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DIPLOMARBEIT

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"Establishment of Reporter Cell Lines for the Characterization of Brachyury and Mesp1 Expression during In Vitro Cardiomyogenesis"

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Contents

Abstract

Heart conditions are still one of the leading causes of death worldwide. As the heart has a very low regenerative potential, the existence of somatic cardiac stem cells opens new perspectives on how to deal with disease and aging.

In order to characterize the Wnt signaling pathway and the expression pattern of Brachyury and Mesp1 during in vitro differentiation we have stably transfected an embryonic stem cell (ESC) line and a cardiovascular progenitor cell (CVPC) line with reporter plasmids (pT-Bra^p-Puro-IRES2-EGFP, pMesP1-EGFP and M50 Super 8x TOPFlash).

Brachyury is one of the best markers of early pan-mesoderm. Its transient expression is found in all nascent mesoderm and is down-regulated as these cells undergo specification. **Mesp1** is a bHLH transcription factor, which is expressed specifically in almost all cardiovascular precursors and is required for cardiac morphogenesis. Mesp1 seems to play a key role during earliest time points of cardiovascular determination and is a promising transcription factor to identify additional direct target genes. **Wnt signaling pathway** is known to induce the formation of mesoderm. Its activation is only transient and it is spatially and temporally regulated. Wnt is a known activator of Brachyury; its direct influence on Mesp1 is not proven yet.

The in vitro model embryoid bodies (EBs) was used to investigate the expression of Brachyury, Mesp1 and Wnt signaling during early embryogenesis, whereas cardiac bodies (CBs) reflected the expression during cardiomyogenesis. The expression levels were reported either by flow cytometry analysis (Brachyury and Mesp1) or by luminiscence (Wnt signaling pathway).

We could demonstrate the transient upregulation of Brachyury and Mesp1 promoter-driven EGFP at day 5 of ESC aggregation and we showed that CVPC derived CBs are five days ahead in differentiation compared to EBs, as both, Brachyury and Mesp1 promoter-driven EGFPs, were found in the beginning of differentiation and disappeared thereafter. Furthermore we demonstrated the responsiveness of the TOPflash reporter cell line to Wnt signaling. This enabled us to test the influence of a GSK-inhibitor (CHIR) and a TGF-β inhibitor (SB 431542) on the expression of the reporter genes.

These experimental data foster the understanding of the transcriptional network regulating heart differentiation and will contribute to the improvement of strategies to efficiently differentiate somatic stem cells into fully functional cardiac muscle cells.

Zusammenfassung

Erkrankungen des Herzens sind immer noch eine der Haupttodesursachen weltweit. Da das Herz ein sehr geringes regeneratives Potential hat, eröffnet die Existenz von somatischen Herzstammzellen neue Möglichkeiten, wie man mit Erkankung und Alterung umgehen kann.

Um den Signaltransduktionsweg von Wnt und das Expressionsverhalten von Brachyury und Mesp1 während der in vitro Differenzierung zu charakterisieren, wurde eine embryonale Stammzelllinie (ESZ) und eine kardiovaskuläre Vorläuferzelllinie (KVZ) mit Reporter Plasmiden (pT-Bra^p-Puro-IRES2-EGFP, pMesP1-EGFP and M50 Super 8x TOPFlash) stabil transfiziert.

Brachyury ist einer der besten Marker für das frühe Pan-Mesoderm. Dessen transiente Expression findet man im gesamten enstehendem Mesoderm und wird herunterreguliert, sobald die mesodermalen Zellen sich zu differenzieren beginnen. **Mesp1** ist ein bHLH Transkriptionsfaktor, der spezifisch in den meisten kardiovaskuläre Vorläufern exprimiert wird, und der für die Entstehung des Herzens notwendig ist. Mesp1 scheint eine Schlüsselrolle in frühen Zeitpunkten der Herzentstehung zu spielen und ist ein vielversprechender Transkriptionsfaktor um weitere direkt beeinflusste Zielgene zu identifizieren. Man weiß, dass der **Wnt Signaltransduktionsweg** die Entstehung des Mesoderms induziert. Seine Aktivierung ist nur vorübergehend und sowohl zeitlich wie auch räumlich reguliert. Wnt ist ein bekannter Aktivator von Brachyury; sein direkter Einfluss auf Mesp1 ist noch nicht nachgewiesen.

Das in vitro Model "Embryoid Bodies" (EBs) wurde verwendet um die Expression von Brachyury, Mesp1 und dem Wnt Signaltransduktionsweg während der frühen Embryogenese zu untersuchen, in "Cardiac Bodies" (CBs) wird die Expression während der Kardiomyogenese widergespiegelt. Die Expressions Werte wurden entweder mittels durchflusszytometrischen Analysen (Brachyury und Mesp1), oder mittels Luminiszenz (Wnt Signaltransduktionsweg) evaluiert.

Im Rahmen dieser Arbeit konnten wir zeigen, dass das Erhöht Grün Fluoreszierende Protein (EGFP), welches von den Promotoren von Brachyury und Mesp1 angeschaltet wurde, am fünften Tag der ESZ Differenzierung transient hochreguliert wurde; zusätzlich konnten wir zeigen, dass die aus KVZ entstandenen CBs fünf Tage in der Entwicklung voraus sind, da das EGFP, angeschalten sowohl vom Brachyury Promoter als auch vom Me-

sp1 Promoter, nur am Beginn der Differenzierung gefunden wurde, und danach abgeschaltet wurde. Zusätzlich konnten wir zeigen, dass die TOPflash Reporter Zelllinie Wnt Signal entsprechend reagierte. Diese Ergebnisse ermöglichten uns den Einfluss eines GSK-Inhibitors (CHIR) und eines TGF-β Inhibitors (SB 431542) auf die Expression der Reportergene zu zeigen.

Diese experimentellen Daten fördern das Verständnis des transkriptionellen Netzwerkes, welches die Herzentstehung reguliert und wird zur Verbesserung von Strategien beitragen, wie man somatische Stammzellen effizient in funktionale Herzmuskelzellen differenzieren kann.

1 Introduction

1.1 The Cardiovascular Disease

By 2030 more than 23 million people will die annually from cardiovascular diseases (CVDs). CVDs are the number one cause of death globally, which equates 30% of all global deaths in 2008 (17.3 million people).

80% of CVD deaths (mainly heart disease and stroke) take place in low- and middle-income countries (WHO 2013).
By reducing risk factors such as tobacco use, unhealthy diet and obesity, physical inactivity, high blood pressure, diabetes and raised lipids most cardiovascular diseases can be prevented.
CVDs include disorders of the heart and blood vessels as listed below:

- coronary heart disease
- cerebrovascular disease
- peripheral arterial disease
- rheumatic heart disease
- congenital heart disease
- deep vein thrombosis and pulmonary embolism

Heart attacks and strokes are usually acute events and are mainly caused by a blockage that prevents blood from flowing to the heart or brain. The most common reason for this is a build-up of fatty deposits on the inner walls of the blood vessels that supply the heart or brain. Strokes can also be caused by bleeding from a blood vessel in the brain or from blood clots (WHO 2013).

The treatment of CVDs is very limited and exists mainly in immediate medical care often followed by a life-endangering open heart surgery. In severe

cases transplantation of the heart is the only possible therapy to survive. Transplantations are burdensome for the patient's physical and psychological well-being. Finding a suitable organ is a long and difficult process. Even after successful transplantation the rejection of the foreign organ has to be avoided by a life-long treatment with immunosuppressive agents. Not only the life expectancy is markedly reduced, also the quality of the patient's life is severely decreased and restricted (Stehlik et al. 2011).

The poor chances of recovery are caused by the very limited regenerative potential of adult hearts. With each heart failure (heart attack, infarction), functional heart cells are lost, which cannot be replaced completely by already described but not yet isolated cardiac stem cells (Hansson, Lindsay, and Chien 2009; Kajstura et al. 2010). In recent years a new approach should be considered as a promising working model - somatic progenitor cells from the adult heart (Taubenschmid and Weitzer 2012). They emerged as a source for cell based treatments of progressive heart failure in the course of a disease or during aging (Leri et al. 2001).

1.2 Stem Cells

1.2.1 Self-renewal and Potency of Differentiation

Stem cells have the capacity to proliferate indefinitely in culture while maintaining the ability to differentiate to form any of the cells of the body. In 1981 the first murine embryonic stem cells (ESCs) were isolated from the inner cell mass (ICM) and cultivated by two independent groups (Evans and Kaufman 1981; Martin 1981); isolation of human ESCs (hESCs) from blastocyts was first achieved in 1998 (Thomson et al. 1998). The knowledge of the unique combination of self-renewal and differentiation suggests that these cells could provide a potentially unlimited source of differentiated cells for the treatment of disease and aging (Johnson et al. 2008).

Stem cells proliferate through symmetric division or self-renew through asymmetric division whilst generating differentiating cell types. Symmetric divisions generate daughter cells with similar fates which leads to an expansion of the stem cell pool, whereas stem cells which divide asymmetrically self-renew whilst also producing differentiating daughter cells and thereby generating cell diversity. The balance between symmetric and asymmetric

divisions must be tightly regulated as excessive symmetric cell division can lead to tumorous overgrowth, whereas precocious asymmetric division results in premature differentiation and underdeveloped organs. A sufficiently large pool of undifferentiated stem cells must be maintained throughout development, to assure the generation of early as well as later born cells, and to repair or regenerate damaged tissue. The tissue homeostasis in the adult organism is obtained by cells, which are kept undifferentiated in niches and serve as a source to replenish differentiating cells after depletion (Egger, Gold, and Brand 2011)

Depending on their differentiation potential, the cells are described by the following terms: totipotent, pluripotent, multipotent and unipotent (Figure 1.1).

Totipotent cells are capable of proliferation, self-renewal and of giving rise to all types of differentiated cells and tissues, including extraembryonic tissues. Totipotency is retained by early progeny of the zygote up to the eight-cell stage of the morula.

Pluripotent cells may differentiate into all types of cells and tissues, except the extraembryonic tissues. Cells dissociated from the inner cell mass (ICM) are pluripotent and contribute to the formation of the three primary germ layers - the endoderm, mesoderm, and ectoderm as well as the primordial germ cells (PGC).

In culture, multipotent cells have been derived from all three embryonic germ layers. These multipotent cells, also described as progenitor cells, are able to give rise to a subset of cell lineages in response to appropriate environmental cues. These cells are often referred to as somatic or adult stem cells (Fischbach and Fischbach 2004; Wobus and Boheler 2005).

Unipotent cells have the capacity to differentiate into only one cell type, for example spermatogonial stem cells in the testis (Donovan and Gearhart 2001).

1.2.2 Pluripotent Stem Cell Lines

So far three types of mammalian pluripotent stem cell lines have been isolated (Figure 1.2): embryonic carcinoma cells (ECCs), the stem cells of testicular tumors; embryonic germ cells (EGCs) derived from primordial germ cells (PGCs) of the post-implantation embryo, and embryonic stem cells (ESCs) derived from pre-implantation embryos (Donovan and Gearhart 2001).

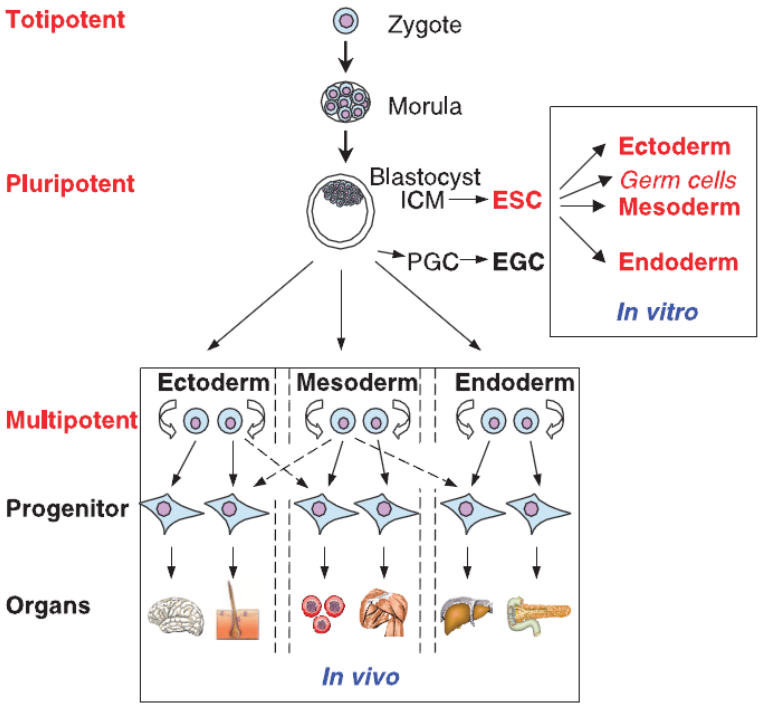


Figure 1.1: Potency of Stem cell
From the zygote to the morula stage, cells are defined as totipotent. At the blastocyst stage the cells of the inner cell mass (ICM) retain the capacity to build up all three primary germ layers (endoderm, mesoderm, and ectoderm) as well as the primordial germ cells (PGCs). Cells derived from the ICM are referred to as embryonic stem cells (ESCs), whereas cells derived from PGCs are referred to as embryonic germ cells (EGCs). Multipotent stem and progenitor cells exist in adult tissues and organs to replace lost or injured cells. Adult stem cells may also develop (transdifferentiate) into cells of other lineages (dashed lines). Progenitor cells differentiate in tissue specific cells contributing to the formation of organs (Wobus and Boheler 2005).

Embryonic Carcinoma Cells (ECCs)

ES cell research dates back to the early 1970s, when the first pluripotent stem cells, embryonic carcinoma (EC) cells (firstly found in teratocarcinomas), were established as cell lines by isolation of testicular germ cell tumors (Kahan and Ephrussi 1970; Rosenthal, Wishnow, and Sato 1970). Teratocarcinomas are gonadal tumors which are composed of undifferentiated ECCs and tissues derived from the three primary germ layers (endoderm, mesoderm and ectoderm) including cartilage, squamous epithelia, primitive neuroectoderm, ganglionic structures, muscle, bone and glandular epithelia. EC cells

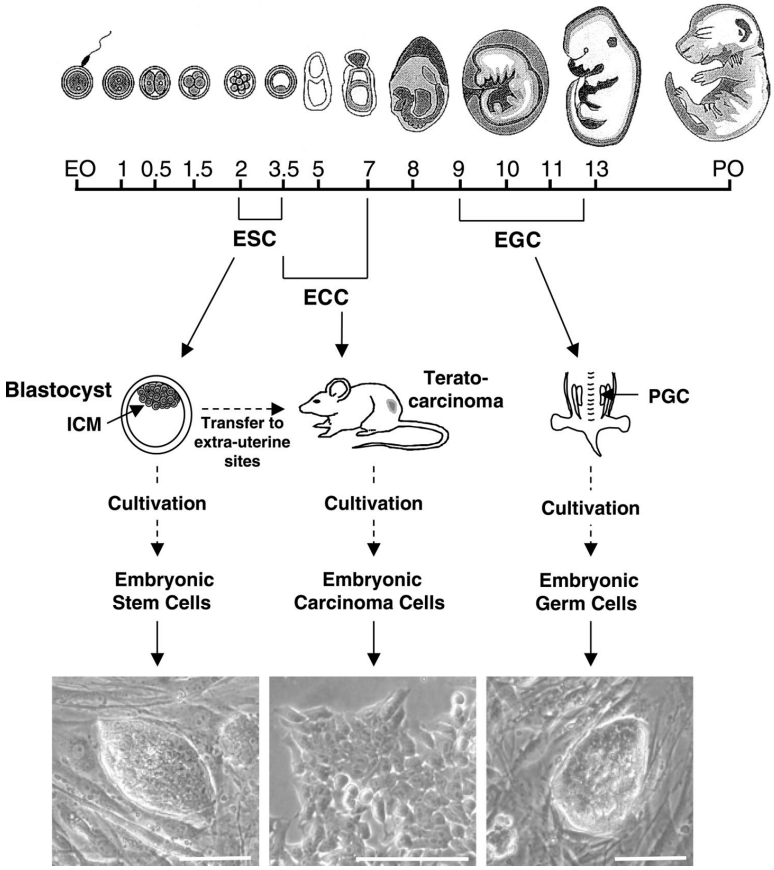


Figure 1.2: Developmental origin of pluripotent embryonic stem cell lines of the mouse. As demonstrated in the scheme the embryonic stem cells (ESC) can be isolated from the blastocyst, embryonic carcinoma cells (ECC) from a teratocarcinoma, and embryonic germ cells (EGC) from primordial germ cells (PGC) respectively. The cell types are isolated at different embryonic stages of the mouse. Bar = 100 μm (Wobus and Boheler 2005).

are derived from the primordial germ cells (PGCs), the embryonic precursors of the gametes. When ECCs are introduced into the ICM of early embryos, they contribute to embryonic development to generate chimeric mice. These cells might show potential alterations as they are derived from teratocarcinomas, hence the next step was the direct in vitro culture of embryonic cells of the mouse (Donovan and Gearhart 2001; Wobus and Boheler 2005).

Embryonic Germ Cells (EGCs)

Murine PGCs were isolated directly from the embryonic gonad and cultivated on feeder cells which led to the establishment of mouse embryonic germ (EG) cell lines. These cells form colonies which are, in most respects, indistinguishable from blastocyst-derived ES cells (Figure 1.2) and are characterized by high proliferative and differentiation capacities in vitro. In contrast to embryonic stem (ES) cells, EG cells retain the capacity to erase gene imprints (Donovan and Gearhart 2001; Wobus and Boheler 2005).

Embryonic Stem Cells (ESCs)

ES cells are derived from the pluripotent inner cell mass (ICM) or epiblast and maintain their pluripotency in culture without any apparent loss of differentiation potential. The maintenance of pluripotency within these cells requires extrinsic factors, either added to the growth medium or provided by growth on a feeder layer of differentiated cells (Smith et al. 1988; Williams et al. 1988). A network of key transcription factors seems to be responsible for this maintenance including the homeodomain transcription factor Oct4 (Nichols et al. 1998; Niwa, Miyazaki, and Smith 2000), the variant homeodomain transcription factor Nanog (Chambers et al. 2003; Mitsui et al. 2003; Kuroda et al. 2005; Hough et al. 2006) and the high mobility group (HMG)-box transcription factor Sox2 (Avilion et al. 2003).

1.2.3 Adult Stem Cell Lines

In the adult soma, stem cells generally are thought of as tissue-specific, able to give rise only to progeny cells corresponding to their tissue of origin. Such tissue-specific stem cells have been described for the adult **bone marrow derived blood cells** (Spangrude, Heimfeld, and Weissman 1988) and **mesenchymal cells** (Friedenstein, Gorskaja, and Kulagina 1976; Friedenstein, Chailakhyan, and Gerasimov 1987; Pereira et al. 1995; Prockop, Gregory, and Spees 2003); **nervous system cells** (PNS: Stemple and Anderson 1993; Morrison et al. 1999; Kruger et al. 2002; and CNS: Uchida et al. 2000; Rietze et al. 2001; Capela and Temple 2002); **heart cells** (Beltrami et al. 2001); **muscle cells** (Mauro 1961); **epidermal cells** (hair bulge - Alonso and Fuchs 2003) and **intestine cells** (Bjerknes and Cheng 1999; Spradling, Drummond-Barbosa, and Kai 2001). For other tissues, including the **liver**

(Forbes et al. 2002; Wang et al. 2003) and the **pancreas** (Bonner-Weir and Sharma 2002; Murtaugh and Melton 2003), the identity of resident stem cell population(s), and even whether regeneration of these tissues in adults is stem cell mediated, is still debated (Wagers and Weissman 2004).

Tissues, such as the blood, skin, gut, respiratory tract and testis, must constantly renew, whereas the majority of cells and tissues in adult mammals exhibit very low turnover under normal circumstances. The response to regenerative pressure diverges greatly in different organs. While the liver for example responds quite well, the heart responds only poorly. Organs, including the heart or the brain, were once thought of as non-renewing, postmitotic tissues, but are now known to have a minor, but existing regenerative potential (Altman and Das 1965; Kajstura et al. 1998; Kuhn, Dickinson-Anson, and Gage 1996; Altman 1969; Rumyantsev and Borisov 1987; Lois and Alvarez-Buylla 1993). Since then the field of adult stem cells grew broader to identify additional tissue-resident adult stem cell populations and evaluate their regenerative potential. Until now the degree to which particular tissues depend upon replenishment of mature cells from relatively undifferentiated stem and progenitor cells is poorly understood, especially the role of endogenous stem cells in tissue homeostasis or responses to damage in other systems remains unclear and has to be further investigated (Wagers and Weissman 2004).

1.2.4 Cardiovascular Progenitor Cells

The mammalian heart was believed to be a post-mitotic organ without any regenerative potential. The number of myocytes was thought to be relatively stable but slowly diminishing throughout an organism's lifetime, without being completely regenerated after injuries (Beltrami et al. 2003). Heart stem cells or cardiovascular progenitor cells (CVPCs) in adult human hearts were first identified by Beltrami and collaborators in 2001 (Beltrami et al. 2001). CVPCs were also found in adult murine hearts (Messina et al. 2004; Tallini et al. 2009).

Mesodermal progenitor cells contributing to the myocardium are supposed to be the first that develop during gastrulation as cardiomyogenesis is the first organ-forming process in embryogenesis. Within the heart, CVPCs have been identified in epicardial tissue (Limana et al. 2007), ventricular tissue (Galvez et al. 2008) (there referred to as mesoangioblasts), and heart

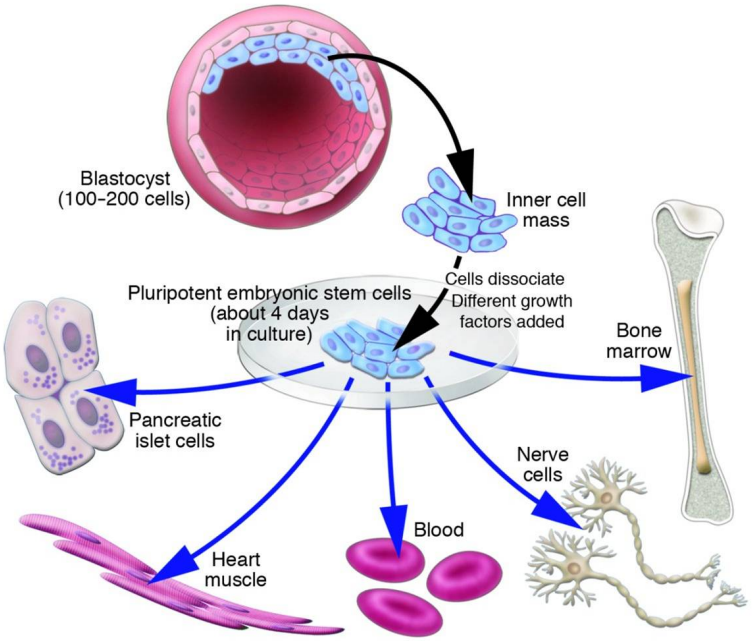


Figure 1.3: Pluripotent Stem Cells
Cells, isolated from the inner cell mass (ICM), have the ability to give rise to all types of cells in the mammalian body including pancreatic islet, heart, blood, nerve and bone marrow cells (Fischbach and Fischbach 2004).

auricles (Gambini et al. 2011). Surface markers were used to identify and isolate CVPCs, such as stem cell antigen-1 (Sca1) (Matsuura et al. 2004; Tateishi et al. 2007; Smits et al. 2009), the drug extruder MDR-1 (Oh et al. 2003), the vascular endothelial growth factor receptor Flk1 (Kattman, Huber, and Keller 2006; Yang et al. 2008), the stem cell factor receptor c-Kit (Beltrami et al. 2003; Bearzi et al. 2007, Bearzi et al. 2009; Tallini et al. 2009; Gambini et al. 2011), and the transcription factor Islet-1 (Isl-1) (Laugwitz et al. 2005; Moretti et al. 2006; Laugwitz et al. 2008).

In vitro CVPCs differentiate only to cardiomyocytes (CMCs), vascular endothelial cells (ETCs), and smooth muscle cells (SMCs) (Srivastava, Cserjesi, and Olson 1995; Bearzi et al. 2007; Oyama et al. 2007; Wu, Chien, and Mummery 2008; Smits et al. 2009).

Early cardiac development is influenced by factors secreted from neighboring extra-embryonic endoderm (Schultheiss, Xydas, and Lassar 1995). The group of Weitzer and others have contributed to this field by demon-

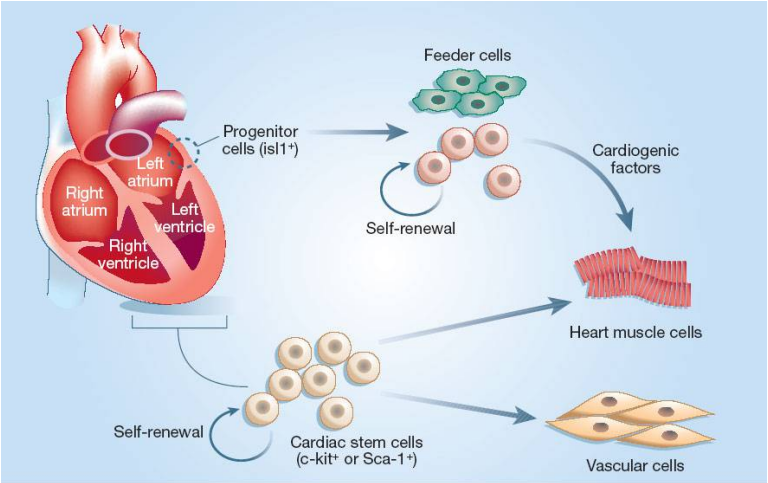


Figure 1.4: Origin of CVPCs
Cardiac progenitors are identified by the expression of the islet-1 gene (isl1*) and are isolated mainly from the heart atrium. Co-culture with feeder cells retains the self-renewal capacity, whereas cardiogenic factors lead to differentiation into mature cardiac cells. Other cardiac stem cells were isolated from the heart which expressed cell-surface proteins (either c-kit or Sca-1) but not isl1*. These markers are also associated with bone-marrow derived stem cells and differentiate not only in cardiac cells (Mummery 2005).

strating the paracrine influence of Leukemia Inhibitory Factor (LIF) (Bader, Al-Dubai, and Weitzer 2000; Bader et al. 2001), Bone Morphogenic Protein 2 (BMP2) (Behfar et al. 2002), and Secreted Protein Acidic and Rich in Cysteine (SPARC) on early cardiomyogenesis (Stary et al. 2005). They showed that SPARC substantially increases the number of cardiomyocytes in CVPCs. LIF maintains self-renewal of CVPC-aggregates but it significantly reduces BMP2 inducted heart cell differentiation.

Although the adult heart is mainly composed of terminally differentiated cells, it is not a terminally differentiated organ as it contains stem cells supporting its regeneration. The existence of these cardiac stem cells or CVPCs opens new opportunities for myocardial repair.

