or identification and validation of new therapeutic targets. In this regard, we also demonstrate that CVPCs respond to biochemical inducers, bringing pre-delivery treatment and subsequent injection into injured cardiac tissue one step closer to therapeutic application.

Recent findings indicate that CVPCs isolated from mammalian hearts are capable of facilitating regeneration of the injured myocardium after acute MI, albeit at rather moderate levels. Nevertheless, isolation, *in vitro* expansion and subsequent injection of patient-specific CVPCs into injured cardiac tissue is suggested to be a promising therapeutic approach for the treatment of CVDs. Since cardiac stem cell therapy harbors the risks of uncontrolled cell growth, inefficient homing or limited differentiation of cells, reactivation of dormant endogenous CVPCs by drug treatment may provide a safer way to induce cardiac repair. For this purpose, a thorough elucidation of signaling pathways involved in cardiac differentiation is necessary to increase the safety and efficiency of cardiac stem cell therapy. Also, the composition of injected cell preparations remains an open question, as mixtures of CVPCs and different immature cardiac cell types may promote regeneration more efficiently than single cell-type injections. In this respect, the geno- and karyotypic stability of CVPCs *in vitro*, in combination with the EB model system, provides a powerful tool for basic cardiac research studies that will further enhance knowledge about the regulatory mechanisms underlying cardiogenesis.

8. References

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9. Abstract

Cardiovascular diseases are the leading death cause worldwide, accounting for 30% of all global deaths. The fact that more people die from cardiovascular diseases annually than from any other cause has led to extensive cardiovascular research in the past. For decades, the heart was believed to be a post-mitotic organ and the inevitable loss of cardiomyocytes after acute myocardial infarction was thought to be irreversible. The recent notion that the heart is capable of intrinsic regeneration via endogenous cardiac precursors has combined the fields of cardiovascular research, developmental and stem cell biology. Various groups have succeeded in the isolation of somatic stem cells from the hearts of different mammalian species, however, so far the obtained cells could not be maintained as self-renewing and phenotypically stable clonal cell lines *in vitro*.

Here we present a simple and robust strategy for the isolation of cardiovascular progenitor cells from both neonatal and adult murine hearts. We use a three component system consisting of somatic heart cells, embryonic stem cells and LIF secreting feeder cells with selectable markers to mimic stem cell niche conditions and foster cardiovascular progenitor cell survival until stable clonal cell lines can be established. We demonstrate that isolated cardiovascular progenitor cells indeed stem from the murine heart and do not arise from cell fusion, transdifferentiation or reprogramming of somatic heart cells. They are capable of self-renewal in a LIF-dependent manner and, in contrast to embryonic stem cells, differentiate preferentially into functional cardiomyocytes, smooth muscle cells and endothelial cells upon LIF deprivation. This indicates a subtle cardiac commitment of these cells, which is consistent with the expression of both stem cell and early mesodermal and cardiac markers in undifferentiated cardiovascular progenitor cells. In addition, using the embryoid body model system we show that cardiovascular progenitor give rise to more functional cardiomyocytes and smooth muscle cells and respond to biochemical induction of cardiac differentiation by cardiogenic small molecules like MK142 or Cardiogenol-C at higher levels compared to embryonic stem cells.

We have cultivated cardiovascular progenitor cells for up to 147 passages so far without any phenotypic and karyotypic changes or the loss of differentiation potential. The stability of these cells *in vitro* along with the feasibility of our isolation method makes them a versatile tool to study regulatory processes in early cardiogenesis.

10. Zusammenfassung

Herz-Kreislauf-Erkrankungen sind die häufigste Todesursache weltweit, 30% aller Menschen weltweit sterben daran. Die Tatsache dass Herz-Kreislauf-Erkrankungen jährlich mehr Todesopfer als alle anderen Krankheiten fordern hat die kardiovaskuläre Forschung in der Vergangenheit stark forciert. Jahrzehnte lang wurde das Herz für ein post-mitotisches Organ gehalten, wodurch der unausweichliche Verlust von Kardiomyozyten nach einem Herzinfarkt irreversibel wäre. Die Entdeckung eines intrinsischen Regenerationspotentials des Herzens durch endogene adulte Herzstammzellen hat die klinische kardiovaskuläre Forschung mit der Entwicklungs- und Stammzellbiologie vereint. Seitdem ist es zwar mehreren Forschungsgruppen gelungen somatische Herzstammzellen aus Herzen verschiedener Säugetiere zu isolieren, jedoch nicht, diese Zellen *in vitro* über längere Zeiträume als phänotypisch stabile, klonale und selbsterneuerungsfähige Zelllinien zu kultivieren.

