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Maintenance Of Calcium Homeostasis in Embryonic Stem Cell-Derived Cardiomyocytes

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1. Introduction

Myocardial infarction is one of the major causes of morbidity and mortality in many developed countries. A potential method for treatment of such disease is the cell replacement therapy which involves the transplantation of cardiomyocytes (CMs). However, CMs are of very limited supply. Embryonic stem cells (ESCs) isolated from the inner cell mass of blastocysts are capable of self-renewal and can differentiate into all cell lineages, including CMs (He et al., 2003; Kehat et al., 2002; Kehat et al., 2001; Moore et al., 2008; Mummery et al., 2003; Ng et al., 2010; Thomson et al., 1998; Xu et al., 2002; Xue et al., 2005). Therefore, ESCs can be an excellent source of CMs for regenerative medicine.

Calcium (Ca^{2+}) is a universal signaling molecule that regulates a wide variety of cellular functions. In CMs, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) plays an important role in the contraction and relaxation of CMs. The $[Ca^{2+}]_i$ is tightly regulated by many proteins, including ion channels, receptors, pumps, and exchangers that are located on the cell surface plasma membrane and on the sarcoplasmic reticulum (SR). The aim of this book chapter is to provide a thorough review on Ca^{2+} handling in ESC-derived CMs.

For each protein of interest, some basic information on the protein was firstly presented. Then, changes in the expression of the protein and their contribution to the Ca^{2+} homeostasis and Ca^{2+} transient as described in human (h) and/or mouse (m) ESC studies were presented. *In vivo* data in mouse embryo studies were also presented for comparison purposes.

The information reviewed in this chapter would be important not only for understanding the basic biology of early differentiating CMs, it would also be important for providing insights into the future uses of ESC-derived CMs for cell replacement therapies.

2.1 Regulation of intracellular calcium level by voltage-operated calcium channels

Voltage-operated Ca^{2+} channels are typically composed of five subunits, namely α_1, α_2, β, δ and γ (Catterall, 1995; Catterall, 2000; De Waard et al., 1996; Dolphin, 2006; Moreno Davila,

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1999). The α_1 subunit forms the ion conduction pore of the channels while the other subunits modulate the functions of the channels. The α_1 subunit is itself a tetramer of proteins, each of which consists of six transmembrane segments (S1-S6). This subunit confers all of the major properties to voltage-operated Ca^{2+} channels in that it contains a voltage sensor at segment S4 and forms a Ca^{2+}-selective pore at segments S5-S6. The other subunits are regarded as to providing ancillary functions to the channels. The α_2 and δ subunits are two products of the same gene; the protein is cleaved into two peptides following translation. Subsequent disulfide bond formation anchors the extracellular α_2 subunit to the membrane via the transmembrane δ subunit. The β subunit is a cytoplasmic protein; it contains an α_1-binding pocket, where specific amino acids in the linker region between segments S1 and S2 of the α_1 subunit bind (Richards et al., 2004). The γ subunit is a monomer, which consists of four transmembrane segments (S1-S4). A diverse range of functionally distinct voltage-operated Ca^{2+} channels are formed by the combinations of different isoforms that have been identified for each of these subunits. The α_1 subunit is encoded by a family of ten distinct genes (the Ca_V gene family), which can be divided into three sub-families; Ca_V1 consists of $Ca_V1.1$-1.4, Ca_V2 consists of $Ca_V2.1$-2.3 and Ca_V3 consists of $Ca_V3.1$-3.3 (Catterall et al., 2005). The α_2/δ and β subunits are each encoded by four genes; $\alpha_2/\delta_{1\text{-}4}$ and $\beta_{1\text{-}4}$ respectively, while the γ subunit is encoded by eight genes; $\gamma_{1\text{-}8}$ (Arikkath and Campbell, 2003; Yang et al., 2011). Voltage-operated Ca^{2+} channels function by regulating the entry of extracellular Ca^{2+} into cells. Two types of voltage-operated Ca^{2+} channels exist in adult CMs (Catterall et al., 2005; Ono and Iijima, 2005). These are T-type Ca^{2+} channels, which open in response to more negative membrane potentials at about >-70mV for very short durations of 0.5 to 2 milliseconds (transient activation), and L-type Ca^{2+} channels, which open in response to less negative membrane potentials at about >-30mV for relatively longer periods of 0.5 to 10 milliseconds (long-lasting activation) (De Waard et al., 1996).

2.1.1 T-type calcium channels

T-type Ca^{2+} channels could be the simplest type of voltage-operated Ca^{2+} channels known thus far. Its structure was initially predicted *in silico* by homology to other α_1 subunits known at the time (Perez-Reyes, 2006). Since expression of the synthetic products was able to reproduce most of the electrophysiological properties observed in its native form, T-type Ca^{2+} channels were thought to consist of only one α_1 subunit; the other ancillary subunits (α_2, β, δ and γ) were considered absent. Although later co-expression studies could describe a role for the other ancillary subunits in the regulating functions of T-type Ca^{2+} channels *in vitro*, presence of these subunits have not been validated *in vivo* (Dolphin et al., 1999; Dubel et al., 2004; Green et al., 2001; Hobom et al., 2000; Lacinova and Klugbauer, 2004). Therefore, further investigations will be required to ascertain the genuine structure of this type of voltage-operated Ca^{2+} channel.

The α_1 subunit of T-type Ca^{2+} channels is encoded by either one of the three Ca_V3 genes, among which $Ca_V3.1$ and $Ca_V3.2$ are expressed in sinoatrial (SA) nodal cells of the heart (Bohn et al., 2000; Perez-Reyes, 2003). The SA node is the primary site, where spontaneous rhythmic action potentials are initiated. At the beginning of each action potential in an SA nodal cell, an influx of extracellular Na^+ into the cytosol, known as the funny current (I_f), first depolarizes the cell to about -50mV, at which T-type Ca^{2+} channels on the membrane open to allow an influx of extracellular Ca^{2+} into the cytosol. This produces a T-type Ca^{2+} current ($I_{Ca,\,T}$), which further depolarizes the cell to about -40mV. L-type Ca^{2+} channels on the membrane then open at this membrane potential to allow a greater influx of extracellular

Ca^{2+} into the cytosol and produce an L-type Ca^{2+} current ($I_{Ca,\ L}$) to a membrane potential until the threshold to produce an action potential is reached. Action potentials generated in the SA nodal cells are conducted to CMs within the working myocardium via gap junctions, ultimately resulting in regular contractions of the adult heart.

