Cardiomyocyte Cell Cycle, Renewal and Isolation

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Doctoral Dissertation

Cardiomyocyte Cell Cycle, Renewal and Isolation

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Articles and manuscripts included in this thesis

This thesis is based on the following articles, referred to in the text by their roman numerals.

I. **Walsh S**, Pontén A, Fleischmann B.K, Jovinge S. Cardiomyocyte cell cycle control and growth estimation in vivo– an analysis based on cardiomyocyte nuclei. 2009. Cardiovascular Research in press 2010.

II. Bergmann O, Bhardwaj R.D, Bernard S, Zdunek S, Barnabé-Heider F, **Walsh S**, Zupicich J, Alkass K,. Buchholz B.A, Druid H, Jovinge S, Frisén J. Evidence for Cardiomyocyte Renewal in Humans. Science 3 April 2009: Vol. 324. no. 5923, pp. 98 – 102

III. Pontén A, **Walsh S**, Malan D, Schéele S, Fleischmann B.K and Jovinge S. Fetal cardiomyocytes are VCAM-1+ CD31- and thus can be isolated by FACS with high purity and cultured in vitro. 2009. Submitted.

IV. **Walsh S**, Pontén A, Fleischmann B.K, Jovinge S. Identification of cardiomyocyte and progenitor populations with cell surface markers: a comparison of fetal, neonatal and adult cardiomyocytes. Manuscript in preparation.

Abbreviations

7-AAD	7-amino actinomyosin
α-MHC	alpha myosin heavy chain
β-actin	beta-actin
β-MHC	beta-myosin heavy chain
BMP	Bone morphogenic protein
BrdU	Bromodeoxyuridine
^{14}C	Cardon 14 or radiocarbon
CCND1	Cyclin D1
CDK2	Cyclin dependant kinase-2
CDK3	Cyclin dependant kinase-3
CDK4	Cyclin dependant kinase-4
cTropI	Troponin-I cardiac isoform
cTropT	Troponin-T cardiac isofrom
DNA	Deoxyribonucleic acid
E2F1	E2F transcription factor 1
eGFP	Enhanced green fluorescent protein
FACS	Fluorescence activated cell sorting
Fgf10	Fibroblast growth factor 10
flk1	Fetal liver kinase-1
FSC	Forward-side scatter
G0/G1	Cell cycle phases Gap 0 and Gap 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA-4	GATA-BINDING PROTEIN 4
isl1	Insulin gene enhancer protein 1
Ki-67	Antigen identified by monoclonal antibody Ki-67
Mesp1	Mesoderm posterior 1 homolog
Nkx2-5	Nk2 transcription factor related locus 2
Q-PCR	(quantitative) polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
S/G2/M	Cell cycle phases Synthesis, Gap 2 and Mitosis
SD	Standard deviation
SSC	Side-scatter
TACC2	Transforming, acidic coiled-coil containing protein 2
WT	Wild Type

Introduction

Cardiogenesis

Overview of cardiac origins and early morphogenesis

The heart is the first organ to develop in vertebrate embryos, and the myocardial cell lineage therefore one of the first specialized cell lineages to evolve from the pluripotent stem cell pool of the gastrulating embryo. The development of the heart from its origins in the pre-cardiac mesoderm into a four chambered pump supporting all pulmonary and blood circulatory requirements is a complex process, in vertebrates occuring through several well established transitions. Classical experimental studies with cultured chick embryos localized regions of cardiogenic cell populations in the blastoderm. The first fate mapping study was performed in the 1940's when Rawles *et al.* identified regions capable of generating beating tissue by grafting components of head-process-stage chick embryos[1]. Subsequent experiments in the 1960's through to the 1980's identified a pre-patterned heart field, regions which were mapped using radioactively labeled transplants, and confirmed the observation of a bilateral heart field[2-4], harboring prospective myocardial cells.

In recent years, our understanding of the origin, development and fate of cardiac cell populations has increased substantially, due in large to the availability of elegant transgenic mouse models and emerging technologies. Marker systems which have contributed significantly to the field include the use of the β-galactisidase labeling assays with the LacZ reporter gene targeted to the α-cardiac actin locus[5], fibroblast growth factor 10 *(Fgf10*) locus[6] and also the Cre-LoxP recombinase system[7, 8] whereby the fate of insulin gene enhancer protein transcription factor (*isl1*)-expressing cells has been investigated[9]. It is now known that cardiac progenitor cells are derivatives of the mesoderm, one of the three primary germ layers formed from uncommitted epiblast cells as they transverse the primitive streak[10, 11]. These cells ingress through the primitive streak region during gastrulation[12] , cardiac progenitor cells have been localized to the anterior region of the streak in both chick and mouse embryos[13, 14], and progress through the streak during gastrulation to form mesodermal and endodermal tissues. The initially generated mesodermal cells exit the posterior region of the primitive streak and contribute predominantly to the haematopoietic and vascular lineages of the blood islands in the yolk sac[10].

Cardiac mesoderm derived from epiblast cells migrate from the streak in an anterior-lateral orientation around embryonic day E6.5, forming two groups of cells either side of the midline. It is at this stage that myocardial markers are first detected. These two separate progenitor cell populations have been coined the precardiac mesoderm[3, 15] or heart field[16, 17] and have been shown to segregate from a common progenitor at

gastrulation[9, 18]. Heart field cells are defined as those that have cardiac developmental potency when explanted and placed into culture[19]. These two cardiogenic regions undergo an additional morphogenesis as cells from both areas bridge the midline and form the cardiac crescent at embryonic day E7.5-E7.75 where differentiated myocardial cells are now observed. The formation of the linear heart tube results from the fusion of the cardiac crescent at the midline, arising in a structure composed of an inner endothelial tube surrounded by a myocardial epithelium. The heart tube then undergoes rightward looping at E8.5, in which the future ventricles become distinct and the atrial and venous precursors are manipulated dorsally and cranially bringing the destined chambers into alignment. The internal geography of the heart becomes complex at this stage, with the formation of endocardial cushions, the precursors of the tricuspid and mitral valves forming in the atrioventricular canal. Endocardial cushions are also responsible for the augmentation of the outflow tract, aortic and pulmonary valves.

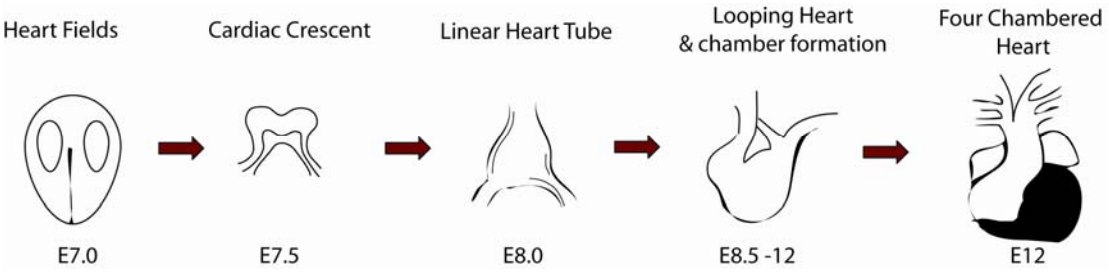

Figure 1. Overview of cardiac morphogenesis in mouse. Myocardial progenitor cells originate in the primitive streak from where they migrate to the anterior of the embryo to form the primary heart fields consisting of two bilateral regions of mesoderm at embryonic day 7 (E7.0). The two heart fields converge to form the cardiac crescent where differentiated myocardial cells are observed at E7.5. The heart tube forms through fusion of the cardiac crescent at the midline, consisting of an endothelial tube surrounded by a layer of myocardial cells at E8.0. Rightward looping then begins E8.5 and by E9.5 the heart has acquired well-defined chambers including the formation of the AV canal forming a boundary between the atrial and ventricular regions of the tube. Endocardial cushion formation preludes the formation of the heart valves the chambers become separated as a result of septation and the outflow tract is remodeled forming the aorta and pulmonary trunk resulting in the familiar four chambered heart E12.

Early cardiogenesis

Studies in lower vertebrate species including frog and chick have revealed an evolutionarily conserved blueprint of heart development, mediated by specific signaling molecules and tissue-specific transcription factors. A myriad of genetic networks regulates the genesis of cardiomyocytes from their mesodermal origin to the fully differentiated, functionally and structurally competent phenotype. The fully developed heart is composed of several diverse cell lineages including cardiomyocytes, endothelial

cells, vascular smooth muscle and fibroblast cells that are derived from distinct subsets of mesoderm during the course of embryonic development.

Precursor cells in vertebrate mesoderm express the T-box transcription factor Brachyury T[20]. Brachyury is considered a marker of mesodermal progenitors which defines the earliest induction of the lineage. However, as these cells progress into the precardiac mesodermal stage of development they begin to express mesoderm posterior 1 and 2 (*Mesp1* and *Mesp2* respectively)[21]. Cells expressing *Mesp1* contribute to all four lineages in the heart. These factors are expressed transiently in the primitive streak and contribute to both primary and secondary heart fields[22], in addition to regulating the migration of cells to the anterior region of the embryo[23]. Brachyury expression diminishes as newly formed mesodermal cells exit the primitive streak and migrate to varying sites in the developing embryo. Another gene associated with mesodermal differentiation is the fetal liver kinase-1 (*flk1*) gene encoding the vascular endothelial growth factors receptor-2. This gene was first identified as a receptor involved in haematopoietic and vascular development in the early embryo. It is expressed in the developing blood islands of the yolk sac[24, 25]. Fate mapping studies have revealed that *flk1* is expressed during the development of the myocyte and skeletal lineages[26]. Additional studies revealed that *flk1* is a broad mesodermal marker, expressed in cells giving rise to the haematopoietic, endothelial, cardiomyocyte and skeletal muscle lineages[27].

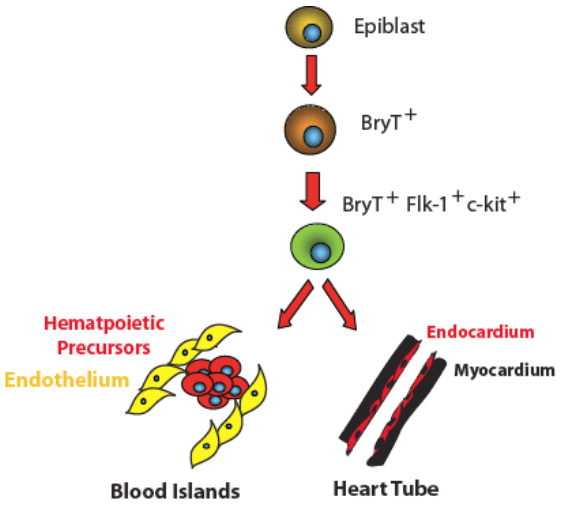

Figure 2. Mesodermal specification. In the developing embryo, embryonic stem cell and epiblast differentiation develops to generate the hematopoietic and vascular lineages and subsequently the development of the Bry+ flk-1+ c-kit+ population which is responsible for the establishment of the myocardial and endothelial cell lineages of the myocardium.

Specification of cardiogenic mesoderm

Once localized in the anterior region, these cells are then exposed to several protein factors including bone morphogenetic proteins or BMPs, members of the transforming growth factor beta (TGFβ) superfamily and FGFs (fibroblast growth factors) which are secreted by adjacent ectoderm and extra-embryonic tissues[28-30]. The mechanisms controlling the temporal aspects of mesoderm induction are poorly understood,

however both BMPs and Wnt signaling exert pivotal roles in the specification of mesoderm to the cardiac lineage[31- 33]. BMP appears to promote cardiac specification[28], application of the *Bmp2* and *Bmp4* to explants of cardiac or non cardiac regions in chick embryos induces the expression of early cardiac markers *GATA-4* (GATA-binding protein 4), *Nkx2-5* (Nk2 transcription factor related locus 2) and *Tbx2* (T-box transcription factor 2), resulting in a beating phenotype, whereas inhibition of BMP signaling blocks expression of *Nkx2-5* and cardiac differentiation [28, 30, 33, 34]. Myocardial transcription factors are thus first detected in the cardiac crescent, where myocardial differentiation is initiated. Wnt signaling exerts differential effects, which appear to be stage specific, and has been reported to act both agonistically or antagonistically depending on the developmental time-frame[35, 36].

These cardiogenic signals appear to be at least partially conserved across species, it is well established that heart formation in *Drosophila* is orchestrated by genetic pathways homologous to those in vertebrates[37, 38]. Active expression of the homeobox gene *tinman* in flies and the related gene *Nkx2-5* in vertebrates are the earliest molecular markers of the cardiac lineage[37]. *Tinman* directly activates transcription of the *Mef2c* gene (Myocyte enhance factor 2c), which directs myocyte differentiation in flies[39], however *Nkx2-5* is not essential for cardiac lineage commitment in mice. In vertebrates, other NK2 homeobox transcription factors may compensate for lack of *Nkx2.5* in determining the myocardial cell fate.