1.2.5 The Stem Cell Niche

Stem cell niches are discrete microenvironments, where stem cells and progenitor cells remain in an undifferentiated and quasi dormant state until external signals stimulate differentiation to specific somatic cells (Hsu and

Fuchs 2012; Hoebaus et al. 2013). The "niche" hypothesis was postulated in 1978 by Schofield, who described it as a physiologically limited microenvironment that supports stem cells (Schofield 1978). Although there were several co-culture experiments done, which supported this hypothesis (Dexter, Moore, and Sheridan 1977; Rios and Williams 1990; Brinster and Zimmermann 1994; Roecklein and Torok-Storb 1995; Sitnicka et al. 1996; Moore, Ema, and Lemischka 1997), only in 2000 the first stem cell niche was located and described in molecular terms by Xie and colleagues in Drosophila melanogaster ovaries (Xie and Spradling 2000) and, in 2001, in Drosophila testis by two independent groups (Kiger et al. 2001; Tulina and Matunis 2001). This was a result of lineage tracing and laser ablation experiments, which were accomplished by several groups over the last decades (Wieschaus and Szabad 1979; Lin and Spradling 1993). The first niches found in mammals were the bulge area of hair follicles, and the intestinal stem cell location near the crypt base, identified by the adult stem cell's ability to retain the BrdU or 3H-thymidine labels (Cotsarelis, Sun, and Lavker 1990; Potten, Owen, and Booth 2002). Recently, there has been significant progress regarding stem cells and their surrounding microenvironments in a variety of mammalian models.

The nature and location of niches varies depending on the tissue type. Its main function is to sustain tissue homeostasis after birth. Adult stem cells, including both germ line stem cells (GSCs) for reproduction and somatic stem cells (SSCs) for organogenesis, reside in these special microenvironments, where they support ongoing tissue regeneration, replacing cells lost due to natural cell death (apoptosis) or injury. Throughout the organism's life span a balance between self-renewal and differentiation must be maintained. This is achieved by environmental regulatory signals on the one hand and intrinsic programs on the other hand (Morrison et al. 1997; Li and Xie 2005).

The common features, structures, and functions of the stem cell niche can be outlined as followed:

The stem cell niche structure differs depending on the tissue they are located in; different cell types can provide the niche environment. The niche can be seen as a physical anchor for stem cells, supported by adhesion molecules, such as E-cadherin (Song and Xie 2002) or N-cadherin (Calvi et al. 2003, Zhang et al. 2003). Extrinsic factors, produced by the niche, control stem cell

fate and number. Numerous signal molecules are involved in the regulation of stem cell behavior, including shh, Wnts, BMPs, FGFs, Notch, SCF, Ang-1, and LIF or Upd through the JAK-Stat pathway. Especially BMP and Wnt signaling pathways have emerged as common pathways for controlling stem cell self-renewal and lineage fate from Drosophila to mammals (Gomes et al. 2002; Ivanova et al. 2002; Park et al. 2002; Ramalho-Santos et al. 2002; Akashi et al. 2003). As many of these regulatory pathways are conserved, it led to the conclusion that orchestration of these signals is essential for proper regulation of stem cell self-renewal and lineage commitment (Li and Xie 2005). The asymmetric structure of the stem cell niche is found in invertebrates and mammals. After division, one daughter cell is maintained in the niche as a stem cell (self-renewal), the other daughter cell leaves the niche to proliferate and differentiate, eventually becoming a functionally mature cell (Li and Xie 2005).

1.3 The Embryoid Body Model System

Embryonic stem cells (ESCs) can be driven into differentiation by specific culture conditions, including the plating of ES cells at a relatively low density (10^4 cells/cm^2), withdrawal of LIF, and addition of BMPs or the teratogen retinoic acid (RA) either in the presence or absence of serum. Using this knowledge an in vitro model mimicking early embryogenesis was established - aggregated ESCs, named embryoid bodies (EBs). EBs provide the environment for lineage commitment to the ectodermal, mesodermal and endodermal fate (Wobus et al. 1984; Desbaillets et al. 2000; Dvash, Ben-Yosef, and Eiges 2006; Yamanaka et al. 2008). These aggregates are prepared in the absence of self-renewal signals, either in hanging drops, in liquid “mass culture”, or in methylcellulose. Especially the hanging drop technique is frequently used, as it enables reproducible aggregation of ESCs. This limits the variations in the developmental outcome, which is highly dependent on cell number (Bader et al. 2001; Boheler et al. 2002; Yamanaka et al. 2008). In contrast to the in vivo cell number of the morula (16 - 64 cells), 600 - 800 embryonic stem cells per EB are needed to assure the formation of the three germ layers (Bader et al. 2001; Dang et al. 2004). Once ES cells have successfully aggregated for 2 days into small spheres of approximately 50 - 100 μm, the irregular surface of the aggregates smooths to resemble (mor-

phologically) morula compaction, but by days 4 - 5 of aggregation, the EB is composed of an inner epiblast-like and an outer primary endoderm-like structure (Figure 1.5) (Yamanaka et al. 2008). Thereafter the aggregated EBs are pseudo-implanted by adhesion to gelatinized tissue culture plates. Occasionally a horseshoe-shaped ridge of cells is formed from the dense center of enlarging EBs (Weitzer 2006). Cells in the area encircled by the horseshoe-shaped ridge of cells will form the primitive mesoderm, identifiable by the expression of Brachyury, an early marker of mesodermal fate (Bader et al. 2001). Hematopoietic cells and spontaneously and rhythmically contracting cardiomyocytes (CMCs) arise from these mesodermal precursors. The first contracting CMCs are found as the heart tube forms at E7.5 to E8.0 from the splanchnic or lateral plate mesoderm. Nonetheless, lineage development in EBs which commences during gastrulation in vivo has until now been mainly considered to be chaotic (Fuchs et al. 2012).

1.4 Cardiomyogenesis in Mammals

Cardiomyogenesis (Figure 1.6 and Figure 1.7) is the first organ-forming process in mammalian embryogenesis, hence the commitment towards cardiac fate is taken early in the developmental process (Van Vliet et al. 2012). Development of the heart tube into the mature four-chambered mammalian heart requires multiple steps that depend on a genetic program (Buckingham, Meilhac, and Zaffran 2005).

In vivo heart development starts around day 7.0 p.c. in mice during gastrulation. The cells of the mesoderm, designated to form the heart, are located in the anterior region of the primitive streak from where they migrate to the anterior-lateral region, now regarded as the primary heart field (Rosenquist 1970; Tam et al. 1997). This horseshoe-like structure (Figure 1.7) fuses and forms a heart tube, which connects with the body through a posterior inflow, or venous pole, and an anterior outflow, or arterial pole. The following remodeling steps lead to the formation of primitive ventricles and atria. Myocardial precursors of the primary heart field support the formation and growth of the atria, whereas cells from the anterior (or second) heart field support the growth of the outflow tract (OT; also referred to as conotruncus) and right ventricle. Non-mesodermal cells from the neural crest (cardiac neural crest cells) participate mainly to the septation of the heart and formation

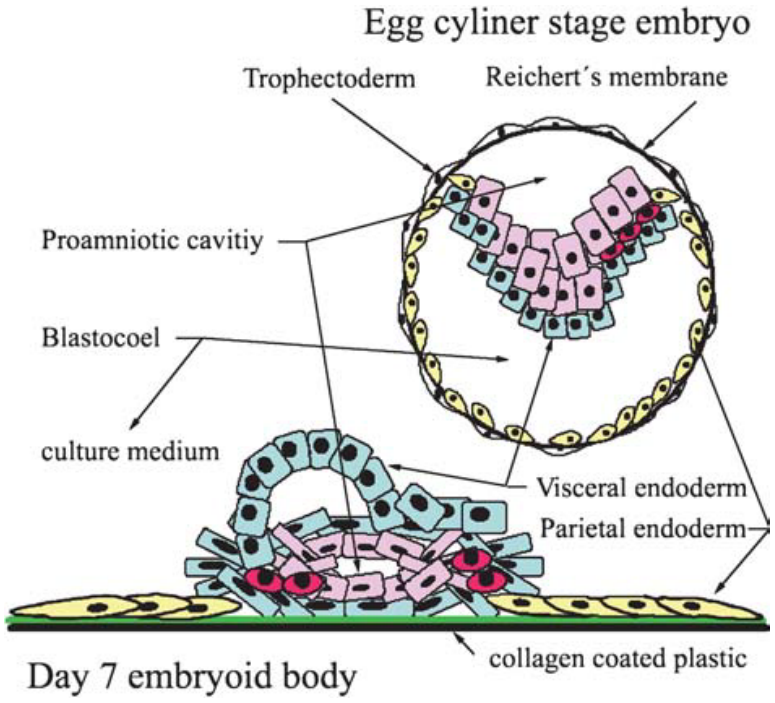


Figure 1.5: Schematic comparison of embryoid body (EB) differentiation and early gastrulation of a murine embryo, highlighting the collagen matrix in green, where the EB is attached, its in vivo pendant is the Reichert's membrane (black line) and, later on, the parietal endoderm (yellow cells). The visceral and embryonic endoderm cells are marked in blue, the primitive ectoderm cells in light pink, and the mesodermal cells in red, respectively (Weitzer 2006).

of the cardiac valves (Kirby, Turnage, and Hays 1985; Xu and Baldini 2007) to build the four-chambered heart of a mature organism.

Induction and specification of cardiac mesoderm seem to be early signals of adjacent endoderm, mainly caused by BMP and FGF. Wnts inhibit cardiogenesis in mammals, by negatively influencing the cardiac crescent cells (Cripps and Olson 2002).

1.5 The Gene Regulatory Network in Cardiogenesis

Cardiomyogenesis is a multi-step process triggered by many different factors as illustrated in Figures 1.6 and 1.7. Beside Mesp1, Nkx2.5 is one of the earliest cardiac markers, which is expressed in cardiogenic mesoderm concomitant with specification of the lineage and is maintained in the heart

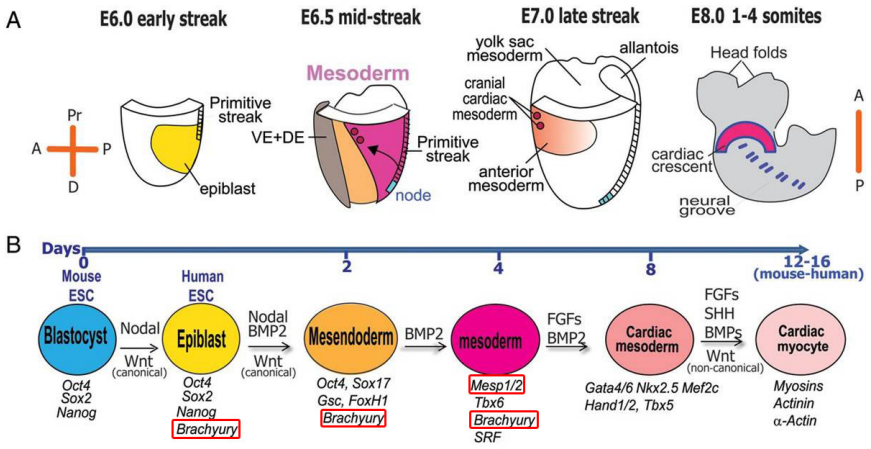


Figure 1.6: Cardiomyogenesis
Comparison of cardiac ES cell differentiation and early embryonic heart development. (A) Temporal progress and embryonic stages of cardiogenesis in mouse embryo. (B) Temporal progress and embryonic stages of ESC differentiating towards the cardiac fate. ExE - extraembryonic ectoderm; VE - visceral endoderm; DE - definitive endoderm; Brachyury and Mesp1 are highlighted in red; the body axes are marked in orange (Van Vliet et al. 2012).

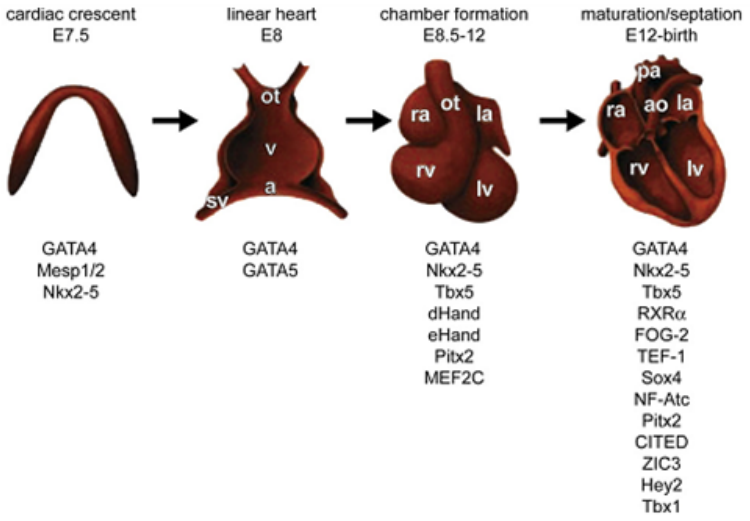


Figure 1.7: Schematic representations of the major stages of murine heart development. The transcription factors are spatio-temporally regulated. a - atria; ao - aorta; la - left atrium; lv - left ventricle; ot - outflow tract; pa - pulmonary aorta/trunk; ra - right atrium; rv - right ventricle; sv - sinus venosus; v - ventricle (Komati 2013).

until adulthood (Lints et al. 1993; Komuro and Izumo 1993). Two independent enhancers of Nkx2.5 contain tandem GATA binding sites that are required for cardiac expression of which one is driven by BMP signaling. This enhancer harbors Smad binding sites that appear to be required for Nkx2.5 expression at all stages of cardiac development (Liberatore et al. 2002).

Besides members of the GATA family also those of Mef2 (especially Mef2c) play key roles in the cardiomyocyte differentiation in mammals. They activate cardiac structural genes and are implicated in the early steps of morphogenesis of the heart tube. GATA-4/5/6 are expressed in the cardiac lineage at various stages of development (Laverriere et al. 1994; Jiang and Evans 1996). Additionally to the regulation of Nkx2.5 expression, they control several downstream cardiac muscle structural genes by forming complexes with transcription factors, including Nkx2.5 and Mef2 (Cripps and Olson 2002). Mef2 mutation assays in mice elucidated its function as a direct activator of myocyte differentiation genes (Lin et al. 1997; Lin et al. 1998).

Morphogenesis and patterning of the mammalian heart is triggered by several transcription factors, including the bHLH factors dHand and eHand (Srivastava, Cserjesi, and Olson 1995). Loss-of-function mutations of mouse dHand result in ablation of the right ventricular chamber (Srivastava et al. 1997). Another important factor expressed in the developing heart is the T-box transcription factor Tbx5. It acts synergistically with Nkx2.5 to activate the atrial natriuretic factor (ANF) and connexin 40 genes (Durocher et al. 1997; Bruneau et al. 2001). Mutations in Tbx5 in mice result in severe abnormalities of the heart including septal defects and conduction abnormalities.

The important role of Brachyury, Mesp1 and the Wnt signaling pathway in cardiomyogenesis is explained hereinafter in more detail, as those were mainly used for further investigations during this thesis.

1.5.1 Brachyury

Brachyury and Early Development in the Mouse

In 1990, Brachyury (T) was the first molecularly characterized T-box gene (Herrmann et al. 1990). It is a highly conserved transcription factor identified in many organisms including frog, fish and mouse (Holland et al. 1995). The first phenotype of Brachyury mutant mice was already described in 1927 by Dobrovolskaia-Zavadskaia, where heterozygous T mice showed a

truncated tail; "T" stands for shorttail (Dobrovolskaia-Zavadskaia 1927). Homozygous $T^{-/-}$ mice are not viable and die shortly after gastrulation. They display severe mesodermal abnormalities, including complete loss of the posterior mesoderm (Gluecksohn-Schoenheimer 1938; Gluecksohn-Schoenheimer 1944). Usually during gastrulation the three primary germ layers become arranged and, amongst others, the primitive streak is formed, which is known to give rise to mesodermal and endodermal cells. As the embryo develops, mesodermal cells leave the streak, migrating to lateral and dorsal positions, and subsequently form axial and paraxial mesoderm structures such as the notochord and somites (Beddington 1982; Tam and Beddington 1992). In $T^{-/-}$ embryos, the primitive streak is condensed and thick compared to wild-type littermates (Chesley 1935; Gluecksohn-Schoenheimer 1938; Gluecksohn-Schoenheimer 1944; Gruneberg 1958). This might be because $T^{-/-}$ mutant embryonic stem (ES) cells are compromised in their ability to migrate away from the primitive streak, and this leads to their accumulation in the primitive streak (Rashbass et al. 1991; Wilson, Rashbass, and Beddington 1993; Wilson et al. 1995; Wilson and Beddington 1997). This accumulation and defects in migration cause severe failures: firstly it eventually leads to loss by programmed cell death (Conlon and Smith 1999); secondly it also affects extra-embryonic mesoderm and the formation of the allantois, which subsequently leads to embryonic lethality at approximately E10.5 as the mutant embryos lack a proper placental connection (Gluecksohn-Schoenheimer 1938; Gluecksohn-Schoenheimer 1944; Wilson, Rashbass, and Beddington 1993). Furthermore $T^{-/-}$ mutants lack notochord in posterior portions of the embryo and numerous other phenotypic abnormalities have been reported (Chesley 1935; Gruneberg 1958; Yanagisawa 1990; Beddington, Rashbass, and Wilson 1992; Herrmann and Kispert 1994). Chimeric and phenotypic analyses have shown that tissues directly affected by loss of Brachyury function are primarily those in which the gene is expressed. All these findings suggest that Brachyury is required during gastrulation for the mesodermal development and it may have a second function in maintaining the differentiated state in the notochord (Showell, Binder, and Conlon 2004).

Function of Brachyury

Brachyury is a transcription factor, which functions cell autonomously, is localized in the nucleus, binds DNA in a sequence-specific manner, and can

regulate transcriptional levels of heterologous and downstream target genes in several different contexts (Kispert and Hermann 1993; Kispert, Koschorz, and Herrmann 1995; Conlon et al. 1996). Brachyury binds as a homodimer the N-terminal region of the protein to a palindromic DNA consensus sequence, known as the T-site or T-box binding element (TBE) (Kispert and Hermann 1993; Conlon et al. 1996); with deletion analyses these two groups were able to map the regions both necessary and sufficient for activation in mouse (Kispert, Koschorz, and Herrmann 1995), Xenopus and zebrafish (Conlon et al. 1996). In mice four domains, two activating and two repressive, were identified. Brachyury acts as a transcriptional activator activating mesoderm-specific genes endogenously and its sequence, expression, and molecular function are evolutionarily conserved (Showell, Binder, and Conlon 2004).

Downstream Targets of Brachyury

Two approaches have been taken to identify direct targets of Brachyury, a candidate and a directed approach. This is very important to fully understand the molecular and cellular level of Brachyury. The candidate approach elucidated embryonic fibroblast growth factor (eFGF) as a target in Xenopus, which is co-expressed in the nascent mesoderm and developing notochord (Isaacs, Tannahill, and Slack 1992; Isaacs, Pownall, and Slack 1994; Isaacs, Pownall, and Slack 1995). Casey and coworkers used the eFGF promoter and demonstrated its activation by Brachyury (Casey et al. 1998). Functional screens conducted in Xenopus and ascidians have identified all other known targets of Brachyury including four highly related homeobox genes Bix1-4 (Tada et al. 1998) which are coexpressed with Xbra (Brachyury gene in Xenopus) in the early mesoderm. Like Xbra, Bix1 and Bix4 can be induced by activin and BMP, both mesoderm growth factors (Tada et al. 1998; Casey et al. 1999). Xwnt-11 was also identified as a potential downstream target of Brachyury (Smith et al. 2000); like Xbra it is required for convergent extension movements in both, Xenopus and zebrafish, and it shows an almost identical expression pattern during early development (Showell, Binder, and Conlon 2004).

Regulation of Brachyury

Besides Eomesodermin and VegT, Brachyury plays an indispensable role in the induction and formation of mesoderm. Its expression has to be precisely regulated temporally and spatially. Most findings were achieved by studies conducted in Xenopus. Early experiments demonstrated that, after treatment with either one of two candidate mesoderm inducing factors: activin A (a TGF-β family ligand) or basic FGF, the expression of Xbra was induced in animal caps as an immediate early response (Smith et al. 1991). This was confirmed by later studies, indicating that both TGF-β and FGF signaling are required for Xbra expression in the embryo (Amaya et al. 1993; Hemmati-Brivanlou and Melton 1992). TGF-β and FGF signaling pathways are necessary for the initiation of Xbra expression; additionally FGF signaling seems to maintain expression during subsequent development. Xbra expression also seems to be maintained by an autoregulatory loop by the co-expression of eFGF (Isaacs, Tannahill, and Slack 1992; Isaacs, Pownall, and Slack 1995; Casey et al. 1998), supported by the finding that eFGF or FGF2 can activate expression of Xbra through the RAS/RAF/MAPK signaling pathway (Smith et al. 1991, Smith et al. 1997; Isaacs, Pownall, and Slack 1994; Schulte-Merker and Smith 1995), whereas inhibition of the FGF/RAS/RAF/MAPK signaling pathway leads to inhibition of Xbra expression (Amaya et al. 1993; Umbhauer et al. 1995). The autoregulatory loop of eFGF and Xbra is not only required during gastrulation but also for the formation of the notochord.

The minimal region both necessary and sufficient for the expression of Brachyury was identified by transgenic studies in mouse and frog (Stott, Kispert, and Herrmann 1993; Clements et al. 1996; Latinkić et al. 1997; Lerchner et al. 2000). This promoter region shows a strong conserved E-box and two canonical Lef1/Tcf1 binding sites, which are involved in the Wnt signaling pathway. Mutations of the Lef1/Tcf1 binding sites and other Wnt deficient embryo studies indicate a requirement for Wnt signaling in the induction of Brachyury (Yamaguchi et al. 1999; Galceran, Hsu, and Grosschedl 2001).

But as Wnt family members cannot induce mesoderm, additional factors must be involved in Brachyury induction, such as members of the TGF-β and FGF families. Additional studies within the Xbra minimal promoter

revealed the existence of FGF and activin responsive elements (Latinkić et al. 1997; Lerchner et al. 2000). Three factors - Goosecoid, Mix.1, and Xotx2 - were shown to bind to these sites and, in further consequence, repress Xbra expression (Latinkić et al. 1997). These three factors are present in the early embryo, and Goosecoid as well as Mix.1 are strongly induced by increasing activin levels. Surprisingly, this region of the Xbra promoter shares no apparent homology to the mouse promoter.

1.5.2 Mesoderm posterior 1 (Mesp1)

Mesp1 and Early Development in the Mouse

Mesp1 was first found in the posterior part of the murine embryonic mesoderm and was therefore called mesoderm posterior 1 (Saga et al. 1996). It is a basic helix-loop-helix (bHLH) transcription factor, which is expressed in nascent mesoderm at the onset of gastrulation (from E6.5), specifically in almost all cardiovascular precursors. Mesp1 is expressed after cells have entered the primitive streak (PS) and is rapidly downregulated as they leave the PS. Saga and co-workers demonstrated that Mesp1-expressing cells that exit the PS are incorporated into the heart field and the head mesenchyme. The definitive demonstration was done using genetic lineage tracing experiments in mice, which revealed that cells that expressed Mesp1 at one point of the development are found in all cardiac lineages including the myocardium, the endocardium, the conduction cells and the epicardium (Saga et al. 1999; Saga, Kitajima, and Miyagawa-Tomita 2000). The data collected by Saga and colleagues indicate that almost all cardiac cells are derived from Mesp1-expressing cells including cardiovascular progenitor cells (CVPCs) of both heart fields (Saga et al. 1999; Saga, Kitajima, and Miyagawa-Tomita 2000; Bondue and Blanpain 2010).

Function and Regulation of Mesp1

Experiments executed with Mesp1/2 knock-down mice revealed that Mesp2, usually expressed at lower levels and at a later point in time, plays a redundant role during the early stages of gastrulation. Like Mesp1, Mesp2 controls the exit of mesoderm precursors out of the PS. In later stages of mesodermal development however, the redundancy is lost, as Mesp1 controls multipotent cardiovascular progenitors migration, whereas Mesp2 is responsible for

somitogenesis (Saga et al. 1997; Kitajima et al. 2000).

Applying human Mesp1 in two-cell stage embryos of Xenopus laevis led to the formation of ectopically functional cardiomyocytes indicating that the expression of Mesp1 is sufficient to drive cells into cardiac lineage (David et al. 2008).

When ESCs are driven into differentiation they mimic early embryonic development, displaying a PS-like stage and expressing the same transcription factors as in vivo (Kattman, Huber, and Keller 2006; Murry and Keller 2008). Mesp1 expression is detected shortly after the onset of Brachyury. According to literature Brachyury is expressed between day 0 and day 7, and its peak is described between day 2 - 4 followed by the expression of Mesp1, between 6 hours and up to 2 days later (Kouskoff et al. 2005; Liu et al. 2007; Ueno et al. 2007; Bondue et al. 2008; Lindsley et al. 2008; Barruet et al. 2011). The core gene regulatory network of cardiovascular differentiation machinery such as Nkx2.5, Gata4, Hand2, and Mef2c are expressed soon after Mesp1 (Bondue and Blanpain 2010). Different groups have shown that Mesp1 promotes cardiomyogenesis only when expressed transiently; it was shown that continuous expression of Mesp1 inhibits cardiac differentiation (Kouskoff et al. 2005; Liu et al. 2007; Ueno et al. 2007; Bondue et al. 2008; Lindsley et al. 2008). Increased Mesp1 expression was proven to promote differentiation of ESCs into all types of cardiomyocytes of both heart fields including atrial and ventricular cardiomyocytes, as well as pacemaker-like cells; furthermore Mesp1 promotes endothelial and smooth muscle cells (Bondue et al. 2008; David et al. 2008; Lindsley et al. 2008). The potential of Mesp1 to promote cardiovascular differentiation is limited to PS derived cells and is dependent on other factors as well. Fibroblasts, for example, could not be induced by Mesp1 (Takeuchi and Bruneau 2009; Ieda et al. 2010).

Wnt signaling has a regulatory effect on cardiac mesoderm formation but its influence on the expression of Mesp1 remains contradictory as described in Section 1.5.3 (Bondue and Blanpain 2010).

Downstream Targets of Mesp1

Bondue and coworkers suggested that Mesp1 drives cells in the cardiac specific lineage through an inductive mechanism (Bondue et al. 2008). To elucidate this assumption, Mesp1 target genes were determined. 423 genes were identified which were differentially regulated 12 hours after the expression

of Mesp1. These were mainly cardiovascular transcription factors such as Hand2, Myocardin, Nkx2.5, Gata4, Mef2c, Tbx20, FoxH1, Foxc1, and Foxc2 (Bondue et al. 2008) and other cardiac structural genes such as Myh6 (α-MHC), Myl1 (MLC1f), Myl2 (MLC2v), Myl7 (β-MHC), and Tnnt2 (cTnT) (Lindsley et al. 2008). Chromatin immunoprecipitation experiments strongly suggest a direct binding of Mesp1 to the promoter regions of Nkx2.5 and Hand2 (Schwartz and Olson 1999; McFadden et al. 2000; Bondue et al. 2008). Myocardin, another key downstream effector of Mesp1, regulates SRF and Mef2c activity and activates cardiac and smooth muscle gene expression (Pipes, Creemers, and Olson 2006).

Mesp1 does not only have an activating function, it was also shown to repress the expression of diverse genes involved in PS formation (Brachyury and FGF8) or early endoderm cell fate specification (Sox17, Nodal, Gsc and FoxA2). Additionally Mesp1 binds to itself, firstly promoting transiently its own expression followed by a long lasting repression of its mRNA expression (Bondue et al. 2008).

Still many questions have to be answered how exactly Mesp1 is involved in cardiac specific differentiation and which and how transcription factors are regulated.