$Ca_V3.1$ mRNA and protein were simultaneously detected as early as embryonic day 14 (E14) after the heart has fully matured *in vivo* within mouse embryos (Cribbs et al., 2001). In a separate experiment, the mRNA level of $Ca_V3.1$ was moderately reduced from E18 when compared to its expression at adult stage (Yasui et al., 2005). Ni^{2+} at a high concentration of 100μM could be used to selectively block 50% of $Ca_V3.1$ T-type channels. The application of Ni^{2+} at this concentration was found to reduce $I_{Ca,\ T}$ by about 40% in CMs derived from mouse embryos at E12.5, indicating that the $Ca_V3.1$ T-type channels were functional by this time-point (Cribbs et al., 2001). In addition, homozygous null $Ca_V3.1^{-/-}$ mice displayed bradycardia, i.e. a reduced rate of cardiac contraction (Mangoni et al., 2006). Expression of $Ca_V3.1$ has also been studied *in vitro*. Its mRNA could be detected as early as day 5 and generally increased up until its last measurement at day 15 post-differntiation in ht7 mouse ESC- (mESC-) derived CMs (Mizuta et al., 2005). In R1 mESC-derived CMs, however, $Ca_V3.1$ mRNA could only be detected at two copies per cell by day 12, with its expression reaching the highest level at day 23 and declining by day 34 post-differentiation; the expression pattern reflected the amplitudes of $I_{Ca,\ T}$ measured at these time-points. (Zhang et al., 2003). A reduction of about 46% in $Ca_V3.1$ expression from day 9.5 to day 23.5 post-differentiation was also reported in EMG7 mESC-derived CMs; this down-regulation was found to be associated with a decrease in the contraction rates of the CMs in that $I_{Ca,\ T}$ was smaller in non-contracting myocytes at day 23.5 when compared to contracting CMs at day 9.5 and that the contraction rate at day 9.5 could be reduced via the application of 10μM efonidipine, an L- and T-type blocker, to the CMs (Yanagi et al., 2007). These data suggested a role for $Ca_V3.1$ in regulating the early contractions observed in the developing embryonic heart; by maintaining these contractions exclusively in developing pacemaker cells, other cells could then develop into non-contracting atrial or ventricular cells.

$Ca_V3.2$ mRNA was detected as early as eight-week gestation in humans (Qu and Boutjdir, 2001). In mouse, the expression of $Ca_V3.2$ was also measured at three time-points; its mRNA was detected as early as E9.5 and then its level was decreased by E18 until it was no longer detected at adult stage (Yasui et al., 2005). Again, $Ca_V3.2$ has been studied *in vitro*. Its mRNA was detected as early as day 5, but peaked at day 6 and gradually declined and was still detectable up until the last measurement at day 15 post-differentiation in ht7 mESC-derived CMs (Mizuta et al., 2005). In line with this finding, $Ca_V3.2$ mRNA level was also found to be down-regulated by about 24% from day 9.5 to day 23.5 post-differentiation in EMG7 mESC-derived CMs (Yanagi et al., 2007). Ni^{2+} at a low concentration of <50μM could be used to selectively block 50% of $Ca_V3.2$ T-type channels. When Ni^{2+} was applied at 40μM to ht7 mESC-derived CMs at day 8 post-differentiation, $I_{Ca,\ T}$ was found to be evidently decreased by about 60%, which signified the presence of functional $Ca_V3.2$ T-type channels by this time-point. Homozygous null $Ca_V3.2^{-/-}$ mice showed no sign of cardiac arrhythmia, but cardiac fibrosis with age-dependent severity (Chen et al., 2003). These data suggested a role for $Ca_V3.2$ in regulating the growth and maturation of the developing embryonic heart.

2.1.2 L-type calcium channels

L-type Ca^{2+} channels are distinguished from other voltage-operated Ca^{2+} channels that open at high membrane potentials for long periods on the basis of their sensitivity to 1, 4-

dihydropyridines (DHPs) (Hess et al., 1984). DHP-binding sites are contained within the α_1 subunit of L-type Ca^{2+} channels, which are encoded by either one of the four genes of the Ca_V1 sub-family. Among these, $Ca_V1.1$ is expressed predominantly in skeletal myocytes, $Ca_V1.2$ in adult CMs and $Ca_V1.3$ exclusively in SA nodal and atrial cells of the heart (Catterall, 2000; Qu et al., 2005). Unlike T-type, L-type Ca^{2+} channels are typical voltage-operated Ca^{2+} channels, which are made up of five subunits. Together with one of each of the $\alpha_2/\delta_{1\text{-}4}$, $\beta_{1\text{-}3}$ and $\gamma_{4,\ 6\text{-}8}$ ancillary subunits, $Ca_V1.2$- and $Ca_V1.3$-encoded α_1 proteins form the functional L-type Ca^{2+} channels that are found within the adult heart (Freise et al., 1999; Hosey et al., 1996; Yang et al., 2011).

