Chapter 4

Overall summary and impact of the study

Two chapters of the thesis summarize significant novel findings which will open new ideas in the field of cardiovascular research. There are multiple areas to explore in cardiac research biology including both its developmental as well as disease repair areas. In developmental aspect, investigations are ongoing to discover the unknown cellular and molecular hierarchies which are associated with consecutive developmental events. Also the key factors and their characteristics, behavior and functional contribution, all are included in cardiac developmental research. Studies in these areas are beneficial to get better information in drug discovery strategies and advancements. And ultimately, it will implement the cardiac science and technology advancement, which would eventually overcome the majority of different clinical problems. Likewise, in aspect of disease repair research, a wide spectrum of studies has been targeted to find out the biologically relevant cardio-protectant natural molecules with minimal off target effects, so that these can be applied directly in cardiology clinics in near future. Cardiac failure is a very common and progressive lethal disease with significant morbidity and mortality (Basson et al., 1997; Karantalis et al., 2012; Schade & Plowright, 2015). According to previously mentioned statistics, cardiovascular investigations are very challenging and demanding area of biological research. Textbook of Colacci and Braunwald has defined heart failure as "*The pathological state or clinical syndrome in which the heart is unable to pump blood commensurate with the requirements of the metabolizing tissues or can do so only with an elevated filing pressure"*. The underlying mechanisms of heart failure and related findings are limited and require more in depth research. Overall, this study has been focused specifically on two important cardiac research areas, namely **epicardial differentiation biology** and **adult aortic valve calcification**.

In vertebrate, in the course of embryogenesis, heart is known as the earliest organ among all the developmental stages. Tubular heart is formed from the precardiac mesoderm and it consists of several cardiac cell types (Banerjee et al., 2019). After development of a contractile myocardium and inner endocardium, the third layer, epicardium is formed enveloping the whole heart. Many errors occur in different

cardiac developmental stages which are responsible for heart malformation resulting in a number of various cardiac abnormalities and diseases during adulthood (Basson et al., 1997; Padang et al., 2012). Latest statistics show the threatening number of cardiac patients and necessary surgeries which are being performed every year in India. Also, dysfunction of the valves, due to mineralization and calcification is projecting about 25% of all types of heart diseases (Padang et al., 2012). Among all kinds of cardiac injuries and diseases, two important and dominant cardiac diseases include myocardial infarction (MI) and calcified aortic valve disease (CAVD) (Peddle, 2014). During cardiogenesis, outer cardiac layer, epicardium is derived from the extracardiac proepicardial organ (PEO), discovered to be situated at the atrio-ventricular junction, near the venous pole of the vertebrate embryonic heart [already mentioned earlier] (Asli et al., 2014; Y. Ishii et al., 2007; Tao, 2012). PEO is a transient, grape like embryonic structure, having migratory cell pool which migrates onto the myocardium to form protective epicardial layer. Next stage of development includes epithelial to mesenchymal transformation (EMT) of Epicardium derived cells (EPDCs) which ultimately differentiates into multiple cardiac lineage specific cells including smooth muscle cells (SMs), fibroblasts, endothelial cells and also cardiomyocytes (CMs) (Gittenberger-de Groot et al., 1998; Lie-Venema et al., 2007). Tbx20 is one of the essential cardiac T-box transcription factor, which is known to express markedly in several cardiac cell types including ventricular myocardium and epicardium (Greulich et al., 2011; Iio et al., 2001; Shelton & Yutzey, 2007; Singh, 2005). In addition, Tbx20 promotes CM cell proliferation during early development (Santanu Chakraborty et al., 2013). Earlier referred reports strongly suggest Tbx20 is actually associated with multifunctional mechanisms with diverse characteristics. Thus, investigation about Tbx20 function and its associated signaling pathways on differentiation of PEO-derived cells and EPDCs will give further implications in cardiac repair or regenerative approaches. The overall data generated from aim/objective 1 reveals that Proepicardial organ (PEO) derived cells have increased differentiation potential compared to embryonic epicardial (eEpi) cells for cardiomyocyte (CM) lineage differentiation and Wnt/β-catenin signaling plays an important role in the differentiation process. In chapter 2, figure 3 E-G significantly depict that PEO-derived cells have shown increased CM specific marker gene expressions, including *Tbx20* compared to EPDCs. It clearly indicates that Tbx20

