CHAPTER 4
DISCUSSION

Cardiovascular diseases affecting millions of lives became number one cause of worldwide mortality among non-communicable diseases (Roger et al. 2012). Main pathophysiology of CVDs includes atherosclerosis, coronary artery disease and myocardial infarction, where death of cardiomyocytes results in scar formation, ventricular remodeling, heart failure and eventual death (Torella et al. 2007). Current pharmacological treatments cannot reverse the damage occurred to the heart and are unable to compensate cardiac dysfunction (Steinhauser et al. 2011). Heart transplantation is the only method existing to address the issue of lost cardiomyocytes but lack of donors is the main hurdle. Henceforth cell based therapies represent an alternative approach that can help promote regeneration of functional myocytes and provide long term solutions (Laflamme & Murry 2011).

For an effective regeneration of the myocardium, transplanted stem cells should at least exhibit properties like ability to differentiate into cardiomyocytes, exert paracrine mechanisms, should stimulate endogenous stem cells, cell-cell interaction and engraftment into the surrounding tissue (Strauer & Steinhoff 2011). Of these, a direct differentiation into cardiomyocytes upon transplantation remains a main problem to generate cardiomyocytes and functional myocardium.

Long lasting belief that heart is a post-mitotic organ, unable to regenerate damaged cells is ruled out by the discovery of endogenous cardiac
stem cells (Beltrami et al. 2003). CDCs are a type of CSCs expanded from the endomyocardial biopsy specimens by explant culture. CDCs are positive for CSC stemness marker- c-kit, mesenchymal or stromal markers (CD 105, CD 90), endothelial markers (CD 34, CD 31) and also express proteins related to cardiac electrophysiology (Smith et al. 2007). CDCs display clonogenic and multilineage potential to give rise to cardiac lineage. Preclinical studies displayed safety and efficacy of CDCs transplantation in a porcine ischemic cardiomyopathy model (Johnston et al. 2009). Furthermore, CDCs exhibited superior activity over bone marrow MSCs, adipose tissue-derived MSCs, and bone marrow mononuclear cells in terms of greatest myogenic differentiation capacity, superior paracrine activity and functional benefits in MI experimentation (Li et al. 2012).

4.1 CULTURE AND EXPANSION OF CEOCS AND CDCS

In order to rejuvenate a functional heart the restoration of cardiomyocytes alone is not adequate, it is necessary to provide protection from the local microenvironment and also able to give rise to other cell types resident in the heart (Menasche 2011). Hence, due to their properties of multilineage capacity and superior paracrine activity, CDCs represent a plausible autologous cell source for efficient cardiac regeneration. In the present study, CDCs were successfully isolated by explant culture method with an intermediate cardiosphere step. From the seeded explants, initial stromal like fibroblast cells were seen in 2-4 days of culture and these cells become confluent by 7-9 days. Above these stromal like cells, phase bright round cells started growing around 7 days on an average and became confluent within 5 days of their initial outgrowth. The size of the explants influenced the outgrowth of cells from the explants. Larger explants of > 4-5 mm size were unable to attach to the dish and shed cells. Hence, it was quiet important to maintain the explant size for a successful outgrowth in short
period of time (Tan et al. 2011). Explant culture has added advantage of generating large number of CEOCs starting from a small amount of heart tissue in comparison to FACS or MACS, which usually yield few cell number and require further expansion to get enough number of cells for the therapeutic purpose. Intermediate cardiosphere step was crucial to minimize fibroblast contamination in the culture and any other mature cells if present, adhere to the poly D Lysine coated plate and the stem cells would form spheres in the suspension within 3-4 days (Smith et al. 2007). CS culture provide a “niche” like environment which usually exist in the myocardium that help CSs to maintain progenitor cells in the core of the sphere and committed cells in the periphery of the sphere. CDCs were expanded from CSs by monolayer culture which acquired elongated spindle shaped structure in the culture.