$Ca_V1.2$ L-type Ca^{2+} channels are responsible for cardiac excitation-contraction coupling (E-C coupling), a mechanism by which an action potential is transformed into contraction in adult CMs. Upon the arrival of an action potential from SA nodal cells, L-type Ca^{2+} channels, which are clustered at transverse tubules (T-tubules), briefly open at the sarcolemma to allow an influx of extracellular Ca^{2+} into the cytosol of the CMs. This only accounts for about 30% of the increase in cytosolic Ca^{2+}, which is maintained and magnified through a process known as calcium-induced calcium release (CICR) (Bers, 2002). CICR is mediated via ryanodine receptor (RyR) Ca^{2+} release channels, which detect the $I_{Ca,\ L}$ and open to release Ca^{2+} from its intracellular stores within the SR into the cytosol of the CMs. This rise enhances binding of Ca^{2+} to troponin within the CMs, ultimately bringing about contractions of the adult heart. Conversely, $Ca_V1.3$ L-type Ca^{2+} channels are responsible for producing $I_{Ca,\ L}$, which constitutes one of the ionic currents to initiate action potentials at the SA node. Hence, these L-type Ca^{2+} channels contribute in maintaining the pacemaker activity of the adult heart (see 'T-type calcium channels' for details).

Consistent with its role in E-C coupling in the adult heart, $Ca_V1.2$ mRNA was detected as early as eight-week gestation in humans, with its level culminating to its maximum at adult stage (Qu and Boutjdir, 2001). $Ca_V1.2$ mRNA was also present *in vitro* at <40 days post-beating in H9.2 human ESC- (hESC-) derived CMs (Satin et al., 2008). In H7 hESC-derived CMs, Ca^{2+} transients that were detected at day 17 post-differentiation could be eliminated by the application of 10μM diltiazem, an L-type specific antagonist; this signified the functionality of L-type channels by this time-point (Zhu et al., 2009). Similar to humans, the expression of $Ca_V1.2$ was also up-regulated from its initial detection at E9.5 when the earliest contractions were observed by about three-fold at E15.5 when the heart has fully matured in mouse embryos; its protein was detected by E12.5 in mouse embryos (Acosta et al., 2004; Xu et al., 2003). In mESCs, patch clamp and Ca^{2+} imaging experiments indicated the functional expression of the $I_{Ca,\ L}$ starting from differentiation day 7, even before the appearance of spontaneous contractions (Kolossov et al., 1998). The current density of I_{Ca} continued to increase to day 10, the day of beginning of spontaneous contractions in most differentiating embryoid bodies (EBs). Thereafter, similar current density was recorded on day 17, the last day of measurement (Kolossov et al., 1998). Homozygous null $Ca_V1.2^{-/-}$ mice died *in utero* by E14.5, probably due to an absence of CICR-regulated contractions, resulting in reduced oxygen supply to the embryos (Seisenberger et al., 2000). Despite the lethality observed, normal rhythmic contractions were sustained in the $Ca_V1.2^{-/-}$ mice until E12.5. For this reason, CICR was initially thought to be non-essential for regulating the early cardiac contractions observed before E12.5 until later time-points. This conclusion was further supported by the fact that both the contractions and Ca^{2+} oscillations of D3 mESC-derived CMs were insensitive to the application of 50nM nisoldipine, a specific L-type channel blocker, at days 8-11 post-differentiation (Viatchenko-Karpinski et al., 1999). In addition,

these cells were insensitive to the application of high K^+ when compared to D3 mESC-derived CMs at day 16 post-differentiation; exposure to high K^+ at this stage would normally lead to immediate hyper-contraction and death of these cells. Albeit these findings, the notion that $I_{Ca,\ L}$ was not required at earlier stages of development was overthrown when evidence for the existence of a compensatory mechanism emerged; the expression of $Ca_V1.3$ was eminently up-regulated at E9.5 and E12.5 by four fold to produce a DHPR-insensitive $I_{Ca,\ L}$ in $Ca_V1.2^{-/-}$ mouse embryos when compared to wild-type (Xu et al., 2003). This has not only reinstated the importance of $I_{Ca,\ L}$ in maintaining cardiac contractions, even at the embryonic stage of the heart, but also appointed a new role for $Ca_V1.3$ to substitute for $Ca_V1.2$ and preserve its role in CICR for E-C coupling in its absence. In contrast with $Ca_V1.2$, $Ca_V1.3$ mRNA was detected by E9.5, with its level elevated only by a modest amount at E15.5 in mouse embryos. Its protein was also detected by E12.5 in mouse embryos (Xu et al., 2003). Analogous to $Ca_V3.1^{-/-}$ mice, homozygous null $Ca_V1.3^{-/-}$ mice developed bradycardia, consistent with its primary role in regulating the pacemaker activity of the heart (Platzer et al., 2000; Zhang et al., 2002). In R1 mESC-derived CMs, the expression of L-type Ca^{2+} channels could be detected as early as day 7 post-differentiation; given that the application of 1μM nifedipine, an L-type channel-specific antagonist, could eliminate Ca^{2+} transients that were normally observed at early (days 9-11) and intermediate (days 13-15), but only partially inhibit those at late (18-21 days post-differentiation) stages, this indicated that L-type channels were, in fact, playing a more dominant role at earlier developmental stages (Fu et al., 2006b).

2.2 Regulation of intracellular calcium level by ligand-operated calcium release channels

2.2.1 Ryanodine receptor (RyR) calcium release channels

RyR-Ca^{2+} release channels of a molecular mass of greater than two Mega-Daltons probably form the largest ion channels known thus far (Lanner et al., 2010). RyR-Ca^{2+} release channels are composed of homo-tetramers of RyR proteins. Owing to its enormous size, structural elucidation of these channels has been a major challenge. Nonetheless, RyR-Ca^{2+} release channels have been predicted to be largely cytoplasmic, with an ion-conducting pore consisting of around four to twelve transmembrane segments.