1.5.3 Wnt Signaling Pathway

Wnt Signal Transduction

Figure 1.8 illustrates a simplified version of the main components of the canonical Wnt signaling pathway. Binding of Wnts to Frizzled receptors results in inhibition of GSK-3β, APC, and Axin proteins, all known to be β-catenin inhibitors. By blocking these inhibitors, β-catenin is stabilized and promotes its translocation into the nucleus. There it initiates together with Tcf/Lef proteins the transcription of its target genes such as Fibronectin, cMyc, Cyclin D1, TCF-1, etc., all involved in development.

The Wnt signaling pathway is inhibited by extracellularly secreted molecules, including members of the secreted Frizzled-related protein family (sFRPs) and Dickkopf-like proteins (Dkk). Wnt signaling is highly regulated during development. Vertebrate genomes contain several different genes encoding Wnts and Wnt antagonists, precisely locally expressed at specific points in time. The complexity of this precisely timed pathway comprises

still a lot of questions to be answered, most likely elucidated by the usage of reporter constructs (Petersen and Reddien 2009).

While some studies indicated a negative influence of Wnt/β-catenin signaling on cardiomyogenesis (Marvin et al. 2001; Schneider and Mercola 2001; Yamashita et al. 2005; Naito et al. 2006), others suggested a positive role in cardiomyogenesis (Keegan et al. 2005; Lindsley et al. 2006). Ueno and colleagues hypothesized that Wnt/β-catenin signaling acts bisphasically, saying that it influences cardiomyogenesis either positively or negatively depending on time. They found out that Wnt/β-catenin signaling before gastrulation promotes cardiac differentiation, whereas signaling during gastrulation inhibits heart formation (Ueno et al. 2007).

In context of this thesis I will mainly focus on the regulatory role of Wnt signaling on Brachyury and Mesp1 expression during cardiomyogenesis.

Wnt's Influence on Brachyury Expression

Brachyury, a transcription factor enriched in precardiac mesoderm, is a first indicator for ongoing cardiomyogenesis. It is usually expressed throughout the primitive streak, the node and the notochord. It was shown that not all Wnts induce Brachyury expression; Wnt3a$^{-/-}$ embryos showed phenotypical similarities with Brachyury$^{-/-}$ embryos, suggesting that Wnt3a plays a role in Brachyury expression. Indeed Wnt3 was identified to play a regulatory role during early mesoderm formation, whereas Wnt3a controls the expression of Brachyury in the paraxial mesoderm and tailbud (Liu et al. 1999; Yamaguchi et al. 1999; Arnold et al. 2000). Both, Wnt3a and Brachyury, are essential for the formation of the anterior - posterior (A-P) body axis, demonstrated by Wnt3a and T mutant phenotypes; as even heterozygous mice show severe phenotypical changes, Wnt3a as well as Brachyury seem to be regulated in a dose dependent manner (Yamaguchi et al. 1999).

Even though the initiation of Brachyury transcription is independent of Wnt3a, as it is expressed prior to the onset of Wnt3a at E7.5, Wnt3a$^{-/-}$ embryos showed a complete lack of Brachyury expression in the anterior half of the primitive streak. This indicates a specific role of Wnt3a on Brachyury expression. Cells originating from this area usually give rise to paraxial mesoderm (Yamaguchi et al. 1999).

Yamaguchi and coworkers were able to demonstrate the direct interaction of Wnt3a with Brachyury by promoter studies. They identified two canonical

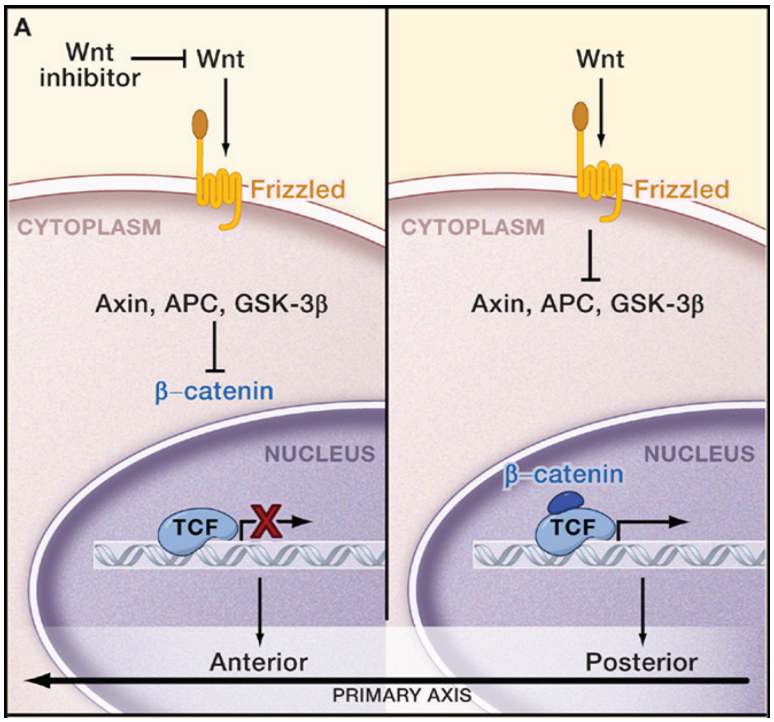


Figure 1.8: The canonical Wnt signaling pathway. (Left panel) Wnt signal inhibition leads to the degradation of β-catenin through interactions with Axin, APC, and the protein kinase GSK-3β. (Right panel) Wnt proteins bind to the Frizzled complex at the cell surface. These receptors repress Axin, APC, and GSK-3β. As a consequence, the degradation of β-catenin is inhibited, it accumulates in the cytoplasm and nucleus, where it interacts with Tcf sites and initiates transcription (Petersen and Reddien 2009).

Lef1/Tcf1 binding sites located within a proximal 500-bp region of the murine T promoter. These sites are essential for this direct interaction of Wnt3a and for the expression of Brachyury in the primitive streak (Yamaguchi et al. 1999).

Ueno and coworkers demonstrated that Brachyury was accelerated expressed, when treated with Wnt-3a during day 3 - 5 of embryoid body (EB) aggregation. Furthermore Wnt-3a dramatically increased the amount of EBs with contracting cardiomyocytes (Ueno et al. 2007).

The findings mentioned above emphazise Wnt3a as the ultimate modulator of mesodermal fates during gastrulation as well as the A-P axis development and its important role on Brachyury expression.

Wnt's Influence on Mesp1 Expression

The influence of Wnt on the expression of Mesp1 is highly debated, but still quite uncertain. Although canonical Wnt signaling is critical for the heart formation during early embryonic development, a direct activation of Mesp1 by Wnt signaling was not found so far. Most likely Mesp1 expression superimposes the expression of Wnt activity (Bondue and Blanpain 2010). Lindsley and coworkers believe Mesp1 acts Wnt independently (Lindsley et al. 2008), others believe Wnt is a downstream target of Mesp1. David and coworkers demonstrated that Mesp1 upregulates the Wnt inhibitor Dkk-1. They could show that Dkk-1 expression in the anterior cardio-cranial mesoderm is Mesp1 dependent, indicating Mesp1s negative influence on Wnt signaling (David et al. 2008). Thinking of the biphasic effect of Wnt on cardiomyogenesis described above, this seems very likely, as Wnt3a has to be downregulated later in development. In 2013 a paper was published by Li and colleagues where they suggest that Oct4 and canonical Wnt Signaling regulate Mesp1 through a Tcf/Lef-Oct4 composite element, which they found on a 6012bp upstream region of the Mesp1 gene. They believe that this is used as a switch from pluripotency to differentiation (Li et al. 2013).

Still there are many questions to be answered to elucidate the interaction of Wnt signaling with Mesp1.

1.6 Working Hypothesis

The regulatory network of factors involved in cardiac differentiation is manifoldly described, but still not fully understood.

To help elucidate this regulatory network we focused on the early mesodermal markers, Brachyury and Mesp1. As described above both genes are required for the formation of the mesoderm and play a role in cardiogenesis. In fact Mesp1 is, so far, the earliest marker of cardiac specific lineage. Another factor known to play an important role in mesodermal fate is the Wnt signaling pathway, whose transient expression is required for heart development.

In former projects our group was able to isolate and propagate CVPCs from adult murine hearts (Section 2.7.3). These cells can be aggregated as cardiac bodies (CBs) and can be used as an in vitro tool mimicking early cardiomyogenesis.

Together with EBs, CBs are useful in vitro models allowing to track the expression of specific reporter genes during early development. We established stable reporter-gene carrying cell lines (ESCs as well as CVPCs) to further investigate the expression of EGFP under the control of the Brachyury and Mesp1 promoter respectively, and the luciferase expression triggered by Tcf/Lef sites (reporting Wnt signaling pathway activation). Stable reporter cell lines, which behave accordingly and reproducibly to wildtype controls set the basis for testing the influence of different factors on the expression of the reporter genes and their influence on cardiomyogenesis on a molecular basis. These results will also help us to understand how somatic stem cells can be efficiently differentiated to fully functional cardiac muscle cells and will help to develop new applications for heart conditions.

2 Materials

2.1 Enzymes

Collagenase	Worthington, USA
DNAse I RNase free	Fermentas, Lithuania
Pancreatin	Sigma, USA
Proteinase K	Fluka, CH
Restriction Enzymes	New England Biolabs, USA
Taq DNA Polymerase	Fermentas, Lithuania
Trypsin	LifeTechnologies, USA

2.2 Chemicals for Cell Culture

β-Mercaptoethanol	Loba, A
DMEM powder	Gibco, USA
DMSO (Dimethylsulfoxide)	Sigma, USA
Fetal Bovine Serum (FBS)	HyClone, USA
Fetal Bovine Serum (FBS)	Gibco, USA
Fetal Bovine Serum (FBS)	Sigma, USA
Trypsin	LifeTechnologies, USA
Gelatine	Difco, USA
Glycine	Applichem, D
Mitomycin C	Acros, B
Penicillin / Streptomycin (P/S)	Gibco, USA
Potassium chloride	Sigma, USA
Potassiumhydrogenphosphate	Fluka, CH

Sodiumhydrongencarbonate	LifeTechnologies, USA
Streptomycin	Sigma, USA

2.3 General Chemicals and Material

Acetic acid	Merck, D
Agarose	Biozyme LE Biozyme, D
APS	Biorad, USA
ß-Mercaptoethanol	Loba Feinchemie, AUT
Bromphenolblue	Sigma, USA
BSA	New England Biolabs, USA
Chloroform	Acros, B
Coomassie Brilliant Blue R250	Merck, D
Dabco	Sigma, USA
dNTPs	Fermentas, Lithuania
Dimethylsulfoxid (DMSO)	Acros, B
Dithiothreitol (DTT)	Acros, B
EDTA	Acros, B
EGTA	Acros, B
Ethanol	Merck, D
Ethidiumbromide	Fluka, CH
Formaldehyde	Merck, D
Glycerine	Merck, D
Glycerophosphate	Merck, D
Glycin	Sigma, USA
Hydrochloric acid (HCl)	Acros, B
Isoamylalcohol	Merck, D
Lithiumchloride	Merck, D
Lipofectamine	Invitrogen, USA
Loading Dye	Fermentas, Lithuania
Methanol	Merck, D
Magnesiumchloride	Fermentas, Lithuania
Magnesiumsulfate	Fluka, CH
Nalgene Filter	Nalagene Labware, USA
PEG6000 and PEG8000	Calbiochem, D
Polyacrylamide	Merck, D

Proteinase Inhibitor	Roche, D
Reverse Transcriptase Buffer	Invitrogen, USA
SDS	BioRad, USA
Sodiumazid	Acros, B
Sodiumbicarbonate	Sigma, USA
Sodiumchloride	Salinen Austria, AUT
Sodiumfluorid	Donauchemie, AUT
Sodiumhydrogencarbonate	LifeTechnologies, USA
Sodiumhydrogenphosphate	Roth, D
Sodiumhydroxide	Merck, D
Sodiumthiosulfate	Merck, D
TEMED	Sigma, USA
Trichloracetic acid	Merck, D
Tris Base	LifeTechnologies, USA
Triton X100	Sigma, USA
Tween-20	Sigma, USA

2.4 Kits

EndoFree Plasmid Maxi Kit	Qiagen, D
ONE-Glo^{TM} Luciferase Assay System	Promega, USA
peqGOLD Gel Extraction Kit	Peqlab, D
peqGOLD Plasmid Miniprep Kit I	Peqlab, D
QIAquick Gel Extraction Kit	Qiagen, D
Wizard^{R} Plus SV Minipreps DNA Purification System	Promega, USA

2.5 Inhibitors and Recombinant Proteins

Chir99021	Axon, NL
MK 142	Prof. Dr. Marko Mikovilovic, TU Vienna
Mouse LIF	Miltenyi Biotec GmbH, D
SB431542	Calbiochem^{R}, D

2.6 PCR Primers

Primer EGFP	**Sequence 5' - 3'**	**Number of nucleotides**	**Tm [°C]**	**Cycles**	**Fragment size [bp]**
Fwd Primer	CACATGAAGCAGCACGACTTCT	22	66.7	32	440
Rev Primer	AACTCCAGCAGGACCATGTGAT	22	66.9	32	440

2.7 Cell Lines

2.7.1 Fibroblasts

- **SNL 76/7 Fibroblasts**: The SNL76/7 cell line was established by Allan Bradley. It derives from the murine STO fibroblast cell line but additionally has a LIF gene and a neomycin-resistance gene inserted into the genome (McMahon and Bradley 1990).

2.7.2 Embryonic Stem Cells

- **W4**: Wild type, isolated by Georg Weitzer from the mouse strain C3H (Lauss et al. 2005).

2.7.3 Cardiovascular Progenitor Cells

Twelve different cardiovascular progenitor cell lines were isolated by Georg Weitzer and Marc Widner from newborn mice carrying a neomycin resistance gene in one allele of the hdac1 locus. The hearts of nine days old mice were extracted, digested with a collagenase/pancreatin solution, co-cultured with embryonic stem cells and feeder cells for 10 passages following a 3T3 protocol. The selection with G418 resulted in the death of murine wild type stem cells and the isolation of twelve different clonal cardiovascular progenitor cell lines. Here the cell line **A5** was used.

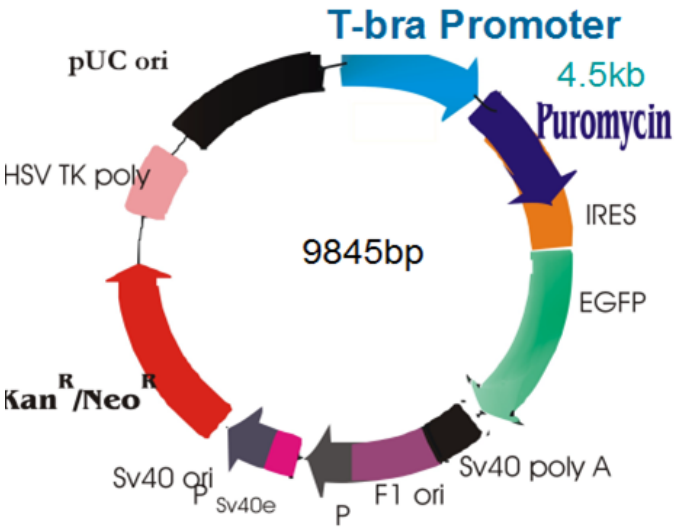


Figure 2.1: pT-Bra^{p}-Puro-IRES2-EGFP

2.8 Bacteria Strains

- **DH5-α**: DH5-α is a competent *Escherichia coli* strain and was used for transformation and plasmid amplification.

2.9 Plasmids

2.9.1 pT-Bra-Puro-IRES2-EGFP

This pT-Bra^{p}-Puro-IRES2-EGFP vector is a reporter plasmid, where the Enhanced Green Fluorescent Protein (EGFP) is under the control of Brachyury 5' UTR (Doss et al. 2010). The plasmid was provided by Prof. Dr. Agapios Sachinidis (Center of Physiology and Pathophysiology, Cologne/Germany).

2.9.2 pMesP1-EGFP

The pMesP1-EGFP vector is a reporter plasmid composed of a MesP1 promoter linked to an EGFP reporter gene. The plasmid was provided by Prof. Dr. Robert David (Medical University, Munich/Germany).

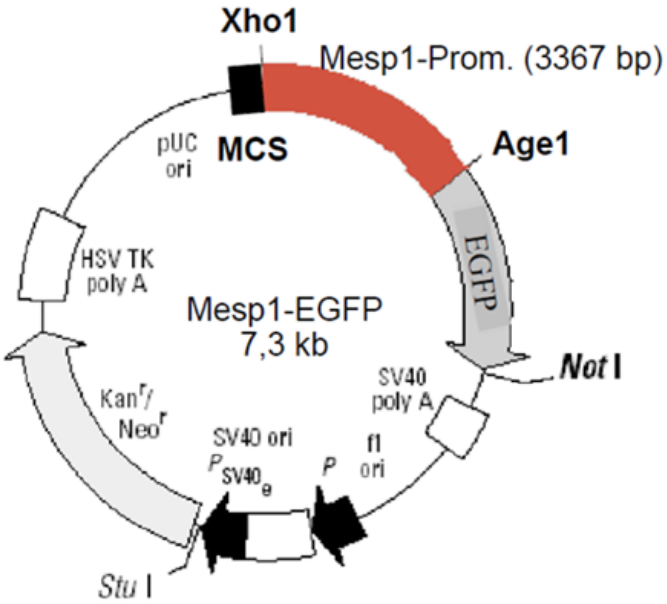


Figure 2.2: pMesP1-EGFP

2.9.3 pMesP1-IRES-EGFP

The pMesP1-IRES-EGFP vector (see Figure 2.3) is an overexpression plasmid containing a hMesP1 fragment which is combined with an Enhanced Green Fluorescent Protein (EGFP) (David et al. 2008). The genes are driven by the strong CMV promoter and get therefore constantly expressed. The plasmid was provided by Prof. Dr. Robert David (Medical University, Munich/Germany).

2.9.4 M50 Super 8x TOPFlash

M50 Super 8x TOPFlash is a luciferase reporter of β-catenin-mediated transcriptional activation. This construct was made by Ajamete Kaykas in the Moon lab. It is the Addgene plasmid # 12456 (Veeman et al. 2003).

2.9.5 M51 Super 8x FOPFlash

M51 Super 8x FOPFlash (TOPFlash mutant) is the negative control of TOPFlash. It has mutated TCF/LEF sites. This construct was made by Ajamete Kaykas in the Moon lab. It is the Addgene plasmid # 12457 (Veeman et al. 2003).

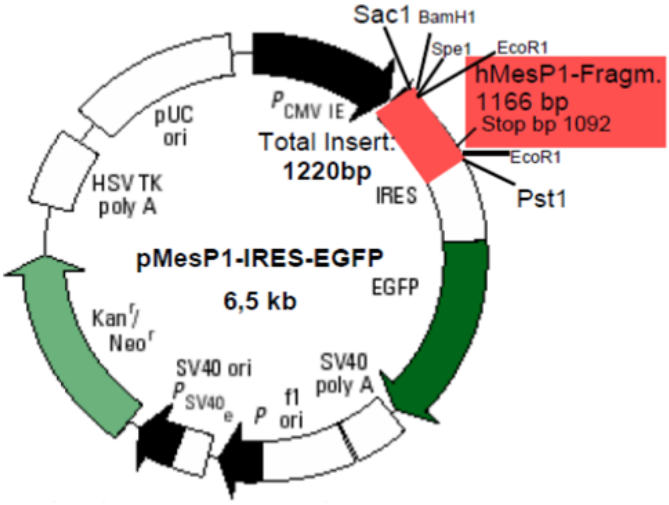


Figure 2.3: pMesP1-IRES-EGFP

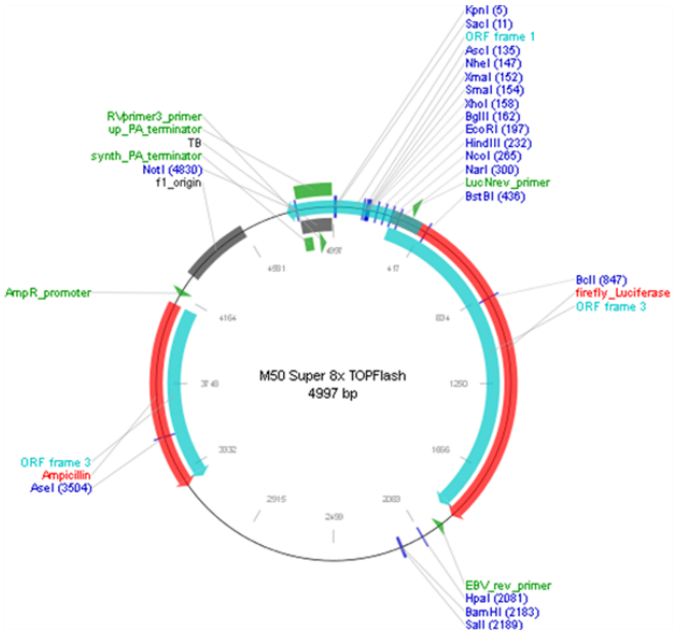


Figure 2.4: M50 Super 8x TOPFlash

2.9.6 pMC1neo-polyA

This plasmid facilitates gene targeting and lineage marking in mammalian stem cells as it contains a neomycin resistance cassette, which can be easily excised by XhoI and SalI. The cassette is under the control of a TK promoter.

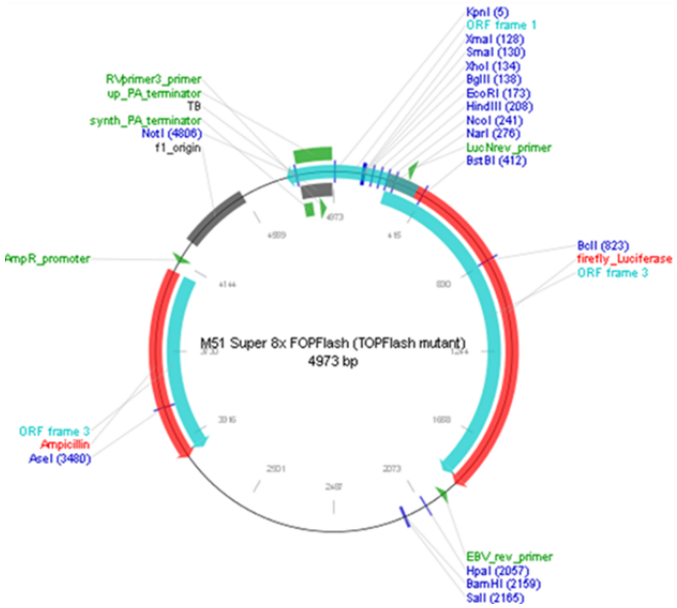


Figure 2.5: M51 Super 8x FOPFlash

Figure 2.6: pMC1neo-polyA

The plasmid is distributed by Stratagene, now an Agilent Technologies company (catalogue number 213201).

2.10 Material for Bacteria Culture

2.10.1 LB Medium

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

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

2.10.2 LB-plates

7.5 g agarose (1.5%) are added to a 500 ml bottle of LB-medium. The bottle is autoclaved and melted for 30 minutes at 180 Watt in the microwave. When the liquid is cooled down below 60°C, antibiotics are added, and the medium is poured into petri dishes. The plates are kept on room temperature to solidify and then stored at 4°C.

3 Methods

3.1 Cell Culture

3.1.1 General Stem Cell Culture Workings

Glass material for stem cell culture should not be contaminated with detergents, because already small leftovers cause death of embryonic stem cells. It has to be cleaned differently than normal laboratory glass ware.

Washing of Glass Bottles for Media and Solutions

Used bottles are filled with tap water containing a few milliliters of 1:3 diluted hypochlorite. The bottles are kept at room temperature for about one hour thereafter they are rinsed with tap water for 10 minutes to get completely rid of hypochlorite. After this step the bottles are flushed once with milliQ-H_2O and are then filled with milliQ-H_2O, kept at room temperature for 24 hours, dried and finally autoclaved at 140°C and 1.4 bar.

Washing of pipettes

After usage, the cotton plug of the glass pipettes is removed and the pipettes are stored in water/hypochlorite containers. When these containers are full, the pipettes are transferred to another container and are rinsed with tap water for at least 4 hours before they are stored in milliQ-H_2O over night. The next day the pipettes are dried at 60°C. A piece of cotton is plugged into each pipette, they are put into the pipette boxes and are baked at 180°C for 8 hours.

3.1.2 Buffer and Media for Cell Culture

10x PBS (Phosphate Buffered Saline) stock solution

- 1.37 M NaCl
- 14.7 mM KCl
- 78.1 mM Na_2HPO_4 x 7 H_2O
- 26.8 mM KH_2PO_4
- Saturated Na_2HPO_4 is added to a final pH of 7.2.
- The solution is sterile filtered (Nalagene Filter, 0.22 μm pore width).

100x GPS (Glutamine-Penicillin-Streptomycin)

- 4.25 g NaCl
- 1.5 g Penicillin
- 2.5 g Streptomycin
- 14.6 g L-(+)-Glutamine
- MilliQ-H_2O is added to a final volume of 500 ml.
- The solution is aliquoted in 50 ml Falcon tubes, stored at -20°C and then kept at 4°C after thawing.

100x β-Mercaptoethanol ($10^{-2} Mol/l$)

- 200 ml 1x PBS
- 144 μl β-Mercaptoethanol (14 M)
- The solution is sterile filtered.
- The solution is aliquoted in 50 ml Falcon tubes, stored at -20°C and then kept at 4°C after thawing.

Trypsin

- 3.5 g NaCl
- 0.5 g D-Glucose
- 0.09 g Na_2HPO_4 x 7 H_2O
- 0.185 g KCl
- 0.12 g KH_2PO_4
- 0.2 g EDTA
- 1.25 g trypsin (Gibco)

- 1.5 g Tris Base
- MilliQ-H_2O is added to a final volume of 500 ml.
- The final pH of 7.6 is achieved with concentrated HCl.
- The solution is aliquoted in 50 ml Falcon tubes, stored at -20°C and then kept at 4°C after thawing.

1% Gelatine Stock Solution

- 10 g Gelatine (Difco) are solute in 1 l milliQ-H_2O. The solution is sterile filtered.

DMEM (Dulbecco's Modified Eagle's Medium)

- A 5 liter Erlenmeyer flask is filled with 4.5 l milliQ-H_2O. Half a DMEM can (Gibco, +4500 mg/l Glucose, -$NaHCO_3$, -Pyruvate #52100-039) is added and dissolved. 18.5 g $NaHCO_3$ are added. Concentrated HCl is added to a final pH of 7.4. MilliQ-H_2O is added to a final volume of 5 l and the medium is sterile filtered into cell culture flasks.
- Contamination can be detected by incubating a sample of each bottle at 37°C over night. After 24 hours the aliquots can be scanned for contaminations under the microscope. If any impurity is seen, the DMEM cell culture flasks have to be sterile filtered again.

Freezing Medium

- 60% DMEM
- 20% Fetal Bovine Serum
- 20% DMSO (Dimethylsulfoxid)

3.1.3 Growing Media

Medium for Fibroblasts (M10Gi)

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

Medium for Embryonic Stem Cells and Cardiovascular Progenitor Cells (M15Hy)

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

Medium for Embryoid and Cardiac Bodies (M15Si)

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

3.1.4 Gelation of Tissue Plates

To coat tissue plates with 0.1% gelatine solution, the solution must cover the ground of the tissue plate needed and they are kept at room temperature for two hours, thereafter the solution is aspirated and the tissue plates are ready for usage. Plates must not become dry.