The developmental role of Nkx2.5 has been extensively investigated - loss-of-function analyses by gene targeting have shown that *Nkx2.5*-deficient mice die at approximately E9.5 owing to abnormal morphogenesis of both the outflow and inflow components of the heart tube and looping of which is completely impaired[40]. Whereas commitment to the cardiac lineage is not affected in *Nkx2-5*-null cardiomyocyte precursors, the expression of several myocardial genes in the heart of *Nkx2-5*-deficient embryos, including that of the genes encoding myosin light chain 2v, atrial natriuretic peptide, brain natriuretic peptide, cardiac *Mef2c*, *eHand*/*Hand1*, N-myc and Iroquois homeobox protein 4 is reduced. These data indicate that *Nkx2-5* still exerts a crucial role in the transcriptional regulation of several sets of cardiac-specific genes[41] . Smad proteins also appear to directly activate early cardiac transcription factor genes, including *Nkx2-5*[42], with *Smad 1, 3* and *5* regulated by BMPs during cardiac induction. It is at this cardiac crescent stage that cardiac precursor candidate cells commit to the cardiac lineage and become cardiac progenitor cells.

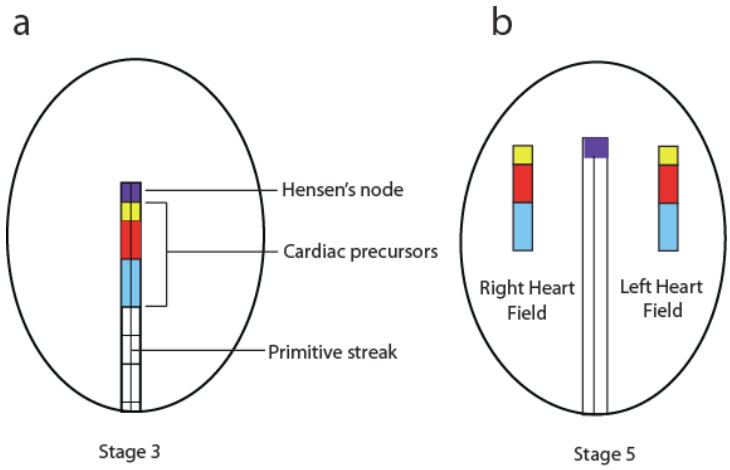

Figure 3. Localization of cardiac precursors during gastrulation. Each region of the heart is composed of a separate region derived from pre-patterned progenitor cells which are localized to the upper portion of the primitive streak and lying posterior to Henson's node. Grafting experiments in the chick embryo have identified these ordered domains of mesoderm at stage 3 in chick embryos (**a**). These different populations of cells are distributed in the same anterior-posterior order as they are later found in the tubular heart. By stage 5 the cardiac progenitors are found in the anterolateral plate mesoderm on either side of the primitive streak (**b**).

Determination and bilateral heart field formation

It is now well established that the heart develops from at least two partitioned progenitor or stem cell pools that have distinct mesodermal origins and regulate cardiac development at distinct periods. The earliest population of cardiac progenitors referred to as the primary heart field originate in the anterior splanchnic mesoderm which gives rise to the cardiac crescent and subsequently the early heart tube, ultimately contributing to the left ventricle, parts of the right ventricle and atria and the atrio-ventricular canal[2, 43].

The second cardiogenic population, referred to as the secondary heart field, is distinguishable at the cardiac crescent stage and is localized both anterior and dorsal to the cardiac crescent of the primary heart field. This was initially identified based on the expression of the *Fgf10* (fibroblast growth factor 10)[6]. Cells expressing *Fgf10* contributed to the myocardium at the anterior pole of the heart. A contribution of this anterior heart field to right ventricular as well as outflow tract myocardium was shown by a recent study[43]. A further contribution of this population of progenitors to the heart was elucidated by studies of the *Isl1* gene (insulin gene enhancer protein, a LIM homeodomain transcription factor). The T-box transcription factor *Isl1* was shown to be a marker of the heart field[44], and reports with Cre-based fate-mapping studies have shown that *Isl1* progenitors contribute predominantly to the derivatives of the second heart field[9, 44, 5], *Isl1*- deficient embryos were shown to lack all the derivatives of the secondary heart field including the outflow tract and right ventricle and both atria[9]. Both primary and secondary heart field progenitors contribute to the right ventricle,

atrioventricular canal, and atria. Recently, indications that *Isl1* is expressed in progenitors of both the primary and secondary heart fields have arisen[45].

Isl1^{+} progenitors retain the capacity for vascular differentiation and may give rise to endothelial and smooth muscle lineages[46, 47], whereas *Nkx2-5*$^{+}$ precursors are more lineage restricted, expression correlates with the loss of multipotency and loss of endothelial[48] and hematpoietic lineage[49] potential in the precardiac mesoderm. The earliest cardiac progenitor expressing *Nkx2-5* also expresses *c-kit*, and is bipotent, capable of differentiating into both the smooth muscle and myocardial lineages[50]. Recently several groups have identified pro-epicardial cells expressing the T-box transcription factor *Tbx18* and Wilm's tumour suppressor protein (*Wt1*) which have been shown to contribute to the fibroblastic, smooth muscle and cardiomyocyte lineages[51, 9].

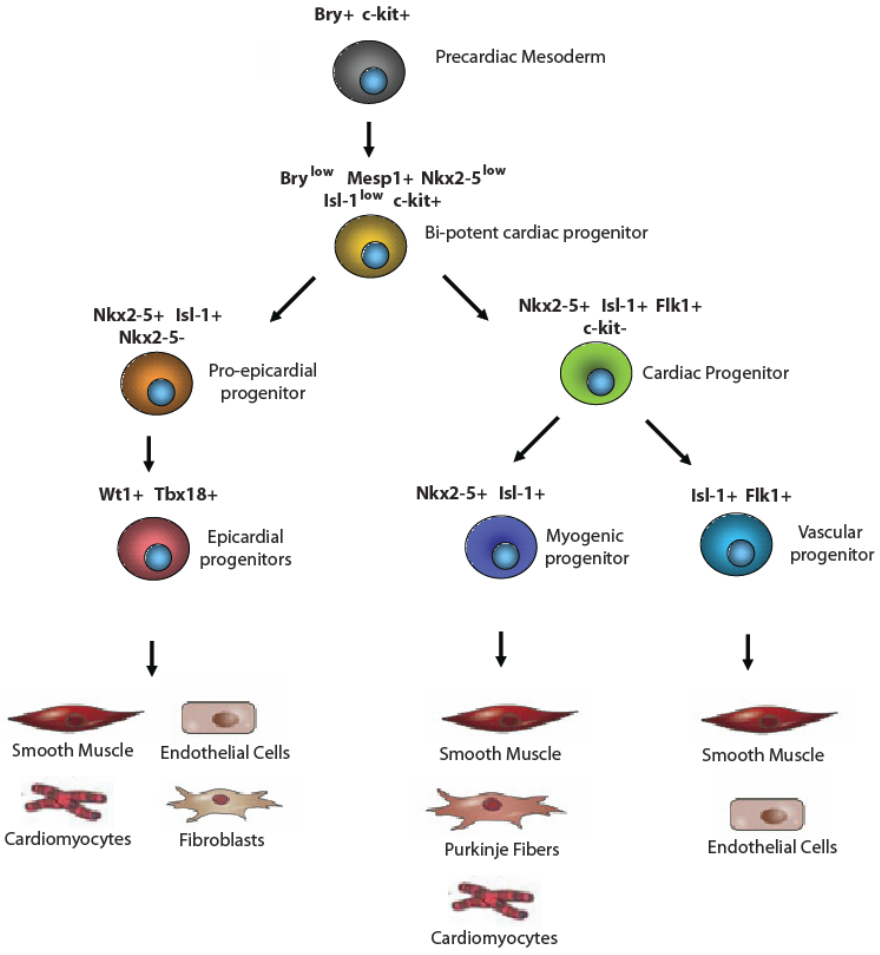

Figure 4. Hierarchy of cardiac progenitor cells and their lineage specification. Brachyury-T expression identifies precardiac mesodermal cells residing in the primitive streak. Ingression through the primitive streak coincides with the expression of *Mesp1*. Cardiac progenitors expressing *Nkx2-5*/*Isl1*/*Flk1* give rise to all three major lineages of the heart, endothelial, smooth muscle and cardiomyocytes. Myogenic progenitors are marked by expression of the transcription factors *Nkx2-5* and *Isl1* only and vascular progenitors by *Isl1* and *Flk1*. Epicardial progenitors have recently been elucidated, marked by the expression of the transcription factors

Wt1 and *Tbx18* giving rise to smooth muscle, endothelium, cardiomyocytes and fibroblasts in the heart. Adapted from Wu *et al* 2006 and Zhou *et al* 2008.

Heart tube formation and looping

The morphogenetic movements that the heart fields undergo in order to form a single heart tube rather than bilateral hearts requires the coordination of many processes and molecular components, including proper cell movements and polarity establishment, specification and differentiation of cardiac precursors. Soon after migration to more anterior positions in the embryo, the cardiac progenitors migrate ventrally as an epithelial sheet towards the anterior ventral midline where they proceed to fuse, proliferate and form a linear heart tube occurring around E8[52-54]. This occurs by the fusion of the paired mesodermal heart fields, consisting of two concentric layers of cells. The outermost layer constituting myocardium and the inner endocardium separated by a layer of extracellular matrix. The tube subsequently becomes attached to the dorsal wall of the pericardial cavity by a dorsal mesocardium and is polarized antero-posterior (AP)[11]. Several requirements for the ventral migration and fusion of the cardiac fields have thus far been identified. These include endodermal signaling induction[55, 56], epithelial organization of the cardiac fields including the migration cues for midline migration[57]. It is at this stage that expression of differentiated cardiomyocyte structural markers appears, including cardiac troponin-T (cTropT), troponin-I (cTropI) and sarcomeric myosin. This gradual expression reinforces the notion that the phases of heart formation—specification, determination, patterning, and differentiation—happen sequentially but with significant temporal overlap.

Dorso-ventral (DV) and antero-posterior patterning are essential for the morphogenesis of the linear heart tube into a fully functional four chambered heart[58]. The linear tube is composed of five segments, each controlled by a specific developmental program and populated by pre-patterned progenitors. These segments are the atrioventricular canal; inflow tract; outflow tract; primitive right ventricle and primitive left ventricle. As development continues, the heart tube elongates at both arterial and venous poles, and begins to undergo a rightward looping[59]. The molecular signals that mediate the interpretation of left-right signals include expression of the *Pitx2* (Pituitary homeobox 2) gene in the lateral plate mesoderm, which is dependant on *Nodal* and is enhanced by the transcription factor *Nkx2-5*[60]. *Pitx2* is expressed in the left portion of the cardiac crescent and in the left side of the linear heart tube [61], and subsequently in the left atrium, ventral portions of the ventricles and left-ventral areas of the outflow tract after looping. Although overexpression of *Pitx2* can affect looping, *Pitx2* expression and heart looping has been shown to be uncoupled, a null mutant exhibits no alteration in looping morphogenesis in contrast to *Nkx2-5* which is indispensible for looping[62], though left-right defects do occur.

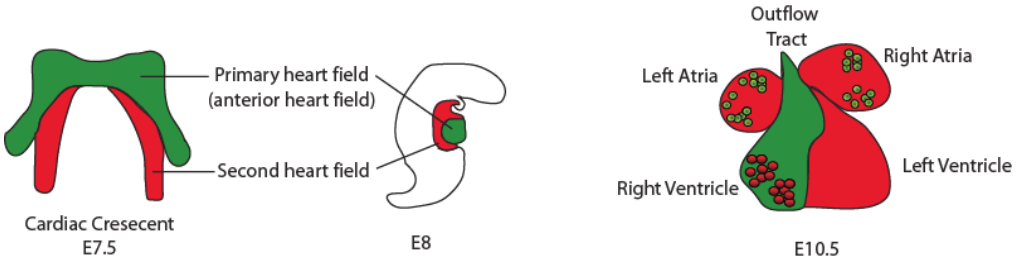

Figure 5. Primary and secondary heart fields. There are two sources of myocardial progenitors in the developing mouse heart. Illustrations depict the relative position and movement of the secondary heart field cells (red) relative to the primary heart field (green) from the cardiac crescent stage through the looping stages of heart development in the mouse. Lineage contributions to the heart at E10.5 are shown.

Chamber formation

Individual cardiac chambers do not become morphologically distinguishable until after cardiac looping, although their fate is programmed much earlier. The basic helix loop helix genes *HAND1* and *HAND2* are expressed predominantly in the primitive right and left ventricular segments of the looping heart during mouse development[63]. Other genes implicated in the looping and early chamber formation process include those encoding transcription factors such as *Cited1*; *Irx1/2/3*, *Tbx20*; gap junction proteins *connexin 43* and *40*; the peptide atrial natuiretic factor (*ANF*) and the cytoskeletal protein *Chisel*[64, 65, 58]. At the completion of looping the heart has assumed a form that closely resembles that of the adult organ. Looping is completed around E12.5 in the mouse.

As looping progresses, the expression pattern of cardiac genes is altered, many genes initially expressed in tubular myocardium become restricted to the presumptive atrial and ventricular promordia[66]. Two hairy-related transcription factors, *Hey1* and *Hey2* are expressed in atrial and ventricular progenitor cells respectively[67, 68], and are critical for the anterior-posterior patterning of the myocardium. The Hey family of transcription factors are regulated by Notch signaling. Iroquois-related homeobox protein *Irx4* has been shown to label ventricular progenitor cells localized in the cardiac crescent, and is expressed exclusively in the ventricular tissue[41]. The specification of atrial development is marked by the expression of *GATA-4* and *Tbx5*, both of which are expressed throughout the cardiac crescent but localized to the outflow tract after tubulogenesis is completed. The orphan nuclear receptor *COUP-TFII* is expressed in atrial precursors, and is required for atrial but not ventricular growth[69].