Three mammalian isoforms of RyRs exist; RyR1 is predominantly expressed in skeletal myocytes, RyR2 in CMs and RyR3 in astrocytes. The isoforms differ in three particular regions, which have been named domains D1-3 (Ma et al., 2004). As mentioned earlier in this chapter, RyR-Ca^{2+} release channels are induced to open in response to Ca^{2+} influx via L-type Ca^{2+} channels to facilitate E-C coupling in muscle cells. In skeletal myocytes, E-C coupling takes place through direct physical interaction between RyR1 and the D2 region, which is located between segments S2 and S3 of the $Ca_V1.1$-encoded α_1 subunit of L-type Ca^{2+} channels. In CMs, however, sequence divergence in the D2 domain between RyR1 and RyR2 means that RyR2 cannot physically interact with the $Ca_V1.2$-encoded α_1 subunit of L-type Ca^{2+} channels; E-C coupling can, therefore, only occur via CICR. RyR-Ca^{2+} release channels are, thus, organized into large arrays at junctions between the SR and the sarcolemma, in close proximity to the $Ca_V1.2$ L-type Ca^{2+} channels in adult CMs (Bers, 2004). Localized Ca^{2+} release events are referred to as Ca^{2+} sparks, which are collectively synchronized by $I_{Ca,\ L}$ to produce large, whole cell Ca^{2+} transients in the CMs.

Release of Ca^{2+} from the SR stores greatly increases the amount of cytosolic Ca^{2+} available to bind troponins in adult CMs. Adult CMs are mostly occupied by bouquets of thick and thin

filaments. Thick filaments are composed of myosin II molecules, while thin filaments are made up of troponin, tropomyosin and actin molecules. Troponin is itself a globular complex, which consists of three subunits, namely troponin T (TnT), troponin C (TnC) and troponin I (TnI). The TnT subunit binds tropomyosin, which coils around strands of actin molecules in the thin filament. Binding of Ca^{2+} to the TnC subunit induces a conformational change in the TnI subunit, thereby removing the steric hindrance on actin from tropomyosin. Binding of myosin II to actin can then occur, inducing a conformational change in the complex. This pulls the actin-associated thin filament pass the myosin II-associated thick filament, resulting in a contracting phenomenon in the CMs.

RyR2 protein was present as early as day 17 post-differentiation in H7 hESC-derived CMs (Zhu et al., 2009). In H9.2 hESC-derived CMs, the application of a puff of 10mM caffeine was able to induce Ca^{2+} release from the SR as early as day 2 post-beating; this signified the functionality of RyR-channels in these cells at this early time-point (Satin et al., 2008). In mouse embryos, RyR2 mRNA was detected as early as E8.5, the level of which continued to increase up until its last measurement at E16.5 (Rosemblit et al., 1999). Ca^{2+} release from the SR was inducible by the application of 10mM caffeine, indicating the functionality of RyR2 channels by E8.5 in mouse embryos. In a different study, RyR2 protein was also detected at E18 and the detection persisted until adult stage (Liu et al., 2002). Homozygous null $RyR2^{-/-}$ mice died at E10, displaying morphological abnormalities in the heart tube (Takeshima et al., 1998). RyR2 expression has also been studied *in vitro* in mESC-derived CMs. RyR2 mRNA and protein were detected as early as day 5 and day 9 post-differentiation in mESC-derived CMs respectively (Boheler et al., 2002; Fu et al., 2006a). Immunohistochemistry revealed a continuous increase in RyR immunofluorescence intensity in differentiation day 15-25 CMs compared with differentiation day 8-11 CMs, suggesting an increasing density of RyRs during cardiac differentiation (Sauer et al., 2001). The application of 10mM caffeine elicited Ca^{2+} transients, the amplitudes of which increased from day 8 to day 17 post-differentiation in mESC-derived CMs (Kapur and Banach, 2007). Hence, RyR2 channels were functional by day 8 post-differentiation in mESC-derived CMs. $RyR2^{-/-}$ knockout R1 mESC-derived CMs exhibited no difference in the amplitudes of Ca^{2+} transients but contractions at a reduced rate from early (days 9-11) to intermediate (days 13- 15) to late (days 18-21 post-differentiation) differentiation stages when compared with wild-type (Fu et al., 2006a; Yang et al., 2002). The applications of 10μM ryanodine and 10mM caffeine were able to inhibit and induce Ca^{2+} release from the SR respectively. Cardiac differentiation was not affected, as indicated by the number of contracting colonies present in the differentiating cultures. Both effects of ryanodine and caffeine were seen to increase with time post-differentiation, indicating that SR Ca^{2+} loads increased during differentiation (Fu et al., 2006a; Sauer et al., 2001). Altogether, these findings suggested a role for RyR to mediate SR Ca^{2+} release, thereby regulating the rate of the earliest contractions observed in the developing embryonic heart, and that this regulation increases with differentiation.

2.2.2 Inositol 1, 4, 5-trisphosphate Receptor (IP_3R) calcium release channels

IP_3R Ca^{2+} release channels represent a type of enigmatic intracellular Ca^{2+} channels in that both its structure and function(s) are not fully understood (Foskett et al., 2007; Taylor et al., 2004; Taylor and Tovey, 2010; Yule et al., 2010). IP_3R-Ca^{2+} release channels can be formed from either homo- or hetero-tetramers of IP_3R proteins. Each subunit of IP_3R proteins contains an IP_3-binding domain at its N-terminus and six transmembrane segments (S1-6) at its C-terminus; the Ca^{2+}-selective pore is formed at segments S5-6. Three mammalian

isoforms of IP_3R exist, namely IP_3R1, IP_3R2 and IP_3R3. These isoforms differ in their binding affinities for IP_3, with IP_3R2 being the most and IP_3R3 being the least sensitive to the ligand (Iwai et al., 2007; Tu et al., 2005). All three IP_3Rs are expressed in human adult CMs, but their subcellular localizations have not been studied (Nakazawa et al., 2011; Uchida et al., 2010). In rat adult CMs, these are expressed at about 50-fold lower than those of RyRs; IP_3R1s are localized around the nuclear envelope and SR, both of which are connected, while IP_3R2s are dispersed throughout the cytosol in a punctate pattern (Bare et al., 2005; Li et al., 2005; Moschella and Marks, 1993). Thus far, the role of IP_3Rs in the adult heart has not been defined, but an up-regulation in their expression has been associated with conditions of cardiac failure in that the mRNA levels of IP_3R1 and IP_3R2 were increased by 123% and 93% respectively in failing compared with normal heart tissues (Ai et al., 2005; Go et al., 1995).

