THE ROLE OF FUNCTIONAL CALCIUM HANDLING DURING THE EARLY STAGES OF MOUSE HEART DEVELOPMENT

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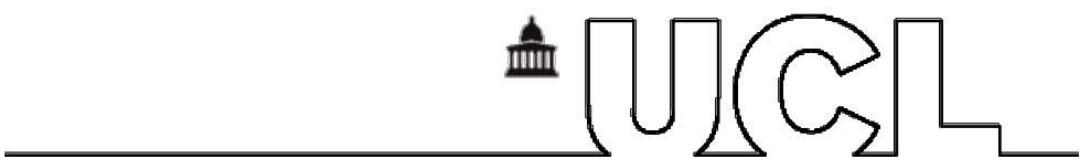

DECLARATION

I, Richard Tyser, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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ABSTRACT

During development the heart is the first organ to form and function in the embryo proper. Along with being fundamental for contraction, Ca^{2+} is also a key signalling molecule known to regulate cardiac genes, however, it is unclear how Ca^{2+}-handling feeds-back onto the initiation of beating and whether this directly impacts on early embryonic heart development per se. The aim of this study was to investigate how initial contractions of the early mouse heart are established and what the downstream consequences of function are on cardiac differentiation and heart development. Using *ex vivo* Ca^{2+} imaging we found evidence of randomly distributed Ca^{2+} transients in the forming cardiac crescent, prior to contraction, suggesting early Ca^{2+} handling is essential for cardiomyocyte differentiation and subsequent heart development. To study the downstream effects of early Ca^{2+} we used a murine embryonic stem cell (mESC) model of cardiomyocyte differentiation which recapitulated heart development *in vivo*. Assessment of Ca^{2+} handling proteins indicated that the Na^{+}-Ca^{2+} exchanger (NCX) was one of the earliest sarcolemmal transporters to be expressed, prior to the L-type Ca^{2+} channel (LTCC). Pharmacological inhibition of NCX revealed an essential early role in establishing and maintaining the first Ca^{2+} transients through to initiation of contraction; a role superseded by the LTCC as differentiation progressed. Upon NCX blockade cardiomyocyte differentiation was inhibited, coincident with the down-regulation of signature cardiac genes and calmodulin kinase signalling, which culminated in the failure of beating cardiomyocytes to form. This study points to a novel mechanism by which form and function are intricately linked such that, from the outset, Ca^{2+} acts to initiate contraction as well as regulate cardiac differentiation and the formation of the heart, adding an important layer onto our current understanding of mammalian cardiovascular development.

ACKNOWLEDGEMENTS

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Gene	Protein	Description	Reference
Oct-4	Octamere-binding TF 4	Pluripotency TF	(Niwa et al. 2005)
Nanog	Homeobox protein Nanog	Pluripotency TF	(Mitsui et al. 2003)
T	Brachyury	Mesoderm TF	(Wilkinson et al. 1990)
Eomes	Eomesodermin	Mesoderm TF	(Costello et al. 2011)
Gsc	Homeobox protein Goosecoid	Gastrulation TF	(Gaunt et al. 1993)
Mesp1	Mesoderm posterior 1 homolog	Cardiac mesoderm TF	(Saga et al. 1999)
Gata-4	Gata-4	CM Differentiation TF	(Arceci et al. 1993)
Nkx2.5	Homeobox protein Nkx-2.5	CM Differentiation TF	(Lien et al. 1999)
Mef2c	Myocyte-specific enhancer factor 2C	CM Differentiation TF	(Edmondson et al. 1994)
Myh6	α-Myosin heavy chain	CM Contraction	(Nakao et al. 1997)
Myh7	B-Myosin heavy chain	CM Contraction	(Nakao et al. 1997)
Tnnt2	Cardiac Troponin T	Contractile regulation	(Cooper & Ordahl 1984)
GFAP	Glial fibrillary acidic protein	CNS intermediate filament	(Eglitis & Mezey 1997)
Slc8a1	Sodium Calcium exchanger 1	CM Ca^{2+} homeostasis	(Nicoll et al. 1990)
Cacna1c	L-type Calcium channel, alpha 1C subunit (Cav1.2)	CM Ca^{2+} influx	(Schultz et al. 1993)
Cacna1g	T-type Calcium channel, alpha 1g subunit (Cav3.1)	CM Ca^{2+} influx	(Perez-Reyes et al. 1998)
Ryr2	Ryanodine receptor type 2	SR Ca^{2+} release	(Otsu et al. 1990)
Atp2a2	Saro(endo)plasmic reticulum ATPase A2	SR Ca^{2+} uptake	(Lompre et al. 1989)
Scn5a	Sodium channel Nav1.5	CM Depolarisation	(Balser 2001)
Itp3r2	Inositol 1,4,5-Trisphosphate Receptor, Type 2	IP_3 activated Ca^{2+} Channel	(Arantes et al. 2012)
Trpc3	Transient receptor potential channel, subfamily C3	Cation sarcolemmal influx	(Sabourin et al. 2011)
Stim1	Stromal interaction molecule 1	Store operated Ca^{2+} release	(Voelkers et al. 2010)

Orai1	Calcium release-activated calcium channel 1	Store operated Ca^{2+} release	(Voelkers et al. 2010)
Tpcn1	Two-pore channel 1	Lysosomal Ca^{2+} release	(Ishibashi et al. 2000)
Tpcn2	Two-pore channel 2	Lysosomal Ca^{2+} release	(Ishibashi et al. 2000)
Slc9a1	Sodium Hydrogen exchanger	pH regulation	(Mahnensmith & Aronson 1985)
Atp1a2	Sodium Potassium ATPase A2	Ion regulation	(James et al. 1999)
Camk2d	Calmodulin kinase type 2 δ	Ca^{2+} activated kinase	(Edman & Schulman 1994)
Pp3ca	Calcineurin, catalytic subunit α	Ca^{2+} activated phosphatase	(Muramatsu et al. 1992)
Nfat	Nuclear factor of activated T-cells	Hypertrophic TF	(Molkentin et al. 1998)
Calm1	Calmodulin Type 1	Ca^{2+} binding messenger	(Stevens 1983)
Calm2	Calmodulin Type 2	Ca^{2+} binding messenger	(Stevens 1983)
Calm3	Calmodulin Type 3	Ca^{2+} binding messenger	(Stevens 1983)
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase	Glycolysis (Housekeeping)	(Murphy & Polak 2002)
Hprt	Hypoxanthine-guanine phosphoribosyltransferase	Purine pathway (Housekeeping)	(Murphy & Polak 2002)

Table of genes

Abbreviation; TF: Transcription factor; CM: Cardiomyocyte; SR: Sarcoplasmic reticulum; CNS; Central nervous system; IP3: Inositol trisphosphate.

1. INTRODUCTION

1.1 Development of the heart

1.1.1 Introduction

The heart is required to continuously circulate blood throughout the body, delivering oxygen and nutrients whilst removing metabolic waste (Figure 1.1a). It is composed of three different layers of tissue including; the endocardium (the internal endothelial lining that is contiguous with the systemic vasculature), the myocardium (the muscular tissue which makes up the majority of the heart) and the epicardium (the epithelial layer surrounding the myocardium) as well as interstitial cells - consisting of cardiac fibroblasts, endothelial cells and vascular smooth muscle cells (Figure 1.1b). The human myocardium is comprised of billions of specialised muscle cells termed cardiomyocytes which have a defined phenotype and form branched interlocking networks that allow the heart to beat in a coordinated and uniform manner. The ability of cardiomyocytes to contract is due to the electrochemical properties of these cells and their ability to regulate the influx and efflux of different ions via sarcolemmal channels and exchangers. As well as being fundamental for contraction, ionic fluxes are also involved in the regulation of gene transcription and have been linked to the pathological progression of diseases such as cardiac hypertrophy and arrhythmias. Whilst cardiomyocytes represent a highly differentiated and specialised population of cells, the transition from pluripotent stem cell to immature cardiomyocyte happens relatively quickly, with the embryonic heart beginning to beat by around a third of the way through gestation. Although formation of immature cardiomyocytes and the initiation of contraction begins during the early stages of embryogenesis, complete maturation of cardiomyocytes does not occur until adulthood with the loss of proliferative capacity and cell plasticity. Over this period there are dynamic changes in gene expression, cell signalling and electrophysiology which ultimately results in the formation of terminally differentiated cardiomyocytes.

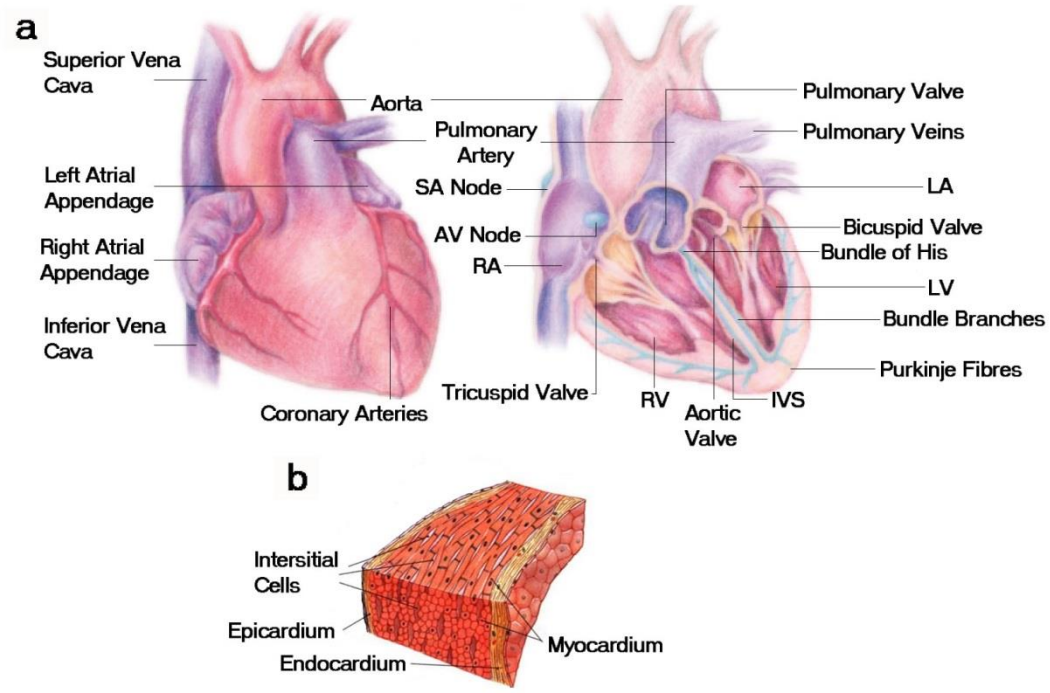

Figure 1.1: Anatomy of the adult heart

Schematic representation of the heart highlighting major anatomical features including the conduction system pathway (a). SA, Sinoatrial node; AV, Atrioventricular node; RA, Right Atria; LA, Left Atria; RV, Right Ventricle; LV, Left Ventricle; IVS, Inter-ventricular septum. Blood enters the heart from the body via the vena cava before being sent to the lungs to be re-oxygenated, it then returns to the heart and is then pumped back around the body via the aorta. Innervation of the heart begins at the SA node, before passing through the atria to the AV node and moving down the intraventricular septum via the left and right branches of the AV bundle block (Bundle of His), activating the Purkinje fibres and triggering co-ordinated contraction of the ventricles. The wall of the heart is composed of the myocardium, endocardium, epicardium as well as interstitial cells, such as fibroblasts, vascular smooth muscle cells and connective tissue (b) (Adapted from (Harvey 2002)(Martini 2006)).

1.1.2 Murine embryogenesis

Embryogenesis is the process by which an embryo forms and develops from a single cell, with the subsequent specification and differentiation of pluripotent progenitor cells to contribute to specialised cell lineages throughout gestation. Embryogenesis is initiated by the fertilisation of an oocyte by sperm, resulting in the formation of a zygote, which undergoes three rounds of cell division to form 8 blastomeres which have no overt morphological differences (Figure 1.2a) (Arnold & Robertson 2009). By embryonic day(E) 3.0 (Morula stage) in the mouse, asymmetrical divisions have begun to occur forming two distinct cellular subpopulations, which segregate to form the inner cell mass and the outer trophectoderm before the embryo cavitates to form the early blastocyst (Sutherland et al. 1990). The inner cell mass is characterised by the expression of the transcription factor Oct-4 which maintains pluripotency in this subpopulation of cells (Niwa et al. 2005). During maturation of the blastocyst, the inner cell mass segregates into the outer most layer of cells, forming the primitive endoderm and giving rise to the yolk sac, the remaining inner cells form the pluripotent epiblast at E4.5 (Chazaud et al. 2006). The epiblast is the founding stage which gives rise to all germ cell lineages and subsequent somatic tissues of the embryo proper. After the formation of the epiblast the embryo undergoes a number of morphological changes to establish both the proximal-distal and anterior-posterior axes via early molecular asymmetries (Beddington & Robertson 1999). Shortly after implantation of the blastocyst into the uterine wall, a cavity forms in the centre of the epiblast and the conceptus elongates along the proximal-distal axis to form the 'egg cylinder' stage embryo. Although molecular evidence for anterior-posterior axis formation becomes evident at around E6.0, the mouse embryo remains morphologically radially symmetrical until the onset of gastrulation at E6.5. At this stage, epiblast cells begin to converge towards the posterior proximal pole of the embryo to form the primitive streak which, over the next 36 hours, initiates gastrulation as the streak elongates and extends towards the distal tip of the embryo (Figure 1.2b) (Lawson et al. 1991). Gastrulation is the morphogenetic process in which cells from the epiblast undergo epithelial-to-mesenchymal transition and ingress at the primitive streak to give rise to the three primary germ layers; endoderm (gastrointestinal and respiratory tracts, pancreas and liver), mesoderm (muscle, blood and vasculature) and ectoderm (nervous tissue and skin), producing the progenitor cells required to form all adult tissue and organs (Figure 1.2c) (Lawson 1999).

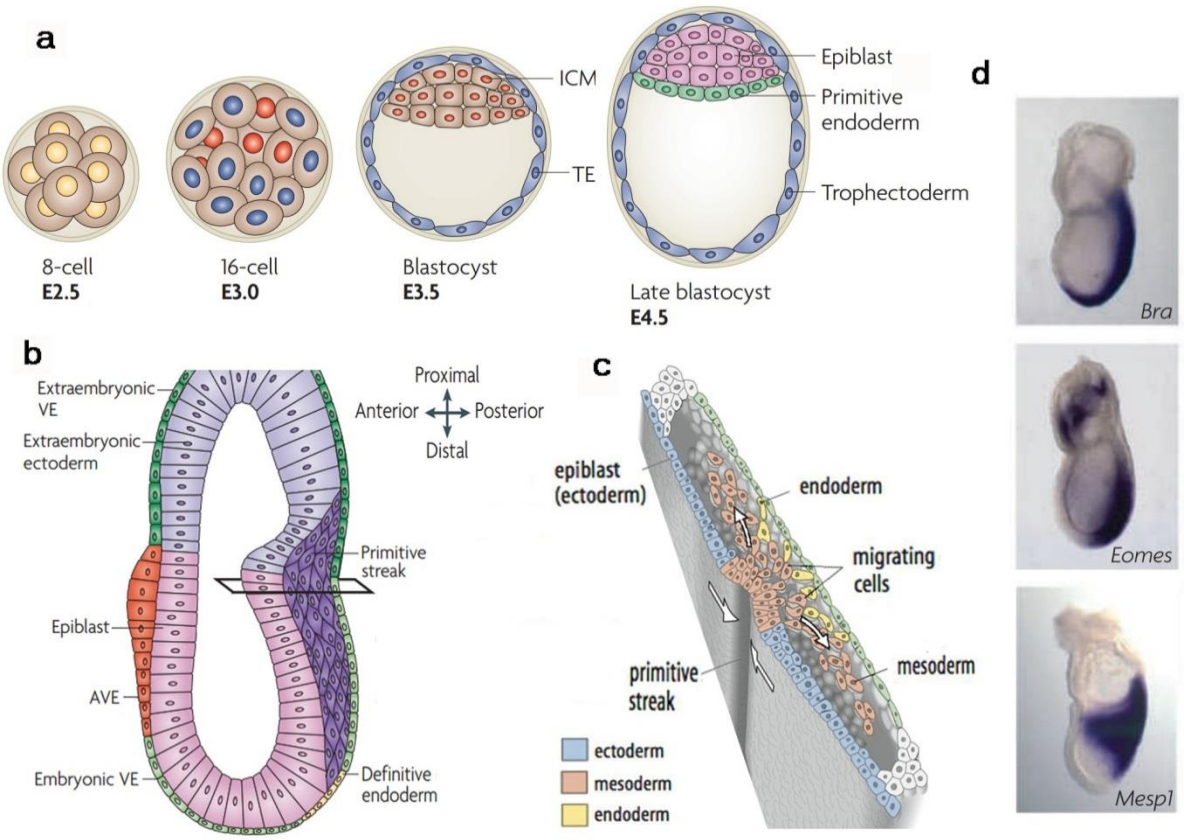

Figure 1.2: Schematic representation of early embryogenesis and lineage segregation in the blastocyst and gastrulation

The primary tissue types of the mouse embryo – trophectoderm (TE), inner cell mass (ICM), epiblast and primitive endoderm- are established before implantation at around embryonic day 4.5 (E4.5). At E2.5, the mouse zygote is formed of eight identical blastomeres, by E3.0 the first binary cell fate decision has occurred to form the trophectoderm or inner cell mass (ICM). The next round of cell divisions generates larger outer TE cells and smaller inner ICM cells, before the primitive endoderm and the epiblast lineages segregate from the ICM at the blastocyst stage (E3.5-E4.5) (a). By E6.25, epithelial cells of the epiblast sheet converge towards the primitive streak, initiating gastrulation and the formation of the embryo proper (b). VE, visceral endoderm: AVE, anterior visceral endoderm. During gastrulation, formation of the nascent mesoderm and endoderm is a result of an epithelial-mesenchymal transition (EMT) and tissue migration through the primitive streak (c). Ectoderm cells will not go through the primitive streak. RNA *in situ* hybridization highlights the localized expression of mesoderm transcriptional regulators at gastrulation (d). Brachyury (Bra) is broadly expressed throughout the entire streak, the node and the notochord. The related T-box gene eomesodermin (Eomes) is restricted to the anterior streak region, whilst the cardiogenic mesoderm marker Mesp1 is expressed in the intermediate primitive streak. (Adapted from (Arnold & Robertson 2009) and (Wolpert et al. 2006))

1.1.3 Heart development

During embryogenesis the heart is the first functional organ to form in the embryo proper, critical in supplying the embryo with sufficient oxygen and nutrients. The commitment and specification of the cardiac cell lineage occurs early in development and due to the fundamental role of the heart early perturbations in this process frequently result in embryonic lethality (Copp 1995). Indeed, congenital heart disease accounts for 10% of all spontaneous abortions and heart defects are present in 1% of all live births (Hoffman & Kaplan 2002).

Specification of cardiac progenitors occurs in the pre-gastrulation mouse embryo at around E6.5 (Auda-Boucher et al. 2000), however, the first morphologically recognisable heart structure does not form until E7.5, when mesodermal derived cardiac progenitor cells delaminated through the primitive streak and migrate in an anterior-lateral direction to below the head folds and form a crescent-shaped epithelium across the midline, the cardiac crescent (Figure 1.3) (for reviews see ((Harvey 2002)(Buckingham et al. 2005)(Bruneau 2008)(Evans et al. 2010)). The cardiac crescent, in which myocardial markers are first detected, is composed of the first heart field (FHF) which contributes primarily to the left ventricle. The crescent migrates towards the midline to form the linear heart tube which is composed of an inner endothelial tube covered by contractile mesoderm derived cardiomyocytes. Elongation and expansion of the linear heart tube occurs via proliferation as well as the addition of cells from the so-called second heart field (SHF) – derived cardiomyocytes to both poles. In conjunction with this expansion, the linear heart tube undergoes rightward looping, with its posterior region moving anteriorly and the future ventricles become distinct and ballooning outwards. During looping the SHF cells from the anterior-dorsal region of the embryo contribute to the atrial regions and systemic venous tributaries which are forced dorsally and cranially so that they are now above the developing ventricles. Following cardiac looping, a large number of complex morphogenetic processes transform the tube into the characteristic four chambered heart. Precursors of the atrioventricular (AV) values (tricuspid and mitral values) form in the AV canal and are composed of cells from the endocardial layer of the heart which migrate and proliferate to form opposing tissue masses called endocardial cushions. Endocardial cushions also form in the outflow tract and are the precursors of the aorticopulmonary septum which divides the outflow tract into the aorta and pulmonary artery as well as giving rise to the aortic and pulmonary values. Neural crest cells originating from the hindbrain migrate into the heart via the outflow cushions and participate in outflow septation as well as initiating neuronal innervation by around E10.5. At this stage the ventricular myocardium undergoes trabeculation, whereby cardiomyocytes along the inner surface of the ventricles extrude into the extracellular matrix (ECM) lying between the myocardium and endocardium layers (the cardiac jelly) to form sheet-like projections resulting in a spongiform layer of myocytes along this inner surface. During the later stages of heart development around E14.5, septation of the heart is

completed forming distinct left and right ventricles and atria. The ECM of the cardiac jelly begins to break down, causing the trabeculae of the ventricles to collapse and thicken into a compact layer of myocardium (myocardial compaction) and development of the conduction system and cardiac pace-making is completed.

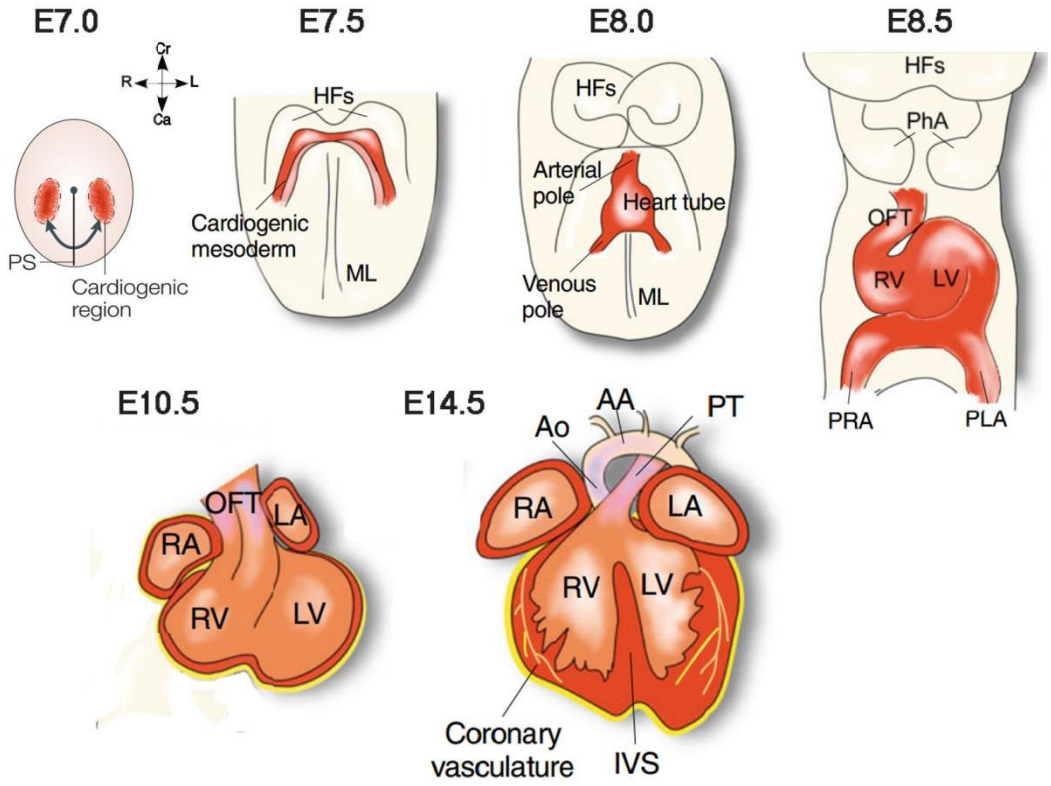

Figure 1.3: Morphogenesis of the developing mouse heart

Myocardial progenitor cells originate in the primitive streak (PS), from where they migrate to the anterior part of the embryo at around E7.0. These progenitor cells go on to form the cardiac crescent at E7.5 which is located below the nascent head folds (HF) and above the midline (ML). The early linear heart tube forms through the fusion of the cardiac crescent (E8.0) before subsequently undergoing looping at E8.5. By E10.5, the chambers of the fetal heart have begun to form with complete septation not occurring until E14.5, when the myocardium has become compacted and the heart has a similar morphology to the adult. RV, right ventricle; LV, left ventricle; RA, right atria; LA, left atria; OFT, Outflow tract; IVS, interventricular septum; AA, aortic arch; Ao, Aorta; PT, pulmonary trunk. (Adapted from (Buckingham et al. 2005)(Laugwitz et al. 2008))

1.1.4 Molecular regulation of cardiomyocyte differentiation

During formation of the heart the underlining changes in cardiac morphology and cellular identity are controlled at the level of gene expression. Stage and cell type-specific patterns of gene expression are reached by cell type-specific transcription factors which bind to specific DNA sequences adjacent to the genes that they regulate. Ultimately, a cascade of transcription factor activation results in the cardiac specification and differentiation of pluripotent progenitor cells to mature cardiomyocytes (for reviews see (Paige et al. 2015)(Bruneau 2002)).

Initially myocardial precursor cells express the mesoderm T-box transcription factor T (Brachyury), which specifies the newly formed mesoderm as it migrates through the primitive streak (Wilkinson et al. 1990). During this stage another T-box transcription factor Eomesodermin (Eomes) activates the basic helix-loop-helix transcription factor mesoderm posterior 1 (Mesp1) (Costello et al. 2011)(van den Ameele et al. 2012)(Figure 1.2d). Mesp1, along with its family member Mesp2, are the earliest markers of cardiovascular specification in the developing embryo and represent the first molecular step towards cardiogenesis (Saga et al. 1999) (Bondue et al. 2011) (Figure 1.4). Mesp1 is first expressed in the FHF which makes up the cardiac crescent, a later wave of Mesp1-positive cells produces cardiac progenitors of the SHF. Although both the FHF and SHF cells express Mesp1, clonal analysis in mice has suggested that these progenitor cells are committed prior to the onset of Mesp1 expression (Devine et al. 2014). The mechanism by which Mesp1 drives cardiac specification is multifaceted in that it promotes the expression of cardiac specific transcription factors whilst also repressing genes that are involved in pluripotency (Bondue et al. 2008). As cardiac differentiation progresses, these early genes become down regulated as the expression of genes encoding cardiac transcription factors such as Nkx2.5, Gata-4 and Mef2c increases. Within the cardiac crescent and during migration to the linear heart tube, the FHF can be identified by the expression of transcription factors such as Tbx5, Nkx2.5 and HCN4 (Paige et al. 2015). As cardiac looping occurs the atrial and ventricular cells selectively proliferate and differentiate leading to cardiac chamber morphogenesis, this process is controlled by a large number of different transcription factors including T-box transcription factors Tbx2, Tbx3, Tbx5 and Tbx20 as well as Nkx2.5, Gata-4, Irx4 and Hand1 which interact with each other to trigger various processes in different parts of the heart. For example, Tbx5 and Tbx20 act in combination with Nkx2.5 and Gata4 to promote specification of the chamber myocardium, whilst in contrast Tbx2 and Tbx3 interact with Nkx2.5 and Gata4 to repress chamber specification in regions such as the outflow tract (Christoffels et al. 2010)(Christoffels et al. 2004). Within the myocardium, the 4 chambers are represented by unique gene expression patterns which define their subsequent phenotype and function in the adult heart. The mouse atria and ventricles express myosin light chain isoforms Mlc2a and Mlc2v, respectively (Small & Krieg 2004), whilst the homeobox gene Irx4 is only expressed in the ventricles, activating ventricular

isoforms of myosin heavy chain and suppressing atrial isoforms (Bao, Science 1999). In the left atria there is a high level of Pitx2 expression which supresses Sino-atrial (SA) node development thus preventing ectopic pacemaker formation (Wang et al. 2010).

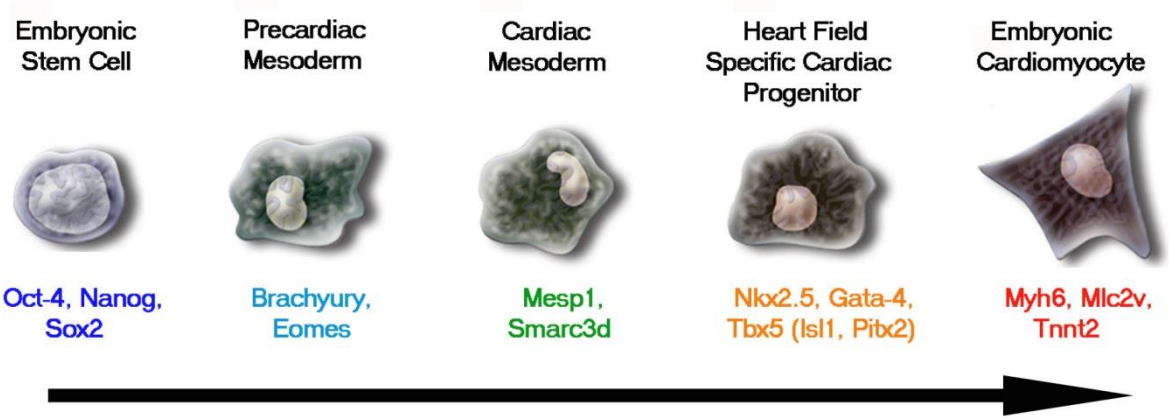

Figure 1.4: Schematic overview of the specific stages and markers during cardiomyocyte differentiation

Simplified model of cardiomyocyte differentiation from pluripotent embryonic stem cells to immature cardiomyocytes via sequential progenitor cell types. Stages are identified by the expression of genes known to encode key transcription factors and cardiomyocyte specific proteins. Genes in brackets represent specific second heart field transcription factors. (Adapted from (Mummery et al. 2012))

1.1.5 Development of cardiac function

Studies of mammalian heart development have typically focused on the molecular basis for cardiac lineage determination and the spatial-temporal allocation of cardiac progenitors whilst insight into the development of cardiac function is limited. It is commonly stated that initiation of contraction begins with the formation of the linear heart tube (Paige et al. 2015), however, initiation of cardiac contraction has been reported prior to this stage in historical studies. Initiation of cardiac function was first studied in the early 20th century using chick (Sabin 1920)(Patten & Kramer 1933) and rat (Goss 1938). Initial studies in rat reported that contractile activity began at E9.5 (equivalent to E8.5 in mice), with contractions having a regular rhythm and a rate of 37 to 42 beats per minute (bpm). Further characterisation described pacemaker-like activity in regions either side of the embryonic midline suggesting contraction was occurring prior to formation of the linear heart tube (Goss 1952). More recent studies in mouse have reported twitching of heart rudiments in embryos around the 3-4 somite stage (E8.0) (Nishii & Shibata 2006). In these studies, the precise determination of when the first cardiac contractions are initiated is difficult due to the variability in staging as assessed using somite number, embryonic day and heart morphology.

By E10.5 the embryonic heart beats at approximately 127 bpm increasing throughout embryonic development to 380bpm at postnatal day 0 before rising to over 600bpm in the adult, highlighting key changes in cardiac physiology during heart development (Keller et al. 1996).

1.1.6 Differentiation of embryonic stem cells into cardiomyocytes

Investigating the cellular biology of early processes during mammalian embryogenesis such as cardiac lineage specification is technically challenging due to the size and accessibility of mammalian embryos. Embryonic stem cells have emerged as a useful *in vitro* tool for studying embryogenesis and differentiation at the cellular level. Mouse embryonic stem cells (mESCs) were first isolated from the inner cell mass of pre-implantation blastocysts in the early 1980's (Evans & Kaufman 1981)(Martin 1981) and could maintain pluripotency *in vitro* when grown on a layer of feeder cells (mouse embryonic fibroblasts, MEFs) and by using leukaemia inhibitory factor (LIF). Cultured mESCs that are injected into blastocysts have the ability to form chimeras (Bradley et al. 1984)(Beddington & Robertson 1989), as previously observed with cells from the inner cell mass (Gardner 1968), giving rise to derivatives of all three germ layers and consequently viable mice. mESCs, therefore, represent a pluripotent population of undifferentiated cells capable of proliferation, self-renewal and the generation of large numbers of differentiated cell progeny. These properties have seen mESCs emerge as a valuable research tool enabling the generation of gene-targeted mice as well as providing an *in vitro* model to study normal and pathological differentiation and development.

Early studies using mESCs have shown that these cells can differentiate into functional cardiomyocytes *in vitro* and recapitulate the pre-gastrulation and post-gastrulation cardiogenic events which occur *in vivo* (Mercola et al. 2011)(Mummery et al. 2012)(Burridge et al. 2012). mESC cardiomyocyte differentiation is initiated by the formation of aggregates, termed embryoid bodies (EBs), which triggers the spontaneous differentiation of cells into all 3 primary germ layers. As the field of mESC cardiomyocyte differentiation has expanded a large number of studies have investigated the parameters which influence cardiomyocyte formation in order to promote differentiation. The differentiation protocols developed for ESC cardiomyocyte formation usually recapitulate the signalling cues that direct cellular specification within the embryo. This is achieved by the addition of the same growth factors such as bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs), or by stimulating the same pathway or receptor using related growth factors such as ActivinA and Nodal (Reissmann et al. 2001)(Kattman et al. 2011). Furthermore there is an increasing number of small molecule modulators for various cardiomyocyte signalling pathways (Willems et al. 2011). These different factors allow the activation and inhibition of developmental pathways and can be used to increase speed and efficiency of ESC-cardiomyocyte differentiation (Chambers et al. 2012).

1.2 Cardiac physiology

1.2.1 Cardiomyocyte electrical excitability

Cardiomyocytes like neurons are excitable cells which, due to their electrochemical properties, can be stimulated to create an electrical impulse, leading to the onset of contraction. Contraction in the adult heart is initiated by the spontaneous electrical activity of the SA node, which generates an action potential that can propagate rapidly throughout the heart, thereby activating cardiomyocytes and generating a coordinated heartbeat (Brown 1982). The sinoatrial node is composed of a specific subtype of cardiomyocytes which are unique in having the ability to automatically generate repetitive action potentials due to the intrinsic expression of ion channels (Mangoni & Nargeot 2008). Action potentials are generated by the movement of ions across the sarcolemmal membrane via ion channels, these ionic fluxes result in membrane potential changes (Figure 1.5). The membrane potential is the difference in electrical charge between the inside and outside of a cell and is the result of differences in extra-cellular and intra-cellular ion concentration as well as sarcolemmal membrane permeability. In non-stimulated cells this potential is termed the resting membrane potential and is negative due to the interior of the cell containing less positive ions relative to the exterior. The resting membrane potential arises due to the sodium/potassium ATPase, which uses ATP to pump 2x K^+ into the cell whilst removing 3x Na^+, and "leaky" potassium channels which allows slow facilitated diffusion of K^+ out of the cell (Glitsch 2001). In mature cardiomyocytes, the resting membrane potential is around -85mV, as calculated by the Goldman-Hodgkin-Katz equation, due to the concentration of intracellular and extracellular ions in the heart as well as the ion transporting enzymes present within the cell membrane (Goldman 1943)(Hodgkin & Katz 1949). A cardiac action potential is generated when the sinoatrial node derived electrical impulse reaches a cardiomyocyte, triggering Na^+ channels to open (increased Na^+ membrane conductance) and thus a rapid influx of Na^+ down its concentration gradient and into the cell leading to membrane depolarisation as the interior of the cell becomes more positive (Grant 2009). The positive membrane potentials reached during depolarisation trigger the activation of both Ca^{2+} and K^+ channels leading to a plateau in the membrane potential at around 10mV, due to the balanced influx of Ca^{2+} and efflux of K^+. Towards the end of this plateau phase, Ca^{2+} channels become inactivated whilst K^+ channels remain open leading to a repolarisation of the cell membrane as K^+ continues to diffuse out of the cell until the resting membrane potential is reached and ionic fluxes become balanced.

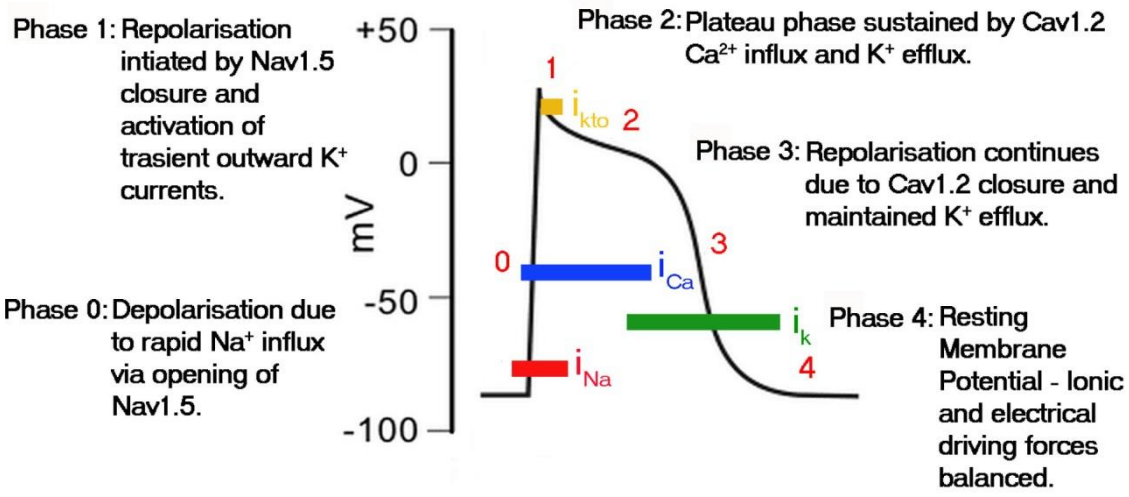

Figure 1.5: Action potential timecourse in a ventricular cardiomyocyte

Schematic diagram to show the typical action potential of a ventricular cardiomyocyte highlighting the different phases of depolarisation and repolarisation as well as the main ionic currents (i) responsible for them.

1.2.2 Cardiac Contraction

In order to generate contraction cardiomyocytes have a highly defined phenotype which allows them to respond to electrical excitation and generate the force needed to pump blood around the body in a coordinated and rhythmic manner. The main contractile components of cardiomyocytes are long filaments which run throughout the cell and are termed myofibrils (Figure 1.6a). Myofibrils are composed of thick (myosin) and thin (actin) filaments which repeat along the length of the cell forming sarcomeres and give cardiomyocytes their characteristic striated appearance (Clark et al. 2002). Upon cardiomyocyte activation, the concentration of cytoplasmic Ca^{2+} increases and binds to troponin. Troponin is a protein complex composed of three subunits, TnC, TnI and TnT, which integrates cytoplasmic Ca^{2+} concentration with the regulation of force generation (Westfall & Metzger 2001). The binding of Ca^{2+} to troponin results in conformational change which prevents tropomyosin from inhibiting contraction by exposing the active sites of the actin filaments (Figure 1.6b)(McKay et al. 1997)(Herzberg & James 1988). Myosin is then able to bind to the exposed actin by forming cross bridges and by alternative attachment and detachment it pulls the actin filaments towards the sarcomere, generating contraction (Vale & Milligan 2000). This cycling process relies on energy produced by ATP hydrolysis and, therefore, cardiomyocytes have large quantities of mitochondria in order to supply the contractile apparatus with sufficient levels of ATP in accordance with a high energy demand. Muscle fibre relaxation occurs when the cytosolic Ca^{2+} concentration decreases and the troponin complex once again blocks contraction by blocking the active sites of actin.

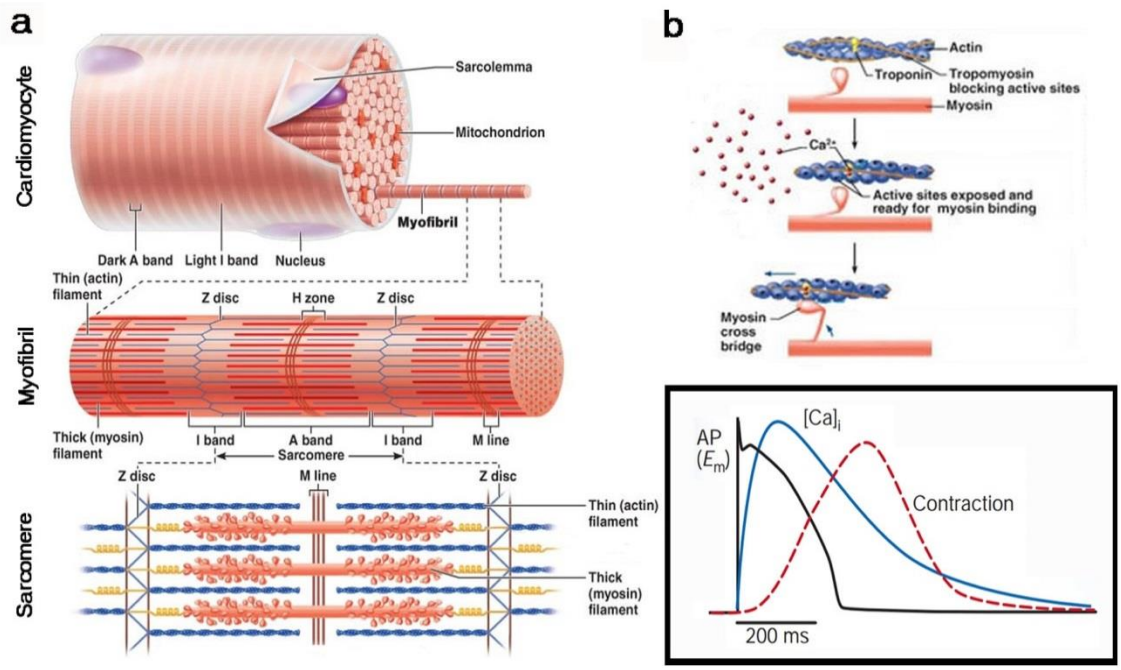

Figure 1.6: Contractile apparatus of cardiac muscle tissue

Myofibrils occupy most of the cardiomyocyte cell volume and are what generate contraction. Myofibrils are composed of sarcomeres and appear striated due to the arrangement of thick (myosin) and thin (actin) filaments, which form the repeating A bands (overlapping thick and thin filaments) and I bands (only thin filaments). Each sarcomere runs between Z discs which are primarily composed of alpha actinin. The H zone represents the region of thick myosin filaments which are not superimposed by thin actin filaments, in the middle of the H-zone is the M-line which is composed of cross connecting elements of the cytoskeleton (a). Contraction occurs via the binding of Ca2+ to troponin which removes the blocking action of tropomyosin, myosin can then bind to actin via cross bridges and trigger contraction (b). Insert shows the timecourse of action potential, Ca2+ influx and contraction measured in rabbit ventricular cardiomyocytes at 37^{o}C. (Figure adapted from (Bers 2002) and (Martini 2006))

1.2.3 Excitation contraction coupling

In the adult heart, coordinated electrical excitation is coupled to physical contraction in a process termed excitation contraction coupling (ECC), which creates the force needed to pump blood around the body (Bers 2002). ECC relies on the second messenger Ca^{2+}, which binds to cardiac myofilaments resulting in contraction. Upon electrical excitation an action potential causes voltage sensitive Ca^{2+} channels to open creating an inward Ca^{2+} current (Figure 1.7). This inward Ca^{2+} current triggers the release of Ca^{2+} from the sarcoplasmic reticulum (SR) through ryanodine receptors (RyR) via a mechanism known as Ca^{2+} induced Ca^{2+} release (CICR) which was first proposed in skinned muscle fibres (Fabiato & Fabiato 1979). In adult cardiomyocytes, the SR is the main intracellular Ca^{2+} store and contributes to between 20-90% of the Ca^{2+} required for contraction depending on species and myocyte type (Shiels & Galli 2014). The release of Ca^{2+} from the SR via RyRs along with the influx of Ca^{2+} through L-

type Ca^{2+} channels increases the cytosolic Ca^{2+} concentration (systolic Ca^{2+} transient) leading to the activation of myofilaments and the generation of cardiac contraction (figure 1.6) (Bers 2002). For relaxation to occur the increase in cytoplasmic Ca^{2+} from the systolic transient needs to be removed. The majority of Ca^{2+} is removed by the sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) which pumps Ca^{2+} back into the SR. A smaller proportion of Ca^{2+} is pumped out of the cell by the Na^{+}/ Ca^{2+} exchanger (NCX1), however, this ratio is again dependent on species and cardiomyocyte subtype (Shiels & Galli 2014).

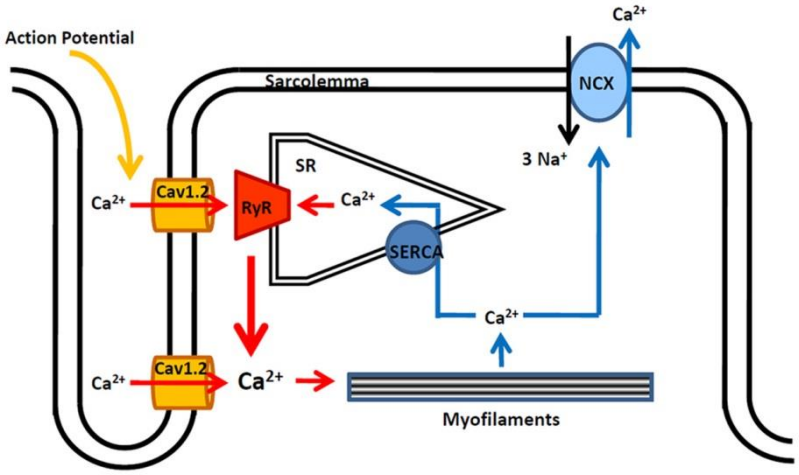

Figure 1.7: Schematic representation of excitation-contraction coupling.

Depolarisation of a cardiomyocyte triggers the opening of Cav1.2 channels and the influx of Ca^{2+}, this leads to the release of Ca^{2+} from the SR, raising the concentration of cytoplasmic Ca^{2+} and resulting in myofilament activation and contraction. Relaxation occurs by removing Ca^{2+} from the cytoplasm via reuptake into the SR via SERCA or by sarcolemmal efflux by NCX1. RyR, ryanodine receptor; PLB, phospholamban; SERCA, Sarco(endo)plasmic reticulum Ca^{2+} ATPase; SR, sarcoplasmic reticulum; NCX1, Na^{+}/ Ca^{2+} exchanger; Cav1.2, L-Type Ca^{2+} channel.

1.2.4 Early embryonic cardiac physiology

Although ECC has been well described in adult cardiomyocytes, the mechanism by which cardiac contraction occurs during early heart development is still poorly understood. Targeted disruption of genes encoding ECC proteins in mice has shown that the early contractile activity of immature cardiomyocytes does not require ECC (Table 1.1). In these transgenic models the immature heart remained contractile until around E10.5 suggesting a different unknown mechanism was occurring. During early cardiogenesis, studies have shown that immature cardiomyocytes have both an excitable membrane (Doevendans et al. 2000), functional sarcoplasmic reticulum (Moorman et al. 2000)(Seki et al. 2003) and express a variety of voltage-activated ion channels and exchangers (Seisenberger et al. 2000)(Linask et al. 2001)(Cribbs et al. 2001), however, the expression and activity of these proteins is distinct from that in adult cardiomyocytes (Liang et al. 2010). In order to explain the mechanism and source

of contractile Ca^{2+} in the early heart, two contrasting mechanisms have been proposed on the basis of experiments with isolated cells and genetically manipulated animals and cells. One line of evidence suggests that myocyte contraction is triggered by sarcolemmal Ca^{2+} influx through voltage activated Ca^{2+} channels with little or no contribution from the SR (Nakanishi et al. 1988)(Takeshima et al. 1998). whilst the opposing view is that early spontaneous Ca^{2+} oscillations originate from the SR, triggering electrical activity as well as contraction (Viatchenko-Karpinski et al. 1999)(Méry et al. 2005)(Sasse et al. 2007). Although in immature cardiomyocytes the exact mechanism for contractile activity and the development of a functional SR is still poorly understood, studies have clearly shown there is a significant difference compared to the adult, with an increased requirement for sarcolemmal Ca^{2+} influx and dynamic changes occurring in channel and exchanger expression and function during development of the heart (Sissman 1970)(Gomez et al. 1994)(Husse & Wussling 1996). This is clearly highlighted by the difference in relative contribution of Ca^{2+} release from the SR vs. sarcolemmal transport between neonates and adult cardiomyocytes (Bers & Bridge 1989)(Haddock et al. 1999).

Protein (gene)	**Tissue of gene modification**	**Cardiac phenotype**	**Reference**
NCX1 (*Slc8a1*)	Germline deletion	Lethality E9 to E10; thin myocardium with reduced cardiomyocytes; no heart beat observed.	(Wakimoto et al. 2000)
		Lethality E9.0 to E9.5; Underdeveloped heart with dilated pericardium; contractile although significantly slower than WT littermates. Mutant and WT hearts indistinguishable at E8.5.	(Cho et al. 2000)
		Lethality E11.5 to E13.5; Lack of a spontaneous beating heart and organised myofibrils.	(Koushik et al. 2001)
Cav1.2 (*Cacna1c*)	Germline deletion	Lethality prior to E14.5; Normal development until E12.5, contractile rate the same as WT littermates.	(Seisenberger et al. 2000)
Cav1.3 (*Cacna1d*)	Germline deletion	Viable embryos. Pronounced bradycardia and arrhythmias at rest in the adult. Altered sinoatrial pacemaker activity.	(Platzer et al. 2000)

RyR2 (*RYR2*)	Germline deletion	Lethality E10.5 to E11.5; Rhythmic contractions at E9.5, although irregularly arranged myocardium. No heart beat at E10.5, anatomically similar to E9.5.	(Takeshima et al. 1998)
SERCA2a (*Atp2a2*)	Germline deletion	No mutant embryos detected.	(Periasamy et al. 1999)
	Deletion in myocardium	Lethality at E11.5; Yolk sac circulation and heartbeat observed until E10.5.	(Andersson et al. 2009)
Nav1.5 (*Scn5a*)	Germline deletion	Lethality E10.5 to E11.5; Some uncoordinated beating at E10.5, however, none observed at E11.5. Reduced ventricular chamber size, trabeculation and cardiomyocytes. Common Atria, endocardial cushions and truncus arteriosus appear normal.	(Papadatos et al. 2002)
Cav3.1 (*Cacna1g*)	Germline deletion	Bradycardia and slowing of atrioventricular conduction.	(Mangoni et al. 2006)
Cav3.2 (*Cacna1h*)	Germline deletion	No effect on heart development or beating frequency. Abnormal coronary function.	(Chen et al. 2003)
TPC1 (*Tpcn1*)	Germline deletion	Mice were viable and bred normally.	(Lin-Moshier et al. 2012)
TPC2 (*Tpcn2*)	Germline deletion	Mice were viable and bred normally.	(Calcraft et al. 2009)

Table 1.1: the role of cardiac channels and exchangers during embryonic heart development

Summary of studies in which proteins required for cardiac physiology and Ca^{2+} handling have been deleted in mice.

1.2.5 The sodium calcium exchanger

The NCX is an electrogenic plasma membrane antiporter responsible for Ca^{2+} homeostasis in a wide range of cell types. In mammals, three different NCX genes have been identified; *Slc8a1* encoding NCX1 (Nicoll et al. 1990), *Slc8a2* encoding NCX2 (Lee et al. 1994) and *Slc8a3* encoding NCX3 (Nicoll et al. 1996). The different isoforms of NCX are expressed in a tissue-specific manner, with NCX2 and NCX3 found in the brain and skeletal muscle whereas NCX1 is the major cardiac isoform although it is ubiquitously expressed (Philipson & Nicoll 2000). In the heart, NCX1 is the predominant mechanism by which Ca^{2+} is removed from the cell following contraction, using the Na^{+} gradient across the cell membrane to remove one Ca^{2+} whilst bring in three Na^{+} which generates an inward current (forward mode activity) (Mechmann & Pott 1986). In adult hearts the inward current generated by NCX1 has been suggested to trigger ectopic action potentials and lead to delayed after depolarisation mediated arrhythmias (Despa & Bers 2013). Whilst NCX1 predominantly favours forward mode activity it can also work in reverse mode to trigger Ca^{2+} influx and Na^{+} efflux. The direction and amplitude of NCX1 depends on the membrane potential and on the internal and external Na^{+} and Ca^{2+}, with depolarised membrane potentials favouring reverse mode activity, resulting in Ca^{2+} influx and Na^{2+} efflux. In the early embryonic heart NCX1 has been suggested to have a predominant role in contraction due to its ability to generate spontaneous action potentials as well as trigger Ca^{2+} influx (Linask et al. 2001). In the developing mouse embryo at E7.0, NCX1 expression is located primarily in the heart-forming mesoderm before being highly expressed in the cardiac crescent and linear heart tube (Koushik et al. 1999). Targeted disruption of the NCX1 gene leads to embryonic lethality between E9.5 and E10.5 (Wakimoto et al. 2000)(Koushik et al. 2001). Mutant mice were significantly smaller by E9.5, although the development of limb buds, cranial neural tube, and somites were unaffected. In terms of cardiac development, the NCX1 KO mice exhibited non-contractile heart tubes with thin ventricular walls that contained fewer cardiac myocytes. In summary, this suggests that NCX1 has a crucial role in cardiac development, not only in contractile function but also in the formation of the heart.

1.2.6 Voltage dependent Ca^{2+} channels

In adult cardiomyocytes, sarcolemmal Ca^{2+} influx is dependent on two types of voltage gated Ca^{2+} channels; L-type (low threshold-type) and T-type (transient-type) channels, which couple membrane depolarisation with Ca^{2+} influx and contraction. L-type Ca^{2+} channels have a relatively large conductance, are activated by depolarised potentials (~-30mV), and contribute most of the Ca^{2+} required for CICR during ECC. On the other hand, T-type Ca^{2+} channels are activated at lower membrane potentials (~-60mV) and become rapidly inactivated (Bers & Perez-Reyes 1999). Whilst L-Type channels are expressed in all contractile cardiac cell types, T-type channels are found principally in pace-maker, atrial and Purkinje cells due to their role in action potential generation (Hagiwara et al. 1988). Voltage-dependent Ca^{2+} channels are formed as complex of

subunits including the main, pore-forming, α-subunit along with other regulatory α2δ, β and γ subunits which determine their physiological characteristics.

L-type Ca^{2+} channels are identified by their pore-forming α-subunit and encoded by 4 different genes; Cav1.1 (α1S), Cav1.2 (α1C), Cav1.3 (α1D) and Cav1.4 (α1F). Whilst Cav1.2 is the predominant ECC L-type Ca^{2+} channel within cardiomyocytes Cav1.3 is also expressed within the heart and contributes to diastolic depolarisation of the SA node, highlighted by SA node dysfunction in mice with genetic ablation of Cav3.1 (Mangoni et al. 2003). In isolated embryonic cardiomyocytes, L-type Ca^{2+} channel activity has been reported to have a fundamental role in the generation of spontaneous action potentials at E8.5 with transcripts for both Cav1.2 and Cav1.3 detectable (Liang et al. 2010)(Acosta et al. 2004). Targeted gene disruption of Cav1.2 in transgenic mice caused embryonic lethality prior to E14.5, although hearts at E12.5 were grossly normal with a normal-rate of beating (Seisenberger et al. 2000). In these Cav1.2 knockout mice it was observed that the expression of Cav1.3 was up regulated at both the RNA and protein level (Xu et al. 2003). These results suggest that up until E12.5, Cav1.2 is dispensable for normal heart development and that contractions prior to E13 are not due to Cav1.2 but occur via compensation or a different unidentified mechanism. In contrast, mice with targeted Cav1.3 gene disruption were viable with no differences in growth or reproduction (Platzer et al. 2000).