might involve cardiomyocyte specification from embryonic epicardial cells *in vitro.* Moreover, Wnt signaling manipulation also results in increased number of CM lineage specification in eEpi derived cells *in vitro*. Figure 4 and 5 have shown at transcript level that CM markers get upregulated after Wnt signaling manipulation which is concomitant with the alterations of non-CM and endothelial lineage specific marker expressions. And very importantly, it has also shown in figure 6 that there are elevated number of CM specific Mf20 positive cells and proliferation specific Ki67 positive cells. So, again it is another evidence of Tbx20 involvement with Wnt signaling in CM differentiation from epicardial cells *in vitro*. Current research in cardiac regenerative biology emphasizes upon the search for the developmental cues shown to be reactivated or induced after adult cardiac injuries in order to repair and/or regenerate the injured heart. Therefore, search for such cardiac progenitor cells, like embryonic epicardial cells with increased potential for other cardiac CM lineage differentiation might provide us further impetus for better cell based repair and regenerative therapy in clinical settings. Cardiac malformation is a major cause of human birth defects and cardiac diseases remain top killer of adults in the developed world (Basson et al., 1997; Plageman & Yutzey, 2005). Recent approaches or proposals have been promoting the potential cardiac stem/progenitor cell-cased therapies (Leucht et al., 2008; Sahara, Santoro, & Chien, 2015). Interestingly, it has been found that cardiac progenitors contribute partly during early stages of vertebrate development and embryonic stem (ES) cell development can be manipulated *in vitro* to differentiate into different types of cardiac cell types (González-iriarte et al., 2002; Kwon et al., 2008; Martin-Puig, Wang, & Chien, 2008; Masters & Riley, 2014). However, molecular detailing that regulates cardiac progenitors is less clear. Our data suggest that canonical Wnt signaling is an essential positive regulator of proepicardial as well as epicardial progenitior differentiation into cardiamyocyte (CM) during cardiogenesis *in vitro*. These findings give important clues for understanding the regulatory molecules that control early differentiation, a prerequisite for future progenitor cell-mediated therapeutics in clinical settings.

Molecular mechanisms underlying CM differentiation from epicardial cells raise several positive and negative cross talks which are further to be elucidated. In our data, Tbx20-linked canonical Wnt signaling results, add more information in this research area. Although our data establish Tbx20-mediated association with Wnt targets in CM

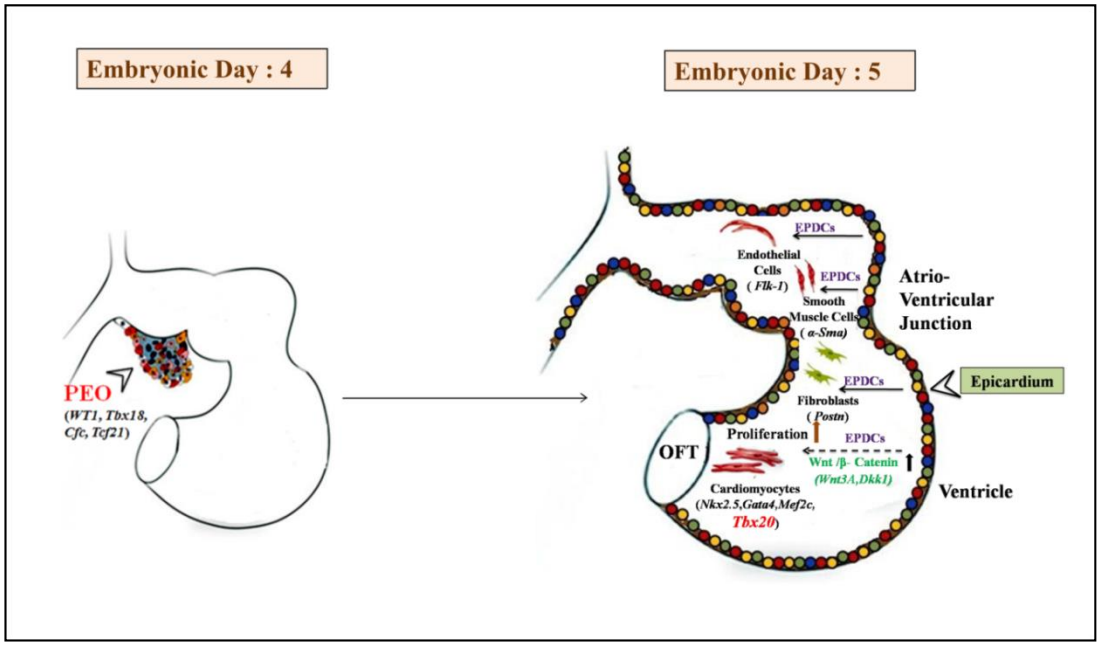
differentiation, still there is requirement of more aspects in order to reveal complete functional network.