Activation of IP_3R-Ca^{2+} release channels is regulated by both the concentration of cytosolic Ca^{2+} ions and the binding of IP_3 to one or more of the IP_3-binding domains (Iino, 1990; Taylor and Laude, 2002). Low concentrations of cytosolic Ca^{2+} ions are known to activate pore opening, while high concentrations inhibit it. IP_3 is a second messenger that is generated via a G protein-coupled receptor-mediated signal transduction pathway. G protein-coupled receptors are transmembrane receptors that are able to sense external stimuli and relay these signals into the cell via their interactions with cytosolic G proteins. G proteins are heterotrimeric complexes, which consist of a GDP-bound G_α subunit and a $G_{\beta\gamma}$ dimer. G protein-coupled receptors are classified into different types according to the isoform of the G_α subunits contained within their G protein-interacting partners. Generation of IP_3 involves the stimulation of a G_q type of G protein-coupled receptors. Upon stimulation with their agonists, a conformational change is induced in the G_q protein-coupled receptors. This causes an exchange of GDP for GTP in the G_α subunits, which then dissociate from their G_q protein complexes. GTP-bound G_α subunits of G_q protein complexes are responsible for activating phospholipase C. It is this enzyme, which hydrolyses phosphatidylinositol 4, 5-bisphosphate (PIP_2), a phospholipid component of the plasma membrane, to ultimately yield IP_3 and another second messenger, diacylglycerol (DAG). IP_3 then binds to and activates IP_3R-Ca^{2+} release channels.

IP_3R mRNA was present at detectable levels in D3 mESCs at the undifferentiated state (Yanagida et al., 2004). Application of 5μM ATP generated a Ca^{2+} transient in the undifferentiated D3 mESCs, which was inhibited by pre-treatment with 75μM 2-APB, an IP_3R blocker. This verified the functionality of IP_3R-channels in these cells. In H9.2 hESCs, immunostaining indicated that both IP_3R1 and IP_3R2 were expressed in hESC-dervied CMs (Sedan et al., 2010; Sedan et al., 2008); and the expression of IP_3R2 was shown to gradually decline with maturation as revealed by quantitative RT-PCR (Satin et al., 2008). The expression of IP_3R was also tested during development *in vivo*; its mRNA was first measured and detected as early as E5.5 in mouse embryos; it was present at high levels by E8.5 and continued to increase until E16.5 when its level started to drop (Rosemblit et al., 1999). These were demonstrated to be functional at two time-points; application of 5μM of IP_3 was able to induce Ca^{2+} release from the SR in CMs derived from mouse embryos at E5.5 and E8.5. Application of 5μM xestospongin C, an IP_3R antagonist, to mouse embryos at E10 diminished its Ca^{2+} spiking; washing the drug out allowed slow recovery of this spiking activity (Mery et al., 2005). Likewise, application of 5μM xestospongin C also abrogated the Ca^{2+} spiking activity observed in CGR8 mESC-derived CMs at day 8-10 post-differentiation. In addition, during the whole course of cardiac differentiation of R1 mESCs, 2-APB

decreased both the amplitude and upstroke velocity of Ca^{2+} transients, with the inhibitory effect decreased as differentiation proceeded, suggesting that IP_3R contributes to the Ca^{2+} transient and its effect decreases with differentiation (Fu et al., 2006b). In an attempt to discriminate between the roles of RyR- and IP_3R2- channels, 50μM ryanodine was first used to block RyR-channels before the application of 5-20μM IP_3-AM to CMs derived from mouse embryos at E10 (Rapila et al., 2008). This was able to induce a Ca^{2+} leak from the SR of the CMs in a concentration-dependent manner, whereby the slopes of the Ca^{2+} transients were elevated, despite its frequency stayed unchanged. In the absence of ryanodine, however, application of 10μM IP_3-AM led to an increase in the frequency of Ca^{2+} transients and, hence, increased contractions in the CMs at E10. These data suggested a role for IP_3R in regulating the rate of the earliest contractions observed in the developing embryonic heart, perhaps by providing a source of Ca^{2+} to bind RyRs so as to increase its open probability for greater Ca^{2+} release from the SR, as suggested by Rapila *et al*. (Rapila et al., 2008) In support of this finding, genetic knockdown of IP_3R in CGR8 mESC-derived pacemaker cells resulted in weak and infrequent contractions, although differentiation was not affected, whilst mice over-expressing the IP_3R2 gene developed mild cardiac hypertrophy by three months of age (Mery et al., 2005; Nakayama et al., 2010).

2.3 Regulation of intracellular calcium level by exchanger and pump

As previously mentioned, upon arrival of cardiac action potential, activation of L-type Ca^{2+} channel followed by CICR increases $[Ca^{2+}]_i$ for the contraction process. Subsequently, the excess Ca^{2+} has to be removed in order to initiate relaxation. In CMs, this Ca^{2+} removal process is mediated by different Ca^{2+} extrusion mechanisms via the action of sodium-calcium exchanger (NCX) and sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA).

2.3.1 Sodium-calcium exchanger (NCX)

NCX contains 9 transmembrane segments with a large loop at the cytoplasmic side (Philipson and Nicoll, 2000). Mutation experiments demonstrated the importance of α-helices in the transmembrane segments for the transport activity of NCX (Nicoll et al., 1996). The large intracellular loop is found to be not essential for the transport; however, it is important for catalyzing the ion translocation reaction and has important regulatory functions (Philipson and Nicoll, 2000). This loop consists of multiple regulatory sites, including the regulatory Ca^{2+} binding site and the exchanger inhibitory peptide (XIP) region. The activity of NCX is known to be regulated by at least 4 factors, including Ca^{2+} (DiPolo, 1979), Na^+ (Hilgemann et al., 1992), PIP_2 (Hilgemann, 1990), and phosphorylation (Iwamoto et al., 1996). Binding of Ca^{2+} to the Ca^{2+} binding site is required for activating the Na^+-Ca^{2+} exchange activity (Giladi et al., 2010; Ottolia et al., 2004; Wu et al., 2010). On the other hand, the XIP region is responsible for the Na^+-dependent inactivation and is involved in the elimination of the Na^+-dependent inactivation process by PIP_2 (Matsuoka et al., 1997). The activity of NCX is also regulated by phosphorylation, with stronger phosphorylation leading to higher NCX activity (Reppel et al., 2007a). Requirements of direct phosphorylation for up-regulation of NCX function by PKA and PKC are still in debate. A recent study by Wanichawan *et al*. demonstrated that the PKA phosphorylation site in full-length NCX1 is inaccessible, suggesting that NCX1 is not a direct substrate of PKA (Wanichawan et al., 2011). On the other hand, another study showed that the activity of NCX1 is dependent on PKC, although direct phosphorylation by PKC is not required (Iwamoto et al., 1998). NCX exists in 3 different isoforms, namely NCX1, NCX2 and NCX3.