T-type Ca^{2+} channels are encoded by 3 different genes; Cav3.1 (α1G), Cav3.2 (α1H), Cav3.3 (α1I), although Cav3.1 (α1G) and Cav3.2 (α1H) are the main channels within heart (Perez-Reyes et al. 1998). The expression of Cav3.1 and Cav3.2 in the heart is developmentally regulated, being high in embryonic and neonatal hearts and decreasing in the adult (Niwa et al. 2004). Functional studies in embryonic cardiomyocytes have shown that T-type Ca^{2+} channel activity is required for the generation of spontaneous action potentials at E8.5 (Liang et al. 2010). Ablation of Cav3.1 in transgenic mice resulted in both bradycardia and a slowing in atrioventricular conduction, however, the ablation of Cav3.2 expression did not affect beating frequency or heart development per se (Mangoni et al. 2006). During *in vitro* differentiation of ESCs to cardiomyocytes it has been reported that the subtype of T-type channel switches from Cav3.2 to Cav3.1 (Mizuta et al. 2010), consistent with findings in the embryonic heart (Niwa et al. 2004) and suggesting that the role of T-type Ca^{2+} channels may vary between developmental stages and cardiomyocyte subsets.

1.2.7 The sarcoplasmic reticulum and ryanodine receptors

The SR is the main Ca^{2+} storage organelle in the adult heart and is responsible for the release of Ca^{2+} upon electrical excitation through RyR2 as well as the removal of cytoplasmic Ca^{2+} via SERCA. The exact timing of functional SR development from the rough endoplasmic reticulum is poorly understood, however, cytosolic Ca^{2+} oscillations originating from the SR have been observed in isolated E8.5 cardiomyocytes (Sasse et

al. 2007), providing support that SR Ca^{2+} release is involved in early contractile activity. Early SR derived Ca^{2+} oscillations are believed to trigger forward-mode NCX1 activity, thereby generating inward currents capable of initiating spontaneous action potentials and contraction. Contrary to these observations, it was reported that RyR2 knockout mice died at around E10.0 due to morphological abnormalities in the heart tube, despite normal contractions occurring in knockouts at E9.5 (Takeshima et al. 1998). Mutants also exhibited delayed development in both size and appearance when compared to control embryos. Moreover, treatment of control myocytes between E9.5 and E11.5 with ryanodine, a compound which locks RyR2 in its open state, did not exert a major effect on spontaneous Ca^{2+} transients. This observation strongly suggests that RyR2 is not required for early embryonic contractions but instead is required for cellular Ca^{2+} homeostasis. The other main ECC related SR protein, responsible for filling the SR with Ca^{2+}, is SERCA2a. Cardiac-specific deletion of SERCA2a at around E8.0, using alpha-MHC promoter driven Cre-lox leads to embryonic lethality at E11.5, potentially highlighting a later role for SR Ca^{2+} stores during heart development.

1.2.8 NAADP signalling and two-pore channels

Two pore channels 1 and 2 (TPC1/2) were identified as novel Ca^{2+} release channels located on lysosomes and activated by nicotinic acid adenine dinucleotide phosphate (NAADP) (Calcraft et al. 2009)(Brailoiu et al. 2009). NAADP is a potent Ca^{2+} mobilising second messenger (Lee 2003)(Lee 1997), known to have signalling roles in the pancreas and T-cells as well as regulating contraction in smooth muscle (for review see (Patel et al. 2011)). Its role was first documented in the fertilisation of sea urchin eggs; where extracellular stimuli were shown to raise NAADP levels leading to an increase in cytosolic Ca^{2+} (Hon Cheung Lee & Aarhus 1995). Levels of NAADP can be very transient, spiking and returning to basal levels within seconds, indicating the dynamic nature of NAADP signalling and making it difficult to assay. TPC1/2 display significant sequence similarity to voltage-gated Ca^{2+} and Na^{+} channels (Ishibashi et al. 2000). Both proteins have been shown to localize to the endo-lysosomal system (Brailoiu et al. 2009), which are known to contain high levels of Ca^{2+} due in part to their acidic pH. NAADP signalling has recently been shown to play a pivotal role in the heart, influencing ECC as well as playing a role in the formation of arrhythmias (Collins et al. 2011)(Nebel et al. 2013). This raises the question of whether lysosomal Ca^{2+} may have a role in early cardiac contraction and signalling, although this is yet to be examined.

1.3 Heart failure and Ca^{2+} signalling

1.3.1 Cardiomyocyte adaption to heart failure

Heart failure is the leading cause of mortality worldwide and is characterized by impaired cardiac function and altered Ca^{2+} homeostasis, this is a result of adverse cardiac remodelling due to pathological stress such as hypertension, myocardial infarction and valvular disease or arising due to genetic defects and structural protein

anomalies. During ventricular remodelling, a combination of myocardial hypertrophy, cell death and interstitial fibrosis occurs (Koitabashi & Kass 2011). Cardiomyocyte hypertrophy is characterized by an increase in cell size without cell division, altered ECC and the induction of fetal cardiac genes such as Mef2c, Nkx2.5, Gata 4 and NFATc1 (Dirkx et al. 2013).

During the progression of heart failure a number of physiological adaptions occur in order to try and preserve cardiac function. Initial cardiomyocyte remodelling is beneficial (as observed during physiological hypertrophy), allowing cardiac output to increase, however, as remodelling progresses "decompensation" occurs, with the ventricular wall reducing in thickness and the heart becoming unable to meet the cardiovascular demands of the body, resulting in sudden death (Levy et al. 1990). During beneficial adaptive hypertrophy Ca^{2+} transients increase in order to raise contraction and cardiac output (Roderick et al. 2007). The increased Ca^{2+} transient amplitude is a result of increased SERCA and RyR2 activity as well as a decrease in Ca^{2+} removal via NCX1. Whilst these initial changes maintain cardiac output they have been suggested to contribute to the progression of heart failure via altered Ca^{2+} signalling mediating the hypertrophic response (Molkentin 2006). As the heart progresses to failure, a significant decrease occurs in Ca^{2+} transient amplitude resulting in decreased contractile activity (Hasenfuss 1998). Underlying this change is a decrease in SERCA activity due to reduced protein expression and increases in the SERCA inhibitor, phospholamban. The decreased ability to pump Ca^{2+} back into the SR leads to an increase in the diastolic concentration of cytoplasmic Ca^{2+} resulting in prolonged cardiomyocyte relaxation as well as an increase in the occurrence of arrhythmias (Gomez 1997). NCX1 expression is also increased during heart failure and is suggested to compensate for the decreased SR Ca^{2+} uptake and increase diastolic Ca^{2+} concentration (Sipido et al. 2000)(Hobai & O'Rourke 2000). During heart failure increased sarcolemmal Ca^{2+} flux, spontaneous RyR2 opening and the inward current generated by increased NCX1 mediated Ca^{2+} efflux cause both early and delayed after depolarisations and result in arrhythmias and sudden cardiac death (Pogwizd et al. 2001).

1.3.2 Pathological Ca^{2+} signalling during heart failure

Whilst numerous intermediate signalling molecules, including mitogen-activated protein kinase (MAPK) and protein kinase B (PKB/AKT), have been implicated in the pathological remodelling associated with heart failure, a widely supported hypothesis is that changes in Ca^{2+} cycling mediate this hypertrophic response via Ca^{2+} dependent signalling pathways, thereby linking cardiac physiology and gene transcription (Molkentin 2006). Two of the most commonly discussed Ca^{2+} dependent second messengers include the Calcium/Calmodulin-dependent kinase II (CaMKII) and calcineurin.

CaMKII is a serine/threonine kinase that binds Ca^{2+} via its EF hands leading to the activation and phosphorylation of downstream targets, including transcription factors such as Mef2c. CaMKII consists of 4 different isoforms which have distinct expression patterns (Tombes et al. 2003). CaMKIIδ is the most abundant CaMKII isoform in the heart (Edman & Schulman 1994). Overexpression of this isoform in mice results in the early onset of hypertrophy whilst ablation leads to protection from hypertrophy and fibrosis (Hoch et al. 1999)(Zhang et al. 2002)(Backs et al. 2009). Activation of CaMKII during cardiac stress results in a broad range of biological effects, including the phosphorylation of ion channels (Cav1.2, RyR2, Nav1.5) and Ca^{2+} handling proteins leading to changes in cardiac physiology and Ca^{2+} homeostasis but it also phosphorylates proteins responsible for the regulation of gene transcription (Anderson et al. 2011). In terms of gene transcription CaMKII is known to be involved in the regulation of many transcription factors including the myocyte enhancer factor 2 (Mef2), cAMP-response element binding protein (CREB) (Sun et al. 1994), the serum response factor (SRF) (Flück et al. 2000) and the activation protein-1 (AP-1) (Antoine et al. 1996). Whilst CaMKII is known to directly regulate these factors it is also known to play an indirect role in transcription by phosphorylating proteins of the epigenetic machinery such as histone deacetylases (HDACs). In a mouse model of heart failure, CaMKII activation resulted in the phosphorylation of the repressor HDAC4 triggering its exit from the nucleus, leading to chromatin remodelling and Mef2c mediated transcription (Figure 1.8).

Calcineurin is a Ca^{2+} dependent protein phosphatase composed of a CaM-binding catalytic subunit (CnA) and a smaller regulatory subunit (CnB), whilst there are three genes known to encode the main catalytic subunit (CnAα, β, γ), the mammalian heart only expresses CnAα and CnAβ (Muramatsu et al. 1992) (Klee et al. 1998). Calcineurin has been identified as a key regulator of cardiac hypertrophy via the de-phosphorylation, and nuclear translocation of the nuclear factor of activated T-cells (NFAT) transcription factor family, resulting in gene transcription. NFAT transcription factors have been shown in genetic mouse models to mediate cardiac hypertrophy (Shimoyama et al. 1999)(Wilkins et al. 2002). Transgenic mice which overexpressed calcineurin demonstrated a striking hypertrophic response that rapidly progressed to dilated heart failure within 2-3months (Molkentin et al. 1998). In terms of calcineurin enzyme activity, a number of studies have reported that in various models of heart failure that calcineurin enzyme activity increases and have suggested that this is a result of abnormal Ca^{2+} handling at the physiological level (De Windt et al. 2000)(Saito et al. 2003)(Zou et al. 2001). In the human, increases in calcineurin activity and expression were associated with failing or hypertrophic hearts (Lim & Molkentin 1999).

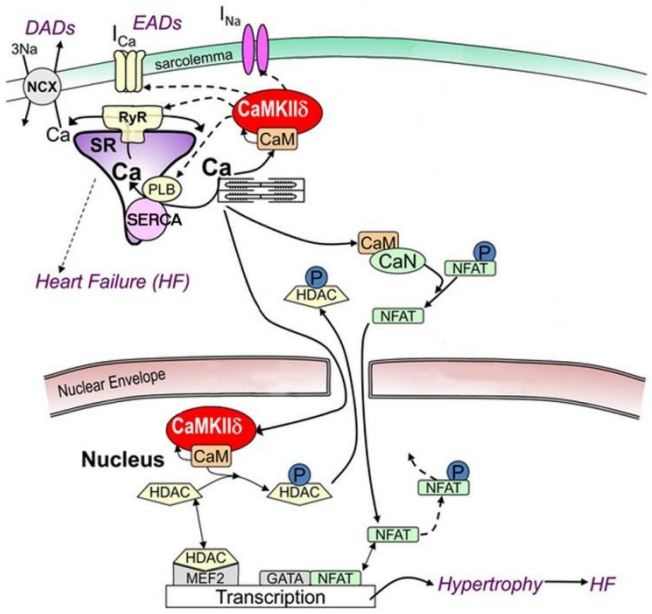

Figure 1.8: Schematic representation of CaMKinaseII and calcineurin signalling.

CaMKIIδ can acutely regulate ion channels (that carry INa and ICa) and Ca^{2+} handling proteins (RyR2 and phospholamban (PLN)), contributing to triggered arrhythmias such as early and delayed after depolarization (EADs and DADs). CaMKII can phosphorylate (P) histone deacetylases (HDAC), and calcineurin (CaN) can dephosphorylate nuclear factor of activated T cells (NFAT), altering nuclear MEF2- and GATA-dependent transcription, which contribute to cardiomyocyte hypertrophy and heart failure (HF). SR, sarcoplasmic reticulum; CaM, calmodulin. (Adapted from (Mattiazzi et al. 2015))

1.4 Thesis rationale, hypothesis and aims

Given the forming mammalian heart contracts from an early stage, this raises the important question of when and how Ca^{2+} transients in cardiac progenitors are initiated during development and to what extent this defines the progression of differentiation and subsequent cardiac morphogenesis. Thus far it has been well documented that contractions in mice are evident during formation of the linear heart tube stage (Navaratnam et al. 1986) (Linask et al. 2001)(Nishii & Shibata 2006), whereas historical studies in the rat have described earlier beating, with pacemaker-like activity observed in lateral regions either side of the embryonic mid-line (Goss 1952). However, it is difficult to conclusively determine when contraction is initiated during heart development due to variation in embryonic staging, as determined by somite number or embryonic day, which do not accurately correlate with early cardiac crescent through to linear heart tube stages of development (Kaufman & Navaratnam 1981). As well as difficulties with staging, current studies have not investigated how initiation of contractile activity within the cardiac crescent relates to the onset of Ca^{2+} transients at the single cell level. In terms of mechanistic insight, a number of studies have performed detailed electrophysiological analysis of immature cardiomyocytes,

however, these studies have relied on dissociated *ex vivo* cardiomyocyte preparations from linear heart tubes and, therefore, do not address or represent cardiac crescent physiology *in vivo*.

The ability of Ca^{2+} to act as second messenger has been well described in the adult heart especially during disease where Ca^{2+} signalling is known to regulate the pathological reactivation of embryonic genes. These studies have begun to correlate changes in cardiac function with changes in cardiac gene expression, however, deciphering the specific role of individual Ca^{2+} influx and efflux proteins is difficult due to the large global Ca^{2+} transients which occur with every contraction. By using a developmental model in which rapid Ca^{2+} transients have not yet developed, I proposed to dissect out the role of specific Ca^{2+} handling proteins in cardiac gene regulation and provide insight into the correlation between form (cardiomyocyte differentiation and morphogenesis) and function (Ca^{2+}-handling and contraction) in the heart. This has wider implications for understanding the mechanistic basis for congenital heart defects, the pathological progression of both cardiac hypertrophy and arrhythmias as well as the establishment of integrated contraction during cell-based approaches to myocardial regeneration during post-ischaemic heart disease.

The aim of this thesis project was to test the hypothesis that early cardiac function is required for cardiomyocyte differentiation and heart development via the integration of functional Ca^{2+} influx and Ca^{2+} signalling pathways. This required analysis of both *in vivo* cardiac crescent formation to determine the onset and mechanism by which early Ca^{2+} influx occurs and *in vitro* ESC-derived cardiomyocyte differentiation, to examine how Ca^{2+} influx may regulate cardiac gene expression. To this end, I sought to address the following:

(i) The temporal onset of both contractile activity and Ca^{2+} transients within the forming cardiac crescent.
(ii) To identify and evaluate the channels and exchangers that are required for Ca^{2+} influx during linear heart tube formation.
(iii) Whether the mechanisms required for Ca^{2+} influx in contraction also play a role in the regulation of cardiomyocyte differentiation.
(iv) Whether early Ca^{2+} influx integrates with known Ca^{2+} signalling pathways to regulate gene expression, during early heart development as analogous to adult cardiac hypertrophy.

2. MATERIALS AND METHODS

2.1 Mouse strains and husbandry

Mice were housed and maintained in controlled environment and all procedures were carried out according to UK Home Office project licence PPL 30/3155 and 30/2887 compliant with the UK animals (Scientific Procedures) Act 1986 and approved by the local Biological Services Ethical Review Process. To obtain wild-type embryos C57BL/6 males were crossed with CD1 females. All mice were maintained in a 12-hour light-dark cycle. Noon of the day finding a vaginal plug was designated E0.5. In order to dissect the embryos, the pregnant females were culled by cervical dislocation and death was confirmed by exsanguination in accordance with the schedule one of the Animal Scientific Procedures Act.

2.2 Embryo collection

Embryos were collected and dissected in M2 media (Sigma) at noon of the desired embryonic day, as determined by vaginal plug. Embryos were collected for cardiac crescent stages 0 – LHT between 23:00 and 03:00 depending on the stage of crescent required, although staging was determined after analysis. Embryos were then processed for the required protocol: mounted in glass bottom petri dishes for DIC imaging; loaded with a Ca^{2+} sensitive dye for Ca^{2+} transient imaging; formaldehyde fixation for immunofluorescence or histological examination; snap-freezing of hearts or whole embryos in liquid nitrogen for RNA extraction. If required, embryos were imaged before further processing using a Zeiss SteREO discovery.V8 microscope with a Zeiss HRc AxioCam attachment and Zen software (all Carl Zeiss).

2.3 Embryo Staging

Given that the stage of cardiac crescent folding and development does not correlate precisely with day of embryonic development, or somite stage, progressive crescent stages were defined based on morphological criteria (Kaufman & Navaratnam 1981). For this purpose the length (left-right axis) and the maximum height (rostral-caudal axis) of the cardiac crescent was measured for each embryo. Embryos were considered to be at the linear heart tube stage once both sides of the cardiac crescent were completely folded and fused. The ratio between width and maximum height

measurements (μm) was used to categorise the embryos from stage 0 through to stage 3 (Table 3.1).

2.4 Live embryo imaging - Differential Interference Contrast and Ca^{2+} imaging

Cardiac contraction in the developing cardiac crescent was imaged on freshly dissected embryos in a mix of 50% CMRL (Life Technologies) supplemented with 10 mM L/glutamine (Sigma-Aldrich) and 50% Knockout Serum Replacement (Life Technologies) using Differential Interference Contrast Imaging on a Spinning Disk Confocal microscope at 37^{o}C and at an atmosphere of 5% CO2+ Air. Images were acquired at 10 frames per second (fps) for no longer than 20 seconds. Ca^{2+} imaging was performed using the fluorescent Ca^{2+} dyes Cal-520 and Rhod-2 AM. Loading of stage 0 to linear heart tube embryos was performed with Cal-520 AM (8 μM) and Rhod-2 AM (4 μM) cultured in 50% CMRL + 50% Knockout Serum Replacement for 15 min at 37^{o}C and an atmosphere of 5% CO_2 + Air. The embryos were then transferred to fresh media and mounted in glass bottomed MatTek dish (MatTek Corporation) with vacuum grease and imaged on a Zeiss 710 LSM confocal microscope at 10 fps with a 20x, 0.6 NA Air Objective. For acute inhibition studies, Ca^{2+} transients were first imaged at baseline before drug-containing media was added. Imaging was performed at 5, 15 and 30 min post drug treatment. Treatments included control (DMSO only), nifedipine (10 μM; Sigma) and 3-Amino-6-chloro-5-[(4-chloro-benzyl)amino]-N-[[(2,4-dimethylbenzyl)-amino]iminomethyl]-pyrazinecarboxamide (CB-DMB) (20 μM; Sigma), all treatments contained the same concentration of DMSO.

2.5 Embryo Culture

Embryos were cultured in the presence of drugs (CB-DMB or nifedipine) for 12 h, from E7.25 prior to cardiac crescent formation using a rolling culture system (Cockroft & Copp 1990). Embryos were cultured in a mix of 50% CMRL and 50% Knockout Serum Replacement at 37^{o}C and with an atmosphere of 5% CO_2+ Air. After culture, embryos where imaged using a Moticam camera (Motic) before being snap frozen for RNA isolation or fixed in formaldehyde for staining.

2.6 Embryonic stem cell culture

Nkx2.5-GFP and *Eomes*-GFP ESCs were maintained in an undifferentiated state by culturing with KO-DMEM supplemented with 2 mM glutamax , 15% embryomax FBS (Millipore), 0.1 mM non-essential amino acids, 1% penicillin - streptomycin (10,000 U/ml penicillin; 10,000 μg/mL streptomycin stock), 0.1 mM β-mercaptoethanol (Sigma) and 1000 U/ml Leukaemia inhibitory factor (Millipore). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37^{o}C and media was replaced every day. ESCs were passaged at 70-90% confluence every 2 days using 0.25% trypsin. All cell culture reagents were from Invitrogen unless otherwise stated.

2.7 Embryonic stem cell cardiomyocyte differentiation

Cardiomyocyte differentiation from ESCs was carried out as previously described using Nkx2.5-GFP (S. M. Wu et al. 2006) and Eomes-GFP (Arnold et al. 2009) cell lines. Differentiation was induced using the hanging drop culture method (Chen et al. 2011). Undifferentiated ESCs were dissociated using 0.25% trypsin and counted using a haemocytometer after being re-suspended in differentiation media containing; 25 mM glucose/DMEM, 20% embryomax FBS, 0.1 mM nonessential amino acids, 1% penicillin – streptomycin (10,000 U/ml penicillin; 10,000 μg/mL streptomycin stock), 0.1 mM β-mercaptoethanol. Approximately 500 ESCs were plated in 20 μl drops of differentiation on the lids of petri dishes (contained warm PBS) using a 50 μl multi-channel pipette and cultured in a humidified atmosphere of 5% CO2 at 37^{o}C as hanging drops throughout the first 4 days of differentiation, allowing embryoid bodies (EBs) to form. At day 4, the EBs were plated onto 0.1 % gelatin coated plates for a further 10 days of culture before collection at day 14 of differentiation. 1 μM CB-DMB and 10 μM nifedipine were added to the differentiation media at day 0. For control experiments, DMSO was added at the same concentration found in the drug containing media. Once the EBs had been plated at day 4, drug-containing media was changed every 2 days up until day 14. For immunostaining, ESCs were cultured on 0.1% gelatin coated glass coverslips before being fixed with 3.7% PFA for 30 minutes on ice. For RNA isolation tissue samples were dissociated for 2 min using 0.25 % trypsin-EDTA at 37 ^{o}C prior to snap freezing. All imaging was performed with a Zeiss Axio Vert.A1 epi-fluorescent microscope with a Zeiss HRc AxioCam attachment and Zen software (Carl Zeiss)

allowing bright field assessment of EB beating and cell outgrowth as well as fluorescent assessment of Nkx2.5-GFP expression.

2.8 Hanging drop model development

The hanging drop method of differentiation is both well established and widely used in the formation of ESC derived cardiomyocytes (Boheler et al. 2002). Whilst establishing the model a number of steps were taken to establish a robust protocol. Two of the major variables known to influence ESC cardiomyocyte differentiation are the starting number of cells and serum. To determine the optimum number of initial cells per 20 µl, hanging drops were created with varying numbers of cells (500, 800 and 1000) per drop. Using the *Nkx2.5*-GFP cell line as a direct read out of cardiomyocyte differentiation, 500 cells per drop led to the largest amount of cardiomyocyte differentiation. Another key variable was serum; I tested the effect of two different serum types, embryomax serum and normal serum on cardiomyocyte differentiation with embryomax serum being the best in terms of *Nkx2.5*-GFP expression and EB morphology. It is important to note that batch variation may have led to variation in speed and efficacy of differentiation, thereby affecting the gene expression profiles between experiments. Therefore, to account for possible variation, multiple experimental repeats were conducted using different serum batches although the same batch was used throughout each individual experiment.

In our initial experiments using the *Nkx2.5*-GFP ESC line, I assessed time points at days 0, 2, 4, 7, 10 and 14, however it became apparent that for future studies it would be informative to increase the number of time points over which specification and differentiation of cardiomyocytes occurred. Therefore samples were collected daily from days 2-7 when using the Eomes-GFP ESC line, allowing more precise determination of the timing of changes in cardiac genes.

2.9 Inhibitor experiments with ESC-derived cardiomyocyte

To conduct bright field inhibitor experiments, ESCs were cultured in hanging drops for 4 days before 1 EB was plated per well of a 0.1% gelatin coated 48 well plate (Falcon) with 250 µl differentiation media. Imaging was then conducted at days 7 and 14 of differentiation using a Zeiss Axio Vert.A1 epi-fluorescent microscope with a Zeiss HRc

AxioCam attachment and Zen software to record brightfield movies of cardiomyocyte beating. Initial recordings were taken at baseline before the same wells were re-imaged 30 min later after the addition of drug (10 µM nifedipine; 10 µM CB-DMB; 30 µM KB-R7943), or vehicle (DMSO).

2.10 Chemical reagents

3-Amino-6-chloro-5-[(4-chlorobenzyl)amino]-N-[(2,4dimethylbenzylamino)(amino)methylene-2-pyrazinecarboxamide (CB-DMB, Sigma) was stored at -80^{o}c as a stock concentration of 10 mM in DMSO and used at a final working concentration as stated. KB-R7943 (Tocris) and SN-6 (Tocris) were stored as a stock concentration of 100 mM in DMSO at -20^{o}c and diluted to final working concentrations of 10 µM and 30 µM, respectively. Nifedipine (Sigma) was stored at a stock concentration of 100 mM in DMSO at -20^{o}c and used at a final working concentration of 500 nM and 10 µM. Ned-K and Ned-19 were also kept at a stock concentration of 100 mM in DMSO at -20^{o}c but used at a working concentration of 30 µM. All Ned compounds used in this study were kindly supplied by Dr Raj Gosain, School of Chemistry, University of Southampton.

2.11 Imaging of Ca^{2+} in ESC-derived cardiomyocytes

To image Ca^{2+} transients in ESC-derived cardiomyocytes, ESCs were cultured in hanging drops for 4 days before ~10 EBs were plated onto 0.1% gelatin coated 35 mm glass bottomed MatTek dishes with 1 ml of differentiation media. At day 7 the cells were loaded with 8 µM Cal-520-AM for 20 min in media without serum at room temperature before being transferred to 37 ^{o}C and an atmosphere of 5% CO_2 + air for a further 10 min. After this, the dye-containing media was removed and differentiation media containing serum was added before imaging. Inhibitor experiments were conducted on embryos as previously described, with a baseline recording taken prior to addition of drug-containing media, subsequent recording were taken after 5, 15 and 30 min incubation. All Ca^{2+} imaging was performed with the Zeiss 710 LSM at 10 fps with a 20x, 0.6 NA air objective.

2.12 Cardiomyocyte isolation

Single ventricular myocytes were isolated from C57BL/6 mice aged between 3 – 4 months. Mice were killed by cervical dislocation before their hearts were quickly removed and the coronary arteries retrogradely perfused via the aorta with a physiological salt solution (PSS) containing (mM): NaCl 134, KCl 4, $MgSO_4$ 1.2, NaH_2PO_4 1.2, Glucose 11.1 and HEPES 10 (pH 7.34 with NaOH at room temperature) using a Langendorff perfused heart apparatus. The hearts were initially perfused at 37 ^{o}C for 5 min with PSS before collagenase type 2 and protease type 14 were added to achieve a final collagenase concentration of 0.3 mg ml^{-1} and a protease concentration of 0.04 mg ml^{-1}. After 6.3 min of digestion, the ventricles were perfused for 10 min with a taurine solution containing (mM): NaCl 115, NaH_2PO_4 1.2, KCl 4, $MgSO_4$ 1.2, $CaCl_2$ 0.1, taurine 50, glucose 11.1, HEPES 10 (pH 7.34 with NaOH at room temperature). The tissue was then minced and single myocytes were obtained by filtering it through 200 µm nylon mesh. Cells were then incubated in taurine solution for 20 min allowing the cells to pellet. The supernatant was then removed and the cells were re-suspended in taurine solution at room temperature before being plated onto laminin-coated coverslips and allowed to attach for 2 h. Dead and non-adhered cells were then removed by rinsing.

2.13 OCT Cryo-embedding and cryosectioning of embryos

E12.5 Embryos were dissected as described and then fixed in 4% formaldehyde/PBS for 2 h on ice, before being washed in PBS and equilibrated in 30% sucrose (VWR) in PBS overnight at 4 ^{o}C. Samples were then transferred to a 50:50 solution of 30% sucrose and OCT (Thermo Scientific Raymond Lamb) and left to rotate at room temperature for 30 min. Embryos were then embedded on their backs in plastic moulds using OCT and frozen in a dry ice and ethanol bath. 7 µm sections were cut using a Bright Model OTF cryostat with 5040 Microtome (Bright Instruments), collected on SuperFrost Plus slides (VWR) and stored at - 80 ^{o}C until use.

2.14 Immunofluorescence of whole mount embryos

To perform whole-mount immunofluorescence, dissected embryos were fixed in 4% formaldehyde/PBS for 1 hour at room temperature. The embryos were then washed 3 times in PBT-0.1% (PBS containing 0.1% Tween-20) for 15 min, permeabilised in PBT-0.25% for 40 min and washed again 3 times in PBT-0.1%. The embryos were

transferred to blocking solution overnight at 4^{o}C. Primary antibodies (Table 2.1) were then added to the solution and incubated o/n at 4^{o}C. The embryos were washed 3 times in PBT-0.1% and incubated overnight 4 ^{o}C in PBT-0.1% with the secondary antibodies (Table 2.2), then subsequently washed 3 times with PBT-0.1% for 15 min and mounted in Vectashield with 4',6-diamidino-2-phenylindole (DAPI; Vectalabs) for at least 24 h at 4 ^{o}C before imaging using a Zeiss 710 LSM.

2.15 Immunostaining of cryosections

Cryosections were processed for immunofluorescence by 2 x 5 min PBS washes to remove OCT. The staining area was then outlined with an ImmEdge™ pen (Vector Laboratories) before samples were permeabilised with 0.5% Triton X-100 (Sigma) in PBS for 5 min. After permeabilisation, sections were blocked for 1 h at room temperature with 10% serum (goat or donkey, depending on the species in which the primary and/or secondary antibodies were raised, both Sigma), 1% BSA (Sigma) in 0.1% Triton X-100/PBS. Incubation with primary antibody, diluted in blocking solution, was carried out in a humidified chamber overnight at 4^{o}C. Following 3 x 5 min washes with 0.1% Triton X-100/PBS, sections were incubated with AlexaFluor secondary antibodies (Invitrogen, 1:200) diluted in blocking solution for 1 h at room temperature. A further 5 x 5 min PBS washes were performed, the penultimate containing DAPI (Cell Signalling Technology) before mounting in 50% glycerol: PBS. Imaging was performed using a Leica SP5 confocal microscope. In all experiments, negative controls for each secondary antibody (no primary antibody) were included to confirm the specificity of the signal.

2.16 Immunofluorescence of ESC-derived cardiomyocytes

ESC-derived cardiomyocytes grown on glass coverslips at different stages of differentiation or unattached EBs were processed for immunofluorescence by removal of growth media, washing in PBS, and fixation in 4% formaldehyde (Sigma) in PBS for 20 min at room temperature. After fixation ESC samples were rinsed with PBS before being permeabilised with 0.1% triton X-100/PBS for 10 min, followed by blocking with 10% goat serum, 1% BSA in 0.1% Triton X-100/PBS for 1 h. Incubation with primary antibodies, diluted in blocking buffer, was carried out overnight at 4 ^{o}C. After incubation with primary antibodies samples were washed 3 times for 10 minutes with

0.1% Triton X-100/PBS and then incubated with secondary antibodies (Supplementary Table 2), diluted in blocking buffer for 1 h at room temperature. After incubation with the secondary antibodies, samples were washed 5 times for 5 minutes in PBS. Samples were mounted using Vectashield mounting media with DAPI 24 hours before imaging using a Zeiss 710 LSM. In all experiments, negative controls for each secondary antibody (no primary antibody) were included to confirm the specificity of the signal.

2.17 RNA extraction using RNAeasy Micro kit

For ESCs (sample sizes of < 1 x10^6 cells), E7.5- E8.5 embryos and for hearts from E9.5 – E12.5, RNA extraction was performed using the Qiagen RNeasy Plus Micro Kit, as per manufacturer's instructions. Cell or tissue pellets were homogenised in lysis buffer containing guanidine-thiocyanate and β-mercaptoethanol, which inactivate RNases, using a 21G needle and 1 ml syringe. Homogenate was initially centrifuged through gDNA eliminator spin columns to remove genomic DNA from the samples. Lysates, in the presence of ethanol, were next loaded onto the spin columns which contained RNA binding silicon membranes. Contaminants were then removed in a series of wash steps before RNA was eluted into clean Eppendorf tubes using RNase-free water. The concentration and purity was determined using a Nanodrop-2000 (Thermo Scientific) spectrophotometer, by measuring absorbance at 260, 280 and 230 nm.

2.18 RNA extraction using Trizol

For hearts from embryos older than E12.5, RNA extraction was performed using Trizol (Invitrogen). RNA extraction from adult hearts required hearts to be powdered using liquid nitrogen and a pestle and mortar. 1 ml Trizol was added to the samples before using a 21G needle and a 1 ml syringe to homogenise the cell pellet/tissue. To allow complete dissociation of nucleoprotein complexes, homogenised samples were left for 5 min at room temperature. After 5 min, 200 µl chloroform (VWR) was added and the samples were vigorously mixed for 15 s before being incubated for 15 min at room temperature. The Trizol/Chloroform mix was next centrifuged at 12,000 x g for 15 min at 4 ^{o}C allowing the aqueous phase (containing the total RNA fraction) to be collected and transferred into a clean 1.5 ml tube. RNA was precipitated by the addition and mixing of isopropanol and left to proceed overnight at -20 ^{o}C. The precipitated RNA was pelleted by centrifugation at 12,000 x g for 10 min at 4 ^{o}C before removal of the

supernatant. Pellets were then washed in 1 ml 75% ethanol in Diethyl Pyrocarbonate (DEPC) treated water to inactive RNases, before being re-pelleted at 7,500 x g for 5 min at 4 ^{o}C, and resuspended in RNase-free water. The concentration and purity of RNA was determined by Nanodrop-2000 as above.

2.19 cDNA synthesis from extracted RNA

Reverse transcription was performed on samples containing 1 µg total RNA. Initially, a pre-cDNA reaction containing 1 µl dNTPs (10 mM of each dNTP; Promega), 0.5 µl random primers (Promega), 1 µg RNA and RNase-free water (total reaction volume equalled 10 µl) was incubated for 5 min at 65 ^{o}C before being cooled to 4 ^{o}C. After incubation, 4 µl 5 x First Strand Buffer (Invitrogen), 2 µl 0.1 M DTT (Promega), 2 µl 25 mM MgCl2 and 1 µl RNasin Plus RNase Inhibitor (Promega) was added to each reaction and the samples were incubated at 25 ^{o}C for 2 min before addition of 1 µl SuperScript III Reverse Transcriptase (Invitrogen). Samples were then incubated at 25 ^{o}C for 10 min, 42 ^{o}C for 50 min and 70 ^{o}C for 15 min, before being cooled to 4 ^{o}C. cDNA was then diluted in water to a final concentration of 4 ng/µl and stored at -20 ^{o}C until use.

2.20 Real-time PCR

The expression of mRNAs for genes of interest (Table 2.3), together with endogenous controls HPRT and/or GAPDH were measured by quantitative real-time PCR using SYBR Green (Applied Biosystems). Each reaction contained: 8 ng cDNA, 0.5 µl of each primer, 6.5 µl water and 12.5 µl 2 x SYBR Green, made up with H_2O to a final volume of 22 µl. Primers were either designed using Primer-BLAST (National Center for Biotechnology Information, National Institutes of Health) or obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank) or previous publications. Where possible, primers were designed to span exon-intron boundaries to prevent any remaining genomic DNA from being amplified, have annealing temperatures around 60 ^{o}C and generate amplicons between 50 bp - 200 bp. The reaction mixture and samples were loaded into either a MicroAmp Optical 96-Well Reaction Plates or MicroAmp Fast Optical 96-Well Reaction Plates and sealed with Optical Adhesive Films (all Life Technologies). Quantification was performed using either an ABI Prism 7000 Sequence Detector or a ViiA 7 Detection System (both Applied Biosystems) using a PCR programme of 95^{o}C 15 minutes followed by 40 cycles of (95^{o}C 15 s melting phase and

60 °C 1 min annealing and extension). Amplification of a single amplicon was confirmed by obtaining dissociation curve (melt curve) profiles as well as using gel electrophoresis to separate the reaction product. Cycle threshold (Ct) values were generated using either RQ Manager V1.2 or Viia7 software (both Applied Biosystems). Relative gene expression levels were obtained using the ΔΔCt method, in which expression of each gene of interest was normalised to Hprt and/or Gapdh (Murphy & Polak 2002), and presented as fold change over a reference sample. Non-template controls were performed by replacing cDNA with water, to test for non-specific amplification.

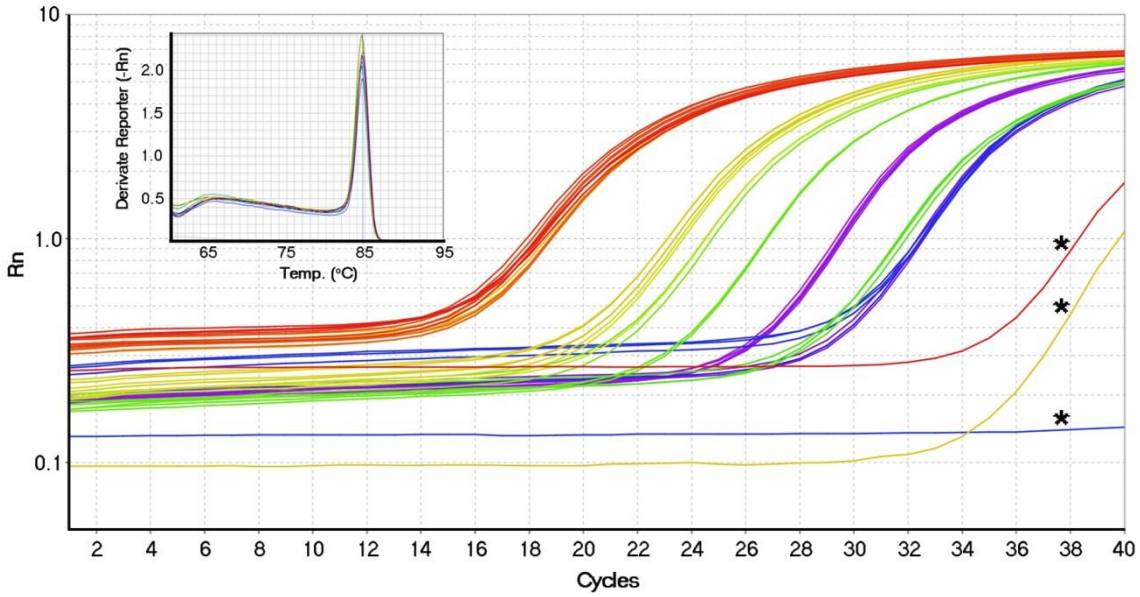

Figure 2.1: Representative real-time PCR amplification and dissociation curve traces

Representative plot of SYBR Green flurosecent signal (Rn = Reporter signal normalised to ROX dye) during RT-PCR amplification, detailing the increase in reporter singal/amplification as the number of PCR cycles increase (*= Non-template control). Insert, representative trace of a dissociation curve, highlighting the formation of a single peak and representing the production of a single amplicon.

2.21 Protein extraction from cells

To prepare protein samples for Western blot analysis, cell culture medium was removed and the cells were washed once with PBS. Protein was extracted on ice using direct lysis of cells with NP-40 extraction buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris (pH8.0)) and 1x protease and phosphatase inhibitor cocktail (Roche). Lysates were collected into pre-cooled 1.5 ml microcentrifuge tubes and incubated on ice for 20 min before being centrifuged at 10,000 rpm for 20 min at 4 °C and the supernatant

collected and stored at -20 ^{o}C. Before freezing a 10 µl aliquot was taken for protein quantification. Protein was quantified using the calorimetric DC protein assay which is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent (Bio-rad).

2.22 SDS-PAGE and Western blot analysis

Protein sample supernatants were prepared for SDS-PAGE analysis by the addition of 4x Laemmli Buffer containing 5% β-mercaptoethanol and boiling at 95 ^{o}C for 5 mins, in order to reduce and denature the sample (Laemmli 1970). Samples were quenched briefly on ice and then 20 µg of protein was loaded into a pre-cast 4-20% gradient SDS-PAGE gel (Biorad). A 4-20% gradient gel was used so that a wide range of protein sizes could be detected on a single gel. In order to make the protein bands run straight all 20 µg samples were made up to a maximum volume of 30 µl using 1x Laemmli buffer. A Precision Plus Protein Kaleidoscope (Biorad) ladder was included alongside samples so that protein size could be assessed. Electrophoresis was performed at 150 V for ~45 min before being electrotransferred (150 mA, 90 min) onto PVDF membranes pre-activated in methanol. Efficient transfer was assessed using Ponceau S solution. Membranes were then washed in TBS + 0.1% Tween-20 (Sigma; TBS-T) to remove Ponceau S and blocked for 1 h with 5% skimmed dry milk dissolved in TBS-T before incubation with primary antibodies diluted in blocking buffer overnight at 4 ^{o}C. Following 2 x 10 min and 3 x 5 min washes with TBS-T, membranes were incubated with HRP-conjugated secondary antibodies (1:2500 in blocking buffer; GE Healthcare) for 1 h at room temperature. Following further TBS-T washes, immunodetection was performed using the enhanced chemiluminescence kit (ECL, GE Healthcare) and a Chemidoc imaging system (BioRad). To re-probe membranes with a second antibody, the first antibody was striped for 15 minutes at room temperature using a glycine stripping solution (200 mM glycine, 3.5mM SDS, 1% Tween20, pH 7.2) before rinsed with TBS-T and re-probed.

2.23 Image Analysis

Tissue motion was visually enhanced using an absolute difference image filter in order to plot measurements of cardiac contractions in the developing cardiac crescent: $p(x_j, y_j) = [p(x_j, y_j) - p(x_{j-N}, y_{j-N})]$ where $p(x_j, y_j)$ is the intensity of a pixel

located at position x,y for frame j, and $p(x_{j-N}, y_{j-N})$ is the same intensity of the same pixel N frames prior (Chen et al. 2015). In summary, pixel displacement, indicative of contractions, was visualized and represented by increasing grey levels within the crescent. Change in pixel intensity was assessed in a selected region, to reveal the contraction dynamics. To normalize the Ca^{2+} transients, background signal was subtracted from all frames of a given movie using ImageJ.

2.24 Statistics

All data involving beat rate and qPCR gene levels were compared using one-way ANOVA followed by a Tukey test for multiple comparisons. In cases where the raw data failed to map to a normal distribution with consistent variance, we performed a log transformation and tested the assumptions for the transformed data. To compare the number of affected embryos upon acute treatments, a Freeman-Halton extension of Fisher exact probability test was applied due to a smaller number of samples. To compare the effect of different treatments on the number of beating EBs, due to a large number of samples, a Chi Square test with a Bonferroni correction for multiple comparisons was performed instead. Principal Component Analysis (PCA) was carried out to directly compare temporal gene expression data from EBs with that derived from embryos, the data of each sample was normalized by assessing the ratio with the maximum value of all samples. A log transformation was applied to the normalized data and the principal components were calculated using R environment. The 3D representation of the PCA was constructed by plotting the 3 first components given that these explained more that 95% of the observed variance. A hierarchical clustering analysis was performed using Ward's minimum variance method (Ward 1963).

2.25: Primary antibodies

Antigen	Host Species	Company	Dilution (application)
Brachyury (C-19)	Goat	Santa Cruz	1:200 (ICC, IF-WM)
CamKII (H-300)	Rabbit	Santa Cruz	1:500 (WB)
Cardiac α-actinin (EA-53)	Mouse	Sigma	1:500 (ICC, IF-Fr)
Cardiac TnT (1C11)	Mouse	Abcam	1:200 (IF-WM, ICC)
Cav1.2 (H280)	Rabbit	Santa Cruz	1:100 (ICC, IF-WM)
Connexin 43	Rabbit	Zymed	1:200 (IF-WM)
Gapdh (6C5)	Mouse	Abcam	1:10000 (WB)
GFP	Chicken	Abcam	1:1000 (ICC)
NCX (π-3)	Rabbit	Swant	1:200 (ICC, IF-FR, IF-WM)
pCamKII (T286)	Rabbit	Abcam	1:500 (WB)

Table 2.1: Primary antibodies

Applications: WB: western blotting; IF-Fr: immunofluorescence of frozen/cryo-embedded sections; ICC: immunocytochemistry; IF-WM: Immunofluorescence of whole-mount tissue.

2.26: Secondary antibodies

Antigen	Host Species	Company	Dilution
Mouse-488	Goat	Life Technologies	1:200
Rabbit-488	Goat	Life Technologies	1:200
Goat-488	Donkey	Life Technologies	1:200
Mouse-555	Donkey	Life Technologies	1:200
Mouse-594	Goat	Life Technologies	1:200
Rabbit-594	Goat	Life Technologies	1:200
Phalloidin Atto-647	N/A	Sigma	1:100

Table 2.2: Secondary antibodies

2.27: Primers for Quantitative RT-PCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon (bp)
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	101
Hprt	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG	122
Oct-4	TGGAGGAAGCCGACAACAATGAGA	TGGCGATGTGAGTGATCTGCTGTA	148
Nanog	CCCTTCCCTCGCCATCACACTG	GGAAGGGCGAGGAGAGGCAGC	172
Brachyury	CATGCTGCCTGTGAGTCATAA	TGTCTGGGAGCCTGGGGTGAT	83
Gooscoid	GAAGCCCTGGAGAACCTCTT	ATCGCTTCTGTCGTCTCCAC	144
Mesp1	GTCTGCAGCGGGGTGTCGTG	CGGCGGCGTCCAGGTTTCTA	189
Gata-4	CCCTACCCAGCCTACATGG	ACATATCGAGATTGGGGTGTCT	118
Nkx2.5	CTATGCCCTGTCCCTCAGAT	CTCCCGGTCCTAGTGTGGAA	139
Mef2c	ACTGGGAAACCCCAATCTTC	ATCAGACCGCCTGTGTTACC	92
Myh6	GCTGGCTGGAAAAGAACAAG	TCTTGCCTCCTTTGCCTTTA	125
Myh7	ACTGTCAACACTAAGAGGGTCA	TTGGATGATTTGATCTTCCAGGG	91
Tnnt2	CAGAGGAGGCCAACGTAGAAG	CTCCATCGGGGATCTTGGGT	119
Sox17	GGAACCTCCAGTAAGCCAGAT	GAGGTGCTGCTCATTGTATCC	87
Gfap	TGCAAGAGACAGAGGAGTGGT	GCGATAGTCGTTAGCTTCGTG	110
Slc8a1	GGACCAACAGCTGGAGAGAG	GGCAAACAGAACCTTCCAGA	152
Slc9a1	CGCCATCATCACCGTCATCT	GGTGGTCCAGGAACTGTGTG	143
Cacna1c	GGAGGGCGTGCATAAGCATT	AGGAAGAGATACTCCACTCGTTC	160
Cacna1g	TGTCTCCGCACGGTCTGTAA	AGATACCCAAAGCGACCATCTT	184
Ryr2	ATTATGAAGGTGGTGCCGTATCA	TTCCACTCCACGCGACTCTTA	88
Atp2a2	TGGGCAAAGTGTATCGACAG	CAGCAGGAACTTTGTCACCA	108
Atp1a2	GAATGGGTTCTACCATCGCG	GCACAGAACCACCACGTGAC	177
Scn5a	CCGCCTTGGACCAAATATGTT	TGACCAGAGACTCAAAGGTGT	64
Itp3r2	TCATCATCGCCTTGATTCTG	CAGTTCCCTGGGTCTCATGT	170
Trpc3	TCGACTACCCCAAGCAAATC	CTCCTTGCACTCAGACCACA	88
Stim1	TGACAGGGACTGTACTGAAGATG	TATGCCGAGTCAAGAGAGGAG	85
Orai1	AACGAGCACTCGATGCAGG	GGGTAGTCATGGTCTGTGTCC	129
Tpcn1	CATGAACTACAGCCGCAAGA	CCTCGCTCCTCACGATAAAG	117
Tpcn2	GAAGCACAGGACCAGGAGAG	AAAGGCCCGGTTCTGAGTAT	92
Camk2d	CTGGCACACCTGGGTATCTT	ATCCCAGAAGGGTGGGTATC	125
Ppp3ca	CTGGCTGCGCTAATGAACCA	GGTCTGACCATAGGATGTCACAC	123
Calm1	TGCTCCGTTCTTCCTTCCTT	TTCCTTGGTTGTGATGGTGC	126
Calm2	GGGCGGAGGAGGAGAATTAG	CTTCAGTCAGTTGGTCAGCC	108
Calm3	AGGCCGACATTGATGGAGAT	AAGAGATCAGGAGAACGGGC	108
NFAT	TCATCCTGTCCAACACCAAA	TCACCCTGGTGTTCTTCCTC	110

Table 2.3: qRT-PCR Primer sequences and amplicon length.

3. CHARACTERISATION OF CARDIAC FUNCTION DURING HEART DEVELOPMENT

3.1 Introduction

It has been well documented that the mammalian heart begins to contract from an early stage, raising the important question of how and when the initiation of function occurs and to what extent this defines and influences the progression of differentiation and subsequent cardiac morphogenesis.

Formation of the mouse heart begins at around embryonic day (E) 7.5, from mesoderm-derived cardiac progenitor cells located in the cardiac crescent, the first morphologically recognisable heart structure (Buckingham et al. 2005). From E7.5 cardiac progenitors migrate to form a linear heart tube, which goes on to develop into the mature septated four chambered heart. Cardiac contractions are widely held to begin with the formation of the linear heart tube between E8.0 and E8.5 (Navaratnam et al. 1986)(Linask et al. 2001)(Nishii & Shibata 2006), but observations from our collaborators, the Srinivas lab, have shown that the cardiac crescent is contractile at stages earlier than previously documented. Further observations during this collaboration revealed single cell slow Ca^{2+} waves within the cardiac crescent prior to the onset of contraction, at around E7.75. In order to study how these early Ca^{2+} transients may influence cardiomyocyte specification and formation, an embryonic stem cell (ESC) model of cardiomyocyte differentiation was used, which recapitulated heart development *in vivo*. ESC - derived cardiomyocytes have provided unique developmental models for understanding the genetic and epigenetic regulation of cardiogenesis and will allow mechanistic insight into the relationship between cardiac contractions coincident with cardiomyocyte specification. In terms of cardiac differentiation, the stages of cellular specification and commitment have been well characterized by the expression of specific genetic markers (Van Vliet et al. 2012). Using these well-defined markers it is possible to study and track the induction and patterning of mesoderm from pluripotent stem call progenitors as well as the specification and maturation of cardiac progenitors.

In this chapter, I will describe two different models of heart development; *In vivo* formation of the murine cardiac crescent as well as *in vitro* ESC cardiomyocyte

differentiation. Using these models I will characterise and compare formation of the cardiac crescent as well as ESC cardiomyocyte differentiation in terms of morphology, gene expression and function. Additionally, early calcium dynamics will be investigated using confocal imaging of *ex vivo* embryos and ESC-derived cardiomyocytes.

3.2 Results

3.2.1 Morphology and sarcomeric formation during heart development

In the heart, cardiomyocytes are the contractile cell type which allows it to perform its essential function; to supply the body with sufficient oxygen and nutrients. To achieve this, cardiomyocytes have a highly defined cellular phenotype and function, which becomes established during development.

To define cardiomyocyte phenotype during development, mouse hearts where imaged from the onset of cardiac crescent formation. When trying to assess formation of the cardiac crescent it became apparent that conventional staging methods did not accurately correlate with specific temporal changes during heart development, instead they represented a range of cardiac morphologies. To precisely characterise formation of the early heart, a system was established which was independent of conventional methods such as somite number or embryonic day. Staging was assessed by measuring both the width and the height of the cardiac crescent (Table 3.1).

Stage	Width/μm	Maximum Height/μm	Width/Height Ratio	Somites	Embryonic Day
0	360 - 390	70 - 80	4.5 - 5.5	0 - 2	E7.5 - E8.0
1	300 - 370	70 - 95	3.0 - 4.2	1 - 3	E8.0 - E8.25
2	230 - 280	95 - 120	2.0 - 2.8	2 - 4	E8.0 - E8.5
3	200 - 230	120 - 140	1.5 - 1.8	3 - 5	E8.25 - E8.5

Table 3.1: Staging criteria of early heart development according to formation of the cardiac crescent

As the crescent matured, the width-to-height ratio decreased with the formation of the linear heart tube. This value roughly correlated with ranges in somite number and embryonic day. The earliest recognisable cardiac crescent could be located between the midline and the head folds at stage 0 (Figure 3.1 a). In terms of size, the stage 0 cardiac crescent was between 360-390 µm in width and 70-80 µm high, with a large width to height ratio around 5. As the heart developed, the height-to-width ratio decreases to around 1.65 at stage 3, with the width of the crescent between 200-230 µm and height around 120-140 µm. Positive immunostaining for cardiac TnT (cTnT) was evident at the earliest stage 0 crescent with expression detected at the cell membrane, albeit without corresponding sarcomeric banding (Figure 3.1 a-e). Sarcomeres manifested later in discrete regions within stage 1 and 2 crescents, indicative of functional cardiomyocytes. Subsequent sarcomere assembly and myofibrillar banding became more ordered by stage 3, and increased through to the linear heart tube stage, consistent with increased cardiomyocyte maturation (figure 3.1 g).

These results revealed that contractile proteins required for cardiac function are expressed and organised at the earliest stages of crescent formation and prior to the onset of contraction. To more accurately correlate changes during early heart development a novel staging method was also defined and characterised.