Development of cardiac function and conductive system

Two migratory cell populations facilitate further cardiac morphogenesis. These are the cardiac neural crest and the epicardial cells which arise from the developing dorsal neural tube and proepicardial epithelium respectively. Cardiac neural crest cells contribute to the septation of the aortic sac, and pharyngeal arteries[70]. Epicardial cells migrate to the surface of the heart and give rise to all cellular components of the vascular

system: smooth muscle cells; fibroblasts and endothelial cells[71]. In addition to contributing cellular elements to the developing heart, these cells also exert influence on surrounding cell populations, in the form of growth factors released by the epicardium which are essential for the proliferation and hypertrophy of cardiomyocytes in the ventricular walls[72]. Epicardium-derived endothelial cells also provide the signals which induce cardiomyocytes to differentiate into purkinje fiber cells[73].

The increasing hemodynamic load exerted on the heart during embryonic development necessitates the proliferation of cardiomyocytes within each chamber. This process is in part mediated by neuregulin growth factors secreted from the endocardium including *ErbB2* and *ErbB4*. These myocardial receptors are required for the formation of the trabeculae[74]. Septation of the cardiac tube into distinct chambers is mediated in part by TGF-β family members[75]. This signaling mediates the transformation of endocardial cells into mesenchymal cells and subsequent migration into the cardiac cushion regions. After re-localization to the cardiac cushions, swellings of the extracellular matrix, differentiation into fibrous tissue of the valves begins. NF-ATc and Smad transcription factors have been shown to be crucial in cardiac valvogenesis[76]. Heart organogenesis culminates with the formation of a four-chambered structure with well developed circulatory and conduction systems.

Cardiomyocyte Growth Patterns

The growth and proliferation of cardiomyocytes occurs in two temporally distinct phases. During embryogenesis, heart mass mainly increases by cell division of cardiomyocytes in a process known as hyperplasia[77] in which karyokinesis and cytokinesis are paralled[78]. This ability of embryonic cardiomyocytes to undergo DNA synthesis and cellular division is in contrast to skeletal muscle cells in which proliferation and differentiation are uncoupled as mutually exclusive processes[79].

It has been suggested that cardiomyocytes loose their ability to proliferate soon after birth[80]. Once cell division ceases in the mammalian heart, growth is achieved exclusively by increases in cell size in a process known as hypertrophy[81]. This transition from hyperplasia to hypertrophy in the early neonatal period is identified in some species by the phenomenon of binucleation. Prior to terminal withdrawal from the cell cycle, cardiomyocytes undergo a final round of incomplete cell division , in which karyokinesis is uncoupled from cytokinesis resulting in failure to complete cell division and culminating in binucleation[78]. In murine hearts, >90% of cardiomyocytes are binucleated. In contrasts to mammals, the cardiomyocytes of lower vertebrates do not loose the ability to divide postnatally. It has been observed in Zebrafish and Newt that heart tissue is regenerated after injury[82, 83]. The molecular mechanisms which control cardiomyocyte cell cycle withdrawal, and cessation of proliferation in adult mammalian hearts is poorly understood.

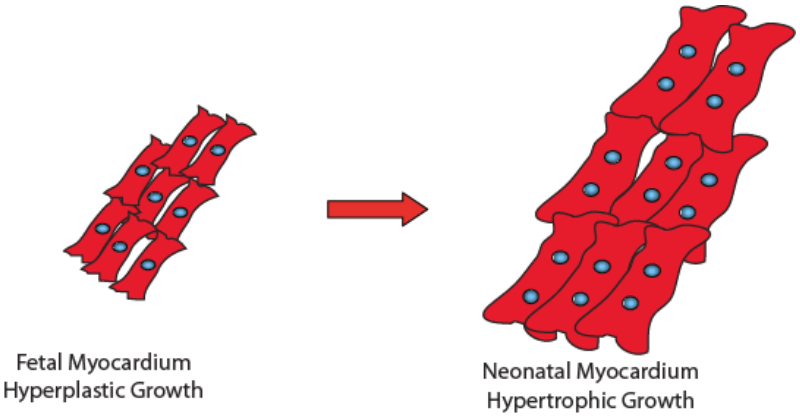

Figure 6. Growth characteristics of cardiomyocytes in development In the embryonic heart increases in cardiac mass are the result of rapid proliferation of mononucleated cardiomyocytes, DNA synthesis and cytokinesis. Shortly after birth in the neonatal period approximately, day 3-4 in mice there is switch in myocyte growth kinetics. Myocytes withdraw from the cell cycle and any increases in cardiac mass are attributable to increases in cell mass as a result of hypertrophy. DNA synthesis occurs in the absence of cytokinesis resulting in binucleation, representing the majority of cardiomyocytes in murine hearts. Thereafter there is terminal withdrawal from the cell cycle in the late neonatal period.

Cell Cycle Overview

Cell proliferation involves cell growth and an ordered sequence of events: replication of the genome, chromosome segregation and cell division. Cell division is regulated by a set of restriction points, the best characterized of these checkpoints are the G1/S and G2/M transitions. A family of serine/threonine protein kinases known as cyclin-dependent kinases (CDKs) are responsible for cell cycle progression and involved in transcriptional control and DNA repair. The core machinery required for cytokinesis are components of the central spindle; RhoA guanosine triphoshatase (GTPase) and its regulators; non-muscle myosin II; actin and direct regulators of its assembly into filaments and factors required for fusion of membrane vesicles.

At the onset of anaphase, proteolytic destruction of cyclins inactivates mitotic kinases which facilitate dephosphorylation and subsequent activation of machinery necessary for mitotic spindle assembly and chromosome segregation. Localization of the central spindle is regulated by the *aurora B* complex[84], and includes the proteins *NuSAP* (nucleolar spindle-associated protein), and tumour suppressor *BRCA2* which affect the progression of cytokinesis[85, 86]. Additionally the cleavage furrow protein anillin has been inextricably linked to cytokinesis[87]. Mammalian cells form a cleavage furrow which is a contractile ring attached to the plasma membrane which partitions the cell into two lobes, consisting of a network of actin and myosin filaments. The assembly of the contractile ring is directed by the RhoA guanosine triphoshatase (GTPase).

A multifaceted response coordinates cell cycle progression with DNA repair, chromatin remodeling, transcriptional programmes and cell death, all mediated by a series of checkpoints. The key components comprising mammalian cell cycle checkpoints are the so called sensors: *ATM* (ataxia-telangiectasia-mutated) and *ATR* (ataxia and rad3

related) kinase Rad9-Hus1-Rad1 clamp complexes mediating the detection of DNA damage[88]. Mediators include *BRCA1*, and *Claspin*[89] which activate the effector kinases of the phosphatidylinositol 3-kinase (*PI3K*) family. Signal transduction is facilitated by the serine/threonine signal transducing kinases, represented by *Chk1* and *Chk2*[89]. Finally a large variety of effector proteins are in operation in checkpoint regulation, including *Cdc25* phosphatases, DNA repair proteins, transcription factors *E2F1* and *p53* and other categories of proteins.

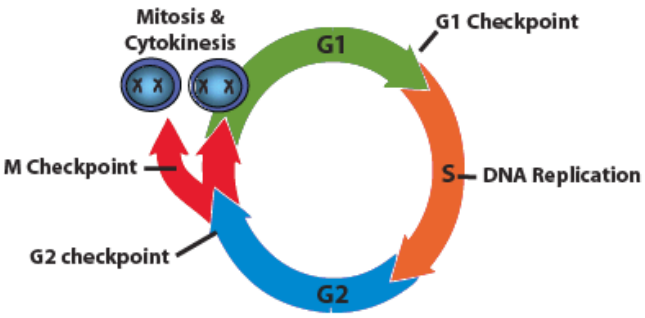

Figure 7. Overview of the cell cycle Cell division consists of two consecutive processes, DNA replication and segregation of replicated chromosomes into separate cells. This progression consists of four distinct phases: G_1 phase, S-phase (synthesis), G_2 phase (collectively known as interphase) and M-phase (mitosis). The progression through each phase is closely regulated by a number of cell cycle control mechanisms and is dependent on the proper progression and completion of the previous phase. Quiescent cells or non-mitotic cells are said to be in G_0 phase.

G1/G0 checkpoint

The first checkpoint occurs prior to entry into S-phase, cells traversing G1 activate the checkpoint transducing kinases *ATM/ATR* and *Chk1/Chk2* complexes which in turn target effectors *Cdc25A* phosphatase and *p53* transcription factor. *p53* stimulates the transcription of several different genes including *p21*, *Mdm2* and *Bax* which result in CDK inhibition and cell cycle arrest and prevents the replication of damaged DNA[90].

Cell cycle progression is coupled with the sequential activation of cyclin-dependent kinases (CDKs). *CDK2* forms active complexes with both cyclin E and cyclin A, *CDK2* is involved in regulating critical molecular events, such as inactivation of pRb initiation of DNA replication , centrosome duplication and histone synthesis[91-93].

Mdm2 regulates *p53* by binding and preventing *p53* transcriptional activity contributing to *p53* degradation by facilitating its ubiquination and thus providing a negative feedback loop [94]. The binding of regulatory proteins can also mediate *p53* ubiquination, *p19* (ARF) protein encoded by the *ARF-INF4* locus binds *Mdm2* and prevents *Mdm2* mediated *p53* proteolysis [95]. When cells are severely damaged, *p53* induces apoptosis by activation of the genes *Bax*, *Fas* and others involved in the oxidative stress pathway[96, 97].

S-Phase checkpoint

The S-phase checkpoint ensures that the onset of mitosis is dependent on the completion of DNA replication. Little is known about S-phase checkpoint control in mammals.

Several studies have demonstrated the suppression of both the initiation and elongation phases of DNA replication[98]. The major pathway involved in the S-phase checkpoint mediated by the *ATM*/*ATR* and activates the cdc25A-cyclinE(A)/Cdk2 cascade[99]. There is also evidence that the *ATM*-mediated phosphorylation of *NBS1* (Nijmegen breakage syndrome 1) is required for S-phase arrest[100], and inhibition of *CDK2* activity leads to the amplification of an *ATM*- and possibly *ATR*-mediated intra-S-phase checkpoint[101].

G2 /M Checkpoint

The G2/M checkpoint prevents cells from initiating mitosis when they experience DNA damage during G2, or if they progress into G2 with unrepaired damage inflicted from previous stages of the cell cycle. A G2 checkpoint ensures that DNA catenations have been sufficiently resolved before mammalian cells enter mitosis. DNA topoisomerase II (*topo II*) is required for chromosome condensation and segregation in eukaryotes[102]. Although these are mitotic processes, their successful completion depends partly on *topo II* activity during DNA replication and in G2-phase. Another key target of the G2 checkpoint is the mitosis promoting cyclin B kinase. Activation after DNA damage is mediated through *ATM*/*ATR* and *Chk1*/*Chk2* mediated signaling.

Eukaryotic cells also arrest in metaphase when microtubule formation is disturbed and also arrest if chromosomes fail to become bi-oriented on the spindle and have not congressed to the metaphase plate[103]. The *Bub2* pathway has been associated with inhibition of mitotic cyclin/Cdk activity and thus prevents spindle disassembly and exit from mitosis. The *Bub2* pathway responds to defects in spindle orientation, spindle localization, spindle damage and DNA damage[104].

Cardiomyocyte Cell Cycle Control mechanisms

Cardiomyocytes exhibit three developmentally determined forms of cell cycle control and expansion, these are: proliferation, binucleation and hypertrophy. There is little evidence for significant postnatal cardiomyocyte proliferation and regeneration. Many forms of cardiac disease are associated with the absence or loss of functional myocardium, injuries acquired after birth such as ischemia/reperfusion through intervention or myocardial infarction all lead to cardiomyocyte loss and a reduction in cardiac function. This comprised function is persistent in adult life which would indicate that the ability of the myocardium to regenerate is diminutive. In contrast the cardiomyocytes from lower vertebrates maintain their proliferative capacity and are capable of postnatal renewal [105]. The cessation of myocyte proliferation in mammalian postnatal life has been attributed to a block in cell cycle[106, 107], however the mechanisms that regulate myocyte hyperplasia arrest remain poorly understood and largely unknown.

Embryonic Cardiomyocytes

Much of the increase in cardiac mass during embryonic development arises predominantly from the proliferation of mononucleated contractile cardiomyocytes. Embryonic cardiomyocytes exhibit formidable expression of cyclins involved in G1, S,

G2 and M-phase. These include Cyclin D1, cyclin D2, cyclin A, B1 and E[108, 109]. The cyclin-dependent kinases *Cdc2*, *CDK2*, *CDK4* and *CDK6* are also highly expressed at this stage of development[110]. These complexes are responsible for the phosphorylation of the *Rb* (retinoblastoma) family members which subsequently initiate the release of *E2F* transcription factors. *E2F* is required for the transcription of genes facilitating G1 exit and DNA synthesis[111]. Conversely, low expression of cyclin inhibitors is observed in embryonic cardiomyocytes, the *INK4* family (including *p19*) and the *Cip*/*Kip* family (including *p21* and *p27*) have a broad function of inhibition of *CDK4*/*6*; *CDK2* and *Cdc2* activity[112].