Selective T-box transcription factors play fundamental roles in cardiac development and disease (Showell et al., 2004). Tbx1, Tbx2, Tbx3, Tbx5 or Tbx20 in mice leads to heart malformations along with several other effects (Greulich et al., 2011), which are previously mentioned in chapter 1. Thus, from those data, our data continue in finding Tbx20 expression showing significant alteration in two consecutive early embryonic stages (E 4 & E 5). Moreover, Tbx20 expression also shows altered expression in effect of canonical Wnt signaling manipulation [figure 4].

Previously many reviews have reported in many ways that epicardium derived cells (EPDCs) are considered as multipotent cardiac progenitors which have contribution in lineage specification (Gittenberger-de Groot et al., 1998; Groot et al., 2010; Smits et al., 2018; Weeke-Klimp et al., 2010). Classically, early cardiac progenitors can be defined as a self maintaining cell population which usually produce a variety of differentiated progeny and also regenerate tissues. On the basis of developmental gene programs, proliferative capacity of the cells after damage and their ability to differentiate into different resident cell types, it has been proved that EPDCs have all kinds of criteria. In adult heart, progenitor cells express such markers (c-kit, Sca-1) on their cell surface (Condorelli et al., 2001; Lepilina et al., 2006; Ruiz-Villalba et al., 2013; Wessels & Pérez-Pomares, 2004). These c-kit and Sca-1 positive cells are considered as cardiac progenitor cells based on their capability to form colonies during development. Here, according to data from this study, isolated epicardial cells have been marked with epicardial progenitor marker Wt1 comparing with c-kit. In addition, Wt1-expressing epicardial cells have been carried forward to study Tbx20 associated function with canonical Wnt pathway in CM differentiation from epicardial cells [figure 4 A]. This *in vitro* cell culture model allows studying directly the effects after Wnt signaling manipulation. Identifying the differences before and after manipulation of Wnt signaling including transcript level expressions of CM lineage markers, helps us to understand better how to unlock one more key of EPDC potential in response upon injury. Our data summarizes with the determination of the Tbx20 molecular factor role in EPDC differentiation into CMs. This will have significant impact to recover cardiac function after certain damages. The distinct role of epicardium in myocardial contribution, is still under investigation and remains unclear. Our study first has been identifying T-box factor, Tbx20 involvement in CM differentiation

instead of proliferation during development. These data further enrich and highlight the cardiac cell biological process, epicardial cell dependent myocardial cell differentiation and associated transcriptional regulatory factors.