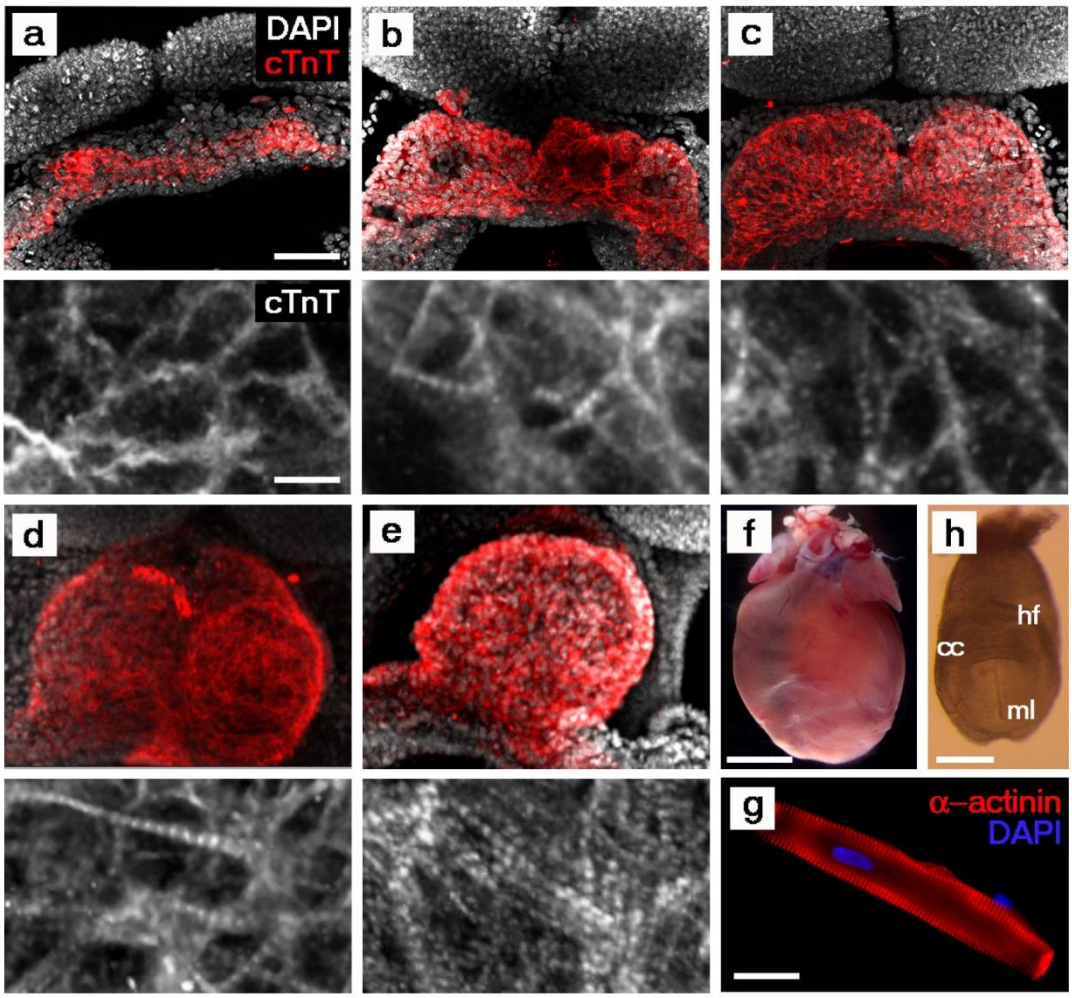

Figure 3.1: Early mouse heart formation and sarcomeric assembly during embryonic development

Maximum intensity projections of cardiac troponin (cTnT) immunostaining revealed progressive differentiation and sarcomeric assembly during stages of cardiac crescent through to linear heart formation (Stage 0 (a); Stage 1 (b); Stage 2 (c); Stage 3 (d); LHT(e)). Data contributed by Antonio A. M. Miranda. Brightfield image of adult mouse heart (f) and immunostaining of α-actinin to visualise sarcomere expression in an isolated, adult mouse-cardiomyocyte (g). Brightfield image of E7.75 embryo (h). cc, cardiac crescent; hf, head folds; ml, midline. Scale bars: a-e, top panel 100µm, bottom panel 10µm; f, 500 µm; g, 25µm; h, 250µm.

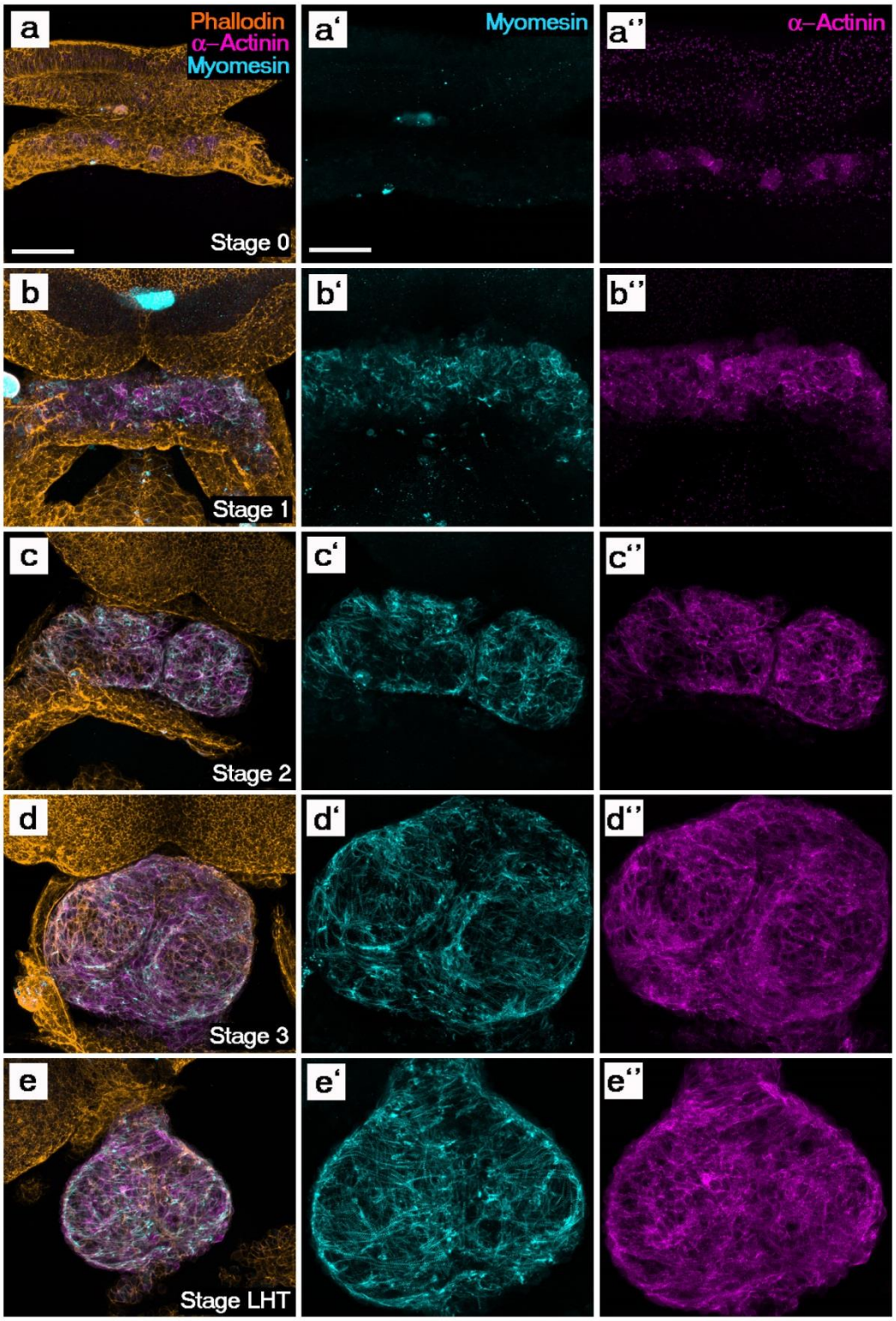

Figure 3.2: Expression and formation of the sarcomere M-band

Maximum intensity projections of immunostaining revealed a progressive increase in the M-band localised protein Myomesin. Myomesin was absent from the stage 0 cardiac crescent however became expressed within stage 1 cardiac crescents and revealed the assembly of sarcomeres as the cardiac crescent developed through to linear heart formation (Stage 0 (a); Stage 1 (b); Stage 2 (c); Stage 3 (d); LHT(e)). Scale bars: a-e, 100 µm and a'-e'', 50µm.

3.2.2 Onset of cardiac contraction within the developing heart

It has been documented that rhythmic cellular contractions in mice are evident prior to formation of the linear heart tube (Goss 1952). Historical studies in rats have described beating even earlier, with pacemaker-like activity observed in lateral regions of the embryonic midline (Hirota et al. 1985). Using differential interference contrast (DIC) microscopy, our collaborator Shankar Srinivas has observed the onset of beating in discrete foci on both the left and right lateral regions of stage 1 cardiac crescents, coincident with sarcomeric banding and earlier than previously described (unpublished work). The initial rate of contraction at stage 1 was around 30 beats per minute (bpm), increasing slightly to 38 bpm by stage 2 with contractions now occurring across the whole crescent (Figure 3.3 a). The discrete beating foci observed at stage 1 contracted at the same rate, indicative of either synchronisation or a shared intrinsic contractile rate (Figure 3.4 a, b). As the cardiac crescent matured towards the linear heart tube there was a significant increase in rate of contraction to around 50 bpm at stage 3, whilst the linear heart tube was beating around 60 bpm. To further investigate the development of cardiac contraction, live-imaging was used to visualise Ca^{2+} transients within the forming cardiac crescent (Figure 3.3 b). In stage 1 cardiac crescents lateral propagation of Ca^{2+} transients, initially from left to right, was observed across the embryonic midline and even through regions of non-contractile tissue, despite the absence of, Connexin 43 (Cx43) expression (Figure 3.4 c). In the adult, Cx43 electrically couples cardiomyocytes via the formation of gap junctions allowing the action potential to propagate quickly between cells. To assess the dynamics of these early Ca^{2+} waves, the time to peak (TTP) fluorescent signal and the time to half maximum (TT1/2M) signal were measured (Figure 3.3 c). TTP and TT1/2M at stage 1 were around 0.49±0.02 secs and 0.33±0.02 secs respectively, unsurprisingly in correlation with the increase in rate of contraction, there was a significant decrease in the TTP and TT1/2M by stage 3 (0.40±0.02 secs; p=0.025 and 0.27±0.01 secs; p=0.025 secs, respectively) (Figure 3.3 d, e).

Overall, these results provided evidence that cardiac contractile function initiates at the earliest stages of heart formation with synchronised propagation of calcium transits across the cardiac crescent.

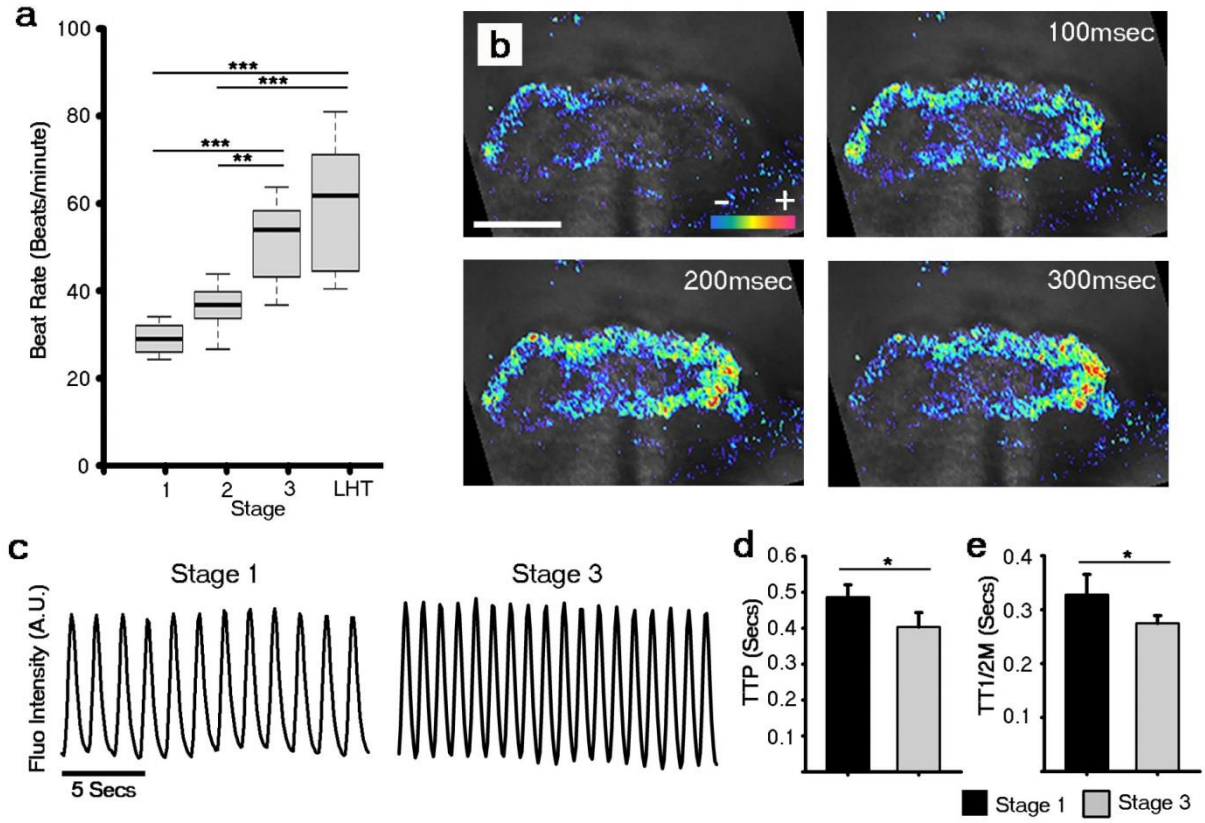

Figure 3.3: Onset of beating and Ca^{2+} transients during cardiac crescent formation

Quantitative analysis from the onset of cardiac contraction at stage 1 of crescent formation to formation of the LHT (stage 1, n=12; stage 2, n=8; stage 3, n=10; LHT, n=7), revealed a significant increase in heart rate from stages 2 to 3 (a); **data courtesy of Antonio A. M. Miranda**. Maximum intensity projection of normalized Ca^{2+} signal following Rhod2-AM loading of stage 1 embryos revealed lateral propagations of transients across the crescent (b). Representative traces of Ca^{2+} transients at stage 1 and 3 of crescent formation (c). Time to peak (TTP) fluorescent signal (d) and time to half maximum (TT1/2M) fluorescence (e), showing a significant decrease in both parameters between stage 1 (TTP, 0.49±0.03 secs; TT1/M 0.33±0.04 secs.) and stage 3 (TTP, 0.40±0.04 secs; TT1/M 0.27±0.01 secs.), n=5. Scale bars: b, 100 µm. All error bars are mean ± standard deviation. Statistics: ANOVA and Tukey test for multiple comparisons (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

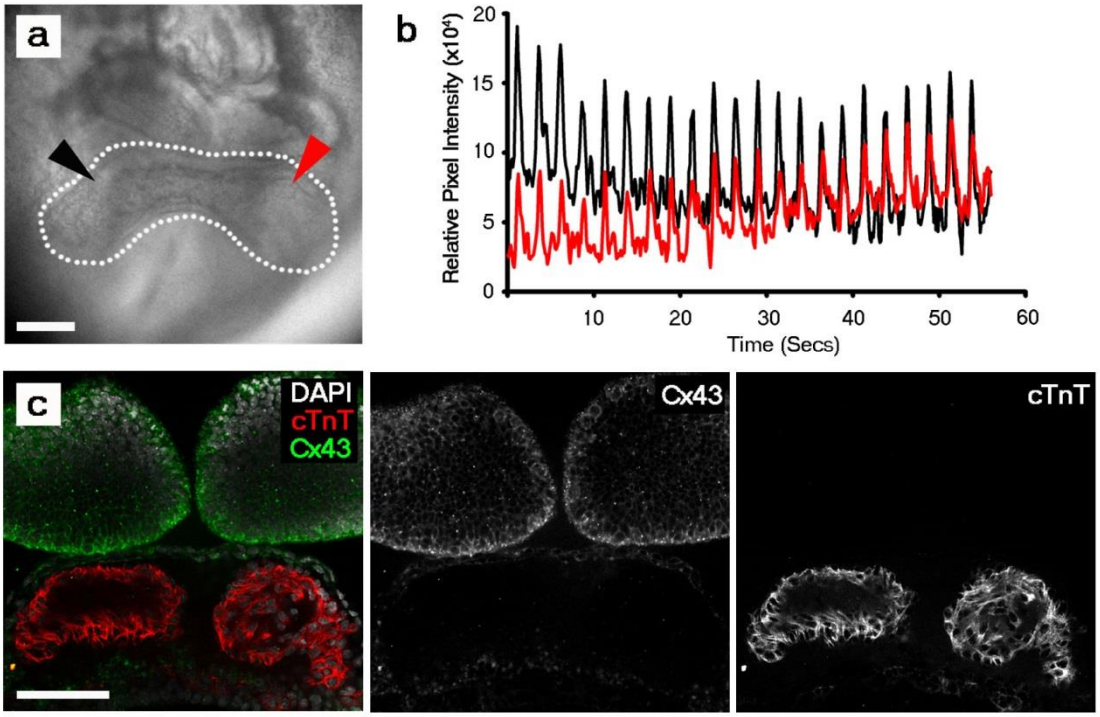

Figure 3.4: Discrete beating foci on either side of the embryonic midline contract at the same rate

DIC image of a stage 1 embryo with foci of beating highlighted on the left (red arrowhead) and right (black arrowhead) of the embryonic midline and cardiac crescent outline highlighted by white dashed line (a). Discrete foci on the left and right of lateral region of the crescent beat at the equivalent frequency (b), indicative of synchronisation across the midline or intrinsic pacing. Immunochemistry revealed a lack of Cx43 expression in the early cardiac crescent (c). Scale bar: a, 100µm.

3.2.3 Development of single cell slow Ca^{2+} waves to propagating fast waves within the early cardiac crescent

Due to the propagation of Ca^{2+} transients within the stage 1 cardiac crescent, stages prior to the onset of contraction were investigated. Using live-embryo imaging of calcium transients in stage 0 non-contractile cardiac crescents stochastic, asynchronous slow Ca^{2+} transients could be observed in single cells (Figure 3.5 a). To characterise the dynamics of these calcium transients the TTP and TT1/2M was measured using the fluorescent signal from single cells within the crescent (Figure 3.5 b). Average time TTP (3.41±2.30secs, n=174) and TT1/2M (2.42±1.86secs, n=169) showed a large amount of variability with ranges in TTP from 0.79 to 11.93 seconds and TT1/2M from 0.39 to 9.66 seconds (Figure 3.5 c, d). Distribution of Ca^{2+} transient rate in terms of TTP (Figure 3.5 e) and TT1/2M (Figure 3.5 f) was positively skewed

with the majority of waves having faster dynamics. There was no correlation (R^2= 0.2987) in the TTP and TT1/2M per single cells, with slower waves having a larger variation in Ca^{2+} dynamics (Figure 3.5 g). Therefore, these results highlighted the presence of pre-contractile Ca^{2+} transients within the developing crescent suggesting that Ca^{2+} handling is crucial for the initial stages of murine heart development and the onset of cardiac function.

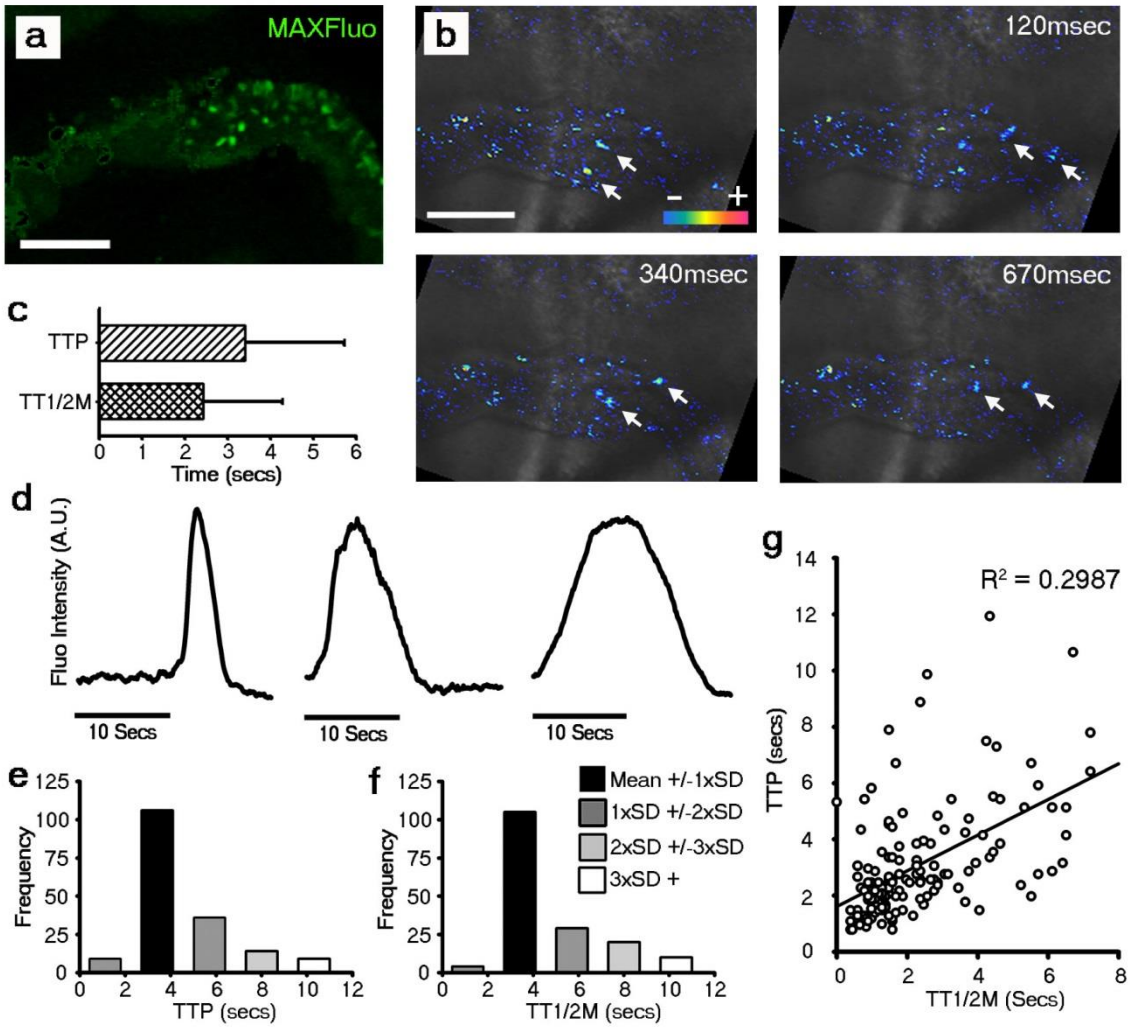

Figure 3.5: Single cell calcium transients precede the onset of contraction within the forming cardiac crescent

Maximum intensity projection of normalised calcium signal following Cal-520 loading of stage 0 embryos revealed asynchronous, sporadic calcium transients (Ca^{2+}) in individual cells prior to beating (a, maximum Cal-520 fluorescence (MaxFluo) and b, highlighted by white arrows). Average time to peak (TTP) (3.41±2.30secs, n=174) and time to ½ maximal (TT1/2M) (2.42±1.86secs, n=169) fluorescent intensity for stage 0 Ca^{2+} transients had a large variation (c, representative traces shown in d), with a positive skewness towards faster dynamics in terms of distribution (e and f). Single cell stage 0 Ca^{2+} transients varied in TTP and time to ½ maximal (TT1/2M) fluorescent intensity (g, n=145), revealing variable Ca^{2+} wave duration as well as

differences in speed of Ca^{2+} influx and efflux. Scale bars: 100 μm. All error bars are mean ± standard deviation.

3.2.4 Cardiac-specific gene expression profiles during mouse heart development

To assess cardiomyocyte differentiation cardiac-specific genes were used as stage related markers; the profiles of these key genes were investigated throughout development using qRT-PCR. To correlate the onset of Ca^{2+} handling and contraction with stage related genes, mRNA form whole embryos at E7.5 (prior to cardiac crescent formation (stage 0)), E8.0 (cardiac crescent (stages 0-3) and E8.5 (linear heart tube) was used (Figure 3.6a-f). A significant reduction in the expression of genes required for mesoderm formation (*Brach-T*; E7.5 vs. E8.5; 6 fold; p=<0.001) and mesoderm specification (*Mesp1*; E7.5 vs. E8.5; 76 fold; p=<0.001) was observed, whilst cardiac specific genes significantly increased over the same time course (*Nkx2.5*; E7.5 vs. E8.5; 11 fold; p=<0.001, *Mef2c*; E7.5 vs. E8.5; 7 fold; p=<0.001, *Tnnt2*; E7.5 vs. E8.5; 80 fold; p=<0.001, *Myh6*; E7.5 vs. E8.5; 150 fold; p=<0.001). During the later stages of development from E9.5, mature myocyte genes significantly increased in the neonatal and adult heart (*Myh6*; E9.5 vs. Adult; 6 fold; p=0.02), whilst early cardiac genes involved in cardiac specification either remained constant (*Mef2c*) or decreased (Nkx2.5; E9.5 vs. Adult; 4 fold; p=0.02) during maturation into the adult (Figure 3.6g-i). Of significance, these data are in agreement with previous reports and allows future correlation between gene expression profiles with *in vitro* ESC-derived cardiomyocyte differentiation.

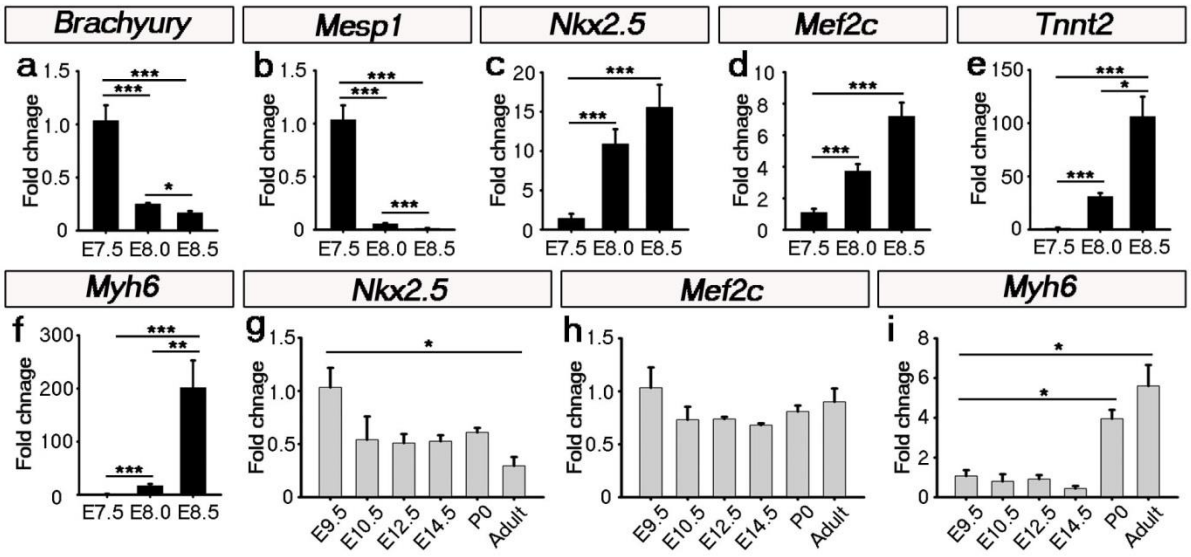

Figure 3.6: Cardiac specific gene expression changes during mouse heart development

Temporal gene expression profiles were assessed on whole embryos by qRT-PCR, normalised to E7.5: down-regulation of mesoderm markers *Brachyury* (a) and *Mesp1* (b) occurred between E7.5 to E8.5 whilst cardiac specific markers *Nkx2.5* (c), *Mef2c* (d), *Tnnt2* (e) and *Myh6* (f) were significantly up regulated by E8.0 and increased further by E8.5. Expression profiles of cardiac specific transcripts during *in vivo* heart development were assessed on isolated hearts using qRT-PCR, normalised to E9.5: whilst *Nkx2.5* (g) and *Mef2c* (h) gene expression remained constant from E9.5 onwards, *Myh6* (i) showed significant up regulation in postnatal day 0 (P0) and adult heart. All error bars are mean ± S.E.M. Statistics: one-way ANOVA and Tukey test for multiple comparisons (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

3.2.5 Nkx2.5-GFP ESC model of cardiomyocyte differentiation

To assess the earliest stages of cardiac specific gene induction and expression a hanging drop model of ESC differentiation was utilized, as previously described (Boheler et al. 2002)(Figure 3.7b). To form embryoid bodies ~500 ESCs were placed into a 20µl drop of differentiation media and cultured for 4 days as hanging drops. After 4 days EBs were plated down and allowed to differentiate for a further 10 days (Figure 3.7c- d). The advantage of this method is that large quantities of EBs and cardiomyocytes can be generated in a reproducible manner. An ESC reporter line containing green fluorescent protein (GFP) under the control of the *Nkx2.5* promoter was used (S. M. Wu et al. 2006), allowing cardiomyocyte differentiation to be easily assessed (Figure 3.7a). At around day 6-7 of differentiation EBs began to express GFP, indicating the formation of *Nkx2.5* positive cardiac progenitor cells. Consequently, by ~day 8 weak rhythmic contractions could be seen in small GFP positive regions. This developed further until strong synchronous beating was observed in large GFP positive regions by ~day 10, as shown in (Figure 3.7e-f). To assess whether beating regions contained muscle-specific cytoskeletal structures, immunohistochemistry was used to determine sarcomeric alpha-actinin localisation. By day 14, striated expression of alpha-actinin was observed in regions containing Nkx2.5-GFP expressing cells, indicating sarcomere formation (Figure 3.8a-b).

qRT-PCR was used to assess the temporal expression of key transcription factors implicated in the transition of pluripotent ESCs to a cardiac committed fate (Van Vliet et al. 2012). Figure 3.9 demonstrates the expression profiles of specific genes throughout the differentiation timecourse. Expression of the pluripotent marker *Oct-4* and *Nanog* decreased from day 0, as would be expected. Genes regulating the specification and patterning of the primitive streak such as *Brachyury* and *Mesp1* were strongly up-regulated at around day 4 of differentiation before being rapidly down-regulated by day 7. Key cardiac transcription factors such as *Nkx2.5* and *Mef2c* began to be expressed at day 7, and peaked at around day 10. mRNA expression of cardiomyocyte structural proteins, such as alpha-MHC (*Myh6*) and cardiac troponin t (*cTnT)*, began at day 7 and increased further thereafter, in accordance with the observed contractile phenotype of these cells. The changes in gene expression between day 4 and 14 of ESC differentiation correlated with those observed during

formation of the cardiac crescent *in vivo* (Figure 3.6), therefore helping to validate the model.

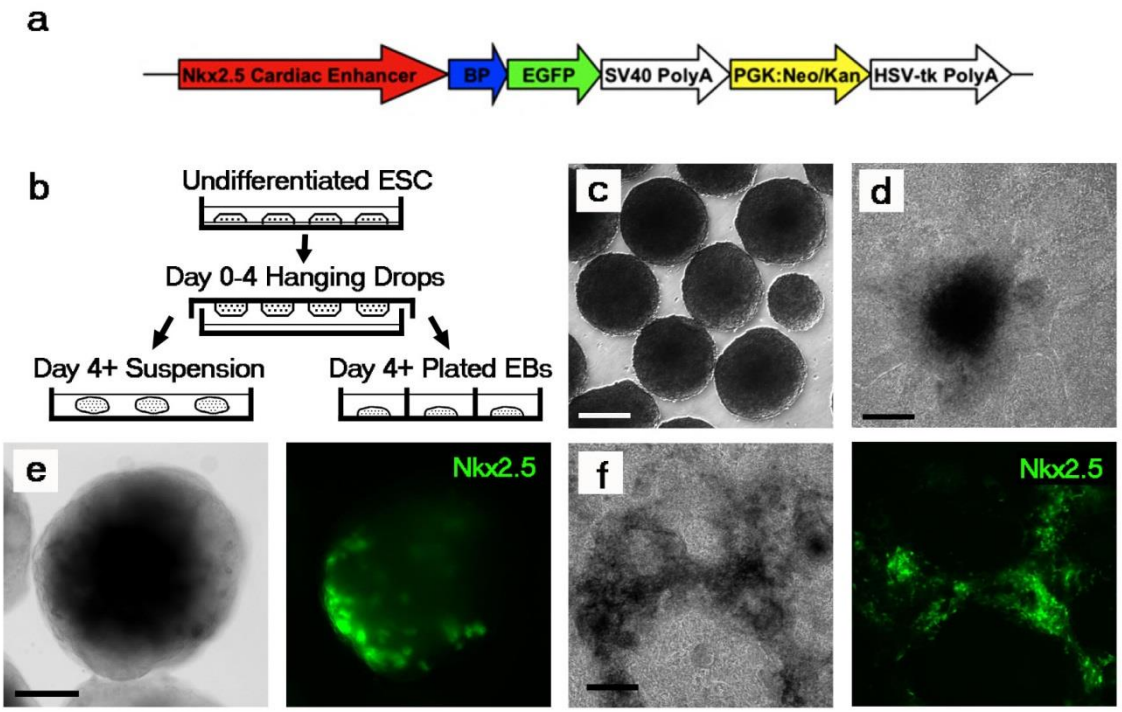

Figure 3.7: *Nkx2.5*-GFP ESC model of cardiomyocyte differentiation

Construct diagram for the Nkx2.5 cardiac enchancer-eGFP plasmid used to create the Nkx2.5-GFP reporter ESC line (Wu et al., 2006) (a). Cardiomyocyte differentiation was achieved using a "hanging drop" method of ESC differentiation (b). Brightfield images show the formation of embryoid bodies (EBs) at day 4 (c) with a large amount of outgrowth occurring by day 7 (d). The *Nkx2.5*-GFP was used to visualise the formation of cardiac progenitors in both EBs (e) and plated-down cultures at day 14 (f). Scale bars: b, c, e, 250µm; d, 100µm.

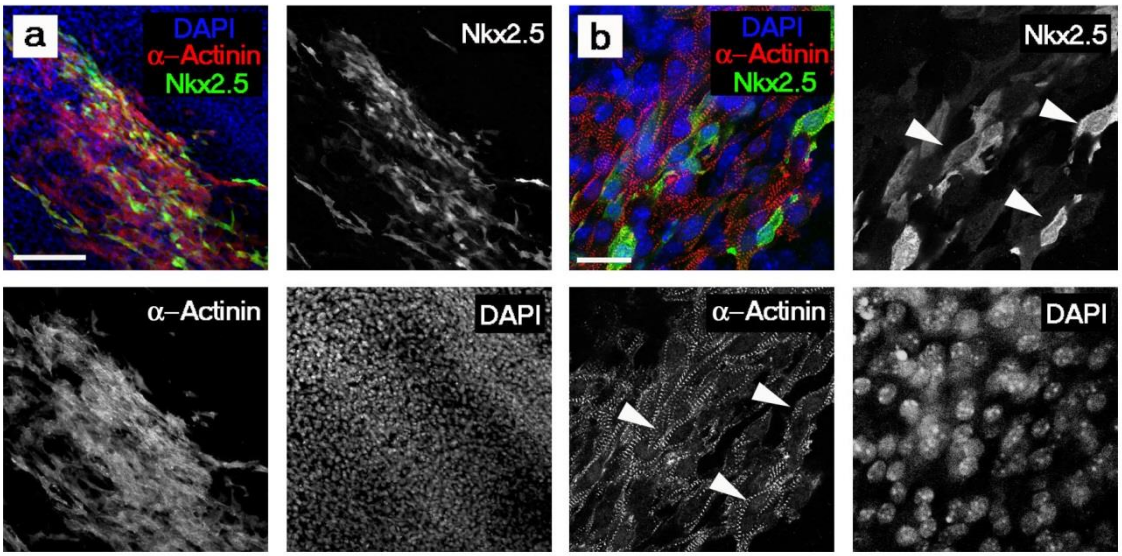

Figure 3.8: Formation of sarcomeres during *Nkx2.5*-GFP ESC cardiomyocyte differentiation

Immunocytochemistry revealed the expression of α-actinin in *Nkx2.5*-GFP positive regions, indicating formation of ESC-derived cardiomyocytes (a); higher magnification images of α-actinin filaments revealed the formation of sarcomeres (b). Arrowheads indicate *Nkx2.5*-GFP positive cells with sarcomeres. Scale bars: a, 100µm; b, 25µm.

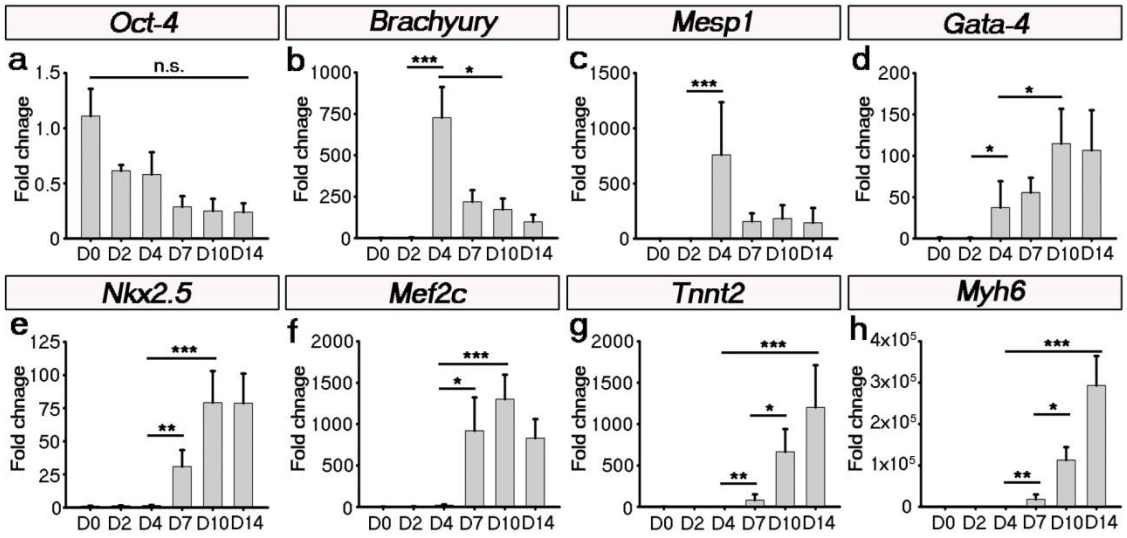

Figure 3.9: Expression of genetic markers during *Nkx2.5*-GFP ESC cardiomyocyte differentiation

Temporal gene expression profiles were assessed during ESC cardiomyocyte differentiation by qRT-PCR and normalised to day 0. Pluripotent marker *Oct-4* decreased throughout the differentiation timecourse (a). Mesoderm markers *Brachyury* (b) and *Mesp1* (c) were significantly up regulated at day 4 of differentiation before being down regulated thereafter. Cardiac specific markers *Nkx2.5* (e), *Mef2c* (f), *Tnnt2* (g), *Myh6* (h) increased throughout differentiation from day 7 whilst *Gata-4* (d) was first up regulated by day 4. N= 5 independent differentiation experiments. All error bars are mean ± S.E.M. Statistics: one-way ANOVA and Tukey test for multiple comparisons (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

3.2.6 *Eomes*-GFP ESC model of cardiomyocyte differentiation

To further characterise the hanging drop model of ESC cardiomyocyte differentiation a second ESC cell line was used. This ESC reporter line contained GFP under the control of the *Eomes* promoter which is known to be expressed by cardiogenic mesoderm and therefore would be useful when trying to identify cardiac progenitors prior to the expression of *Nkx2.5* (Arnold et al. 2009)(Figure 3.10a). At around day 4 of differentiation there was strong expression of the *Eomes*-GFP reporter in EBs, indicating formation of cardiogenic mesoderm (Figure 3.10b). By day 7 the levels of GFP had decreased with GFP only being observed in a very small population of cells at day 14, suggesting that cells had undergone differentiation and no longer expressed *Eomes* (Figure 3.10c-d). As a result of this differentiation, beating was observed between day 6 and 7 within small foci, with strong synchronous beating occurring across large regions by day 14. To assess whether beating regions contained muscle

specific cytoskeletal structures, immunohistochemistry was employed to determine sarcomeric alpha-actinin localisation. Results revealed clear striated expression of alpha-actinin in small regions at day 7, indicative of sarcomere formation and correlating with the onset of beating (Figure 3.10e). Beating rate was assessed using bright field movies. At day 7 of differentiation small regions of ESC derived cardiomyocytes were beating at 32.5±1.1 bpm, increasing to 36.0±1.3 bpm by day 14 (Figure 3.11a). Further analysis using Ca^{2+} reporter dyes showed large propagating calcium waves occurring between cells (Figure 3.11b) as well as slow calcium waves occurring within single cells (Figure 3.11c), similar to that observed in the early embryo (Figure 3.5b).

qRT-PCR was again used to assess the temporal expression of key transcription factors implicated in the transition of pluripotent ESCs to a cardiac committed fate. Figure 3.12 demonstrates the gene expression profiles throughout the differentiation timecourse. Pluripotent markers (*Oct-4*) decreased as expected throughout the differentiation protocol. Genes regulating the specification and patterning of the primitive streak such as *Eomes*, and *Brachyury* had increased by day 3 before being rapidly down regulated whilst *Mesp1* was strongly up regulated at day 4. Key cardiac transcription factors such as *Nkx2.5* and *Mef2c* began to be expressed at day 5, with *Gata-4* already being up-regulated by day 4. mRNA expression of *Myh6* and *cTnT*, initially began at day 5 and 6 respectively and increased further thereafter, correlating with both the observed contractile phenotype as well as the gene expression changes in the early embryo (Figure 3.6).

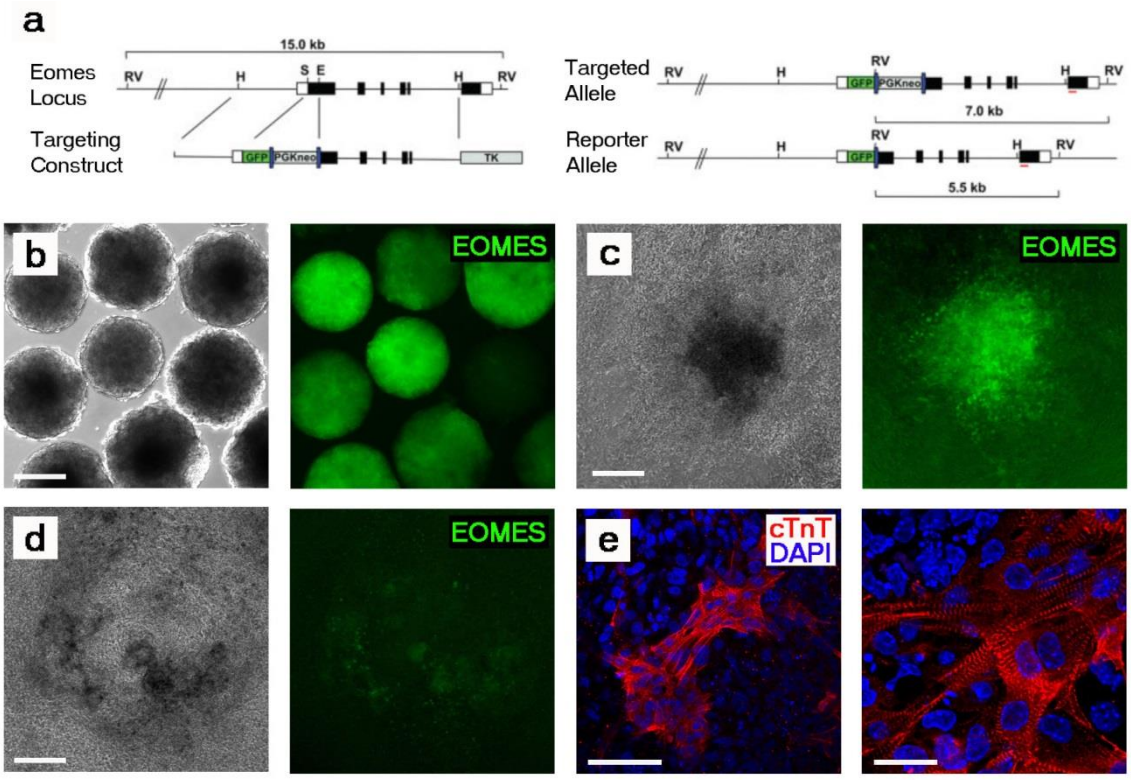

Figure 3.10: *Eomes*-GFP ESC model of cardiomyocyte differentiation

Strategy to introduce eGFP into the first coding Exon 1 of the Eomes locus by homologous recombination in ES cells (a). An EGFP.pA cassette was placed into the transcriptional start site of the endogenous Eomes locus, followed by a removable, LoxP-flanked (blue arrows) selection cassette (PGK.neo). Brightfield and epi-fluorescence images of *Eomes*-GFP expression in day 4 embryoid bodies (b) and day 7 (c) and 14 (d) plated EBs, revealing a decrease in *Eomes* expression during cardiomyocyte differentiation. Immunostaining of day 7 plated EBs revealed the striated expression of cardiac Troponin T and the formation of ESC derived cardiomyocytes with nuclear staining (blue; d, left panel); higher magnification of cTnT filaments revealed the formation of sarcomeres (e, right panel). Scale bars: a, b, c 250µm; d, left panel 100µm, right panel 25µm.

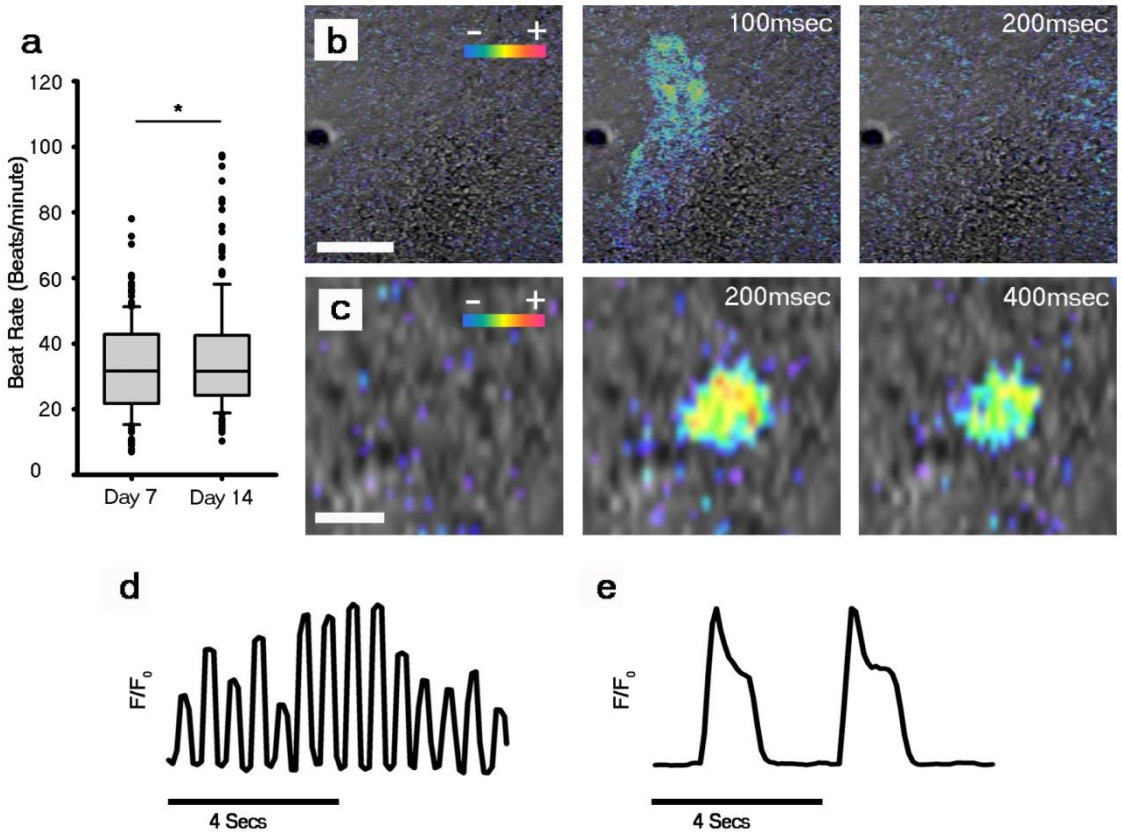

Figure 3.11: Onset of beating and Ca^{2+} transients during *Eomes*-GFP ESC cardiomyocyte differentiation

Beating was first observed in day 7 (D7) *Eomes*-GFP ESC-derived cardiomyocytes. Rate of beating was assessed using bright field movies and calculated at day 7 (n=176 EBs) and day 14 (n=180 EBs), revealing a significant increase between the two time points (a). Cal520 calcium imaging of day 7 ESC-derived cardiomyocytes revealed large fast propagating Ca^{2+} waves (b) as well as slow Ca^{2+} transients in single cells (c). Representative Cal-520 traces for the time series shown in figure 3.11b (d) and 3.10c (e). Scale bars: b, 100µm; c, 10µm. All error bars are mean ± standard deviation. Statistics: ANOVA and Tukey test for comparisons (* $p<0.05$).

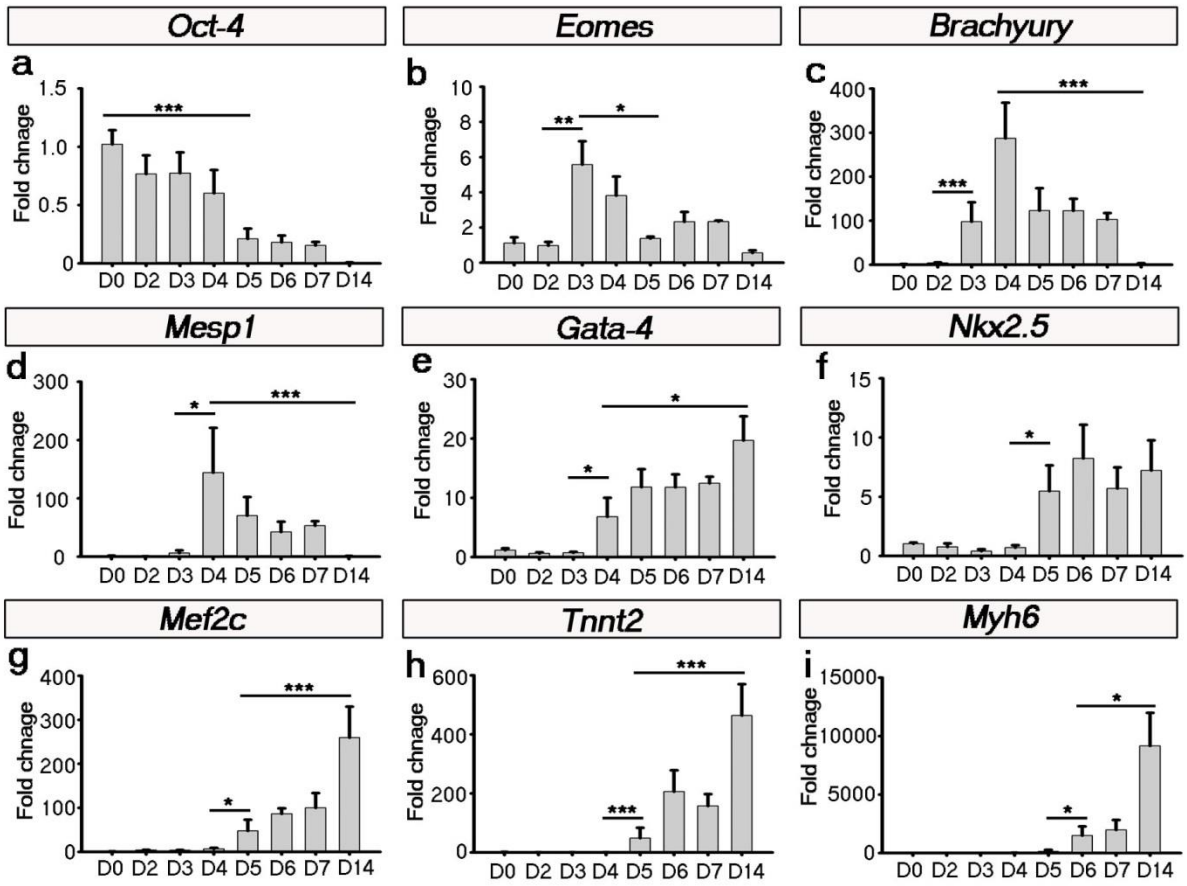

Figure 3.12: Expression of genetic markers during *Eomes*-GFP ESC cardiomyocyte differentiation

Temporal gene expression profiles were assessed during ESC cardiomyocyte differentiation by qRT-PCR and normalised to day 0. Pluripotent marker *Oct-4* decreased throughout the differentiation timecourse (a). Early mesoderm markers *Eomes* (b) and *Brachyury* (c) were significantly unregulated at day 3 of differentiation whilst *Mesp1* (d) increased at day 4 before beginning down regulated thereafter. Cardiac progenitor marker *Gata-4* (e) was first up regulated at day 4 of differentiation whilst other progenitor markers such as *Nkx2.5* (f), *Mef2c* (g) increased from day 5. Mature cardiomyocyte markers increased from day 5 (*Tnnt2*) and day 6 (*Myh6*) with further increases throughout differentiation. N= 5 independent differentiation experiments. All error bars are mean ± S.E.M. Statistics: one-way ANOVA and Tukey test for multiple comparisons (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

3.2.7 Comparison of ESC differentiation and *in vivo* gene profiles

In order to map the gene expression changes that occurred during ESC differentiation onto those occurring during *in vivo* heart development a principal component analysis and cluster analysis of qRT-PCR data was performed (Figure 3.13). The expression profiles of 12 cardiac related genes (*Brachyury*, *Mesp1*, *Gata-4*, *Nkx2.5*, *Mef2c*, *Tnnt2*, *Myh6*, *Cacna1c*, *Slc8a1*, R*yr2*, *Ppp3ca*, *Camk2d*) were used to compare embryonic stages E7.5 to E8.5 with *Eomes*-GFP ESC-derived cardiomyocytes from days 0-7

inclusive and day 14 of differentiation. This analysis revealed no clustering between days 0-3 of ESC differentiation, which would be predicted to represent a significantly earlier developmental stage than E7.5. In contrast, there was correlation between the expression profiles of ESC days 6 and 7 with E8.0, when beating was established in both ESC-derived cardiomyocytes and the embryonic heart proper. The most mature phenotypes, day 14 and E8.5, also correlated strongly together. Thus, gene expression profiles during the onset of contraction in both embryos and ESC cardiomyocyte formation correlated, and appropriately reflected stages of cardiac progenitor specification and differentiation, allowing comparison of both models in parallel.

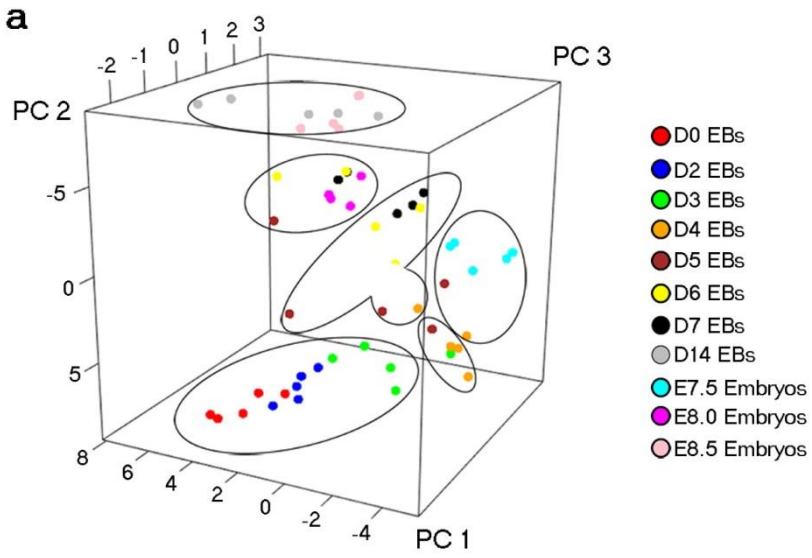

Figure 3.13: Principle component analysis comparing the gene expression during in vivo heart development and ESC-cardiomyocyte differentiation

Principal component analysis (PCA) was conducted using 12 different genes (*Brachyury*, *Mesp1*, *Gata-4*, *Nkx2.5*, *Mef2c*, *Tnnt2*, *Myh6*, *Cacna1c*, *Slc8a1*, *Ryr2*, *Ppp3ca*, *Camk2d*) to compare embryonic stages E7.5 to E8.5 (chapter 3) with days 0-14 of ESC induced cardiomyocyte differentiation. Groups were calculated by hierarchical clustering: E7.5 embryos clustered weakly with day 4/5 EBs, E8.0 embryos clustered with day 6/7 EBs and E8.5 embryos clustered with day 14 EBs representing mature cardiomyocytes within each model (a). **Experiments conducted by R.Tyser, data analysed by A.M. Miranda.**

3.3 Discussion

Studies of mammalian heart development have historically focused on the origin and spatio-temporal allocation of cardiac progenitors and cardiovascular lineage determination. Whilst studies into the identification and regulation of cardiac cell types are important for improved understanding of congenital heart disease, insight into the initiation and development of cardiac function is crucial to investigate the impact of function on subsequent cardiac development in terms of differentiation, maturation and organogenesis. In this chapter, a method for the precise staging of early morphological changes in the cardiac crescent has been established and correlated with the onset of contractile function by using live imaging. In parallel, two different ESC lines have been used to assess cardiomyocyte specification and differentiation, as evidenced by GFP expression, visible contraction, and cardiac specific gene expression, which closely mimicked those seen *in vivo*.