3.1.5 Fibroblasts

Culture of SNL76/7 Fibroblasts

Embryonic stem cells grow on mitotically inactivated SNL76/7 fibroblasts, referred to as feeder cells, which produce LIF and therefore keep the embryonic stem cells in an undifferentiated state. The SNL76/7 cell line derives from the STO fibroblast cell line but additionally has a LIF gene inserted into the genome. The cells are cultured on 10 cm cell culture plates at 37°C and 5% CO_2. Usually they are fed with M10Gi once a week (change of color of the medium from pink to yellow indicates acidification and consequently depletion of nutrients). SNL cells are split up to 1:8 ratios when they reach confluency.

Thawing of Fibroblasts

The cells are stored in 2 ml cryotubes in liquid nitrogen at -196°C. After removal from the liquid nitrogen, the cells are thawed as fast as possible in

a 37°C water bath and transferred to a 15 ml Falcon tube. M10Gi medium (approx. 8 ml) is added drop by drop, after each drop the tube is gently shaken. If the addition of the medium is applied too fast, the cells will rupture due to too fast decrease of osmotic pressure. The cells are then centrifuged for 7 minutes, 1000 rpm at room temperature. The supernatant is aspirated and the cell pellet is resuspended in 5 ml fresh M10Gi. The cells are transferred onto a 10 cm cell culture dish. The cells are dispersed by moving the plate in a shape of an eight and are then incubated at 37°C and 5% CO_2 .

Splitting of Fibroblasts

Before splitting, the medium is aspirated and the cells are washed with 1x PBS. After removal of the PBS 1 ml trypsin is added and the plate is incubated at 37°C for 5 - 10 minutes. The detached cells are resuspended in fresh M10Gi medium and split either 1:6 or 1:8. One part of the cell suspension is transferred onto a new 10 cm tissue plate and mixed with fresh M10Gi medium.

Production of Feeder Cells

For production of feeder cells, the SNL76/7 cells are mitotically inactivated with Mitomycin C. Thus they can not divide anymore but still produce all factors needed for self-renewal. The medium of the 10 cm culture plate is aspirated except 4 ml. After addition of 80 μl Mitomycin C solution (0.5 mg/ml) the plate is incubated at 37°C for 3 - 4 hours. The medium is aspirated and the cells are washed twice with 1x PBS. 1 ml trypsin is added and the plates are incubated at 37°C for 5 - 10 minutes. The detached cells are resuspended in 5 ml M10Gi and transferred into a sterile 15 ml Falcon tube which is centrifuged for 7 minutes, 1000 rpm at room temperature. The supernatant is aspirated and the pellet is resuspended in 10 ml M10Gi. The cell number is determined with a cell counter. A final concentration of 3.5 x 10^5 cells/ml is achieved by dilution. These cells are seeded onto gelled tissue culture plates as described in Table 3.1. The plates are incubated at 37°C and can be used the next day when the cells have adhered to the plate.

Table 3.1: Amount of Feeder Cells [ml]

Plate size	Amount of feeder cell suspension [ml]
10 cm	12
6 cm	4
24 well	0.5
48 well	0.3
96 well	0.2

3.1.6 Embryonic Stem Cells (ESCs) and Cardiovascular Progenitor Cells (CVPCs)

Culture of Embryonic Stem Cells and Cardiovascular Progenitor Cells

The cells are incubated at 37°C and 5% CO_2, are fed every 24 hours with M15Hy and are split at approximately 90% confluency in the desired ratio (mostly 1:2 and 1:3).

Thawing of Embryonic Stem Cells and Cardiovascular Progenitor Cells

The cells are stored in 1 - 2 ml cryotubes in liquid nitrogen at -196°C. Two hours before thawing, the feeders of a 24-well plate, on which the cells will grow, are fed with 2 ml M15Hy. After removal of the desired cells from liquid nitrogen they are thawed as fast as possible in a 37°C water bath and transferred to a 15 ml Falcon tube. M15Hy medium (approximately 6 ml medium/1 ml cell suspension) is added drop by drop, after each drop the tube is gently shaken. The cells are centrifuged for 7 minutes, 1000 rpm at room temperature. The supernatant is aspirated and 1 ml M15Hy of the pre-fed feeder cells is transferred to resuspend the cell pellet. The singularized cells are transferred back onto the feeder cells and are dispersed by moving the plate in a shape of an eight before they are incubated at 37°C and 5% CO_2. After 24 hours the cells adhere to the feeder cells and the medium can be changed.

Splitting of Embryonic Stem Cells and Cardiovascular Progenitor Cells

ESCs/CVPCs are split when they reach 80 - 90% confluency. Two hours before splitting the feeder cells as well as ESCs/CVPCs are fed with fresh M15Hy (2 ml M15Hy for feeder cells and 1 ml for ESCs/CVPCs). After two hours the supernatant of ESCs/CVPCs is aspirated and the cells are washed with 1x PBS. The PBS is aspirated and 200 μl trypsin are added to the cells. The plate is incubated at 37°C for 15 - 20 minutes. The detached cells are resuspended in 1 ml medium from the pre-fed feeder cells and 400 μl (1:3 ratio; 600 μl - 1:2 ratio) of the suspension are transferred onto the feeder cells. The plate is incubated at 37°C and 5% CO_2 for at least 24 hours.

Freezing of Embryonic Stem Cells and Cardiovascular Progenitor Cells

Two hours before freezing, the ESCs/CVPCs are fed with 1 ml M15Hy. After aspiration of the medium the cells are washed with 1x PBS. 200 μl trypsin are added to the cells and the plate is incubated for 15 - 20 minutes at 37°C. The cells are resuspended in 800 μl M15Hy. 1 ml of freshly prepared freezing medium is added drop by drop, after each drop the Falcon tube is gently pivoted. The cell suspension is apportioned to two cryotubes, the tubes are placed in a thick-walled styrofoam container and are put in the -80°C freezer for at least 48 hours, to reassure a slow freezing of the cells of maximally -1°C/min. Thereafter they are transferred into liquid nitrogen tanks.

3.1.7 Embryoid Bodies and Cardiac Bodies

Production of Embryoid Bodies and Cardiac Bodies

24 hours before production of embryoid bodies (EBs) or cardiac bodies (CBs) the desired embryonic stem cells or cardiovascular progenitor cells (cultivated on a 24-well plate), respectively, are split in a 1:2 ratio to obtain cells in an ideal condition. Two hours prior to making EBs/CBs, the ESCs/CVPCs are fed with 1 ml M15Si medium. Meanwhile one 6 cm tissue culture plate per cell line is gelled (0.1% gelatine solution for 2 hours). After two hours the medium is aspirated, the cells are washed once with 1xPBS, before they are trypsinized for 15 - 20 minutes. The trypsinized cells are suspended in 800 μl

M15Si medium and are transferred onto the gelled plates (these are filled up with M15Si to a final volume of 4 ml). Feeder cells adhere to the gelled plate within 45 - 60 minutes and can therefore be separated from ECSs/CVPCs. This is necessary as the feeder cells would hinder the ECSs/CVPCs from differentiating. After 45 - 60 minutes the supernatant is collected in a 15 ml Falcon tube, and the cells are spun down (1000 rpm, 7 min). The supernatant is discarded; the pellet is dissolved in 10 ml M15Si medium. The cell number is determined with a cell counter. The cells are diluted to a concentration of 4 x 10^4 cells/ml (for EBs) and 4.5 x 10^4 cells/ml (for CBs). If the aggregation of cells is limited, the concentration might be doubled.

The bottom of sterile bacteria plates (6 cm or 10 cm) is covered with autoclaved milliQ-H_2O. 20 μl drops are pipetted onto the lid of the bacteria plate (approximately 100 drops per lid $\cong$ 2 ml of the cell suspension per lid). The lid is gently and swiftly turned and placed on the plate containing the water. The plate with the hanging drops is carefully placed in the incubator and incubated at 37°C and 5% CO_2. The day of aggregation is referred to as day 0. On day 4.5 (EBs) and day 4.7 (CBs) the lid is rinsed with about 8 ml M15Si medium on a gelatinized 10 cm tissue culture plate and the embryoid and cardiac bodies are dispersed by gently rocking in two different directions. The plate is incubated at 37°C and 5% CO_2 for at least 24 hours without moving them until the EBs/CBs adhere to the plate.

Culture of Embryoid Bodies and Cardiac Bodies

Embryoid and cardiac bodies are fed every third day with fresh M15Si, however the old medium is kept partly and fresh medium is added to the old one. On day 7 and day 10, 3 ml of old medium are kept and 8 ml of fresh M15Si are added. On day 13, day 16, day 19 and day 22, 4 ml of old medium are kept in the plate and 10 ml of fresh M15Si are added. From day 25, 5 ml old medium are kept in the plate and 12 ml of fresh M15Si medium are added.

Trypsinization of Embryoid and Cardiac Bodies

The M15Si medium is aspirated; the plates are washed twice with 1xPBS and 1 - 2 ml trypsin are added. The plates are incubated at 37°C for 20 minutes. The cells are suspended in 5 ml M15Si medium, spun down at 1000

rpm for 7 minutes and then resuspended in an appropriate amount of M15Si medium (see Section 3.6.1 and Section 3.7.1).

3.2 Cloning Systems

3.2.1 Production of Competent *E.Coli*

A colony of *E.Coli* DH5-α is picked and transferred to a flask containing 2 ml LB medium and is incubated over night. The bacteria culture is diluted with 98 ml LB medium in a 250 ml flask and is incubated until an $OD_{600} = 0.3$ - 0.6 is reached (2 - 4 hours). The culture is centrifuged at 4°C for 10 minutes and 1000 x g. The supernatant is aspirated and the pellet is resuspended in 10 ml TSB. The solution is kept on ice for 10 minutes and then aliquoted (500 μl) in pre-cooled Eppendorf tubes. The bacteria are shock-frozen in liquid nitrogen and then stored at -80°C.

TSB (sterile filtered, stored at 4°C)

- 10% PEG6000 or PEG8000
- 5% DMSO
- 10 mM $MgCl_2$ x 6 H_2O
- 10 mM $MgSO_4$ x 7 H_2O
- LB medium

3.2.2 Transformation of Competent *E.Coli*

10 μl of the plasmid DNA are pipetted to 150 μl of competent *E. Coli* DH5-α. The suspension is kept on ice for 30 minutes. 450 μl TSB-G (TSB + 20 mM glucose) medium are added and the mixture is shaken (1300 rpm) at 37°C for 60 minutes. 100 - 300 μl of transformed bacteria are plated on antibiotic selective LB-agar plates and incubated over night at 37°C. The next day single colonies can be picked.

3.2.3 Glycerin Stocks of *E.Coli*

Glycerinstocks are used for long-term storage of bacteria containing a specific plasmid. A single colony of competent *E.Coli* transformed with the plasmid of interest is picked and transferred into a cuvette tube containing 2 - 5

ml antibiotic selective medium. The bacteria are incubated and shaken over night at 37°C. The next day, 900 μl of the overnight culture are mixed with 100 μl sterile 87% Glycerol and are transferred to a cryotube. The bacteria are shock-frozen in liquid nitrogen and are then stored at -80°C. After 2 - 3 days the glycerine stocks are validated by

- Plating on LB plates containing appropriate antibiotics.
- Inoculation of liquid medium.
- Plasmid preparation (Miniprep) and restriction digestion.

Minipreparation (Promega Wizard® Plus SV Minipreps)

A colony is picked from an LB plate (containing antibiotics) and is incubated over night at 37°C in 2 ml liquid LB medium (containing antibiotics). The next day the bacterial culture is transferred into a 2 ml Eppendorf tube and harvested by centrifuging 5 minutes at 10,000 x g ($\cong$ 11,000 rpm). The supernatant is poured off and the inverted tube is plotted on a paper towel to remove excess media. 250 μl of Cell Resuspension Solution are added and the cell pellet is completely resuspended by vortexing or pipetting. To avoid shearing of chromosomal DNA vortexing is not applied after this step. Now 250 μl of Cell Lysis Solution are added and mixed by inverting the tube 4 times. The cell suspension is incubated at room temperature until it is clear (approximately 1 - 5 minutes). After 5 minutes at most 10 μl of Alkaline Protease Solution are added and mixed by inverting the tube 4 times. The mix is incubated for 5 minutes at room temperature. Alkaline protease inactivates endonucleases and other proteins released during the lysis of the bacterial cells that can adversely affect the quality of the isolated DNA. 5 minutes of incubation should not be exceeded as nicking of the plasmid DNA may occur. Immediately 350 μl of Neutralization Solution are added and mixed by inverting the tube 4 times. After this step the bacterial lysate is centrifuged at maximum speed (around 14,000 $\times$ g) in a microcentrifuge for 10 minutes at room temperature. The cleared lysate (approximately 850 μl) is transferred to the prepared Spin Column by decanting without transferring any of the white precipitate with the supernatant (If the white precipitate is accidentally transferred to the Spin Column, the Spin Column content is poured back into a sterile 1.5 ml microcentrifuge tube and centrifuged

for another 5 - 10 minutes at maximum speed. The resulting supernatant is transferred into the same Spin Column that was used initially for this sample. The Spin Column can be reused but only for this sample.). The supernatant is centrifuged at maximum speed in a microcentrifuge for 1 minute at room temperature then the Spin Column is removed from the tube and the flowthrough is discarded from the Collection Tube. The Spin Column is reinserted into the Collection Tube. 750 μl of Column Wash Solution are added, previously diluted with 95% ethanol, to the Spin Column. Again it is centrifuged at maximum speed in a microcentrifuge for 1 minute at room temperature. The flowthrough is discarded. The washing step is repeated with 250 μl of Column Wash Solution. The flowthrough is discarded. After this step the empty tube is once again centrifuged at maximum speed in a microcentrifuge for 2 minutes at room temperature to get rid of excess fluids. The Spin Column is transferred to a new, sterile 1.5 ml microcentrifuge tube and the plasmid DNA is eluted by adding 100 μl of Nuclease-Free Water to the Spin Column. The tube is centrifuged at maximum speed for 1 minute at room temperature in a microcentrifuge. The eluted DNA can be stored at -20°C or below.

3.2.4 Restriction Digest of Ligation Plasmids

For the overhang cloning, the backbone vector is linearized by a single cutter restriction enzyme. The insert of interest is cut out of another plasmid with restriction enzymes producing correspondent restriction sites (overhangs) of the backbone vector. Because of the sticky ends the insert can be ligated into the backbone vector. A standard restriction digest mixture is described below:

x μl	DNA (approx. 2 μg)	
10 μl	restriction enzyme buffer (10x)	
x μl	restriction enzyme I (1 U/μg DNA)	
(x μl	restriction enzyme II (1 U/μg DNA))	[insert excision only]
1 μl	BSA (100x)	
x μl	ddH_2O (fill up to total volume)	
100 μl	Total	

Mostly, 1 hour of digestion is sufficient to achieve a high restriction efficiency. Depending on the enzymes used a digestion can be prolonged for up to 24 hours.

3.2.5 Purification of Plasmid DNA for Ligation

After restriction digestion the fragments have to be examined if the digestion was complete. This is done by loading a sample onto a 0.8% agarose gel and checking for expected bands. A linearized backbone plasmid should only exhibit one band, whereas a successful digestion of the insert plasmid is displayed by two calculated fragments. Both fragments need further purification steps.

Dephosphorylation of Backbone DNA

To hinder the backbone vector to religate, the phosphate groups on its 5' ends have to be removed. This is achieved by the use of the calf intestinal alkine phosphatase (CIP). 0.5 units of CIP per μg vector DNA dephosphorylates the 5' ends within an hour at room temperature. Thereafter the backbone vector has to be purified by phenol/chloroform extraction. Thereby one volume of DNA suspension is mixed with one volume of phenol (as phenol is very toxic this extraction has to be done in the laboratory hood). The mixture is vortexed and centrifuged at 1400 rpm for 8 minutes. The upper aqueous phase containing the DNA is transferred into an Eppendorf tube and the same volume phenol/chloroform (1:1) is added. Again the mixture is vortexed thoroughly and centrifuged at 1400 rpm for 7 minutes. The upper clear phase is transferred into a new Eppendorf tube and the same volume chloroform is added. The solution is vortexed thoroughly and centrifuged at 1400 rpm for 5 minutes. After transfer of the aqueous upper phase into a new Eppendorf tube it is mixed with $\frac{1}{2}$ volume ammonium acetate solution and 3 volumes 96% EtOH (-20°C). The solution is incubated at -20°C overnight. The next day the mixture is centrifuged at 4000 rpm for 10 minutes. Now the DNA is precipitated and its pellet is washed twice with 70% EtOH (-20°C) (centrifugation at 4000 rpm for 5 min for each wash step). The pellet is air-dried for about 10 minutes and resuspended in 100 - 500 μl ddH_2O (volume depending on the size of the pellet). Now the DNA is ready for ligation.

Isolation of Insert

The insert is purified by gel extraction. We used the peqGOLD Gel Extraction Kit. The whole restriction digest is loaded on a 0.8% agarose gel. The DNA fragment of interest is identified under UV light and excised using a sterile scalpel and transferred into an Eppendorf tube. 1 g gel is equivalent to 1 ml. Considering this, the equal volume of binding buffer is added to the gel slice. The tube is incubated for 7 minutes at 55°C - 65°C. Every 2 - 3 minutes the tube is mixed or vortexed until the agarose is completely dissolved. Before loading the solution to the PerfectBind DNA Column for the adsorption of the DNA to the membrane, the color of the solution has to be controlled. Red or orange color indicates a too high pH which can be lowered by the addition of 5 μl 5 M Na-acetate; the solution should appear in light yellow. 750 μl of solution can be loaded to the column. Then the tube is centrifuged at 10,000 x g for 1 minute. If the solution is more than 750 μl the step has to be repeated. The flowthrough can be discarded. The loading step is followed by several washing steps to remove any contaminants. Therefore 2 - 3 washing steps (1x with 300 μl Binding Buffer (optional), 2x with 600 μl CG Wash Buffer) are performed. Before each centrifugation (10,000 x g for 1 minute) the mixture is incubated for 2 - 3 minutes. The flowthrough is always discarded. The column is dried by centrifugation at 10,000 x g for 1 minute. The elution of the DNA is the last step. The column is transferred into a new Eppendorf tube, 30 - 50 μl Elution Buffer or ddH_2O are added and the DNA is eluted by centrifugation at 5,000 x g for 1 minute. Now the insert DNA is ready for ligation.

3.2.6 Ligation of Backbone and Insert DNA

Backbone and insert DNA are mixed in a 1:3 ratio. It is important to calculate the correct ratios taking into account the concentration and the size of backbone and insert DNA. The mixture of backbone and insert DNA is incubated at 45°C for 10 minutes to fuze cohesive ends, then the ligation preparation is put on ice for 5 minutes. Thereafter T4 DNA ligase and ligase buffer are added. Usually the ligation is finished after 1 hour at room temperature, but as we experienced some problems, we performed our ligation over night, we started on ice and left the ice box at room temperature, that the ice could slowly melt and the ligation mix slowly warmed up to room

temperature.

Following reaction mixture is assembled:

x μl	Backbone DNA
x μl	Insert DNA
2 μl	Ligase buffer (10x)
1 μl	T4 DNA Ligase
x μl	ddH_2O (fill up to total volume)
20 μl	Total

3.2.7 Negative Controls

Two negative controls are done in parallel to the actual experiment. To obtain comparable results the amount of the utilized DNA has to be equal in all mixtures. Negative control #1 is simply a linearized vector, neither dephosphorylated nor ligated. Negative control #2 is a linearized and dephosphorylated vector. The same reaction mixture is used as mentioned above except for the insert DNA.

Negative control #1 is used to check for complete digestion (test of restriction efficiency), negative control #2 is used to test the efficiency of the calf intestinal alkine phosphatase (CIP). Only vectors which were completely dephosphorylated can not religate after addition of ligase (test of phosphatase efficiency).

3.2.8 Transformation of Ligated Plasmids

After ligation, all batches (ligation plus negative controls) are transformed in competent DH5-α bacteria. Each reaction tube (20 μl) is added to 150 μl freshly thawed DH5-α bacteria. The solution is incubated on ice for 30 minutes. Then 450 μl TSB-G is added and the bacteria get incubated at 37°C for 1 hour. The transformed bacteria are now plated onto LB-plates containing the appropriate antibiotics. The plates are incubated at 37°C over night. Bacteria, which incorporate the correctly ligated plasmid, form colonies within 16 hours.

3.2.9 Selection and Validation of Transformants

If no (or at least hardly any) colonies are grown on the negative control plates, colonies of the ligation plate are picked and transferred into a cuvette tube containing 2 ml antibiotic selective LB medium. The bacteria are incubated and shaken over night at 37°C. The next day the plasmids are isolated by minipreps (as described in Section 3.2.3). To check whether the plasmid incorporated the insert, restriction digestions are performed. Digestion with one or more restriction enzymes ensures the uptake of the insert if actual fragment size matches the predicted one. Positive identified clones can be further propagated as described below.

3.2.10 Plasmid Preparation for Electroporation

To electroporate mammalian cells with plasmid DNA, large amounts of DNA are needed. The plasmids are propagated in bacteria and purified with Qiagen EndoFree Plasmid Maxi Kit, where a higher concentration of plasmid DNA is achieved.

Maxiprep (Qiagen EndoFree Plasmid Maxi Kit)

A toothpick is dipped in a glycerin stock and the bacteria on its tip are transferred to an antibiotic-selective LB-agar plate and incubated at 37°C over night. The next day, a single colony is picked and transferred into a tube containing 2 ml antibiotic-selective LB medium. The tube is shaken at 37°C over night and then 150 μl of the tube are transferred into a 500 ml flask containing 100 ml antibiotic-selective LB medium. The flask is incubated and shaken at 37°C over night. The overnight culture is centrifuged for 15 minutes at 4°C and 8,500 rpm. The supernatant is discarded; the pellet is resuspended in 10 ml Buffer P1 and transferred to a 50 ml Falcon tube. 10 ml of Buffer P2 are added, the tube is inverted and kept at room temperature for 5 minutes. 10 ml of chilled Buffer P3 are added, the tube is inverted 4 - 6 times. The lysate is poured into the barrel of QIAfilter Cartridge and kept at room temperature for 10 minutes (The plunger is not inserted yet!). After 10 minutes the cap from the QIAfilter Cartridge outlet nozzle is removed, the plunger is inserted and the cell lysate is filtered into a new 50 ml tube. 2.5 ml of ER buffer are added; the tube is inverted about 10 times and kept on ice for 30 minutes. A QIAGEN-tip 500 is equilibrated with 10 ml Buffer

QBT and the column is emptied by gravity flow. Then the filtered lysate is applied to the QIAGEN-tip and left until it is emptied by gravity flow. The tip is washed two times with 30 ml Buffer QC. Finally the DNA is eluted with 15 ml Buffer QN into a new flask. To precipitate the DNA 10.5 ml isopropanol are added, mixed and centrifuged for 30 minutes at 13,500 rpm and 4°C. The supernatant is decanted. The DNA pellet is washed with 5 ml 70% ethanol and the tube is again centrifuged for 10 minutes at 4°C and 13,500 rpm. The supernatant is decanted; the pellet is air-dried for 5 - 10 minutes and dissolved in 600 μl TE-buffer. The DNA concentration is measured with NanoDrop and the Plasmid DNA is stored at 4°C.

3.3 Electroporation

3.3.1 Linearization of Vector DNA

25 μg of linearized DNA are used per electroporation. Dependent on the DNA concentration of the maxiprep an appropriate digestion of DNA is made in a final volume of 250 μl. The single cutters need to linearize the vector without cutting the construct of interest. 1 U restriction enzyme per μg DNA is sufficient. The digestion is made over night and purified by phenol extraction.

3.3.2 Preparation of Embryonic Stem Cells (ESCs) and Cardiovascular Progenitor Cells (CVPCs)

ESCs/CVPCs (80% confluent) are split onto feeder cells (10 cm plates) in a 1:2 ratio one day before electroporation. After 24 hours the cells are fed with fresh M15Hy medium two hours prior to electroporation. Then the medium is aspirated and the cells are washed twice with 1xPBS. The cells are trypsinized with 1 ml of trypsin for 15 minutes at 37°C. The cells are suspended in 5 ml M15Hy medium, centrifuged (1000 rpm for 7 minutes), the supernatant is discarded and the pellet is suspended in 10 ml 1xPBS. After determining the cell number with Thoma cell counter, the cells are once again centrifuged and suspended in the correct volume of 1xPBS to achieve a final concentration of 1.1 x 10^7 cells/ml.

3.3.3 Electroporation

- 900 μl of cell suspension (equals 1 x 10^7 cells) are mixed with 25 μg of the linearized vector in an electroporation cuvette and let stand for 5 minutes at RT.
- Meanwhile the Bio-Rad Gene Pulser II is set at 230 V and 500 μF.
- The cuvette is placed in the electroporation holder with the foil electrode in contact with the metal holding clip.
- A single pulse (230 V and 500 μF) is applied (press both buttons until a sound is audible). The time constant should read between 5.6 and 7.0 milliseconds. This ensures sufficient perforation of the cells without bursting and enabling the uptake of DNA.
- The electroporated cells should rest for 5 minutes at RT and are thereafter plated onto pre-fed feeder dishes. The cells should be well suspended, without destroying the feeder layer.
- After seeding the cells need to recover for 24 hours in non-selective medium.

3.3.4 Selection of Monoclonal Colonies

Addition of Antibiotics

24 hours after electroporation selective medium is added. The antibiotic of choice depends on the resistance gene of the recombinant fragment. Usually G418 (a synthetic analogue of neomycin) is used at a concentration of 180 μg/ml, whereas the concentration of puromycin is 1 μg/ml. The selective medium is changed every day (between 10 to 14 days) until resistant colonies are visible to the naked eye.

Picking of Colonies

2 - 3 hours prior to picking, feeder cells on a 96-well plate should be fed with 100 μl M15Hy. As many wells are fed as colonies are picked (usually 10 are sufficient). The 10 cm plate containing the colonies is also pre-fed (without antibiotics). The colonies are counted and preferentially preselected under the fluorescence microscope if green fluorescence is visible [only if cells contain an EGFP reporter contruct!]. Before picking 30 μl trypsin are put in an empty (no feeder cells, not gelled) 96-well plate. All instruments used

for picking must be sterilized with 70% EtOH (20 μl pipette, microscope, working space). The 10 cm plate is washed twice with 1xPBS, and then 6 ml 1xPBS are put on the plate, which is now ready for picking.

For picking a colony the pipette is set at 10 μl, the trigger has to be pushed before carefully surrounding the colony of choice with the tip of the pipette. After detaching the colony from the bottom of the plate, the trigger has to be released quickly so that the colony can be sucked up. The colony is transferred into a well of the 96-well plate filled with 30 μl trypsin and is suspended. The colonies should not be trypsinized longer than one hour (minimum 15 minutes). Afterwards 120 μl of M15Hy medium are added, the cells are suspended and transferred onto the pre-fed 96-well feeder plate. Selection with antibiotics can be started 24 hours after picking.