4.2 CHARACTERIZATION OF CEOCS AND CDCS

Wide range of markers were utilized to identify CSCs based on the type of isolation and region of isolation from the heart (Koudstaal et al. 2013). In this study, CEOCs and CDCs were characterized using reverse transcriptase PCR and immunofluorescence analysis for the presence of cardiac progenitor markers, early cardiac transcription markers and mesenchymal markers. Reverse transcriptase PCR results demonstrated that both CEOCs and CDCs were consistent in the expression of Isl-1 marker. Isl-1 is a homeodomain transcription factor present in the secondary heart field from which outflow tract of the heart develops. Previously it was thought that Isl-1 would express in embryonic stage, but later it was identified in the adult heart also and found to co-express along with c-kit (Serradifalco et al. 2011, Ye et al. 2012). CEOCs also expressed Vimentin, which confirm the presence of cardiac fibroblasts in the initial outgrowth of explants. Further, absence of Vimentin expression in CDCs prove that intermediate CS step is
essential to eliminate fibroblasts contamination in CDCs (Smith et al. 2007). Presence of CD 90, Nkx 2.5 and GATA 4 expression in CDCs confirm that CDCs were more committed to cardiomyogenic lineage. c-kit expression was prominent in CEOCs and reduced in CDCs as seen from the results obtained from both PCR and immunofluorescence results. Our results were in agreement with the previous reports which display similar kind of expression of these markers in CEOCs and CDCs (Messina et al. 2004, Smith et al. 2007, Tateishi et al. 2007). Altogether, the results obtained from both the analyses suggest that CDCs were of heterogeneous in nature, displaying c-kit, CD 90, CD 105, Nkx 2.5 and GATA 4 markers while CEOCs were positive for c-kit, CD 90, GATA 4 and Vimentin.

Immunofluorescence analysis of CEOCs surrounding the explant using Z-stack method displayed c-kit expression only in the freshly shed CEOCs validating that no cardiomyocyte contamination was present. A study by Davis et al. (2009) has revealed that freshly shed outgrowth cells from heart explants were of non-cardiomyocyte origin by MerCreMer-Z/EG bi-transgenic mouse model containing GFP labelled cardiomyocytes. This supported our result of c-kit expression in CEOCs surrounding the explant.

Immunofluorescence analysis further demonstrated that CDCs strongly expressed CD 105 while c-kit expression was limited. CDCs also expressed low levels of early transcription factors - Nkx 2.5 and GATA 4 proving that CDCs were committed to cardiomyogenic lineage. Studies revealed that CDCs express primarily CD 105 marker (75-95%) and very low percentage of cells positive for c-kit (1-5%) (Li et al. 2010, Mishra et al. 2011). Moreover, Smith et al. (2007) proposed that presence of mesenchymal sub population in CDCs might help c-kit subpopulation during expansion by providing physical support or acting in a paracrine manner. Altogether, these results display that CEOCs and CDCs contain subpopulations of
mesenchymal like cells and cardiac progenitor cells which could effectively contribute to cardiac regeneration.

4.3 CDCS DIFFERENTIATION

CDCs displayed encouraging results in preclinical and clinical trials (Chimenti et al. 2010, Chugh et al. 2012, Makkar et al. 2012). When injected into the myocardial infarct border of porcine MI model, CDCs occupied the infarcted zone and acted in paracrine fashion by releasing growth factors into the surrounding environment (Chimenti et al. 2010). Randomized, first-in-human phase I clinical trial, Stem Cell Infusion in Patients with Ischemic cardiomyopathy (SCIPIO) using c-kit+/Lin- CDCs in MI patients displayed improvement in left ventricular ejection fraction (LVEF) of about 12.3% with no major adverse cardiac events or mortality rate (Bolli et al. 2011, Chugh et al. 2012).

CADUCEUS, phase I randomized clinical trial displayed reduction in scar size, increased viable myocardium with improved regional function, but no notable LVEF change was observed (Malliaras et al. 2014). Hence, understanding cellular and molecular mechanisms involved in stem cell maintenance, differentiation and ability in regeneration has to be properly scrutinized in in vitro conditions that may provide an opportunity for developing a better therapeutic outcome in vivo.