3.3.1 Methods to stage heart development in the mouse embryo

Dynamic changes were observed in both morphology and function during cardiac crescent formation, which required accurate and precise staging. Current methods for staging embryos include somite number and embryonic day/days post coitum, however, these did not directly correlate with heart morphology or function (Kaufman & Navaratnam 1981). During dissections, it became apparent that there was a large variation in the age (embryonic day) of embryos within a single litter as well as in the crescent morphology relative to somite number, suggesting current staging methods do not accurately represent early heart development.

Somites are bilateral-paired blocks of mesoderm, which develop to form skeletal components such as bone, cartilage and muscle. Somitogenesis produces somites at regular intervals from around E7.5-8.5 of development. In chick, these intervals occur regularly (every 90 minutes), however, in mice the interval between somite formation varies (Tam 1981). Although somite number is widely used to stage embryos at early developmental time points, previous studies have noted that heart development is not strictly synchronous with other developmental processes including that of somite formation (Biben & Harvey 1997).

To determine embryonic day, mice were mated in the evening, and the morning of vaginal plug formation was counted as 0.5 days post-coitum (embryonic day), making the assumption that conception occurred in the middle of the night. This method works well for later stages of gestation, when the developmental processes are less dynamic and embryos within a litter have become more uniform. However, at earlier stages of embryonic development the large variation between individual embryos per litter make this approach inappropriate and sub-optimal. Therefore, hearts were grouped based on width and height measurements, enabling the precise characterisation of the onset and changes in cardiac function during early development.

3.3.2 Initiation of the first heartbeat

Formation and organisation of sarcomeres was investigated using immunofluorescence to detect the striated myofilament pattern associated with the overlapping of thick (myosin) and thin (actin) filaments. In the developing heart at stage 1 striated cTnT expression clearly revealed the formation of sarcomeres, suggesting that immature cardiomyocytes had the ability to contract. However, in stage 0 crescents it was apparent that despite expression of cTnT, myofilaments had not become organised into sarcomeres. At this time-point, cTnT expression was localised to the cell membrane, previously shown to be the site of cardiac myofibril assembly. Myofibrils originate, as fibrillar bundles of actin filaments with closely spaced Z-bodies before maturing into sarcomeres with regularly spaced z-lines (Clark et al. 2002). This data reveals that formation of the contractile apparatus occurs during the transition between stage 0 and stage 1 and indirectly implies that contraction could be occurring within stage 1 of the cardiac crescent. Although contractile apparatus was detected at the protein level by immunostaining it does not allow the onset of contraction to be determined. For function to occur, immature cardiomyocytes would also need the ability to raise intracellular Ca^{2+} concentrations to stimulate the contractile machinery.

The difficulty when trying to determine when cardiac contraction is first established is that previous studies have used conventional staging methods, making comparisons difficult due to reasons previously discussed. Using this new staging the earliest

observed contractions in the developing cardiac crescent could be precisely characterised. In these experiments, the mouse strains used were C57Bl/6 males crossed with CD-1 females, to ensure large litter sizes. On average C57Bl/6 females have 6-8 pups per litter whilst CD-1 females have around 12 (data not shown). In these mice with mixed background, contractile function began at stage 1 of crescent formation, correlating with the appearance of sarcomeres. To confirm these findings along with rule out any strain dependent variation, a number of different mouse strains were assessed, including; pure bred CD-1, C57Bl/6 and a Hex-GFP transgenic line (mixed CBA/J and C57BL6 background)(data not shown). Although slight differences were observed in the rate of embryo development, onset of beating occurred at the same stage of cardiac crescent formation in all strains examined.

Early contractile activity correlated with obvious, propagating Ca^{2+} transients from stage 1. These initially propagated across the symmetric cardiac crescent from left to right, which suggests that there is a functional break in left-right symmetry prior to the appearance of morphological left-right differences. This early synchronisation suggests connectivity across the cardiac crescent, as well as the formation of a pacemaker region from which Ca^{2+} waves propagate. Cardiomyocytes are electrically coupled by gap junctions - clusters of channels composed of connexins. In the heart, there are three major subtypes: Connexin-40 (Cx-40), Connexin-43 (Cx-43) and Connexin-45 (Cx-45) (Jansen et al. 2010). In the developing cardiac crescent, expression of Cx-43 by immunohistochemistry was not detected, suggesting connectivity is occurring via Cx-40 or Cx-45 or does not require coupling via gap junctions. It have previously been reported that Cx-45 is the predominant gap junction prior to chamber formation (Kumai et al. 2000) and therefore would be important to characterise.

The basis for the cardiac pacemaker activity in the adult is a controversial and much debated subject with two different hypotheses proposed (Lakatta & DiFrancesco 2009). The “funny current” hypothesis proposes that regularly repeating changes in inward membrane Na^+ current (I_f) are responsible for pacemaker activity (Brown et al. 1979). In opposition, the “calcium clock” hypothesis proposes that intracellular changes in Ca^{2+} concentration determine pacemaker activity (Bogdanov et al. 2001). Applying these two hypotheses to the early heart, which lacks a defined pace making region, pace making could be initiated by either a distinct population of cells within the

cardiac crescent which have an HCN4 derived I_f "funny" current or by cells simply gaining the ability to generate intracellular calcium transients to produce contraction. Previous studies have shown that mouse embryos lacking HCN4 contract rhythmically and survive to adulthood despite a heart rate that is reduced by ~50% (Stieber et al. 2003), blockade of HCN4 in E8.5 cardiomyocytes resulted in a reduced action potential frequency, but did not prevent action potential generation (Liang et al. 2010). Together, this data would suggest that I_f is not essential for initiating cardiac contraction. However, since it is also known that HCN4 is expressed in the early heart, and has recently been suggested to label a population of cardiac progenitor cells (Liang et al. 2013)(Spater et al. 2013), and therefore the role of HCN4 and I_f cannot be ruled out. In contrast, genetic knock out or pharmacological inhibition of calcium handling proteins such as Cav1.2, RyR2 or NCX in mice leads to a lack of heart beat along with embryonic lethality (Seisenberger et al. 2000)(Takeshima et al. 1998) (Wakimoto et al. 2000), suggesting that Ca^{2+} channels are fundamentally required for cardiac development. However, determining whether this phenotype is due to an inability to initiate contraction or simply a loss of pace maker activity has yet to be determined.

3.3.3 Calcium transients precede function in the developing heart

When examining embryos at stage 0 of development, it was evident that single cell Ca^{2+} transients preceded the onset of contraction. This finding raises the questions as to the role of Ca^{2+} transients in the initiation of contraction and development of cardiac function. It also suggests that Ca^{2+} handling is fundamental to the initial formation of the heart, with signalling pathways independent of mechanical force regulating early cardiomyocyte differentiation. The role of Ca^{2+} signalling pathways in the heart is not a new concept; it has been clearly shown in the adult heart that Ca^{2+} signalling has a key role in regulating gene expression during pathological hypertrophy and remodelling (Molkentin 2006). One of the major challenges in the field is separating direct Ca^{2+} signalling pathways from indirect mechanical force pathways induced by regular contraction.

The influence of mechanical force on cardiomyocyte formation and maturation has been well studied, however mechanotransduction signalling is extremely complex. Stretch is known to directly affect the activity and expression of several ion channels

and gap junctions (Jacot et al. 2010), and has a role in cardiomyocyte maturation (Granados-Riveron & Brook 2012), but its role in cardiomyocyte specification and early formation is more difficult to assess. In one study, applying stretch to embryonic stem cells was shown to promote differentiation by increasing expression of cardiac specific genes such as *Mef2c* and *ACTN2* (encodes the protein α-sarcomeric actinin) (Schmelter et al. 2006), although in a second study using human embryonic stem cells, direct stretch was shown to reduce differentiation (Saha et al. 2006). By investigating the role of Ca^{2+} transients prior to contraction it may be possible to elucidate distinct roles for direct Ca^{2+} versus mechanical signalling pathways on cardiomyocyte differentiation.

TTP and TT1/2P measurements were employed to quantify the speed at which intracellular Ca^{2+} increased and decreased (Lindner et al. 2002). These measurements are used in mature cardiomyocytes to assess Ca^{2+} transients due to the fast upstroke and slow decay in Ca^{2+} levels during contraction. The Ca^{2+} waves observed at stage 0 were significantly slower than the propagating waves observed at stage 1, as determined by time to peak (TTP) and time to half maximum (TT1/2M). This raises the question as to how the rate of propagation changes between stages 0/1 as well as how co-ordination of single cell Ca^{2+} transients becomes established. After completing TTP and TT1/2M analysis at stage 0, the rate of Ca^{2+} influx and efflux showed significantly different dynamics compared to the adult. Due to the slow rise in Ca^{2+} levels, in future it may be better to use a more comparable measure of Ca^{2+} rise and decay, such as time from 50% max to peak compared with TT1/2M or comparing the increase and decrease from 10% to 90% fluorescence, although this could be debated.

Ca^{2+} transients at stage 0 had a large variation in both their TTP and TT1/2M. The underlying basis for this variation is most likely due to differences in the expression of proteins which regulate cellular electrophysiology and Ca^{2+} handling, such as channels and exchangers. The increase in the correlation between the TTP and TT1/2M as Ca^{2+} transient speed increased may suggest that differences in the cells' electrochemical properties and related protein expression become more uniform as development progresses. Two possibilities might explain the variation observed in Ca^{2+} transients at stage 0. First, the differences seen reflect the stage of cardiomyocyte differentiation with direct correlation between cellular electrophysiology and the expression of specific genes which control cardiomyocyte lineage, suggesting each cell follows a

predetermined pattern of cardiac Ca^{2+} handling protein expression. Alternatively the electrophysiology and expression of Ca^{2+} handling proteins fluctuates within individual cells in the stage 0 cardiac crescent until a required function/physiology is reached, similar to that seen during the formation of the neuromuscular junction (Prieto-Godino et al. 2012), where cellular physiology acts as signalling entity to feedback and control gene transcription. Further studies will be needed to make any conclusions as to the mechanisms by which these early Ca^{2+} transients are both initiated and regulated.

3.3.4 Technical limitations of using Ca^{2+} dyes to image transients in *ex vivo* embryos

Although the use of dyes allowed the assessment of live calcium dynamics within the developing heart for the first time, this approach has a number of drawbacks which are important to address. The advantage of the Ca^{2+} dye Rhod-2 is that the wavelength of light it emits is around 580 nm allowing it to be combined with other fluorescent reporters such as GFP, however, it also loads to mitochondria and has a relatively high Kd value (570 nM) (Paredes et al. 2008). The Kd value determines how tightly Ca^{2+} binds to the indicator, the high Kd of Rhod-2, therefore, means it may be less sensitive than other Ca^{2+} dyes. Cal-520 is a novel Ca^{2+} dye which has a lower Kd value (320 nM) as well as a better signal-to-noise ratio and does not load to mitochondria (Tada et al. 2014). Using Cal-520, better dye loading and brighter Ca^{2+} signals could be achieved making its use preferable to Rhod-2. The problem with high affinity Ca^{2+} dyes is that, although they have brighter signals, their strong Ca^{2+} buffering capacity may affect physiological Ca^{2+} dynamics and signalling. Unfortunately Cal-520 is not a ratiometric dye and as such the concentration of Ca^{2+} within the cell as well as the efficiency of dye loading has not been assessed.

A second caveat with the use of Ca^{2+} dyes is the degree of tissue penetration. To allow penetration and loading in this case, the overlying endoderm had to be removed, disrupting the cardiac environment and preventing long-term culture. If the endoderm is not removed it takes up the dye and the fluorescent Ca^{2+} signal from the heart is blocked. Surgical removal of endoderm to gain access to the myocardium for *ex vivo* Ca^{2+} imaging could, therefore, influence the contractile activity of the cardiac crescent. Endoderm is known to promote the specification of mesoderm to cardiomyocytes (Murry & Keller 2008) by controlling extracellular signalling molecules, which directly

influence transcription, leading to lineage specific differentiation (Cai et al. 2013). Endoderm has also been shown to have a role in the left-right patterning of cardiac mesoderm (Viotti et al. 2012). Although the endoderm and mesoderm are known to interact, the influence of the endoderm on early cardiac function has not been investigated. To date, our initial observations made at stages 1-3 of crescent formation using DIC imaging suggested that accidental perturbations of the endoderm (made during dissection) affected function, however, further experiments are required to confirm this observation.

An approach which may circumvent the problem of dyes would be to use transgenic mice which express genetically encoded calcium indicators (GECI). GECIs were developed over the last decade to image Ca^{2+} dynamics *in vivo*, mainly in the mouse brain to assess neuronal activity (Tian et al. 2009). One type of GECI is GCaMP, which is based on the calmodulin Ca^{2+} sensing domain and the fluorophore EGFP to assess Ca^{2+} levels. The most recently developed variant GCaMP6f has properties similar to Cal-520 in terms of kinetics, making it suitable for early embryo imaging (Chen et al. 2013). Using a Cre dependent GECI would make it possible to express the Ca^{2+} reporter specifically within the cardiac crescent and image transients without removing the endoderm. A potential Cre line to drive GECI expression would be the Mesp1-Cre transgenic mouse line, as it is one of the earliest cardiovascular markers (Saga et al. 1999).

3.3.5 ESC model of cardiomyocyte differentiation

ESCs have provided an *in vitro* model to study both normal and pathological development in early mouse and human embryos. The pluripotent nature of ESCs gives them the ability to form any embryonic cell type (Beddington & Robertson 1989), making them an important tool when investigating the delineation of early cell fate decisions such as mesendoderm segregation into endoderm and mesoderm. Attempting to study these early cell fate decisions *in vivo* is difficult due the embryonic lethality associated with early knockout (KO) of genes. Working with ESCs, therefore, makes it possible to carry out fine mechanistic studies on pure cell populations which have proved difficult when conducting genetic/epigenetic studies in the embryo.

Using ESCs as a model also has a number of limitations, including a lack of cross-talk between tissues, loss of germ layer spatial organisation, variation in the extent of different cell lineages formed and difficulty in controlling extracellular stimuli. *In vitro* differentiation of ESCs is limited in terms of studying morphogenetic events, such as the spatial organisation of germ layers and the interactions between different tissue types, such as the role of overlying endoderm in cardiac crescent formation. This also means it is difficult to mimic morphogen gradients or reveal true morphogenetic actions of growth factors such as BMP2, a key growth factor involved in cardiogenic mesoderm specification (Schultheiss et al. 1997). The pluripotent nature of ESCs can also lead to misleading results as non-physiologically relevant differentiation can occur due to the plasticity of these cells.

The hanging drop method of cardiomyocyte differentiation allowed reproducible formation of contractile cardiomyocytes in around 70% of embryoid bodies. Although all experiments followed the same sequence of gene marker expression there was a significant degree of variation in the relative mRNA levels between experiments. This variation could be due to a number of different variables, including the levels of growth factors and signalling molecules within the culture media. Using the hanging drop method of differentiation EBs contained a mixed population of cells, representing multiple differentiation pathways. The cell type heterogeneity within the EBs will lead to variations in the growth factors and signalling molecules released by different cell types and, therefore, influence cardiogenic specification and maturation. Another variable affecting the levels of growth factors is the serum used, therefore, serum batch variation may affect EB cell heterogeneity and cardiomyocyte formation. Other methods which may be more appropriate in terms of reducing the variation in cardiomyocyte differentiation and producing a purer population of cardiomyocytes include models of directed differentiation using chemically defined media, as well as small molecules to promote cardiomyocyte formation and reduce the myriad of other potential cell types (Kattman et al. 2011).

3.3.6 Comparisons between the *Nkx2.5*-GFP and *Eomes*-GFP ESC lines

Although both ESC models (*Nkx2.5*-GFP and *Eomes*-GFP) led to the production of beating cardiomyocytes it is interesting to note the differences observed between the

two. Overall the *Eomes*-GFP cell line was the more reliable model in terms of cardiomyocyte formation and temporal onset of beating relative to the *Nkx2.5*-GFP ESC line, however, the *Nkx2.5*-GFP model had stronger cardiac gene expression and provided an easy fluorescent read-out of cardiomyocyte differentiation.

The main difference between the models was in the speed at which differentiation occurred. Using the *Nkx2.5*-GFP ESCs beating was observed at around day 8 of differentiation whilst Eomes ESCs had already started to contract around day 6/7, comparable with the differences observed in formation of sarcomeres. It is difficult to compare the two ESC models directly in terms of temporal gene expression profiles, due to differences in the time-points when samples were collected, although both followed similar trends. However, there were clear differences in the overall level of gene expression relative to day 0. Despite the *Nkx2.5*-GFP cell line taking longer to form functional cardiomyocytes, the maximum expression for each gene was much greater in this model compared to the *Eomes*-GFP Esc line. Expression of mesoderm genes, such as *Brachyury* and *Mesp1,* were greater in the *Nkx2.5*-GFP model compared to the *Eomes*-GFP model at day 4 (*Brachyury* ~2.7x and *Mesp1* ~5.3x); cardiac progenitor genes such as *Nkx2.5* and *Mef2c* were ~10.9 and ~5.0 fold greater in the *Nkx2.5*-GFP model respectively, at day 14, whilst the mature marker *Myh6* was ~32.0 fold higher. The differences between the two models as differentiation proceeded suggests that mesoderm specification is greater in the Nkx2.5-GFP model leading to more cardiomyocyte differentiation due to an increased number of cardiac progenitors.

The most likely reasons for the variation seen between the *Eomes*-GFP and *Nkx2.5*-GFP ESCs during differentiation are due to differences in the original cell line used, the method used to make each, the effect of the inserted transgene and how each had been previously maintained (Fedorov et al. 1997). The Nkx2.5-GFP ESC line was created by injecting CJ7 ES cells with a vector containing eGFP under the control of a murine Nkx2.5 promoter and a regulatory region (S. M. Wu et al. 2006)(Lien et al. 1999). This is in contrast to the knock in Eomes-GFP ESC line which used a CCE ES line (Robertson et al. 1986) to insert a eGFP reporter cassette into the translational start site of the endogenous Eomes gene leading to the deletion of 500bp within exon 1 (Arnold et al. 2009). The difference in starting cell line as well as the method of eGFP

reporter insertion is likely to significantly influence downstream gene expression and cardiomyocyte differentiation.

The RT-PCR method used for these studies measured relative changes in gene expression compared to a calibrator. The calibrator chosen for the timecourse experiments was the undifferentiated day 0 ESCs, meaning that all changes in gene expression are relative to the starting population of cells and are not an absolute quantification. Using day 0 as the calibrator, therefore, means that any differences in the starting population of cells would affect the relative quantification of gene expression at later stages of differentiation. Although every effort was made to keep the starting populations of ESCs pluripotent, any difference in the number of pluripotent versus committed cells would not only affect differentiation but would also affect the relative analyse of gene expression.

Overall, both models followed similar trends and led to the production of ESC-derived cardiomyocytes, however, it is important to take into consideration the different levels of gene expression when making conclusions in future experiments.

3.3.7 Formation of cardiomyocytes

Both ESC lines led to the production of beating cardiomyocytes with gene expression profiles following the well documented stage-specific timecourse of ESC differentiation from the pluripotent state to defined cardiomyocytes as determined using established markers (Van Vliet et al. 2012)(Li et al. 2015). In terms of validation, it was striking to see that the initial rate of beating in both the ESC model and the embryonic cardiac crescent were similar at around 30 bpm, refer to Figure 4.5 a, suggesting that the *in vivo* and *in vitro* models of cardiomyocyte development correlate in terms of function. This observation further suggests that immature developing cardiomyocytes have an intrinsic rate at which they initially start beating as discussed previously. There was also a significant increase in the rate of beating as ESC-derived cardiomyocytes matured between day 7 and day 14 which suggests increases in contractile rate underlie cardiomyocyte maturation independent of external cues and pacemaker activity.

Another important finding which again validated the ESC model and was consistent with observations *in vivo* was the identification of individual differentiating ESC cells which were able to generate Ca^{2+} transients prior to contraction. At day 7 these were located in close proximity to small contractile regions, despite not being contractile themselves. Ca^{2+} dye imaging, revealed Ca^{2+} oscillations within single cells which had slow dynamics in terms of transient wave duration, again suggesting a potentially fundamental role for Ca^{2+} handling in cardiomyocyte differentiation. When observing these cells it was clear to see that they had a small and round morphology suggestive of a more undifferentiated phenotype. To further characterise these cells it would be important to investigate stages earlier than day 7, whilst also using a cell line which would permit identification of the origin prior to contraction such, as a *Mesp1*-GFP ESC line (Bondue et al. 2011). The ability of cells to handle Ca^{2+} prior to contraction suggests that calcium signalling has a key role in the regulation of cardiac specific genes. ESC models will therefore allow mechanistic insight into this regulation.

3.4 Summary

In this chapter, crescent morphology, cardiac function and gene expression changes have been examined during the early stages of heart formation allowing the correlation of form and function in the developing cardiac crescent. Along with this characterisation, Ca^{2+} transients were observed in single cells within the cardiac crescent prior to the onset of contraction, suggesting that Ca^{2+} signalling could be acting as an independent signalling entity to contraction during early heart development. This chapter has also described the formation and differentiation of cardiomyocytes using two different ESC lines. Correlation of these findings with *in vivo* results has validated the ESC differentiation and generated a stage matched model of ESC cardiomyocyte formation, allowing the mechanisms involved in heart development to be more reliably investigated *in vitro*. In the following chapters, the regulation of early cardiac function will be investigated as well as the downstream influence Ca^{2+} transients have on gene expression, cardiomyocyte differentiation and heart development.

4. CHARACTERISATION OF POTENTIAL CHANNELS AND EXCHANGERS REQUIRED FOR Ca^{2+} TRANSIENTS DURING FORMATION OF THE HEART

4.1 Introduction

In the adult heart, coordinated electrical excitation is coupled to physical contraction in a process termed excitation contraction coupling (ECC). ECC relies on the second messenger Ca^{2+}, which binds to cardiac myofilaments resulting in contraction and creating the force needed to pump blood around the body (Bers 2002). Although this mechanism of contraction has been well described in the adult, this study aimed to investigate how Ca^{2+} handling occurs during formation of the cardiac crescent. Targeted disruption of genes encoding ECC proteins has shown that prior to E13.5, contraction can occur without the need for ECC, via a non-defined mechanism. Whilst the functional significance of the SR in immature cardiomyocytes is debated it is widely excepted that sarcolemmal Ca^{2+} flux is a predominant source of Ca^{2+} required to generate early contractile activity. Whilst characterising the development and initiation of cardiac function, large changes were observed in Ca^{2+} transient dynamics during the onset of contraction (Chapter 3). We, therefore, sought to understand the potential mechanisms which may be regulating the concentration of intracellular Ca^{2+}.

This chapter will examine both the *in vivo* and *in vitro* expression of genes and proteins involved in cardiomyocyte Ca^{2+} handling and signalling, focusing on the earliest stages of cardiomyocyte differentiation. It will also assess the functional role of sarcolemmal channels and exchangers using acute pharmacological inhibition during both early murine heart development (cardiac crescent formation) and ESC- derived cardiomyocyte differentiation.

4.2 Results

4.2.1 Temporal variability in the expression of Ca^{2+} related genes during late heart development

To characterise the expression of ECC genes as well as other Ca^{2+} related genes during maturation of the developing heart, mRNA expression was measured using qRT-PCR and correlated with key stage specific transcription factors implicated in cardiac differentiation as described in chapter 1. During maturation of the heart key ECC genes were significantly elevated in the postnatal (*Cacna1c*; 16.0 fold ±1.1; p=0.002; *Ryr2*; 8.3 fold ±1.1; p≤0.001; *Atp2a2*; 3.7 fold ±0.3; p=0.004; Figure 4.1b-d) and adult heart samples (*Cacna1c*; 11.5 fold ±3.0; p=0.008; *Ryr2*; 12.0 fold ±1.1; p≤0.001; *Atp2a2*; 8.0 fold ±1.9; p≤0.001) compared to the embryonic heart at E9.5. In contrast to *Cacna1c* and SR related ECC genes, *Slc8a1* (encoding NCX1) did not change from E9.5, suggesting that *Slc8a1* was maximally expressed in the heart by E9.5 (Figure 4.1 a) (Reppel, Sasse, et al. 2007). A number of other Ca^{2+} related genes were also assessed. *Scn5a*, the main Na^{+} channel involved in cardiomyocyte depolarisation (Haufe et al. 2005) was significantly elevated in the P0 (3.8 fold ±0.3; p=0.007; Figure 4.1 e) and adult heart (4.3 fold ±0.7; p=0.005) compared to E12.5. *Itp3r2*, encoding IP_3R, a channel known to be involved in Ca^{2+} release from the endoplasmic reticulum (Perez et al. 1997) was significantly increased in the postnatal (6.9 fold ±0.2; p=0.002; Figure 4.1 f) and adult heart (4.1 fold ±0.2; p=0.002) compared to the E9.5 heart. The final calcium channels studied were the two pore channels 1 and 2 (TPC1/2), encoded by *Tpcn1* and *Tpcn2*, known to release calcium from lysosomal stores (Calcraft et al. 2009; Brailoiu et al. 2009), which remained unchanged between E9.5 and the adult stages (Figure 4.1 g, h). Together these results reveal that whilst mature ECC genes increase from E9.5 into the adult, expression of the NCX1 encoding gene, *Slc8a1,* remains unchanged throughout the later stages of heart development suggesting NCX1 is maximally expressed by E9.5.

In order to investigate NCX1 protein expression and localisation, immunohistochemistry was performed on E12.5 heart cryosections. Cardiomyocytes were labelled using alpha-actinin staining and examined by confocal microscopy. NCX1 was expressed in both the ventricle and atria and exhibited an anticipated membrane localisation as shown in Figure 4.2. Overall these findings indicate that NCX1 is strongly

expressed in the heart by E9.5 and that the level of gene expression is maintained throughout development, this was in contrast to other cardiac specific Ca^{2+} handling genes which increased in a similar manner to mature cardiomyocyte markers such as α-MHC (Figure 3.7).

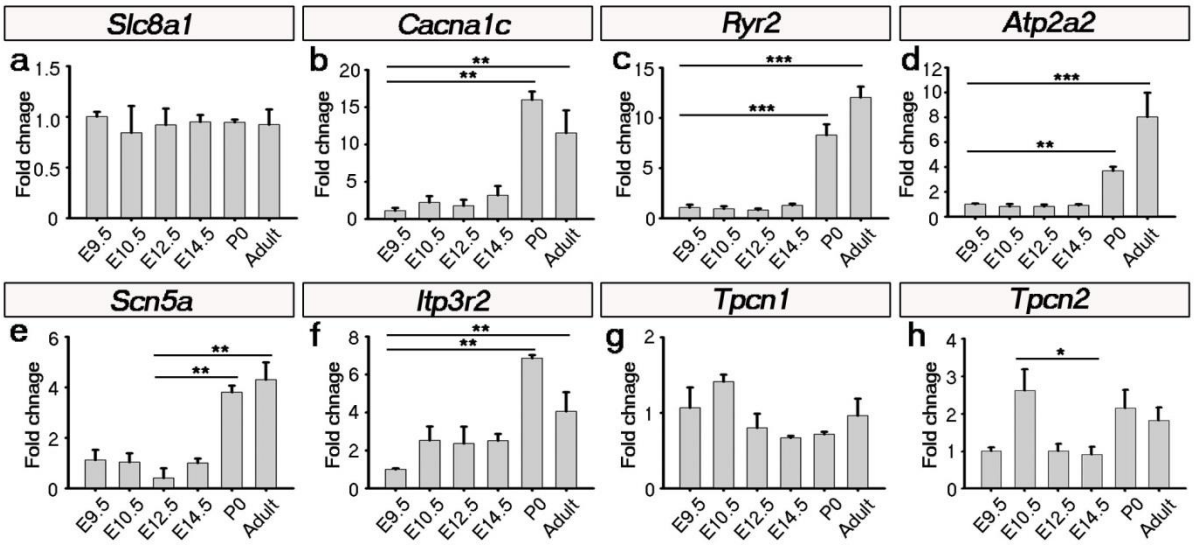

Figure 4.1: Temporal expression patterns of Ca^{2+} genes during *in vivo* cardiomyocyte maturation

Gene expression was assessed during maturation of the heart from E9.5 using qRT-PCR on isolated mouse heart samples. ECC genes including Cacna1c (b), Ryr2 (c), Atp2a2 (d) as well as Scn5a (e) and Itp3r2 (f) significantly increased in the postnatal day 0 and adult hearts samples, whilst Slc8a1 (a), Tpcn 1 (g) and 2 (h) remained constant from E9.5. All error bars are mean ± S.E.M. Statistics: one-way ANOVA and Tukey test for multiple comparisons (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

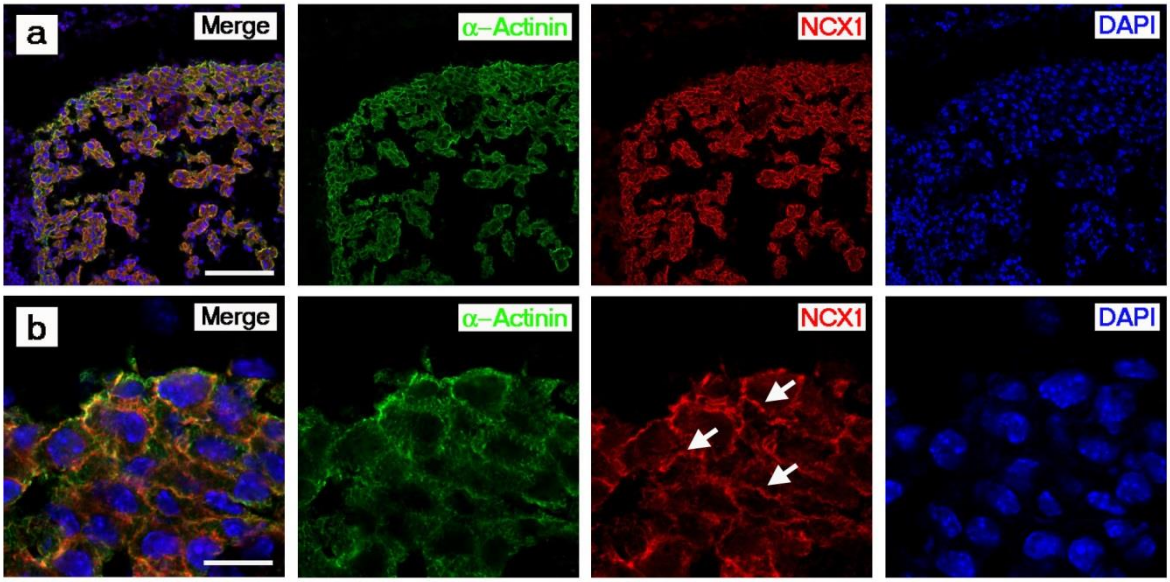

Figure 4.2: Localised expression of NCX1 in E12.5 mouse hearts

Immunocytochemistry imaging revealed the expression of NCX1 in α-actinin positive E12.5 mouse hearts (a); higher magnification images revealed membrane localised expression of NCX1 (b). Arrows highlight membrane localised expression. Scale bars: a, 100µm; b, 15µm.

4.2.2 Expression of NCX1 and LTCC during cardiac crescent formation

Due to the early expression of *Slc8a1* (which encodes NCX1) at E9.5 and the findings, in chapter 3, on function within the cardiac crescent it was important to study time points earlier than E9.5. Using immunohistochemistry the expression of the main sarcolemmal ECC proteins NCX1 and Cav1.2 was assessed. In the late stage2/3 cardiac crescent, expression and localisation of both NCX1 and Cav1.2 was detected and corresponded with cTnT expression (Figure 4.3a, b). However, whilst NCX1 was clearly detectable within the early cardiac crescent at stage 0, Cav1.2 was absent (Figure 4.4 e, f). To identify a transcriptional difference that could explain the difference in protein levels, RNA was collected and analysed from whole embryos at E7.5 (prior to crescent formation), E8.0 (cardiac crescent) and E8.5 (linear heart tube). At these early time points there was a significant increase in *Slc8a1* expression between days E7.5 and E8.0 (2.2 fold ±0.3; $p \leq 0.001$; Figure 4.4 a) whilst *Cacna1c* (which encodes Cav1.2) expression was unchanged (Figure 4.4 b). By E8.5 there was significant up regulation in both *Slc8a1* (12.1 fold ±0.5; $p \leq 0.001$) and *Cacna1c* (3.7 fold ±0.9; $p \leq 0.01$). The early

expression of membrane localised NCX1 expression within the stage 0 cardiac crescent therefore correlates with the increase in *Slc8a1,* between E7.5 and E8.0.

RyR2 and HCN4 mRNA levels were also studied because of their role in adult cardiac physiology. RyR2 is responsible for Ca^{2+} release from the SR and is fundamental for contraction within the mature heart (Bround et al. 2012), however, its role in early cardiac function is debated (Rapila et al. 2008). HCN4 is functionally required for the spontaneous pacemaker activity of the heart (Difrancesco 2010) although the mechanism for pacemaker activity in the developing cardiac crescent is unknown. HCN4 has also been reported to demarcate a source of cardiac progenitors during formation of the heart. *RyR2* and *Hcn4* were found to be significantly up-regulated between E7.5 and E8.5 (*Ryr2*; 9.4 fold ±1.5; p≤0.001; *Hcn4;* 4.8 fold ±0.5; p=0.001 Figure 4.4 c, d). At E8.0 *Ryr2* was significantly up regulated compared to E7.5 (3.1 fold ±0.1; p=0.003), whilst *Hcn4* was unchanged. Therefore, during formation of the cardiac crescent and transition to the linear heart tube both RyR2 and HCN4 increase, potentially resulting in changes in cardiac physiology.

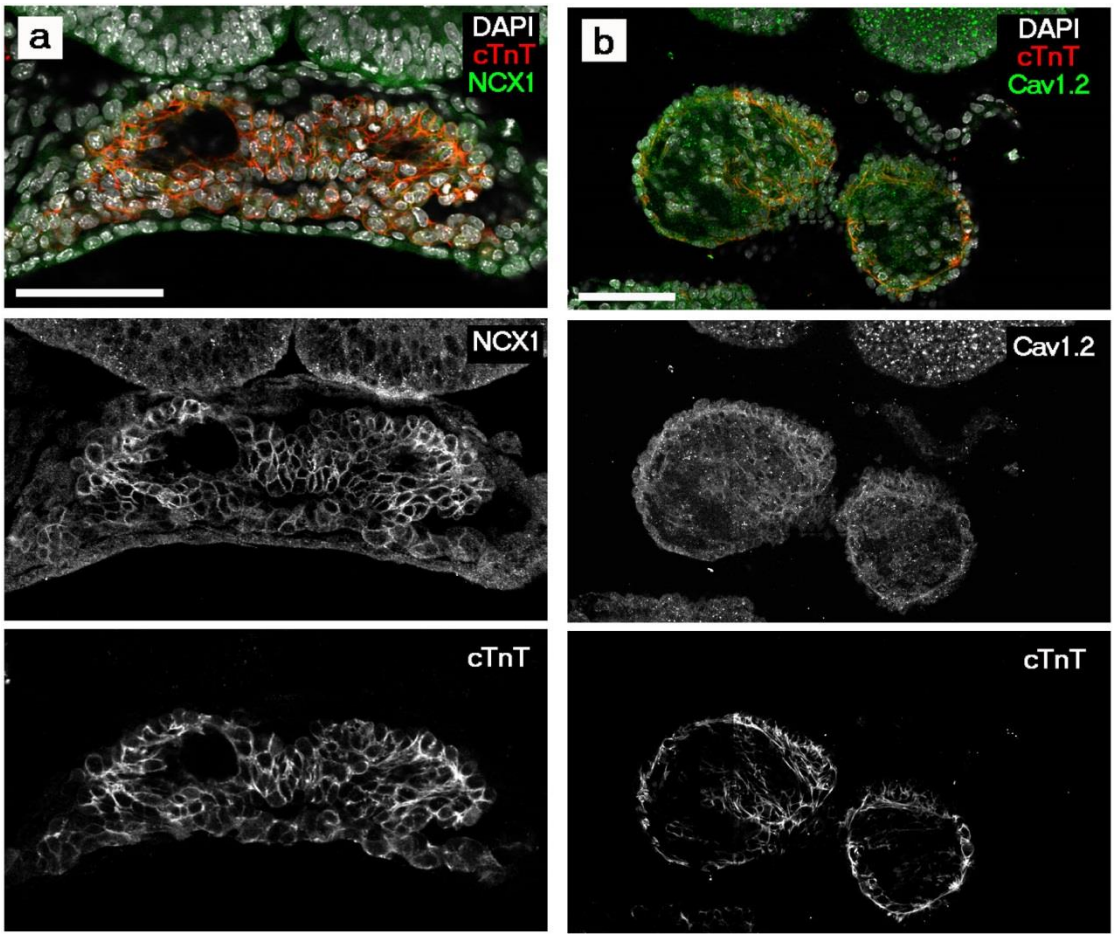

Figure 4.3: Membrane localised expression of NCX1 and Cav1.2 in stage 2 cardiac crescent

Using whole mount immunohistochemistry and confocal imaging of embryos it was possible to detect the expression of both NCX1 and Cav1.2 within stage 2 cardiac crescents, as demarcated by cTnT expression. Scale bars: 100µm.

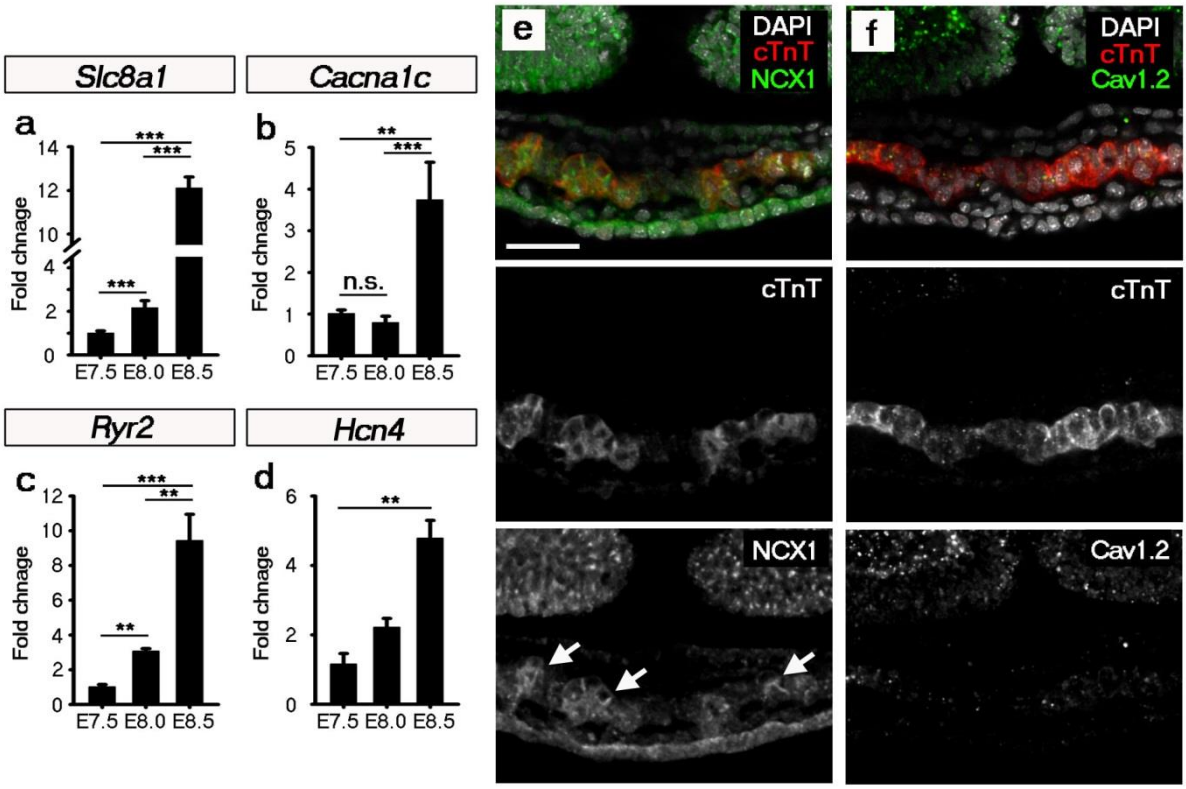

Figure 4.4: Temporal expression patterns of NCX1 and Cav1.2 during formation of the cardiac crescent

Temporal gene expression profiles were assessed on whole embryos by qRT-PCR, normalised to E7.5: significant up-regulation of both Slc8a1 and RyR2 occurred by E8.0 increasing further at E8.5, whilst Cacna1c and Hcn4 didn't significantly increase until E8.5. Immunostaining revealed the expression of NCX1 (arrows, e) but not Cav1.2 (f) in the cardiac crescent at stage 0 as marked by the expression of cTnT. All error bars are mean ± S.E.M. Statistics: ANOVA and Tukey test for multiple comparisons (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). Scale bars: 50µm.

4.2.3 Temporal variability in the expression of genes related to Ca^{2+} regulation during Nkx2.5-GFP ESC differentiation

To enable the identification of potential proteins involved in the regulation of the earliest Ca^{2+} transients and heart function, gene expression profiles of functionally relevant channels and exchangers were initially investigated using *Nkx2.5*-GFP ESC cardiomyocyte differentiation. Figure 4.5 Shows that apart from *Atp2a2* (encoding SERC2a) which was unchanged (Figure 4.5 d), there was a significant up regulation in the main ECC Ca^{2+} handling genes over the 14 day timecourse (*Slc8a1*; 373.1 fold ±111.6; $p\leq0.001$; *Cacna1c*; 76.1 fold ±10.9; $p\leq0.001$; *Ryr2*; 5.6 fold ±1.4; $p=0.025$; Figure 4.5 a, b, c). NCX1 mRNA was significantly up regulated by day 4 of

differentiation, compared to levels at day 0 (5.7 fold ±0.9; p=0.011), however, there was no change in the mRNA levels of the sarcolemmal Ca^{2+} channel, Cav1.2, or the SR Ca^{2+} channel, RyR2, over the same timescale. Other channels involved in adult heart function included Nav1.5 and Cav3.1 (encoded by *Scn5a* and *Cacna1g*, respectively). Nav1.5 is responsible for the depolarisation of cardiomyocytes upon electrical excitation and therefore has a key role in triggering Ca^{2+} influx and generating contraction. Cav3.1 is a T-type sarcolemmal Ca^{2+} channel reported to be involved in pace maker activity and Ca2+ influx during early heart development. Following formation of embryoid bodies, *Scn5a* was up-regulated by day 2 of differentiation (8.7 fold ±2.6; p=0.001; Figure 4.5 e), increasing further at day 14 (13.8 fold ±4.0; p≤0.001), whilst C*acna1g* was not up regulated until day 14 (19.0 fold ±13.5; p=0.021; Figure 4.5 i). The lysosomal Ca^{2+} channels TPC1 and TPC2 (encoded by *Tpcn1* and *Tpcn2*) had different expression profiles during ESC differentiation, *Tpcn1* was significantly elevated at day 7 (*Tpcn1*; 3.5 fold ±0.5; p=0.005), whilst *Tpcn2* expression remained constant throughout (Figure 4.5 g, h). Other Ca^{2+} channels investigated included the Transient receptor potential channel 3 (TRPC3 encoded by *Trpc3*), the IP_3R channel (encoded by *Itp3r2*) and the store-operated related channels Stim1 and Orai1 (encoded by *Stim1* and *Orai1*) which have been reported to be involved in the regulation of pacemaker activity, pathological Ca^{2+} influx and signalling during cardiac hypertrophy as well as ESC cardiomyocyte differentiation (Bush et al. 2006) (Arantes et al. 2012) (Voelkers et al. 2010). Over the 14 day protocol, *Trpc3* expression was elevated at day 7 of differentiation (*Trpc3;* 28.9 fold ±11.5; p=0.016; Figure 4.5 j), whilst, Itp3r2 did not significantly increase until day 10 (18.4 fold ±5.0; p=0.037; Figure 4.5 f). *Stim1* and *Orai1* remained constant throughout the differentiation protocol (Figure 4.5 k, l). Overall this data reveals a gene expression timecourse for certain channels and exchangers involved in cardiomyocyte physiology and highlights the early expression of NCX1 prior to mature cardiomyocyte marker expression (Figure 4.3).

Immunohistochemistry was used to investigate NCX1 protein expression and localisation. NCX1 was observed in *Nkx2.5*-positive cells and localised to the membrane at day 7 (Figure 4.6 b). NCX1 membrane expression was also present in cells that did not express *Nkx2.5*-GFP. By day 14, it was observed that all *Nkx2.5*-GFP positive ESC derived cardiomyocytes strongly expressed membrane localised NCX1

protein, whist regions which were *Nkx2.5*-GFP negative did not express NCX1 (Figure 4.6 a). This data confirms the expression of NCX1 protein during ESC differentiation and reveals a heterogeneity in the cell types which express membrane localised NCX1.

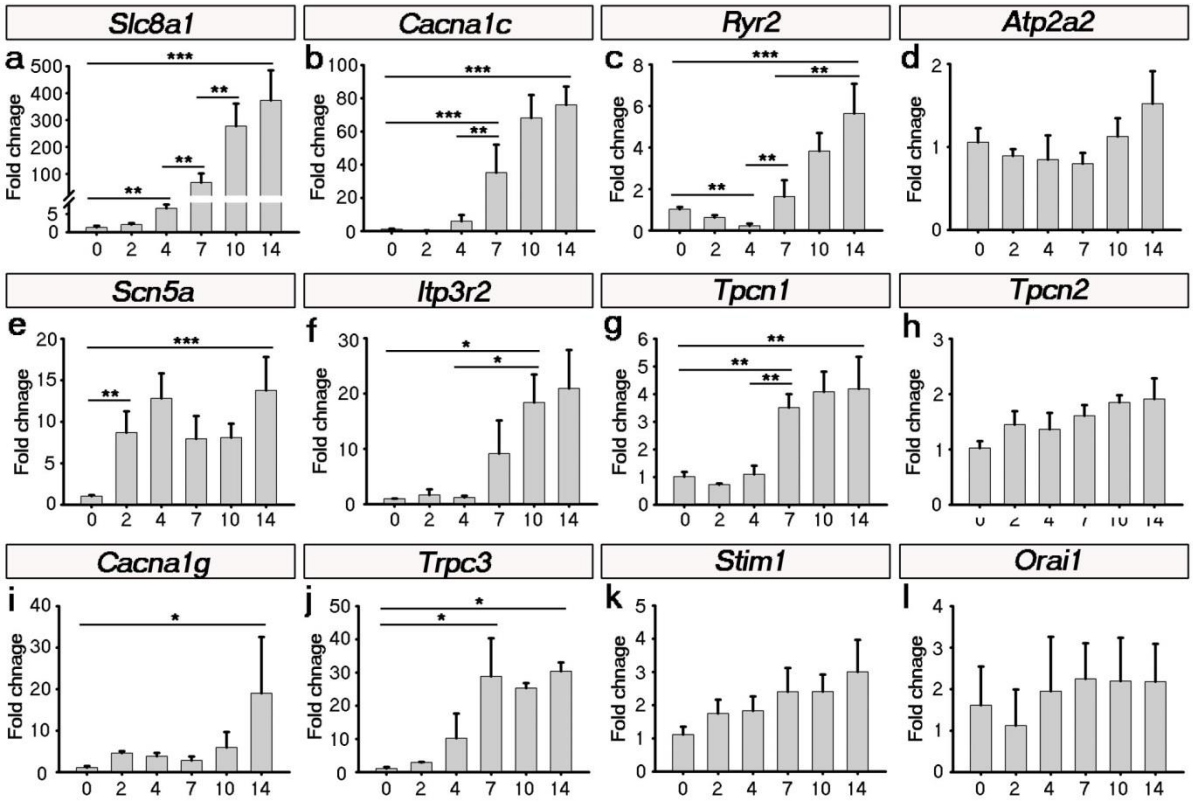

Figure 4.5: Temporal expression patterns of Ca^{2+} genes during Nkx2.5-GFP ESC cardiomyocyte differentiation

Gene expression was assessed during ESC cardiomyocyte differentiation using qRT-PCR. ECC genes including Slc8a1 (a), Cacna1c (b) and Ryr2 (c) increased throughout differentiation from between day 4 and 7 whilst Atp2a2 (d) remained constant. Scn5a (e) became significantly up-regulated by day 2 of differentiation, with expression levels remaining constant throughout the rest of differentiation. Itp3r2 (f), Tpcn1 (g), Cacna1g (i) and Trpc3 (j) all increased as differentiation progressed towards day 14. Tpcn2 (h), Stim1 (k) and Orai1 (l) remained constant through Esc cardiomyocyte differentiation. All error bars are mean ± S.E.M. Statistics: one-way ANOVA and Tukey test for multiple comparisons (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

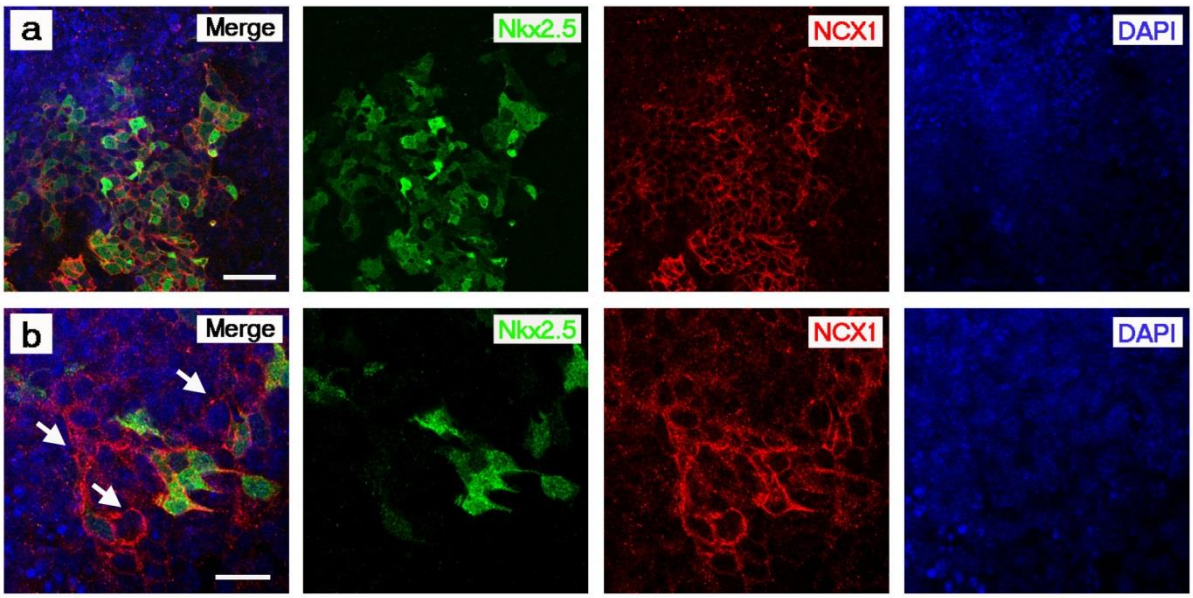

Figure 4.6: Membrane localised expression of NCX1 during Nkx2.5-GFP cardiomyocyte differentiation

Immunocytochemistry revealed the expression of NCX1 (red) in Nkx2.5 -GFP (green) positive regions at day 14 (a). By day 7 membrane localised expression of NCX1 was observed in cells which expressed Nkx2.5-GFP but also in cells which were Nkx2.5-GFP negative (arrows) (b). Scale bars: a, 50µm; b, 25µm.

4.2.4 Temporal variability in the expression of Ca^{2+} related gene expression during EOMEs-GFP ESC differentiation

To confirm the results gathered from the *Nkx2.5*-GFP ESC line and to investigate with higher temporal resolution the expression changes of Ca^{2+} handling genes occurring during ESC differentiation experiments were repeated using the *Eomes*-GFP ESC line. During differentiation both *Slc8a1* and *Cacna1c* were up-regulated by day 14 (*Slc8a1*; 373.4 fold ±40.0; p≤0.001; *Cacna1c*; 124.4 fold ±21.0; p≤0.001; Figure 4.7 a, d), however, there was a significant increase in *Slc8a1* by day 2 (4.0 fold ±0.8; p=0.004; Figure 4.7 b) whilst *Cacna1c* was not up-regulated until day 4 of differentiation (5.9 fold ±1.9; p=0.009; Figure 4.7 e). *Ryr2* expression was significantly up-regulated by day 14 of differentiation compared to day 0 (6.1 fold ±1.3; p=0.043; Figure 4.7 c), in contrast to *Atp2a2* (encoding SERC2a) remained unchanged throughout differentiation (Figure 4.7 f). Other genes related to cardiomyocyte physiology included *Scn5a* (encoding Nav1.5) and *Cacna1g* (encoding Cav3.1) which were both significantly up-regulated by day 2 (*Scn5a*; 4.6 fold ±0.9; p≤0.001; *Cacna1g;* 4.7 fold ±1.2; p≤0.001; Figure 4.7 h, i), whilst *Scn5a* stayed relatively constant throughout subsequent differentiation, *Cacna1g* became significantly up-regulated at day 14 compared to day 7 (91.2 fold ±10.5; p≤0.001; Figure 4.7 g). *Atp1a2* (encoding Na^+/K^+ATPaseα2) was first up-regulated at day 4 (6.8 fold ±2.2; p=0.014; Figure 4.7 k) before increasing further at day 14 (45.7 fold ±16.4; p≤0.001; Figure 4.7 j), in contrast *Slc9a1* (encoding NHE1) remained constant throughout differentiation from day 0-4 before increasing at day 14 (2.8 fold ±0.5; p=0.003; Figure 4.7 l).

Overall this data reveals that NCX1, Cav3.1 and Nav1.5 gene expression is up regulated prior to the onset of contraction and formation of cardiomyocytes and significantly earlier than other cardiomyocyte related channels and exchangers.