Hier stellen wir eine simple und robuste Strategie zur Isolierung von Herzstammzellen aus Herzen von frisch geborenen und adulten Mäusen vor. Wir verwenden ein Drei-Komponenten-Kultursystem bestehend aus somatischen Herzzellen, embryonalen Stammzellen und LIF sekretierenden Feeder Zellen mit selektierbaren Markern, um eine künstliche *in vitro* Stammzellnische zu simulieren, die das Überleben und Wachstum der Herzstammzellen ermöglicht, bis stabile, klonale Zelllinien etabliert werden können. Wir zeigen, dass die isolierten Herzstammzellen tatsächlich aus dem murinen Herz stammen und nicht durch Zellfusion, Transdifferenzierung oder Reprogrammierung entstandene Zellkultur-Artefakte sind. LIF ist notwendig und ausreichend für die Selbsterneuerung von Herzstammzellen, im Gegensatz zu murinen embryonalen Stammzellen differenzieren Herzstammzellen jedoch bei LIF-Entzug überwiegend zu funktionellen Kardiomyozyten, glatten Muskelzellen und Endothelzellen, den drei Hauptzelltypen des Herzens. Dieses limitierte *in vitro* Differenzierungspotential und die gleichzeitige Expression von Stammzell-, frühen mesodermalen und Herzzellmarkern in undifferenzierten Herzstammzellen deuten darauf hin, dass diese Zellen bereits auf die Differenzierung zu Herzzellen eingeschränkt sind. Zusätzlich demonstrieren wir unter Verwendung des embryoid body Modellsystems, dass Herzstammzellen in einem größeren Ausmaß zu funktionellen Kardiomyozyten und glatten Muskelzellen differenzieren und stärker auf biochemisch induzierte Herzdifferenzierung

durch kardiogene Substanzen wie MK142 oder Cardiogenol-C reagieren als embryonale Stammzellen.

Wir konnten Herzstammzellen bisher über 147 Passagen kultivieren, ohne Veränderungen im Phänotyp, Karyotyp oder Differenzierungspotential der Zellen zu beobachten. Die Stabilität der Herzstammzellen *in vitro* in Kombination mit der relativ einfachen Durchführbarkeit der Isolierung macht diese Zellen zu einem vielseitigen Instrument, um regulatorische Prozesse in der frühen Herzentwicklung untersuchen zu können.

11. Curriculum Vitae

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SCIENTIFIC SYMPOSIA - POSTERS AND TALKS

Philipp Heher, Sonja Gawlas, Christiane Fuchs and Georg Weitzer. "Desmin promotes Nkx2.5 expression during early cardiomyogenesis via temporal restricted interaction with the *Nkx2.5* gene."

(Poster presentation at the 3rd annual meeting of the Austrian Association of Molecular Life Sciences and Biotechnology, Salzburg, Austria, 28th to 30th of September, 2011)

Philipp Heher and Georg Weitzer. "Desmin, a modulator of transcription in cardiomyogenesis?"

(Short talk at the 3rd annual meeting of the Austrian Association of Molecular Life Sciences and Biotechnology, Salzburg, Austria, 28th to 30th of September, 2011)

PUBLICATIONS

Julia Hoebaus, **Philipp Heher**, Teresa Gottschamel, Matthias Scheinast, Harmen Auner, Diana Walder, Marc Wiedner, Jasmin Taubenschmid, Maximilian Miksch, Thomas Sauer, Martina Schultheis, Alexey Kuzmenkin, Christian Seiser, Juergen Hescheler, and Georg Weitzer (2012). *Embryonic Stem Cells Facilitate the Isolation of Persistent Clonal Cardiovascular Progenitor Cell Lines and Leukemia Inhibitor Factor Maintains their Self-Renewal and Myocardial Differentiation Potential In Vitro*. Cells Tissues Organs, accepted for publication on November 12th 2012, DOI: 10.1159/000345804.

AWARDS

Prize for best Short Talk (awarded at the 3rd annual meeting of the Austrian Association of Molecular Life Sciences and Biotechnology, Salzburg, Austria, 28th to 30th of September, 2011)