NCX1 is referred as the cardiac NCX isoform as it is highly expressed in CMs but only in a lesser extent in other tissues such as brain and kidney (Lee et al., 1994).

NCX is classified as a secondary active transporter, which uses the energy stored in the electrochemical gradient in Na^+ to extrude Ca^{2+} out of the cells, while the electrochemical gradient of Na^+ is maintained by the Na^+/K^+-ATPase. Under normal condition, NCX operates in the forward mode in which it constitutively brings 3 Na^+ into the cells and extrudes 1 Ca^{2+} in each translocation cycle. The forward mode is stimulated in response to a rise in $[Ca^{2+}]_i$, and it serves to bring $[Ca^{2+}]_i$ back to normal level. In CMs, the primary role of NCX is to extrude Ca^{2+} after excitation under normal physiological conditions (Philipson and Nicoll, 2000). Some studies also showed that NCX functions in shaping the cardiac action potential. Application of NCX blocker KB-R7943 leads to shortening of plateau phase of cardiac potential (Spencer and Sham, 2003); similarly, CMs from NCX knockout mice also has a shorter AP when compare to wild-type mice (Pott et al., 2005), while induced over-expression of NCX leads to a longer plateau (Wang et al., 2009). On the other hand, NCX also operates in the reverse mode in response to membrane depolarization in CMs. During the depolarization phase of action potential when the $[Ca^{2+}]_i$ has not reached the peak of Ca^{2+} transients, the reverse mode is predominant (Sah et al., 2003). Ca^{2+} influx via the reverse mode of NCX has been suggested to act synergistically with Ca^{2+} influx via L-type Ca^{2+} channels to trigger Ca^{2+} release from the SR as $I_{Ca,\,L}$ are small at depolarized membrane potential (Sah et al., 2003). In addition, NCX can positively regulate SR Ca^{2+} load via the reverse mode action (Hirota et al., 2007). Interestingly, under pathophysiological conditions such as cardiac failure, NCX also operates in the reverse mode to allow additional Ca^{2+} influx for contraction in order to compensate for the reduction in Ca^{2+} release from SR (Gaughan et al., 1999).

NCX is found to be essential for embryo development. Several studies showed that NCX1 is expressed restrictedly in the embryonic heart during early development. NCX knockout are embryonic lethal at ~9-11 days post coitum with immature heart development (Cho et al., 2000; Koushik et al., 2001; Reuter et al., 2003; Wakimoto et al., 2000). Molecular studies demonstrated that NCX mRNA expresses before the appearance of spontaneously beating mESC-CMs; the expression persists thereafter in the CMs (Fu et al., 2006b). It is suggested that expression of NCX at that early stage is essential for early EC-coupling as SR is not well-developed at that stage, NCX is hence essential for maintaining the proper Ca^{2+} homeostasis even in the very early stage cardiac development (Reppel et al., 2007a; Reppel et al., 2007b).

Two approaches were used to demonstrate the functional expression of NCX in ESC-CM. In Otsu *et al.* (Otsu et al., 2005), function of NCX was indirectly assessed by using high concentration of NCX blocker KB-R7943. Application of KB-R7943 induced sustained elevation of $[Ca^{2+}]_i$, and this elevation increased as differentiation of mESC-CMs proceeded (Otsu et al., 2005). Apart from using pharmacological blocker, direct measurement of NCX activity was performed by using patch-clamping. I_{NCX} was found to be increased as hESC-CMs developed from day 7+40 to day 7+90 (Fu et al., 2010). However, CMs derived from murine embryonic heart at late stage (E16.5) showed a significantly lower I_{NCX} density when compared to CMs at early stage (E10.5) (Reppel et al., 2007a), probably due to the high phosphorylation status of NCX in early stage. Consistently, upon differentiation, the proportion of Ca^{2+} extrusion by NCX declined from day 9 to day 17 in mESC-CMs (Kapur and Banach, 2007). The discrepancy between different studies on the absolute functional expression of NCX as development proceeds is unknown. Nonetheless, it is clear that NCX is important for Ca^{2+} extrusion in differentiating CMs and the decreased contribution by

NCX to Ca^{2+} extrusion as development proceeds may be explained by the gradual development of SERCA on the SR.

In the study by Fu *et al*., the basal $[Ca^{2+}]_i$ of mESC-CMs in both early and late developmental stages was increased after applying Na^+-free solution, suggesting that NCX is functional in maintaining Ca^{2+} homeostasis (Fu et al., 2006b). Interestingly, Na^+-free solution completely blocked the Ca^{2+} transients in CMs from late developmental stage but not the CMs from early developmental stage, suggesting that NCX starts to regulate Ca^{2+} transients only in the late developmental stage (Fu et al., 2006b). Similar results were obtained from hESC-CMs (Fu et al., 2010). Basal $[Ca^{2+}]_i$ was marginally increased in CMs at day 7+90 after applying Na^+-free solution, but the same was not observed in CMs at day 7+40. In addition, irregular Ca^{2+} transient pattern was observed in day 7+90 CMs treated with Na^+-free solution (Fu et al., 2010). These suggested that the contribution of NCX to Ca^{2+} transients becomes more important as development proceeds in ESC-CMs.