Neonatal Period and Binucleation

Cardiomyocytes proliferate rapidly throughout fetal development but loose this ability shortly after birth in the neonatal period in most mammals, occurring day 3-4 in mice. Prior to terminal withdrawal from the cell cycle, myocytes undergo a final bout of DNA synthesis, however karyokinesis becomes uncoupled from cytokinesis resulting in the formation of binucleated cardiomyocytes. Cytokinesis is the final step of cell division and is responsible for the equal portioning of cytoplasm between daughter cells to complete mitosis[113], DNA synthesis results predominantly in binucleation in the neonatal period. The process of nuclear division without the completion of cellular division is known as acytokinetic mitosis. Cytokinesis is a complex process involving regulatory and cytoskeletal components. To complete cell division, fully differentiated embryonic cardiomyocytes must first disassemble their myofibril complex completely[79], which occurs in two steps. Firstly Z-disc and thin (actin)-filament proteins followed sequentially by M-band disassembly occurs, reminiscent of sarcomere disassembly during muscle wastage[114]. A gradual increase in the complexity of myofibrils on cardiomyocytes is observed as terminal differentiation progresses beyond the postnatal phase[115].

The formation of binucleated myocytes has been associated with failure in formation of the actin-myosin contractile ring in the cleavage furrow at the final step of cytokinesis[116] However, this is not the case, as the actin-myosin ring does form during the process of binucleation in rat hearts[117]. A more recent study has demonstrated that there is defective focusing of the cleavage furrow regulator protein anillin in the mid-body region which results in the failure of cardiomyocytes to undergo abscission[118]. In the hearts of rodents the accumulation of binucleated myocytes begins day 3-4 and is completed by the third postnatal week with >90% of cardiomyocytes binucleated[80, 119]. Roughly 25% of cardiomyocytes are binucleated in humans at birth, and this proportion stays constant throughout life[120]. Another study has shown that there is a distinct and differential decrease in cyclin kinase activity in myocytes during the neonatal period. Specifically, an increase in *cdc2* activity in the neonatal stage, corresponding to the binucleation period[110], and a decrease in *CDK4* and *CDK2* activity were observed.

An increase in the *INK4* family member *p16* during the neonatal period is detected, which decreases in adult life, representing a potential marker of cardiac senescence[121]. *p16* functions as a stabilizer of the tumor suppressor protein *p53* as it can interact with,

and sequester, *Mdm2* responsible for the degradation of *p53* and inhibits *CDK4* and *6* activity necessary for *Rb* phosphorylation. Despite these considerations, the exact basis for this failed cytokinesis and resulting binucleation is unknown.

Adult Cardiomyocytes and Cell Cycle Withdrawal

Terminal differentiation of cardiomyocytes occurs shortly after birth. This process is characterized by the up-regulation of tissue-specific genes including the structural proteins α-myosin heavy chain (MHC) and cardiac actin in the late perinatal period. Embryonic and neonatal cardiomyocytes predominantly express β-myosin heavy chain (MHC) and skeletal actin[122]. Another hallmark of terminal differentiation in myocytes is that the majority do not reenter the cell cycle in response to mitogens or physiological stress. This observation is reinforced by clinical observations - primary myocardial tumours are rarely observed in adults and myocardial regeneration is not observed in disease or injury that result in myocyte loss. There is however evidence that cardiomyocytes may reenter the cell cycle in human hearts in response to injury[123]. Despite this and other manifestations of cell cycle re-entry[124 - 126], there is still significant controversy concerning the level at which DNA synthesis occurs in healthy or injured hearts [127, 128]. There have even been suggestions that in the absence of ongoing myocyte proliferation in adult rodent life, the entire population of ventricular myocytes would dissipate within five months, such is the inherent rate of apoptosis in the myocardium[129]. Labeling assays utilizing triated thymidine have revealed only minute levels of DNA synthesis in normal (0.0005%) and injured (0.008%) adult mouse ventricular cardiomyocytes[130]. Restricted cell cycle re-entry may occur in human versus mouse hearts, with 1-4% of cardiomyocytes observed as being mitotically active[126] in response to injury. The fate and of these proliferating cells remains unresolved and the issue of species-specific idiosyncrasies pertaining to myocyte proliferative capacity remain unresolved. The discovery of mitotic cardiomyocyte nuclei in a minority of cardiomyocytes could very well be an observation of endoreduplication, a plausible explanation for DNA synthesis. Indeed, many of the variations in cell cycle activity can be traced to flaws in the identification of cardiomyocyte nuclei in histological sections, and the sensitivity of the assay used[131]. Currently the most sensitive method for determining the age of cells is ^{14}C dating with a resolution of 1-2 years[132], making it unsuitable for studies in rodents. We and others have established with ^{14}C dating that human cardiomyocytes are generated into adulthood[133], albeit at exceptionally meager levels, and certainly proliferation of this degree has little impact under circumstances of injury to the functionality of the myocardium.

The relinquished ability to proliferate in the late neonatal period is accompanied by changes in cell cycle regulatory molecules. The protein expression of cell cycle mediators cyclins D1, D2, D3, B1 and E and their affiliated kinases is downregulated in adult cardiomyocytes[108, 110]. This down-regulation has also been shown to be reciprocated by the up-regulation of cell cycle inhibitors[134]. *p107* pocket protein expression parallels that of *Rb*, highest in embryonic and completely down-regulated in adult myocytes, with a role in inhibiting cell cycle progression through regulation of *E2F* responsive genes[135].

Myocardial Infarction and loss of function

Cardiovascular disease is the leading cause of death and illness in the developed world[136]. Atherosclerosis is a progressive disease associated with the accumulation of lipids and fibrous elements in the coronary arterial system supplying the myocardium. The accumulation of lipids with an atheroma and subsequent inflammatory response can result in the thrombosis of a ruptured fibrous plaque cap. When this plaque ruptures, blood coming into contact with the tissue factor coagulates and generates thrombus formation. If this thrombus occludes a vessel persistently, the result is an acute myocardial infarction. Acute myocardial infarction due to thrombosis of an atherosclerotic coronary artery has shown to result in irreversible and irreplaceable loss of myocardium[137].

Subsequent to coronary artery occlusion, comprehensive myocyte necrosis and ventricular wall remodeling transpires. There is migration of macrophages and endothelial cells to the border and infarct zones and the necrotic tissue is initially replaced with granulation tissue[138, 139]. This initial deposition of proteoglycans, osteopontin and fibronectin is succeeded by myofibroblastic deposition of collagen and the initial matrix becomes resorbed. The resulting apoptosis of this granulation tissue results in the formation of scar tissue, composed mainly of collagen matrix[140]. As this process develops there is further thinning of the ventricular wall and alteration in chamber dynamics through ventricular remodeling. The associated impairment and reduction of myocardial function frequently results in heart failure, aneurysm formation and mortality[141, 142].

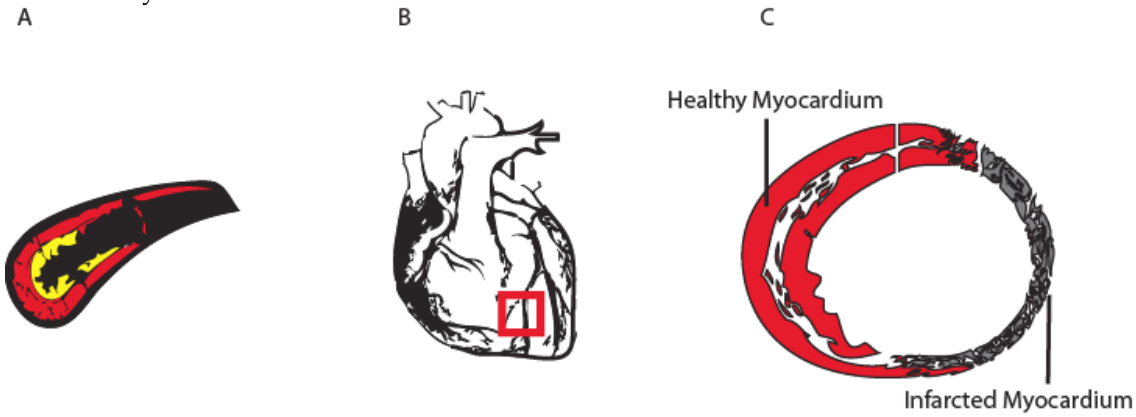

Figure 8. Myocardial infraction The occlusion of coronary arteries by atherosclerosis (A) and thrombus formation results in acute myocardial infraction (B). Extensive cardiomyocyte necrosis and remodeling of the ventricular wall occurs. The necrotic tissue is initially replaced with granulation tissue and eventually this is replaced by collagen deposition resulting in scar tissue formation. Healthy left ventricular myocardium and injured myocardium with scar tissue formation and necrotic tissue (C) Progressive ventricular remodeling and wall thinning are associated with loss of cardiac function and congestive heart failure.

Cardiac Regeneration

It is accepted that the heart's inherent ability to repair itself is insufficient to regenerate tissue damaged by trauma such as ischemic conditions leading to cardiac infarction. In humans, the kinetics of cardiomyocyte renewal as established by ^{14}C dating is estimated at an annual turnover of 1% in the left ventricle at the age of 20, decreasing to 0.3% at the age of 75[133]. The magnitude of myocyte turnover at the mean ages when myocardial infractions generally occur as a result of coronary artery disease is therefore diminutive.

The withdrawal from the cell cycle of adult cardiomyocytes contributes much to this dilemma, even though some mitosis has been observed in human myocardium following injury[126], this proliferation is of too little magnitude to enhance the biological function of the damaged heart. Somatic stem cells which migrate to heart tissue and differentiate into cardiomyocytes have been reported [143, 144], yet these events are still insufficient to reverse pathological conditions. Even pools of resident stem cells in the heart have been reported [145, 146], colloquially named 'cardiac stem cells' or CSCs. It is claimed that these 'stem cells' reside in interstitial niches within the myocardial tissue and that the heart is actually regulated by a stem cell compartment. Despite the potential of these cells to contribute to the formation of new myocardium, the proliferative and integrative requirements are colossal. In order to cause heart failure, an infarct must manifest itself as an area of necrosis and scar tissue in 25% of the ventricular tissue[147]. The myocyte deficit of such an infarction would therefore be in the order of one billion cardiomyocytes. Not only do future therapies need to address the restoration of lost myocytes, but also their integration and synchrony with the electrophysiological and electromechanical elements of the myocardium. Several cell sources and types have been investigated for their potential to reverse pathological damage. Diverse cell types have been transplanted into infracted hearts, and there is evidence that many are equipotent. Nevertheless, most of the benefit observed is uncoupled from the graft cells beating in synchrony with the host myocardium, but a so-called improvement in the passive mechanics of the infarct zone. This usually manifests itself as donor cell engraftment in the border zone of the infarct region and subsequent strengthening or contributions to the infracted ventricular wall, which results in improved mechanical properties[148] of the insulted myocardium. Other side-benefits have also included paracrine effects whereby donor cell production of signaling molecules may initiate or promote survival of native cardiomyocytes[149].

<u>Cell Based Repair</u>

Bone Marrow transplantation

Both hematopoietic stem cells and other marrow components have been investigated as therapeutic agents for myocardial infarction[150, 151]. Several studies have investigated the potential of these hematopoietic cells to transdifferentiate into cardiomyocytes post transplantation in infracted myocardium. A study by Orlic *et al.* documented extensive myocardial regeneration in infarcted hearts following transplantation with c-kit$^+$ enriched hematopoietic stem cells[150]. However, further investigations failed to detect any transdifferentiation events despite the use of various transgenic approaches[151-154]. Such

evidence suggests that there is little contribution of hematopoietic cells to ventricular remodeling and that no significant transdifferentiation to the cardiac lineage, occurs following transplantation. Fusion derived cardiomyocytes have been observed following transplantation, but at a low frequency[153]. Despite this several clinical trails have been conducted with BMT as a therapy for myocardial infarction[155-157], but currently only short term benefits are observed, in studies with 6 month follow-ups, no long-term benefit in cardiac performance was perceived despite the use of several assessment methods including electrocardiogram-gated single-photon-emission computed tomography (SPECT), echocardiography, and MRI[158].

Embryonic Stem Cells

Many groups have shown that cardiomyocytes can be obtained consistently from embryonic stem cells (ESCs)[159] or induced pluripotent stem cells (iPS). Several studies have achieved directed differentiation of mouse and human ESCs into cardiomyocytes[160, 161]. Myocytes may be derived in sufficient quantities for research, but not therapeutic strategies, usually representing less than 1% of the cells in a differentiating culture[162]. The versatility of ES cells in their capacity to generate and differentiate into many lineages is a powerful resource but also their ruination. Teratomas are probably the most considerable risk associated ESC derived therapies, and have been reported following transplantation[163]. Many groups have adopted a strategy of pre-differentiation and subsequent enrichment involving cardiac specific promoters[164] and non-genetic methods including surface marker proteins[165] and even mitochondrial fluorescent dyes[166]. Although potentially providing an unlimited source of cardiomyocytes, the adequate purification methods for these cells prior to cellular therapy is still in relative infancy.

Skeletal Myoblasts

One of the first cell types proposed for cellular therapy following myocardial infarction were skeletal muscle-derived progenitor cells, also known as myoblasts. It has been shown that autologous skeletal myoblasts can differentiate into striated muscle cells within damaged myocardium[167]. Despite hopes of transdifferentiation, there is evidence that these cells retain commitment to the to the skeletal muscle lineage[168]. Several negative aspects of transplantation with this cell type include observations of electromechanical incompatability with host myocardium[169] and the presence of mature skeletal myofibers in infract zones months post transplantation. However, improvement of left ventricular function is observed following grafting[170] in several clinical trials although several patients have been documented to develop arrhythmias, and it is unclear if these were associated with the cell therapy.