Selection and Propagation of Monoclonal Cells

ESCs/CVPCs are grown on the 96-well plate until they are confluent. Then they are split onto 48-well plates (whole cell suspension of 96 wells $\cong$ 1:3.5 ratio). When the cells are confluent at the 48-well plate 50 μl (of 250 μl) are given on a gelled 96-well plate (no feeder cells) with 200 μl of differentiation medium (M15Si), the rest is split 1:4 on a 24-well plate (125 μl). If EGFP reporter genes have been transfected, the 96-well plate is investigated under the fluorescence microscope the following days to identify positive clones by their specific fluorescence (EGFP emits green fluorescence). When the cells on the 24-well plate are confluent all monoclonal cell clones are frozen, except 1 - 3 clearly positive clones, which are split (1:2 or 1:3) on 24 wells and referred to as new cell lines, starting at passage one. These monoclonal cell lines are propagated and used for further investigations.

If no definite clone can be identified, half of the confluent 24 well is frozen, whereas the other half is plated on a gelled 48-well plate, fed with 430 μl M15Si plus 70 μl CHIR 99021 (3 μMol/l). CHIR mimics Wnt signaling and consequently should activate responsive reporter constructs, detectable either by fluorescence (EGFP reporter gene) or luminiscence (luciferase reporter gene).

If there are still no clearly positive clones identified, the DNA has to be isolated and the presence of the transgene must be verified by PCR.

3.4 DNA Extraction

Before lysis, the cells are washed twice with 1x PBS. 200 μl Lysis Buffer are added to each 48 well, followed by incubation at 60°C for 24 hours in a humidity chamber. The next day 400 μl of a 75 mM NaCl solution (diluted in 96% EtOH) are added to each 48 well. The plate is tightly sealed and put in a -20°C freezer over night. In this step the DNA gets precipitated. After 24 hours the DNA should adhere to the bottom of the 48-well plate. The supernatant can be discarded by simply flipping the 48-well plate. The wells are washed twice with 400 μl 70% EtOH (again flipping the plate). Thereafter the pellets are dried for 15 minutes at room temperature. The DNA pellet is dissolved in 150 μl milliQ H_2O and is stored at -20°C. It can now be used as a template for PCR.

Lysis Buffer

- 10 mM Tris-HCl pH 7.5
- 10 mM EDTA pH 8.0
- 10 mM NaCl
- 0.5% Sarcosyl
- 1 mg/ml Proteinase K (added shortly before usage)

3.5 Polymerase Chain Reaction (PCR)

The PCRs were performed with a Biometra T-Personal PCR Cycler. The PRC products were analyzed by gel electrophoresis on 2% agarose gels. The PCR reaction mix of the only primer pair used (see Section 2.6) and the cycling program is listed below:

ddH_2O	38.75 μl
10x buffer	5 μl
25 mM $MgCl_2$	3 μl
10 mM dNTPs	1 μl
Forward Primer	0.5 μl
Reverse Primer	0.5 μl
DNA	1 μl
Taq Polymerase	0.25 μl
Total	50 μl

PCR Program:

94°C	1 min	
94°C	1 sec	
62°C	45 sec	30 x
72°C	1 min	
72°C	4 min	

3.6 Fluorescence Cytometry

All our experiments were done with BD FACSCalibur flow cytometer. The settings were tailored to our needs. The enhanced green fluorescent protein (EGFP) has an excitation maximum of 488 nm and an emission maximum of 509 nm. Propidium iodide (PI), when bound to nucleic acids, has an excitation maximum of 535 nm and an emission maximum of 617 nm. PI is used to stain dead cells, by entering through ruptured cell membranes and intercalation in DNA. According to these fluorophores two gates were drawn with the help of Thomas Sauer as seen in Figure 3.1. The settings (see Figure 3.2) were kept throughout all experiments and can be found in Section 3.6.2. To investigate and better understand the expression of Brachyury and Mesp1 promoter-driven EGFP during embryogenesis/cardiomyogenesis, up to 9 points in time were measured in each testseries. EBs generated from W4 ESCs and CBs generated from A5 CVPCs were used as working model. The percentage of EGFP expressing cells at day 0, 1, 3, 4, 5, 7, 9, 11, 13, 15 of EB/CB development was captured. Usually only one time point out of day 11, 13 or 15 was measured. In Figure 3.2 a typical visual output of a test series can be found. In (a) the negative control W4 ESCs showed hardly any EGFP positive cells, indicating the gate for GFP was set correctly, whereas W4-MesP-R ((b)-(g)) showed an up and down regulation of EGFP positive cells during differentiation.

Two successive measurements were done. Firstly only the percentage of EGFP expressing cells was captured, followed by the addition of 1 μl PI and subsequent capture of PI positive cells, which account for dead cells. This had to be done in a second step, as PI quenches the EGFP signal.

3.6.1 Preparation of Cells

Collection of Hanging Drops (d0 - d4)

In the first four days, while cells aggregate in the hanging drops, the cells for fluorescence analysis are collected by simply mingling the hanging drops on the lid. One milliliter of the cell suspension is sucked up and transferred into a FACS measuring tube. The cells are dispersed by gently pipetting them up and down. This cell suspension is promptly measured.

Collection of EBs/CBs (d5 - d15)

The aggregated cells (EBs/CBs) are rinsed onto a gelled tissue culture plate where they get stuck and start to differentiate. To collect EBs/CBs, the plates are washed twice with 1xPBS, before they are incubated for 20 minutes with 1 ml trypsin at 37°C. Trypsin is inactivated by the addition of 5 ml M15Si medium. The EBs/CBs are now detached and their cells are singularized. The cell suspension is transferred into a 15 ml Falcon tube and the cells are spun down for 7 minutes at 1000 rpm. The supernatant is discarded and the pellet is suspended in 1 ml M15Si medium by pipetting the cells up and down. Depending on cell density either the whole volume (usually d5) or only 100 - 500 μl (d7 - d15) are transferred into a FACS measuring tube. The measuring tubes are always filled up to a final volume of 1 ml. The cell suspension is promptly measured.

3.6.2 Measurement

Settings of FACSCalibur Flow Cytometer

The actual settings can be found in Table 3.2. Only four parameters were used and are therefore listed. Forward-scattered light (FSC) is proportional to cell-surface area or size. Side-scattered light (SSC) is proportional to cell granularity or internal complexity. In our case FCS-H/SSC-H captures the maximum height of our signal. Fluorescence 1 and 2 (FL1 and FL2) indicate two fluorescences measured. In our experiment these settings are adapted to EGFP and PI. The detectors are voltage dependent as the voltage strengthens the signal and makes it detectable. The Amplification Gain is another tool to enhance the signal, its effect multiplies the voltage and was only used for the forward scatter. The mode of FCS and SCC is linear by de-

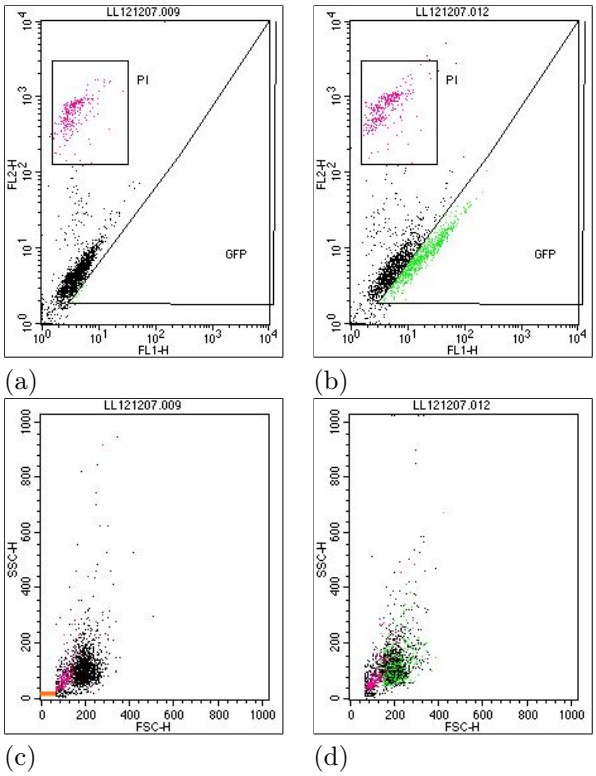


Figure 3.1: Fluorescence flow cytometry experimental setting illustrating the gates for EGFP (GFP - marked in green) and PI (marked in red). (a) W4 ESCs (control) and (b) W4-MesP-R (EGFP expressing cells).
The size of embryonic stem cells is illustrated in (c) W4 ESCs (control) and (d) W4-MesP-R. The threshold is marked in orange.

fault, whereas it is logarithmic for fluorescence as the difference of weakly or strongly fluorescent cells differs much more. On the scale of FCS and SCC are therefore 0 - 1,000 canals found, whereas fluorescence is captured in 0 - 10,000 (10^4) canals. Furthermore we determined a threshold for FCS (primary parameter), which had the value 53. The threshold defines the size of events we consider as large enough to be cells and to get rid of waste (cell fragments, contaminations ...). The threshold is illustrated in Figure 3.2 as the gap between y-axis and the cloud of dots (marked in orange). Each dot equals an event/cell. We captured 10,000 events for each measuring point.

The gates (as seen in Figure 3.1) are set manually by an experienced user (Thomas Sauer in our case). To get comparable results we used the same template for all our experiments.

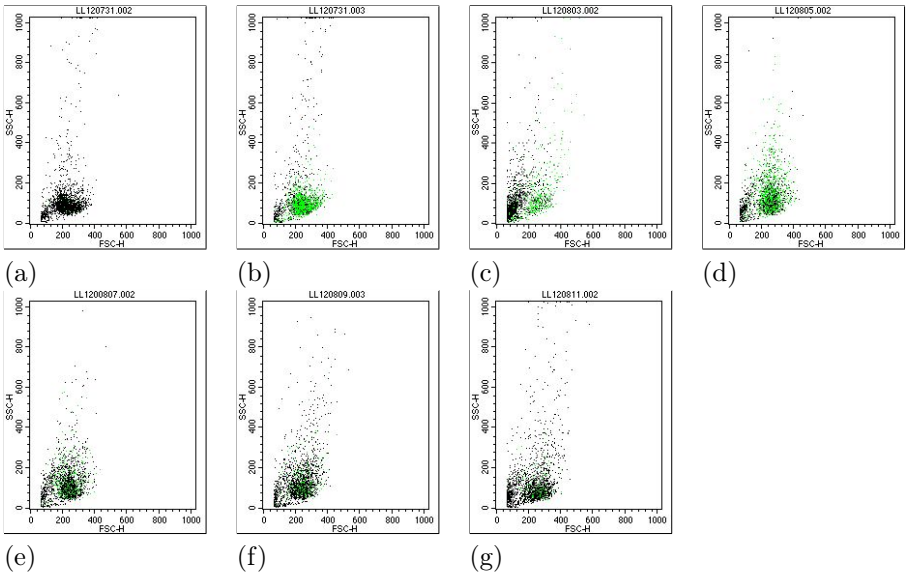


Figure 3.2: Graphical output of fluorescence analysis of W4 ESCs (a) at day 0 and cells from W4-MesP-R c4 derived embryoid bodies at (b) d0, (c) d3, (d) d5, (e) d7, (f) d9 and (g) d11.

Table 3.2: Fluorescence flow cytometer settings

Parameter	Detector	Voltage	AmpGain	Mode
P1	FSC	0.10	6.00	Lin
P2	SSC	350	1.00	Lin
P3	FL1	456	1.00	Log
P4	FL1	456	1.00	Log

Actual Measurement

The measuring tube containing 1 ml cell suspension is analyzed by BD FACS-calibur cytometer. We were always using the same template (EGFP and PI gates) and the same settings as described in Section 3.6.2. After capturing the percentage of EGFP expressing cells, 1 μl PI is added, the tube is gently pivoted and the cells are again measured. In the second run the amount of dead cells is evaluated. The evaluation has to be done in a subsequent step as PI quenches the amount of EGFP positive cells. One measurement lasts between 20 to 120 seconds, depending on cell density.

3.7 Luminiscence

All our experiments are done with Victor^3 V multilabel plate reader to capture luminiscence. The peak emission wavelength of firefly luciferase is about 560 nm. The according filter (filter A, slot 7) of Victor^3 V is used automatically. We adopt the program 'luminiscence' of Wallac 1420 software (version 3.4) which also displays the visual output. Four points in time of each test series (d3, d5, d7 and d11) were evaluated. 100 μl cell suspension are mixed with 100 μl ONE-Glo$^T M$ reagent in a nontransparent 96-well plate. The inside of the wells is coated in white, which augments luminiscence of each well without dispersing it in the neighboring wells. Three minutes after addition of ONE-Glo$^T M$ reagent the luminiscence can be captured.

3.7.1 Preparation of Cells

Collection of Hanging Drops (d0 - d4)

The cells are pooled as described in Section 3.6.1. 100 μl of the cell suspension are transferred onto a nontransparent 96-well plate. 100 μl ONE-Glo$^T M$ reagent was mixed with the cells and after three minutes the cell are measured.

Collection of EBs/CBs (d5 - d15)

The aggregated cells (EBs/CBs) are collected as described in Section 3.6.1. 100 μl of the cell suspension are transferred onto a nontransparent 96-well plate. 100 μl ONE-Glo$^T M$ reagent was mixed with the cells and after three minutes the cells are ready for measurement.

3.7.2 Measurement

The PC as well as Victor^3 V multilabel plate reader are switched on. The nontransparent 96-well plate was put in the dedicated slot of Victor^3 V. Then the software Wallac 1420 was opened and the settings for luminiscence are loaded. The plate is evaluated threefoldly, using the same settings throughout the experiment.

4 Results

The overall goal of this thesis was to establish stable reporter cell lines to further investigate cardiomyogenesis. Stably transfected cell lines are a powerful and useful tool to reproducibly detect and measure the influence of different factors on the activity of reporter genes, in our case key regulators of cardiac development. This offers great opportunity to better understand embryogenesis, its triggers and how to positively influence the formation of cardiomyocytes.

4.1 Production of Stable Reporter Cell Lines

All plasmids were transfected by electroporation (Section 3.3) in W4, an embryonic stem cell (ESC) line and A5, a cardiovascular progenitor cell (CVPC) line (Section 2.7).

4.1.1 Brachyury Reporter Assay

The heart arises from the mesoderm and is the first organ formed during embryogenesis (Van Vliet et al. 2012). The Brachyury gene is an early marker of nascent mesoderm, and found throughout invertebrate development (reviewed by Showell, Binder, and Conlon 2004). For our studies we wanted to investigate its expression in murine ESCs as well as CVPCs. Hence reporter cell lines were established by stably integrating the pT-Bra^{p}-Puro-IRES2-EGFP in both, ESCs and CVPCs. The plasmid was kindly provided by Prof. Dr. Agapios Sachinidis, who created this fully functional EGFP reporter construct driven by the Brachyury promoter (Figure 2.1). The plasmid pT-Bra^{p}-Puro-IRES2-EGFP was propagated in DH5-α bacteria, isolated and purified

Table 4.1: Electroporation settings for W4/A5

Cell line	Transfected vector	Milliseconds (ms)	Actual volts (V)
W4	pT-Bra^p-Puro-IRES2-EGFP	7.2	244
A5	pT-Bra^p-Puro-IRES2-EGFP	7.3	250
A5	pT-Bra^p-Puro-IRES2-EGFP	7.1	246
W4	pMesP1-EGFP	6.8	246
A5	pMesP1-EGFP	6.9	252
W4	pMesP1-IRES-EGFP	7.3	244
A5	pMesP1-IRES-EGFP	7.8	246
W4	pTOPflash	7.6	250
A5	pTOPflash	7.1	246
W4	pFOPflash	7.2	246
A5	pFOPflash	6.9	246

with the Qiagen EndoFree Plasmid Maxi Kit as described in Section 3.2. A concentration of 543.15 ng/μl of pT-Bra^p-Puro-IRES2-EGFP (pT-Bra) was obtained. 1 x 10^7 cells of W4, an ESC cell line, and A5, a CVPC cell line, respectively, were transfected with 25 μg of the linearized DNA. 46 μl of pT-Bra^p-Puro-IRES2-EGFP DNA solution were linearized with the NEB restriction enzyme Afe I and used for every electroporation.

The electroporation was administered with the Bio-Rad Gene Pulser II as described in Section 3.3.3, the actual settings can be found in Table 4.1.

The electroporated cells were allowed to recover for 24 hours in non-selective medium. The linearized vector should be randomly integrated into the genome. As the pT-Bra vector contains a neomycin resistance gene cassette, Geneticin (G418), which is a synthetic analogue of neomycin, was used as selective antibiotic in a final concentration of 180 μg/ml in the W4 (ESC line). In contrast, the A5 cell line contains already a neomycin cassette in its genome so following strategies were considered:

1. Increase of G418 concentration, based on the assumption that duplication of the neomycin cassette boosts the resistance against the antibiotic.
2. Double selection with G418 and Puromycin, as the construct also contains the puromycin cassette, which is only expressed under the influence of the Brachyury promoter.

Table 4.2: Concentrations of antibiotics (AB)

Cell line	**Transfected vector**	**Concentration of AB (μg/ml)**	**Antibiotic (AB)**
W4	pT-Bra^p-Puro-IRES2-EGFP	180	G418
A5	pT-Bra^p-Puro-IRES2-EGFP	540 - 900	G418
A5	pT-Bra^p-Puro-IRES2-EGFP	1	Puromycin
W4	pMesP1-EGFP	180	G418
A5	pMesP1-EGFP	900	G418
W4	pMesP1-IRES-EGFP	180	G418
A5	pMesP1-IRES-EGFP	540 - 900	G418
W4	pTOPflash	180	G418
A5	pTOPflash	540	G418
W4	pFOPflash	180	G418
A5	pFOPflash	540	G418

After 11 days under selective pressure (see Table 4.2) approximately 430 colonies on the W4 plate were counted of which 35 were picked. In contrast no colony was formed on puromycin selected A5 plate, but approximately 600 colonies were formed on the G418 selected plate, of which 16 clones were picked and transferred onto a 96-well plate. The master plates were allowed to recover for 24 hours in non-selective medium and were frozen the next day. The picked colonies were propagated as described in Section 3.3.4. The cells were regularly screened for green fluorescent colonies under the fluorescence microscope. As none of the colonies could be identified positively (by its green fluorescence), an aliquot of each monoclonal cell culture was transferred onto a gelled 96-well plate when they were split from a 48 well to a 24 well. These aliquoted cells were then driven into differentiation (M15-Si medium, no feeder cells). The Brachyury cell line started to emit green fluorescence once the promoter of the construct was turned on. EGFP is under the control of the Brachyury 5' UTR, hence its green fluorescence simulates Brachyury activation. Accordingly cells that appear in green in an undifferentiated state are very likely deregulated. Possibly the construct is under the influence of a housekeeping promoter (strong promoter, which is constantly active), which turns on the construct independently of Brachyury gene specific induction.

Brachyury is usually expressed between day 2 and day 8 of differentiation. This is visualized in this thesis by the vector construct harboring the Brachyury promoter coupled to an enhanced green fluorescent protein. After 4 days some colonies of the W4 ESCs containing the Brachyury construct - from now on referred to as W4-bra-R - could be positively identified (detectable green fluorescence) - clone number 3, 8, 12 and 22. When their undifferentiated counterparts of the 24-well plate reached confluency, all of them were frozen ($\frac{1}{2}$ well per cryotube), except $\frac{1}{2}$ of # 3, 8, 12 and 22, which were further propagated. These four monoclonal cell lines provided the basis for further experiments.

None of the A5 containing the Brachyury construct, now referred to as A5-bra-R, showed any explicit fluorescence. To stronger induce differentiation CHIR 99021 was added in a final concentration of 3 μMol/l. CHIR is a GSK inhibitor and hence mimics the activation of Wnt signaling. Brachyury is known to be driven by this pathway, consequently it should get activated upon CHIR induction. Still no significant fluorescence could be detected. There are at least two possible explanations for this:

1. The CHIR used was kept as a solution (in DMSO) for more than two years at 4°C, therefore it was likely, that it had lost its function (CHIR is stable for 2 years at -20°C when dissolved in DMSO).
2. CHIR was added after several days, where the cells have already started differentiating. As Brachyury is an early marker of nascent mesoderm, and CVPCs are approximately five days ahead in development compared to ESCs, it is likely that Brachyury was already downregulated at this point in time.

Although we selected with G418 at high concentrations (up to 900 μg/ml) we could not be sure that our colonies had integrated the Brachyury construct. To check this we isolated the DNA of the cell clones and screened for EGFP positive clones by PCR. Positive results are a strong indication that the construct was integrated, even though giving us no further information about site or completeness of integration. We identified four clones (#2, 3, 13, 16) containing at least the EGFP gene; out of those, clone #2 and #13 were further investigated.

4.1.2 Mesp1 Reporter Assay

The Mesp1 reporter plasmid (Figure 2.2) helps to elucidate the regulation pattern of the Mesp1 gene, which is, until now, the earliest known marker of cardiac specific lineage. Whether or not there is a direct interaction between Wnt signaling and Mesp1 is highly debated, but not proven yet (reviewed by Bondue and Blanpain 2010). An indirect upregulation of Mesp1 expression however is very likely, as Mesp1 is driven by Brachyury, which in turn gets activated by Wnt signaling.

W4 ESCs as well as A5 CVPCs were electroporated with 25 μg of linearized pMesP1-EGFP plasmid DNA. The plasmid was kindly provided by Prof. Dr. Robert David. pMesP1-EGFP was propagated in DH5-α *E.coli* and purified with Qiagen EndoFree Plasmid Maxi Kit at a final concentration of 266.3 ng/μl. 93 μl of the plasmid DNA solution were linearized with NEB restriction enzyme StuI. All further steps were done as described in Section 4.1.1. The Bio-Rad Gene Pulser II settings and the concentrations of the antibiotics can be found in Table 4.1 and Table 4.2, respectively. The ESCs which have integrated the pMesP1-EGFP plasmid are now referred to as W4-MesP-R, and the CVPCs as A5-MesP-R. After 12 days 10 clones from each plate were picked (165 in total of W4-MesP-R and 51 in total of A5-MesP-R), transferred and further propagated. The 96-well plate monolayer (differentiating cells) revealed that clone #2, 4, 6, 9 and 10 of the W4-MesP-R were significantly green as well as clone #1 of A5-MesP-R.

In addition cells which overexpress the Mesp1 protein under the control of the CMV promoter were established but not further used in this thesis.

4.1.3 Mesp1 Overexpression

Again both cell types (W4 and A5) were electroporated with 25 μg of linearized pMesP1-IRES-EGFP plasmid DNA. pMesP1-IRES-EGFP was propagated in DH5-α *E.coli* and purified with Qiagen EndoFree Plasmid Maxi Kit at a final concentration of 795.25 ng/μl. 31.4 μl of the overexpression plasmid DNA solution was linearized with NEB restriction enzyme Mfe I. All further steps were done as described in Section 4.1.1. The Bio-Rad Gene Pulser II settings and the concentrations of the antibiotics can be found in Table 4.1 and Table 4.2, respectively. After 14 days 18 clones were picked from W4 +

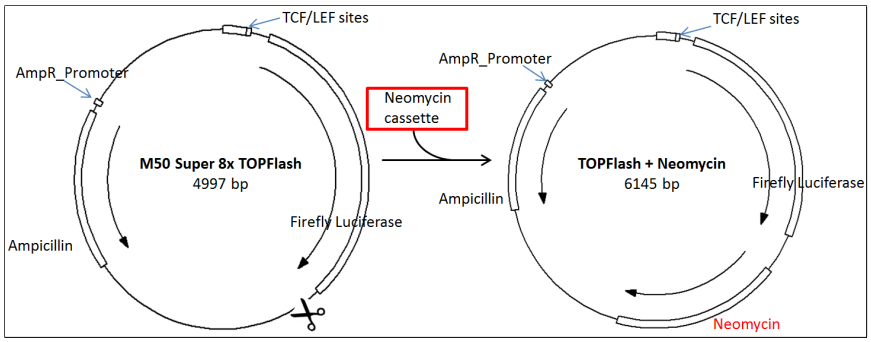


Figure 4.1: Construction of TOPflash reporter plasmid.
On the left the original vector is displayed. On the right site TOPflash vector containing the neomycin cassette is shown.

pMesP1-IRES-EGFP plate (approx. 600 in total), now referred to as W4-Mesp-ExpEGFP and 15 clones were picked from the A5 + pMesP1-IRES-EGFP plate (approx. 500 in total), now referred to as A5-Mesp-ExpEGFP. These clones were propagated and screened for positive (= green fluorescent) clones. Clone #2 and 3 of W4-Mesp-ExpEGFP and clone #1 and especially #14 of A5-Mesp-ExpEGFP were positive. All of the cell lines were frozen and no further experiments were done in the context of this thesis.

4.1.4 Wnt Signaling Reporter Assay

Wnt signaling pathway plays a substantial role in early embryogenesis, especially in mesoderm formation (Section 1.5.3). Its influence during cardiomyogenesis was reflected by using the M50 Super 8x TOPFlash (TOPflash) reporter plasmid. The M51 Super 8x FOPFlash (FOPflash) reporter plasmid, a TOPflash mutant was used as negative control. Both are luciferase reporters of β-catenin-mediated transcriptional activation illustrated in Figure 2.4 and Figure 2.5.

Insertion of Neomycin Cassette

As both plasmids originally did not contain a selective cassette suitable for eukaryotic cells we first had to genetically modify the plasmids. We excised the neomycin cassette from pMC1neo-polyA with XhoI/SalI and linearized the TOPflash/FOPflash vector with SalI. TOP/FOPflash vectors were dephosphorylated, ligated with the neomycin cassette and transformed into

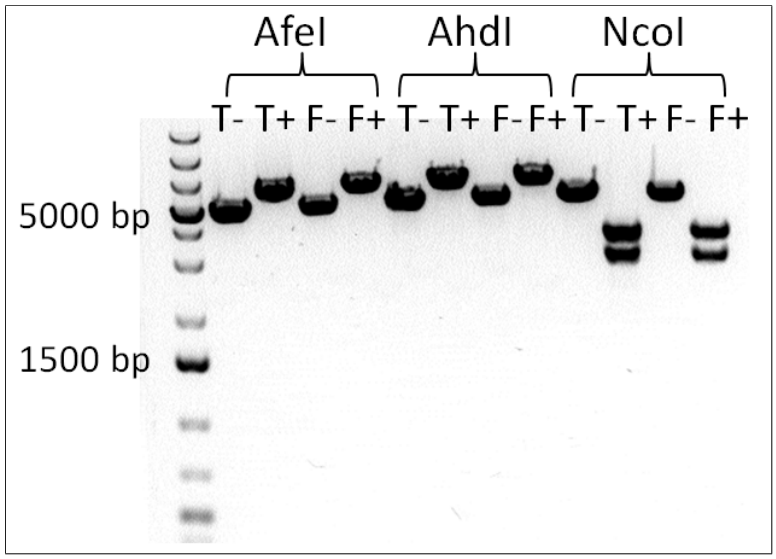


Figure 4.2: Digestion of TOP/FOPflash with AfeI, AhdI and NcoI
'T-/F-' stands for TOP/FOPflash plasmid, without neomycin cassette; 'T+/F+' stands for TOP/FOPflash plasmid including the neomycin cassette. Standard is GeneRuler^{TM} 1 kb Plus DNA Ladder.

competent DH5-α bacteria. Figure 4.1 shows the TOPflash vector before and after uptake of the neomycin cassette (FOPflash is very similar despite some mutations in the TCF/LEF site, and therefore not illustrated here). As the negative controls hardly showed any colonies, colonies from the TOP/FOPflash plates (which should have integrated the insert) were picked, propagated, purified and validated as described in Section 3.2.9. One FOPflash colony as well as several colonies of TOPflash were positively identified. One of each kind was further propagated and purified with Qiagen EndoFree Plasmid Maxi Kit. TOPflash had a final concentration of 1000,5 ng/μl and, FOPflash of 653 ng/μl. Figure 4.2 shows different digestions of TOP/FOPflash with and without the neomycin cassette. AfeI and AhdI are single cutters of the TOP/FOPflash backbone. Linearized TOP/FOPflash vectors have a length of approx. 5 kb, whereas TOP/FOPflash vectors containing the 1.1 kb neomycin cassette have a length of 6.1 kb. NcoI cuts once in the TOP/FOPflash backbone and once within the neomycin insert. Hence NcoI linearizes TOP/FOPflash plasmids and cuts TOP/FOPflash plasmids containing the neomycin cassette into two fragments (3.3k and 2.8k).