2.3.2 Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)

Details of the structure and function relationship of SERCA have been extensively reviewed (Periasamy et al., 2008; Periasamy and Huke, 2001; Toyoshima, 2008; Toyoshima and Inesi, 2004; Wuytack et al., 2002). SERCA is a single polypeptide with ~1000 amino acid residues located on the ER/SR membrane. It consists of 10 transmembrane (M) domains and 3 cytosolic domains, including actuator (A) domain, phosphorylation (P) domain, and nucleotide-binding (N) domain. A domain regulates the Ca^{2+} binding and release. N domain is connected to the P domain; it contains the adenosine binding site and forms the catalytic site. On the other hand, the γ-phosphate reacts with an amino acid residue in the P domain. SERCA utilizes the energy derived from ATP hydrolysis to pump Ca^{2+} against concentration gradient from the cytosol to the lumen of ER/SR. Two Ca^{2+} are transported by hydrolysis of one ATP in each catalytic cycle.

Regulation of SERCA is mainly achieved by the action of SR membrane proteins phospholamban and sarcolipin (Asahi et al., 2003a; Edes and Kranias, 1987; MacLennan et al., 2002; MacLennan and Kranias, 2003; Simmerman and Jones, 1998; Traaseth et al., 2008). De-phosphorylated form of phospholamban interacts with SERCA and inhibits the pumping activities by decreasing the Ca^{2+} affinity of SERCA. Phospholamban exists in monomeric or pentameric form, while the monomeric form is inhibitory. Phosphorylation of phospholamban favors the formation of pentameric form, which in turns relieves the inhibitory effect on SERCA. Phosphorylation of phospholamban is regulated by cAMP-dependent protein kinase (Schwinger et al., 1998) and Ca^{2+}/calmodulin-dependent kinase (Ji et al., 2003). Sarcolipin is a shorter homolog of phospholamban (Hellstern et al., 2001). Unlike phospholamban, sarcolipin has no obvious phosphorylation site (Odermatt et al., 1997); therefore, the effect of sarcolipin on SERCA inhibition is mainly controlled by altering the expression level of sarcolipin (Odermatt et al., 1998). Sarcolipin interacts directly with SERCA and inhibits its function by decreasing the Ca^{2+} affinity of SERCA. Sarcolipin can also exert its superinhibitory effect on SERCA by forming the tertiary complex phospholamban-sarcolipin-SERCA (Asahi et al., 2002; Asahi et al., 2003b). In addition, sarcolipin stabilizes the SERCA-phospholamban complex in the absence of phospholamban phosphorylation and also inhibits phospholamban phosphorylation (Asahi et al., 2004).

SERCA plays a vital role in Ca^{2+} cycling between SR and cytosol, and this is important for EC-coupling. Over-expression of SERCA2a improved cardiac contractility by increasing SR Ca^{2+} loading and frequency of Ca^{2+} transients (Baker et al., 1998; He et al., 1997; Maier et al.,

2005; Prasad et al., 2004). On the other hand, homozygous SERCA2a knockout mice are embryonic lethal (Periasamy et al., 1999), while heterozygous knockout mice are alive and able to reproduce (Shull et al., 2003). Ji *et al*. has reported that the content of SR Ca^{2+} stores and the amplitude of Ca^{2+} transients were decreased by 40-60% and ~30-40%, respectively, in heterozygous CMs (Ji et al., 2000). Interestingly, heterozygous CMs showed a reduced phospholamban expression, an enhanced phospholamban phosphorylation, and an upregulated NCX expression. However, these changes in Ca^{2+} handling proteins were not sufficient to compensate the effects on contractility by the loss of SERCA2a, indicating that SERCA2a is a critical regulator in controlling the E-C coupling of CMs (Ji et al., 2000). Apart from genetic manipulation of the expression level of SERCA, function of SERCA can also be assessed by using the pharmacological blocker thapsigargin. Acute application of thapsigargin caused the decrease in Ca^{2+} transient amplitude, rate of decay of Ca^{2+} transients, and duration of action potential in isolated ventricular myocytes (Kirby et al., 1992), again suggesting the involvement of SERCA in the E-C coupling of CMs.

In vertebrates, SERCA is encoded by three genes, including the SERCA1, SERCA2, and SERCA3. Alternative splicing of the transcripts from these genes produces more than 10 SERCA isoforms. In CMs, SERCA2a and SERCA2b are expressed, with SERCA2a being the predominant form (Periasamy and Kalyanasundaram, 2007).

SERCA2a mRNA is present before initial contraction of mESC-CMs, but has no obvious change in expression level during further differentiation (Fu et al., 2006b). From embryo studies, SERCA2 protein increased from E9.5 to E18 in mouse heart (Liu et al., 2002). In human, it was reported that SERCA2a protein level remained steady between 8 to 15th week gestation, and started to increase afterwards (Qu and Boutjdir, 2001). Role of SERCA in regulating Ca^{2+} transients in ESC-CMs has been studied by several groups. Zhu *et al*. demonstrated that SERCA inhibitors, including thapsigargin and cyclopiazonic acid, reduced ~70% amplitude of Ca^{2+} transients in hESC-CMs, but had no effect on the time of decay (Zhu et al., 2009). In case of mESC-CMs, thapsigargin reduced both the amplitude and decay of Ca^{2+} transients, but exerted similar inhibitory effect on CMs from the 3 developmental stages (Fu et al., 2006a; Fu et al., 2006b). These findings are therefore consistent with the mRNA expression level of SERCA during mESC-CM differentiation. However, the contribution of Ca^{2+} removal by SERCA is estimated to be more important as differentiation proceeds based on Ca^{2+} imaging experiments (Kapur and Banach, 2007). Therefore, the role of other SR Ca^{2+} handling proteins cannot be neglected. For example, expression level of calsequestrin increases as SR matures (Fu et al., 2006a). This increases the capacity of SR Ca^{2+} load and may account for the requirement of greater contribution of Ca^{2+} removal by SERCA in later developmental stage of mESC-CMs.