When injected, differentiation of uncommitted/unstimulated stem cells fate couldn’t be predicted under in vivo conditions and might provoke local inflammatory cells or fibroblasts that result in ‘a scar within a scar’ (Balsam et al. 2004, Burlacu et al. 2008). Hence, it was crucial to direct the cells towards determined fate through an alternative strategy called as pre-implantation differentiation.
The strategy of co-culturing stem cells with neonatal or adult cardiomyocytes for differentiation was limited in application as it was difficult to separate the co-cultured cells with the side effects of allogenic cells when transplanted (Badorff 2003, Heng et al. 2004).

To overcome this hurdle, pretreatment of CDCs to differentiate to cardiomyocytes using cytokines, growth factors and certain chemicals under in vitro conditions may help for efficient cardiac regeneration (Heng et al. 2004). In recent times, small molecule mediated stem cell differentiation have added advantages of being economical, safe and effective. Moreover, biological activities can be easily controlled with little side effects and also helps to understand the signaling pathways involved in generating differentiated cells (Ding & Schultz 2004, Thal et al. 2012, Naeem et al. 2013).

Aza has enhanced the spontaneous cardiomyocyte differentiation from embryoid bodies when ESCs were stimulated by the combination of hanging drop culture and Aza (Yoon et al. 2006). This effect might be due to the influence of Aza on the methylation status of cardiomyogenic related genes in the differentiation of ESCs to cardiomyocytes. Hou et al. (2013) has demonstrated that bone morphogenetic protein – 2 (BMP-2) in combination with Aza could significantly augment differentiation of bone marrow MSCs to cardiomyocytes and very less damage to the cells was observed. BMP-2 has neutralized the cytotoxicity effect of Aza and suggested that this combination of induction method was safe and efficient in generation of cardiomyocytes.

Pennarossa et al. (2013) has demonstrated that exposure of adult human skin fibroblasts cells to Aza for 18 hours was sufficient to induce differentiation to pancreatic cells without provoking any cytotoxicity. This could be explained by the fact that Aza prevent DNMT activity which was
bound to DNA there by prompting methylation. It was also shown that about 5% of Aza could induce 85-90% of the genome demethylation within 2 hours of cells exposure to it. These studies proved that Aza didn’t cause cytotoxicity and moreover, combination of Aza with other inducing agents may enhance the differentiation action.

Interleukin-1β is a proinflammatory cytokine which usually get activated during myocardial damage. Recently, it was shown that IL-1β along with Aza displayed synergistic effect in inducing MSCs differentiation to cardiomyocytes (Khajeniazi et al. 2016). Gao et al. (2014) has demonstrated that the combination of Aza+SalB+CLM was successful in generation of cardiomyocytes and expression of structural proteins like cardiac troponin, alpha-cardiac actin and connexin 43 was observed. Moreover, it was also found that Wnt/β-catenin was downregulated in the differentiation process which was evident by the decreased expression of β-catenin.

AA, a common cofactor in metabolic reactions, plays a crucial role in mediating proliferation and differentiation of stem cells by influencing ECM secretion, collagen synthesis via MEK-ERK1/2 pathway and upregulating the late-stage markers of cardiogenesis (Takahashi t al. 2003, Choi et al. 2008). Further, Potdar, PD & D'Souza SB (2010) has demonstrated that AA promoted the growth and expansion of MSCs without affecting their usual expression of MSC related markers and also the differentiation ability. AA has induced matured cardiac phenotype in ESCs and iPSCs along with the generation of action potentials between the cells (Cao et al. 2012).

In view of i) CDCs intrinsic commitment to cardiac lineage ii) ability of Aza to induce mesodermal lineage, cardiomyogenesis specifically and iii) ability of AA in promoting cardiomyogenic lineage, the present study was intended to examine the effects of Aza alone and in combination with AA
in driving CDCs to cardiomyogenic lineage and to investigate the role of Wnt signaling in differentiation.

CDCs were treated with Aza alone, AA alone and Aza+AA combination to investigate their differentiation induction potential. Preliminary examination showed that AA exerted its effect mainly on proliferation, but no significant induction of differentiation rate was observed (data not shown). Hence, Aza alone and Aza+AA combinations were chosen for further differentiation and functional analysis.