Figure 1: Proposed Model



***[Figure 1:** At embryonic day4 (E:4), a cluster or group of cells, better known as PEO has been marked by arrowhead in looped heart. A day after (E5), PEO migrate onto and envelop the whole myocardium forming epicardium, also indicated by the arrowhead. Subsequently, EPDCs start to invade through the myocardium and contribute to several cardiac lineage specific cells including but not limited to endothelial, smooth muscles, fibroblasts and cardiomyocytes. Overall, our data have been proposing and establishing a model which reveal a distinct transcriptional regulatory mechanism interconnecting Wnt/ β-catenin signaling and Tbx20 with CM differentiation and proliferation of embryonic PEO derived cells and epicardium derived cells during cardiogenesis.]*

Although there is wealth of data focusing on the involvement of T-box genes (including Tbx20) in controlling developmental mechanisms in the early embryo, little is known about the genetic networks associated with molecular pathways (C.-L. Cai,

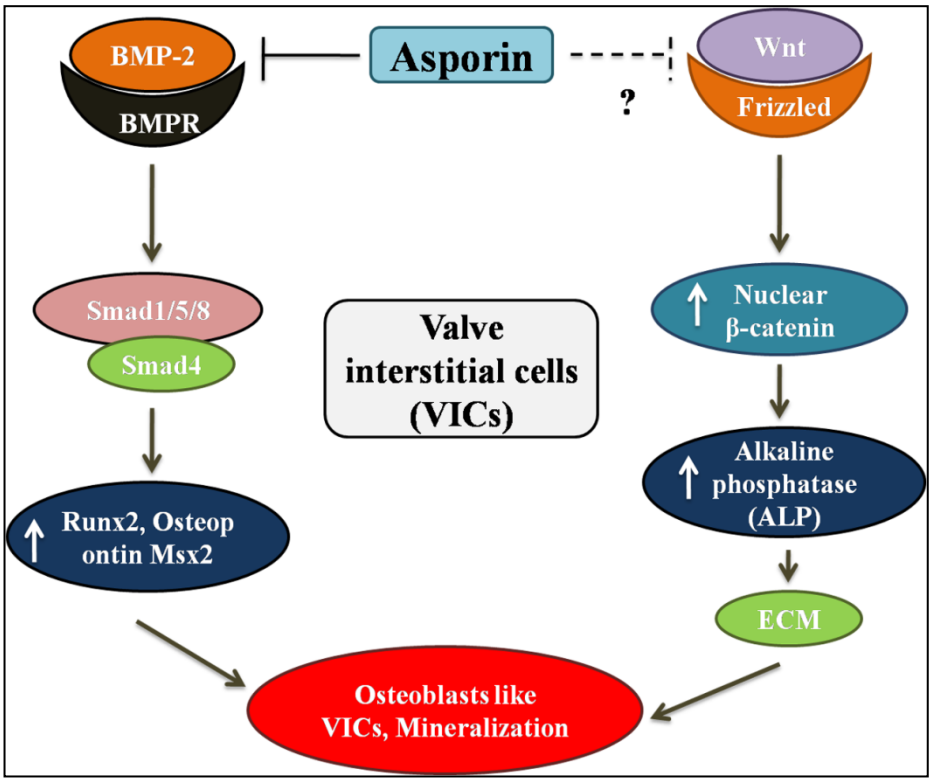
2005; Santanu Chakraborty, 2014; Greulich et al., 2011; C. Liu et al., 2008; Meganathan, Sotiriadou, Natarajan, Hescheler, & Sachinidis, 2015; Plageman & Yutzey, 2005; Shelton & Yutzey, 2007; T. Shen et al., 2013). There is no doubt that this Tbx20 study and further future research proposals will be able to find out greater reports with innovative approaches in these areas.