Electroporation of TOP/FOPflash

25 μg of both vectors were linearized with AseI (NEB) for electroporation. The cells (W4 and A5) were electroporated with the Bio-Rad Gene Pulser II. It was set at 230 V and 500 μF. The actual values and antibiotic concentrations can be found in Table 4.1 and Table 4.2. In contrast to the experimental settings above (see Section 3.3.3), we plated only 600 μl (1 ml in total) of the electroporated cells onto a 6 cm plate instead of a 10 cm plate, because we always had observed plethora of colonies. The cells that inserted the construct in their genome are now referred to as W4-Top, A5-Top, W4-Fop and A5-Fop. After 12 days colonies on every plate were detected. We picked all visible colonies: 6 x W4-Top, 9 x A5-Top, 8 x W4-Fop and 9 x A5-Fop. All cells were transferred onto 96-well plates and were further propagated. As these cells could only be identified by luciferase assays, another way of detection (luminiscence) had to be chosen as described below in Section 4.3.

4.2 Characterization of Stable Cell Lines by Fluorescence Cytometry

All experiments were done with BD FACSCalibur flow cytometer. The used software was "cellquest pro", the settings are listed in Section 3.6 and were used throughout all experiments. 10,000 events were measured, quantifying the amount of EGFP positive cells as well as that of propidium iodide (PI) positive cells. Enhanced green fluorescent protein (EGFP) has an excitation maximum of 488 nm and an emission maximum of 509 nm. PI enters through ruptured cell membranes and consequently intercalates in DNA of dead cells only. By binding to nucleic acids it fluoresces with an excitation maximum of 535 nm and the emission maximum is 617 nm.

We were interested in the expression of Brachyury and Mesp1 during early embryogenesis and cardiomyogenesis. Therefore a working model was needed, which mimics these processes. As our cell lines had stably integrated the promoter constructs, we used the in vitro models embryoid bodies (EBs) for ESCs and accordingly cardiac bodies (CBs) for CVPCs. Both Brachyury and Mesp1 are early markers of mesodermal development and, Mesp1 even more, are involved in cardiac specific development. Both are upregulated only transiently at the beginning of embryogenesis hence we decided to quantify

the amount of EGFP positive cells during the first 15 days of EB/CB development. With minor deviations the following points in time were chosen for measurement: d0, d1, d3, d4, d5, d7, d9, d11/13/15. For each point in time one plate of EBs/CBs was quantified.

All cell lines (W4-bra-R, A5-bra-R, W4-MesP-R and A5-MesP-R) were expressing EGFP under the control of the 5' UTR of Brachyury or Mesp1. But as the constructs were inserted randomly in the wildtype (wt) genome (W4 and A5) we further had to test, whether the site of insertion hinders the cells to develop normally. Depending on the site of integration the construct could be driven by a housekeeping promoter (a strong promoter, which is constantly active), which would turn on the construct non specifically and therefore lead to a deregulation of the reporter gene. Differences in morphology of the established cell lines compared to wildtype cells (control) during development were examined using EBs/CBs plates of each cell line and of control (wt) (see Figure 4.4 and Figure 4.5). In total 9 plates of EBs/CBs were needed for one test series (one plate for every measure point plus two plates to investigate morphology). The preparation of cells before measurement of the EGFP fluorescence are found in Section 3.6.1; the actual measurement was done as described in Section 3.6.2.

4.2.1 Fluorescence of Brachyury positive Cells

Several independent test series (TS) were done for W4-bra-R clones #3 (1 TS), #8 (3 TS), #12 (2 TS) and #22 (2 TS); and A5-bra-R clones #A (2 TS) and #B (2 TS).

W4-bra-R

Three of the four investigated clones showed a similar expression pattern: W4-bra-R c3, c8 and c12, whereas W4-bra-R c22 seemed to be deregulated. The clones #3, 8 and 12 showed an EGFP peak at d0 and at d5. In Figure 4.3 the expression of EGFP under the control of the Brachyury promoter of clone 3, 8 and 12 were normalized to day 5. The clone 3 was chosen for further experiments as these EBs developed normally (see Figure 4.4 (b)) and they showed the expected trend of Brachyury activation as described in Section 1.5.1.

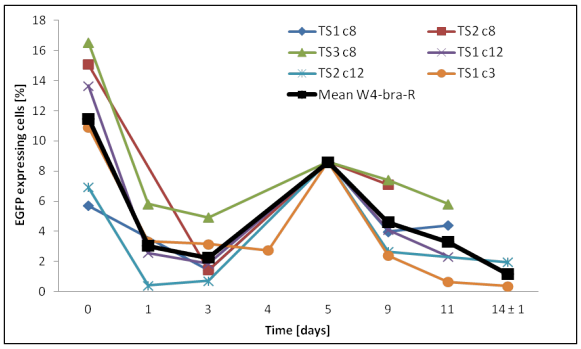


Figure 4.3: Percentage of cells expressing EGFP under the control of the Brachyury 5' UTR in W4 ESC derived embryoid bodies. Data of individual test series (TS) and clones (c) were normalized to the value measured on day 5.

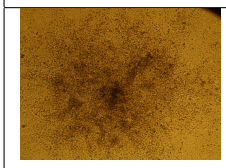
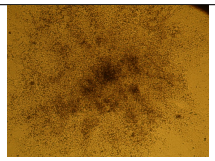
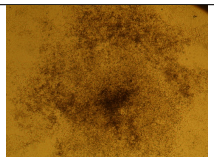
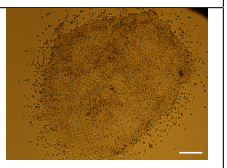
W4 wt	W4-bra-R c3	W4-MesP-R cA	W4-MesP-R cB
			
(a)	(b)	(c)	(d)

Figure 4.4: Morphology of embryoid bodies generated from (a) W4 wild-type, (b) W4-bra-R c3, (c) W4-MesP-R cA and (d) W4-MesP-R cB at day 7. Phase contrast images. Bar = 200 μm.

A5-bra-R

A5-bra-R c2 and c13 were renamed, as they got mixed up after thawing. As we could not assure, which clone was #2 and which one was #13 (these two were chosen because of positive PCR results), we decided to rename them "A" and "B", as still some aliquots of #2 and 13 are stored in liquid nitrogen. Although both clones showed a similar behavior in fluorescence cytometry, they developed differently. Figure 4.5 demonstrates that A5-bra-R cB derived CBs (c) developed similarly to the control (a) whereas A5-bra-R cA derived CBs (b) stayed compact and never developed beating cardiomyocytes. The percentage of EGFP positive cells was decreasing from day 0 to day 3 and stayed low during the rest of the experiment which is illustrated in Figure 4.6.

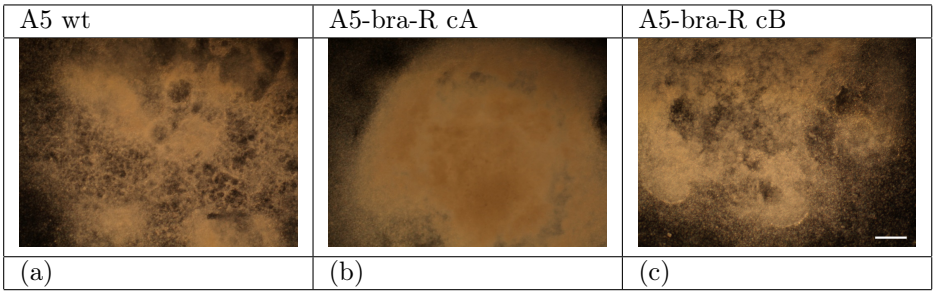


Figure 4.5: Morphology of CBs derived from (a) A5 wildtype, (b) A5-bra-R cA and (c) A5-bra-R cB at day 10. Pictures were made by phase contrast microscopy. Bar = 200 μm.

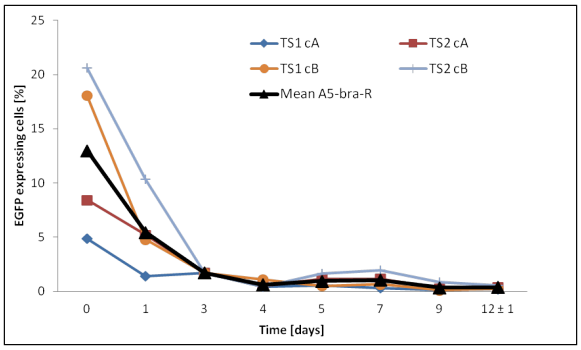


Figure 4.6: Percentage of cells expressing EGFP under the control of the Brachyury 5' UTR in A5 CVPC derived cardiac bodies. Data of individual test series (TS) and clones (c) were normalized to the value measured on day 3.

Comparison W4-bra-R and A5-bra-R

In Figure 4.7 the mean of EGFP expressing cells of W4-bra-R was compared to that of A5-bra-R. In this figure we adapted the graph of W4-bra-R at day 0. According to literature Brachyury is only transiently upregulated around day 4, hence our high percentage of EGFP positive cells at day 0 (in ESC derived EBs) is unexpected. In 2012 Diekmann and collaborators demonstrated unspecific fluorescence of stably integrated reporter genes and they managed to reduce this background fluorescence by using specific miRNAs (Diekmann et al. 2012). Taking this into consideration we decided to omit our actual mean value of day 0 assuming that it displays an unspecific fluorescence and replaced it by a more reasonable one (lowest value of all test series). Therefore Figure 4.7 reflects an idealized version of the EGFP

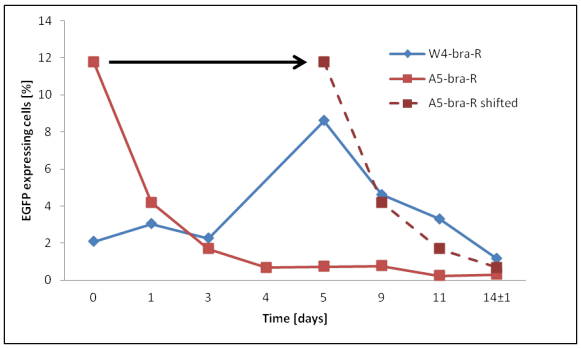


Figure 4.7: The mean percentage of cells expressing EGFP under the control of the Brachyury 5' UTR in W4 ESC derived embryoid bodies compared to the mean percentage of cells expressing EGFP under the control of the Brachyury 5' UTR in A5 CVPC derived cardiac bodies. The black arrow and the dotted red graph indicate the working hypothesis that the developmental stage of CVPCs is approximately 5 to 7 days ahead of ESCs.

expression. CVPCs are approximately 5 to 7 days ahead of ESCs, illustrated by a shift of the graph of A5-bra-R from d0 to d5 (dotted line). We suggested that the graph of A5-bra-R (CVPCs) equates the decline of the peak of the W4-bra-R (ESCs) at day 5. These graphs represent the expression of Brachyury promoter-driven EGFP. The transient expression of Brachyury around day 4 of embryogenesis is found even earlier in CVPC derived CBs, as these cells are some days ahead in differentiation compared to ESCs and are already dedicated to the mesodermal lineage. Brachyury gets downregulated quickly after this specification (Section 1.5), hence only the decline of Brachyury promoter-driven EGFP could be found in the CBs.

4.2.2 Fluorescence of Mesp1 positive Cells

Several test series (TS) were done for W4-MesP-R clones #4 (3 TS), #A (1 TS), #B (1 TS); and A5-MesP-R clones #1 (2 TS).

W4-MesP-R

Clone #4 was chosen, which showed a significant green fluorescence even in the undifferentiated state (see Figure 4.8). Our experiments revealed that this clone was deregulated (see Figure 4.4) as it did not differentiate properly. Therefore, we decided to choose clones #5 and #8, which were not as highly fluorescent at day 0 as clone #4. W4-MesP-R c5 and c8 were renamed, as

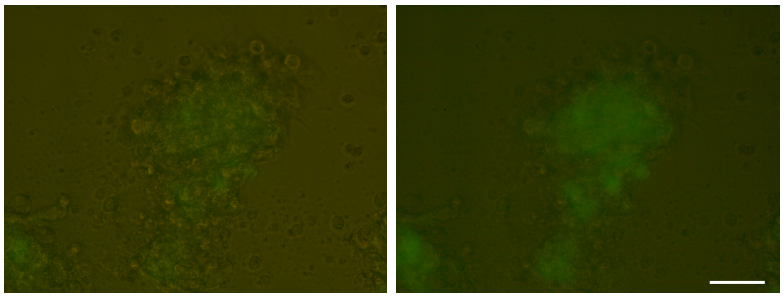


Figure 4.8: EGFP expression under the control of the Mesp1 5' UTR in colonies of undifferentiated W4 ESCs. Fluorescence microscopical image overload on phase contrast image. Bar = 50 μm.

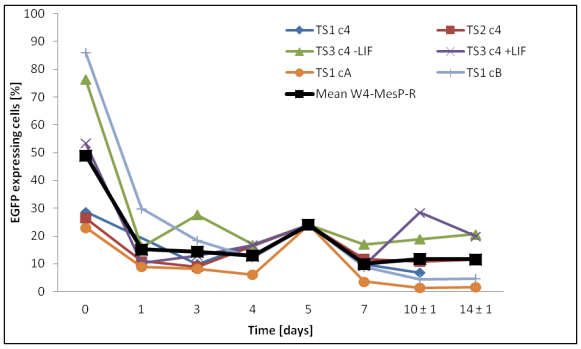


Figure 4.9: Percentage of cells expressing EGFP under the control of the Mesp1 5' UTR in W4 ESC derived embryoid bodies. Data of individual test series (TS) and clones (c) were normalized to the value measured on day 5. Clone 4 in TS3 was also cultivated in the presence of LIF for 2 passages before aggregation of ESCs to EBs on day 0 (+LIF) and compared to control (-LIF). In black the mean of all test series is shown.

they got mixed up after thawing. Since we could not assure, which clone was #5 and which one was #8, we decided to rename them into "A" and "B", as still some aliquots of #5 and #8 are stored in liquid nitrogen.

Similar to clone 4, clone B did not differentiate properly, easily recognizable by its smaller size compared to the control (see Figure 4.4 (d)) and it never developed beating cardiomyocytes. The percentage of EGFP expressing cells of W4-MesP-R cA was comparatively low at day 0 but reached the maximum at day 5 (Figure 4.9). Since clone A developed normally (Figure 4.4 (c)) it was chosen for further analysis.

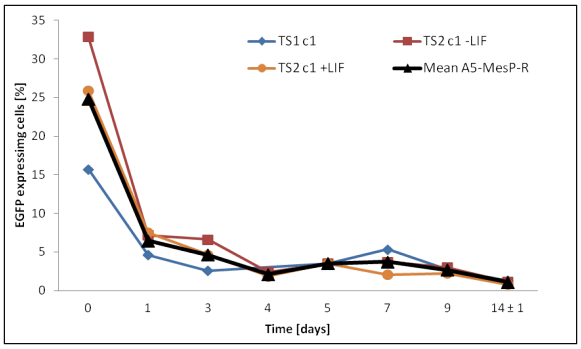


Figure 4.10: Percentage of cells expressing EGFP under the control of the Mesp1 5' UTR in A5 CVPCs derived cardiac bodies. Data of individual test series (TS) of clone 1 were normalized on day 5. Clone 1 in TS2 was also cultivated in the presence of LIF for 2 passages before aggregation of CVPCs to CBs on day 0 (+LIF) and compared to control (-LIF). In black the mean of all test series is shown.

A5-MesP-R

Green fluorescence was only detected in clone #1 and, thus further analyzed. Similar to the A5-bra-R clones we saw a decline of fluorescence from d0 on, as shown in Figure 4.10.

Comparison W4-MesP-R and A5-MesP-R

When we compared the mean of EGFP expression under the control of the Mesp1 promoter in differentiating W4-MesP-R clones with that of A5-MesP-R clones we could draw a similar picture as for Brachyury driven EGFP expression (Figure 4.11). Brachyury induces Mesp1 which is subsequently and also only transiently upregulated around day 4, similar as described in Section 4.2.1. Again we changed the value of the graph of W4-MesP-R at day 0 (we took the value of day 1 instead). Therefore Figure 4.11 reflects an idealized version of EGFP distribution. We assumed that CVPCs (A5-MesP-R) are 5 - 7 days ahead in development compared to ESCs, consequently the expression of Mesp1, visualized by the Mesp1 promoter-driven EGFP expression, was found already at d0 in CVPC derived CBs. The shift of the expression is illustrated by the dotted graph.

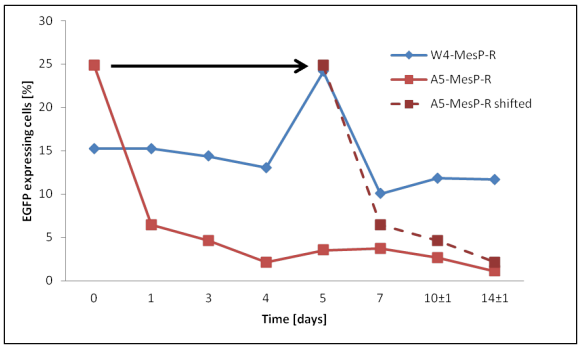


Figure 4.11: The mean percentage of cells expressing EGFP under the control of the Mesp1 5' UTR in W4 ESC derived embryoid bodies compared to the mean percentage of cells expressing EGFP under the control of the Mesp1 5' UTR in A5 CVPC derived cardiac bodies. The black arrow and the dotted red graph indicate the working hypothesis that the developmental stage of CVPCs is approximately 5 to 7 days ahead of ESCs.

4.2.3 Influence of LIF on Brachyury/Mesp1 driven EGFP Expression

The expression of Brachyury and Mesp1 is transiently and usually peaks around day 4 in ESCs. We got a significant signal at day 0 as well, therefore we suspected that the feeder cells did not produce enough LIF to keep the cells in an undifferentiated state. Considering this we assumed that some of our cells were already driven into differentiation and hence account for the EGFP expression in cells at day 0. To confirm this we generated new experimental settings. For the control test series we used the same set-up as before, additionally, two test series were supplemented with 20 U/ml leukemia inhibitory factor (LIF). One series was supplemented throughout the whole experiment, the other only until the production of EBs/CBs (d0). The addition of LIF was started two passages before production of EBs/CBs. The different setups were named '-LIF' (no supplements), '± LIF' (supplemented till d0) and '+LIF' (constantly supplemented).

These experiments were done with clone #4 of W4-MesP-R and with clone #1 of A5-MesP-R. The tests were done in parallel and the same points in time were investigated as described above (d0, d1, d3d, d4, d5, d9 and d13).

W4-MesP-R c4

Having a closer look on W4-MesP-R c4, LIF did not show any influence on the EGFP expression at day 0. The cells continuously treated with LIF were blocked in differentiation (Figure 4.13). Hence we concluded that the concentration of LIF was high enough to keep the cells in an undifferentiated state. As the -LIF and the ± LIF experiments did not differ at day 0 of EB aggregation, we assumed that the feeder cells sufficiently provided LIF to prohibit differentiation. The unexpected EGFP expression at d0 of W4-bra-R and W4-MesP-R clones seemed not to be caused by lack of LIF. We examined the morphology (see Figure 4.13) of our EBs at day 6. W4-MesP-R c4 independently of LIF treatment did not develop normally. W4-MesP-R c4 derived EBs - LIF and ± LIF looked alike, but were significantly smaller compared to W4 control EBs. W4-MesP-R c4 derived EBs + LIF were mainly undifferentiated and thus appeared more dense. None of the W4-MesP-R c4 EBs developed beating cardiomyocytes. W4-MesP-R c4 cells showed high expression levels of EGFP even in an undifferentiated state and did not develop normally, which was a strong indication that the site of insertion led to a deregulation of development. Taking this into account the experiment should be repeated with a cell clone, which develops normally to proof whether or not the maximum at day 0 is caused by a lack of LIF. As mentioned above another possible explanation of the unexpected signal at day 0 is an unspecific fluorescence of reporter constructs (Diekmann et al. 2012). It would have been too time consuming to proof this in the context of this thesis, as new, genetically modified, reporter constructs would have been needed.

A5-MesP-R c1

The influence of LIF on A5-MesP-R c1 can be found in Figure 4.14. All three test series showed a similar trend - a decline of EGFP expressing cells starting from day 0.

The morphology of CBs in Figure 4.15 shows the unaffected development of A5-MesP-R c1 - LIF and A5-MesP-R c1 ± LIF compared to A5 wildtype control. Under the influence of LIF, A5-MesP-R c1 derived CBs were blocked in differentiation (dense center composed of undifferentiated cells) and never displayed beating cardiomyocytes. As we assume that CVPCs are 5 - 7 days

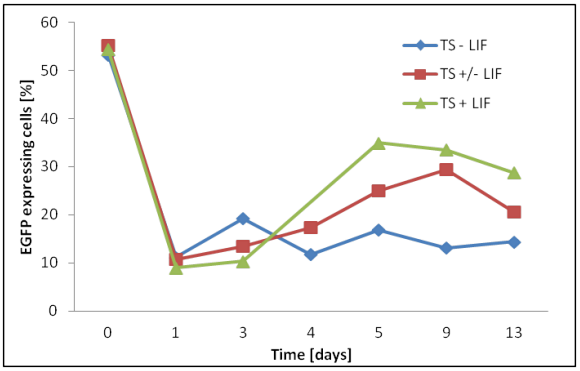


Figure 4.12: Influence of LIF on the expression of Mesp1 in differentiating W4 ESCs. LIF was added for 2 passages before aggregation of cells (±) and further given throughout the experiment while ESCs aggregated to EBs (+).

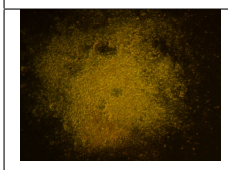
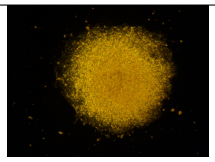
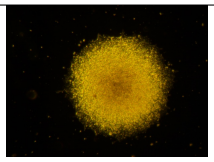
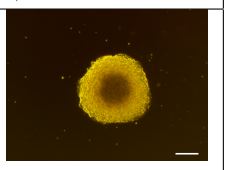
W4 wt d6	W4-MesP-R c4 - LIF	W4-MesP-R c4 ± LIF	W4-MesP-R c4 + LIF
			
(a)	(b)	(c)	(d)

Figure 4.13: Morphology of embryoid bodies derived from (a) W4 wildtype, (b) W4-MesP-R c4 - LIF, (c) W4-MesP-R c4 ± LIF and (d) W4-MesP-R c4 + LIF at day 6. LIF was added for 2 passages before aggregation of cells (±) and further given throughout the experiment while ESCs aggregated to EBs (+). Bar = 200 μm.

ahead of ESCs the maximum at day 0 equates the decline of the peak of ESC derived EBs at day 5. Considering this we would always get a maximum of EGFP expressing cells at day 0, independent of LIF supplementation, hence the results were not indicative whether LIF concentration was sufficient or not.

4.2.4 Cell Death during ESC and CVPC Differentiation

To evaluate the percentage of dead cells in developing EBs and CBs, a cell sample using propidium iodide (PI) was analyzed at the same measure points as for EGFP expression. PI intercalates in DNA of dead cells and could be captured by its specific emission wavelength. PI was added shortly before

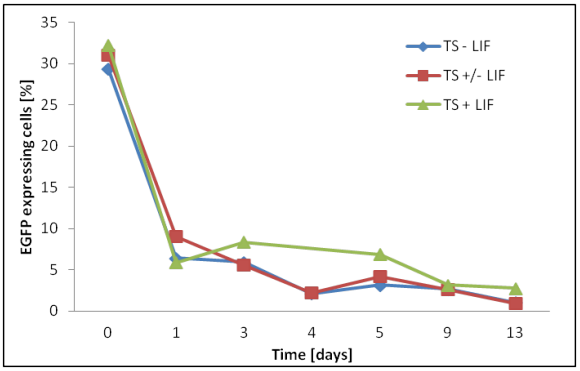


Figure 4.14: Influence of LIF on the expression of Mesp1 in differentiating A5 CVPCs. LIF was added for 2 passages before aggregation of cells (±) and further given throughout the experiment while CVPCs aggregated to CBs (+).

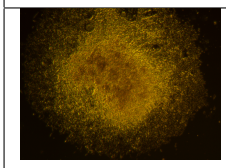
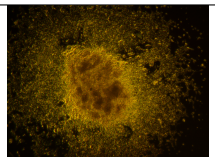
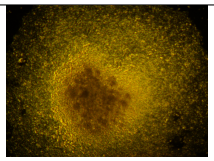
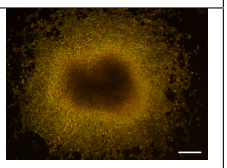
A5 wt d6	A5-MesP-R c1 - LIF	A5-MesP-R c1 ± LIF	A5-MesP-R c1 + LIF
			
(a)	(b)	(c)	(d)

Figure 4.15: Morphology of cardiac bodies generated from (a) A5 wildtype, (b) A5-MesP-R c1 - LIF, (c) A5-MesP-R c1 ± LIF and (d) A5-MesP-R c1 + LIF at day 6 of CB development. LIF was added for 2 passages before aggregation of cells (±) and further given throughout the experiment while CVPCs aggregated to CBs (+). Bar = 200 μm.

measurement.

The percentages of dead cells of W4 and A5 reporter cell lines (Brachyury and Mesp1) were evaluated and depicted by their means (Figure 4.16). Generally one trend was observed. At day 0 cells are in a good condition. After production of EBs/CBs at day 1 there was a significant increase in dead cells (up to 50%) likely caused by stress the cells experience while making the EBs/CBs. In the first four days the cells measured were harvested from mingled hanging drops. Most of these cells contribute to the formation of the EB/CB, but a hanging drop also contains not aggregated and dead cells. After 4 days the cells were rinsed and adhered to a gelatinized culture plate. To collect these cells, the plate was washed twice before trypsinisation, hence

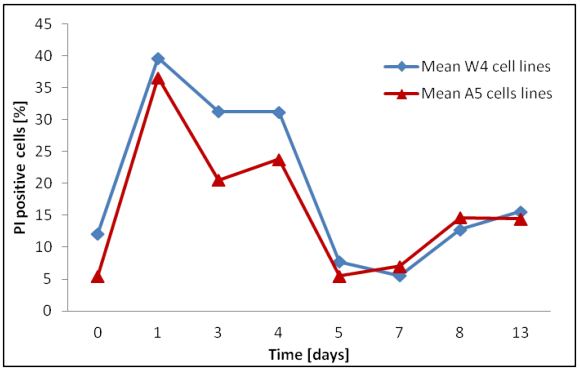


Figure 4.16: Percentage of propidium iodide (PI) positive cells in W4 ESC derived EBs and A5 CVPC derived CBs representing aggregation (d1 - d4) and differentiation (d5 - 13).

the dead cells were very likely rinsed off. Some fluctuation of PI stained cells throughout the experiment may be caused by rough treatment, minor changes in time of trypsinisation or other handling aberrations. After day 1 the amount of dead cells dropped in all cell lines until day 5 (A5) or day 7 (W4) and started to ascend again from these points in time.