To summarize for the whole chapter, Figure 1 represents a summary of the relative contributions of different proteins responsible for regulating Ca^{2+} transients in early differentiating ESC-CMs as development proceeds.

3. Conclusion

In summary, early differentiating ESC-CMs have already developed a scheme for regulating their $[Ca^{2+}]_i$ for E-C coupling. The relative contributions of the proteins that regulate Ca^{2+} transients alter upon the maturation of CMs. By comparing the regulation of $[Ca^{2+}]_i$ in ESC-CMs and that in adult CMs, we can obtain important insights into the potential strategies for 'fine-tuning' ESC-CMs to better-suit different therapeutic and research purposes.

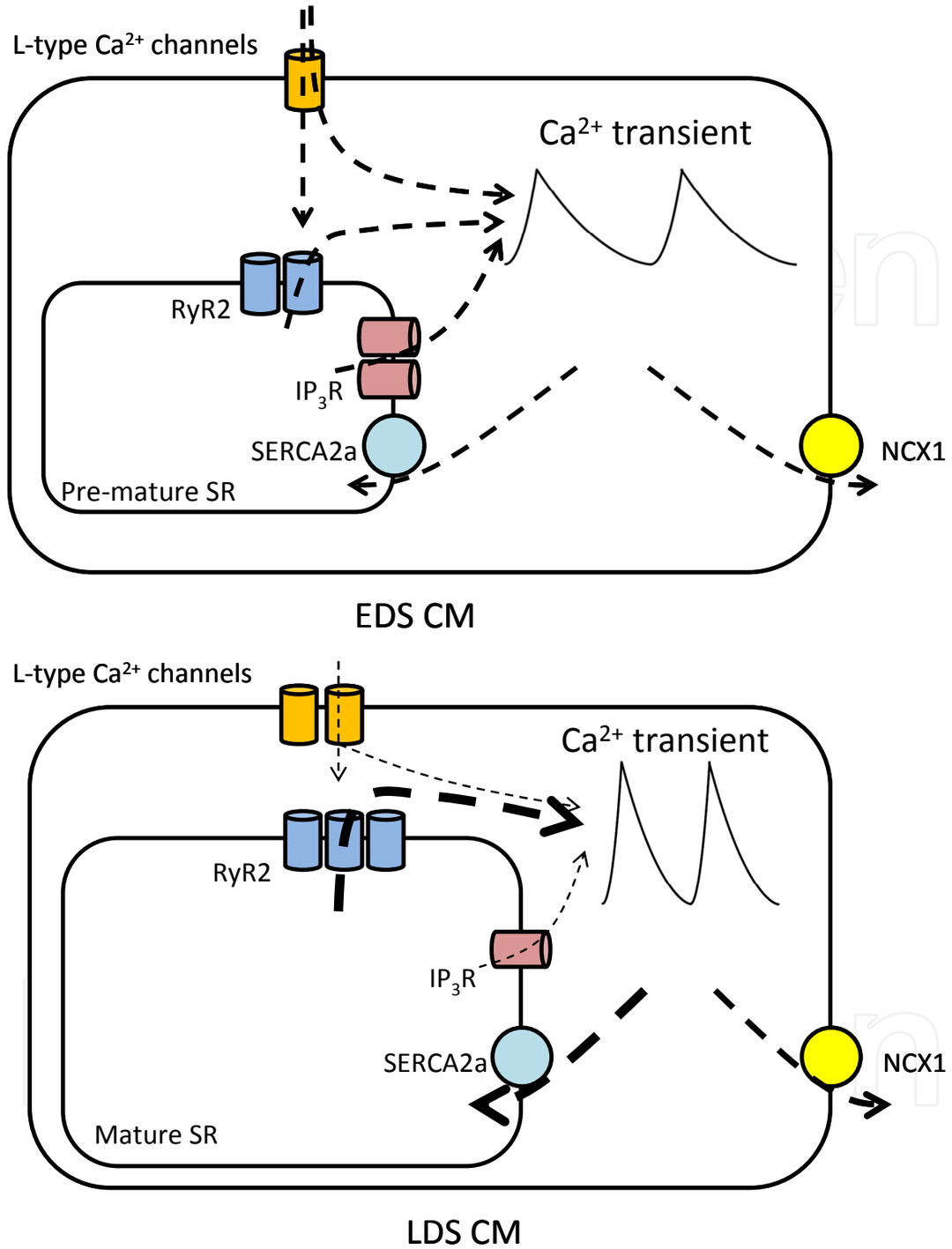

Fig. 1. Summary of the regulation of Ca^{2+} transients in CMs at early differentiation stage (EDS) and late differentiation stage (LDS). Number of a particular protein represents the relative changes in the expression level of that particular protein as differentiation proceeds. Thickness of the arrows represents the relative contribution of a particular path to the Ca^{2+} transients as differentiation proceeds.

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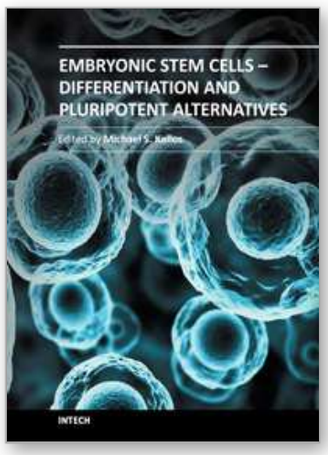

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The ultimate clinical implementation of embryonic stem cells will require methods and protocols to turn these unspecialized cells into the fully functioning cell types found in a wide variety of tissues and organs. In order to achieve this, it is necessary to clearly understand the signals and cues that direct embryonic stem cell differentiation. This book provides a snapshot of current research on the differentiation of embryonic stem cells to a wide variety of cell types, including neural, cardiac, endothelial, osteogenic, and hepatic cells. In addition, induced pluripotent stem cells and other pluripotent stem cell sources are described. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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