During vertebrate valvulogenesis, embryonic mesenchymal cells (MSCs) of out flow tract (OFT) cushions are known as the precursor cells of mature adult aortic valvular cusps (Lincoln et al., 2004). Embryonic, undifferentiated MSCs of endocardial cushion get specified into mature aortic valves with dynamic extracellular matrix (ECM) (Santanu Chakraborty et al., 2008; Rutkovskiy et al., 2017). Next developmental event includes the stratification of mature aortic valve leaflets into elastin-rich atrialis/ventricularis, proteoglycan-rich spongiosa and highly organized collagen fiber-rich fibrosa. Key event of calfic aortic valve disease (CAVD) is adult aortic valve calcification which occurs due to gradual thickening of valvular cusps in effects of matrix mineralization. CAVD results in compromised heart function due to narrowing of the path of unidirectional blood flow. Previous studies demonstrate that biological phenomenon of valvular mineralization and calcification can be compared the similarities with osteoporosis (Lincoln et al., 2006; Rajamannan et al., 2003). Because there are already previous reports which have shown that shared gene regulations and signaling pathways exist between valvular calcification and bone osteogenesis mechanisms. Asporin or PLAP1 (Periodontal ligament associated protein 1) is one type of small leucine reach proteoglycan (SLRP) which plays essential role in negative regulation of periodontal cell mineralization and bone cell calcification (Satoru Yamada et al., 2007). Asporin is reported to be expressed in adult murine aortic valve tissue (Santanu Chakraborty et al., 2008). Also, several other reports have suggested that Asporin functions through BMP2/Smad1/5/8 signaling pathway and inhibits mineralization and calcification. It deactivates BMP2/pSmad1/5/8 signaling via competitive binding with BMP receptor (Song et al., 2015; Tomoeda et al., 2008). Wnt/β-Catenin signaling is directly associated with bone cell mechanisms. Because, previous reports have revealed that Wnt signaling gets activated in bone cell lineage differentiation and promotes osteogenesis (Alfieri et al., 2010b; Gu et al., 2014). So far, no such study has been targeted on Asporin role determination and associated signaling mechanisms to regulate aortic valve

mineralization and calcification. The overall data generated from aim/objective 2 reveals decreased level of *Asporin* mRNA and protein in cultured adult AVICs with induced mineralization and concomitant increased level of Wnt/β-catenin signaling. Asporin, a small leucine rich proteoglycan negatively regulates bone cell differentiation, and is also expressed in adult mouse aortic valves with unknown functional significance. In contrast and as hypothesized, overexpression with recombinant Asporin protein (rhAsp) significantly decreases AVICs matrix mineralization, detected by Alizarin red S staining, associated with reduced levels of Wnt/β-catenin signaling (figure 5). Again, in figure 6, recombinant Wnt3a (rhWnt3a) induced AVICs mineralization level also gets reduced by overexpression of Asporin protein. Alternatively, when Wnt signaling is inhibited by Xav939 treatment, then mineralization level is not changed but addition of rhAsp results in the reduction of mineralization level in adult AVICs in culture (figure 7). Therefore, in human heart valve disease, the search for natural molecules with anti-calcific properties like Asporin and its associated regulatory signaling might provide us future implications for better drug based clinical treatments against aortic valve mineralization and calcification. Overall, our data is highly suggestive of *Asporin* might be a novel bio-molecular target to treat patients with CAVD over current cusp replacement surgery. For CAVD research area, as such there are no data on PLAP-1/ Asporin in aortic valve mineralization and calcification. Most of the Asporin reports have been based on bone cell and periodontal cell biology (Gruber et al., 2009; Tomoeda et al., 2008; Satoru Yamada et al., 2007). Thus, in this report, in adult aortic valve mineralization and calcification, Asporin related data and findings of chapter 3 (aim 2), will also be able to detect and reveal valvular cardio protective mechanisms and drug discoveries. There are such emerging evidences that extracellular matrix proteoglycan components directly take part in heart valve matrix mineralization and subsequent calcification (Gruber et al., 2009; L. Svensson et al., 1995; S Yamada et al., 2006; Satoru Yamada et al., 2008). Associated signaling includes Bmp/smad and canonical Wnt signaling which are proved to be involved in valve mineralization (T. Cai et al., 2016; Glass et al., 2005; Hu, 2004; Regeneration, 2009; Santanu Chakraborty, Michelle D. Combs, 2011; Satoru Yamada et al., 2007). Thus, Here, determination of Asporin (leucine rich proteoglycan) function has been focused and Wnt targets dependent Asporin involvement has been analysed. Actually, valvular interstitial cells (VICs) are known