4.3 Assessment of Wnt Signaling in EBs and CBs for the Characterization of Cell Lines by Luciferase Assays

We measured luciferase activity in ESCs and CVPCs which were stably transfected with TOPflash and its negative control FOPflash. TOPflash clones harbor Tcf/Lef sites which account for a β-catenin-mediated transcriptional activation; generally they are used for measuring activities of canonical Wnt signaling. To identify clones which showed activation of Wnt signaling, a luciferase assay was carried out.

Once the cells were confluent on a 24-well plate they were split in a 1:2 ratio. One half was further propagated on a 24-well plate. The other half was again divided by two and seeded onto gelled 48 wells. One well of the 48-well plate was supplemented with 2.5 μMol/l CHIR 99021 (a freshly prepared solution), the other remained untreated. After 24 hours the cells were analyzed with a Victor^3 V multilabel reader. The cells were trypsinized and suspended in one milliliter of fresh medium. 100 μl of cell suspension

was mixed with 100 μl ONE-Glo^{T}M reagent and luminiscence was captured by Victor^3 V after three minutes. As CHIR is a GSK inhibitor (it induces migration of β-catenin to the nucleus) it mimics Wnt signaling and should activate the TOPflash constructs of the W4 ESCs and A5 CVPCs; in contrast the FOPflash constructs should not show any luminiscence regardless of whether CHIR was added or not (as the Tef/Lef sites of this construct are mutated and not longer functional). A5-Top clones (especially #2 and #6) showed a great activation when treated with CHIR 99021 (Figure 4.17). W4-Top clones did not show such an intense activation but clone #3 and #5 were significantly enhanced, determined by the limit of detection (LOD). The LOD was estimated from the mean of the blanks (x_B) and the standard deviation of the blanks (s_B). Blanks equaled the value of empty wells.

LOD = x_B + 3 x s_B.

W4-Fop clone #3 and A5-Fop clone #6 showed some irregular behavior and were therefore omitted. The other Fop clones did not show any significant signal, as expected. Based on this data W4-Top clone #3 and #5; A5-Top clone #2 and #6; W4-Fop clone #7 and A5-Fop clone #4 were selected and propagated for further experiments, whereas the others were frozen and stored in liquid nitrogen.

4.4 Regulation of the Brachyury and Mesp1 Expression in EBs and CBs

4.4.1 Influence of CHIR and SB on Expression of W4-bra-R and W4-MesP-R derived EBs

To investigate the influence of Wnt and TGF-β signaling on Brachyury and Mesp1 expression in EBs, the most promising candidates of the validation experiments were chosen: W4-bra-R c3, and W4-MesP-R cA. With these clones the influence of several substances on their development was investigated:

1. CHIR 99021 is a GSK inhibitor that mimics Wnt signaling. Brachyury is directly, Mesp1, more likely, indirectly regulated by the Wnt signaling. Hence we suggested that the EGFP expression, which is driven by either the promoter of Brachyury or Mesp1, respectively, would rise.
2. SB 431542 is a potent and selective inhibitor of activin receptor-like kinase (ALK) receptors which act on the TGF-β pathway. ALK4 and

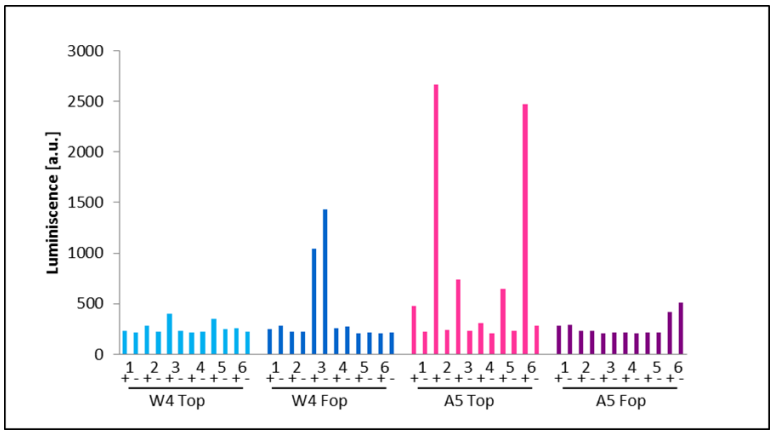


Figure 4.17: Luciferase Activity in undifferentiated W4 ESCs and A5 CVPCs with non-homologously integrated β-catenin responsive reporter genes (Top). To demonstrate specificity of luciferase activity Wnt-signaling was mimicked by the addition of 2.5 μMol/l CHIR 99021. As control cells were used with a non-homologous integrated β-catenin responsive reporter gene (Fop). Clones 1 - 6 of W4-Top, W4-Fop, A5-Top and A5-Fop were measured 24 hours after addition of 2.5 μMol/l CHIR (+). The induction was compared to untreated controls (-).

ALK7 are responsible for the phosphorylation of Smad1 and in further consequence SB 431542 inhibits TGF-β induced epithelial to mesenchymal transition (ETM) (Halder, Beauchamp, and Datta 2005). It should not directly influence the Brachyury and Mesp1 promoter-driven EGFP expression, as these are not known to be induced by the TGF-β pathway.

A similar test setting was used as described in Section 3.6, but only four points in time for fluorescence flow cytometry measurements: d3, d5, d7 and d11 were chosen.

The experiment was done in three parallel test series: W4-bra-R/W4-MesP-R control (no supplements), W4-bra-R/W4-MesP-R + 2.5 μMol/l CHIR 99021 and W4-bra-R/W4-MesP-R + 2 - 4 μMol/l SB 431542. Both substances were added to the cells before the aggregation of ESCs (d0). As cells treated with 4 μMol/l SB showed significantly more dead cells compared to our other test series (see Figure 4.19 (b) and Figure 4.20 (b)) we assumed that the dose of SB was partly toxic and we reduced the concentration to 2 μMol/l when the cells were transferred to gelatinized culture plates (d4). Furthermore we investigated the influence of CHIR and SB on the morphol-

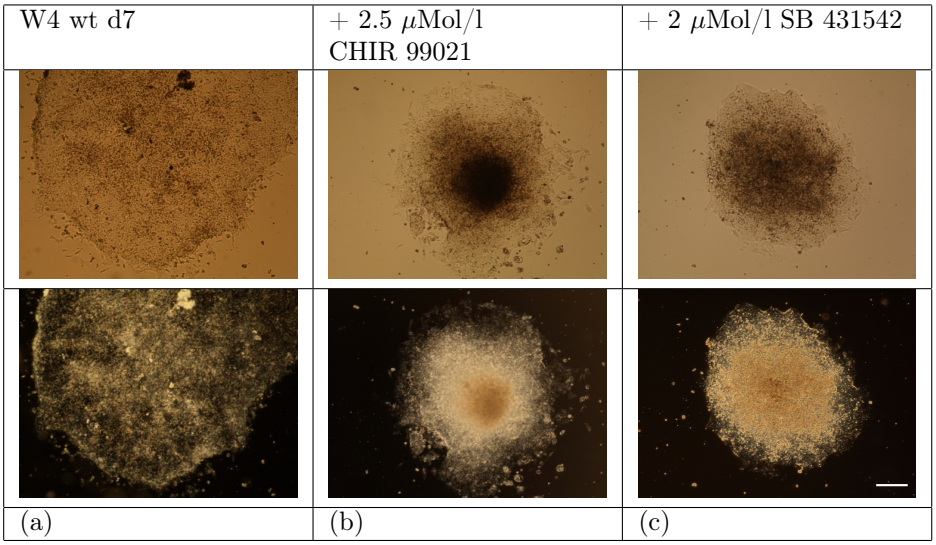


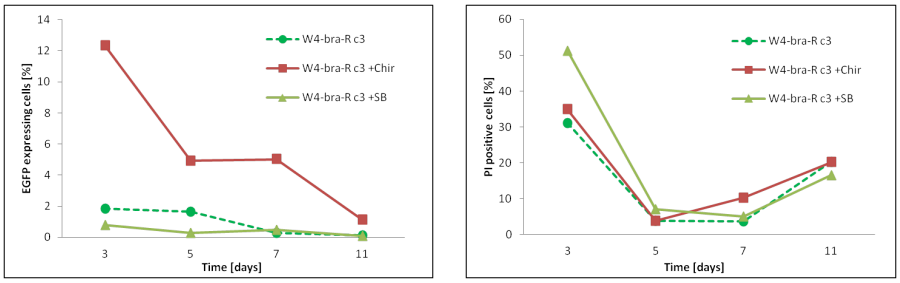
Figure 4.18: Morphology of W4 EBs in the presence of 2.5 μMol/l CHIR 99021 and 2 μMol/l SB 431542 (both added at d0 of ESCs aggregation). Photomicrographs were taken at day 7 after aggregation; first row, light transmission microscopy; second row, dark field microscopy. Bar = 200 μm.

ogy of EBs. EBs treated with CHIR or SB did not develop normally hence we concluded that the addition of CHIR and SB led to a deregulation of differentiation of our cell lines. All embryonic cell lines (W4 wildtype, W4-bra-R, W4-MesP-R, W4-Top, W4-Fop) showed a similar morphology (untreated, treated with CHIR or SB), representatively only W4 wt is shown in Figure 4.18.

As these experiments were very time consuming only one experiment was carried out in the context of this thesis.

W4-bra-R c3

CHIR had a significant positive effect on the expression of Brachyury promoter-driven EGFP in ESC derived EBs, whereas SB slightly inhibited EGFP expression (Figure 4.19). Non-treated cells were used as control. In former experiments we showed that W4-bra-R cell lines had a maximum of EGFP positive cells at day 5, correlating with the known transient upregulation of Brachyury (Figure 4.3). This peak was not detected in this experiment. Considering this discrepancy it was likely that we missed the peak as only day 3, 5, 7 and 11 were measured. A possible explanation would be an accelerated



(a) (b)

Figure 4.19: (a) Influence of CHIR and SB on Brachyury expression in ESC derived EBs. b) Cell death in presence of CHIR and SB, respectively, in ESC derived EBs. PI ... propidium iodide.

expression of Brachyury. This was demonstrated by Ueno and coworkers, who overexpressed Wnt3a, another factor which activates β-catenin; this led to an accelerated expression of several Wnt dependent factors including Brachyury and Mesp1 (Ueno et al. 2007).

The Amount of PI stained (dead) Brachyury ESCs

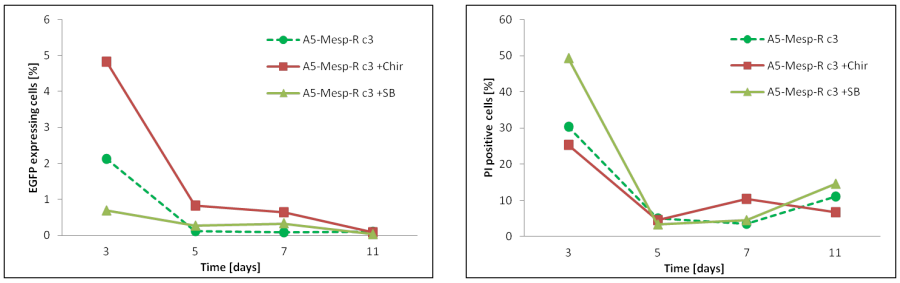
To test the influence of CHIR or SB on cell mortality we determined the percentage of dead cells by using propidium iodide (PI), which can be detected via fluorescence flow cytometry.

All test series showed a similar trend, but there was a big discrepancy at d3 (Figure 4.19 (b)). Cells treated with SB showed significantly more dead cells at day 3 of ESCs aggregation. As mentioned above we thought that the dose was too high and decided to reduce the concentration of SB from 4 to 2 μMol/l starting at day 4. At day 5 the lowest percentages of dead cells where found in all three test series. Non-treated cells were used as control.

The decline in EGFP expression seemed not to be caused by an increase in dead cells before day 5. As the percentage of dead cells started to rise again at day 7, we could not exclude that this caused (at least partly) the decrease of EGFP expressing cells.

W4-MesP-R cA

In order to determine whether or not CHIR and SB were influencing the expression of EGFP driven by the Mesp1 promoter, we analyzed W4-MesP-R cA derived EBs by fluorescence flow cytometry (Figure 4.20 (a)) at day 3,



(a) (b)

Figure 4.20: (a) Influence of CHIR and SB on Mesp1 expression in ESC derived EBs. b) Cell death in presence of CHIR and SB respectively in ESC derived EBs. PI ... propidium iodide.

5, 7 and 11 after aggregation. CHIR had a positive effect on the expression of Mesp1 promoter-driven EGFP in ESC derived EBs, although not as strong as found in the Brachyury cell line. SB inhibited EGFP expression (Figure 4.19). Non-treated cells were used as control.

As demonstrated in former experiments the expression of EGFP in W4-MesP-R derived EBs peaked at day 5, correlating with the transient up-regulation of Mesp1 (Figure 4.9). Although the direct interaction of Mesp1 with Wnt signaling is still unclear, at least an indirect influence is likely as demonstrated by Ueno and coworkers, who found an accelerated expression of Mesp1, after inducing Wnt signaling (Ueno et al. 2007).

The Amount of PI stained (dead) Mesp1 ESCs

Similar to the Brachyury cell line, the decline in EGFP expression seemed not to be caused by an increase in dead cells before day 5. As the percentage of dead cells started to rise again at day 7, we could not exclude that this caused (at least partly) the decrease in EFGP expressing cells.

4.4.2 Influence of CHIR, SB and MK on Expression of A5-bra-R and A5-MesP-R derived CBs

To investigate the influence of Wnt and TGF-β signaling on Brachyury and Mesp1 expression in CBs, we chose the most promising candidates of Section 4.2 for further experiments: A5-bra-R cB and A5-MesP-R c1. With these clones we investigated the influence of CHIR 99021, SB 431542 and MK 142

on their development. MK 142 is a newly synthesized small molecule, obtained from Prof. Dr. Marko Mikovilovic (TU Wien), supposed to activate the formation of cardiomyocytes.

We used identical test settings as described in Section 4.4.1. The experiment was done in four parallel test series: A5-bra-R/A5-MesP-R control (no supplements), A5-bra-R/A5-MesP-R + 2.5 μMol/l CHIR 99021, A5-bra-R/A5-MesP-R + 2-4 μMol/l SB 431542 and A5-bra-R + 1 μMol/l MK 142. All substances were added at day 0 of aggregation of CVPCs.

Furthermore we investigated the influence of CHIR, SB and MK on the morphology of CBs. A5-bra-R/A5-MesP-R derived CBs treated with either CHIR or SB did not develop normally. Neither of these CBs developed beating cardiomyocytes over the period measured. A5-bra-R/A5-MesP-R derived CBs treated with MK and untreated controls differentiated normally, developing the first beating cardiomyocytes at day 10. Hence we concluded that the addition of CHIR and SB led to a deregulation of differentiation, whereas MK did not seem to influence differentiation. All cardiovascular progenitor cell lines (A5 wildtype, A5-bra-R, A5-MesP-R, A5-Top, A5-Fop) showed a similar morphology (untreated, treated with CHIR, SB or MK), representatively only A5-Top wt is shown (Figure 4.21).

As these experiments were very time consuming only one experiment was carried out in the context of this thesis.

A5-bra-R cB

We wanted to evaluate if CHIR, SB or MK influence the expression of EGFP under the control of the Brachyury 5' UTR (Figure 4.22 (a)). None of these substances showed a significant effect compared to the control (non-treated cells). During the whole experiment the amount of EGFP positive cells stayed below 1%. Although we assumed a trend - an upregulation of Brachyury promoter-driven EGFP expression between d5 to d7 - the experiment has to be repeated to confirm this assumption. Cells treated with MK seemed to be hardly induced, whereas cells treated with CHIR showed the highest activation.

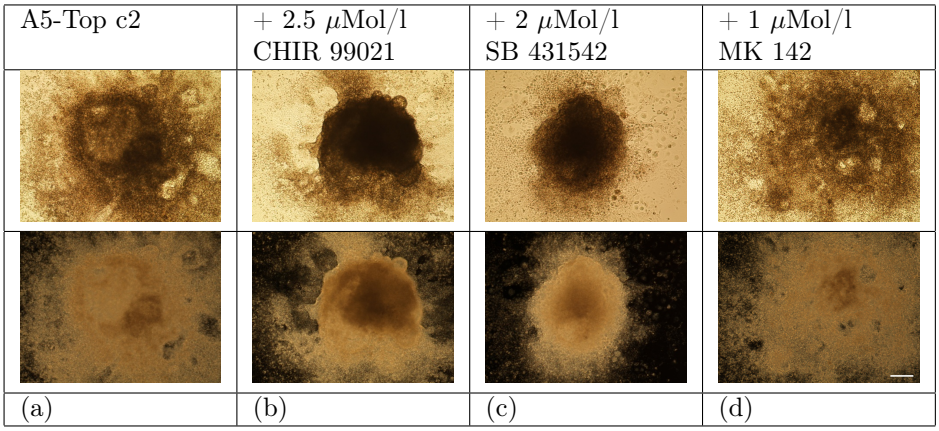


Figure 4.21: Morphology of A5-Top c2 CBs in the presence of 2.5 μMol/l CHIR 99021, 2 μMol/l SB 431542 and 1 μMol/l MK 142 (all added at d0 of CB production). Photomicrographs were taken at day 7 after aggregation; first row light transmission microscopy, second row dark field microscopy. Bar = 200 μm.

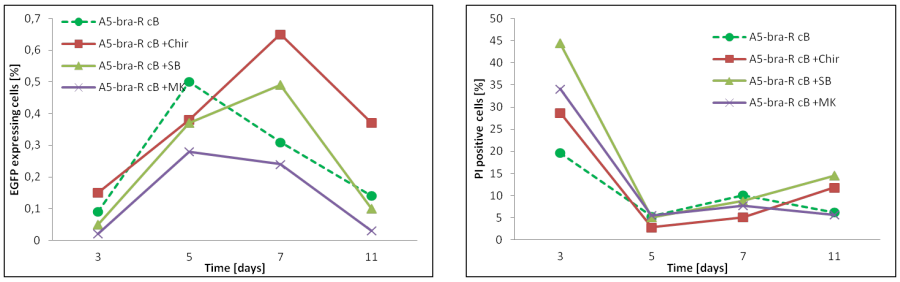
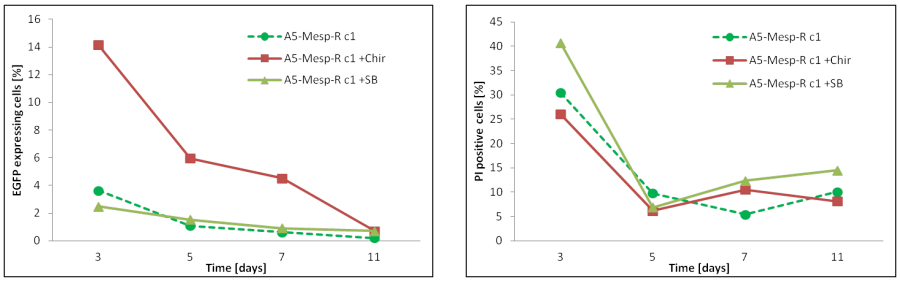


Figure 4.22: (a) Influence of CHIR, SB and MK on Brachyury expression in CVPC derived CBs. b) Cell death in presence of CHIR and SB respectively in CVPC derived CBs. PI ... propidium iodide.

The amount of PI stained (dead) Brachyury CVPCs

To explore whether CHIR, SB or MK kills cells we determined the percentage of dead cells by addition of propidium iodide (PI). All test series showed a similar trend, despite the discrepancy at d3 (Figure 4.22 (b)). Cells treated with CHIR and MK showed significantly more dead cells at day 3 of CVPC aggregation compared to control; cells treated with SB showed even twice as much dead cells than the control although the concentration of SB was reduced by half. At day 5 the lowest percentages of dead cells were found in



(a) (b)

Figure 4.23: (a) Influence of CHIR and SB on Mesp1 expression in CVPC derived CBs. (b) Cell death in presence of CHIR and SB respectively in CVPC derived CBs. PI ... propidium iodide.

all three test series. Non-treated cells were used as control.

SB seemed to cause increased cell death independent of its concentration. Even after reducing the SB concentration, the highest amount of dead cells in all performed experiments was found. This finding indicates that the deregulation of TGF-β signaling induced by SB 431542 in developing cells is rather responsible for the increased cell death than the concentration, as this concentration was already used in former projects. The regulation of TGF-β is a precisely defined process during early embryogenesis; aberrations lead to deregulations and severe non-viable phenotypes (reviewed by Wu and Hill 2009).

A5-MesP-R c1

In order to test if CHIR or SB influence the expression of Mesp1 promoter-driven EGFP, we analyzed A5-MesP-R c1 derived CBs by fluorescence flow cytometry (Figure 4.23 (a)) at day 3, 5, 7 and 11 after aggregation. CHIR had a significant positive effect on the expression of EGFP driven by the Mesp1 promoter. It slowly decreased over time and reached basal levels at day 11. SB did not influence the expression of EGFP. Non-treated cells were used as control.

The amount of PI stained (dead) MesP CVPCs

The influence of CHIR or SB on cell mortality was the same as described in Section 4.4.2.

The decline in EGFP expression seemed not to be caused by an increase

in dead cells before day 5. As the percentage of dead cells started to rise again at day 7, we could not exclude that this caused (at least partly) the decrease of EFGP expressing cells.

4.4.3 Comparison of Brachyury and Mesp1 Expression in W4 ESCs and A5 CVPCs

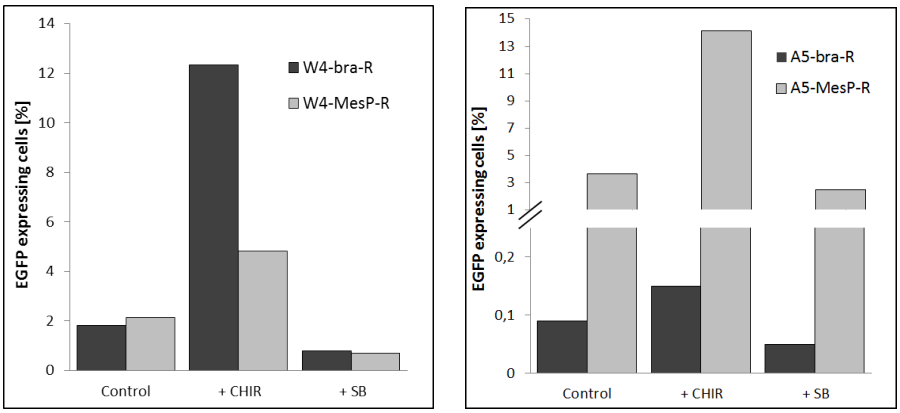
Comparing the percentage of EGFP positive cells of the Brachyury to the Mesp1 cell line at day 3 it was found that, under the influence of CHIR, the expression of Brachyury promoter-driven EGFP was highly upregulated in ESC derived EBs but low in CVPC derived CBs, although this is very likely due to a failure of this particular experiment, as the percentage of EGFP expressing cells stayed below 1%, unlike in former experiments, where at least day 3 was always above 1% (Figure 4.10).

The expression of EGFP under the control of the Mesp1 promoter was highly upregulated in CVPC derived CBs, and only slightly upregulated in ESC derived EBs (Figure 4.24(c)). SB always seemed to have a repressive influence on the percentages of EGFP positive cells compared to the control.

These results perfectly fit the current view, that Brachyury, as an early mesodermal marker, is only transiently upregulated at the very beginning of embryogenesis, while Mesp1 is until now the earliest marker of cardiac-specific lineage (Figure 1.6) and gets upregulated shortly after, hence the expression of EGFP driven by the Brachyury promoter was almost exclusively found in ESC derived EBs, whereas the highest expression of Mesp1 promoter-driven EGFP was found in CVPC derived CBs.

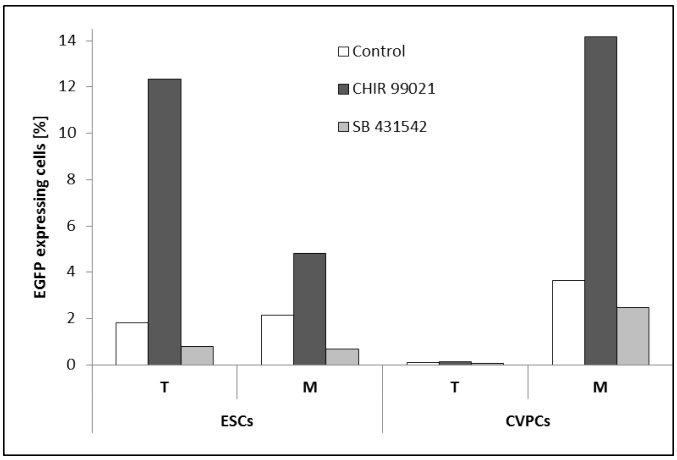
4.5 Assessment of Wnt Signaling in EBs and CBs by TOPflash Luciferase Analysis

To test the influence of CHIR, SB and MK on Wnt signaling in ESC derived EBs and CVPC derived CBs, respectively, the previously established TOPflash clones and their negative controls (FOPflash clones) were used. The TOPflash reporter cells harbor Tcf/Lef sites, which monitor the activity of Wnt signal transduction pathways in cultured cells. The Tcf/Lef-responsive luciferase construct contains two sets (with the second set in the reverse orientation) of three copies of the Tcf binding site (wild type) up-



(a)

(b)



(c)

Figure 4.24: (a) Comparison of Brachyury and Mesp1 expression in W4 ESCs under the influence of CHIR and SB; controls are untreated cells (b) Comparison of Brachyury and Mesp1 expression in A5 CVPCs under the influence of CHIR and SB; controls are untreated cells (c) Comparison of Brachyury and Mesp1 expression in W4 ESCs and A5 CVPCs under the influence of CHIR and SB; controls are untreated cells.

stream of the thymidine kinase (TK) minimal promoter and a luciferase open reading frame. FOPflash is used as negative control, harboring mutated Tcf/Lef sites.

After stable transfection of the ESC and the CVPC line, as described in Section 4.1.4, EBs/CBs were chosen as a working model to investigate the regulation of Wnt signaling during early in vitro embryogenesis and cardiomyogenesis.

The handling of the cells can be found in Section 3.7. We evaluated the

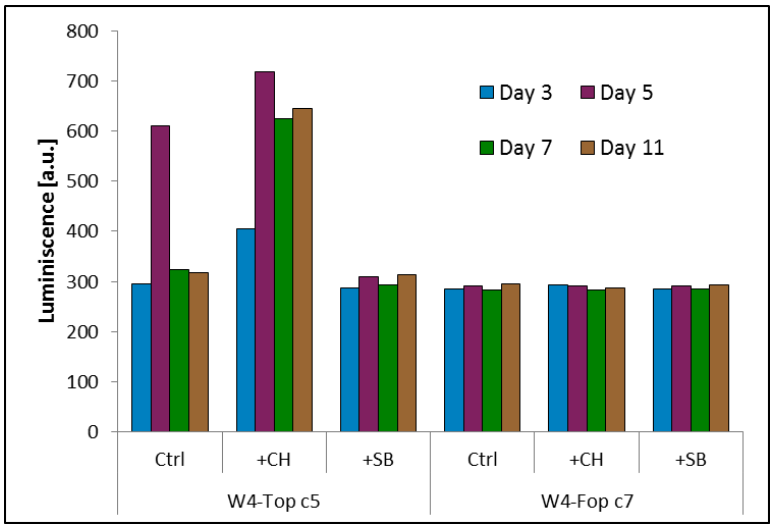


Figure 4.25: Luciferase Activity in differentiating W4 derived embryoid bodies with non-homologously integrated β-catenin responsive reporter genes (Top). Untreated cells (Ctrl) and cells under the influence of CHIR 99021 (+CH) and SB 432542 (+SB) were measured at day 3, 5, 7 and 11. As negative controls cells were used with a non-homologous integrated mutated β-catenin responsive reporter gene (Fop).

luminiscence of the TOPflash/FOPflash constructs at four points in time of each test series (d3, d5, d7 and d11). As these experiments were very time consuming only one experiment was carried out in the context of this thesis.