Aza could inhibit DNMT-1 in a short span of time to selectively activate gene expression (Haff 1995). Hence, we believe that exposure of CDCs to Aza for 24 hours was adequate to reactivate cardiac specific genes. Moreover, LDH cytotoxicity assay substantiated that Aza did not exert any significant cell death for the above mentioned period during the cultured period. It was proposed that Aza treatment make cells less responsive to other micro environmental inductive factors that might modulate differentiation process (Rosca & Burlacu 2011). Nonetheless, Aza is known to induce some cytotoxicity apart from its inducing effect, while AA exerts its effect on proliferation with variance in differentiation ability (Choi et al. 2008).

4.4 MORPHOLOGICAL AND GROWTH ANALYSIS OF DIFFERENTIATED CDCS

The present study demonstrated that Aza+AA synergistically induced structural and functional changes in CDCs leading their differentiation towards cardiomyogenic lineage in in vitro conditions. Morphological analysis clearly demonstrated that CDCs had shifted from basic fibroblast like morphology to long, elongated structures with interconnections. In Aza treated group, CDCs became elongated with connections with adjoining cells. It was observed that Aza+AA clearly prompted CDCs to form distinct cell clusters along with intercellular connections with branches after 14 days of treatment.
Aza treated adult bone marrow MSCs attained an elongated stick like appearance with formation of myotube like structures along with connections with the surrounding cells (Antonitsis et al. 2007). Zhang et al. (2009) has revealed that Aza at 10 µM concentration has successfully induced differentiation of human first trimester MSCs into cardiomyocytes and the cells have developed myotube like structures with string-bead like nuclei. In line with these reports, our results demonstrated that morphologically CDCs have been shifted from progenitor stage to a myogenic lineage. In line with the study by Yoon et al (2005), we believe that seeding high cell number was also a critical factor in obtaining differentiated cells as shown in the present report.

Growth curve analysis further substantiated that Aza exhibited effect on CDCs proliferation when compared to control cells. Presence of AA in Aza+AA treatment group has increased the proliferation of the treated cells. This displayed that AA showed its effect chiefly on proliferation of the cells (Choi et al. 2008). CFU-F assay demonstrated that colony formation ability was reduced in treated CDCs. Aza+AA treatment resulted in less number of colonies, but the colonies size was big with notable interconnections between the cells. Taken together, morphological analysis, growth curve analysis and CFU-F assay displayed that distinct morphological changes were observed with notable intercellular connections in both the treatment groups. Aza+AA had found effective in bringing up the notable changes when compared to Aza group.

4.5 CHARACTERIZATION OF DIFFERENTIATED CDCS BY IMMUNOFLUORESCENCE, REAL TIME PCR AND WESTERN BLOT ANALYSIS

In this study, Aza, AA and Aza+AA were utilized to analyze the best protocol to induce differentiation of CDCs to cardiomyocytes. CDCs were cultured for 14 days with these three induction protocols and regular
change of culture medium with morphological analysis were performed. Results from immunofluorescence, real time PCR and western blot substantiated that Aza+AA treatment group had higher myogenic induction potential and could effectively mediate CDCs towards cardiomyogenic lineage than Aza alone.

Aza+AA treatment has significantly suppressed the expression of CDC specific progenitor marker, CD 105 at the end of the treatment period when compared to Aza treatment alone. Immunofluorescence analysis indicated that CD 105 expression decreased by 20-fold in Aza+AA group ($p = 0.0003$) than Aza group with over all significance of $p \leq 0.05$ when compared to control. At the mRNA level also Aza+AA group exposed a 9 fold decrease in expression ($p=0.018$) when compared to control. This outcome suggest that with overall decrease of CD 105, CDCs were successfully directed from a progenitor to cardiomyogenic phase.

GATA 4 is an early cardiac transcription factor belongs to the Zinc-finger transcription GATA family members. GATA 4 is a key regulator in cardiomyogenesis and it differentially control various stages of cardiogenesis by interacting with other GATA factors or with other co-factors in the myocardium (Charron & Nemer 1999). The homeobox transcription factor, Nkx 2.5 is an evolutionarily conserved factor which play an essential role in transcriptional regulation both during early cardiogenesis and also in mature hearts (Akazawa & Komuro 2005, Planat-Bernard et al. 2004).