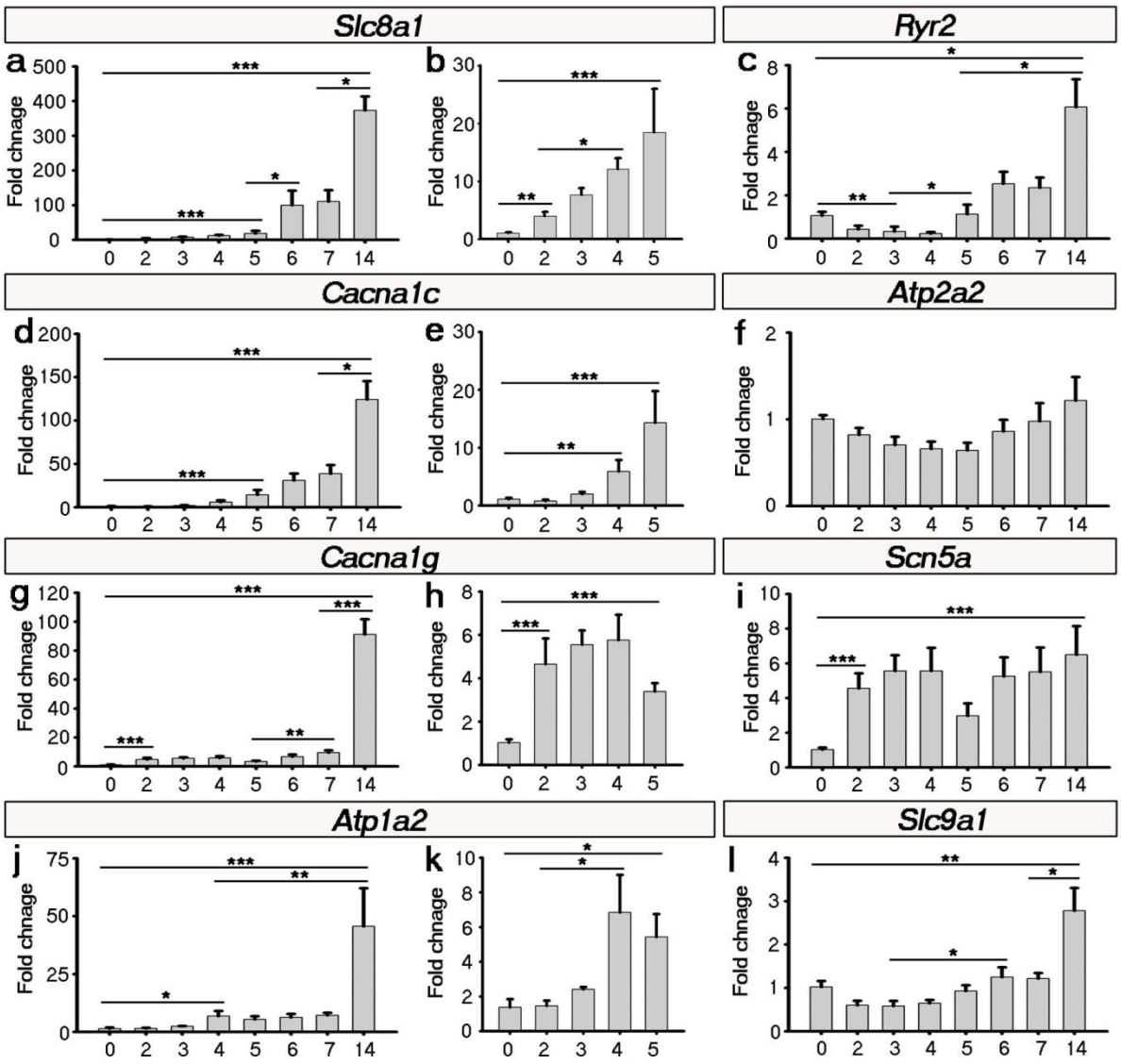

Figure 4.7: Temporal expression patterns of Ca^{2+} genes during Eomes-GFP ESC cardiomyocyte differentiation

Gene expression was assessed during ESC cardiomyocyte differentiation using qRT-PCR. Slc8a1 (a, b), Scn5a (i) and Cacna1g (g,h) genes were significantly up-regulated by day of differentiation, with both Slc8a1 and Cacna1g increasing again at later time points. Cacna1c (d, e) and Atp1a2 (j, k) mRNA levels became significantly elevated by day 4 of differentiation compared to day 0. The gene Atp2a2 (f) remained constant throughout differentiation whilst Ryr2(c) and Slc9a1 (I) became up-regulated by day 5 and 6, respectively, before increasing further at day 14. All error bars are mean ± S.E.M. Statistics: one-way ANOVA and Tukey test for multiple comparisons (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

4.2.5 Expression of NCX1 and Cav1.2 during Eomes-GFP cardiomyocyte differentiation

Although relative mRNA levels give an approximate indication about protein levels, it was important to directly measure and localise protein expression. In *Eomes*-GFP ESCs at day 7 of differentiation, both NCX1 and Cav1.2 were expressed, but whilst NCX1 was consistently co-expressed with cTnT (Figure 4.8 a), there were clear cTnT+ve foci which were negative for Cav1.2 (Figure 4.8 b). Further immunohistochemical analysis showed that whilst Brachyury positive cells did not express NCX1, the latter was found in close proximity to Brachyury positive cells at day 7 (Figure 4.9). At day 4 there was clear expression of Brachyury when imaging whole mount EBs as well as distinct puncta of NCX1 which looked to be expressed at the membrane and were not detectable in secondary antibody negative controls (Figure 4.10).

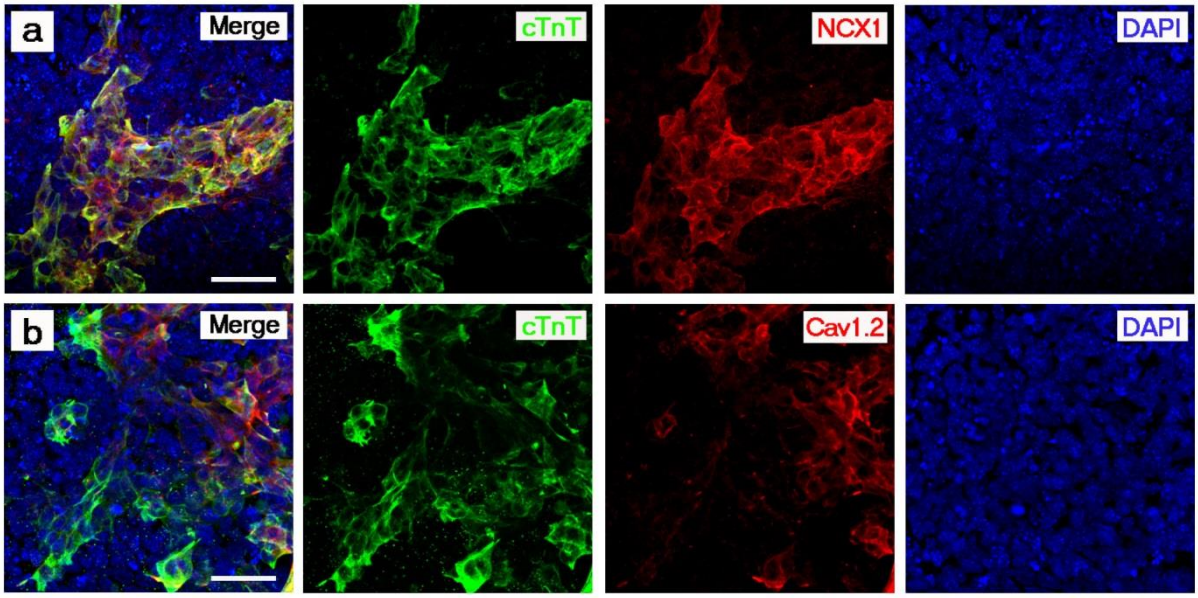

Figure 4.8: Expression of NCX1 and Cav1.2 at day 7 of Eomes-GFP ESC cardiomyocyte differentiation

Immunocytochemistry at day 7 of ESC-cardiomyocyte differentiation revealed the complete overlap of NCX1 (green) and cTnT (red) expression (a), whereas Cav1.2 (green) expression overlapped in certain regions with cTnT (red) but not all regions expressed both Cav1.2 and cTnT (b) as seen with NCX1. Scale bars: 50μm.

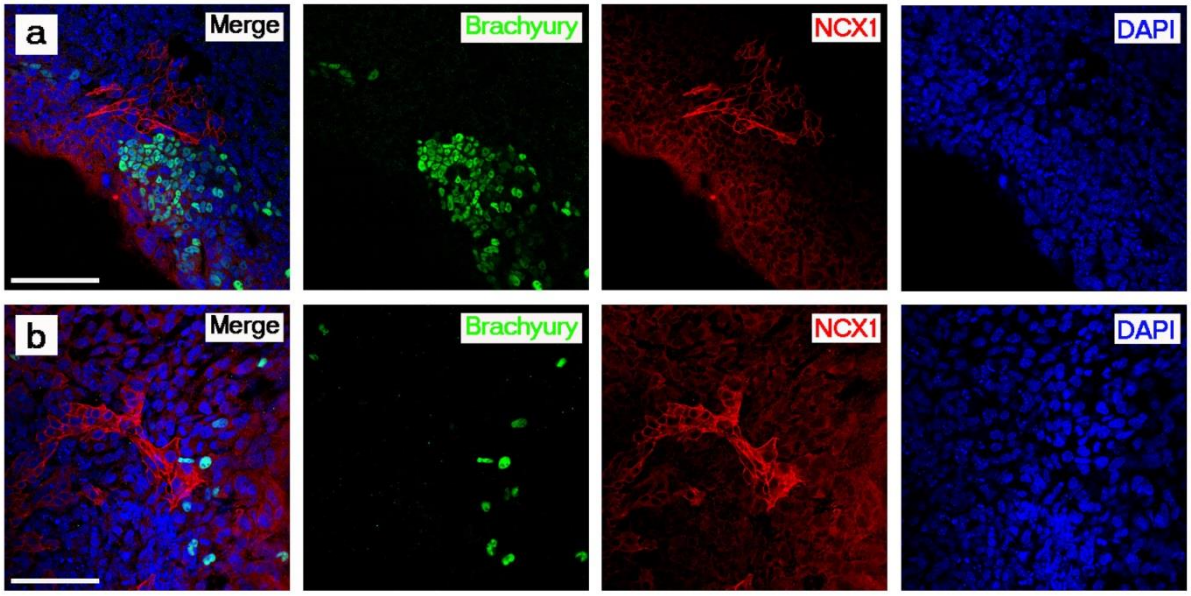

Figure 4.9: Expression of NCX1 and Brachyury at day 7 of Eomes-GFP ESC cardiomyocyte differentiation

At day 7, immunocytochemistry of ESC-cardiomyocytes revealed NCX1 (red) expressing cells sitting adjacent to Brachyury positive cells (green) suggesting a potential interaction between the two cell types. Scale bars: 100µm.

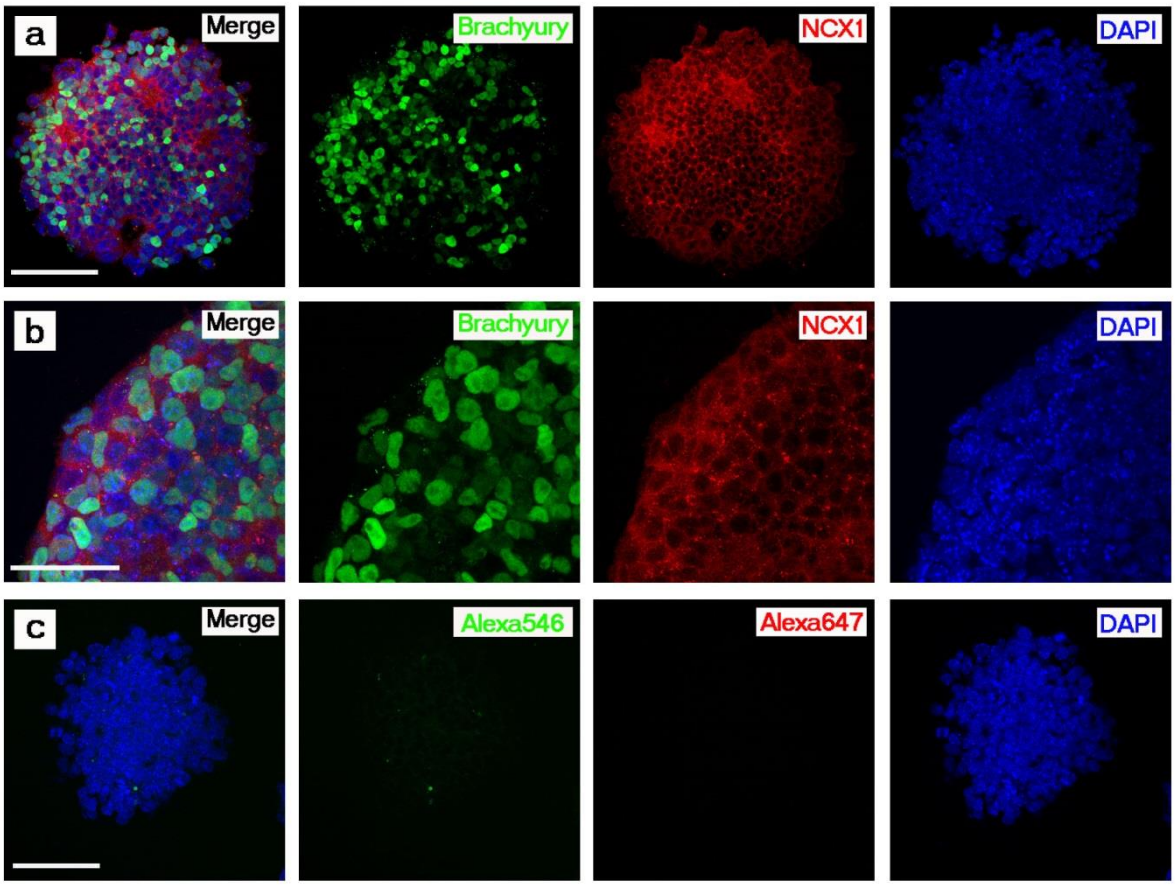

Figure 4.10: Expression of NCX1 and Brachyury at day 4 of Eomes-GFP ESC cardiomyocyte differentiation

Immunostaining of day 4 embryoid bodies revealed the expression of Brachyury (green) positive cells as well as discrete puncta of NCX1 (red) expression (a, b), which were not detected when secondary antibodies were added alone as a negative control (c). Scale bars: a, c, 100µm; b, 50µm.

4.2.6 NCX1 & Cav1.2 are required for Ca^{2+} influx during cardiac crescent maturation

To directly investigate the role of NCX1 and Cav1.2 in establishing and maintaining cardiac contractions, pharmacological blockade using the specific inhibitors CB-DMB or KB-R7943 to block NCX1 activity was employed (Secondo et al. 2009)(Kimura et al. 1999) and nifedipine to inhibit Cav1.2 (McDonald et al. 1994), on embryos maintained *ex-vivo.* Embryos were isolated and imaged for contractile activity by differential interference contrast imaging (DIC) whilst Ca^{2+} transients were assessed in parallel with confocal imaging of the Ca^{2+} dye Cal-520. Acute treatment (5, 15 and 30 min) with either NCX1 inhibitors (CB-DMB and KBR7943) or the L-type Ca^{2+} channel inhibitor (Nifedipine), affected the embryos in a stage dependent-manner when compared to

baseline recordings (Figure 4.11a, Figure 4.11b). Inhibition of either NCX1 or Cav1.2 prevented Ca^{2+} transients at stage 1/2, when contraction was first established, however, at later stages only Cav1.2 inhibition prevented Ca^{2+} transients (3 and LHT). Inhibition of both NCX1 and Cav1.2 led to an initial confinement of Ca^{2+} transients to the right-side of the crescent (within approximately 5 min which persisted through to 15 min) followed shortly afterwards by complete loss of Ca^{2+} signal (Figure 4.11c, Figure 4.11d). At stage 3 and later, only Cav1.2 was required for Ca^{2+} transients and contractile activity, since NCX1 inhibition no longer had any effect.

This data highlights a stage dependent role for NCX1 and Cav1.2 in the generation of Ca^{2+} transients and contraction during formation of the cardiac crescent, thus providing a novel mechanism by which early Ca^{2+} influx is regulated.

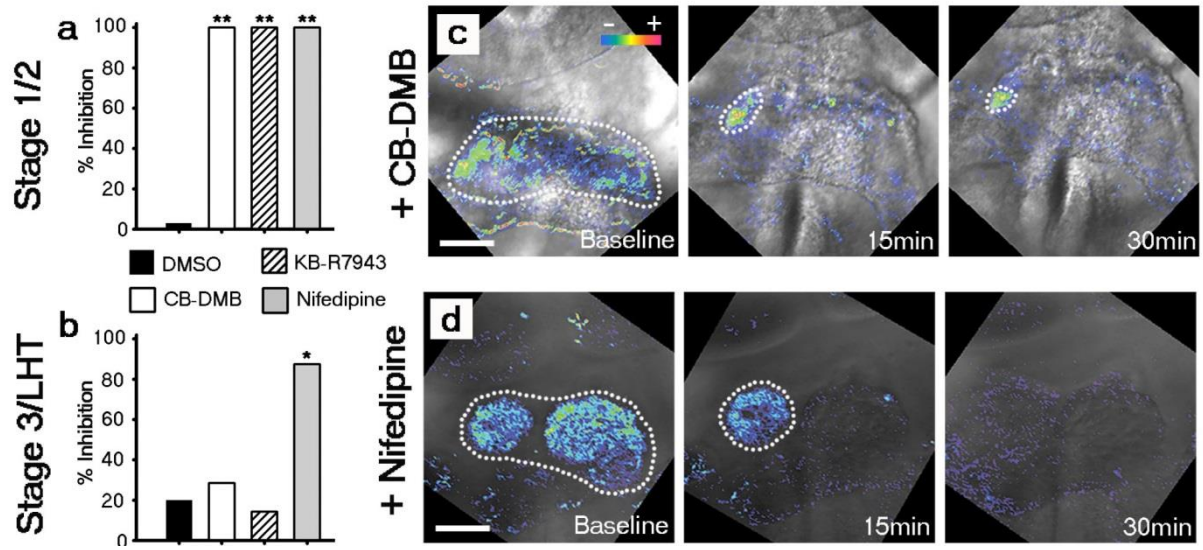

Figure 4.11: NCX1 and Cav1.2 inhibition prevents Ca^{2+} transients in a stage dependent manner during cardiac crescent maturation

Inhibition of Ca^{2+} transients occurred upon treatment of embryos between stage 1 and LHT with either NCX1 inhibitors CB-DMB, KB-R7943 or the Cav1.2 inhibitor Nifedipine, relative to DMSO control (a, b). Inhibition of NCX1 with either CB-DMB (20 µM) or KB-R7943 (30 µM) affected only stage 1 and 2 embryos(a), whereas inhibition of Cav1.2 with nifedipine (10 µM) effected both stages 1 and 2 and the later stage 3/LHT (b; stage1/2: DMSO, n=5; CB-DMB, n=7; KB-R7943, n=6; nifedipine, n=6;stage3/LHT: DMSO, n=5; CB-DMB, n=14; KB-R7943, n=7; nifedipine, n=8). Stage1-2 embryo time series of calcium transients at different time points of either CB-DMB or Nifedipine treatment, revealed a confinement to the right side of the embryo prior to complete block (c, d). All scale bars 100µm. Statistics: Freeman-Halton extension of Fisher exact probability test (* $p<0.05$; ** $p<0.01$;*** $p<0.001$).

4.2.7 Inhibition of NCX1 & Cav1.2 prevents Ca^{2+} transients during EOMEs-GFP ESC cardiomyocyte differentiation

To determine whether NCX1 or Cav1.2 are involved in ESC derived cardiomyocyte Ca^{2+} influx, pharmacological inhibitor experiments were performed at day 7 and day 14 of ESC differentiation. ESC derived cardiomyocytes were treated for 30 minutes with CB-DMB, KB-R7943 or Nifedipine. Similar to observations made in the embryo, NCX1 inhibition affected ESC-derived cardiomyocytes in a stage dependent manner whilst Cav1.2 was required for contractile function at all stages examined (Figure 4.12a, Figure 4.12b). Inhibition of NCX1 at day 7 with CB-DMB or KB-R7934 blocked beating in ESC-derived cardiomyocytes, however, by day 14 of cardiomyocyte differentiation acute administration of CB-DMB had no effect on beating whilst inhibition with KB-R7943 was significantly reduced. This was in contrast to inhibition of Cav1.2 with Nifedipine which prevented beating at both day 7 and 14. Consistent with these findings both CB-DMB and Nifedipine restricted the propagation of Ca^{2+} waves at day 7 of differentiation after 5 minutes of treatment before completely preventing wave propagation after 30 minutes (Figure 4.12c, Figure 4.12d).

Overall the relative effects of CB-DMB, KB-R7943 and Nifedipine were consistent with those observed in the developing cardiac crescent and suggest that both NCX1 and Cav1.2 have stage-dependent roles in Ca^{2+} influx during cardiomyocyte differentiation.

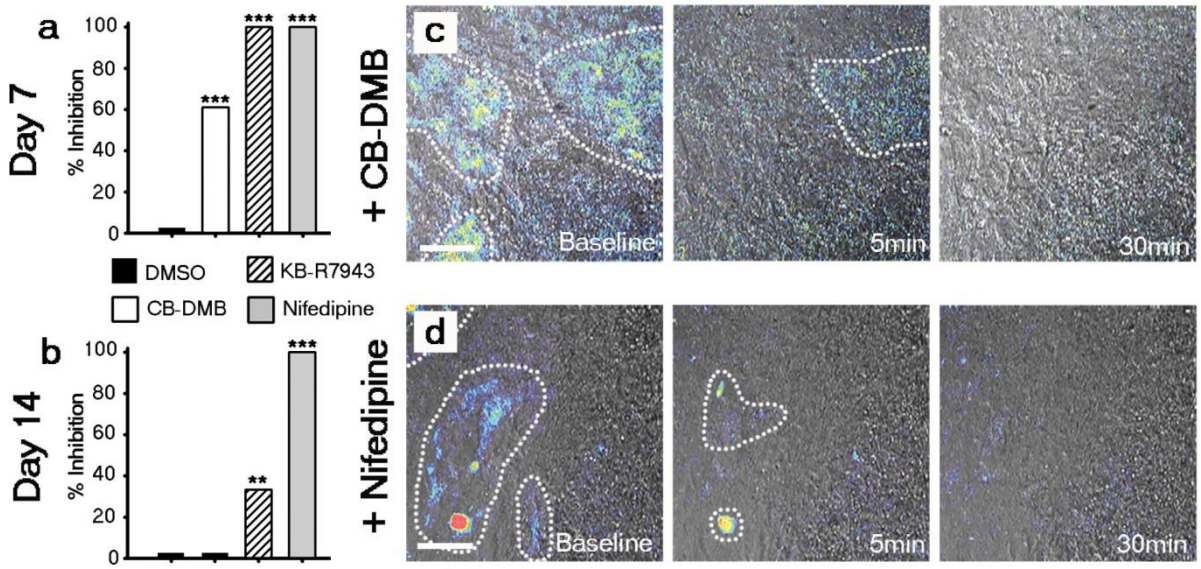

Figure 4.12: NCX1 and Cav1.2 inhibition prevents Ca^{2+} transients in a stage dependent manner during ESC-derived cardiomyocyte differentiation

Contraction was assessed in ESC-derived cardiomyocytes at different days of differentiation after treatment with the same channel blockers as used in the mouse embryo. Inhibition of NCX1 with CB-DMB (10 µM) significantly reduced contractions only in day 7 cardiomyocytes (a), whereas KB-R7943 (30 µM) affected cardiomyocytes at both day 7 and 14 (a, b). Inhibition of LTCC with nifedipine (10 µM) significantly reduced contractions in both day 7 and 14 cardiomyocytes and to a much greater extent than KB-R7943 at the later stage (b; day 7: DMSO, n=38; CB-DMB, n=36; KB-R7943, n=7; nifedipine, n=10; day 14: DMSO, n=25; CB-DMB, n=36; KB-R7943, n=15; nifedipine, n=15). Time series of day 7 ESC-derived cardiomyocyte calcium transients at different time points of either CB-DMB or Nifedipine treatment, revealed confinement of Ca^{2+} transients prior to complete block (c, d), equivalent to that observed in the treated embryos (Figure 4.11). All scale bars100µm. Statistics: Freeman-Halton extension of Fisher exact probability test (* $p<0.05$; ** $p<0.01$;*** $p<0.001$).

4.2.8 Ca^{2+} influx via NCX1 is required for single cell Ca^{2+} transients at stage 0 of crescent formation

Taking into account the earlier expression of NCX1 relative to Cav1.2 at stage 0, the possibility that NCX1 might be pivotal in generating the single-cell, slow Ca^{2+} transients observed at stage 0 of crescent formation was tested. Treatment of stage 0 embryos, prior to the onset of contraction for 15 min with CB-DMB to inhibit NCX1 resulted in a significant inhibition of slow Ca^{2+} transients compared to application of DMSO or inhibition of Cav1.2 with Nifedipine (DMSO, 32% ± 6% n=8; CB-DMB, 87% ± 8% n= 6; nifedipine, 43% ± 10% n=8 embryos per treatment; $p\leq0.01$; Figure 4.13a, Figure 4.13b, Figure 4.13c).

This data suggests that NCX1 is the predominant mechanism for Ca^{2+} influx in pre-contractile cardiomyocytes at stage 0 of cardiac crescent formation and suggests that NCX1 function is required for the establishment of cardiac function during formation of the heart.

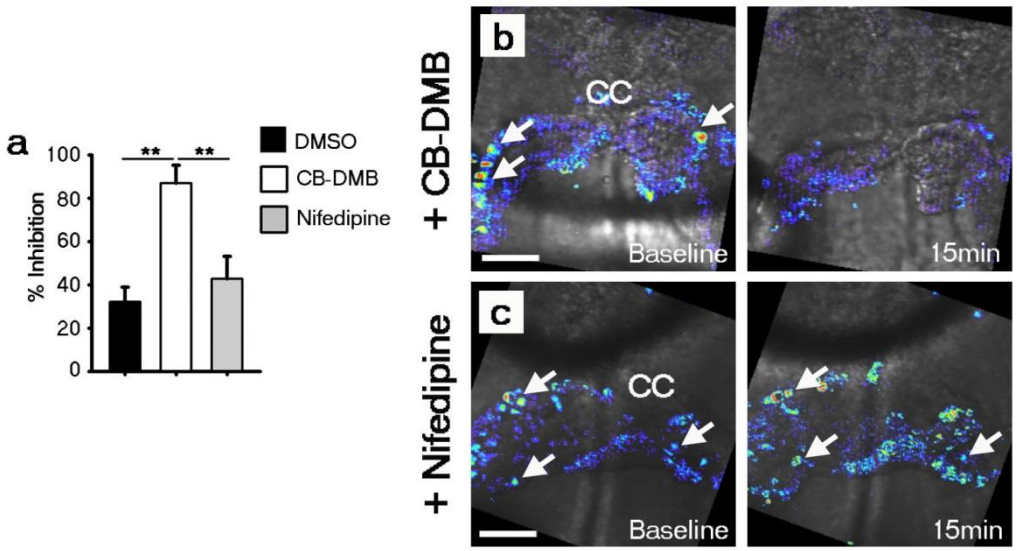

Figure 4.13: NCX1 activity is required for Ca^{2+} transients prior to the onset of contraction in the murine stage 0 cardiac crescent

Treatment of stage 0 embryos prior to the onset of beating with CB-DMB (20 µM) resulted in inhibition of slow asynchronous Ca^{2+} transients (a, b) whereas treatment with nifedipine (10 µM) had no discernible effect on the slow transients compared to DMSO (a, c; DMSO, n=8; CB-DMB, n=6; Nifedipine, n=8), supporting the earlier role for NCX1 in initiating Ca^{2+} handling prior to beating. cc, cardiac crescent. All scale bars100µm. Statistics: ANOVA and Tukey test for multiple comparisons (** $p<0.01$).

4.3 Discussion

Due to the early function observed in both *in vivo* and *in vitro* models it was important to assess the potential mechanisms by which Ca^{2+} could be regulated in immature cardiomyocytes. Previous studies in both the adult and E8.5+ embryonic heart have described and characterised a number of Ca^{2+} handling proteins which have multiple roles in function, signalling and gene expression (Liang et al. 2010)(Sabourin et al. 2011)(Méry et al. 2005). These studies were the basis for initial investigations into the Ca^{2+} handling proteins involved in early heart development specifically cardiac crescent formation and function. Studies into the mechanisms of embryonic heart function have typically focused on stages from the linear heart tube (E8.5) onwards. This thesis has shown that cardiac contraction is established prior to that described in previous studies, with single cell Ca^{2+} transients preceding contraction in the developing cardiac crescent. Therefore to further characterise these observations it was important to address the potential mechanisms which may be involved in regulating early function and Ca^{2+} transients. Using published studies as well as our own expression analysis, NCX1 and Cav1.2 represent potential sarcolemmal proteins involved in early cardiac function. This chapter has shown that both NCX1 and Cav1.2 have a dynamic role in regulating Ca^{2+} influx. Although these experiments have focused on NCX1 and Cav1.2 it is important to note that other channels could potentially contribute to early Ca^{2+} transient dynamics including T-type calcium channels, SR Ca^{2+} channels such as ryanodine receptors or IP_3receptors and lysosomal Ca^{2+} channels, however studying all of the potential channel-based mechanisms that might influence early heart function in the embryo was beyond the scope of this PhD.

4.3.1 Maturation of cardiomyocyte function

During cardiomyocyte maturation a large number of genetic, phenotypic and functional changes occur, including increases in the number of mitochondria (Khryapenkova et al. 2008)(Kasahara et al. 2013) as well as the formation of t-tubules (Brette & Orchard 2003). In terms of function, the embryonic mouse heart at E10.5 has been reported to beat at ~127bpm, increasing to 300bpm at neonatal stages, whilst the adult heart beats at over 500bpm (Keller et al. 1996). In the neonatal and adult heart, the mRNA expression of ECCs proteins such as RyR2, SERCA2a, Nav1.5 and Cav1.2 became elevated, corresponding with increases in heart function. This

correlation would suggest that in order to raise heart rate, increases in ECC protein expression need to occur. The maturational changes observed in both function and ECC gene expression also correlated with the expression of mature structural cardiomyocyte markers (Figure 3.7). Whether changes in these Ca^{2+} handling proteins are a downstream consequence of differentiation or whether they can directly regulate gene expression and maturation is still open for debate. In terms of ECC maturation, knockout studies have suggested that functional ECC develops around E10.5-11.5, however the exact timing of embryonic lethality varies depending on the specific ECC gene knockout. Homozygous RyR2 gene inactivation in transgenic mice causes embryonic lethality between E10.5-11.5 (Takeshima et al. 1998). Embryos with homozygous Cav1.2 gene inactivation developed normally until E12.5, however, no viable embryos were detected at E14.5 (Seisenberger et al. 2000), whilst homozygous NCX1 gene inactivation led to lethality at ~E9.5 ((Wakimoto et al. 2000)(Cho et al. 2000)(Koushik et al. 2001)). During embryonic heart development (E9.5 – E14.5), there were no changes detected in the mRNA expression of RyR2, Cav1.2 or NCX1. Due to the embryonic lethality associated with the disruption of these genes, this data would suggest that RyR2, Cav1.2 and NCX1 are already expressed prior to E9.5, however, it is likely that they may individually be able to trigger contraction independent of ECC and therefore allowing embryos survive past E9.5. These experiments highlight a significant role for ECC during the later stages of heart development, although, they suggest that in the early heart, contraction occurs via a different mechanism.

4.3.2 Expression of NCX1 occurs during early cardiomyocyte differentiation

In the adult heart, NCX1 is one of the major Ca^{2+} removal pathways allowing relaxation of adult cardiomyocytes after contraction (Reuter & Seitz 1968)(Nicoll et al. 1990). *Slc8a1*, the gene which encodes NCX1, was expressed at similar levels throughout development of the heart from E9.5, suggesting that increases in *Slc8a1* were not required during maturation within the post natal and adult heart and that initiation of expression occurred at the earliest stages of heart development. This observation fits with previous studies which have shown the functional expression of NCX1 in E8.5 cardiomyocytes (Reppel, Sasse, et al. 2007). By analysing whole embryos, it was observed that *Slc8a1* mRNA first became elevated at E8.0 compared to E7.5. This up regulation in *Slc8a1* was further supported by the membrane-localised expression of

NCX1 within the stage 0 cardiac crescent. In the ESC model of differentiation NCX1 was also the first ECC gene to be up regulated at around day 2 of differentiation. Unsurprisingly at day 7 all cTnT +ve ESC-derived cardiomyocytes expressed localised NCX1 corresponding with a mature phenotype and supporting the early expression of NCX1. Together this data suggests that NCX1 is one of the first sarcolemmal Ca^{2+} handling proteins to be expressed during differentiation and may be required for the earliest observed pre-contractile Ca^{2+} transients.

Whilst using the Nkx2.5-GFP reporter line it was possible at day 7 to observe NCX1 +ve regions which didn't express Nkx2.5-GFP. This would suggest that during differentiation, NCX1 is expressed in cells prior to cardiac commitment (Nkx2.5-GFP expression) and may have a role in the specification of mesoderm into cardiac progenitors. Alternatively, NCX1 may be expressed in other cells types within the ESC culture. However, due to the juxtaposition of the NCX1 +ve Nkx2.5-GFP -ve cells and NCX1 +ve Nkx2.5-GFP +ve cells, it seems likely that they originate from a common progenitor and are not derived from other lineages (ectoderm or endoderm) (Z. Wang et al. 2012). At earlier stages of differentiation (day 4) prior to Nkx2.5-GFP, punctated expression of NCX1 could be observed within EBs, potentially at the cell membrane. This would suggest that NCX1 expression, although not yet distributed throughout the entire membrane, is expressed prior to cardiomyocyte specification. Whether these NCX1 puncta are functional and represent discrete membrane signalling domains (Schulze et al. 2003) or whether NCX1 is none-functional and is still being trafficked to the membrane is unclear (Feldmann et al. 2009).

To attempt to determine the earliest cardiac progenitor population which expressed NCX1, a Brachyury antibody was used to locate cells of the mesoderm lineage. Although Brachyury +ve cells did not express membrane localised NCX1 it was interesting to note that NCX1 +ve cells could be found adjacent to Brachyury +ve cells, potentially suggesting communication between the two different cell types. Communication between different cell types during differentiation can have both feed forward positive effects, enhancing and promoting cardiomyocyte differentiation (Lavine et al. 2005), or alternatively it can repress mesoderm gene expression in progenitor cells (Mercola et al. 2011)(Lian et al. 2013). To be able to determine the earliest population of cardiac progenitors which express NCX1 it would be important to

try other markers such as Mesp1 or Gata-4, since their mRNA expression profiles corresponded with the increase in NCX1 expression (Table 3.8 and 3.11).

4.3.3 Cav1.2 expression during cardiomyocyte differentiation

In the adult heart, Cav1.2 is the main sarcolemmal Ca^{2+} influx pathway required for ECC. During cardiac crescent formation, the up regulation of *Cacna1c* (the gene which encodes Cav1.2) did not occur until E8.5 (after the increase in NCX1) with mRNA levels remaining unchanged between E7.5 and E8.0. This mRNA expression analysis was supported by immunohistochemical of whole mount embryos which showed that Cav1.2 was expressed within cardiac crescent at stage 2, but was absent at stage 0. Together this data revealed that the expression of Cav1.2 is preceded by NCX1 within the developing cardiac crescent. This difference in expression suggests that NCX1 is the main sarcolemmal protein responsible for the Ca^{2+} influx required to generate Ca^{2+} transients prior to contraction within the stage 0 heart. Whilst at later stages of cardiac crescent formation both Cav1.2 and NCX1 have a functional role in sarcolemmal Ca^{2+} flux and contraction. Although this data provides an insight into the dynamics of sarcolemmal ECC gene expression, levels of both channel and exchanger mRNA and protein do not directly correlate with function. Channels are known to have a greater rate of ion flux in comparison to exchangers meaning even with reduced expression Cav1.2 could perform a similar amount of Ca^{2+} flux compared to NCX1 (Gadsby 2009). To definitively understand the mechanisms for function, electrophysiological recordings of direct channel and exchange function would be needed.

To investigate further the expression of Cav1.2 especially in relation to NCX1, temporal changes in mRNA expression and protein localization during ESC cardiomyocyte differentiation were examined. Expression of *Cacna1c* did not increase until day 4 of differentiation in the Eomes-GFP model, 2 days later than the initial increase in *Slc8a1*. Expression of Cav1.2 protein by immunohistochemical analysis could be observed in cTnT+ve cells at day 7 of differentiation however there was also a population of cTnT+ve cells which did not express Cav1.2. This difference could be due to the maturity of the ESC-derived cardiomyocytes. Previous data has shown Cav1.2 expression occurs later than other functionally related cardiac genes such as NCX1 and suggests that these cTnT +ve Cav1.2 –ve cells represent less terminally differentiated

cells. Alternatively the variation in expression could be due to differences in cardiomyocyte cell type within cTnT+ve regions. In the adult heart there are multiple subtypes of cardiomyocytes (ventricular and atrial) which express different levels of various Ca^{2+} channels. In ventricular cardiomyocytes L-type channels are the main sarcolemmal Ca^{2+} channel subtype, however in the atria another Ca^{2+} channel (T-Type) is suggested to be more predominant. The role of other Ca^{2+} channels in heart function during development has also been described (Ferron et al. 2002)(Sabourin et al. 2011)(Méry et al. 2005). These findings suggest that cTnT+ve cells which do not express Cav1.2 may express other types of Ca^{2+} channels allowing Ca^{2+} influx and contraction to occur and suggest that the cTnT+ve regions of cells is heterogonous in terms of Ca^{2+} influx mechanisms.

4.3.4 The role of sarcolemmal Ca^{2+} release during cardiomyocyte differentiation

The mechanism by which Ca^{2+} transients are generated prior to the development of ECC is poorly understood, with debate surrounding the relative contribution of Ca^{2+} from internal sarcoplasmic stores (Sasse et al. 2007)(Rapila et al. 2008). Previous immunofluorescence showed that both the sarcolemmal ECC proteins NCX1 and Cav1.2 are expressed within the cardiac crescent. To begin to investigate the role of the SR during cardiomyocyte differentiation, the expression of ECC genes responsible for SR Ca^{2+} regulation was assessed. During early development of the heart, *Ryr2* (encoding the ryanodine receptor – RyR2) expression was elevated during formation of the cardiac crescent (E8.0) and further increased during progression to the linear heart tube (E8.5). The increases in *Ryr2* expression suggest that the SR is capable of releasing Ca^{2+} within the cardiac crescent. However, during ESC cardiomyocyte differentiation, *Ryr2* expression did not increase until later stages of differentiation and, although significant, these changes were relatively small suggesting SR Ca^{2+} release via RyR2 was less fundamental during ESC cardiomyocyte differentiation. The development of a functional SR from the rough endoplasmic reticulum is poorly understood. Cytosolic Ca^{2+} oscillations originating from the SR have been observed in isolated E8.5 cardiomyocytes (Sasse et al. 2007), providing support for the role of SR Ca^{2+} release in early contractile activity. However, contrary to these observations, treatment of control myocytes between E9.5 and E11.5 with ryanodine, a compound which locks the RyR2 in an open state, did not exert a major effect on spontaneous Ca^{2+} transients

(Takeshima et al. 1998). The observation that RyR2 KO transgenic mice have beating linear heart tubes at E9.5 again suggests SR Ca^{2+} release is not required for early contractile function. The late onset of *Ryr2* expression in the ESC model would suggest that SR Ca^{2+} release does not influence early cardiomyocyte differentiation, however, this data cannot unequivocally exclude a role for RyR2 mediated Ca^{2+} release within the cardiac crescent until further functional studies are conducted. The relativity low level and late expression of Ryr2 in the ESC models compared to the embryo would suggest that the functional relevance of SR Ca^{2+} stores and RyR2 mediated Ca^{2+} release is different *in vitro* compared to *in vivo*. This difference could either be due to the relative immaturity of ESC-derived cardiomyocytes (Li et al. 2015) and that RyR2 expression correlates with maturity, alternatively the mechanism by which ESC-derived cardiomyocytes and embryonic hearts regulate contraction may be fundamentally different.

4.3.5 Sodium regulation during cardiomyocyte differentiation

In adult cardiomyocytes, ECC couples electrical depolarization with contraction. Depolarisation is initiated by the sodium channel, Nav1.5 (encoded by the gene *Scn5a*), which, once open, leads to Na^{+} influx which in a feed-forward manner further depolarises the membrane (Bers et al. 2003)(Aronsen et al. 2013). Although not directly involved in Ca^{2+} regulation it was interesting to investigate Nav1.5 gene expression to understand the electrophysical properties of the early heart and how other ions may be regulated. Unsurprisingly, as observed with the ECC components, Nav1.5 mRNA expression increased during postnatal and adult stages correlating with the maturation of cardiomyocytes. In terms of ESC cardiomyocyte differentiation, Nav1.5 was one of the first genes to be up regulated at around day 2 and had a dynamic expression profile during differentiation, as also observed in the embryo from E9.5 (Domínguez et al. 2008), suggesting a potential role for Nav1.5 and Na^{+} influx during early differentiation. Nav1.5 is known to have a role in heart development, as shown in Nav1.5 KO embryos which die around E10.5 with restricted ventricle development (Papadatos et al. 2002). Studies in zebrafish have shown that knock down of Nav1.5 leads to defects in specification of pre-cardiac mesoderm (Chopra et al. 2010) and a reduction in cardiomyocyte number, implying that proliferation of embryonic cardiomyocytes requires Nav1.5 activity (Bennett et al. 2013).

The correlation between the expression profiles of different channels and exchangers during differentiation, suggests that multiple ion fluxes may interact to regulate function. In guinea pig ventricular cardiomyocytes, the local accumulation of Na^+ via Nav1.5 can trigger reverse mode NCX1 activity leading to Ca^{2+} induced Ca^{2+} release from the SR (Leblanc & Hume 1990). In the *Eomes*-GFP model of cardiomyocyte differentiation, increases in Nav1.5 mRNA corresponded with increases in NCX1 mRNA at day 2, suggesting an interaction between the two proteins and that early Na^+ influx via Nav1.5 may be capable of regulating NCX1 activity. Together this data suggests a complex mechanism by which multiple proteins and exchangers can indirectly regulate different intracellular ions to effect downstream function and signalling. Na^+ is also known to activate transcription and gene expression via its ability to activate salt-inducible kinase 1 (Popov et al. 2012) suggesting a direct mechanism by which early Na^+ influx could regulate differentiation.

The early expression of both Scn5a and NCX1 suggests early increases in the concentration of intracellular Na^+, we, therefore, wanted to investigate whether other proteins involved in Na^+ removal were regulated during differentiation such as Na^+/K^+ ATPase. The Na^+/K^+ATPaseα2 is a ATP-dependent exchanger which removes Na^+ from the cell whilst bringing in K^+. Overexpression of Na^+/K^+ATPaseα2 has been shown to attenuate pathological cardiac hypertrophy in the adult by regulating intracellular Na^+ and Ca^{2+} levels (Correll et al. 2014). During ESC differentiation, *Atp1a2* (encoding Na^+/K^+ATPaseα2) was first up regulated at day 4 of differentiation before increasing further by day 14. This finding would fit with the hypothesis that the concentration of intracellular Na^+ becomes elevated between day 2 and 4, increasing the requirement for Na^+ removal. Another Na^+-related gene studied was *Slc9a1* which encodes Na^+/H^+ exchanger (NHE1), a regulator of intracellular pH which functions to transport protons out of the cell and bring Na^+ in. NHE1 activity has also been reported to mediate Ca^{2+} oscillation via acidification and increases in the concentration of intracellular Na^+ (Nakamura et al. 2012). As Ca^{2+} transients are initiated and function develops, an increase in metabolism would occur in order supply the cardiomyocytes with enough energy for contraction to occur (Abel & Doenst 2011). Increases in metabolism and ATP production are known to decrease intracellular pH (Poole-Wilson 1982), therefore NHE1 may be require to regulate these changes in pH. Initially the expression of NHE1

in the *Eomes*-GFP ESC model of differentiation was examined and a small increase in expression was observed between day 3 and 6 with a further increase at day 14. This relatively minor increase in expression suggests that NHE1 does not play a major role in cardiomyocyte formation although a role for NHE1 cannot be excluded as protein expression or activity has not been assessed.

4.3.6 T-Type Calcium channel

Although Cav1.2 is the predominant calcium channel in cardiomyocytes contributing to most of the Ca^{2+} required for ECC, other sarcolemmal Ca^{2+} channels are expressed in the heart, for example T-type Ca^{2+} channels (Cav3.1 and Cav3.2), are reported to have a role in pacemaker activity (Hagiwara et al. 1988)(Zhou & Lipsius 1994). T-type Ca^{2+} channels have also been suggested to have a role in heart development as they are observed at higher densities in embryonic (Mizuta et al. 2010)(Ferron et al. 2002) and neonatal myocytes (Nuss & Marban 1994)(Leuranguer et al. 2000) than in mature ventricular myocytes. It has been suggested that Ca^{2+} entry via T-type Ca^{2+} channels may serve as a current injector to initiate depolarisation during heart development (Zhang et al. 2003)(Schroder et al. 2006). This study focused on the expression of *Cacna1g*, the gene known to encode the α1g subunit of Cav3.1. In the Nkx2.5-GFP cells, significant up regulation of *Cacna1g* occurred at day 14 of differentiation, although there was also a small increase at day 2 which did not reach significance. In the *Eomes*-GFP model, the expression profile of Cav3.1 was similar, with a large increase at day 14, although in this model *Cacna1g* was also significantly up regulated at day 2 of differentiation. This data suggests that Cav3.1 may be expressed at very early stages of ESC differentiation and is consistent with a previous study that demonstrated the functional expression of Cav3.1 in ESC-derived cardiomyocytes (Zhang et al. 2003). Together this could suggest that T-type Ca^{2+} channels may be involved in linking spontaneous electrical activity and Ca^{2+} handling with transcriptional activity during cardiac differentiation, as observed in neonatal cardiomyocytes (Ruchon et al. 2012).

4.3.7 Inositol trisphosphate receptor type 2 (IP_3R2)

The main mechanism for Ca^{2+} release from the SR in the adult heart is via RyR2 channels, however, IP_3 receptors can also release Ca^{2+} from the SR in a number of

different cell types including cardiomyocytes (Berridge 2009). IP_3R-mediated Ca^{2+} oscillations are some of the first signals known to occur during development, essential in activating unfertilized oocytes (Ciapa & Chiri 2000). Although the functional role of IP_3R receptors in regulating heart rate is controversial, accumulating evidence suggests they may be involved in the rhythm control of non-cardiac pacemaker tissue as well as in the embryonic heart (Méry et al. 2005)(Ju et al. 2012). IP_3R-dependent nuclear calcium signalling is also an important mechanism involved in cardiomyocyte excitation-transcription coupling, both in the adult heart during cardiac hypertrophy (X. Wu et al. 2006)(Nakayama et al. 2010) and in neonatal cardiomyocytes (Arantes et al. 2012)(Ibarra et al. 2013). ESC-derived cardiomyocyte studies have shown that application of Xestospongin-C or 2-amino-ethoxydiphenyl borate (2-APB), IP_3-receptor antagonists, prevented spontaneous beating (Ferreira-Martins et al. 2009). In single E9.5 cardiomyocytes, IP_3 receptors have been shown to regulate the frequency of SR Ca^{2+} oscillations and thus heartbeats (Sasse et al. 2007).

We first examined IP_3R gene expression during the later stages of embryonic development from E9.5 and found elevated *Itp3r2,* which encodes the main cardiac isoform of IP_3R channels, within the P0 and adult heart. In the *Nkx2.5*-GFP ESC model of cardiomyocyte differentiation *Itp3r2* was up regulated at around day 7 of differentiation, however, the increase was not statistically significant until around day 10. Although this data does not exclude a role for *Itp3r2* in the earliest calcium transients, the IP_3R gene expression profile in comparison to other Ca^{2+} channels and cardiomyocyte markers suggests that other Ca^{2+} handling proteins may be more important for early embryonic heart function.

4.3.8 NAADP, Two-pore channels and lysosomal calcium stores

NAADP is the most potent Ca^{2+}-releasing second messenger known. Extensive studies have demonstrated that NAADP can stimulate Ca^{2+} release from lysosomes via Two-pore channels 1 and 2 (TPC1 and TPC2). TPC1/2 were first cloned in 2000 (Ishibashi et al. 2000) and displayed significant sequence similarity to voltage-gated Ca^{2+} and Na^{2+} channels. Both TPC1 and 2 localize to the endo-lysosomal system which is known to contain high levels of Ca^{2+} (Brailoiu et al. 2009). More recently NAADP has been shown to be involved in atrial cardiomyocyte contraction and arrhythmias (Collins et al.

2011)(Nebel et al. 2013). Therefore it is a potential source of Ca^{2+} that has not been investigated to-date in cardiac development. NAADP signalling was therefore investigated as a potentially novel regulator of early Ca^{2+} signalling during ESC differentiation. NAADP levels were not quantified during ESC differentiation due to difficulties with assaying this nucleotide (Graeff & Lee 2002), instead TPC1 and 2 expression was assessed.

Throughout heart development, gene expression of *Tpcn1* and *Tpcn2* (encoding TPC1 and TPC2) was maintained from E9.5, suggesting both early expression and a potential physiological role throughout development. In terms of ESC differentiation, *Tpcn1* expression began to increase from day 4 whilst levels of *Tpcn2* remained constant throughout differentiation. The specific localisation and function of TPC1 and TPC2 are reported to be slightly different. TPC1 is primarily located in endolysosomes and believed to cause localised Ca^{2+} transients whereas TPC2 localises solely within lysosomes and triggers globalised Ca^{2+} transients (Patel et al. 2011). This may indicate that TPC1 and TPC2 are regulated in a cell-type specific manner as previously mentioned, which may account for the variation in mRNA levels during development. Although absolute gene expression levels were not quantified, Ct values for TPCs in both the *in vivo* heart samples and ESCs were very similar (TPC1; *in vivo* = ~25cts, *in vitro* = ~25cts. TPC2; *in vivo* = ~28cts, *in vitro* = ~29cts.), suggesting similar mRNA expression levels and hence cellular requirement. To determine protein levels, western blots and immunohistochemistry needs to be performed however currently available TPC antibodies are of low quality, rendering this difficult.

Whilst, TPC1 and TPC2 have been strongly reported to be involved in Ca^{2+} release from acidic stores, recent studies have suggested that TPC1 and TPC2 are actually Na^{+} selective (X. Wang et al. 2012), although this is strongly debated (Marchant & Patel 2013). If TPC1 and TPC2 were Na^{+}-selective channels they could potentially regulate cardiomyocyte differentiation via direct Na^{+} related signalling pathways or indirectly by activating other Na^{+} regulated exchangers such as NCX, similar to the potential function of Nav1.5.

4.3.9 Store operated calcium release

Transient receptor potential channels (TRPC) are store-operated sarcolemmal Ca^{2+} channels that can be activated by signalling molecules, internal Ca^{2+} store depletion and membrane stretch. Studies in chick hearts have shown that TRPCs can regulate both contractility and pacemaker activity during development (Sabourin et al. 2011). TRPC3 is a cardiac-specific isoform, shown to be up regulated in murine hypertrophy (Bush et al. 2006). Inhibition of TRPC3 with the specific inhibitors, GSK2332255B and GSK2833503A , inhibited the onset and progression of pathological hypertrophy in mice (Seo et al. 2014). Expression of TRPC3 increased at day 7 of ES cardiomyocyte differentiation correlating with the expression of mature cardiac specific markers suggesting TRPC3 may not be essential for cardiomyocyte formation and early function in this model, but may play a role in maturation. This would fit with the observation that TRPC3 global KO mice survive to adulthood (Hartmann et al. 2008).

Another important stored-operated Ca^{2+} channel is Orai1, which is activated upon decreases in the concentration of Ca^{2+} in the endoplasmic via an interacting protein Stim1. Stim1 and Orai1 have been shown to regulate hypertrophic growth in cardiomyocytes (Voelkers et al. 2010) as well as having a role in regulating cardiomyocyte function (Collins et al. 2013). In the *Eomes*-GFP ESC model *stim1* and *orai1* were not up regulated during cardiomyocyte differentiation, suggesting that they do not play a role in heart formation. This is further supported by Stim1 (Oh-Hora et al. 2008) and Orai1 (Gwack et al. 2008) KO mice surviving until at least the late stages of embryonic development.

4.3.10 Cav1.2 is required for cardiomyocyte contraction during cardiac crescent development

Cav1.2, a voltage-dependent Ca^{2+} channel, is activated by membrane depolarisation allowing Ca^{2+} to cross the sarcolemmal membrane via an electrochemical gradient. In the adult heart, Cav1.2 is the predominant source of Ca^{2+} influx, triggering SR Ca^{2+} release and contraction (Reuter 1979)(Lederer et al. 1990) (Ertel et al. 2000). In these current studies, Cav1.2 was found to have a fundamental role in generating Ca^{2+} transients from the onset of contraction, although it was not involved at the earliest

stages exemplified by the pre-contractile Ca^{2+} transients observed during cardiac crescent formation.

In adult cardiomyocytes, Cav1.2 is activated during action potential depolarisation, at a membrane potential of -40mV, allowing Ca^{2+} influx until repolarisation occurs. In cardiomyocytes at E10.5 the resting membrane potential is reported to be around -40mV, which is significantly greater than that in adult cardiomyocytes and suggests constant Cav1.2 activation or that smaller depolarisation currents are sufficient to trigger activity and Ca^{2+} influx. In addition to the membrane potential, a number of other factors can affect Cav1.2 activity, including intracellular Ca^{2+} levels, auxiliary channel subunit composition as well as regulation by different signalling molecules including calmodulin kinase II and A-kinase anchoring proteins (AKAPs) (for review see (Harvey & Hell 2013)). Due to the dynamic regulation of Cav1.2 it is difficult to assess the kinetics of this channel within the stage 1 cardiac crescent, however, it is likely that the properties of this channel in the embryonic heart are significantly different from that in the adult.

To generate contraction, Ca^{2+} must bind and activate myofilaments; this activation is facilitated by rapid increases in the concentration of intracellular Ca^{2+} (Bers 2000)(De Tombe 2003). In intact adult cardiomyocytes, the concentration of free intracellular Ca^{2+} required for half-maximal myofilament activation is around 600 nM, however due to Ca^{2+} buffering, greater levels of Ca^{2+} influx are required in order for free cytosolic Ca^{2+} to reach the threshold for contraction (Berlin et al. 1994)(Trafford et al. 1999). Ca^{2+} is buffered by a number of different intracellular moieties which compete with troponin to bind Ca^{2+} (Gao et al. 1994). Studies have shown that to raise intracellular Ca^{2+} concentrations from around 100 nM to peak systolic levels of around 1 µM, the actual Ca^{2+} concentration needs to increase by over 100 µM due to Ca^{2+} buffering (Trafford et al. 1999). This suggests that Cav1.2 is fundamentally required to generate the large Ca^{2+} fluxes required for contraction in adult cardiomyocytes. In embryonic cardiomyocytes, however, the concentration of Ca^{2+} required for contraction may be reduced due to a number of different cellular properties including; cell size, the expression of Ca^{2+} buffering proteins as well as altered sensitivity of myofilaments to Ca^{2+}. These differences may potentially allow Cav1.2-mediated Ca^{2+} influx to initiate contraction independently of SR activity. The functional impact of Cav1.2 inhibition

correlated with an increased time-to-peak (TTP) observed between stage 0 and stage 1 (chapter 3) and would suggest increased Cav1.2 kinetics, in terms of rate of flux, were responsible for the increased rate of Ca^{2+} transients. This embryo-adult difference suggests that Cav1.2 expression facilitated contraction by enabling a greater concentration of Ca^{2+} to enter the cell, therefore, raising the intracellular Ca^{2+} concentration to a level capable of triggering myofilament activation.

Overall this data reveals that Ca^{2+} channel activity is fundamental for contraction at stage 1 of cardiac crescent development and suggests that Cav1.2 is essential for the generation of the large inward Ca^{2+} fluxes in order to raise intracellular Ca^{2+} levels sufficiently to active myofilaments and generate contraction.