Mesenchymal Stem Cells

Bone marrow mononuclear cells contain a rare population of adult stem cells, which *in vitro* can adhere to tissue culture plastic, and give rise to fibroblastic, spindle-shaped colonies. These multipotent, non-hematopoietic cells are referred to as bone marrow

mesenchymal stem cells (MSC). Bone marrow derived MSC can differentiate to oesteoblast, chondrocyte and adipocyte lineages [171]. In addition, it was reported that these mesenchymal cells also had the ability to differentiate into cardiomyocytes *in vitro* [172]. This ability and their reported allotolerance and subsequent local immunosuppressive functions [173] and homing ability [174] in comparison with other cells types considered for cardiomyoplasty, makes them promising candidates for cell therapy. It should be noted that a report found *in vitro* expanded MSC differentiating into bone-forming osteoblasts – rather than cardiomyocytes - in transplanted mouse hearts [175]. Preventing the differentiation of multipotent cells into undesirable cell types and lineages is a major consideration.

Paracrine Effects in Cell Based Repair

The paracrine mechanisms associated with progenitor cells and identification of cell-derived paracrine factors which mediate improvements in cardiac function are largely unknown and under intense investigation by many groups. The infusion of cytokines rather than cell transplantation could provide the necessary signals to induce proliferation and regeneration of resident progenitor and stem cells and even mature fully differentiated myocytes. This cytokine mediated mobilization is routinely used in the hematopoietic field. Several studies have focused on the role of G-CSF to mobilize progenitor cells to the heart following infarction[176, 177], showing significant regeneration in murine models. However, subsequent studies provide evidence that no regeneration occurs following G-CSF treatment[153] and that these cells contribute little to damaged myocardium. Nevertheless a co-transplanting strategy, in which fibroblasts together with ES cell- derived cardiomyocytes were introduced into the infracted hearts of mice resulted in 6-10 fold cardiomyocyte yield, indicating that paracrine factors may be necessary for cardiomyocyte engraftment[154]. The protein periostin has been shown to induce the proliferation of cardiomyocytes *in vivo* following myocardial infarction[178]. Another cytokine shown to regulate proliferation of cardiomyocytes is the TNF-related weak inducer of apoptosis (*TWEAK*). Stimulation of rat neonatal and adult cardiomyocytes with *TWEAK* resulted in DNA synthesis, proliferation and increased cardiomyocyte numbers[179].

Resident Myocardial Progenitors

Several groups have suggested the existence of a small resident progenitor population in the heart, with cardiomyogenic potential, which also exhibit the stem cell properties of self-renewal and multipotency. A definitive marker for these so-called cardiac stem cells (CSCs) has not yet been identified, but many potential candidates have been reported. Two surface markers associated with hematopoietic stem cells have been described in cell populations occurring in the heart, stem cell antigen-1 (Sca-1) and c-kit. Sca-1 expressing cells have been isolated from hearts of mice and injected intravenously into mice following myocardial infarction[180], yielding donor derived cardiomyocytes expressing cardiac markers by mechanism of fusion and transdifferentiation. A population of c-kit expressing cells with cardiomyogenic potential has also been isolated

from the rat heart, and after expansion, transplanted into ischemic myocardium[181]. Consequently these cells were reported to differentiate into cardiomyocytes, and endothelial and smooth muscle lineages. The engrafted cells exhibited cardiac myosin and improved ventricular function. Side-population cells, identified by their ability to exclude the Hoechst dye were first described in the bone marrow and thereafter in the adult mouse heart[182], and when co-cultured with heterogeneous cardiac cell populations, expression of α-actinin sarcomeric protein was induced. Some side population cells express c-kit and /or Sca-1 and like their c-kit$^+$ and Sca-1$^+$ counterparts are able to generate cardiomyocytes *in vitro* and *in vivo* [183].

Another population of cardiac stem cells expressing islet-1 were detected in mouse, rat and human neonatal and adult hearts, albeit in small numbers and in adult limited to the right atrium[184]. These cells have the ability to self renew *in vitro* on cardiac mesenchymal feeder cells and can be stimulated to differentiate into cardiomyocytes. Lineage tracing experiments have previously shown that *Isl1* expressing cells can differentiate into endothelial, smooth muscle, right ventricular and atrial lineages during normal cardiogenesis[185]. The role that *Isl1* progenitors exert in formation of heart lineages is understood, however their contribution to regeneration in the postnatal period is unknown.

All of these reported cardiac stem cells are distinct from one another in their expression of surface markers. Sca-1 and c-kit double positive cells are not observed at any stage in the myocardium as they are in early hematopoiesis. Additionally, the *Isl1* population of progenitors expresses neither the c-kit nor Sca-1 surface markers, so effectively all these groups represent particular or temporally distinct populations with somewhat overlapping commitment and differentiation potential to the cardiogenic lineages. The exact lineage relationship between these adult cardiac progenitors and their embryonic cardiac precursor counter-parts is largely unknown. Nevertheless, these populations are rare and it is unlikely that they contribute major benefit as a result of regeneration and proliferation in ischemic myocardium . The epigenetic modifications that can result from the isolation and expansion of these cells *in vitro* prior to transplantation are also of concern.

Models and Methods

Flowcytometry & Cardiomyocyte Isolation

In all four papers, evaluation and purification of cardiomyocytes is investigated by FACS analysis, and thus this platform was instrumental to all the studies. Flowcytometry is a powerful tool for cellular analysis allowing us to recognize populations and subpopulations of cells within a tissue based on the detection of fluorescently conjugated antibodies to surface or intracellular markers. The cellular structure, rigidity and composition of the heart make it a troublesome target for cytometric analyses. The presence of multiple classes of gap junctions and cell adhesion molecules do not facilitate the dissociation of the heart to a single cell suspension. We have invested considerable energy in optimizing the FACS of cardiomyocytes and their subsequent purification. Single cell suspension of cardiac tissue was achieved by several rounds of enzymatic dissociation with Liberase Blendzyme 3 (Roche) or with collagenase IV. In paper II we have purified human cardiomyocytes from cardiac biopsies and their nuclei, and in papers I, III and IV we have isolated myocytes from both wild type and transgenic mouse models.

The potential for cardiac cells to form aggregates or doublets which have not dissociated in entirety or which form conglomerates in suspension prior to FACS is a reality of cardiac cell population FACS. These 'hitch-hiking' cells could potentially scew analyses, particularly for the sensitive microarray analysis in papers I and IV. Therefore we operated a stringent doublet discrimination strategy as described previously[186]. Many cardiomyocytes in the late neonatal and adult periods are binucleated and it is difficult to distinguish a binucleated cell from two aggregating mononucleated cells (of which one could be a non-cardiomyocyte) in the flowcytometer. Singlet cardiomyocytes were defined as a function of forward scatter width (FSC-W) and forward scatter height (FSC-H). All sorts were performed on a FACS Aria with a 100μm Nozzle. The Purity of flowcytometrically sorted cardiomyocytes was confirmed by reanalyzing the sorted populations. For viable myocyte isolation cells were also stained with 7-amino actinomyosin (7-AAD) to exclude dead cells. We also include several lineage specific antibodies allowing non-myocyte depletion including PECAM-1 (pan-endothelial), CD45 (pan-haematopoietic) and PDGFRβ (fibroblast and smooth muscle specific), in this way we can further enrich for the cardiomyocyte lineage.

Validation

We have used the FACS platform extensively to purify cardiomyocytes and their nuclei from mouse (papers I, III and IV) and human (paper I). The purity and specificity of all these isolations have been substantiated by other, complimentary methods. In paper II, the isolation of human cardiomyocyte nuclei by flowcytometry was verified by western blot for cardiomyocyte-specific transcription factors *Nkx2.5* and *GATA4* and cardiac specific structural proteins. It was demonstrated that these nuclei could be isolated with great sensitivity even when mixed with nuclei preparations from other tissues. In papers I and III we isolated both fixed (paper III) and viable cardiomyocytes (papers I

and III) from mouse hearts. Re-analysis was performed by FACS and immunohistochemistry for cardiac specific markers. In papers I, III and IV we have also validated the FACS isolations by molecular analysis with QPCR.

Human heart tissue

To establish the kinetics of cardiomyocyte renewal, extensive human myocardial tissue was procured from cases admitted during the period 2005-7 to the Department of Forensic Medicine, Karolinska Institutet, after receiving consent from relatives and ethical permission from the Karolinska Institutet Ethical Committee.

Accelerator mass spectrometry

Cardiomyocyte nuclei were purified by FACS based on the intra-nuclear expression of cardiac Troponin-T (cTropT) and c-Troponin-I (cTropI). Accelerator mass spectrometry (AMS) analysis was performed at the Center for Accelerator Mass Spectrometry at Lawrence-Livermore National Laboratory, California, USA. Extracted DNA from cardiomyocyte nuclei in water was transferred to quartz AMS combustion tubes, evaporated in a lyophilizer, excess copper oxide (CuO) was introduced to each sample, the air was evacuated completely, and then sealed off with a H_2/O_2 torch. In order to completely combust all carbon to CO_2, samples were then put into a 900°C furnace for 3.5 hours. The CO_2 evoked was purified, trapped, and subsequently reduced to graphite in the presence of an iron catalyst. CO_2 samples greater than 500 μg were split and the $\delta^{13}C$ measurement was attained by stable isotope ratio mass spectrometry. All of the ^{14}C data are reported as decay corrected $\Delta^{14}C$ [187].

α-MHC-eGFP mouse model

There are no surface markers which are specific to cardiomyocytes that can be exploited for their enrichment. The difficulty in isolating purified populations of viable cardiomyocytes has been remedied by the availability of transgenic mouse strains expressing cardiac specific markers under various reporter genes**.** In this study we used enhanced GFP-transgenic DBA mice with GFP expression driven constitutively by the α-MHC promoter to isolate viable cardiomyocytes. This strain has allowed us to isolate cardiomyocytes from different developmental time-points which were used for global gene expression analysis (papers I and IV).

Cell cycle analysis

Given the issues and caveats associated with myocyte nuclear identification, we opted for a FACS based purification method and subsequent nuclei isolation, along with traditional immunohistochemical techniques. Purification of cardiomyomyocytes lessened the subjectivity associated when attempting to score adult cardiomyocyte DNA synthesis. We developed a reliable assay of cardiomyocyte FACS based analysis, whereby we could successfully eliminate the subjective aspects of cardiomyocyte nuclear identification. To monitor cardiomyocyte cell division mice were injected with the thymidine analog bromodeoxyuridine (BrdU) (paper I). BrdU is incorporated during

DNA synthesis in dividing cells. Immunohistochemistry allows detection of cells that have undergone DNA synthesis during the period in which BrdU is administered and present in the system of the animal. FACS was also used as a method for BrdU detection in cardiomyocyte nuclei. Mice were injected with 50mg/kg i.p. twice daily for two weeks prior to sacrifice in the case of adult time-points and 4 hours prior to sacrifice for other time-points (Paper I). Ki-67, another marker of proliferation, was also assayed by FACS and immunohistochemistry.

Aims

Heart disease results from the loss of cardiomyocytes following myocardial damage and is a leading cause of death worldwide. Stimulating the endogenous regeneration of cardiomyocytes is an attractive therapeutic strategy. There has been conflicting observations in the field pertaining to the mitotic capacity and turnover kinetics of cardiomyocytes in both mouse and human. The studies in this thesis sought to accurately establish the turnover of cardiac myocytes and to identify gene expression patterns associated with the three developmentally distinct developmental periods: fetal mitotic, neonatal hypertrophic and adult terminally differentiated cardiomyocytes. We aspired to identify key cell cycle genes with therapeutic potential and also surface markers that would facilitate non-genetically mediated isolation of cardiomyocytes for manipulation and cell therapy.

More specifically the aims were:

Article I To investigate cardiomyocyte turnover in mouse hearts throughout development and the genes associated with the switch from hyperplasia to hypertrophy and binucelation.

Article II To investigate cardiomyocyte turnover in human hearts

Article III To investigate a non-genetic method to isolate fetal cardiomyocytes based on surface marker expression

Article IV To investigate potential surface markers for the isolation of developmentally distinct populations of cardiomyocytes

Summary and discussion of articles

Article I

Cardiomyocyte cell cycle control and growth estimation *in vivo* – an analysis based on cardiomyocyte nuclei

Rationale

Adult mammalian cardiomyocytes are traditionally viewed as being permanently withdrawn from the cell cycle. While some groups have reported none, others have reported extensive mitosis in adult myocardium under steady state conditions. Recently a highly specific assay of ^{14}C dating in humans has suggested a continuous generation of cardiomyocytes in the adult, albeit at a very low rate. Mice represent the most commonly used animal model for these studies but its short life-spam make it unsuitable for ^{14}C studies. Herein we investigate the cellular growth pattern for murine cardiomyocyte growth under steady-state conditions, addressed with new analytical and technical strategies and furthermore relate this to gene expression patterns.