Durocher et al. (1997) has demonstrated that Nkx 2.5 and GATA 4 usually co-express in early cardiogenesis events. Through structure/function analysis, it was shown that GATA 4 binded to C-terminus auto repressive domain of Nkx 2.5 and activated Nkx 2.5 by causing conformational change, proving that cooperative interaction between these two factors was important for early cardiomyogenesis (Durocher et al. 1997).
In relation with above, we too observed Nkx 2.5 and GATA 4 expression in differentiated cells. Immunofluorescence analysis displayed augmentation of 15-fold both in Nkx 2.5 and GATA 4 expression levels in Aza+AA treatment group compared to Aza alone. Whereas, RNA levels demonstrated 40-fold increase in GATA 4 expression compared to Nkx 2.5 which showed a 20-fold increase in Aza+AA treated CDCs (p ≤ 0.001 between Aza+AA and Aza groups on 14th day of the treatment). Protein expression analysis also confirmed a 10-fold enhanced GATA 4 expression in the presence of AA. Takahashi et al. has revealed that AA significantly induced GATA 4 expression in cardiac differentiation of ESCs. In accordance with this, we hypothesize that AA might have showed synergistic effect in relative upregulation of GATA 4 in Aza+AA treated CDCs.

α-actinin is a major part of sarcomere in the cardiomyogenic cells during second heart tube formation of embryonic heart. α-sarcomeric actinin is a cardiac structural protein that plays a central role during physiological and pathophysiological conditions of the heart. Re-expression of α-sarcomeric actinin during cardiac disease emphasizes its crucial role in both development and disease (van Eldik & Passier 2013).

Immunofluorescence and protein expression analysis in the study further revealed that there was a significant increase in the expression of cardiac structural protein, α-Sarcomeric actinin in Aza+AA group than Aza (p≤0.0001). GATA transcription factors are involved in the expression of many genes, which encode for contractile proteins like cardiac troponin-T and cardiac alpha actin (Shirinsky et al., 2008). Arminan et al. (2008) has revealed that there existed a direct correlation between the expression of α-Sarcomeric actinin expression and nuclear translocation of Nkx 2.5 and GATA 4.
4.6 INVolVEMENT OF WNT/B-CATENIN PATHWAY IN CDCS DIFFERENTIATION

Several studies have reported that the Wnt/β-catenin pathway would be suppressed when the stem cells are directed to cardiomyocyte like cells. Hlaing et al. (2014) has revealed that in 1,25-vitamin-D3 driven differentiation of H9C2 cells to cardiomyocytes, canonical Wnt pathway was down regulated which was evident by increased expression of Casein kinase-1-α1. Moreover, Non-canonical Wnt pathway was activated by increased expression of Wnt 11. In GSK-3β overexpressing bone marrow MSCs, it was evident that GSK-3β enhanced cardiomyocyte differentiation with distinct upregulation of Cardiac troponin T. Also, when injected into MI mice model, these MSCs were shown to upregulate c-kit positive cardiac progenitor cells, enhanced capillary density and improved paracrine action in the myocardium (Cho et al. 2011).

In this study, we also explored the role of Aza and AA in modulating Wnt/β-catenin pathway. Wnt/β-catenin signaling coordinates stem cell maintenance, proliferation and cell-fate decisions at both fetal and adult life. β-catenin is the direct downstream regulator of the Wnt/β-catenin pathway and its proteolytic regulation is the crucial step in modulating the signaling cascade. RNA expression results revealed that the expression of β-catenin and cyclin D1 levels was greatly repressed by 14 days of treatment by synergistic effect of Aza+AA (p ≤ 0.01).