4.5.1 Evaluation of W4-Top and W4-Fop

In order to determine the influence of CHIR and SB on ESC derived EBs we chose the most promising clone after transfection with the TOPflash (W4-Top c5) and FOPflash (W4-Fop c7) construct, respectively. Three test series were done in parallel. W4-Top/Fop control (no supplements), W4-Top/Fop + 2.5 μMol/l CHIR 99021 and W4-Top/Fop + 2 - 4 μM SB 431542. Both substances were added before aggregation of ESCs (d0). Again the concentration of SB was reduced, due to the high mortality of cells treated with SB.

To determine the baseline (LOD), the readouts of empty wells and wells containing only the cell suspension were used as blanks.

Under the influence of CHIR, W4-Top c5 derived EBs showed slightly upregulated activity of luciferase at day 3 and stayed approximately two

fold upregulated to the end of our test series (d11) (Figure 4.25). SB supplemented W4-Top c5 derived EBs stayed at baseline levels throughout our measurements. The control test series was upregulated only at day 5.

As the control reflected the natural behavior of this clone, the supplemented cells should be compared to the control. Taking this into account CHIR had a positive regulatory effect on W4-Top derived EBs, whereas SB seemed to have a negative regulatory effect on the expression of the TOPflash construct, particularly at day 5. To confirm this data the experiment has to be repeated.

As expected, no luciferase activity of the W4-Fop c7 derived EBs was observed.

4.5.2 Evaluation of A5-Top and A5-Fop

Testing the influence of CHIR, SB and MK on CVPC derived CBs was done as described in Section 4.5.1. We chose the most promising candidates (A5-Top c2 and A5-Fop c4) according to responsiveness of the luciferase reporter construct (Section 4.3).

Under the influence of CHIR, A5-Top c2 derived CBs, harboring the luciferase reporter gene induced by Tcf/Lef sites, showed the highest intensity of luminiscence (Figure 4.26). The most considerable upregulation (more than 51-fold higher than basal level and 4-fold higher than control) was found at day 5. Throughout the experiment the activity of luciferase stayed above the basal level. SB treated CBs showed unexpected behavior. Except for day 5, SB seemed to activate the reporter constructs. This was very surprising, as SB is known to act on the TGF-β pathway, but not on the Wnt signaling pathway. Furthermore we did not find this regulatory effect in ESC derived EBs, where SB showed, if at all, a slightly negative effect. As these experiments were only carried out once, they have to be repeated to confirm these findings. MK seemed to have a minor positive effect only on A5-Top c2 derived CBs which lead us to the assumption that this small molecule does, if at all, negligibly influence the Wnt signaling pathway.

As expected throughout the whole experiment, the FOPflash construct of the A5-Fop cells never rose above basal levels. This was most likely due to the fact that the construct exhibits only mutated Tcf/Lef sites and could therefore not respond, but it can not be excluded, that the clones did not take the DNA construct up, because A5 CVPCs carry a neomycin resistance gene

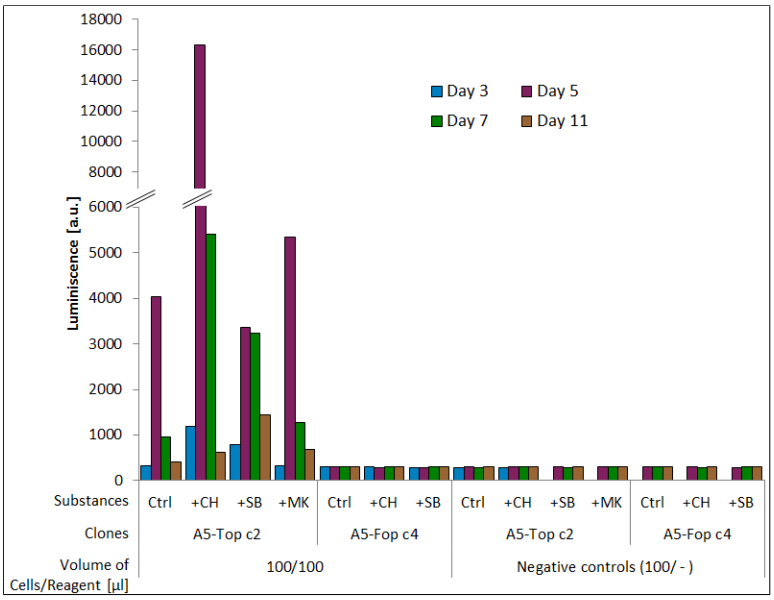


Figure 4.26: Luciferase Activity in differentiating A5 derived cardiac bodies with non-homologously integrated β-catenin responsive reporter genes (Top). Untreated cells (Ctrl) and cells under the influence of CHIR 99021 (+CH), SB 432542 (+SB) and MK 142 (+MK) were measured at day 3, 5, 7 and 11. As negative controls cells were used with a non-homologous integrated β-catenin responsive reporter gene (Fop). 100/100 stands for 100 μl cell suspension plus 100 μl ONE-Glo$^T M$ reagent; 100/- stands for 100 μl cell suspension only (negative control).

naturally. This could only be proven by isolating the DNA and searching for the presence of the FOPflash construct.

These experiments were done only once, thus they have to be repeated to confirm these data. Although we tried to equal the handling of the cells, errors can not be completely excluded. Furthermore Victor^3 V measures only actual luminiscence, which of course is dependent on cell number hence variations of luminiscence may occur.

4.5.3 Reduction of Working Volume

All luciferase experiments were carried out with 100 μl cell suspension plus 100 μl ONE-Glo$^T M$ reagent as suggested in the user manual. To economize our means we reduced the working volume by half and ran a parallel test series with 50 μl of A5-Top/A5-Fop cell suspension and 50 μl ONE-Glo$^T M$ reagent. As demonstrated in Figure 4.27 still a similar distribution of luminis-

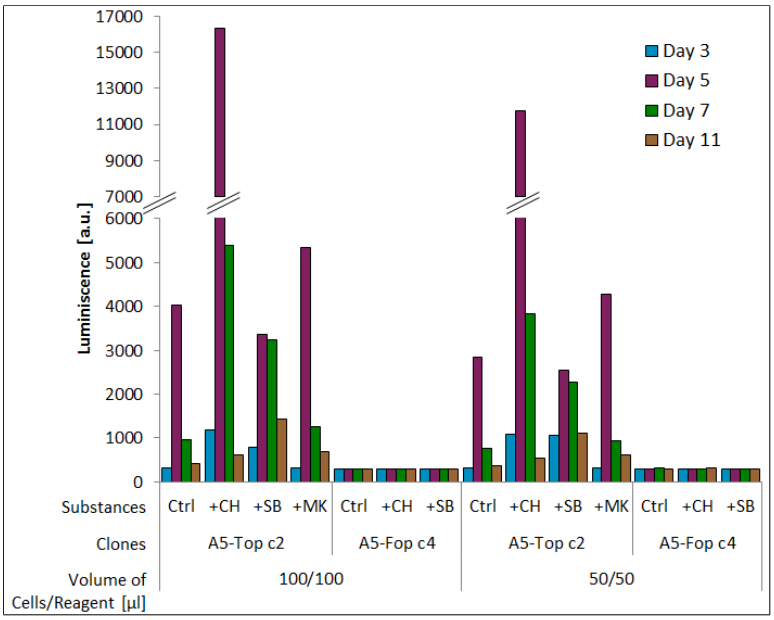


Figure 4.27: Luciferase Activity in differentiating A5 derived cardiac bodies with non-homologously integrated β-catenin responsive reporter genes (Top). Untreated cells (Ctrl) and cells under the influence of CHIR 99021 (+CH), SB 432542 (+SB) and MK 142 (+MK) were measured at day 3, 5, 7 and 11. As negative controls cells were used with a non-homologous integrated β-catenin responsive reporter gene (Fop). 100/100 stand for 100 μl cell suspension plus 100 μl ONE-Glo^TM reagent; 50/50 stand for 50 μl cell suspension mixed with 50 μl ONE-Glo^TM reagent

cence as with 100/100 was detected. The highest activation of the reporter construct was found when treated with CHIR. In general we found only a slight reduction of luminiscence values. For later experiments 50 μl of cell suspension and 50 μl ONE-Glo^TM reagent would be sufficient.

4.5.4 Comparison of W4-Top and A5-Top

In general it seemed that β-catenin responsive reporter CVPCs were significantly more inducible by CHIR than ESCs. Wnt signaling is known to play a distinct role in the formation of mesoderm and in early cardiogenesis (Klaus et al. 2007), explaining its much higher occurrence in CVPC derived CBs. The CVPCs seemed to be more responsive and hence more dependent on Wnt signaling than ESCs. The EBs/CBs treated with CHIR 99021 or SB 431542 showed irregular differentiation and got stuck in development,

whereas the controls as well as the A5-Top c2 + MK developed normally and formed beating cardiomyocytes (Figure 4.18 and Figure 4.21). Thus we conclude that the site of insertion of Top in A5 and W4 did not influence development and the reporter genes were responsive to Wnt signaling. In both cases, W4 and A5, the transient expression of Wnt as well as the TGF-β pathway is indispensable for differentiation and is precisely regulated during embryogenesis as described in literature (Wnt signaling reviewed by Petersen and Reddien 2009; TGF-β signaling reviewed by Wu and Hill 2009). Overstimulation or repression of these pathways, mimicked by the addition of CHIR and SB, led to deregulation of the cells, notable by significantly smaller and more dense EBs/CBs, which got blocked in differentiation and never developed beating cardiomyocytes.

5 Discussion

5.1 Essence of the Fluorescence Experiments

Our findings showed nicely the different expression patterns of Brachyury and Mesp1 promoter-driven EGFP in ESC derived EBs and CVPC derived CBs.

While Brachyury is only transiently expressed in developing EBs around day 5, what we demonstrated in Figure 4.3, we only found a decline of EGFP expressing cells in CBs (Figure 4.6), indicating that CVPCs are approximately 5 days ahead in development compared to ESCs. This assumption was reinforced by the experimental data we got from the Mesp cell line. Mesp1 is known to be induced by Brachyury thus it is also transiently expressed in ESCs slightly later (Figure 4.9). As Mesp1 is the earliest marker of cardiac specific mesoderm, its expression at the very beginning of CB differentiation was expected and actually found as demonstrated in Figure 4.10.

ESCs are derived from the inner cell mass, they are pluripotent and are able to differentiate into all three germ layers. Brachyury and Mesp1 are found to be required to drive stem cells into the mesodermal fate, hence the cells expressing EGFP under the control of Brachyury/Mesp1 5'UTR were likely representing nascent mesoderm. As Mesp1 induces the formation of the heart, this makes it suitable to use it as an early marker of cardiac fate.

CVPCs on the other hand consist solely of late mesodermal cells, and differentiate in vitro only to cardiomyocytes (CMCs), vascular endothelial cells (ETCs), and smooth muscle cells (SMCs) (Section 1.2.4). Brachyury is only transiently upregulated in nascent mesoderm before the developmental state of CVPCs; upon differentiation Brachyury is downregulated in developing CBs and will not reappear thereafter (Section 1.5.1). Mesp1 is mainly ex-

pressed at the beginning of cardiomyogenesis, which is reflected in developing CVPCs (Figure 4.10). When these cells undergo differentiation, Mesp1 gets downregulated in ETCs and SMCs, but will reappear in developing CMCs (Section 1.5.2). In Section 5.4, we show some experimental data which perfectly fit this hypothesis, illustrated by the intensity of the EGFP positive fraction of the Brachyury and Mesp1 cell lines.

5.2 Stability of EGFP in Fluorescence Analysis

Green fluorescent protein (GFP) is widely used as a reporter for gene expression studies. This bright and stable fluorophore has a low toxicity, and enables its quantitative detection without disrupting the cell. The half-time of GFP is 26 hours (Corish and Tyler-Smith 1999), hence it accumulates to high levels allowing its detection. This high stability makes the protein insensitive to dynamical changes in gene expression (Kitsera, Khobta, and Epe 2007). EGFP is a GFP variant where different sites of the chromophore were mutated to make the protein 20 - 35 times brighter than wild-type GFP, its halftime though remains the same (Cormack, Valdivia, and Falkow 1996). In our fluorescence reporter assays we assume that the EGFP is coexpressed with the promoter of our gene of interest (GOI) by creating a fusion protein. The stability of this fusion protein might not correlate with the stability of the corresponding protein of interest. Both of our GOIs (Brachyury and Mesp1) are known to be expressed only transiently, hence the expression of EGFP under the control of the 5' UTR of Brachyury and Mesp1, respectively, might reflect a prolonged and not actual expression pattern of these genes. The mRNA as well as the fusion protein itself are likely to differ in stability and sensitivity.

Nonetheless we could demonstrate that the expression of EGFP corresponded with the expression pattern of our GOIs as described in literature (Section 1.5.1 and 1.5.2). This indicated at least the responsiveness of our reporter constructs to Brachyury and Mesp1 activation, respectively. To investigate the gene expression more precisely, we could increase the sensitivity of our reporter constructs by the use of modified GFPs. The group of Epe and others managed to destabilize the GFP and decreased its half-time significantly down to 5 - 2 hours enabling its use to detect dynamical changes in gene expression (Loetscher, Pratt, and Rechsteiner 1991; Li et al. 1998;

Kitsera, Khobta, and Epe 2007).

5.3 Unexpected Fluorescent Signal in Undifferentiated ESCs

All our ESC derived EBs gave a significant EGFP signal at day 0 of aggregation, which was unexpected, as both, Brachyury and Mesp1, are known to be upregulated transiently around day 4. Our first assumption was that insufficient LIF concentrations might account for this maximum. Stem cells are usually co-cultivated on feeder cells, which produce LIF, to keep cells in a pluripotent state (Hoebaus et al. 2013). If feeder cells provide too little amounts of LIF, cells might start to differentiate even before the production of EBs. Genes involved in differentiation would get upregulated, including Brachyury and Mesp1, as well as their corresponding reporter constructs which would be detectable by green fluorescence at day 0.

We performed the "LIF experiment" (Section 4.2.3) with a clone, which had a strong fluorescent signal at day 0 and was blocked later in development, which made us aware that we chose a deregulated clone. Thus the results were not conclusive as the clone did not differentiate properly, but they indicated that our cells were sufficiently provided with LIF, as supplemented cells had the same percentage of EGFP expressing cells compared to their non-treated controls. Self-evidently this has to be confirmed using a clone, which is not deregulated. A possible explanation of the strong signal at day 0 is described below.

The maximum at day 0 was found in all investigated cell clones, independently, whether cells developed normally in EBs/CBs or not. Interestingly the percentage of the EGFP positive cells was even higher in deregulated clones, suggesting an unsuitable site of insertion of the reporter construct. We assumed that the construct is under the control of a housekeeping promoter, making it unresponsive to reporter gene specific regulation. This erroneous site of insertion also blocked proper development, easily noticeable by the morphology of the EBs (Figure 4.4).

In 2012, Diekmann and coworkers demonstrated unspecific fluorescence of their reporter constructs specifically in undifferentiated mouse ES cells (Diekmann et al. 2012). The maximum fluorescence of our ES cell lines at day 0 may also be caused by this unspecific fluorescence. Diekmann and

colleagues were able to reduce this background fluorescence by using specific microRNAs (mir). They identified two microRNAs which are important regulators of gene expression, murine mmu-mir-294, mainly expressed during the state of pluripotency and mir-302, primarily expressed during early differentiation. They constructed a lentiviral vector containing a mmu-mir-294 target site (mirT294 site) downstream of the GFP2 open reading frame (ORF) and GFP2 was under the control of a tissue-specific promoter fragment. They were able to reduce the expression of GFP2 specifically in undifferentiated mouse ES cells. This stage specific knock-down was reversed at the onset of differentiation notable as the miRNA was highly expressed in ES cells and decreased upon differentiation (Diekmann et al. 2012). Diekmann and collaborators argued, besides the prevention of unspecific fluorescence, this technique would thereby allow to sort cells more precisely and more uniformly and reduce the risk of cells dedicated to other lineages or, even worse, harboring embryonic potential, which might lead to tumor formation in follow-up experiments (Diekmann et al. 2012). This approach could be a new possibility for us, to eliminate the unexpected high fluorescent signal at day 0. As we would have to create new reporter constructs including this mirT294 site, the establishment of these cell lines would be a time consuming but surely interesting and promising new approach and would facilitate new experimental issues such as the isolation of pure reporter gene expressing populations of differentiating cells.

5.4 Intensity of EGFP Signaling in Cells

The interpretation of the data revealed not only the percentage of EGFP expressing cells, but also the intensity of this fluorescent fraction. We analyzed one test series (TS) of W4-bra-R c3 and W4-MesP-R cA, two TS of A5-bra-R cB and three TS of A5-MesP-R c1. We calculated the mean of the TS of A5-bra-R cB and of A5-MesP-R c1 as illustrated in Figure 5.1.

Days, where the percentage of EGFP positive cells was elevated, did not always correlate with days where the intensity of the investigated fraction was enhanced as well. We calculated the mean and the median of the intensity of the EGFP positive cells. In general the mean of these test series showed higher values than the median, suggesting an unequal distribution of a small but intensively fluorescent fraction during the experiment, especially at day

1 and day 5 of aggregation.

At day 5 we observed a maximum of highly fluorescent cells in all test series except A5-bra-R cB. In ESC derived EBs this fraction of intensively green cells correlates with the transient upregulation of Brachyury and Mesp1, respectively, suggesting, that these cells express EGFP according to the activation of the promoters of Brachyury and Mesp1.

In CVPC derived CBs, Brachyury promoter-driven EGFP did not display significant changes in fluorescence intensity, correlating the current view, that Brachyury is only transiently upregulated in nascent mesoderm before the developmental state of CVPCs as described above.

In contrast Mesp1 is the first marker of cardiac fate and therefore expressed slightly later in development. The small fraction of highly fluorescent cells at day 5 of aggregation (Figure 5.1 (d)) might be caused by developing cardiac muscle cells, which are known to express Mesp1.

The highest intensity of EGFP expressing cells was found at day 1, unlike the maximum of EGFP expressing cells, which was detected at day 0 (Figure 4.3 and Figure 4.9). We assumed that this intensity maximum reflected a recovery peak as a result of the stress the cells experience after formation of EBs/CBs. 24 hours later the cells had recovered from the rough handling, such as trypsinization, which might have quenched the fluorescent signal at day 0.

However, like the unexpected maximum number of EGFP expressing cells at day 0, we assumed that the high intensity of EGFP expression in these cells at day 1 is also due to the stem cell specific effect of reporter constructs as described by Diekmann and colleagues (Diekmann et al. 2012).

5.5 The Expression Peak of Brachyury in Developing EBs

The reproducibility of experimental data is dependent on many different factors. We found the maximum of Brachyury promoter-driven EGFPs mostly at day 5 of aggregation. Due to handling aberrations and/or different protocols this peak might be shifted by one or two days. According to literature the expression of Brachyury is found between day 2 and day 8 of ESC differentiation (Section 1.5). This discrepancy of the described maxima of Brachyury expression might be caused by unequal in vitro differentiation protocols used

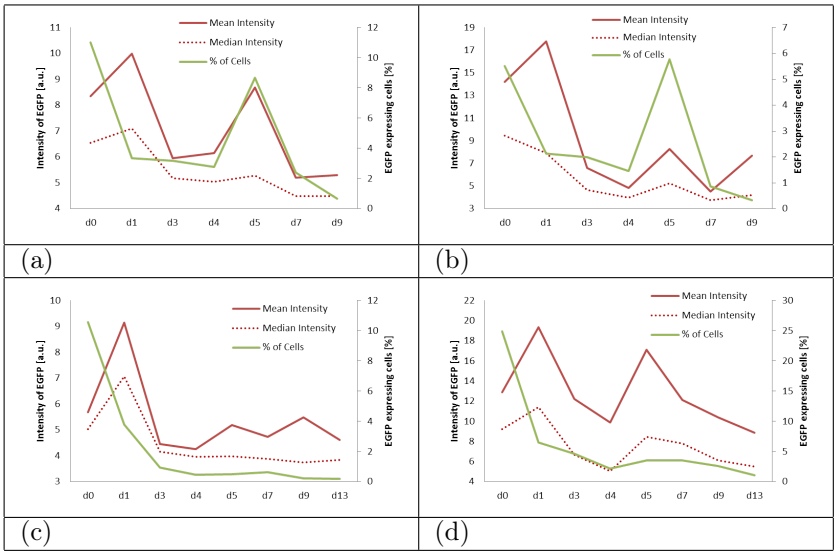


Figure 5.1: Percentage of EGFP expressing cells and the intensity of the fluorescent signal in EBs derived from ESCs of (a) W4-bra-R c3 and (b) W4-MesP-R cA; and CBs derived from CVPCs of (c) A5-bra-R cB and (d) A5-MesP-R c1.

or whether a group investigated the gene expression on the mRNA or the protein level.

5.5.1 Differences of In Vitro Differentiation Protocols

Most groups, including us, used EBs as an in vitro differentiation model. There are several different protocols how to create EBs (Section 1.3). Depending on the method the onset of differentiation may be slightly delayed. Even within a group the reproducibility of results is error-prone. As previously described by our group the initial cell number plays an important part in the formation of EBs (Bader et al. 2001). The ideal ES cell number lies between 150 and 1000 ES cells per hanging drop, but should always be similar to produce comparable results. EBs composed of less or more cells were not able to aggregate and hence failed to develop. Although we tried to work reproducibly, we could not assure minor deviations in cell number or handling aberrations.

5.5.2 Differences in Detection

The expression of a gene can be measured by different assays. Some groups evaluate the expression pattern of a gene by its mRNA levels, whereas others measure the protein levels. Our group, for instance, evaluates the protein level using a reporter gene. As mentioned above the expression of a fusion protein (GOI combined with a reporter gene) might not reflect the natural expression pattern of the gene. EGFP has a half time of 26 hours, hence once it is expressed, it fluoresces more than 24 hours within the cell. As some of our measure points were taken within 24 hours the value is not only composed of newly expressed fluorescent proteins, but also of proteins expressed within the last days. This may account for a shift and a broadening of the signal.

Adachi and colleagues demonstrated only recently that the mRNA levels might significantly differ from the protein levels of the same gene (in their case NFAT5)(Adachi et al. 2012). Taking this into account we should also examine both, mRNA levels as well as protein levels, to better understand post-transcriptional and post-translational modifications.

5.6 Essence of the Luminiscence Experiments

We could clearly demonstrate a difference in Wnt signaling intensity between ESC derived EBs and CVPC derived CBs. The β-catenin responsive reporter EBs and CBs were induced at day 5 of aggregation. But in contrast to EBs, where the induction was two-fold higher compared to baseline, the induction of CBs was even 13-fold higher (Figure 4.25 and Figure 4.26) than the baseline. This induction was further enhanced by the addition of CHIR, a GSK inhibitor, which mimics Wnt activation. The continuous treatment with CHIR led to a deregulation of the EBs/CBs (Figure 4.18 and Figure 4.21). Unlike the untreated controls, which showed only a transient upregulation of Wnt signaling at day 5, the CHIR treated ESC derived EBs were constantly two-fold upregulated throughout the experiment, whereas the intensity of luminiscence in CVPC derived CBs fluctuated, but was always higher than the control.

SB 431542 (a TGF-β inhibitor) blocked Wnt signaling in EBs at day 5; in CBs the influence was rather surprising. Apart from day 5, the endogenous activation of Wnt signaling was significantly increased in CBs. As SB is an inhibitor of TGF-β, and not known to influence Wnt signaling, these results

are currently not interpretable. The distinct influence of SB on Wnt signaling in EBs/CBs (inhibition of Wnt signaling in EBs; induction of Wnt signaling in CBs) has to be confirmed by repeating the experiment, as this was only done once.

The morphological investigation of the clones revealed the negative influence of CHIR and SB on EB/CB differentiation. The permanent supplementation of CHIR or SB led to a block in early differentiation. Overstimulation of the Wnt signaling pathway as well as blockage of the TGF-β signaling pathway account for this deregulation. This was predictable as both pathways are known to be required and precisely regulated during embryogenesis.

5.7 Stability of Luciferase in Luminiscence Analysis

Luciferase cDNAs were first isolated from the firefly Photinus pyralis in 1987 (Wet et al. 1987). The 62-kDa monomeric protein encoded by the firefly luciferase gene generates a bioluminiscent signal by catalyzing the oxidation of its substrate luciferin (Leclerc et al. 2000). In contrast to the quite long half-time of EGFP (26h) the decay rate of the luciferase enzyme is only about 3 - 4 hours in mammalian cells. This makes it more suitable for detecting dynamic changes of gene expression (Thompson, Hayes, and Lloyd 1991). Leclerc and coworkers were able reduce the half time by more than fourfold compared to that of the wild-type luciferase (Leclerc et al. 2000). As we had at least 24 hours (mostly 48 hours) between our measure points, it is likely that we missed a maximum of luminescent cells due to the quite rapid decay rate of luciferase. This might also explain the unexpected behavior of SB treated cells, which might delay Wnt signaling, and hence account for increased values of luminiscence.

Lastly, we always have to keep in mind that evaluation of luminiscence is always dependent on cell number, although we tried to equal the handling of the cells, errors can not be completely excluded and might account for fluctuations of luminiscence.

6

Conclusion and Outlook

By using the newly established stable reporter cell lines, we could demonstrate the different expression pattern of Brachyury, Mesp1 and Wnt signaling during EB and CB differentiation. Our results confirmed the hypothesis, that CVPCs are approximately five days ahead in development compared to ESCs.

The addition of CHIR to differentiating EBs/CBs clearly enhanced the expression of the reporter genes but led to a block in development, this negative effect on differentiation was also found after the addition of SB 431542. These findings underpin the importance of the spatially and temporally regulated Wnt and TGF-β signaling pathways during early in vitro embryogenesis and cardiomyogenesis.

In the Discussion part (Section 5) we provide some approaches, how to get rid of the unexpected EGFP expression in undifferentiated ESCs. The elimination of this unwanted signal would increase the reliability of the established cell lines.

The newly established reporter-gene carrying cell lines can be used as efficient working tools to test the effect of factors, known to affect cardiac development, such as SPARC, Nodal or BMP2, on the expression of Brachyury and Mesp1. Furthermore the regulatory mechanisms of the Wnt signaling pathway can be investigated by identification of new factors influencing this pathway and by identification of new target factors.

The answer to these questions would promote the understanding of the transcriptional network regulating heart differentiation and would help to elucidate regulatory mechanisms which foster efficient differentiation of somatic stem cells into fully functional cardiac muscle cells.

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7

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