Results

To address the proliferative capacity of cardiomyocytes in the heart, we performed nuclei isolation on purified populations of myocytes and subsequently FACS analysis with BrdU and 7-amino actinomyosin (7-AAD) staining. No detectable BrdU-positive nuclei were observed in adult myocardium suggesting that cardiomyocyte DNA synthesis completely ceases around the 3 week time point in mouse left ventricular tissue. Myocyte turnover was also assessed by measuring the fraction of Ki-67$^+$ nuclei, by three weeks of age no proliferative activity was observed. The frequency of cardiomyocyte nuclei determined to be in the cell cycle was congruous regardless of the proliferation marker assessed by FACS (**Fig. 4A**). Proliferation was undetectable after 21 days indicating terminal differentiation and cell cycle inhibition. These frequencies were also compared to traditional immunohistochemical techniques.

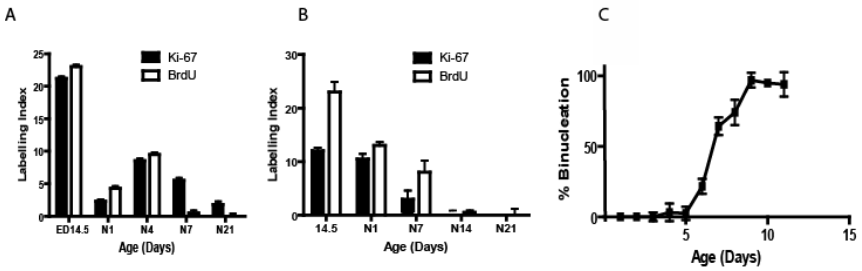

Figure 4. Cell cycle kinetics Comparsion of Bromodeoxyuridine (BrdU) labeling and Ki-67 staining of isolated myocyte nuclei by FACS (**A**). ED14.5 and IHC (**B**) and binucleation index of cardiac myocytes (**C**). Data represented as mean ± SD.

We isolated viable myocytes from eGFP-transgenic mice with GFP expression driven by the cardiomyocyte α-MHC promoter, allowing us to examine the gene expression patterns between cardiomyocytes of embryonic, neonatal and adult origin (**Fig. 5A-D**).

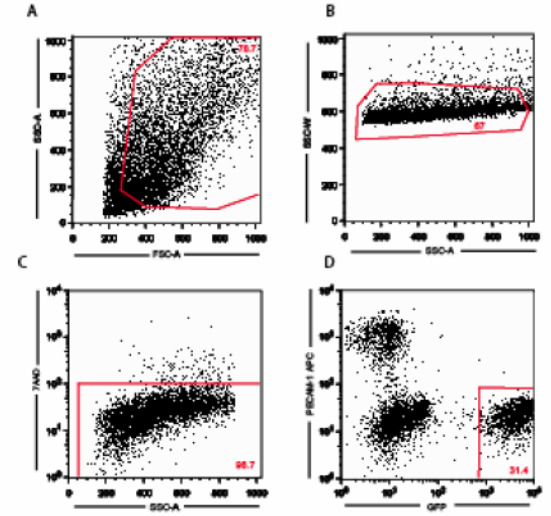

Figure 5. Isolation of viable cardiomyocytes. Cardiomyocytes were labeled in the hearts of α-MHC-EGFP transgenic mice, which were isolated by flow cytometry. Flowcytometry profiles of neonatal transgenic myocardium single cell preparation. Size gating (**A**); doublet discrimination (**B**) viability staining with 7AAD (**C**). $EGFP^{+}$ $CD31^{neg}$ population was sorted (**D**).

Based on the raw microarray data, a gene list was generated, whereby embryonic, neonatal and mature cardiomyocyte stage specific genes were identified. Our micro-array confirmed that several cell cycle regulators are differentially expressed - adult cardiomyocytes differ from their neonatal and fetal counterparts in that *CDK2* (**Fig. 6E**) , *CDK3* (**Fig. 6F**) and the cyclin *D1*/*CDK4* complex(**Fig. 6G-F**), are solely down-regulated in adult cardiomyocytes.

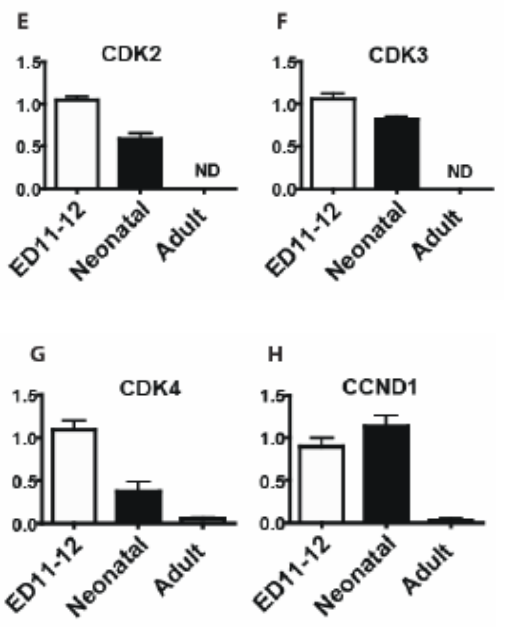

Fig 6. Cell cycle control in neonatal cardiomyocytes Quantitative RT-PCR data of indicated genes in FACS-sorted cardiomyocytes from embryonic (ED11-12), neonatal (N3-4) and adult (10 weeks) time-points. All data were normalized to the expression of GAPDH and β-actin housekeeping genes. Expression levels in embryonic cardiomyocytes was set to 1 as reference. Results represent mean (SEM) values from two independent experiments, with PCR analysis in each experiment performed in triplicates. ND = no detectable expression after 45 cycles of PCR.

Several G1/S transition regulators were expressed during neonatal development, coupled with the down-regulation of key kinetochore structural proteins necessary for completion of mitosis, specifically the down-regulation of anillin. These expression patterns were synchronous with G2/M modulators *E2F1* (**Fig. 6J**) and *WEE1* (**Fig. 6K**), evidence of a complex multi-gene checkpoint control of cytokinesis during the switch from hyperplasia to hypertrophic growth during neonatal cardiomyocyte development.

Discussion

Cardiomyocyte turnover during development has been a major issue of dispute. In this study we attempted to reconcile the diametrically opposed findings of myocyte kinetics in the mouse. While some groups have reported none, others have reported extensive myocyte turnover in the adult rodent heart[125]. One of the major methodological caveats in the assessment of myocyte turnover has been the correct identification of cardiomyocytes, or more specifically the nuclei of cardiomyocytes. This is evident by the many opposed findings obtained by the same method in the same species. Many assays to establish myocyte mitotic activity have relied heavily on traditional immunohistochemical techniques utilizing both BrdU and Ki-67. Although BrdU immunohistochemistry has advantages, it also has many defects which potentially result in erroneous conclusions[188]. As BrdU is not an indicator of cell division *per se*, but of DNA synthesis, mitotic evaluation by independent means must be demonstrated. Although Ki-67 is a reliable marker of mitosis because it is expressed, albeit at different levels, throughout all stages of the cell cycle, there remains the identity crisis of the cardiomyocyte.

Microscopic artefacts are potentially misleading when attempting to evaluate cardiomyocyte nuclei in histologic sections and utilizing markers for DNA synthesis/mitosis is somewhat problematic when analyzing cell populations that exhibit exceptionally low proliferative activity. Herein we re-evaluated the findings of earlier studies, with the intention of improving the specificity of the estimates of cardiomyocyte kinetics. The most specific marker of cardiomyocytes is the cardiac isoform of Troponin-T, which was exploited for the FACS based purification of cardiomyocyte populations and subsequent nuclei analysis with proliferation markers. This approach allowed us to eliminate many of the subjective aspects of cardiomyocyte nuclei identification. We observed no DNA synthesis or mitotic activity in the cardiomyocytes of mice after the third postnatal week. Our results support the hypothesis that there is no DNA synthesis in adult murine cardiomyocytes under normal physiological conditions.

Cardiomyocyte DNA synthesis in the early postnatal period was predominantly associated with binucleation and acytokinetic mitosis during the period of hypertrophic growth. This rapid switch from hyperplasia to myocyte hypertrophy provided the natural model in which to examine the molecular regulation and mechanisms that govern cell proliferation and hypertrophy. We generated a transcriptional profile for hyperplastic, hypertrophic and adult myocytes that encompasses stage-specific and stage-independent markers for these developmentally distinct populations. Gene

analyses identified 32 genes whose expression was predicted to be unique to day 3-4 neonatal myocytes, compared to embryonic or adult cells. Several key genes central to the processes of binucleation and polyploidy were identified including the kinetochore protein anillin and microtubule interacting protein *TACC2* which were differentially expressed at the neonatal period. These genes could be potential candidates for manipulation and induction of proliferation in a therapeutic setting.

Article II

Cardiomyocyte renewal in humans

Rationale

It has been difficult to establish whether there is any significant cardiomyocyte turnover after birth in humans or if cardiomyocytes are generated also later in life. We have taken advantage of the integration of ^{14}C, generated by nuclear bomb tests during the Cold War into DNA to establish the age of cardiomyocytes in humans.

Results

We first carbon dated hetergenous left ventricle myocardial cells, including cardiomyocytes and other cell types, to determine the extent of postnatal DNA synthesis in the healthy human heart. DNA was extracted and ^{14}C levels measured by accelerator mass spectrometry. The cellular birth dates can be inferred from determining at what time the sample's ^{14}C level corresponded to the atmospheric levels (**Fig. 1A**). ^{14}C levels from all individuals born around or after the nuclear bomb tests corresponded to atmospheric levels several years after the subjects' birth (**Fig. 1B**), indicating substantial postnatal DNA synthesis. Analysis of individuals born before the period of nuclear bomb tests allows for sensitive detection of any turnover after 1955, due to the dramatic increase in ^{14}C levels. In all studied cases (born up to 22 years before the onset of the nuclear bomb tests), ^{14}C concentrations were elevated compared to the pre-nuclear bomb test levels (**Fig. 1C**). Thus, DNA is synthesized many years after birth, indicating that cells in the human heart do renew into adulthood.

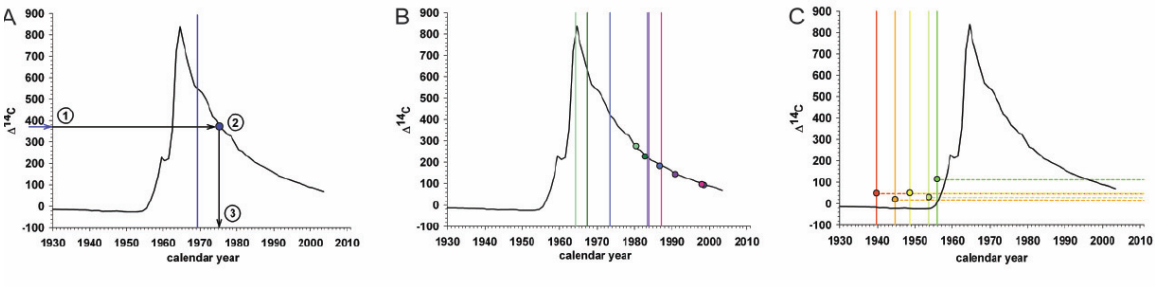

Figure 1. Cell turnover in the heart (A) Schematic figure demonstrating the strategy to establish cell age by ^{14}C dating. The black curve in all graphs shows the atmospheric levels of ^{14}C over the last decades (data from [189]). The vertical bar indicates the date of birth of the individual. The measured ^{14}C concentration (1) is related to the atmospheric ^{14}C level by using the established atmospheric ^{14}C bomb curve (2). The average birth-date of the population can be inferred by determining where the data point intersects the x-axis (3). ^{14}C levels in DNA of cells from the left ventricle myocardium in individuals born after (B) or before (C) the nuclear bomb tests correspond to times points substantially after the time of birth, indicating postnatal cell turnover. The vertical bar indicates the date of birth of each individual and the similarly colored dots represents the ^{14}C data for the same individual. For individuals born before the increase in ^{14}C levels, it is not possible to directly infer a time point as the measured level can be a result of incorporation during the rising and/or falling part of the atmospheric curve, why the level is indicated by a dotted horizontal line.

We developed a strategy to isolate cardiomyocyte nuclei by flow cytometry. We found that the well characterized cardiomyocyte specific proteins cardiac troponin I and cardiac troponin T have evolutionarily conserved nuclear localization signals and are partly localized in the nuclei of cardiomyocytes (**Fig. 2**)

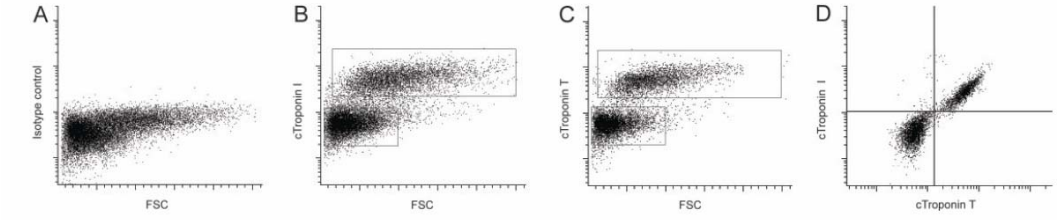

Figure 2. Isolation of cardiomyocyte nuclei (A-C) Flow cytometric analysis of cardiomyocyte nuclei from the left ventricle of the human heart with an isotype control antibody or antibodies to the cardiomyocyte specific antigens cTroponin I or T. (D) cTroponin I and T are present in the same subpopulation of heart cell nuclei.