In the absence of Wnt ligands, destruction complex binds to N-terminal region of β-catenin and phosphorylates it leading to degradation by the ubiquitin–proteasome system. Therefore, active and unbound β-catenin will be maintained at very low levels in the cytoplasm (Aberle et al. 1997, Ikeda et al. 1998).
In the present study, we have analyzed two forms of β-catenin, phospho and non-phospho (active) forms by protein expression analysis and found that phospho β-catenin expression was high in the differentiated cells, especially in Aza+AA treated one. Phospho β-catenin expression would be perceived only if it gets phosphorylated at serine 33, 37 and threonine 41 sites. GSK-3β mediates the phosphorylation of these sites on β-catenin which leads to poor accumulation of non-phospho β-catenin in the nucleus and consequent inhibition of the Wnt pathway (Amit et al. 2002). Study by Minami et al (2012) and Bhuvanalakshmi et al (2017) using Wnt inhibitors has demonstrated that Wnt inhibition was crucial in directing stem cells towards cardiomyogenic lineage. In line with this, outcomes from our study further conclude that Aza+AA might synergistically mediate CDCs differentiation by phosphorylation of β-catenin at the sites, serine 33, 37 and threonine 41.

4.7 RELATIONSHIP BETWEEN β-CATENIN AND CARDIOMYOCYGENIC MARKERS

Anton et al. (2007) demonstrated that Wnt signaling pathway sustained stemness and positively regulated proliferation of stem cells and negatively regulated differentiation in a stage and dose dependent manner. Additionally, there are reports validating that augmentation of cardiac specific markers, Tbx5 and GATA 4 during cardiac differentiation were mediated by down regulation of β-catenin (Zelarayan et al. 2008). This data support the results obtained in our study that there was a negative correlation between GATA 4 and β-catenin during cardiomyogenic differentiation.
Over expression of β-catenin repressed expression of both Nkx 2.5 and GATA 4 in a study that involve anti Nkx2.5 miRNA and Wnt ligands (Liu et al. 2009). β-catenin and GATA 4 possess NKX2-5 binding elements (NKEs) in their promoter regions to which Nkx 2.5 binds and modulate their expression during cardiogenesis. Luciferase reporter assay along with mutational analysis of the NKEs demonstrated that Nkx2.5 negatively regulate β-catenin and upregulate GATA 4 (Riazi et al. 2009). In accordance with this, our results also propose that Aza and AA induce CDC differentiation by negatively influencing Wnt pathway.

Rosca & Burlacu (2011) revealed that in adult stem cells, the cardiomyogenic genes would be acting at a lower level and when the promoting agent like Aza was added to the cells, these genes might trigger in their expression and would be influenced by conditions prevailing during differentiation. Based on this, we could state that upregulation of cardiac specific genes after combined treatment of Aza+AA might be because of promoting effect instead of inductive effect on myogenic gene expression.

Aza was primarily used in MSCs as demethylating agent to induce cardiomyogenic lineage and variable results were obtained (Makino et al. 1999, Antonitsis et al. 2007, Burlacu et al. 2008, Naeem et al. 2013). Irrespective of wide known facts of Aza in cardiomyogenesis, little is known about AA being directly involved in stem cell differentiation (Cao et al. 2012, Choi et al. 2008). We are the first to show the synergistic effect of Aza+AA in directing CDCs to cardiomyogenic lineage with down regulation of Wnt/β-catenin pathway (Mundre et al. 2017).
4.8 MITOCHONDRIAL MEMBRANE POTENTIAL AND CALCIUM IMAGING OF DIFFERENTIATED CDCS

Evaluation of functional properties of cardiomyocytes obtained from CDCs is crucial to check if the cells are capable of propagating action potentials in between the cells that lead to contraction of the cells. In this study, we have performed mitochondrial membrane potential and intracellular Calcium signaling to assess the functional properties of cardiomyocytes obtained from CDCs differentiation.

A characteristic feature of cardiomyocytes is the presence of high network of mitochondria that help cells to meet energy demands, maintain calcium homeostasis and provide mechanical strength to the heart to function normally (Kurz et al. 2010). Hence mitochondrial function also plays a crucial role during the differentiation of stem cells to cardiomyocytes (Chung et al. 2007).

During ATP synthesis, proton motive force developed across inner mitochondrial membrane during electron transport leads to generation of mitochondrial membrane potential (MMP) (Mathur et al. 2000). Maintenance of MMP is essential for the normal function of the cells with high-energy requirement like beating cardiomyocytes. Hence, MMP acts as an indicator of the active mitochondria and energetics.