4.3.11 Directionality of NCX1 activity

In the adult heart NCX1 is the main Ca^{2+} efflux pathway, working in combination with SERCA to remove Ca^{2+}, after contraction. Although NCX1 works predominantly in "forward mode" (Ca^{2+} efflux), it can also work in "reverse mode" (Ca^{2+} influx), due to its electrogenic nature and sarcolemmal ionic gradients, resulting in Ca^{2+} influx (Blaustein & Lederer 1999)(Leblanc & Hume 1990). These results reveal that NCX1 is fundamental for the generation of Ca^{2+} transients prior to contraction in early developing cardiomyocytes and suggests that in the stage 0 cardiac crescent NCX1 functions in reverse mode to trigger Ca^{2+} influx. In mature cardiomyocytes, the importance of NCX1-mediated Ca^{2+} influx is widely debated, although studies have shown that Ca^{2+} entry via "reverse mode" NCX, triggered by sodium influx during an action potential, can work synergistically with Cav1.2 to trigger Ca^{2+} release from the SR (Larbig et al. 2010). In the developing heart (~E8.5), however, "forward mode" NCX1 has a predominant role in action potential generation (Liang et al. 2010) as well as potentially contributing to early Ca^{2+} influx by working in "reverse mode" (Reppel, Reuter, et al. 2007). Previous studies have characterised the functional expression of NCX1 from E8.5-E9.5 (post-linear heart tube formation) in mouse (Linask et al. 2001)(Reppel, Sasse, et al. 2007)(Liang et al. 2010), coincident with cardiac looping morphogenesis, which is later than the stages in which onset of NCX1 expression and associated cardiac function is observed.

The directionality of NCX1 activity is dependent on the concentration gradient of intra- and extra-cellular Ca^{2+} and Na^{+} in addition to the membrane potential (Reppel, Reuter, et al. 2007). The reversal potential determines the membrane potential at which NCX1 activity is balanced and varies depending on the intra and extracellular concentrations of Na^{+} and Ca^{2+}. Under normal physiological conditions, the concentration of intracellular Na^{+} is ~8 mM whilst Ca^{2+} is ~100 nM (Bers et al. 2003), which is significantly lower than the extracellular concentrations (Na+ = ~140mM; Ca^{2+} = ~1.5mM). As a result the reversal potential, calculated using the Nernst equation, for NCX1 is around -26mV. In terms of NCX1 function this means that, if the membrane potential was lower than -26mV, "forward mode" activity (Ca^{2+} efflux) would occur, however at membrane potentials above -26mV "reverse-mode" activity(Ca^{2+} influx) would predominate.

Adult cardiomyocytes have a resting membrane potential of around -85mV, which favours "forward-mode" NCX1 activity and the removal of Ca^{2+}, however, during development the resting membrane potential of immature cardiomyocytes is known to be significantly higher. Embryonic cardiomyocytes at around E10.5 have been shown to have a resting membrane potential of around -40mV (Sasse et al. 2007)(Rapila et al. 2008), which would increase the likelihood and potential functional relevance of "reverse mode" NCX1 activity. The conditions that favour reverse mode NCX1 activity include less depolarised membrane potentials as well as a high concentration of intracellular Na^{+}, which are likely to be present in immature cardiomyocytes. Although the membrane potential of stage 0 cardiomyocytes has not been measured, it can be assumed to be greater than -40mV meaning NCX1 activity would be more susceptible to changes in the concentration of intracellular Ca^{2+} and Na^{+}. Collectively this suggests that within the developing heart the directionality of NCX1 activity will be highly variable due to electrochemical properties of immature cardiomyocytes and the dynamic ionic fluxes occurring during formation of the cardiac crescent.

4.3.12 The role of NCX1 in cardiomyocyte function

Inhibition of NCX1 function with either CB-DMB or KB-R7943 blocked single–cell, slow Ca^{2+} transients in pre-contractile cells at stage 0, suggesting that "reverse mode" NCX1

activity was prevented due to an inhibition in Ca^{2+} influx. It has been reported that KB-R9743 preferentially blocks "reverse mode" NCX1 activity (Hoyt et al. 1998)(Iwamoto 2004) further supporting a role for NCX1 in Ca^{2+} influx, although the ability of pharmacological NCX1 inhibitors to block specific modes of NCX1 function is debated. One possibility was that the inhibition of spontaneous Ca^{2+} transients was due to cytosolic Ca^{2+} overload occurring secondary to the inhibition of NCX1 acting in its forward mode of operation and transporting Ca^{2+} out of the cell. However, arguing against this is the fact that there were no changes observed in the baseline Cal-520 signal over the imaging timecourse. This may further support a role for "reverse mode" NCX1 function in early Ca^{2+} transients.

Although NCX1 inhibition directly blocked Ca^{2+} transients suggesting NCX1 mediated Ca^{2+} influx was inhibited, it could also be suggested that this phenotype was alternatively due to forward mode activity. It is widely accepted that "forward mode" NCX1 function removes one Ca^{2+} in exchange for three Na^{2+} (Blaustein & Lederer 1999), overall resulting in one extra positive charge entering the cell leading to production of a net inward current. In the adult, the NCX1-derived, inward current can trigger ectopic action potentials leading to arrhythmias such as delayed after depolarisations, whilst at ~E8.5 NCX1-derived, inward current has been suggested to contribute to depolarisation and action potential generation (Liang et al. 2010). This suggests that inhibition of NCX1 could block Ca^{2+} transients by preventing action potential generation and depolarisation resulting in the inhibition of Ca^{2+} influx via other Ca^{2+} channels. This mode of inhibition is most likely to occur in the stage 1 cardiac crescent when both CB-DMB and nifedipine inhibited Ca^{2+} transient generation, since "forward mode" NCX1 activity could trigger the opening of Cav1.2. In the stage 0 cardiac crescent, however, nifedipine did not affect Ca^{2+} transient activity, further supporting a role for "reverse mode" NCX1 Ca^{2+} influx triggering Ca^{2+} transients. Alternately at stage 0 "forward mode" NCX1 activity could still generate action potentials and depolarisation but may activate unidentified Ca^{2+} channels however due to the slow Ca^{2+} dynamics observed at this stage this is unlikely. A third possibility in terms of NCX1 function is that within the stage 0 cardiac crescent NCX1 could be responsible for both the Ca^{2+} influx and efflux, correlating with the slow dynamics of stage 0 Ca^{2+} transients. It was shown in chapter 5 that the Na^{2+} channel

Nav1.5 was expressed at the earliest stages of cardiomyocyte differentiation and could act as a potential mechanism for increasing intracellular Na^+ concentrations and driving initial NCX1-mediated Ca^{2+} transients. As “reverse mode” NCX1 activity triggers Ca^{2+} influx the intracellular concentration of Ca^{2+} would increase raising the reversal potential of NCX1 and triggering Ca^{2+} efflux via “forward mode” activity. This “forward mode” NCX1 activity may also go on to regulate the next Ca^{2+} transient by activating Nav1.5 and triggering further Na^+ influx.

4.3.13 Dynamic requirement for NCX1 and Cav1.2 during cardiac crescent formation

During formation of the heart from the pre-contractile cardiac crescent (stage 0) to the linear heart tube stage-dependent changes in the role of both NCX1 and Cav1.2 were revealed. Similar differences were also observed in the ESC cardiomyocyte differentiation model between days 7 and 14. These findings correlate with the increases in rate of contraction and changes in Ca^{2+} transient dynamics during formation of the cardiac crescent (chapter 3) as well as the increased expression of NCX1 prior to Cav1.2. The influence of NCX1 and Cav1.2 on Ca^{2+} transients occurs in a dynamic way, with the transition from cardiac crescent to LHT taking around 12 hours suggesting that changes in NCX1 activity occur very rapidly. These differences are most likely due to the stage-dependent gene expression of NCX1 and Cav1.2 as discussed in chapter 5, however a number of other factors are likely to determine the functional significance of NCX1 and Cav1.2 during initiation of cardiac function.

One of the main factors regulating Cav1.2 and NCX1 function is the difference in kinetics of channels and exchangers. Typically channels have around a 1000-fold greater rate of ion flux compared to exchangers (Bers 2001). At stage 0 of crescent formation inhibition of Cav1.2 had no effect on pre-contractile Ca^{2+} transients whilst NCX1 was required for their initiation. The ability of CB-DMB to inhibit pre-contractile Ca^{2+} waves suggests that the slow dynamics of these stage 0 Ca^{2+} transients are due to the slow kinetics of NCX1 activity. The lack of contraction at stage 0 may be due to two different reasons. One possibility is that the lack of sarcomere formation at stage 0 may prevent contraction, meaning NCX1 derived Ca^{2+} transients cannot trigger contraction due to the immature contractile apparatus. Alternatively, NCX1-derived Ca^{2+} transients may not be sufficient to raise intracellular Ca^{2+} levels and trigger

activation of myofilaments. The inability of NCX1 mediated Ca^{2+} transients to generate contraction may be exacerbated by Ca^{2+}-binding proteins buffering free Ca^{2+} during initial Ca^{2+} influx and could suggest that intracellular Ca^{2+} has to reach a threshold before contraction can occur.

As the cardiac crescent matured from stage 1 to the linear heart a number of changes were observed including, the onset and increase in rate of contraction (chapter 3), the expression of Cav1.2 along with a loss in ability of NCX1 blockade to prevent Ca^{2+} transients. The onset and increase in contraction correlate with the kinetics of Cav1.2 and its increased expression as previously discussed, however the loss of "reverse-mode" NCX1 activity and Ca^{2+} influx may be due to a number of interlinked factors. As cardiomyocytes mature their resting membrane potential decreases leading to a change in NCX1 activity (Reppel, Sasse, et al. 2007). Between stage 0 and stage 3 there is likely a reduction in resting membrane potential sufficient to prevent "reverse-mode" NCX1 activity and therefore Ca^{2+} influx. The loss of NCX-mediated Ca^{2+} influx may also be due to the role of NCX1 in Ca^{2+} homeostasis. The increased expression and function of Cav1.2 would trigger greater Ca^{2+} influx, therefore, raising intracellular Ca^{2+} concentration leading to increased "forward–mode" NCX1 activity in order to prevent Ca^{2+} overload. NCX1-mediated Ca^{2+} efflux may be more fundamentally relevant in the embryonic setting compared to the adult due to the lack of a mature sarcoplasmic reticulum, making the balance between Cav1.2 mediated Ca^{2+} influx and NCX1 Ca^{2+} efflux critical. Elevated Ca^{2+} removal via NCX1 could also result in an increased rate of contraction due to the inward depolarising current generated by "forward-mode" NCX1 activity having a pacemaker like function. In terms of pacemaker activity it is likely that the role of NCX1 in action potential generation is superseded by the expression of HCN4 along with decreases in the membrane potential as cardiomyocytes mature.

In conclusion, NCX1 appears to be fundamental to the initiation of pre-contractile slow Ca^{2+} transients, however, its role becomes superseded by Cav1.2 during crescent maturation and as contraction rate increases. In order to determine the mechanisms underlying these functional changes, further electrophysiological studies would be required.

4.3.14 Restriction of Ca^{2+} wave propagation in the cardiac crescent

Whilst assessing the effect of pharmacological inhibition on wave propagation in the cardiac crescent at stages 1-3, a confinement of Ca^{2+} transients was observed after 5 min of drug application before complete inhibition. CB-DMB, KB-R7943 and Nifedipine all caused a similar effect in confining Ca^{2+} waves to the right side of the embryo. This would suggest a cellular heterogeneity across the cardiac crescent in terms Ca^{2+} transient generation. This heterogeneity could represent cardiomyocyte maturity or lineage specific differences. As cardiomyocytes mature, the role of pace maker currents such as the I_f HCN4 derived "funny current" and the T-type type current become more predominant in controlling the rate of contraction. This non-inhibited region may, therefore, represent a population of cells which have matured so that Ca^{2+} transient generation is more resistant to inhibition. Alternatively, this observation could be explained by the cardiac crescent being made up of different cardiomyocyte sub-populations which have different electrochemical properties. In the adult, different regions of the heart have different electrophysical properties due to the channels and exchangers they express. For example, HCN4 and T-type Ca^{2+} channels are expressed at greater levels in the atria compared to the ventricles. It has been shown that during first and second heart field formation both lineages express the cardiogenic mesoderm marker, *Mesp1,* however, these cells are already committed to form distinct populations of cardiomyocytes which give rise to different regions of the heart. In contrast, differences in channel expression between the two ventricles or the two atria may be due to left-right signalling as confinement of Ca^{2+} transients occurring in an asymmetrical manner. Expression of genes involved in left-right patterning such as Pitx2 may control the expression of genes responsible for the electrophysiological heterogeneity and left-right differences. Pitx2 plays a crucial role in morphology of the forming heart as well as being shown to control cardiac transcription factors such as *Mef2c* and *Gata-4* but also ion channels including HCN4 and repolarising potassium channels such as Kcna1, Kcnk2 (Wang et al. 2010). In addition to a role in heart development, Pitx2 has also been implicated as causative of atrial fibrillation in the adult (Sinner et al. 2011), highlighting a potential role for left-right patterning in manifesting via the electrophysiological properties across the adult heart.

Overall, this data reveals that in the stage 1 cardiac crescent, the response to pharmacological inhibition is not uniform, with confinement of Ca^{2+} waves to a specific region. This observation would suggest that across the symmetrical cardiac crescent there is heterogeneous ion channel and exchanger expression, however what determines this electrophysiological asymmetry is still unknown.

4.3.15 Pharmacological and technical limitations when assessing NCX1 and Cav1.2 function

Whilst CB-DMB, KB-R7943 and nifedipine have been reported as specific inhibitors to NCX1 and L-type Ca^{2+} channels respectively caution must be taken when interpreting this data in regards to off-target effects. To reduce the chance of off-target effects when using NCX1 inhibitors two different specific inhibitors of NCX1 were used. Both CB-DMB and KB-R7943 have been shown to directly inhibit NCX1 function, however, it has been published that at high concentrations they can inhibit L-type Ca^{2+} channel activity (Secondo et al. 2009). Using the whole embryo in order to image Ca^{2+} transients in the forming heart prevented the precise concentration of inhibitor reaching the cardiac crescent from being known, although the concentration is likely to be significantly lower than that calculated in the media due to mounting of the embryos, the tissue surrounding the heart and diffusion of the media. These observations revealed that both KB-R7943 and CB-DMB had the same stage-dependent effect on Ca^{2+} transients; with no inhibition observed at the late stages of cardiac crescent formation, in contrast to application of nifedipine which continued to inhibit Ca^{2+} transients at the later stages of development. Together this data would strongly support the specific nature of both CB-DMB and KB-R7943 when inhibiting NCX1 function, especially in ruling out off-target inhibition of L-type Ca^{2+} channels. Nifedipine is widely used as a specific L-type Ca^{2+} channel inhibitor, however, it could also inhibit other L-type Ca^{2+} channels such as Cav1.1. Although the focus of these studies was on the L-type channel Cav1.2, other L-type Ca^{2+} channels, such as Cav1.3, may also influence Ca^{2+} transients and contraction within the cardiac crescent, however, this was not addressed in this study.

When analysing Ca^{2+} transients at stage 0 application of DMSO, as a vehicle control, inhibited Ca^{2+} transients, whilst from stage 1 onwards it had no effect. The inhibition observed at this stage under control conditions (DMSO) is most likely due to the model

used and the technical difficulties associated with Ca^{2+} dye loading, fine dissection and removal of the endoderm as previously discussed. The fact that application of DMSO resulted in a decrease in the number of cells with Ca^{2+} transients would suggest that the endoderm has a role in maintaining early Ca^{2+} transients. This again highlights the need for a new approach when trying to investigate the initiation of Ca^{2+} transients during heart development such as transgenic Ca^{2+} reporter lines which would allow Ca^{2+} imaging to be conducted without removing the overlying endoderm.

4.4 Summary

In this chapter the expression of a number of different Ca^{2+} related channels and exchangers during cardiomyocyte differentiation and heart formation has been characterised. The aim of this study was to try and identify potential Ca^{2+} handling mechanisms which may have a role in both cardiac function as well as influencing downstream signalling pathways required for cardiac gene expression and cardiomyocyte differentiation. This chapter has focused on the sarcolemmal protein NCX1 as having a major role in pre-contractile Ca^{2+} transients due to its early expression and previously reported role in embryonic cardiomyocyte function. The functional significance of NCX1 and Cav1.2 has been investigated during the initiation of cardiac function with similar roles for NCX1 and Cav1.2 observed in both the mouse embryo and ESC-derived cardiomyocytes. These results revealed that NCX1 is responsible for Ca^{2+} influx in stage 0 Ca^{2+} transients whilst its role is superseded by Cav1.2 as cardiomyocytes mature, highlighting a dynamic role for these proteins during the initiation of heart function. In the following chapters, how these early Ca^{2+} influx pathways may regulate cardiomyocyte gene expression and differentiation to feedback onto early heart development will be investigated.

5. CHRONIC INHIBITION OF NCX1, CAV1.2 AND TPC1/2 INHIBITS CARDIOMYOCYTE DIFFERENTIATION

5.1 Introduction

During formation of the cardiac crescent dynamic changes were observed in cardiac function, the expression of Ca^{2+} handling proteins and the mechanism by which Ca^{2+} transients are initiated. NCX1 was found to be a predominant mechanism for sarcolemmal Ca^{2+} influx and transient generation prior to contraction a role superseded by Cav1.2 as the cardiac crescent matured. Whilst transgenic models of NCX1 and Cav1.2 loss of function have shown that these proteins are required for viable embryo development they have not revealed how early Ca^{2+} handling via NCX1 or Cav1.2 effects cardiomyocyte differentiation. In this chapter we will examine how pharmacological inhibition of NCX1 and Cav1.2 effects cardiomyocyte differentiation using both ESC cardiomyocyte differentiation and embryo culture models.

5.2 Results

5.2.1 CB-DMB and nifedipine have half-lives suitable for long term culture experiments

To assess the role of NCX1 and Cav1.2 mediated Ca^{2+} influx pharmacological inhibition was used during ESC cardiomyocyte differentiation and embryo culture. To determine the most suitable inhibitors for long-term culture, it was important to determine the activity of CB-DMB, KB-R7943 and nifedipine over extended periods of culture, due to the differentiation protocol requiring up to 4 days without a media change. To determine the most stable NCX1 and Cav1.2 inhibitors, drug activity was measured by assessing the percentage of beating EBs at day 7 after 30mins of application. Inhibitors were either freshly made or maintained in solution at 37^{o}C for 2 or 4 days. The inhibitory effect of CB-DMB, nifedipine and DMSO was unchanged when either 2 day or 4 day old drug-containing-media was added, compared to fresh drug containing media (Figure 5.1a & b, Figure 5.1d). This was in contrast to KB-R7943 which when made fresh inhibited contraction but had no effect after 4 days at 37^{o}C (Figure 5.1c).

Overall this data suggests that for long-term culture the most suitable NCX1 and Cav1.2 inhibitors would be CB-DMB and nifedipine respectively based on their stability and continued inhibition after 4 days in culture.

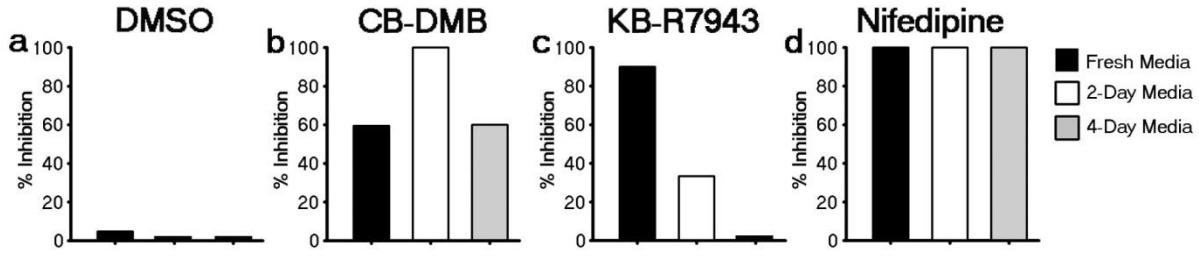

Figure 5.1: Pharmacological activity of CB-DMB and nifedipine is maintained after 4-days in culture

Contraction was assessed in ESC-derived cardiomyocytes at day 7 of differentiation after 30mins culture with NCX1 and Cav1.2 channel blockers cultured for different lengths of time. Application of DMSO did not inhibit contraction when using fresh, 2-day old and 4-day old media (a, fresh, n=42; 2-day=2; 4-day n=9). Inhibition of NCX1 with CB-DMB (10 µM) reduced contractions when fresh, 2-day old and 4-day old drug containing media was added (b, fresh, n=37; 2-day=5; 4-day n=6), whereas fresh KB-R7943 (30 µM) completely inhibited contraction but its ability to inhibit contraction was reduced when 2-day old drug containing media was added and had no effect when 4-day old drug was applied (c, fresh, n=10; 2-day=3; 4-day n=3). Inhibition of LTCC with nifedipine (10 µM) significantly reduced contractions when fresh, 2-day old and 4-day old drug containing media was added (d, fresh, n=15; 2-day=2; 4-day n=6).

5.2.2 Inhibition of NCX1 prevents *Nkx2.5*-GFP ESC cardiomyocyte differentiation

Pharmacological inhibition was used to establish the role of NCX1 and Cav1.2 in cardiomyocyte specification and differentiation. For these experiments, Nkx2.5-GFP EBs were plated at day 4 of differentiation on gelatin-coated 12 well plates. In cells treated throughout differentiation with 1 µM CB-DMB, expression of *Nkx2.5* was significantly reduced as assessed by number of GFP positive cells present at day 14 (Figure 5.2e-h). In terms of phenotype, the inhibition of NCX1 had no effect on the formation of EBs or cell outgrowth, although at day 14 the morphology of original EBs was more defined compared to the DMSO control. To establish the stage at which NCX1 inhibition affects cardiac differentiation mRNA expression of stage specific markers was measured. Cells treated with 1 µm CB-DMB, a concentration known to inhibit the NCX1 function (Secondo et al. 2009), from day 0-14 exhibited significantly reduced expression of the myocyte markers *Myh6* and *Tnnt2* (Cooper & Ordahl 1984)(Nakao et al. 1997)(*Myh6*; 0.031 ±0.02; p≤0.001; *Tnnt2;* 0.122 ±0.04; p≤0.001;

Figure 5.3a, Figure 5.3b) as well as inhibiting expression of the early cardiac progenitor markers *Nkx2.5* and *Mef2c* (Edmondson et al. 1994)(Lien et al. 1999)(*Nkx2.5*; 0.242 ±0.04; p≤0.001; *Mef2c;* 0.278 ±0.11; p=0.006; Figure 5.3c, Figure 5.3d). The endoderm marker *Sox17* was also down regulated (0.268 ±0.10; p=0.04; Figure 5.3j) whilst *GFAP* and *Goosecoid* were unchanged (Eglitis & Mezey 1997)(Gaunt et al. 1993)(Figure 5.3i, Figure 5.3k). In contrast, NCX1 inhibition significantly increased the expression of the mesoderm marker *Brachyury* (Wilkinson et al. 1990)(7.30 ±2.27; p≤0.001; Figure 5.3f). Although the cardiogenic mesoderm marker *Mesp1* was up-regulated this was not significant (Saga et al. 1999)(3.35 ±1.03; Figure 5.3e). Pluripotent markers *Oct-4* and *Nanog* (Niwa et al. 2005) (Mitsui et al. 2003) were also both up-regulated (*Oct-4*; 3.60 ±1.04; p≤0.001; *Nanog;* 2.75 ±0.34; p=0.003; Figure 5.3g, Figure 5.3h) compared to the DMSO control. These findings indicate that NCX1 inhibition may be playing a role in the specification of mesoderm to cardiac progenitors.

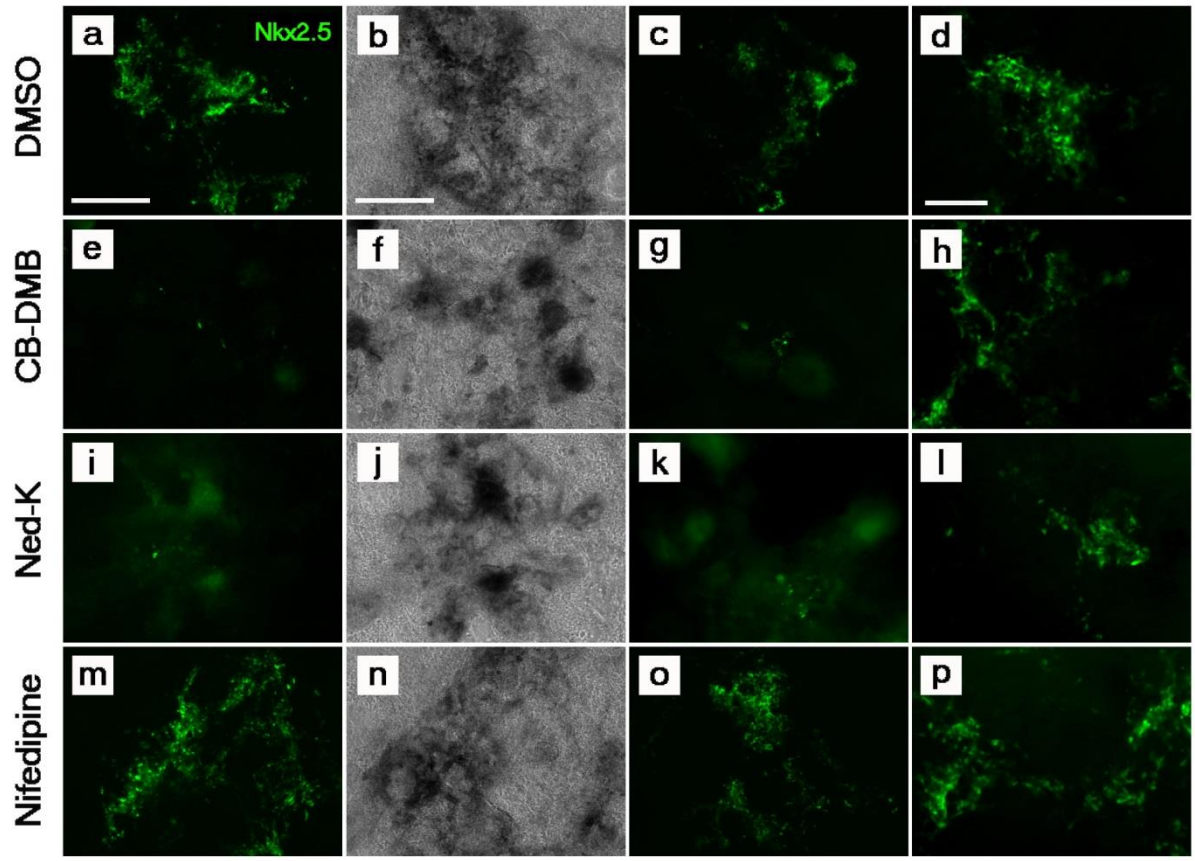

Figure 5.2: *Nkx2.5*-GFP ESC derived cardiomyocyte formation is reduced at day 14 after culture with 1 µM CB-DMB and 30 µM Ned-k throughout differentiation, but was not affected by 500 nM nifedipine

At day 14 control *Nkx2.5*-GFP cardiomyocyte differentiation revealed extensive GFP+ expression after culture with DMSO from day 0-14 (a-d) as compared to a loss of GFP+ cells when treated from day 0-14 with CB-DMB (day 0-14) to inhibit NCX1 (e-h) or Ned-K to inhibit NAADP signalling (i-l). Inhibition of Cav1.2 with 500 nM nifedipine had no effect on *Nkx2.5*-GFP expression at after 14 days of differentiation (m-p). Fluorescence (a, c-e, g-i, k-m, o, p) and bright field (b, f, j, n) images shown for each treatment group; bright field confirmed the cultures were grossly unaffected by treatment. Scale bar: a-c, e-g, i-k, m-o = 500 µm; d, h, l, p = 200 µm.

In order to determine the time point at which NCX1 inhibition was repressing differentiation, ESCs were treated from day 0-4 with 1µm CB-DMB before being cultured in normal control media from day 4 to 14. This treatment regime led to a reduced number of *Nkx2.5*-GFP positive cells forming, although there were more in total when compared to treatment from day 0-14 (Figure 5.4e-h). Expression of *Myh6*, *Tnnt2* and *Nkx2.5* mRNA was found to be significantly decreased by day 14 when compared to DMSO treatment (*Myh6*; 0.086 ±0.04; p=0.019; *Tnnt2;* 0.215 ±0.04; p=0.001; *Nkx2.5*; 0.328 ±0.06; p≤0.001; Figure 5.3a-b, Figure 5.3d), although to a lesser extent than observed when ESC where treated with 1 µm CB-DMB throughout differentiation. In contrast, Mef2c expression was not significantly reduced at day 14 when treated from day 0-4 with 1 µm CB-DMB (Figure 5.3c). In terms of early markers at day 14, *Mesp1, Brachyury*, *Oct-4* and *Nanog* expression were significantly up-regulated compared to DMSO control (*Mesp1*; 4.86 ±0.43; p=0.018; *Brachyury;* 3.62 ±0.55; p=0.012; *Oct-4*; 2.19 ±0.21; p=0.002; *Nanog*; 2.21 ±0.34; p=0.02; Figure 5.3e-h), although *Brachyury*, *Oct-4* and *Nanog* were not elevated to the same extent as 1 µm CB-DMB treatment from day 0-14. There were significant differences at day 14 in the expression levels of *Myh6*, *Mef2c* and *Nanog* compared to day 0-14 1 µm CB-DMB treatment (*Myh6*; p=0.031; *Mef2c;* p=0.035; *Nanog*; p=0.008; Figure 5.3a, Figure 5.3c, Figure 5.3h). Therefore, whilst blocking early NCX1 function during mesoderm pattering and cardiomyocyte specification (day 0-4) reduced cardiomyocyte differentiation it did not completely prevent it, as demonstrated by the increases in cardiomyocyte-specific mRNA levels and the expression of *Nk2.5*-GFP at day 14. This highlights a potential early role for NCX1 in mesoderm to cardiomyocyte specification.

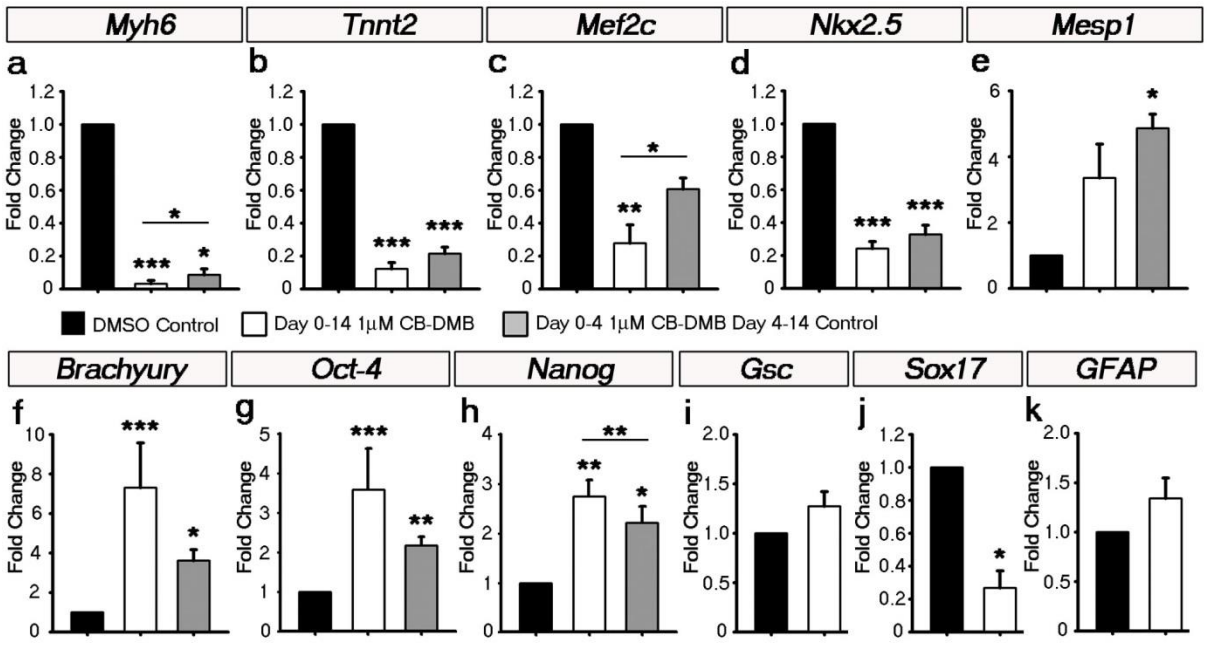

Figure 5.3: Inhibition of NCX1 with 1 µM CB-DMB prevents cardiomyocyte specific gene expression and promotes early ESC markers at day 14 of *Nkx2.5*-GFP ESC differentiation

Following chronic exposure of *Nkx2.5*-GFP ESC for 14 days to CB-DMB (1 µM) there was a significant decreases in the cardiac genes *Myh6* (a), *Tnnt2* (b), *Mef2c* (c) and *Nkx2.5*(d), whilst early ESC genes *Brachyury* (f), *Oct-4* (g) and *Nanog* (h) were up regulated compared to DMSO control. The endoderm marker *Sox17* was also down regulated (j), whereas *Mesp1* (e), *GSC* (i) and *GFAP* (k) were not significantly different. Treatment from day 0-4 with CB-DMB and subsequent culture in control media until day 14 resulted in the same effect on gene expression as that observed with treatment from day 0-14 although there was a significant increase in the expression of *Myh6* (a) and *Mef2c* (c) compared to treatment from day 0-14. Whilst most genes showed a similar expression pattern at day 14 when treated from day 0-4 compared when treated throughout the mesoderm gene Mesp1 (e) became significantly up regulated. All error bars are mean ± S.E.M (n=5). Statistics: one-way ANOVA and Tukey test for multiple comparisons (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

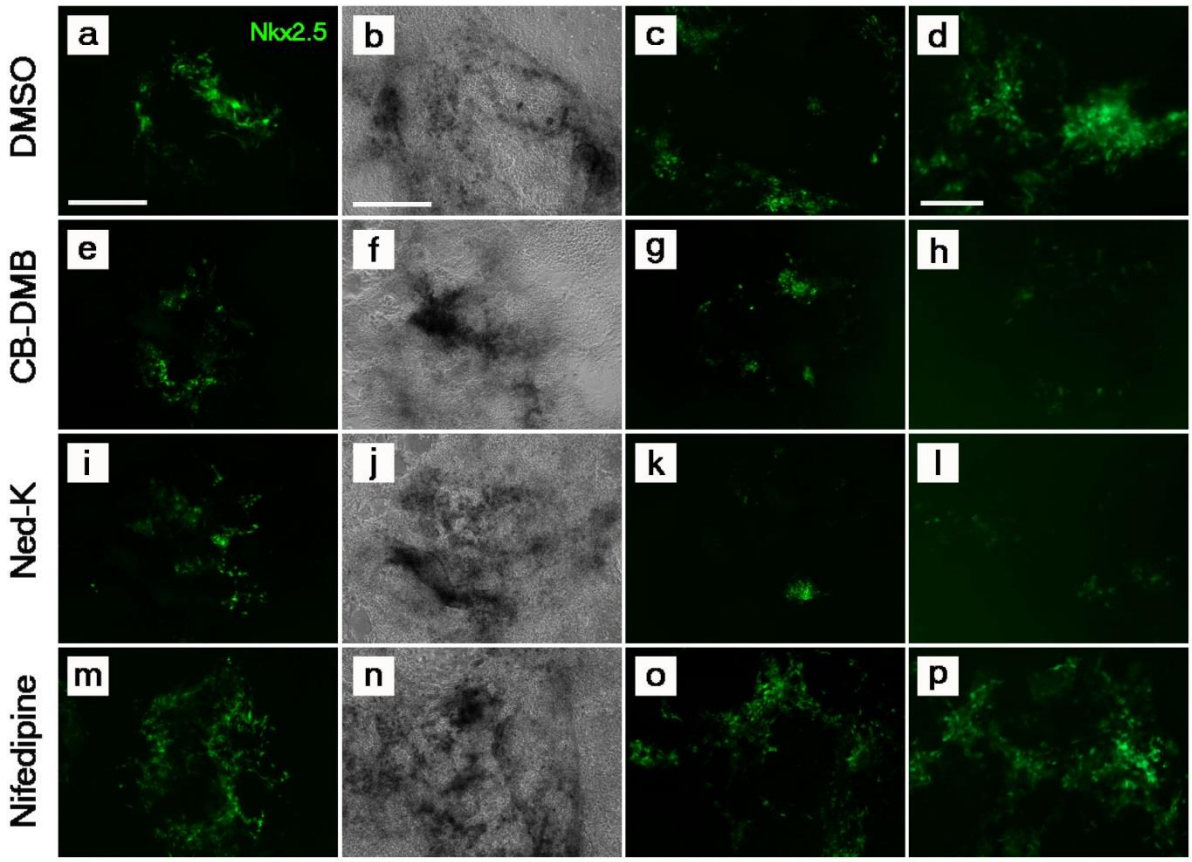

Figure 5.4: *Nkx2.5*-GFP ESC derived cardiomyocyte formation is reduced at day 14 after culture with 1 µM CB-DMB and 30 µM Ned-k from day 0-4, but was not affected by 500 nM nifedipine

Nkx2.5-GFP cardiomyocyte differentiation revealed extensive GFP+ expression after treatment with DMSO from day 0-4 (a-d) before culture for 10 days in control media, as compared to a significant reduction in GFP+ cells when treated from day 0-4 with CB-DMB (maintained in control media from day 4-14) to inhibit NCX1 (e-h) or Ned-K to inhibit NAADP signalling (i-l). Inhibition of Cav1.2 with 500 nM nifedipine had no effect on *Nkx2.5*-GFP expression after 14 days of differentiation (m-p). Fluorescence (a, c-e, g-i, k-m, o, p) and bright field (b, f, j, n) images shown for each treatment group; bright field confirmed the cultures were grossly unaffected by treatment. Scale bar: a-c, e-g, i-k, m-o = 500 µm; d, h, l, p = 200 µm.

5.2.3 Low dose inhibition of Cav1.2 has no effect on *Nkx2*.5-GFP cardiomyocyte differentiation

Inhibition of Ca^{2+} influx by nifedipine was used to assess the role of Cav1.2 in myocyte differentiation. Treatment of Nkx2.5-GFP ESCs with 500 nM nifedipine, a concentration previously shown to inhibit Cav1.2 function (Thiel et al. 2008), from either day 0 to 14 or day 0 to 4 had no effect on the levels of GFP positive cells compared to vehicle control at day 14 (Figure 5.2m-p, Figure 5.4m-p). Treatment with nifedipine did not prevent either the formation of EBs or the outgrowth of cells. Furthermore, application

of nifedipine had no effect on expression of the stage-specific markers when compared to control (Figure 5.5). Together these results indicate that Cav1.2 inhibition with 500 nM nifedipine has no effect on cardiomyocyte differentiation in this model.

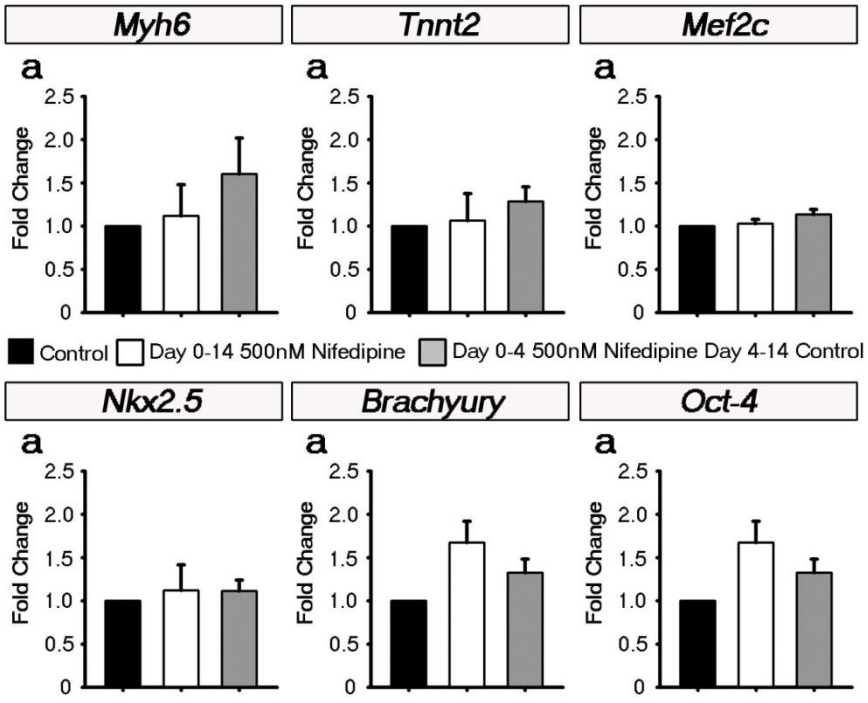

Figure 5.5: Inhibition of Cav1.2 with 500 nM nifedipine did not affect cardiomyocyte gene expression or effect early ESC markers at day 14 of *Nkx2.5*-GFP ESC differentiation

Chronic exposure of *Nkx2.5*-GFP ESC from day 0-14 and day 0-4 with nifedipine (500 nM) did not affect the expression of cardiomyocyte genes *Myh6* (a), *Tnnt2* (b), *Mef2c* (c) and *Nkx2.5*(d) or early ESC genes including *Brachyury* (e) and *Oct-4* (f) compared to DMSO control at day 14. All error bars are mean ± S.E.M (n=5). Statistics: one-way ANOVA (all results showed no significance).

5.2.4 Inhibition of NAADP signalling prevents *Nkx2.5*-GFP ESC cardiomyocyte differentiation

To assess the requirement for NAADP-stimulated release of lysosomal Ca^{2+} stores via TPCs during cardiomyocyte differentiation the inhibitor Ned-K was employed (Calcraft et al. 2009). Ned-K is based on the inhibitor Ned-19, which inhibits TPC function by blocking both Ca^{2+} release and NAADP binding (Naylor et al. 2009). Application of Ned-K from day 0-14 of *Nkx2.5*-GFP ESC differentiation reduced the expression of GFP positive cells in comparison to DMSO treated control samples (Figure 5.2i-l). Treating cultures with Ned-K from day 0-14 significantly reduced expression of the mature

cardiomyocyte markers *Myh6* and *Tnnt2* (*Myh6*; 0.014 ±0.01; p≤0.001; *Tnnt2;* 0.063 ±0.02; p≤0.001; Figure 5.6a-b), as well as significantly inhibiting the cardiac progenitor markers *Nkx2.5* (0.20 ±0.05; p=0.001; Figure 5.6d) and *Mef2c* (0.18 ±0.064; p≤0.001; Figure 5.6c). Inhibition of NAADP signalling had no significant effect on *Mesp1*, *Brachyury*, *Goosecoid* and *GFAP* mRNA levels (Figure 5.6e-f, Figure 5.6i, Figure 5.6k) whilst *Sox17* was significantly down regulated (0.36 ±0.13; p=0.033; Figure 5.6j). In contrast pluripotent markers *Oct-4* and *Nanog* were significantly up-regulated at day 14 (*Oct-4*; 2.86 fold ±0.60; p=0.004; *Nanog;* 2.21 ±0.31; p=0.002; Figure 5.6g-h). In terms of phenotype, inhibition of NAADP signalling had no effect on the formation of EBs or cell outgrowth. Treatment of ESCs with Ned-K from day 0-4 inhibited the number of GFP-positive cells, although these numbers were greater than when Ned-K was present throughout days 0-14 (Figure 5.4i-l). Similarly, mRNA levels of *Myh6*, *Tnnt2* and *Nkx2.5* and were significantly reduced when Ned-K was present from day 0-4 (*Myh6*; 0.058 ±0.03; p=0.014; *Tnnt2;* 0.185 ±0.03; p=0.002; *Nkx2.5*; 0.288 ±0.05; p=0.006; Figure 5.6a-b, Figure 5.6d), although expression was significantly greater than that observed when ESC were treated from day 0-14 with Ned-K (*Myh6*; p=0.01; *Tnnt2*; p=0.004; *Mef2c*; p=0.013; *Nkx2.5*; p=0.032. Figure 5.6a-d). Day 0-4 inhibition of TPC1/2 significantly up-regulated mRNA levels of early ESC genes including *Brachyury* (3.69 ±1.2; p=0.021; Figure 5.6f)*, Mesp1* (5.04 ±0.4; p=0.028; Figure 5.6e)*, Oct-4* (2.21 ±0.3; p=0.018; Figure 5.6g) and *Nanog* (1.71 ±0.1; p=0.018; Figure 5.6h)*,* although the level of *Nanog* expression was significantly reduced compared to treatment throughout differentiation (p=0.012). Overall these results reveal that TPC1/2 inhibition prevented cardiomyocyte formation, suggesting a novel role for NAADP signalling and lysosomal Ca^{2+} release during differentiation.

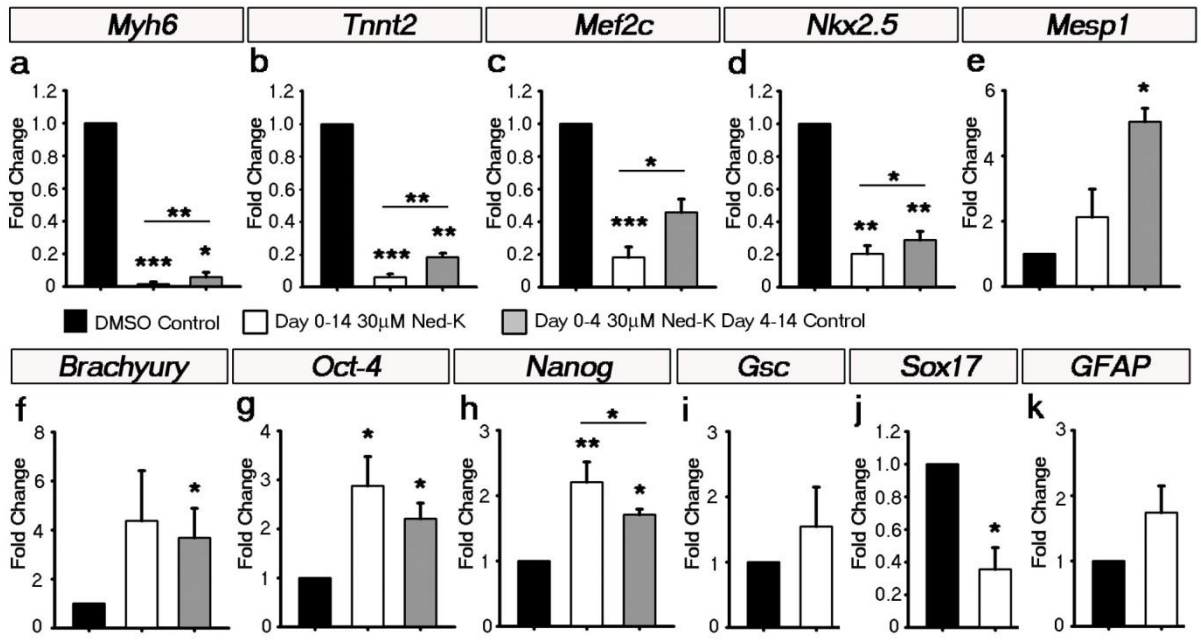

Figure 5.6: Inhibition of NAADP signalling with 30 μM Ned-K prevents cardiomyocyte gene expression and promotes early ESC markers at day 14 of *Nkx2.5*-GFP ESC differentiation

Exposure of *Nkx2.5*-GFP ESC to Ned-K (30 μM) for 14 days resulted in a significant decrease in cardiac genes *Myh6* (a), *Tnnt2* (b), *Mef2c* (c) and *Nkx2.5*(d), whilst early ESC genes, *Oct-4* (g) and *Nanog* (h) were up regulated compared to DMSO control. The endoderm marker *Sox17* was also down regulated (j), whereas *Brachyury* (f), *Mesp1* (e), *Gsc* (i) and *GFAP* (k) were not significantly different from the DMSO control samples. Treatment from day 0-4 with Ned-K before subsequent culture in control media until day 14 resulted in similar overall effect on gene expression when treated throughout although there were significant increases in the expression of *Myh6* (a), *Tnnt2* (b), *Mef2c* (c) and *Nkx2.5* (d) as well as a decrease in *Nanog* expression (h) when compared to treatment from day 0-14. Whilst most genes showed a similar expression pattern at day 14 when treated from day 0-4 compared to treatment throughout the mesoderm genes Mesp1 (e) and Brachyury (f) became significantly up regulated. All error bars are mean ± S.E.M (n=5). Statistics: one-way ANOVA and Tukey test for multiple comparisons (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

5.2.5 Inhibition of NCX1 prevents *Eomes*-GFP ESC cardiomyocyte differentiation

To confirm the above observations made using the *Nkx2.5*-GFP cell line experiments were repeated using the *Eomes*-GFP ESC line. Due to the absence of cardiomyocyte specific GFP reporter readout in this model it was not possible to directly asses cardiomyocyte formation using GFP expression, instead the percentage of spontaneously beating EBs was measured after drug removal. Inhibition of NCX1 led to a significant decrease in the number of beating EBs compared to DMSO at both day 7 (10% vs. 68%; $p\leq0.001$; Figure 5.7a) and day 14 (22% vs. 77%; $p\leq0.001$; Figure 5.7b) and was consistent with the inhibition observed in the *Nkx2.5*-GFP ESC line. The

difference in percentage of beating EBs corresponded with differences in gene expression. In terms of phenotype, the inhibition of NCX1 had no effect on the formation of EBs or cell outgrowth (Figure 5.8e-h) and the number of cells was not significantly different from DMSO treated EBs at day 4 (Figure 5.8m) and 7 (Figure 5.8n). Mature cardiomyocyte markers *Myh6*, *Myh7* and *Tnnt2* were all significantly down regulated at both day 7 (*Myh6*; 0.485 ±0.15; p=0.003; *Tnnt2*; 0.624 ±0.16; p=0.039; Figure 5.9a-c) and day 14 of differentiation (*Myh6*; 0.585 ±0.08; p=0.002; *Myh7*; 0.445 ±0.05; p=0.002; *Tnnt2*; 0.571 ±0.06; p≤0.001; Figure 5.9a-c). Cardiac progenitor markers *Mef2c* and *Nkx2.5* were significantly down regulated at day 14 of differentiation (*Mef2c*; 0.600 ±0.05; p≤0.001; *Nkx2.5*; 0.807 ±0.09; p=0.039; Figure 5.9d and Figure 5.9e), whilst *Gata-4* expression was unchanged at both day 7 and day 14 (Figure 5.9f). Mesoderm markers *Mesp1* and *Brachyury* showed no difference at day 7 or day 14 except for *Brachyury* which was significantly up-regulated at day 14 (3.75 ±1.78; p=0.04; Figure 5.9h), whilst the pluripotent marker *Oct-4* was unchanged (Figure 5.9i). Gene expression of Ca^{2+} handling proteins NCX1 (*Slc8a1*) and Cav1.2 (*Canca1c*) were significantly down regulated at day 7 (*Slc8a1*; 0.503 ±0.13; p=0.001; *Canca1c*; 0.809 ±0.09; p≤0.001; Figure 5.9j and Figure 5.9k) and day 14 of differentiation (*Slc8a1*; 0.764 ±0.09; p=0.037; *Canca1c*; 0.780 ±0.05; p=0.003; Figure 5.9j and Figure 5.9k), most likely representing the decrease in cardiomyocyte formation.

To assess further the role of NCX1 during early differentiation in *Eomes*-GFP ESCs, EBs were treated with CB-DMB from day 0-4 before being cultured in control media until day 14. At day 7, the percentage of day 0-4 CB-DMB treated EBs was significantly reduced compared to DMSO (CB-DMB 25%; DMSO 66%; p≤0.001; Figure 5.7a), whilst at day 14 there was no difference between treated and control ESCs (CB-DMB 48%; DMSO 59%; Figure 5.7b). Thus, removal of NCX1 inhibition allowed differentiation to proceed normally and cardiomyocytes could form after 10 days in normal media. Overall these results recapitulate the effect of CB-DMB treatment when using the *Nkx2.5*-GFP ESCs and confirm the role of NCX1 in ESC cardiomyocyte differentiation using two different ESC lines.