We extracted DNA from cardiomyocyte nuclei and measured the ^{14}C concentration in genomic DNA. In all individuals born before the onset of the nuclear bomb tests the ^{14}C level in cardiomyocyte genomic DNA was higher than the pre-bomb atmospheric levels, demonstrating DNA synthesis after 1955. All individuals born near or after the time of the nuclear bomb tests had ^{14}C concentrations in cardiomyocyte DNA corresponding to several years after their birth, indicating postnatal cardiomyocyte DNA synthesis. We

observed in individuals born after the nuclear bomb tests that the difference between the birth date of the individual and the date corresponding to the ^{14}C level in cardiomyocyte DNA increased with the age of the individual demonstrating that cardiomyocyte DNA synthesis is not restricted to a limited period in childhood but continues in adulthood (**Fig. 3**).

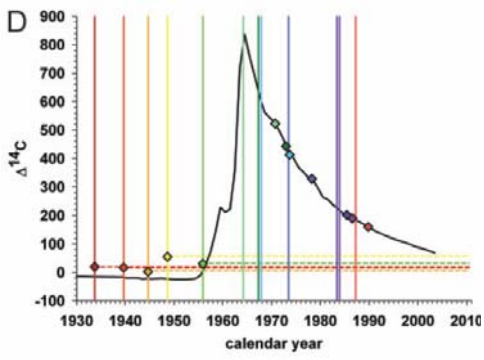

Figure 3. Cardiomyocyte turnover in adulthood
^{14}C values corrected for the physiologically occurring polyploidisation of cardiomyocytes during childhood for individuals born before and after the bomb spike, calculated based on the individual average DNA content per cardiomyocyte nuclei. The ^{14}C content is not affected in individuals where the polyploidization occurred before the increase in atmospheric levels. The vertical bar indicates year of birth, with the correspondingly colored data point indicating the delta ^{14}C value.

Polyploidization of cardiomyocyte DNA occurs in a well defined spatial manner during childhood [190] [191]. By subtracting the polyploidization-associated ^{14}C incorporation from the measured value in each case, we could estimate polyploidization-independent ^{14}C values. Mathematical modeling of ^{14}C data was performed and the best fit was found with an inverse-linear declining turnover rate (**Fig. 4B**), where younger cardiomyocytes were more likely than old to be replaced This model predicts that cardiomyocytes are renewed at a rate of approximately 1% per year at the age of 20 and 0.3% at the age of 75 (**Fig. 4B**).

Discussion

A highly specific isolation strategy was employed in this study which eliminated any subjective aspects of cardiomyocyte identification. Throughout human fetal life there is extensive proliferation of myocytes which slows dramatically at birth and the 30- to 50-fold increase in heart mass in adulthood[192] is attributable to myocyte hypertrophy and non-myocyte population proliferation. Throughout the first decade of life, DNA synthesis occurs in the absence of cell division resulting in ~25% of cardiomyocytes in the human heart becoming binucleated. Cardiomyocyte ploidity also increases throughout the first decade of life with the majority of myocyte nuclei being polyploidy.
DNA from cardiomyocytes was found to be younger than the patients' chronological age through cardbon-14 dating in all instances establishing that there is DNA synthesis after birth in humans.
DNA synthesis as a result of binucleation was eliminated as a source of error, as binucleation in humans is typically complete by the age of 10[120] and individuals who were adults at the time of nuclear testing nevertheless exhibited a turnover of myocytes. Additionally, misconceptions owing to polyploidization were addressed by sorting

cardiomyocyte nuclei based on DNA content. The ^{14}C content of diploid nuclei remained younger than the patients' chronological age. Taken together we can suggest that multinucleation and polyploidization are not sufficient to explain the postnatal DNA synthesis. Through mathematical modeling we have established that the kinetics of this gradual turnover is estimated at an annual turnover of 1% in the left ventricle at the age of 20 which decreases to 0.3% at the age of 75. This study dismissed the myth that human cardiomyocyte turnover is widespread, however the fact that it occurs at all could possibly be exploited for therapeutic means and may offer targets for pharmaceutical strategies to stimulate myocyte replacement following infarction or as a complement to future cell therapies.

It is unknown if stem or progenitor cells are responsible for the new generation of cardiomyocytes after birth or if they derive from preexisting cells that re-enter the cell cycle. The origin of these cycling myocytes is difficult to establish in humans. Nuclei were purified based on the presence of mature myocyte specific proteins *cTropT* and *cTropI* and therefore immature or progenitor populations were not assessed. It is also possible that these progenitor populations are perhaps localized in a particular zone or niche within the myocardium. Nevertheless, the inherent ability of the heart to regenerate itself is insufficient for functional improvement following myocardial infarction.

The evidence presented herein also suggests that human and mouse cardiomyocytes of a similar developmental stage share common features such as neonatal polyploidisation, binucleation and growth arrest in adult. However the period of each developmental phase differs immensely between these species e.g. while the neonatal phase of bi-nucleation and polyploidisation in humans lasts for the first decade of life this phase seems to be completed in mice within three weeks. Our studies of DNA synthesis in adult mouse cardiomyocytes could not detect proliferation above background levels suggesting that mouse data may not reflect human cell turnover. This is unsurprising given the enormous physical differences and variations in physiological requirements between the two species.

Article III

Fetal cardiomyocytes are VCAM-1+ CD31- and thus can be isolated by FACS with high purity and cultured *in vitro*

Rationale

Any potential cellular therapy for heart disease involving cardiomyocytes would require the elimination of contaminating non-myocyte cells. A strategy that facilitates a non-genetic method of isolating embryonic cardiomyocytes would also be a powerful tool for the study of such cells. Cardiomyocyte purification is limited by the absence of specific surface markers or cell labeling techniques.

Results

The surface marker protein VCAM-1 was identified as a potential candidate for the purification of mouse embryonic cardiomyocytes based on its expression in the developing myocardium. VCAM-1 expression was specific to the myocardium from E10.5 until E13, and was co-localized with the expression of the myocyte specific structural protein cTrop-T. VCAM-1 expression was not observed in skeletal muscle, epithelial and endothelial tissues of the developing heart (**Fig. 1**). Thereafter, beginning E14, VCAM-1 is downregulated and became undetectable in adult myocardium.

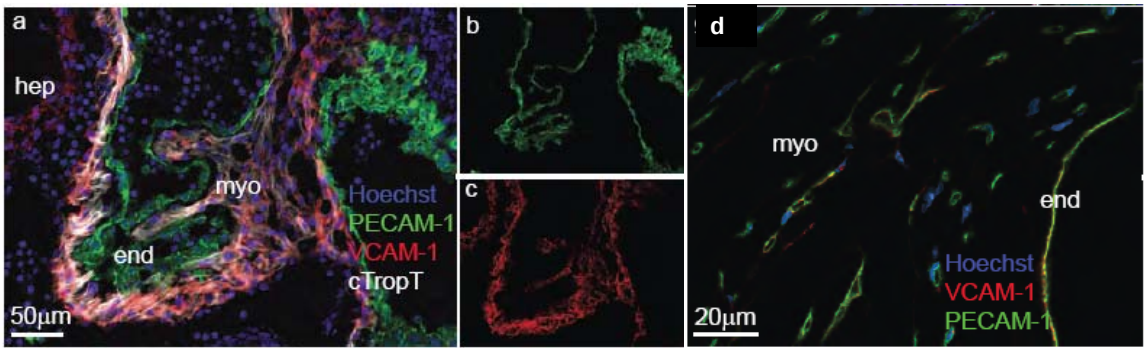

Figure 1. VCAM-1 expression in mouse embryonic myocardium VCAM-1 is expressed and co-localized with Troponin-T in the developing myocardium at E10.5, but not with endothelial tissue (**a-c**). Conversely in the adult heart, VCAM-1 expression was observed with endocardium and vascular endothelium (**d**)

After enzymatic cardiac dissociation, VCAM-1 was used to purify viable embryonic cardiomyocytes by FACS, and by this method >97% of VCAM-1^{+} PECAM-1^{-} sorted cells from E10.5-E11.5 embryos were also cTropT positive upon reanalysis. We sorted cells from embryonic hearts at E10-11 due to the limitation of cell number at E9 (where VCAM-1 expression is highest) and lower specificity due to downregulation at E12. Sorted cells were cultured and expression of cardiac specific structural markers and transcription factors was confirmed including *α-MHC*, *BNP*, *MLC2a* and *ML2v* and *NKX2-5* and *MEF2c*. Cultured cells also began beating. These biological observations were also confirmed by patch clamping to demonstrate the physiological functionality and integrity of these sorted embryonic cardiomyocytes.

Additionally we sought to develop a culture method for these sorted cells that would allow their expansion *in vitro*. We subsequently improved the traditional culture techniques of mouse cardiomyocytes by seeding sorted cells on pre-plated irradiated embryonic cardiac fibroblasts or gelatin. Fibroblasts have been shown to be essential for cardiomyocyte proliferation[193]. Cultured VCAM-1^{+} cardiomyocytes could be expanded *in vitro* and were mitotically active as identified by BrdU and Ki-67 cell proliferation assays (**Fig. 6**). Beating myocytes co-cultured with irradiated fibroblasts could be kept in culture for 15 days which has not previously been reported for mouse cardiomyocytes.

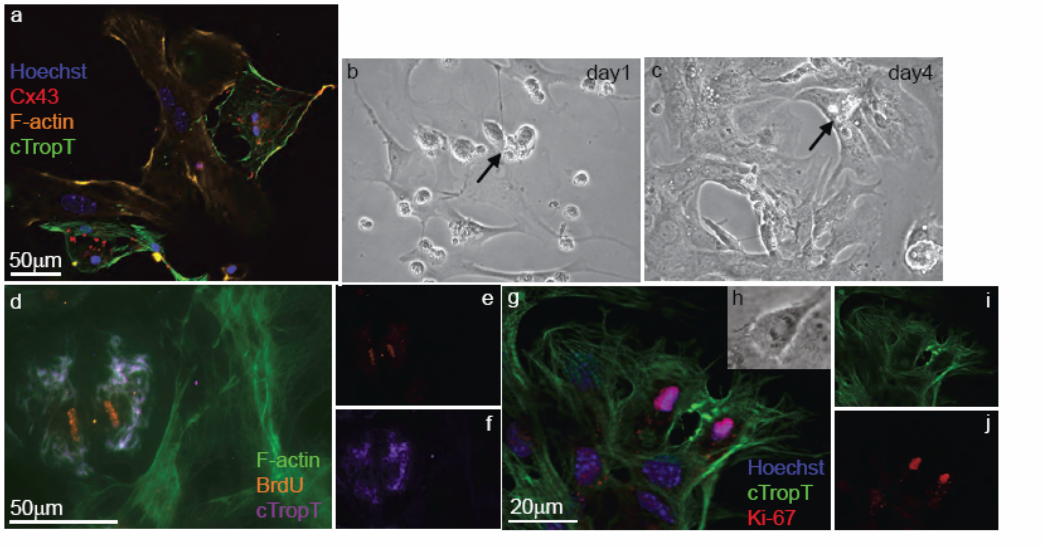

Figure 6. Sorted embryonic cardiomyocytes proliferate in co-culture with irradiated embryonic fibroblasts Sorted cells after 4 days in culture expressing myocyte specific markers Connexin 43 and cardiac Tropnin-T (**a-c**). Sorted cells were mitotically active as assessed by BrdU incorporation and Ki-67 expression *in vitro* (**d-J**).

Discussion

In this study a novel non-genetic surface marker based purification method was established for embryonic cardiomyocytes. By FACS sorting based on the expression VCAM-1 we achieved an enriched fraction of cardiomyocytes with greater than 97% purity. These sorted cardiomyocytes were viable, mitotically active and functionally adept, expressing myocyte specific markers. This purification method is not dependent of a reporter system and allows the enrichment of wild-type fetal cardiomyocytes by surface marker expression. VCAM-1 expression is co-localized with cTropT at the embryonic time-point and not in the endothelium. However, in the neonatal period there is a change in expression and VCAM-1 becomes widely expressed in the endothelium in the late neonatal period into adulthood where once it was absent. It is unknown when this switch in endothelial VCAM-1 expression occurs.

Sorted VCAM-1^{+} PECAM-1^{-} cells from fetal hearts formed beating colonies when seeded on gelatin and embryonic cardiac fibroblast feeders. This technique will be a valuable tool for utilizing populations of fetal cardiomyocytes for cell culture, genetic manipulation, RNA or protein expression assays. These cells were also rapidly expanded *in vitro* co-culture with embryonic irradiated fibroblasts as feeder cells for up to two weeks. This is a reliable, reproducible method for the isolation of embryonic cardiomyocytes. VCAM-1 expression was highest from E9-E12 but lower as development proceeds beyond E13.

VCAM-1 was also utilized to isolate cardiomyocytes from ESC colonies, although some PECAM-1 positive cells also expressed VCAM-1, perhaps representing immature

mesenchymal cells or other undifferentiated cell types. ES cells represent an ideal stem cell source for *ex vivo* generation and expansion of cardiomyocytes, however, studies are still required to assess the long-term teratoma potential and tumour-free engraftment. The major challenge for the clinical development of ES cell–derived cardiomyocytes has been the development of safe and efficient methods for cellular enrichment *in vitro*. This simple isolation technique should prove useful as a non-genetic method for enrichment and facilitate further investigation. Cellular replacement approaches with these FACS isolated cardiomyocytes will have to be assessed, and if possible co-transplantation with cardiac fetal fibroblast is a prospective therapy for myocardial infraction.