Changes in MMP can be determined by JC-1 dye, which forms aggregates inside the cells with active mitochondria that emits red fluorescence. Decrease in MMP is indicated by the formation of monomers in the cell that gives a green fluorescence. In our study we revealed that active
mitochondria was present in Aza+AA treated CDCs which exhibited highest red fluorescence than Aza and control CDCs. We conclude that Aza+AA were successful in deriving cardiomyocytes with functional and active mitochondria.

In a report, it was revealed that there was an upregulation of aerobic mitochondrial metabolism like high oxygen consumption, increase in intracellular ATP (Chen et al. 2008). Increased DNA and protein copy number in mitochondria when MSCs were allowed to differentiate to osteogenic lineage. These results propose that there exist a synergistic regulation of mitochondrial biogenesis and antioxidant enzymes during the differentiation induction. In line with this, we hypothesize that presence of AA in our differentiation induction medium might have further enhanced the mitochondrial potential of the Aza+AA treated CDCs.

In the heart, \( \text{Ca}^{2+} \) homeostasis is fundamental for excitation-contraction coupling and the contractility of functional cardiomyocytes (Bers 2002). During depolarization of cardiac membrane, \( \text{Ca}^{2+} \) from sarcoplasmic reticulum (SR) will be released into the cytoplasm of the cell which leads to increase in cytoplasmic \( \text{Ca}^{2+} \) concentration (Rovetti et al. 2010). However, this intracellular increase in \( \text{Ca}^{2+} \) is transient because sodium-calcium exchanger sends Ca2+ back into the SR. This transient rise and fall in \( \text{Ca}^{2+} \) concentration is sufficient to maintain synchronous contractions in the cardiomyocytes (Stern et al. 2013).

A recent report suggest that functional \( \text{Ca}^{2+} \) transients were observed in ventricular myocytes derived from small molecule mediated differentiation of human ESCs (Karakikes et al. 2014). Hence, an elevated
intracellular $\text{Ca}^{2+}$ amounts indicate that cells were functional and exhibit excitation - contraction synchronous with the surrounding cells. In this study, Calcium signaling analysis using Fluo-4 AM revealed enhanced intracellular $\text{Ca}^{2+}$ signal levels in Aza+AA treated CDCs which prove that cells were successfully differentiated to cardiomyocytes and were functional in nature. Moreover, observation of spontaneous beating in Aza+AA treated CDCs further emphasize the role of $\text{Ca}^{2+}$ signaling in beating of the cells and CDCs were successfully differentiated to cardiomyocytes. MMP also influences $\text{Ca}^{2+}$ influx into mitochondria by $\text{Ca}^{2+}$ uniporter which further stimulates ATP production according to the energy need of the cell (Hansford & Zoroy 1998). Both MMP and $\text{Ca}^{2+}$ signaling act simultaneously under normal metabolic conditions. In accordance with this, our report also represent increased MMP and $\text{Ca}^{2+}$ signaling in the differentiated cells.
The whole theme of the present work was demonstrated as a schematic representation in Figure 4.1. It displays the efficacy of Aza+AA in mediating cardiomyogenic differentiation of CDCs effectively by upregulating the cardiac specific genes at both mRNA and protein levels along with enhanced MMP and Ca$^{2+}$ levels. Low levels of Cyclin D1 further support that Aza and Aza+AA treatments slowed down the proliferative capacity of CDCs, directing them towards cardiac lineage. High expression of phospho β-catenin and low Cyclin D1 levels exhibit the down regulation of Wnt/β-catenin pathway during the differentiation process of CDCs. Even though Aza was successful in mediating differentiation, its efficiency was increased in the presence of AA.
Overall, these outcomes suggest that pre-treatment of CDCs with Aza followed by AA supplementation has a synergistic effect in generation of cardiomyocytes, making them an ideal candidate for cardiac regenerative therapeutics. It was also proved that AA enhanced the effect of Aza in differentiation of CDCs to cardiomyocytes. Aza+AA promoted CDCs cardiac lineage commitment by down regulating Wnt signaling pathway. Further in vivo experiments are prerequisite to evaluate the role of Wnt/β-catenin pathway in Aza+AA mediated differentiation. Thus, stimulation and differentiation of CDCs in in vitro conditions may increase the chances of effective myocardial regeneration under in vivo environment.