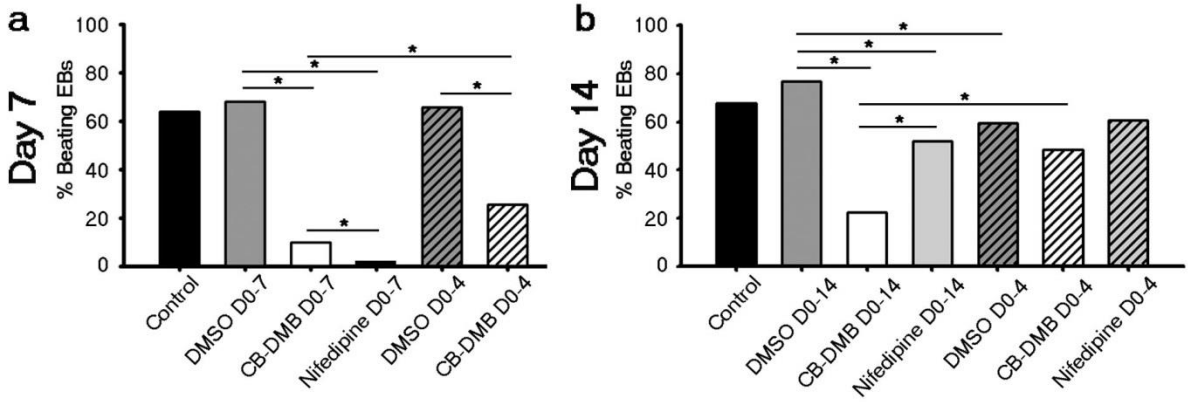

Figure 5.7: Inhibition of NCX1 with 1 µM CB-DMB and Cav1.2 with 10 µM nifedipine reduced the formation of *Eomes-GFP* ESC derived cardiomyocyte at day 4 and 7

Chronic exposure of embryoid bodies to CB-DMB (1 µM) or nifedipine (10 µM) resulted in a significant decrease in the incidence of beating EBs at both day 7 (Control, 64% (n=233); DMSO, 68% (n=603); CB-DMB, 10% (n=363); nifedipine, 0% (n=83) and day 14 (Control, 68% (n=183); DMSO, 80% (n=196); CB-DMB, 22% (n=152); nifedipine, 52% (n=71)) although at day 14 there was significantly greater percentage of beating EBs when treated with nifedipine compared to CB-DMB (a). Initial treatment from day 0-4 with CB-DMB resulted in a decreased number of beating EBs compared to DMSO at day 7 (Day 0-4 DMSO 66%, (n=105); Day 0-4 CB-DMB 25%, (n=102)) however there were significantly more beating EBs compared to treatment from day 0-7 (a). By day 14, treatment from day 0-4 with both CB-DMB and nifedipine had no effect on the percentage of beating EBs compared to the DMSO control (DMSO, 59% (n=207); CB-DMB, 48% (n=172); nifedipine, 60% (n=33)) as well as there being a significant increase in the percentage of beating EBs when comparing EBs treated with CB-DMB from day 0-4 B to treatment throughout. Statistics: Fisher's exact test with a Bonferroni correction for multiple comparisons. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

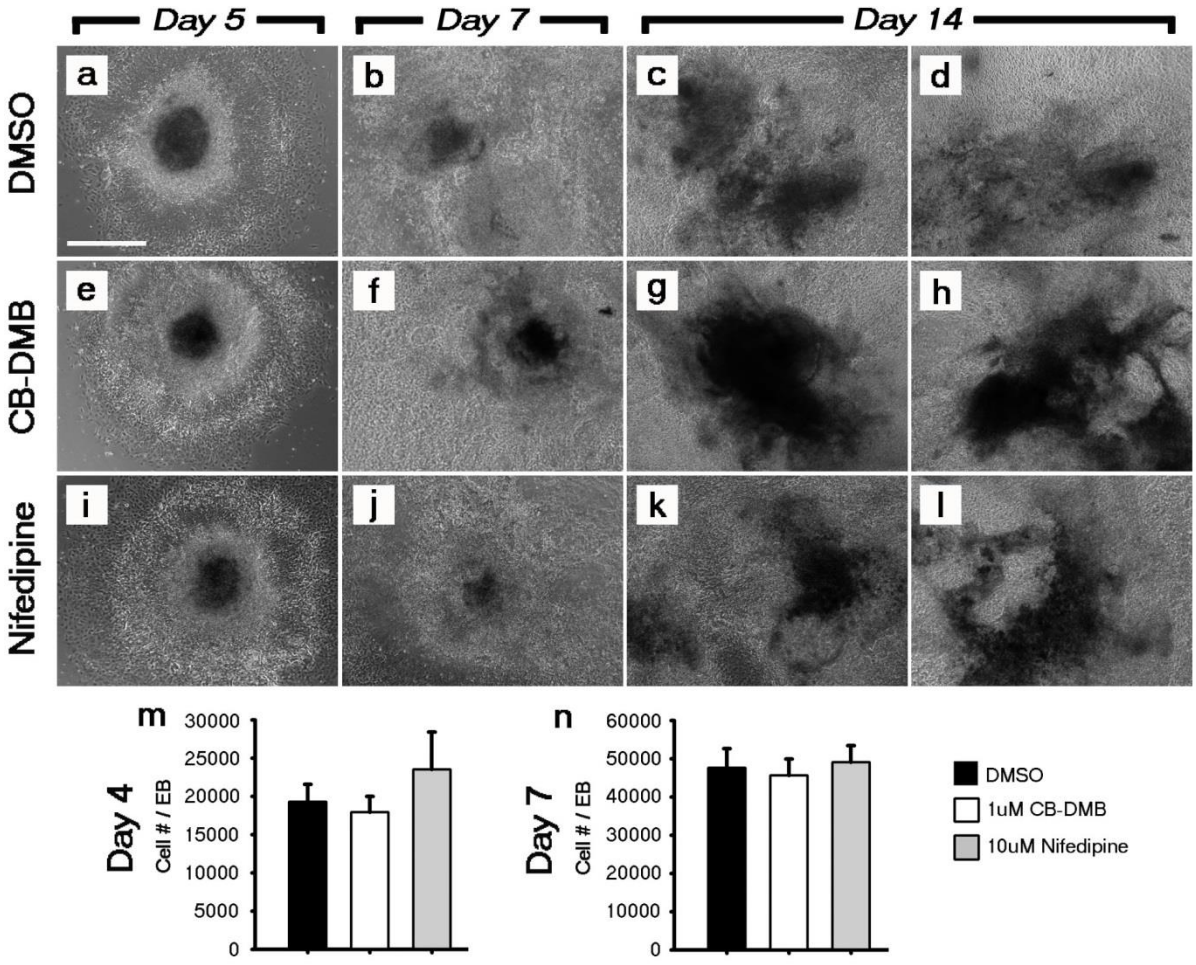

Figure 5.8: Inhibition of NCX1 with 1 µM CB-DMB and Cav1.2 with 10 µM nifedipine did not effect embryoid body formation or cell outgrowth during *Eomes-GFP* ESC differentiation

Treatment of *Eomes*-GFP ESCs with 1 µm CB-DMB or 10 µm nifedipine did not prevent EB formation or cell outgrowth. Bright field images confirmed that cultures were grossly unaffected by CB-DMB or nifedipine at all stages of differentiation as was the number of cells per EB at both day 4 (m, DMSO, 20744 cells/EB (n=9); CB-DMB, 19184 cells/EB (n=7); nifedipine, 26359 cells/EB (n=3)) and day 7 (n, DMSO, 46800 cells/EB (n=10); CB-DMB, 44922 cells/EB (n=8); nifedipine, 50761 cells/EB (n=3)). Scale bars: 500µm. All error bars are mean ± S.E.M. Statistics: one-way ANOVA and Tukey test for multiple comparisons (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

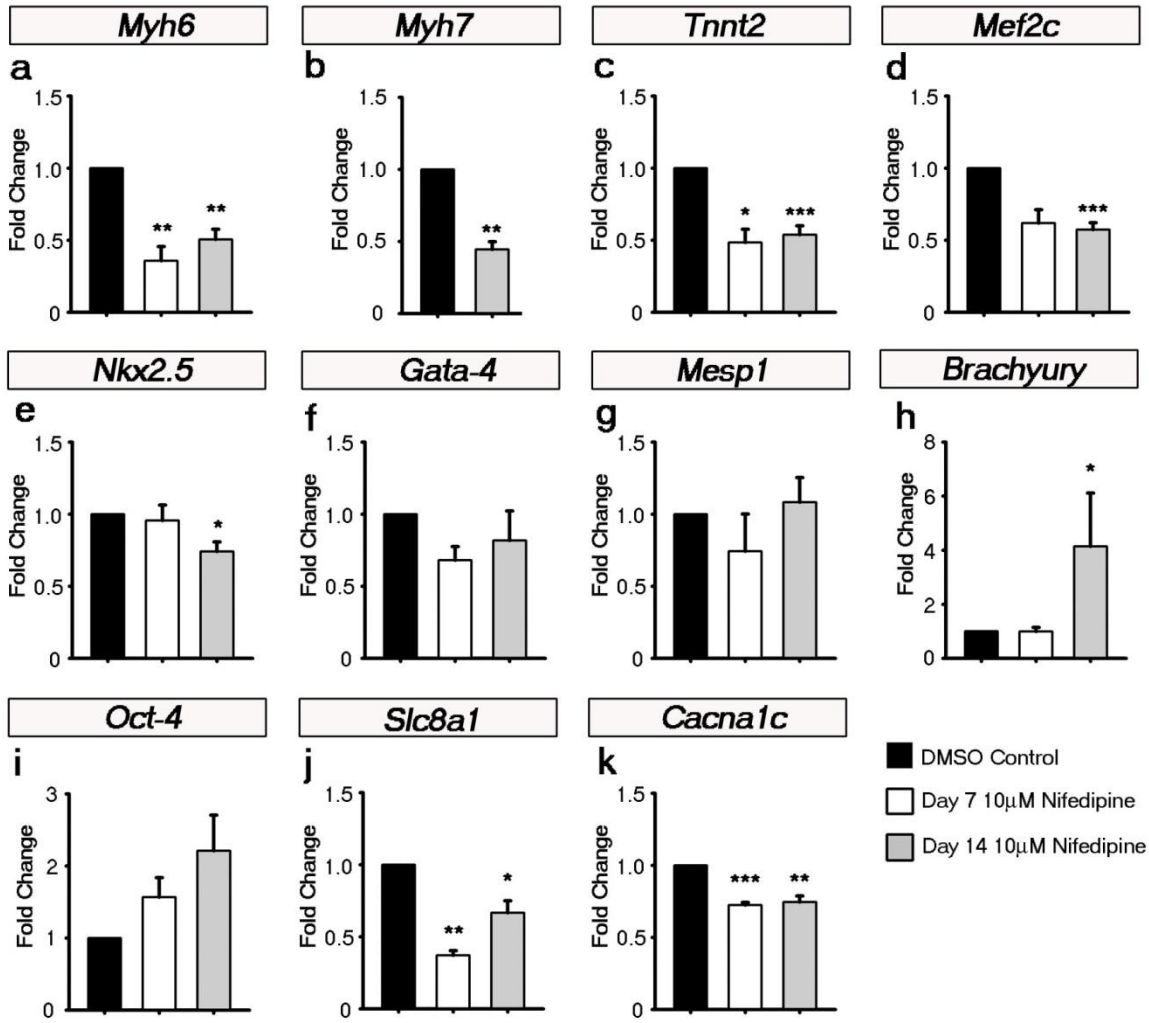

Figure 5.9: Inhibition of NCX1 with 1 μM CB-DMB prevents cardiomyocyte specific gene expression during *Eomes*-GFP ESC differentiation

Treatment of *Eomes*-GFP ESCs throughout differentiation with CB-DMB (1 μM) resulted in a down regulation of cardiac genes *Myh6* (a, day 7, n=6; day 14, n=9), *Tnnt2* (c, day 7, n=4; day 14, n=9), *Slc8a1* (j, day 7, n=4; day 14, n=9) and *Cacna1c* (k, day 7, n=4; day 14, n=9) at both day 7 and 14 of differentiation compared to the DMSO control whilst *Myh7* (b, day 14, n=5), *Mef2c* (d, day 7, n=4; day 14, n=9), *Nkx2.5* (e, day 7, n=6; day 14, n=9) where significantly reduced by day 14. The cardiac progenitor gene, *Gata-4* (f, day 7, n=4; day 14, n=6), cardiac mesoderm marker, *Mesp1* (g, day 7, n=4; day 14, n=9) and pluripotent maker, *Oct-4* (i, day 7, n=4; day 14, n=9) were unaffected by treatment with CB-DMB at both day 7 and 14 although the early mesoderm marker *Brachyury* was significantly up-regulated by day 14 (h, day 7, n=6; day 14, n=8). All error bars are mean ± S.E.M. Statistics: Student's paired t-test (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

5.2.6 Inhibition of Cav1.2 reduces differentiation *of Eomes*-GFP ESC cardiomyocyte

Although experiments using 500 nM nifedipine did not reveal an effect on cardiomyocyte differentiation in the *Nkx2.5*-GFP model, recently published work suggested that Cav1.2 does have a role in ESC cardiomyocyte differentiation (Nguemo et al. 2013). In this study, the authors used a higher concentration (10 µm) than previously tested in this study (500 nM), therefore to further asses the role of Cav1.2 experiments repeated using 10µm nifedipine. Treatment of EBs with nifedipine prevented the appearance of beating EBs at day 7 (nifedipine 0%; DMSO; 68%; $p \leq 0.001$; Figure 5.7a), with a significant reduction in the number of beating EBs at day 14 compared to DMSO (nifedipine 52%; DMSO; 76%; $p \leq 0.001$; Figure 5.7b), although there were still significantly more beating EBs at day 14 compared to CB-DMB treatment (nifedipine 52%; CB-DMB 22%; $p \leq 0.001$). In terms of phenotype, there was no difference in EB formation, cell outgrowth (Figure 5.8i-l) or cell number per EB at day 4 or day 7 (Figure 5.8m-n). The expression of cardiomyocyte marker genes (*Myh6*, *Tnnt2*, *Mef2c*, *Nkx2.5*, *Slc8a1*, *Cacna1c*) were unaffected at both day 7 and day 14 of treatment (Figure 5.10a, Figure 5.10c-e & Figure 5.10j-k) whilst *Myh7* and *Gata-4* were significantly down-regulated at day 14 (*Myh7*; 0.425 ±0.06; p=0.002; *Gata-4*; 0.652 ±0.12; p=0.037; Figure 5.10b and Figure 5.10f). Mesoderm marker, *Brachyury*, and pluripotent marker, *Oct-4,* were also unaffected (Figure 5.10h-i) whilst *Mesp1* was significantly down-regulated by day 14 (0.550 ±0.12; p=0.043; Figure 5.10g), although unchanged at day 7. Overall 10µm nifedipine led to delayed cardiomyocyte formation but did not inhibit differentiation to the same extent as NCX1 inhibition.

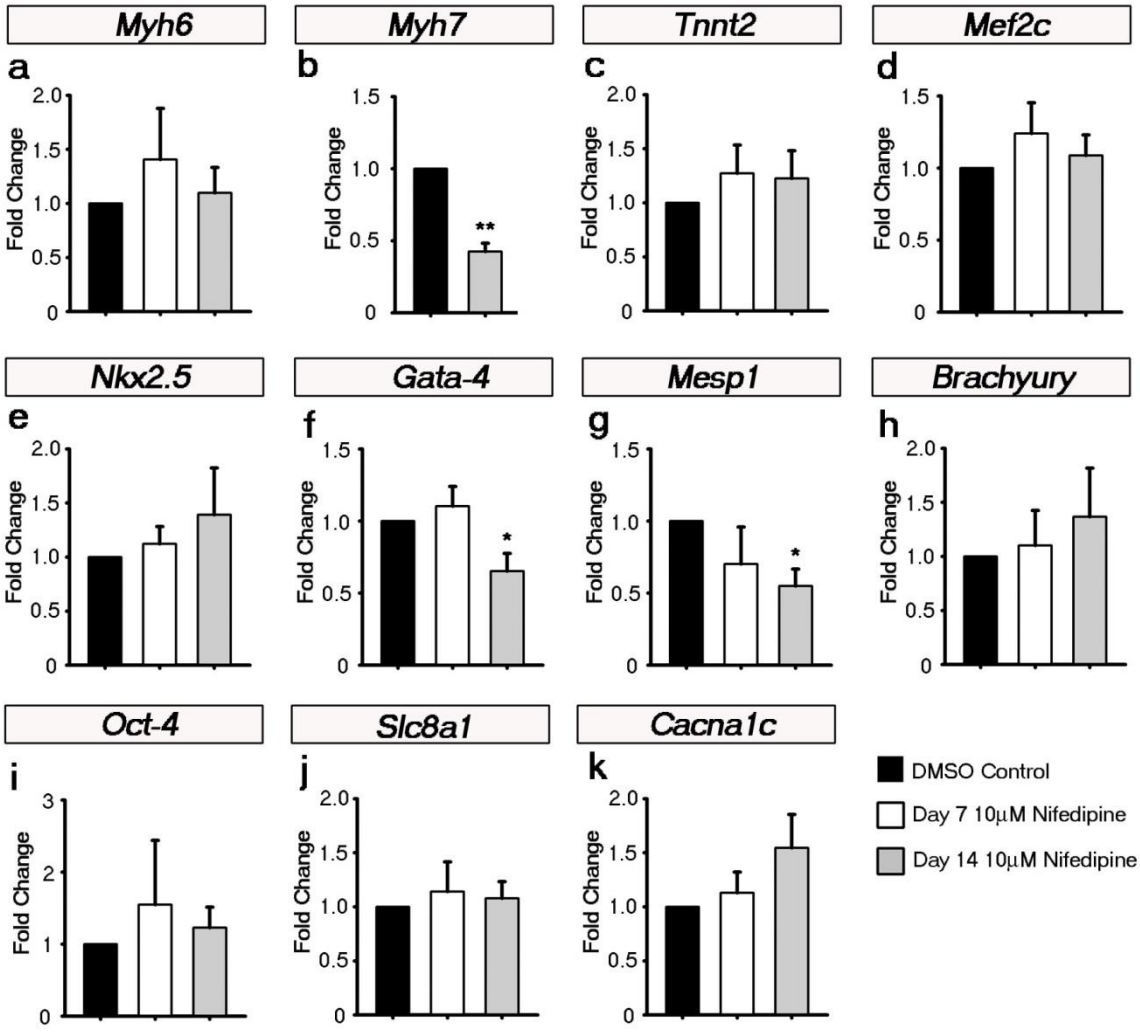

Figure 5.10: Inhibition of Cav1.2 with 10 μM nifedipine prevents cardiomyocyte specific gene expression during *Nkx2.5*-GFP ESC differentiation

Nifedipine treatment of *Eomes*-GFP ESCs throughout differentiation had no effect on the expression of *Myh6* (a), *Tnnt2* (c), *Mef2c* (d), *Nkx2.5* (e), *Brachyury* (h), *Oct-4* (i), *Slc8a1* (j) or *Cacna1c* (k) compared to DMSO control at day 7 or 14. In contrast *Myh7* (b), *Gata-4* (f) and *Mesp1* (g) were all significantly down regulated by day 14 of differentiation although these genes were still unaffected by treatment with nifedipine at day 7. All error bars are mean ± S.E.M (n=5). Statistics: Student's paired t-test (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

5.2.7 Inhibition of NCX1 and Cav1.2 during cardiac crescent formation *ex vivo*

To characterise the effect of drug inhibition in the embryo proper, *ex vivo* culture was used. Embryos were collected at E7.25 prior to the formation of the cardiac crescent and cultured in their yolk sacs using rolling culture. During 12 h culture with DMSO, cells arising from the lateral plate mesoderm coalesced into a distinct crescent structure with respect to the overlying neural ectoderm (head folds) and endoderm

(Figure 5.11a). When embryos were treated with 3 µm CB-DMB cells failed to coalesce into distinct cardiac crescent structures as observed by actin staining (Figure 5.11c). After culture, embryos were collected and gene expression was assessed. Inhibition of NCX1 with CB-DMB led to a significant down-regulation in *Tnnt2*, *Mef2c*, *Nkx2.5*, *Slc8a1* (*Tnnt2*; 0.54 ±0.11; p=0.038; *Mef2c;* 0.74 ±0.05; p=0.012; *Nkx2.5*; 0.48 ±0.08; p=0.004; *Slc8a1*; 0.77 ±0.04; p=0.043; Figure 5.12b-e), whilst *Myh6* and *Cacna1c* were not significantly affected (Figure 5.12a & Figure 5.12f). Thus overall the down-regulation in cardiac gene expression correlated with the morphological differences observed and the failed crescent formation following NCX1 inhibition. This was is in contrast to treatment with 10 µm nifedipine, in which the crescent developed normally and progressed to the equivalent stage as untreated embryos at E8.0 (Figure 5.11b). This was further supported by the gene expression profile of treated embryos which showed no differences when compared to embryos treated with DMSO (Figure 5.12g-l).

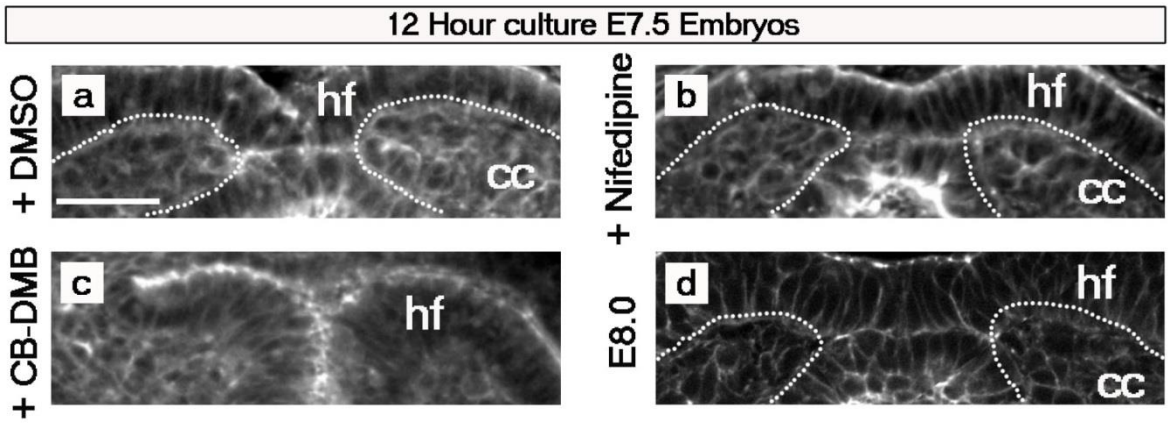

Figure 5.11: Inhibition of NCX1 with CB-DMB prevents formation of the cardiac crescent during embryo culture from E7.25

E7.25 embryos were dissected and cultured for 12 hours in media containing either DMSO, nifedipine (10 µM) or CB-DMB (3 µM) and stained for actin (a-c). Embryos developed normally in culture, as indicated by head fold formation, coalescence of the cardiac crescent (a-c) and addition of somites (not shown) relative to dissected stage-matched control embryos at E8.0 (d). Embryos cultured in CB-DMB were delayed in terms of cardiac crescent formation (c) compared to either DMSO alone (a), nifedipine-treated (b) or E8.0 dissected embryos (P). White dashed line highlights cardiac crescent. cc, cardiac crescent; hf, headfolds. All scale bars 50µm.

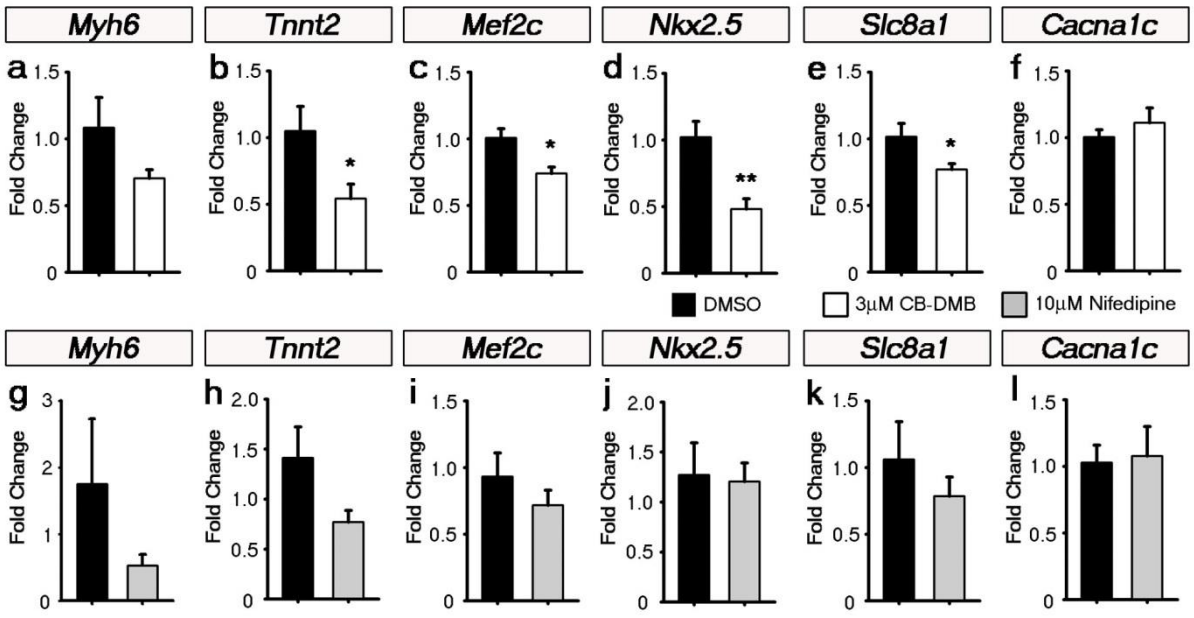

Figure 5.12: Inhibition of NCX1 with CB-DMB prevents cardiomyocyte specific gene expression during formation of the cardiac crescent from E7.25

Cultured E7.25 embryos in the presence of either CB-DMB or nifedipine for 12 hours, revealed that CB-DMB significantly down-regulated the expression of both late *TnnT2* (b) and early *Mef2c* (c) and *Nkx2.5* (d) cardiac genes as well as *Slc8a1* (e) which encode NCX1, coincident with impaired cardiac crescent formation, whilst *Myh6* (a) and *Cacna1c* (f) were unaffected. Whereas, nifedipine treatment did not affect cardiac gene expression after 12 hours of culture (g-l). All error bars are mean ± S.E.M (n=5). Statistics: Student's paired t-test (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

5.3 Discussion

We have previously shown that both NCX1 and Cav1.2 are expressed within the developing heart and are required for Ca^{2+} transients and contraction during formation of the cardiac crescent. The early expression and function of these proteins would suggest that not only are they crucial for contraction, but that they may have downstream effects in terms of gene expression and differentiation. In this chapter, I have shown that NCX1, Cav1.2 and NAADP signalling via TPCs are required for the specification and functional differentiation of cardiomyocytes suggesting these channels and exchangers are fundamentally required for cardiomyocyte development.

5.3.1 Inhibition of NCX1 prevents the formation of cardiomyocytes

NCX1 is one of the first ECC proteins to be expressed during development; becoming up-regulated prior to RyR2 and Cav1.2 as well as being required for Ca^{2+} transients

prior to contraction during cardiac crescent formation. Using long-term pharmacological inhibition with CB-DMB we assessed the role of NCX1 during ESC-derived cardiomyocyte differentiation in two different ESC lines (*Nkx2.5*-GFP and *Eomes*-GFP). By assessing the expression *Nkx2.5*-GFP as well as the percentage of beating EBs we observed a significant reduction in the formation of cardiomyocytes when NCX1 was inhibited from day 0-14. Between day 7 and day 14 further increases in the percentage of beating EBs were modest when treated with DMSO (~1.1 fold increase) or CB-DMB (~2.2 fold increase) treatment, suggesting that by day 7 the maximal number of EBs had undergone cardiomyocyte differentiation, however, the size of beating regions continued to increase. In terms of NCX1 inhibition this would suggest that cardiomyocyte differentiation was completely prevented by day 7, rather than being delayed. The decrease in cardiomyocyte differentiation by NCX1 was also reflected in the down regulation of cardiac progenitor and cardiomyocyte specific genes. Overall these findings suggested that NCX1 is required for cardiomyocyte specification and differentiation. Alternatively, the decrease in cardiomyocyte formation could be due to inhibition of cell growth, decrease in proliferation or cell death. Culture with CB-DMB throughout differentiation did not reduce the number of cells per EB, nor did it affect the gross morphology of EBs or inhibit cell out growth, this suggests that proliferation and cell viability were not affected by CB-DMB treatment. Furthermore, we did not observe increases in intracellular Ca^{2+} during acute treatment, suggesting that there was no Ca^{2+} overload secondary to NCX1 inhibition. To conclusively rule out increased cell death as a mechanism for reduced cardiomyocyte formation it would be important to perform TUNEL staining to assess levels of apoptosis, especially within GFP-positive cardiac progenitors.

Although cardiomyocyte differentiation was significantly reduced at both day 7 and 14, the formation of cardiomyocytes was not completely inhibited as demonstrated by the observation of GFP+ cells within the cultures as well as the small percentage of beating regions which developed during CB-DMB treatment. The residual development of some cardiomyocytes would also explain why cardiac marker gene expression was not more strongly down-regulated in comparison to the decrease in the percentage of beating EBs. The inability of CB-DMB to completely inhibit cardiomyocyte differentiation may suggest incomplete inhibition of NCX1 activity with 1 µm CB-DMB

or that a specific sub-cardiac lineage does not require NCX1 for cardiomyocyte differentiation. Published studies which directly measured NCX1 generated current have shown that 1µm CB-DMB inhibits around 80% of NCX1 activity (Secondo et al. 2009), suggesting some cells may escape inhibition and go on to differentiate, which would correlate with the 20% of beating EBs observed at day 14. An alternative reason could be that certain genes required for cardiomyocyte differentiation are not regulated by NCX1 function or that heterogeneity in the expression of Ca^{2+} handling proteins within cardiac progenitors may compensate for a loss in NCX1 function.

Our observations that NCX1 inhibition leads to reduced cardiomyocyte differentiation is consistent with the early embryonic lethality observed in NCX1 transgenic mice around E9.5-11 (Cho et al. 2000)(Wakimoto et al. 2000)(Koushik et al. 2001). Mouse transgenic lines in which NCX1 is disrupted have shown that NCX1 is required for heart development however it is difficult to make conclusions about the role of NCX1 with certainty due to the large variation in the cardiac phenotype observed between different transgenic lines despite them having used the same targeting strategy. The first transgenic model published showed that disruption of NCX1 resulted in embryonic lethality between E9.5-10.5 (Wakimoto et al. 2000). At E9.5, hearts had reduced ventricular wall thickness due to fewer cardiomyocytes and increased apoptosis. In addition, whilst 70% of NCX1 knock out hearts lacked a heartbeat, the remaining 30% were able to contract with slow arrhythmic heartbeats. In comparison, another transgenic NCX1 knockout mouse survived until E11.0 with normal heart morphogenesis, although these mice lacked a heartbeat and had disorganised myofibril assembly (Koushik et al. 2001). Although the early embryonic lethality associated with a lack of NCX1 supports these findings, transgenic models may not be the most suitable model for examining the early role of NCX1 due to the variable penetrance and consequently in the observed cardiac phenotype. These differences may be due to other Ca^{2+} channels or different NCX1 isoforms compensating for the loss of NCX1 function due to its fundamental role in heart formation. In terms of compensation we assessed whether pharmacological inhibition of NCX1 might result in a direct up regulation of the NCX1 gene itself (Figure 5.9), however, NCX1 was down regulated, suggesting this was a consequence of decreased cardiomyocyte formation and not the result of compensation.

Overall this data shows that inhibition of NCX1 with CB-DMB significantly decreased the extent of cardiac gene expression and cardiomyocyte differentiation suggesting NCX1 has a fundamental role in the formation of cardiomyocytes and early heart development.

5.3.2 Inhibition of Cav1.2 delays ESC-derived cardiomyocyte differentiation

The L-type Ca^{2+} channel, Cav1.2 is the main route of sarcolemmal Ca^{2+} influx in mature cardiomyocytes (Bers 2000). Expression of Cav1.2 mRNA increased throughout the ESC differentiation from day 5 and was functionally relevant for the initiation of contractile activity within the stage 1 cardiac crescent. To assess the role of Cav1.2 on cardiomyocyte differentiation, a selective inhibitor was used to block channel function during ESC differentiation. Initial experiments with 500 nM nifedipine had no effect on cardiomyocyte gene expression or apparent differentiation. 500 nM nifedipine was used to investigate the role of Cav1.2 during differentiation due to nifedipine having a low IC50 value (~5 nM) in adult and ESC derived cardiomyocytes (Zheng et al. 1992)(Brown 2009)(Kang et al. 2012). However, whilst conducting these initial experiments it was reported that 10µM nifedipine blocked cardiomyogenesis by inhibiting mesodermal commitment (Nguemo et al. 2013). In the *Eomes*-GFP model, treatment with 10 µm nifedipine throughout differentiation led to a significant decrease in the percentage of beating EBs at day 7 and 14. The difference in effects between 500 nM and 10 µm nifedipine could be due to 500 nM not completely inhibiting Cav1.2 during differentiation. Incomplete inhibition could be caused by the formation of EBs preventing drug penetration. Another potential reason could be that 10 µM nifedipine inhibits other Ca^{2+} channels such as T-type channels or other L-type channel subunits, which were shown to be up-regulated during cardiomyocyte differentiation (chapter 4). High concentrations of nifedipine has been shown to inhibit T-type channel activity in both oocytes and HEK293 cells as well as in thalamic neurons (Shcheglovitov et al. 2005).

Although there was a significant decrease in the overall formation of cardiomyocytes when treated with nifedipine from day 0-14, the number of beating EBs increased 50 fold between days 7 and 14. This was also reflected in the difference between NCX1 and Cav1.2 inhibition during differentiation; at day 7 nifedipine significantly reduced

the percentage of beating EBs, whilst at day 14 there was a significant increase relative to CB-DMB treatment. An increase in the percentage of beating EBs suggests that inhibition of Cav1.2 with nifedipine does not totally prevent cardiomyocyte formation but delays the rate of commitment or maturation. One explanation for the ability of ESCs to differentiate during Cav1.2 inhibition could be that other types of Ca^{2+} channels compensate for the lack of Cav1.2 function. Alternatively, Cav1.2 may only regulate certain cardiac specific genes which are not essential for cardiomyocyte formation. Transgenic mice with targeted Cav1.2 gene disruption died at around E13.5, however, at E12.5 their hearts were normal and beating at wild type rates, which the authors suggested explained as due to an unidentified Ca^{2+} channel and not an L-type Ca^{2+} channel (Seisenberger et al. 2000). This suggests that Cav1.2 is not fundamental for the formation of cardiomyocytes and that other Ca^{2+} channels may be more critical for early function.

In terms of gene expression, inhibition of Cav1.2 throughout differentiation with 10 µm nifedipine did not inhibit expression of the majority of cardiac-specific genes assessed including mature markers *Myh6* and *Tnnt2* as well as cardiac progenitor genes *Nkx2.5* and *Mef2c*, although it did significantly inhibit *Myh7* and *Gata-4*. *Myh7* encodes the embryonic isoform of myosin heavy chain, β-MHC, which is expressed during embryonic heart development prior to the adult isoform α-MHC (*Myh6*), as well as being re-expressed in the diseased heart. The fact that nifedipine treatment inhibited *Myh7* expression and not other mature cardiomyocyte genes, suggests that Cav1.2 may directly regulate *Myh7* expression, potentially due to Cav1.2-mediated signalling mechanisms or via its role in contraction. The complete loss of beating EBs at day 7 may be due to the decreased levels of *Myh7* preventing contraction, however, up regulation of *Myh6* at later stages may compensate and allow contraction to occur. The other cardiac gene inhibited by nifedipine treatment was *Gata-4,* an early cardiac marker of specification stages, suggests that Cav1.2 directly regulates *Gata-4* gene expression via a mechanism independent from that controlling other early markers such *Nkx2.5* and *Mef2*. Since *Gata-4* has also been shown to be regulated by mechanical force (Schmelter et al. 2006), the lack of beating during nifedipine treatment might explain the decreased expression.

5.3.3 The role of NCX1 and Cav1.2 in pluripotent and mesoderm gene expression during ESC differentiation

To investigate the stage at which NCX1 and Cav1.2 inhibition affected cardiomyocyte differentiation we studied the expression of mesoderm and pluripotent genes. Inhibition of NCX1 surprisingly resulted in the up-regulation of pluripotent markers *Oct-4* and *Nanog* as well as the lateral plate mesoderm marker *Brachyury* whilst *Mesp1* a cardiogenic mesoderm marker was unaffected. Due to the decreased expression of cardiac progenitor markers this would suggest that inhibition of NCX1 prevents the formation of cardiac progenitors but does not block mesoderm commitment.

The up-regulation of early differentiation genes could potentially be caused by NCX1 having a direct role in the regulation of mesoderm formation or may indirectly affect mesoderm gene expression due to decreased cardiac progenitor formation. In chapter 5, we observed membrane-localised expression of NCX1 adjacent to Brachyury positive cells which suggests NCX1 activity indirectly feeds back to inhibit differentiation of neighbouring cells or up regulates mesoderm genes. Alternatively, in a cell autonomous manner, NCX1 could also have a direct signalling role at earlier stages of cardiomyocyte differentiation suggested by the punctate expression of NCX1 within day 4 EBs.

Indirectly, the reduced amount of cardiomyocyte differentiation may result in a loss of repressive feedback signals. For example, *Mesp1* can repress genes involved in pluripotency and early mesoderm induction, such as *Brachyury* and *Goosecoid*, by binding to the start codon of these genes and preventing transcription (Bondue et al. 2008). The observed up regulation of mesoderm and pluripotent markers therefore may be due to a loss of repressive signals arising from the reduced cardiac progenitor formation. In contrast, Cav1.2 inhibition did not affect pluripotency or mesoderm gene expression but did result in significantly less expression of *Mesp1* at day 14 of differentiation but not day 7 suggesting that Cav1.2 does not play a role in early cardiac differentiation, but is down regulated due to a consequence of delayed differentiation.

Overall, this data shows that NCX1 has a role in the expression of pluripotent and mesoderm genes and suggests that NCX1 activity is essential for the progression of

mesoderm to cardiac progenitors. In contrast Cav1.2 potentially plays a later role in the maturation of cardiomyocytes acting once cardiac progenitors have formed.

5.3.4 Inhibition of NCX1 blocks cardiomyocyte formation during mesoderm specification

To assess the consequences of early NCX1 inhibition on cardiomyocyte formation, EBs were treated with CB-DMB for the first 4 days of differentiation before subsequent culture in control media until day 14. In the *Nkx2.5*-GFP ESC line, treatment from day 0-4 with CB-DMB inhibited the formation of cardiomyocytes, however, to a lesser extent than treatment from day 0-14. At day 14 the expression of cardiac progenitor and mature cardiomyocyte genes was significantly reduced compared to DMSO control, however, there was a significant up-regulation in *Myh6* and *Mef2c* expression at day 14 when treated with CB-DMB from day 0-4 compared to treatment from day 0-14. In the *Eomes*-GFP line, treatment from day 0-4 with CB-DMB significantly reduced the percentage of beating EBs at 7, however, by day 14 there was no difference in comparison to treatment with DMSO. Together this data further suggests that NCX1 plays an early role in the specification of cardiac progenitors and that removal of NCX1 inhibition enables normal differentiation to proceed. In comparison, inhibition with nifedipine from day 0-4 had no effected on the percentage of beating EBs at day 14 compared to treatment with DMSO from day 0-14 suggesting Cav1.2 does not influence early cardiomyocyte differentiation or mesoderm specification, supporting previous observations.

5.3.5 Variation in the response of *Nkx2.5*-GFP and *Eomes*-GFP ESC lines to NCX1 inhibition

Despite the fact that inhibition of NCX1 resulted in a similar phenotype in the *Nkx2.5*-GFP and *Eomes*-GFP ESCs, there were a number of differences between the two lines. Inhibition of NCX1 in the *Nkx2.5*-GFP line resulted in a more pronounced response to culture with CB-DMB than the *Eomes*-GFP ESC, with stronger down regulation of cardiac genes as well as greater up regulation of pluripotent and mesoderm markers. These differences are most likely due to the variation observed in the extent of cardiomyocyte differentiation between the two cell lines (Chapter 4). During differentiation, the *Eomes*-GFP line resulted in beating EBs 1 to 2 days earlier than in the *Nkx2.5*-GFP line, suggesting cardiac progenitor formation occurred more rapidly.

Earlier differentiation may allow more cells to escape NCX1 inhibition due to mesoderm specification occurring whilst cells were still in hanging drops. The dense EBs formed between day 0-4 could potentially restrict drug penetration, also during the hanging drop culture drug containing media was not changed until day 4, meaning drug activity may have decreased over this timescale. In contrast, the slower rate of mesoderm specification and cardiac progenitor formation in the *Nkx2.5*-GFP line would mean there was longer exposure to NCX1 inhibition. The difference in rate of differentiation may also explain why at day 14, inhibition from day 0-4 in the *Eomes*-GFP line had no effect on percentage of beating EBs whilst in the *Nkx2.5*-GFP line there was still a significantly reduced number of *Nkx2.5*-GFP positive cells. In addition, the *Nkx2.5*-GFP line had a relatively higher level of cardiac gene expression during differentiation compared to the *Eomes*-GFP line, which may explain why NCX1 inhibition more strongly reduced gene expression in the former.

5.3.6 The role of NCX1 and Cav1.2 during *ex vivo* embryo culture crescent formation

Using ESCs as a model of cardiomyocyte differentiation this thesis has shown that NCX1 is required for the formation of cardiac progenitors whilst Cav1.2 play a role in cardiomyocyte maturation. To address the roles of Cav1.2 and NCX1 in a more physiological setting we adopted an *ex vivo* embryo culture approach. By isolating E7.25 embryos and culturing them for 12 hours with either CB-DMB or nifedipine we could investigate formation of the cardiac crescent. Culture with CB-DMB resulted in decreased expression of both cardiac progenitor and mature cardiomyocyte genes and ultimately failed coalescence of cardiac cells to form a crescent. Treatment did not adversely affect embryo viability as normal addition of somites was observed. When culturing embryos with CB-DMB the concentration of drug was increased from 1 µM to 3 µM relative to the ESC cultures due to the embryos being cultured in their yolk sacs which could potentially decrease access of the drug and prevent NCX1 inhibition. In comparison, culture with 10 µM nifedipine did not affect embryo development and had no significant effect on cardiac gene expression or cardiac crescent formation. The high concentration of nifedipine used meant we did not increase the concentration of nifedipine for these experiments, although to conclusively rule out a role for Cav1.2 in cardiac crescent formation it would be important to determine a positive control for nifedipine activity *ex vivo*.

The embryo culture experiments provided further evidence to support the observations made using ESC models that NCX1 plays a fundamental role in cardiac progenitor specification whilst Cav1.2 is not required for the initial formation of cardiomyocytes but is likely more involved in later stage myocyte differentiation and maturation.

5.3.7 Potential mechanisms involved in NCX1 and Cav1.2 regulation of differentiation

In chapter 6, we that revealed both NCX1 and Cav1.2 played a role in cardiomyocyte Ca^{2+} influx and contraction, with NCX1 required for Ca^{2+} influx prior to the onset of beating. This difference in the functional roles for NCX1 and Cav1.2 may explain the observed differences between CB-DMB and nifedipine inhibition. Overall both NCX1 and Cav1.2 inhibition reduced the formation of cardiomyocytes suggesting, that Ca^{2+} influx is not only critical for early function but also has a downstream role in signalling and gene regulation. The ability of NCX1 to regulate cardiac progenitor formation may be due to its ability to regulate Ca^{2+} influx at the earliest stages of cardiac crescent, whilst the functional role of Cav1.2 at later stages of crescent formation suggests an involvement in cardiomyocyte maturation.

Ca^{2+} is key second messenger, known to be responsible for signalling in a large number of different cell types. In the adult heart, Ca^{2+} signalling has been strongly implicated in hypertrophy, acting as key regulator of gene expression and disease progression. During cardiac hypertrophy a number of foetal genes become up-regulated, such as *Myh7*, *Nkx2.5* and *Mef2c*, these changes in gene expression correlate with the development of abnormal Ca^{2+} transients. In the diseased heart there is a distinct change in the properties of excitation contraction coupling, with a decrease in SR Ca^{2+}uptake due to decreased SERCA expression along with increased sarcolemmal Ca^{2+} flux due to increases in NCX1 expression (Rourke et al. 1999). Downstream Ca^{2+} signalling pathways involved in hypertrophy include the calcineurin and calmodulin kinase pathways (Shimoyama et al. 1999) (Hoch et al. 1999). It is, therefore, likely that Ca^{2+} influx, via reverse mode NCX1, at the earliest stages of cardiomyocyte differentiation may directly regulate cardiac progenitor gene expression via Ca^{2+}-dependent signalling pathways.

Inhibitors of NCX1 and Cav1.2 not only prevented Ca^{2+} influx at stage 1 but also inhibited early contractile activity, thus reducing the mechanical force applied to the cardiac progenitors and early forming cardiomyocytes. Studies have shown in mouse embryonic stem cells that the expression of cardiac differentiation markers such as *Gata-4* and sarcomeric α-actinin are increased after static stretching for 2 hours (Schmelter et al. 2006). *Gata-4* was one of the only genes to be affected by nifedipine treatment at day 14 suggesting that a lack of contraction due to loss Cav1.2-dependant Ca^{2+} influx may influence gene expression via mechanical force signalling. In contrast, *Gata-4* was unaffected during NCX1 inhibition further suggesting that NCX1 and Cav1.2 function via different mechanisms to control gene expression.

In chapter 6, we showed that at later stages of cardiac crescent formation only nifedipine prevented contraction which suggests that Cav1.2 has a role in maturation process of cardiomyocytes via mechano-signalling compared to NCX1. Studies in which cultures were selected for beating colonies and cardiomyocyte markers such as α-MHC and *Nkx2.5*, revealed that these contractile myocytes not only continued to increase expression of these markers when continuously stretched at 1.0Hz but were able to synchronously beat upon implantation into infarcted rat hearts (Gwak et al. 2008). This suggests that delayed cardiomyocyte maturation in the presence of nifedipine is due to the lack of contraction not regulating feed forward signalling pathways required for the expression of contractile proteins and maturation of cardiomyocytes via mechano-signalling.

5.3.8 NAADP signalling and lysosomal Ca^{2+} release via TPCs is require for specification and differentiation of cardiomyocytes

To investigate the role of TPC1/2 in development, the recently identified Ned compounds were used in ESC differentiation experiments. These compounds are known to inhibit NAADP signalling and suppress lysosomal Ca^{2+} release, as shown in pancreatic beta cells (Cancela et al. 1999)(Naylor et al. 2009). Ned-K is a recently identified Ned compound suggested to be more specific and potent in the inhibition of TPC1/2. Although we have mainly used Ned-K in these studies, application of the previously published inhibitor Ned-19 had the same effect as Ned-K inhibition in preliminary experiments confirming these results (data not shown). The fluorescent properties of Ned-19 and Ned-K enable localisation of the compound to be observed.

From day 4 of differentiation speckled fluorescence could be observed throughout the cytoplasm of culture ESCs, suggesting localisation of the drug to the endo-lysosomal system, however, these preliminary observations using the auto fluorescence properties of Ned inhibitors need to be confirmed via other methods and co-localisation experiments.

Inhibition of TPC1/2 with 30 µM Ned-K from day 0-14 had a significant effect on both the number of Nkx2.5-GFP+ve cells as well as the expression of cardiac specific genes. Inhibition from day 0-4 significantly reduced GFP expression, but did not completely prevent formation of Nkx2.5-GFP+ve cells at day 14, in contrast to day 0-14 treatment. This was reflected by the increased expression of cardiac genes, notably those marking cardiac progenitors. Pluripotent markers *Oct-4* and *Nanog* as well as *Brachyury* were also increased compared to DMSO control at day 14, suggesting that TPC1/2 Ca^{2+} release may have a role in undifferentiated cells and mesoderm formation, similar to that seen with NCX1 inhibition. The parallels observed between inhibition of NCX1 and TPC1/2 suggests either a potential interaction between the two proteins, or that they regulate cardiomyocyte differentiation via similar/convergent pathways. The ability of both NCX1 and TPC1/2 to regulate intracellular Ca^{2+} levels suggests both proteins are capable of regulating cardiomyocyte differentiation via downstream Ca^{2+} signalling. Transgenic knockout of TPC1/2 in mice does not affect embryo viability (Lin-Moshier et al. 2012)(Calcraft et al. 2009) suggesting that compensation can overcome TPC1/2 disruption during development, further supporting TPC1/2 and NCX1 could act via the same signalling pathway and may suggest a degree of functional redundancy. Alternatively, TPC1/2 have also been described as Na^{+} selective channels (X. Wang et al. 2012), which suggests that Na^{+} release via TPC1/2 may lead to Ca^{2+} influx via the activation of reverse mode NCX1 activity due to increases in intracellular Na^{+}.

The activity of TPC1/2 is regulated via NAADP signalling which is reported to be the most potent Ca^{2+}-mobilizing second messenger (Lee 2003) and has previously been shown to regulate skeletal muscle differentiation via TPCs (Aley et al. 2010). The inhibition of cardiomyocyte differentiation during treatment with Ned-K suggests that NAADP signalling is required for heart development. To assess in more detail the dynamics of NAADP signalling it will be crucial to directly measure the levels of NAADP

throughout differentiation, using an assay such as the biochemical cycling assay (Graeff & Lee 2002).

In summary, these findings show for the first time that TPC1/2 and NAADP signalling have a role in the early determination of cardiac progenitors, with early inhibition being sufficient to delay the subsequent progression of differentiation. Taken together these findings suggest that lysosomes may act as early intracellular Ca^{2+} stores and support the hypothesis that early Ca^{2+} signalling is crucial for cardiac development.

5.4 Summary

In this chapter we have examined how inhibition of Ca^{2+} handling proteins influences cardiomyocyte differentiation and heart formation. We have shown that NCX1, Cav1.2 and TPC1/2 are required for cardiomyocyte formation although inhibit differentiation at stages and potentially in different ways. In the following chapter we investigate the signalling mechanism which may link NCX1 and Cav1.2 function with cardiomyocyte differentiation.

6. Ca^{2+}-DEPENDENT SIGNALLING REGULATES CARDIOMYOCYTE DIFFERENTIATION

6.1 Introduction

Previous chapters have revealed that both NCX1 and Cav1.2 are required for the formation of cardiomyocytes; subsequently we wanted to investigate the potential downstream signalling pathways which may link channel and exchanger activity with cardiomyocyte differentiation. The second messenger Ca^{2+} is known to play a key role in a wide range of different signalling pathways, controlling a variety of cellular functions. In the adult heart, Ca^{2+} signalling contributes to disease progression and the reactivation of foetal cardiac genes during pathological remodelling. Due to our observations that both NCX1 and Cav1.2 are required for early sarcolemmal Ca^{2+} influx during the initiation of contractile activity in heart we sought to investigate whether early changes in Ca^{2+} handling directly integrated with Ca^{2+} dependent signalling networks known to regulate developmental cardiac genes, which in turn could induce cardiomyocyte differentiation.

6.2 Results

6.2.1 Reduced external Ca^{2+} inhibits *Eomes*-GFP ESC Cardiomyocyte differentiation

Ca^{2+} signalling is involved in a variety of different intracellular signalling pathways via the allosteric regulation of many enzymes and proteins (Berridge et al. 2000). To investigate whether the decrease in cardiomyocyte formation associated with NCX1 and Cav1.2 inhibition was mediated by changes in Ca^{2+} influx, ESCs were cultured in reduced extracellular Ca^{2+}. In previous ESC experiments the culture media contained 1.8 mM Ca^{2+} which is similar to the physiological extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) and widely used in most cell culture media, therefore, for these experiments the concentration of Ca^{2+} was lowered to 0.1 mM and 1.0 mM, in order to reduce the extracellular Ca^{2+} concentration and sarcolemmal Ca^{2+} influx. Exposure of ESCs to 0.1 mM Ca^{2+} throughout differentiation resulted in a significant decrease in the number of beating EBs compared to 1.8 mM media at both day 7 (0.1 mM Ca^{2+}, 2% (n=60); 1.8 mM Ca^{2+}, 25% (n=61). p=<0.001) (Figure 6.1a) and day 14 (0.1 mM Ca^{2+}, 29% (n=80); 1.8 mM Ca^{2+}, 82% (n=91). p=<0.001) (Figure 6.1b). Chronic exposure to 1.0 mM Ca^{2+} significantly reduced the percentage of beating at day 14 compared to 1.8 mM Ca^{2+}

(1.0 mM Ca^{2+}, 59% (n=65) vs. 1.8 mM Ca^{2+}, 82% (n=91). p=0.0018) but not at day 7 (1.0 mM Ca^{2+}, 11% (n=55) vs. 1.8 mM Ca^{2+}, 25% (n=61). p=0.09), whilst beating was significantly greater at day 14 than when ESCs were cultured in 1.0 mM Ca^{2+} compared to 0.1 mM Ca^{2+} media (p=<0.001). To investigate the role of Ca^{2+} during cardiomyocyte specification, ESCs were treated with 0.1 mM Ca^{2+} from day 0 - 4, before being returned to normal levels of Ca^{2+} for the remaining 10 days of differentiation. Under these conditions there was a significantly lower percentage of beating EBs compared to culture from days 0-14 in 1.8 mM Ca^{2+} (Day 0-4 0.1 mM – day 4-14 1.8 mM Ca^{2+}, 50% (n=40); Day 0-14 1.8 mM Ca^{2+}, 82% (n=91). p=<0.001) . In contrast, when ESCs were treated with 1.8 mM Ca^{2+} from day 0-4 , before being cultured in 0.1 mM Ca^{2+} until day 14, there was no difference in comparison to treatment with 1.8 mM Ca^{2+} from day 0-14, however, there was significantly more beating EBs when compared to culture with 0.1 mM Ca^{2+} from day 0-14 (Day 0-4 1.8 mM – day 4-14 0.1 mM Ca^{2+}, 74% (n=38); Day 0-14 1.8 mM Ca^{2+}, 82% (n=91). Day 0-14 0.1 mM Ca^{2+}, 29% (n=80). p=<0.001). To exclude the possibility that the effect of low Ca^{2+} was due to reduced cell proliferation, the total number of cells per EB were assessed. At both day 4 and day 7 there was no difference in the number of cells per EB when cultured with 0.1 mM or 1.8 mM Ca^{2+} containing media (Day 4; 0.1 mM Ca^{2+}, 9688 cells/EB; 1.8 mM Ca^{2+}, 12894 cells/EB. Day 7; 0.1 mM Ca^{2+}, 37230 cells/EB; 1.8 mM Ca^{2+}, 46833 cells/EB) (Figure 6.1c, Figure 6.1d), this correlated with brightfield images of EBs in which the phenotype and cell outgrowth was similar under all conditions (Figure 6.1e-j). Together this data reveals that $[Ca^{2+}]_o$ plays a fundamental role in the formation of ESC-derived cardiomyocytes, with reduced concentrations inhibiting cardiomyocyte differentiation but not preventing overall cell growth or proliferation. It also suggests that the concentration of $[Ca^{2+}]_o$ is fundamental in the specification of cardiac progenitors occurring between days 0-4.

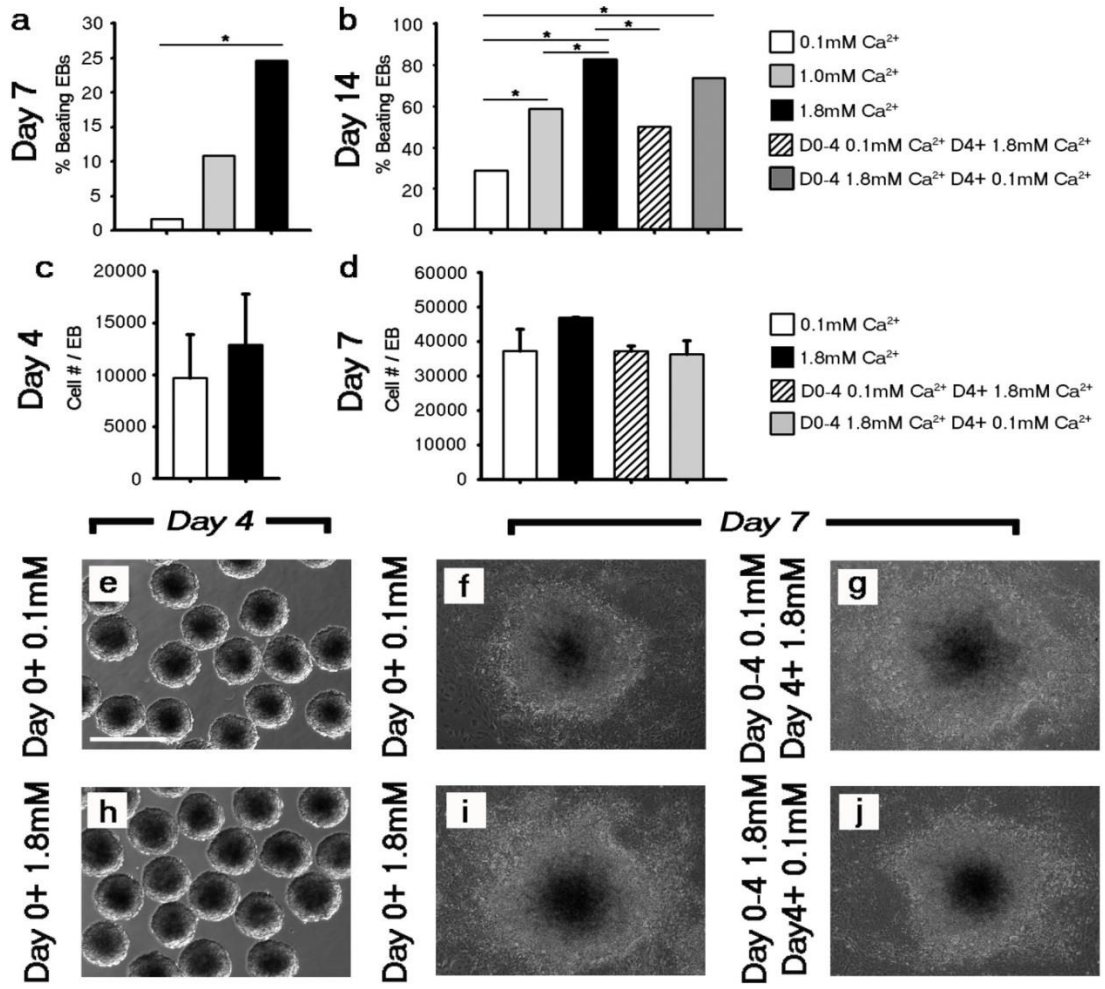

Figure 6.1: Reducing the external concentration of Ca^{2+} inhibits Eomes-GFP cardiomyocyte differentiation

Chronic exposure of embryoid bodies to 0.1 mM external Ca^{2+} resulted in a significant decrease in the incidence of beating EBs at day 7 (a, 1.8 mM Ca^{2+}, 25% (n=61); 1.0 mM Ca^{2+}, 11% (n=55); 0.1 mM Ca^{2+}, 2% (n=60)). At day 14, 0.1 mM and 1.0 mM external Ca^{2+} reduced the percentage of beating EBs although there was a significantly greater percentage of beating EBs when treated with 1.0 mM Ca^{2+} compared to 0.1 mM (b, 1.8 mM Ca^{2+}, 82% (n=91); 1.0 mM Ca^{2+}, 59% (n=65); 0.1 mM Ca^{2+}, 29% (n=80)). Initial treatment from day 0-4 with 0.1 mM Ca^{2+} before subsequent culture until day 14 in 1.8 mM Ca^{2+} resulted in a decreased number of beating EBs compared to control (Day 0-4 0.1 mM - Day 4-14 1.8 mM Ca^{2+}, 50%, (n=40); Day 0-4 1.8 mM - Day 4-14 0.1 mM Ca^{2+}, 74%, (n=38)), however, there was a significant increase in the percentage of beating EBs when cultured in 1.8 mM Ca^{2+} form day 0-4 before culture in 0.1 mM Ca^{2+} until day 14 when compared to culture with 0.1 mM Ca^{2+} from day 0-14 (b). Treatment of Eomes-GFP ESCs with reduced concentrations of external Ca^{2+} did not affect the number of cells per EB at both day 4 (c, 0.1 mM Ca^{2+}, 9688 cells/EB; 1.8 mM Ca^{2+}, 12894 cells/EB) or day 7 (d, day 0-7 0.1 mM Ca^{2+}, 37230 cells/EB; day 0-7 1.8 mM Ca^{2+}, 46833 cells/EB; day 0-4 0.1 mM Ca^{2+}, day 4-7 1.8 mM Ca^{2+}, 37157 cells/EB; day 0-4 1.8 mM Ca^{2+}, day 4-7 0.1 mM Ca^{2+}, 36283 cells/EB) or prevent EB formation and cell outgrowth. Bright field images confirmed that cultures were grossly unaffected by reduced external Ca^{2+} concentration at all stages of differentiation (e-j).Scale bars: 500µm. All error bars are mean ± S.E.M. Statistics: Fisher's exact test with a Bonferroni correction for multiple comparisons. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

6.2.2 Acute inhibition of NCX1 and Cav1.2 has no effect on cardiac gene expression of ESCs

To assess whether inhibition of NCX1 or Cav1.2 played an acute role in regulating cardiac gene expression, RT-PCR was employed to assess the levels of signature genes after treatment for 4 hours with NCX1 and Cav1.2 inhibitors. At day 4 of differentiation, prior to cardiomyocyte beating, treatment with NCX1 inhibitors did not affect the expression of mesoderm genes (*Brachyury* & *Mesp1*) or cardiac-specific genes (*Gata-4*, *Mef2c* and *Myh6*)(Figure 6.2). Expression of *Nkx2.5* was unchanged in all treatments at day 4 except when treated with 10μm CB-MB, which caused it to be significantly up-regulated (1.96-fold, p=0.043 (n=4)). This experiment was also repeated at day 7 of differentiation, after the formation of beating cardiomyocytes (Figure 6.3). Expression of *Mesp1*, *Nkx2.5*, *Mef2c* and *Myh6* were all unchanged after 4 hours in the presence of NCX1 or Cav1.2 inhibitors (CB-DMB, KB-R, SN-6 & nifedipine). This data shows that the short term application of NCX1 and Cav1.2 inhibitors does not acutely effect the expression of cardiomyocyte specific genes at day 4 or day 7, and suggests that NCX1 and Cav1.2 do not directly regulate cardiac gene expression.