Article IV

Identification of cardiomyocyte and progenitor populations with cell surface markers: a comparison of fetal, neonatal and adult cardiomyocytes

Rationale

This study sought to fish and investigate surface markers for the isolation of cardiomyocyte populations and discern any variations in cardiomyocyte lineages during the process of maturation and differentiation. We combined transcriptional profiling of cardiomyocytes from distinct developmental stages, fetal, neonatal and adult with immunocytological assays to identify membrane markers for the enrichment and potential chatacterization of mycocyte populations.

Results

Previously we isolated viable myocytes from eGFP-transgenic mice with GFP expression driven by the cardiomyocyte α-MHC promoter, allowing us to examine the gene expression patterns between cardiomyocytes of embryonic, neonatal and adult origin[119]. Embryonic, neonatal and mature cardiomyocyte stage specific genes were identified. Genes were clustered by cellular location as biological description in Gene Ontology (GO). 118 embryonic 152 neonatal and 239 adult membrane associated markers highly enriched for the presence of membrane targeting motifs including signal peptide and /or transmembrane domain were identified.

We identified commercially available antibodies and performed immunofluorescence staining on frozen cardiac tissue to verify these candidate surface markers were co-localized with cardiac specific proteins cTropT. We observed clear evidence of surface expression of novel markers by immunohistochemistry. We subsequently investigated if we could isolate cardiomyocyte populations based on the expression of these surface markers by FACS (**Fig. 5**). Candidate markers from each developmental time-point were combined with lineage markers for the endothelial, hematopoietic, fibroblast and smooth muscle lineages and populations sorted for re-analysis and validation by molecular means.

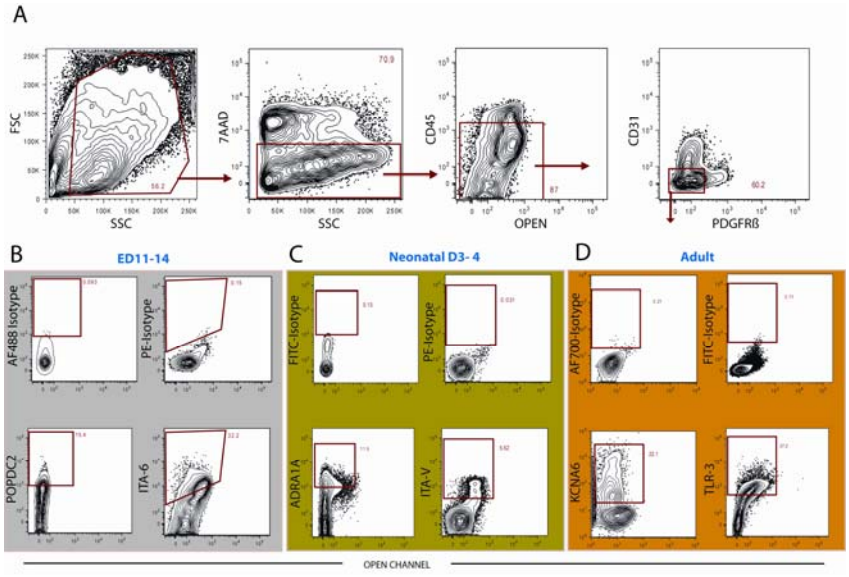

Figure 5. Fluorescence-activated cell sorting (FACS) analysis of a subset of potential cardiomyocyte surface markers identified in this study. Single cell cardiac preparations from fetal (ED11-14.5), neonatal day 3-4 and adult (10 weeks) were stained with antibodies against CD45, CD31 and PDGFRβ constituting the major mature cardiac lineage markers. Cell preparations were also stained with 7-AAD and only live cells sorted. Based on this phenotypic analysis viable cells which were lineage negative enriched (CD45neg PDGFRβneg CD31neg) were defined (A) and cellular subsets were sorted based on the expression of candidate surface markers for each time-point. Surface marker sorting based on isotype controls (top panels B-D) and sorting profiles (bottom panels B-D). Flow cytometric profiles are representative of more than 3 individually stained cardiac preparations.

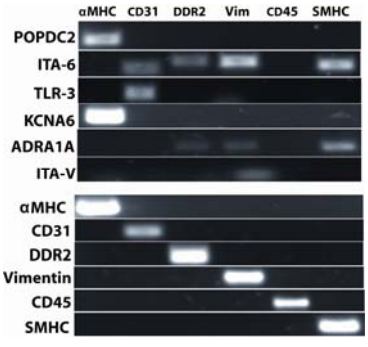

Figure 6. Gene expression analysis of sorted cell fractions with candidate surface markers.
Indicated cell types were bulk sorted based on surface marker expression directly into lysis buffer. RNA was extracted from sorted cardiac populations POPDC2, ITA-6, TLR3, KCNA6, ADRA1A and ITA-V and subjected to quantitiative RT-PCR for myocyte specific structural protein *αMHC*, endothelial marker *CD31*, fibroblast marker *DDR2*, fibroblast and smooth muscle specific protein Vimentin *Vim*, pan-hematopoietic marker *CD45* and smooth muscle specific *SSMHC*. Agarose gel electrophoresis of RT–PCR products (**Top Panel**). RNA from embryonic heart tissue was used as control (**bottom panel**).

The popeye domain containing 2 (*POPDC2*) and the potassium voltage-gated channel subfamily A member 6 (*KCNA6*) antibodies appeared to effectively isolate myocyte populations when combined with other lineage excluding antibodies (**Figure 6** ***top panel***). Both of these isolated fractions were negative for CD31, DDR2, vimentin, CD45 and SMHC contamination according to our RT-PCR analysis.
Other candidate markers proved inadequate for the purification of cardiomyocytes, despite the lineage exclusion strategy implemented when FACS sorting. *ITA-6*, *TLR-3*, *ADRA1A* and *ITA-V* sorted populations were found to be deficient in myocyte specific *αMHC* but other lineage markers were abundant. (**Fig. 6**). *ITA-6* and *TLR-3* were both positive for endothelial marker *CD31* and both *ITA-6* and *ADRA1A* were positive for several lineage markers including fibroblast, and smooth muscle lineages. This perhaps demonstrates the variability of surface marker retention following enzymatic dissociation of tissue and the sensitivity of the antibodies used in this study.

Discussion

We have identified a core set of genes that mark cardiomyocytes and determined the cell surface marker subset by gene annotation and in doing so isolated several novel myocyte markers that could potentially be utilized in the purification or isolation for therapeutic treatments. This study we hope, will expand the potential antigens that can be utilized to differentiate myocytes from non-myocytes and cardiac progenitors from mature differentiated myocytes. Surface marker expression forms the basis for characterization and isolation of many mature and progenitor and or stem types. Herein we combine immunotranscriptional profiling of purified populations of embryonic, neonatal and adult cardiomyocytes to fish genes encoding potential surface marker proteins.
We have profiled several potential surface markers for developmentally distinct subsets of myocytes and tested commercially available antibodies by immunohistochemical screening. Several antibodies that appeared to be co-localized with cardiac specific markers such as cTropT in frozen sections were often less suitable for FACS analysis and/or sorting. Frequently there was absence of correlation between transcriptional profiles and protein levels as detected by IHC and FACS. Following the FACS sorting of candidate populations after major myocardial lineage exclusion of endothelial, hematopoietic and fibroblastic cell types, bulk sorting purity and identity of populations was by verified by RT-PCR. Of all the surface markers assayed to date, only two proved capable of myocyte isolation from a heterogenous myocardial cell population. The embryonic marker *POPDC2* fraction and the adult marker *KCNA6* fraction were free of contamination of other lineages when assessed by RT-PCR. Nevertheless, further morphological and physiological parameters of these populations will have to be investigated to confirm our findings. It would be of interest to investigate the culture potential of these fractions and if they can be expanded *in vitro*. A number of these

markers may prove useful in isolating embryonic myocytes and removing these from ESC culture.

Current work is focused on screening additional antibodies to other putative cardiomyocyte surface markers identified in this study, and should hopefully deliver a resource that may be used in a variety of applications.

Sammanfattning på Svenska

Hjärtsjukdom som orsakas av skador på hjärtmuskelceller är en av de vanligaste dödsorsakerna världen över. En framtida önskvärd behandling vore att kunna stimulera kroppens egna förmåga att återskapa hjärtmuskelceller, eller att isolera hjärtmuskelceller framställda i cellkultur.

Arbete I är fokuserat på studier rörande tillväxtmönstret hos hjärtmuskelceller under normala förhållanden hos möss. Graden av nybildning och omsättning av hjärtmuskelceller fastlogs exakt och genuttryck specifika för olika tidpunkter i hjärtats utveckling identifierades. Resultatet visade att det inte fanns någon signifikant nybildning av hjärtmuskelceller i hjärtat på vuxna djur.

I arbete II fokuseras studierna på omsättningen av hjärtmuskelceller hos människa. Här har vi kunnat dra fördel av den inkorporering av radioaktivt kol (^{14}C) i kromosomalt DNA som inträffade under kalla krigets provsprängningar med atomvapen. Denna radioaktiva inmärkning gjorde det möjligt att exakt mäta åldern på hjärtmuskelceller hos människor. Matematiskt kunde vi bestämma att hjärtmuskelceller förnyades med en frekvens på ungefär 1% per år vid 20 års ålder och 0.3% vid 75 års ålder.

I arbete III och IV ligger fokus på att utveckla strategier för att isolera embryonala (III och IV) eller mer utmognade (IV) hjärtmuskelceller. Vi har upprättat en genetiskt oberoende FACS-baserad teknik där ytmarkören VCAM-1 används för att rena fram tidiga embryonala hjärtmuskelceller (III). Ytterligare ytmarkörer undersöks för deras potential att kunna definiera och isolera hjärtmuskelceller även från andra tidpunkter (IV).

Den här avhandlingen tillför ny kunskap inom forskning som rör kinetik och celldelning hos hjärtmuskelceller. Dessutom har vi identifierat nyckelgener som är involverade i cellcykel-kontroll av hjärtmuskelceller och som utgör potentiella kandidater för en terapeutisk behandling och manipulation. Utöver detta har vi utvecklat en genetiskt oberoende FACS-baserad isoleringsmetod för embryonala hjärtmuskelceller. För alla tänkbara cell-baserade transplantationer till ett skadat hjärta kommer det krävas att celler som inte är hjärtmuskelceller kan elimineras. En strategi där en genetiskt oberoende metod används för att rena fram hjärtmuskelceller skulle även kunna utgöra ett viktigt redskap för att studera dessa celler.

Achoimre Gaeilge

Tá galar chroí mar an bpríomh chúis bhás sa domhan, agus tarlaíonn sé nuair atá damáiste miócairdeach déanta agus cailiú cill na chroí, na 'cardiomyocytes'. Is feidir linn an damáiste a cheartú tríd na cardiomyocytes a spreagadh le atghinúint a dhéanamh, nó foinse *in vitro* a úsáid chun iad a ionadú. Tá na dhá straitéis seo an suimiúil le teiripí nua a aimsiú. Chun na teiripí seo a chuir chun cinn, rinne muid iniúchadh in san athnuachan i cardiomyocytes a dtarlaíonn i gcroí luch sutha, nuabherithe agus fásta. Fuair muid amach nach dtarlaíonn athnuachan cardiomyocytes i gcroí luch in aon chur. I gcroí daoine, d'aimsigh muid go dtarlíonn a lán athnuacha i groí suthach, ach go laghdaíonn sé seo de réir a chéile i rith an tsaoil. Léirigh muid go ndéanann cardiomyocytes i gcroíthe daoine fásta athnuacha.

Rinne muid iarracht na comhartaí atá tabachtach san athnuachan i cardiomyocytes a aimsiú. Bhain muid úsáid as luch géiniteach atá na cardiomyocytes ag leiriú GFP. Bhí muid in ann na cardiomyocytes a aonrú agus rinne muid 'micro-array' orthu, chun comhartaí géiniteach a aimsiú atá riachtanach le athnuachan a dhéanamh chun an bpróiséis ina gcáilíonn cill suthach an cumas athnuachan agus iad ag fás a tuiscint. D'aimsigh muid cúpla géin atá tabhachtach dun phróiséis seo agus is feidir a shaothrú chun teiripí nua a aimsiú.

Cuardaigh muid suaitheantais seachtracha ar cardiomyocytes gur feidir a úsáid chun iad a aonrú as daonra measctha, gan úsáid a bhaint as suaitheantas géiniteach, mar is gnáth. D'aimsigh muid gur suaitheantas seachtrach iontach é VCAM-1 le cardiomyocytes a aonrú.Tabharann an tráchtas seo eolas nua don réimse miócairdiach. Aon teiripí nua do galar croí atá chun an chroí a dheisiú, beidh modh le cardiomyocytes a aonrú riachtanach. Cuireann an eolas seo ar fail modh nua le taighde a dhéanamh ar cardiomyocytes, gan úsáid a bhaint as modhanna géiniteach.

Articles Not Included in this Thesis

Walsh S, Nygren J, Pontén A, Fleischmann B.K, Jovinge S. Bone marrow contribution to non-haematopoietic lineages in a W^{41}/ Dmd^{mdx} deficient mouse model. 2009.
Manuscript

Ariane Tormin, Jan Claas Brune, **Stuart Walsh**, Johan Richter, Xiaolong Fan, Stefan Scheding
CD146 Expression in Primary Bone Marrow MSC Progenitor/Stem Cells is Dependent on their In Situ Location
Submitted

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