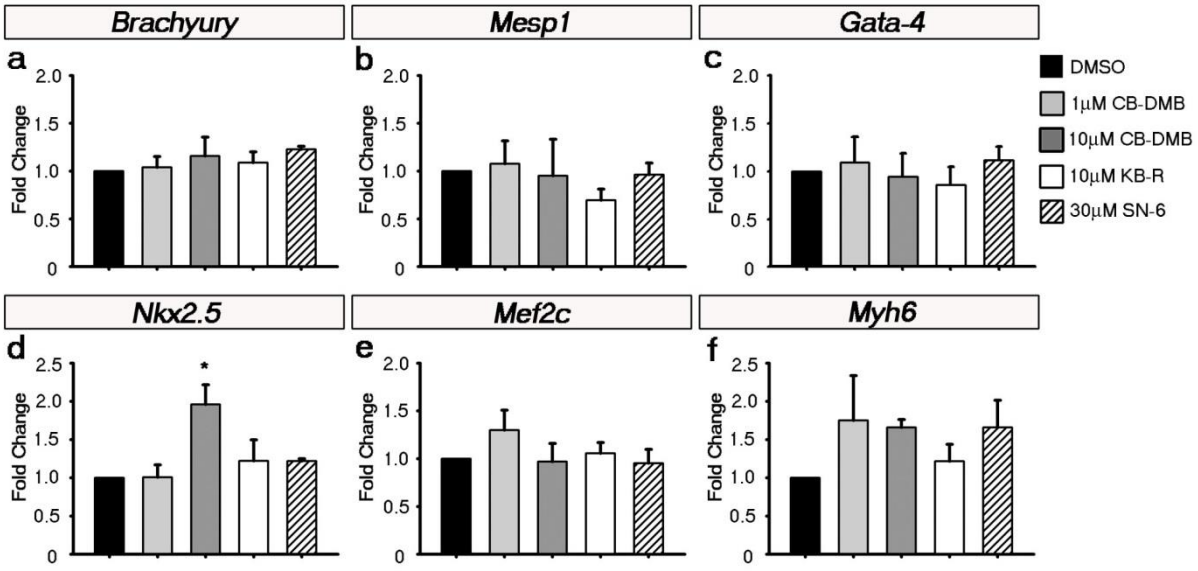

Figure 6.2: Inhibition of NCX1 at day 4 of *Eomes*-GFP for 4 hours does not affect cardiac related gene expression

Inhibition of NCX1 with CB-DMB, KB-R7943 or SN-6 for 4 hours at day 4 of *Eomes*-GFP ESCs differentiation had no effect on the expression of *Brachyury* (a), *Mesp 1* (b), *Gata-4* (c), *Mef2c* (e) or *Myh6* (f), whilst Nkx2.5 was unaffected by all inhibitors expect doe 10 µM CB-DMB which caused a small but significant increase in expression (d). All error bars are mean ± S.E.M (DMSO n=5, 1 µM CB-DMB n=5, 10 µM CB-DMB n=5, 10 µM KB-R7943 n=3, 30 µM SN-6 n=4). Statistics: one-way ANOVA and Tukey test for multiple comparisons (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

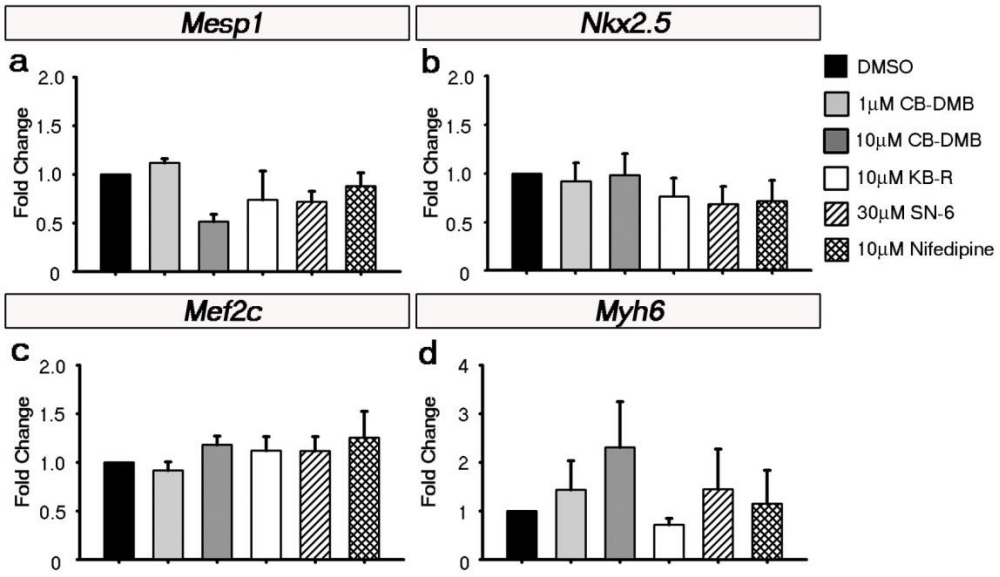

Figure 6.3: Inhibition of NCX1 or Cav1.2 at day 7 of *Eomes*-GFP for 4 hours does not affect cardiac related gene expression

Inhibition of NCX1 with CB-DMB, KB-R7943 or SN-6 as well as Cav1.2 with Nifedipine for 4 hours at day 7 of differentiation had no effect on the expression of *Mesp 1* (a), *Nkx2.5* (b), *Mef2c* (c) or *Myh6* (d). All error bars are mean ± S.E.M (n=3). Statistics: one-way ANOVA and Tukey test for multiple comparisons (* p<0.05; ** p<0.01; *** p<0.001).

6.2.3 CaMKIIδ and Calcineurin gene expression during ESC Cardiomyocyte differentiation

Two of the main enzymes involved in Ca^{2+} regulated signalling pathways within the heart include calmodulin kinase II (CaMKII) and calcineurin (Molkentin 2006). Whilst there are a number of different isoforms and subunits for each of these enzymes, this study focused on the CaMKII delta isoform and the alpha subunit of calcineurin, which are the predominant isoforms expressed in the heart (Edman & Schulman 1994)(Muramatsu et al. 1992). During *in vivo* heart development, the genes *Camk2d* (encoding CaMKII Delta) and *Ppp3ca* (encoding alpha calcineurin subunit), showed no significant change between E7.5 and E8.5 (Figure 6.4a, Figure 6.4d). During *Eomes*-GFP ESC differentiation *Camk2d* was significantly up-regulated at day 5 compared to day 0 of differentiation (2.7 fold ±0.3; p=<0.001) with expression increasing until day 14 (15.7 fold ±2.3; p=<0.001) (Figure 6.4b). *Ppp3ca* gene expression also increased throughout differentiation (18.6 fold ±3.9; p=<0.001), becoming significantly elevated from day 4 (2.5 fold ±0.6; p=0.036) (Figure 6.4e). This data demonstrates that the expression of

genes related to Ca^{2+} signalling increase throughout ESC cardiomyocyte differentiation in a similar manner to cardiomyocyte specific markers (Chapter 4).

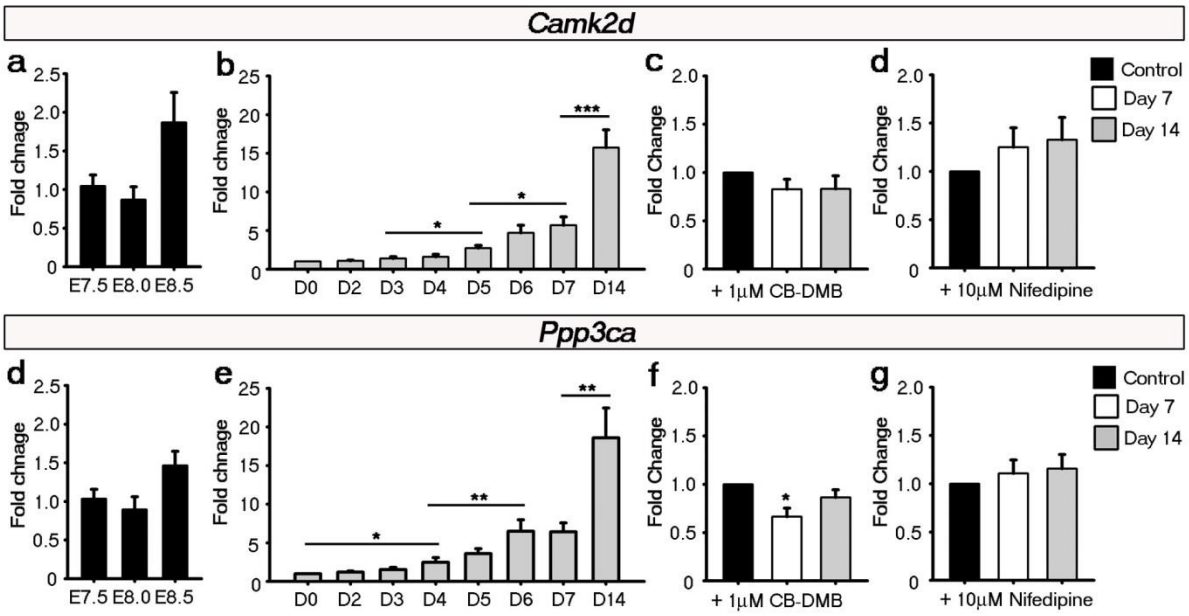

Figure 6.4: Gene expression of CaMKinaseII and Calcineurin increase throughout cardiomyocyte differentiation but are unaffected by NCX1 or Cav1.2 inhibition

Temporal gene expression of Camk2d (a) and Ppp3ca (d) were assessed on whole embryos by qRT-PCR, normalised to E7.5; no significant differences were observed between E7.5 and E8.5 (n=5). Gene expression was also assessed during *Eomes*-GFP cardiomyocyte differentiation with Camk2d (b) and Ppp3ca (e) increased over the 14 day timecourse with significant up regulation in Camk2d by day 5 and Ppp3ca by day 4 (n=5). Treatment throughout differentiation with either CB-DMB or Nifedipine did not affect expression of Camk2d at either day 7 (n=4) or day 14 (n=6) whilst Ppp3ca was significantly down regulated during CB-DMB treatment at 7 whilst being unaffected by inhibition at day 14. Inhibition of Cav1.2 with Nifedipine did not affect Ppp3ca expression at either day 7 or 14. All error bars are mean ± S.E.M. Statistics: ANOVA and Tukey test for multiple comparisons (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

6.2.4 Effect of Cav1.2 and NCX1 on genes involved in known Ca^{2+}-related signalling pathways

To assess whether Ca^{2+} signalling genes were affected by inhibition of NCX1 or Cav1.2, expression levels were assessed after long-term treatment with CB-DMB or nifedipine. Chronic exposure to CB-DMB significantly decreased the expression of both calcineurin (*Ppp3ca*) (0.7 fold ±0.1; p=0.04) and *NFAT* (0.8 fold ±0.1; p=0.03) at day 7 of differentiation (Figure 6.4f, Figure 6.5d). *NFAT* remained significantly decreased at day 14 when treated with CB-DMB (0.8 fold ±0.1; p=0.045), in contrast to *Camk2d* which

was unaffected by treatment at day 7 and day 14 (Figure 6.4c). The expression of the intermediate messenger calmodulin was also assessed as it is a Ca^{2+}-dependent regulator of both CamKII and calcineurin. Calmodulin is encoded by three genes (*Calm1-3*), however, none of these were affected by CB-DMB treatment at day 7 or 14 (Figure 6.5a-c). Similarly, no difference was observed in the expression of the *Calm1-3* when treated with Nifedipine at both day 7 and 14 (Figure 6.5e-h). Overall this data suggests that inhibition of NCX1 and Cav1.2 affected Ca^{2+} signalling-related genes in different ways, implicating these two proteins in effecting different signalling pathways during ESC cardiomyocyte differentiation.

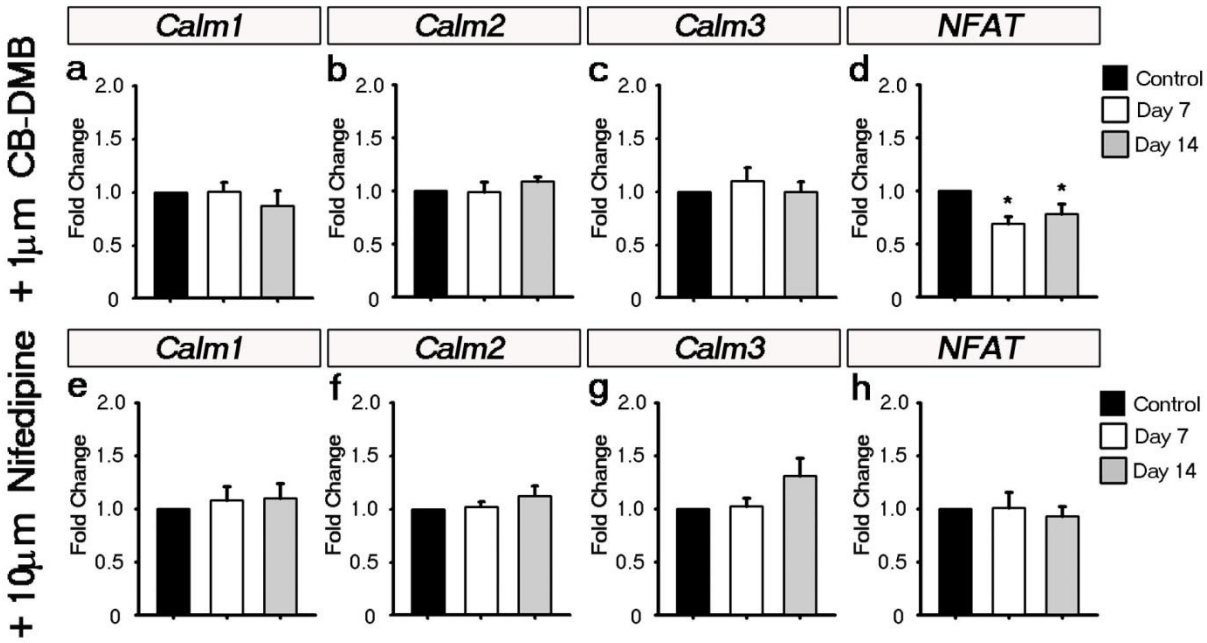

Figure 6.5: Inhibition of NCX1 or Cav1.2 does affect expression of Calmodulin genes whilst NCX1 inhibition reduces NFAT expression during *Eomes*-GFP cardiomyocyte differentiation

Chronic treatment of *Eomes*-GFP ESCs with either 1 μM CB-DMB (a-c) or 10 μM Nifedipine (e-g) did not affect the expression of Calmodulin genes *Calm1*, *Calm2* or *Calm3* at either day 7 or day 14 of differentiation. Treatment with 1 μM CB-DMB did, however, significantly reduce the expression of NFATc1 at both day 7 and 14 whilst 10 μM Nifedipine had no effect. All error bars are mean ± S.E.M (day 7, n=4; day14, n=6). Statistics: ANOVA and Tukey test for multiple comparisons (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

6.2.5 Inhibition of NCX1 reduces CaMKII activity during cardiomyocyte differentiation of *Eomes*-GFP cells

In order to more accurately assess how sarcolemmal Ca^{2+} influx may regulate Ca^{2+} signalling during ESC differentiation, the enzyme activity of CaMKII was investigated due to its role in the regulation of Mef2c during adult pathological hypertrophy (X. Wu et al. 2006). Upon Ca^{2+} binding, CaMKII auto-phosphorylates at specific residues leading to constitutive and increased enzyme activity. Using specific antibodies it is possible to detect the auto-phosphorylated form of CaMKII and, with scanning densitometry, assess enzyme activity. Treatment of *Eomes*-GFP ESCs with CB-DMB or nifedipine throughout differentiation had no effect on the ratio of activated phospho-CaMKII (pCaMKII) levels compared to total CaMKII, relative to GAPDH expression, at day 4 (mean+ SEM: DMSO, 0.93±0.33; CB-DMB, 1.09±0.07; nifedipine, 0.98±0.28; n=3)(Figure 6.8a, Figure 6.6b). However, at day 7 inhibition of NCX1 with CB-DMB significantly reduced the ratio of pCaMKII / total CaMKII in comparison to treatment with DMSO, whilst treatment with Nifedipine had no effect (mean+ SEM: DMSO, 1.18±0.13; CB-DMB, 0.69±0.15; nifedipine, 1.13±0.06. DMSO vs. CB-DMB, $p \leq 0.01$; CB-DMB vs. nifedipine, $p \leq 0.05$; n=3.) (Figure 6.6c, Figure 6.6d). This data reveals that NCX1 can regulate CaMKII activity in a stage-dependent manner, whilst Cav1.2 inhibition does not influence this pathway.

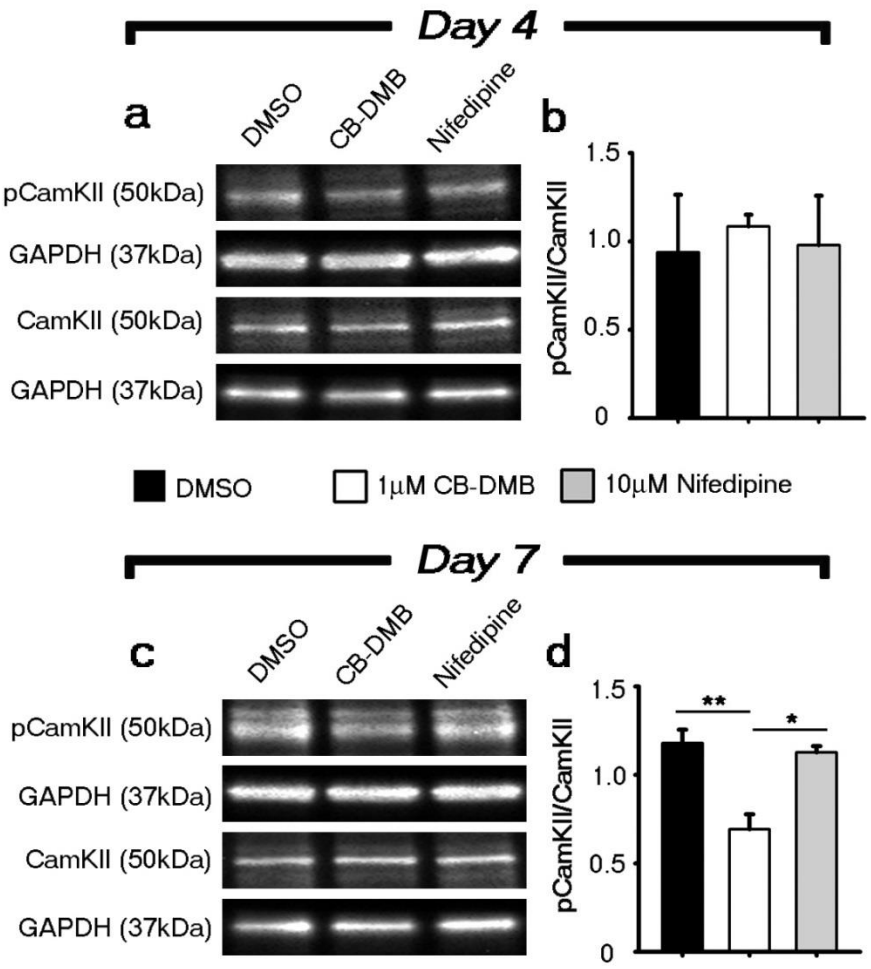

Figure 6.6: Inhibition of NCX1 reduces the activation of CaMKinaseII at day 7 of *Eomes*-GFP cardiomyocyte differentiation

Exposure of cultured ESC derived-cardiomyocytes NCX1 and Cav1.2 inhibitors effected downstream Ca^{2+} signalling via alterations in the levels of phosphorylated CaMKII (pCaMKII). The ratio of pCaMKII:total CaMKII was unaffected by chronic treatment with DMSO, 1 μM CB-DMB or 10 μM Nifedipine at day 4(a, b), whilst at day 7 1 μM CB-DMB significantly decreased the ratio pCaMKII:total CaMKII compared to DMSO or Nifedipine (c,d). All error bars are mean ± S.E.M (n=3). Statistics: ANOVA and Tukey test for multiple comparisons (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

6.3 Discussion

6.3.1 The concentration of external Ca^{2+} influences cardiomyocyte differentiation

The ability of NCX1 and Cav1.2 to mediate early Ca^{2+} transients, as well as inhibit cardiomyocyte formation suggests that Ca^{2+} may be fundamental in integrating early functional activity with gene regulation during heart development. It was, therefore, important to assess how global changes in extracellular Ca^{2+} influenced myocyte differentiation in order to determine whether inhibition via NCX1 and Cav1.2 could be mediated by Ca^{2+} alone. To investigate this *Eomes*-GFP ESCs were cultured in media with reduced external Ca^{2+} concentrations in comparison to the 1.8 mM Ca^{2+}, used in previous experiments. In adult murine cardiomyocytes Ca^{2+} is driven into the cell upon the opening of Ca^{2+} channels by the sarcolemmal concentration gradient, which is a result of the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) being significantly lower (~0.1mM) than the extracellular concentration ($[Ca^{2+}]_o$) (1.0m-1.8mM). Lowering external Ca^{2+} would, therefore, decrease the concentration gradient across the sarcolemmal membrane and reduce the driving force required for Ca^{2+} influx. In terms of NCX1 function, reducing external Ca^{2+} to 0.1 mM would increase the reversal potential of NCX1 from -26mV to around 43mV and lead to greater forward mode NCX1 activity, therefore, reducing the ability of NCX1 to work in reverse mode and facilitate Ca^{2+} influx.

Reducing external Ca^{2+} led to a significant decrease in cardiomyocyte differentiation, as assessed by the percentage of beating EBs at both day 7 and 14. In order to determine whether the reduction in beating EBs was due to a decrease in the formation of cardiomyocytes or a consequence of reduced Ca^{2+} on contractile function, EBs that had been cultured in low Ca^{2+} were returned to control (1.8 mM Ca^{2+}) media for 2 hours prior to being assessed. By day 14, the percentage of beating EBs cultured in 0.1 mM Ca^{2+} was similar to that when NCX1 was inhibited, suggesting that both treatments may act via the same pathway, and that inhibition of cardiomyocyte differentiation via NCX1 could be mediated by Ca^{2+} influx. Culture with 1.0 mM Ca^{2+}, which may more be physiologically relevant than 1.8 mM in terms of the *in vivo* $[Ca^{2+}]_o$ in the mouse heart (Kashimura et al. 2010), had an intermediate effect on cardiomyocyte differentiation compared to 1.8 mM and 0.1 mM Ca^{2+}. Together this suggests that the concentration of external Ca^{2+} directly relates to the formation of

cardiomyocytes, with increased $[Ca^{2+}]_o$ promoting cardiomyocyte differentiation. Significantly, low $[Ca^{2+}]_o$ from day 0-4 reduced the percentage of beating at day 14 even after 10 days in control 1.8 mM Ca^{2+}. In contrast, maintaining EBs in 1.8 mM Ca^{2+} from day 0-4 before culturing them from day 4-14 in 0.1 mM Ca^{2+} had no effect on the percentage of beating compared with culture in 1.8 mM from day 0-14. These findings suggest that Ca^{2+} is crucial for the early specification and formation of cardiac progenitors again consistent with the phenotype of NCX1 inhibition. However, once cardiac progenitors have formed and cardiomyocyte differentiation has begun, the requirement for Ca^{2+} becomes altered, which may be due to changes in the balance between sarcolemmal Ca^{2+} influx and the release of Ca^{2+} from intracellular stores such as the SR or lysosomes.

Ca^{2+} is a major second messenger involved in the regulation of cell growth and proliferation (Berridge 1995), it was, therefore, important to assess whether proliferation was inhibited by the reduced $[Ca^{2+}]_o$. In terms of phenotype, EBs at day 4 of differentiation were similar when cultured in low 0.1 mM and 1.8 mM Ca^{2+}, this was also reflected in the number of cells per EB at day 4 and 7. Overall, this would suggest that although $[Ca^{2+}]_o$ was reduced, the concentration was still sufficient to maintain normal cell proliferation and growth, however, a larger sample size would be required to conclusively determine this due to the substantial variation observed between experiments. A more accurate method for assessing the proliferation status during culture would be to use a dye such as BrdU, which incorporates itself into newly synthesised DNA and allows assessment of cell division.

Overall this data shows that reducing the concentration of external Ca^{2+} inhibits cardiomyocyte formation and correlates with observations made using pharmacological inhibitors of NCX1, Cav1.2 and TPC activation. Together these results highlight a role for Ca^{2+} in the regulation of cardiomyocyte differentiation and suggest that the Ca^{2+} influx associated with early functional activity is required for subsequent heart development.

6.3.2 Hypertrophic Ca^{2+} signalling during cardiomyocyte differentiation

Cardiac hypertrophy is the abnormal enlargement of the heart due to increases in cardiomyocyte size as well as changes in other components of the heart such as the

extracellular matrix. Hypertrophy is an adaptive response to pressure or volume overload (e.g. hypertension), mutations in sarcomeric proteins or the loss of contractile mass from a prior myocardial infarction, leading to an increased risk of heart failure, arrhythmias and sudden cardiac death (Frey et al. 2004). A widely supported hypothesis is that changes in Ca^{2+} cycling mediate this hypertrophic response (Molkentin 2006) via the onset of abnormal Ca^{2+} transients and the reactivation of fetal cardiac genes, such as Mef2c, Nkx2.5 and β-MHC, via Ca^{2+}-dependent signalling pathways. In terms of cardiac physiology, hypertrophy leads to significant changes in ECC with a decrease in SR function and increased sarcolemmal Ca^{2+} flux, due to reduced SERCA2a levels as well as increased NCX1 expression (Rourke et al. 1999). The increased requirement for sarcolemmal Ca^{2+} flux during hypertrophy is similar to the physiology observed within the early embryo, as discussed in chapter 6, suggesting that changes in Ca^{2+} dynamics during formation of cardiomyocytes may integrate with known hypertrophic Ca^{2+} signalling pathways. Ca^{2+} and calmodulin – dependent signalling pathways such as CaMKII and calcineurin which in turn have been strongly implicated in the induction of cardiac hypertrophy and the reactivation of fetal genes (Frey & Olson 2000)(Bassel-Duby & Olson 2003).

Calcineurin is a Ca^{2+}-dependent protein phosphatase, composed of a CaM-binding catalytic subunit and a smaller regulatory subunit and has been identified as a key regulator of cardiac hypertrophy via the de-phosphorylation and activation of the transcription factor NFAT. CaMKII is a serine/threonine kinase that binds Ca^{2+} via its EF hands leading to the activation and phosphorylation of downstream targets, including transcription factors such as Mef2c (X. Wu et al. 2006). Gain- and loss-of-function studies in genetically altered mice have demonstrated that CaM, CaMKII and calcineurin are all necessary to regulate pathologic cardiac hypertrophy (Molkentin 2004)(Backs et al. 2009)(Mishra et al. 2010), however, little is known about their role during embryonic heart formation. This study has focused on the gene expression of the predominant cardiac isoform of calcineurin, the catalytic α subunit (encoded by ppp3ca) and the CaMKIIδ isoform (encoded by camk2d). Over the course of ESC cardiomyocyte differentiation both calcineurin and CaMKIIδ increased suggesting an elevated requirement for Ca^{2+}-dependent signalling as differentiation progressed. In terms of gene expression profiles *ppp3ca* (encoding calcineurin subunit a) became

significantly up regulated at day 4 of differentiation whilst *camk2d* (encoding CaMKIIδ) increased at day 5. These increases correlated with the up-regulation of cardiac progenitor markers Nkx2.5 and Mef2c observed at the same time points in chapter 4 and suggesting that both calcineurin and CaMKIIδ are required during the formation of ESC derived cardiomyocytes.

The increased gene expression of CaMKIIδ and calcineurin observed during the formation of ESC-derived cardiomyocytes was not reflected during formation of the cardiac crescent between E7.5 and E8.5 *in vivo*. The lack of change in expression of these genes suggested that CaMKIIδ and calcineurin are not required for the formation of cardiomyocytes *in vivo*. In terms of calcineurin, this data would fit with previous studies which have shown that calcineurin and its downstream target NFAT are not required for the formation of the linear heart tube but instead are involved in the maturation of cardiomyocytes (Schulz & Yutzey 2004). Although this data shows that CaMKIIδ and calcineurin do not increase during formation of the cardiac crescent, it does not exclude them from having a role in signalling over this timecourse as gene expression does not directly correlate with protein levels or enzyme activity. Both CaMKII and calcineurin/NFAT are expressed within the early embryo and are known to have a role in lineage specification and the integration of Ca^{2+} signals during fertilisation (Li et al. 2011)(Knott et al. 2006), suggesting that these proteins are likely to be expressed prior to E7.5. This would also fit with the expression profiles of ESC differentiation marker genes (Chapter 4), as both CaMKIIδ and calcineurin were initially up regulated when mesoderm markers such as Brachyury and Mesp1 were maximally elevated during gastrulation at E6.5. It is likely that other non-cardiac specific isoforms of these enzymes, such as CaMKIIγ or CaMKIV, may have more predominant roles during early embryonic development and contribute to the formation of the cardiac crescent potentially compensating for the later expression of the cardiac isoforms. Overall whilst CaMKIIδ and calcineurin gene expression does not increase during *in vivo* formation of the heart these signalling pathways are present and potentially active within the embryo at E7.5, however, samples prior to E7.5 would need to be analysed in order to compare the *in vivo* and ESC models when using relative gene expression or by assessing enzyme activity at the protein level to examine Ca^{2+} dependent signalling during *in vivo* cardiomyocyte formation.

6.3.3 Specific roles of NCX1 and Cav1.2 in Ca^{2+}-dependent signalling

Activity-dependent gene expression triggered by Ca^{2+} entry into cardiomyocytes is reported to be responsible for the progression of cardiac diseases but whether specific sources of Ca^{2+} act distinctly or merely supply Ca^{2+} to a common pool remains uncertain. To begin to assess how Cav1.2 and NCX1 inhibition may influence Ca^{2+} dependent signalling pathways, gene expression of related genes were examined after chronic inhibitor treatment. CaMKII and calcineurin signalling is regulated by the upstream Ca^{2+} binding second messenger calmodulin which is encoded by three genes; *Calm1*, *Calm2*, *Calm3*. Inhibition of NCX1 and Cav1.2 had no effect on the expression of all three calmodulin genes. The inability of CB-DMB or nifedipine to affect calmodulin suggests that calmodulin is not regulated by sarcolemmal Ca^{2+} influx, however, the expression of calmodulin in all eukaryotic cells (Stevens 1983) potentially masks any changes in cardiomyocyte specific gene levels and makes it difficult to conclude how NCX1 or Cav1.2 may regulate calmodulin.

The expression of calcineurin and NFAT genes was significantly down regulated by chronic inhibition of NCX1 suggesting the calcineurin pathway may be regulated by NCX1 mediated Ca^{2+} flux, however, this decrease could also represent the reduced formation of cardiomyocytes during NCX1 inhibition. By day 14 of CB-DMB treatment the significant reduction in the relative expression of calcineurin was lost, potentially due to up-regulation of calcineurin in the small proportion of cardiomyocytes that form during CB-DMB treatment or because of increased expression in other cells types other than cardiomyocytes as differentiation progresses. In contrast, the gene expression of NFAT remained down regulated at day 14 which is likely to be a consequence of the significant decrease in calcineurin and NFAT gene expression at day 7. NFAT is known to positively feedback to regulate its own expression, therefore, any decrease in NFAT would exacerbate the inhibitory effect in comparison to control. Whilst NFAT is not required for the formation of cardiomyocytes the decrease in NFAT expression suggests that NCX1 could also regulate cardiomyocyte maturation as well as formation. In contrast inhibition of Cav1.2 did not affect the expression of either calcineurin or NFAT, suggesting that NCX1 and Cav1.2 inhibit cardiomyocyte formation via different signalling pathways, as will be discussed later.

Mef2c is one of the main transcription factors regulated by CaMKII activity via the phosphorylation and removal of repressive HDACs (histone deacetylases) from the nucleus thereby altering chromatin structure and allowing Mef2c mediated transcription (X. Wu et al. 2006). Inhibition of NCX1 throughout ESC differentiation caused significant decreases in the expression of Mef2c, however, removal of NCX1 inhibition at day 4 resulted in the recovery of Mef2c expression (Chapter 5) suggesting that CaMKII may be required for Mef2c regulation during ESC cardiomyocyte differentiation. Upon Ca^{2+} binding, CaMKII is known to auto phosphorylate at specific residues, leading to constitutive activation and an increase in kinase activity (Ishida et al. 1996). Using specific antibodies for total CaMKII and auto-phosphorylated CaMKII a significant decrease was observed in the auto-phosphorylation of CaMKII at day 7 of ESC differentiation when NCX1 was inhibited in comparison to DMSO or Cav1.2 inhibition. This was in contrast to CaMKIIδ gene expression which was not effected by NCX1 inhibition and, therefore, highlights the importance of performing direct assays to assess signalling dynamics. The inability of NCX1 inhibition to reduce CaMKIIδ gene expression even when other cardiac specific genes were down regulated (Chapter 5) could suggest that CaMKIIδ is up-regulated in the small percentage of cardiomyocytes that form during NCX1 inhibition as a potential compensatory mechanism for the overall decrease in CaMKII enzyme activity, however, this was not conclusive at the protein level. When assessing enzyme activity using western blot analysis it was not possible to measure the activity of the cardiac CaMKIIδ isoform alone as current antibodies detect the phosphorylation state of all CaMKII isoforms. At day 4, NCX1 inhibition had no effect on CaMKII auto-phosphorylation, despite treatment from day 0-4 inhibiting cardiomyocyte differentiation (Chapter 5), thus suggesting that CaMKII signalling is not involved in the early signalling required for cardiac progenitor specification and that other Ca^{2+} related signalling pathways are required. The inability of CB-DMB to reduce CamKII auto-phosphorylation at day 4 also corresponds with the timecourse of CaMKIIδ gene expression, as significant increases in CaMKIIδ expression did not occur until day 5 of differentiation, suggesting CaMKII signalling may not be occurring prior to day 5. The ability of CamKII to impact on cardiomyocyte differentiation would also be influenced by the expression of its downstream targets. In terms of cardiac related genes regulated by CamKII, Mef2c expression did not increase until between days 5 and 7 during ESC differentiation suggesting that even if

CamKII was active prior to day 4 it would not be required for the regulation of Mef2c. That said, CaMKII activity may have a role in the initiation of Mef2c activity, via its ability to phosphorylate and remove repressive HDACs (Zhang et al. 2002) thereby altering the epigenetic landscape and allowing Mef2c mediated transcription to occur.

The inability of Cav1.2 inhibition to reduce CaMKII activity suggests that whilst both Cav1.2 and NCX1 can prevent Ca^{2+} transients at day 7,the specific source of sarcolemmal Ca^{2+} determines the Ca^{2+} dependent signalling pathway regulated. The differences in NCX1 and Cav1.2 regulation of CaMKII activity potentially correlate with the differences observed in NCX1 and Cav1.2 function during the initiation of Ca^{2+} transients and the timecourse of NCX1 and Cav1.2 expression during formation of cardiomyocytes (Chapter 4). Chapter 4 showed that NCX1 was up regulated before Cav1.2 at both the RNA and protein level, whilst NCX1 mediated Ca^{2+} influx preceded Cav1.2 activity suggesting that NCX1 could influence Ca^{2+} signalling at earlier stages of cardiomyocyte formation and lead to stronger downstream effects at day 7.

In chapter 5 it was suggested that Cav1.2 may have a more predominant role in controlling cardiac signalling via mechanical stretch at later stages of differentiation, this is further supported by the observation that inhibition of Cav1.2 with nifedipine did not affect CaMKII activity. Contraction can trigger mechanical signalling via a number of different mechanisms, including-stretch activated Ca^{2+} channels but also via Ca^{2+}-independent signalling pathways such as the Akt and MAPK pathways working through G-protein coupled receptors and integrins. It could, therefore, be suggested that Cav1.2 may be involved in signalling pathways that are not directly controlled by Ca^{2+}. To investigate alternative pathways it would be interesting to inhibit contraction during ESC differentiation without affecting either Ca^{2+} transients or actin/cytoskeletal dynamics.

Another factor which may influence the signalling properties of NCX1, Cav1.2 and TPC1/2 could be their subcellular localisation and the formation of micro-domains. In adult cardiomyocytes cellular micro-domains are known to regulate the signalling properties of different pathways, and localisation of Ca^{2+} to specific micro-domains determines how Ca^{2+} regulates downstream signalling (Ibarra et al. 2013). This suggests that in early cardiac progenitors NCX1 and Cav1.2 are located within different

micro-domains and, therefore, regulate Ca^{2+} signalling in different ways. On the other hand, discrete Ca^{2+} signalling domains may have not yet formed in early cardiac progenitors allowing signals which become restricted in the adult to propagate throughout the cytoplasm and act at the nucleus. Micro domains can be created by the formation of signalling complexes made up of a number of different interacting proteins such as A kinase anchoring proteins (AKAPs) and the cytoskeleton. It has been shown that both NCX1 and Cav1.2 can form macromolecular signalling complexes with a range of different signalling molecules including protein kinases (PKA and PKC), protein phosphatases (PP2A and PP1) and AKAPs suggesting that the signalling properties of these two proteins could be dynamically regulated by the associated proteins expressed throughout differentiation (Schulze et al. 2003)(Harvey & Hell 2013).

Overall this data shows that NCX1 is capable of regulating CaMKII activity suggesting that early sarcolemmal Ca^{2+} flux has a role in signalling, that could potentially regulate gene transcription and differentiation, however, there are a wide range of other mechanisms by which NCX1 and Cav1.2-mediated Ca^{2+} handling could regulate the signalling and gene transcription required for cardiomyocyte differentiation.

6.3.4 Alternative Ca^{2+} signalling mechanisms during cardiomyocyte formation

Whilst CaMKII and calcineurin have been strongly implicated in Ca^{2+}-dependent signalling pathways in the heart a number of more novel mechanisms may be capable of directly linking Ca^{2+} influx with gene transcription. In the brain, Cav1.2 has been reported to acutely control gene expression over a period of 5mins via the transcription factor CREB (cAMP response element-binding protein) (Ma et al. 2014), whilst in isolated cardiomyocytes the acute activation of EPAC, a guanylyl exchange protein, triggers Ca^{2+} mediated HDAC nuclear export within 60 minutes (Pereira et al. 2012). To determine whether NCX1 or Cav1.2 had an acute role in the regulation of cardiac gene expression during differentiation. ESCs were treated for only 4 hours such that any changes in expression would be due to direct signalling effects and not as a consequence of cell division or protein turnover. Neither treatment affected cardiac-related gene expression after 4 hours of inhibition suggesting NCX1 or Cav1.2 did not acutely regulate transcriptional activity and that NCX1 and Cav1.2 indirectly influence

cardiac gene expression via other cellular process such as metabolism. However, due to the ESC model used, acute inhibition is likely to be masked by the myriad of cell types present and the large variation in maturity of cardiomyocytes during differentiation. Previous studies which have investigated the acute role of Ca^{2+} channel activity and gene regulation have typically used pure populations of single cells. Although ESCs have been staged by the overall level of gene expression at various days of differentiation, ESC derived cardiomyocytes do not represent a pure population of cells at the same stage of differentiation. Therefore, if a specific gene or subset of cells was acutely affected by inhibition the response could potentially be masked by other cells which already express that gene or by cells which do not require NCX1 or Cav1.2 for the expression of that particular gene.

As well as Ca^{2+} acting via downstream signalling molecules to regulate transcription within the nucleus, Ca^{2+} also directly binds certain transcription factors to regulate activity. DREAM (Downstream Regulatory Element Antagonistic Modulator) binds to DNA at low Ca^{2+} concentrations and represses transcription; upon direct Ca^{2+} binding DREAM dissociates from DNA allowing transcription to occur (Carrión et al. 1999). In neonatal cardiomyocytes, DREAM has been shown to regulate the expression of *Cacna1c* (encoding Cav1.2) creating a physiological feedback mechanism enabling cardiomyocytes to adjust Ca^{2+} influx via Cav1.2 to the concentration of intracellular Ca^{2+} (Ronkainen et al. 2011). The early influx of Ca^{2+} during cardiac differentiation may, therefore, be capable of directly regulating the binding and transcriptional activity of certain transcription factors, such as DREAM, and thereby indirectly regulate the expression of downstream cardiac specific genes

Cardiomyocyte signalling and the regulation of gene transcription could also be regulated by the electrophysical properties of NCX1- and Cav1.2-mediated sarcolemmal ion flux. Electrical charge is known to affect interactions between nucleotides as well as playing a key role in protein folding (Snodin et al. 2015). A novel mechanism termed excitation-transcription coupling has been described in sympathetic neurons which links channel activity due to membrane depolarisation with activation of the nuclear transcription factor CREB independently of Ca^{2+} influx (Wheeler et al. 2008)(D'Arco & Dolphin 2012). In Drosophila, electrochemical cues have been shown to regulate Wnt signalling complexes at the plasma membrane. The

binding and recruitment of key Wnt signalling molecules, dishevelled and frizzled, was regulated by both pH and electrical charge with the membrane localisation of dishevelled being dependent on Na^+/H^+ exchanger activity at the plasma membrane (Simons et al. 2009). These studies highlight the role that electrical charge can have on cellular interactions and adds a new level of complexity to cellular signalling. Similar mechanisms may be occurring in early cardiomyocytes due to the electrogenic nature of the NCX1 and the role of Wnt signalling during cardiomyocyte differentiation (Gessert & Kühl 2010) as well as the inward current generated by Cav1.2 activation.

Although these studies have highlighted CaMKII signalling as a key pathway, which can potentially integrate NCX1 Ca^{2+} handling and gene regulation, as well as provided some preliminary insight into the other Ca^{2+} signalling pathways involved, a much broader study would be required to further examine the role of Ca^{2+} signalling in cardiomyocyte differentiation.

6.4 Summary

In summary, we have shown that reducing extracellular Ca^{2+} prevents cardiomyocyte differentiation in a manner similar to that observed during NCX1, Cav1.2 and TPC1/2 inhibition suggesting Ca^{2+} is fundamental for cardiomyocyte differentiation. We have also begun to investigate how NCX1 and Cav1.2 activity integrates with specific Ca^{2+} signalling pathways during ESC cardiomyocyte differentiation, revealing a role for NCX1 in the regulation of CaMKII activity and highlighted differences in the mechanisms by which NCX1 and Cav1.2 can regulate downstream Ca^{2+} signalling, however, as discussed above further studies are needed to address this in more detail.

7. CONCLUDING REMARKS

In order for the adult heart to supply the body with sufficient oxygen and nutrients it relies on the coordinated influx and efflux of Ca^{2+} via ECC. In the failing heart, the hypertrophic response is mediated by alterations in Ca^{2+} handling and Ca^{2+} dependent signalling pathways, leading to changes in transcription and thereby linking cardiomyocyte function with gene expression. It is well documented that the heart is the first organ to form and function in the embryo proper, with contractile activity occurring prior to the formation of mature cardiomyocytes. This highlights a potential link between early heart function and subsequent cardiomyocyte differentiation. Whilst contractile activity is known to regulate cardiac gene expression via mechanical force, only a small number of studies have begun to investigate how early function though Ca^{2+} dynamics may regulate cardiomyocyte differentiation during the initial stages of heart development..

To begin to understand how function may integrate with heart formation, it was essential to precisely characterise the temporal development of the heart from the earliest specification of pre-cardiac mesoderm to linear heart tube stage in order to assess when and how contraction was initiated. To this end we developed a staging method focusing on murine cardiac crescent development, which allowed us to accurately correlate morphogenesis and differentiation with channel/exchanger expression and contractile activity. Whilst characterising the formation of the cardiac crescent we observed single cell Ca^{2+} transients prior to the onset of contraction, suggesting that Ca^{2+} dynamics, independent of contractile activity, may regulate cardiomyocyte differentiation during early heart development. Moreover, we identified NCX1 as a mechanism for generating pre-contractile Ca^{2+} transients via its ability to trigger Ca^{2+} influx through reverse mode of action, a role superseded by the expression of L-type Ca^{2+} transients.

Whilst the dynamic nature of immature cardiomyocyte electrophysiology has previously been described, and NCX1 suggested to be responsible for the initiation of cardiac function, we believe our findings represent the first observation of NCX1-meditead pre-contractile Ca^{2+} transients in the intact mammalian heart. Inhibition of sarcolemmal Ca^{2+} handling via NCX1 and Cav1.2 as well as Ca^{2+} release form

intracellular lysosomal stores via TPCs caused a significant reduction in ESC cardiomyocyte differentiation, although to varying degrees, highlighting a requirement for Ca^{2+} during early differentiation of the forming heart. Inhibition of NCX1 had the most significant effect on cardiomyocyte formation (most likely due to its early expression and function) and was capable of regulating CaMKII auto-phosphorylation, something that was not observed during Cav1.2 inhibition. This finding implicates distinct sources of Ca^{2+} as modulators of specific signalling pathways during cardiomyocyte differentiation and is extremely relevant in the diseased adult heart in which NCX1 is up-regulated and SR function decreases.

This study is by no means a complete investigation, and has raised further questions which will require future experiments to address, as described throughout the individual chapters. In terms of early cardiomyocyte physiology, further insight will be gained by fully characterising the electrophysiological and molecular basis of pre-contractile individual cell Ca^{2+} transients, as well as their lineage identity at both the whole tissue and single cell level. This data would allow us to address the heterogeneity observed in the dynamics of single cell Ca^{2+} transients prior to contraction as well as determining how single cell Ca^{2+} transients develop into co-ordinated propagating Ca^{2+} waves across the cardiac crescent. In parallel, it will be essential to assess the potential off-target effects of pharmacological inhibition. Whilst a wider range of drugs could be employed, it would also be appropriate to study genetic animal models. As detailed in Table 1.1, a number of different mouse models have been previously described which ablate relevant cardiac channels and exchangers and result in embryonic lethality due to cardiac defects. Using the methods and staging we have applied in this thesis, it would be interesting to investigate early cardiac crescent formation in these models. In addition, due to the dynamic nature of embryonic cardiomyocyte electrophysiology and the fundamental requirement for early heart function during development, it would be relevant to examine whether compensation occurs in order to maintain this early function. Whilst gene deletion studies would allow us to more accurately define the specific roles of certain Ca^{2+} handling and signalling molecules, overexpression studies would also be useful in implicating specific pathways required for the formation of the heart. We would hypothesise from our data, that overexpression of both NCX1 and CaMKII would lead

to enhanced cardiomyocyte differentiation. Whilst we began to look at potential signalling pathways required for the integration of function and gene transcription, it will be essential to further explore these and other more novel pathways. This thesis has also highlighted the need for using pure populations of specific cardiac progenitor cell types as well as direct readouts of signalling activity in order to make any definitive conclusions regarding Ca^{2+} signalling and cardiac gene transcription.

Insight into how heart form (muscle differentiation and morphogenesis) and function (Ca^{2+}-handling, contraction and downstream gene expression) are related during development of the cardiac crescent has a number of potential clinical implications. Firstly, these studies are highly relevant in helping to understand the basis of congenital defects, but more importantly the analogous roles of Ca^{2+} in the early heart and adult hypertrophy with regards to cardiac function and foetal cardiac gene induction to promote physiological or pathological myocyte growth, respectively, mean these studies have a much broader context. Investigating how contractile activity arises during heart development and is coordinated across individual cells of the cardiac crescent will give insight into cardiac arrhythmias and the role of abnormal Ca^{2+} handling in the reactivation of developmental genes during disease, potentially informing on the development of new therapies to prevent pathological gene reactivation. Gaining mechanistic insight into cardiomyocyte formation will also aid with understanding how best to produce and integrate *de novo* cardiomyocytes from both stem and progenitor cell types during cardiovascular regenerative medicine, and will be crucial to any tissue reparative strategy in which *de novo* cardiomyocytes are generated in order to restore the muscle of a diseased heart.

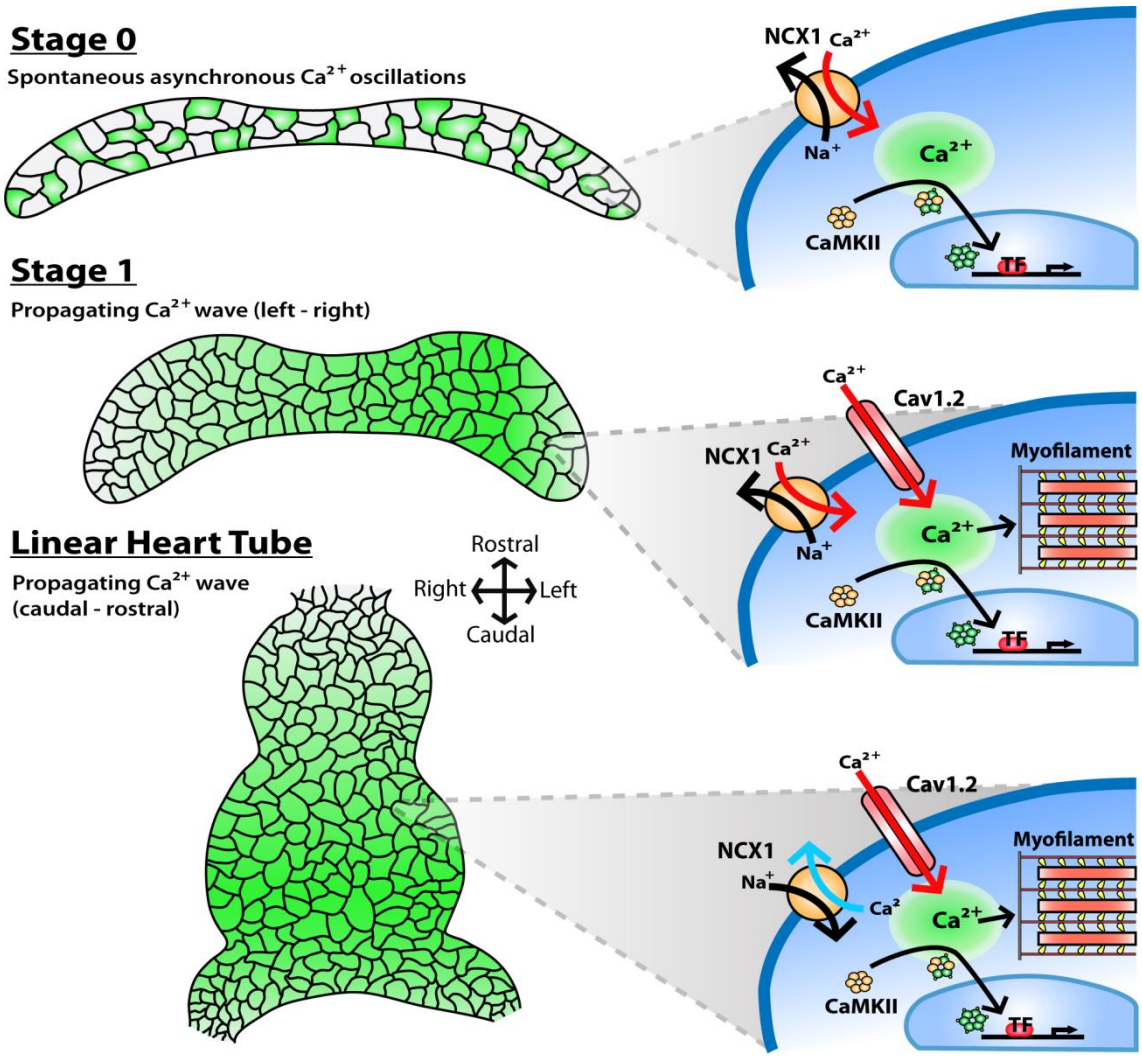

Figure 7.1: Schematic overview summarising the onset of Ca^{2+} transients within the developing cardiac crescent and the role of NCX1 and Cav1.2 in regulating Ca^{2+} influx.

Ca^{2+} transients were initially observed within stage 0 cardiac crescents as spontaneous asynchronous oscillations before the onset of propagating Ca^{2+} waves at stage 1. Within the stage 0 cardiac crescents, Ca^{2+} transients were mediated by NCX1, however no contraction was observed, thus correlating with the absence of sarcomere formation. As the cardiac crescent developed sarcomeres could be detected at stage 1, with both NCX1 and Cav1.2 having a role in the generation of propagating Ca^{2+} transients. At stage 3, and within the linear heart tube, NCX1's role in Ca^{2+} transient initiation was lost and transients were solely generated by Cav1.2.

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