to be differentiated into osteoblasts due to several physiological as well as environmental factors. Asporin mRNA is found to be expressed in adult heart valve, which has been reported in Chakraborty et al report. As per previous reports, Asporin is involved in negative regulation of periodontal ligament cell mineralization via Bmp pathway (other references also in chapter 1 and 3). We have shown in this study that Asporin mRNA got decreased in artificially induced osteogenic avian adult VICs. Thus, it indicates that Aporin is associated with the maintenance of VIC characteristics and control to osteoblast differentiation in valve cell also. Significant numbers of researches have revealed interesting shared signaling pathways that control heart valve differentiation, also with maturation of cartilage, tendon and bone. Key pathways which are shown to regulate osteogenesis mechanisms are Bmp/smad pathway, canonical wnt pathway and also notch pathway. Our study has also shown the marker gene expression profile of these signaling factor genes before and after induction of osteogenesis. Those results have been verified with previous reports and carried on further experiments. We have studied about Asporin role dependent on canonical Wnt signaling. For this we have performed overexpression of Asporin protein on adult VICs in culture and carried out further experiments to study Asporin function. Here, it is shown that addition of human recombinant Asporin protein reduces VICs mineralization level in culture. Also we have manipulated canonical Wnt signaling to determine Asporin dependent result. There we have got the data which shows that Wnt signaling mediated osteogenesis also get reduced in effects of recombinant Asporin protein treatment. This part of our work has been centered on Asporin function as an anti-mineralization molecule in adult AVICs induced with osteogenesis in culture. Studies in adult cardiac diseases, associated with aortic valve calcification are actively searching for factors with inhibitory effect on disease progression involved in the pathology of valvular calcification. In human calcific valve disease, it will be really beneficial to identify naturally expressed factor/molecule with inhibitory effect on induction of aortic valve calcification process for better adult aortic valve protection and function. Therefore, search for such biologically active molecules, like Asporin with increased inhibitory effects on osteogenic gene induction might provide us future impetus for natural biomolecule based clinical treatments against CAVD patients in clinical settings.

Figure 2:Proposed Model



*[**Figure 2:** Previously, it is already shown that during osteogenesis process, Asporin functions via BMP pathway through binding with BMP receptor in valve interstitial cells (VICs). We are proposing that Asporin might control osteogenesis via competitive binding with Wnt receptor in valvular calcification regulatory mechanisms.]*

Conclusion:

Epicardial biology is now an emerging field in cardiovascular development and disease research. Regarding the pluripotent characteristics of the EPDCs as well as its overall revealed importance in heart development, this study has provided such significant results supporting and discovering new information and potential of EPDCs. Elucidating detailed transcript level expressions of embryonic epicardial events will not only help us to give more understanding in heart morphogenesis, but also unveil novel insights in the epicardial differentiation mechanisms underlying in cardiac diseases. Moreover, our data are also suggestive of future EPDC research and

focus on revealing the hierarchies associating the positive effect of EPDC progenitor cells in new CM formation after injury, improving cardiac function. A balanced population of EPDCs with complementary paracrine and differentiation properties might ultimately lead to a promising cell therapy treatment of injured heart. Investigation on Asporin will implicate or enrich in CAVD research area to inhibit aortic valve calcification and discoveries of new valve-protective drugs. Those Asporin data will open new directions to reduce procalcific effects including induction of proresorptive aortic valve tissue regulators. Also, new hopes can be drawn that expectedly, Asporin research will be reducing the annual and global rate of valve transplantation in near future.

Therefore, this study has focused on two different aspects of cardiovascular research. Firstly, proepicardial organ and epicardium derived cells have been studied as potent cardiac progenitor cell population, ultimately that will be implemented in the field of cellular repair and regenerative therapies clinically in near future. Thus, data generated from first aim of this study will be valuable to find more strategies in EPDC based regenerative approaches. Likewise, data generated from second aim of this study has been centered on Asporin as natural cardio-protectant molecule in aortic valve mineralization. Here, investigation on anti-calcific molecule, Asporin will definitely bring more avenues to overcome a major burden of calcific aortic cusp replacement surgeries. Therefore, this study will be able to unveil novel insights on treatment strategies against CAVDs. Overall, studies on both epicardial differentiation biology and aortic valve mineralization will enrich our understanding on cardiovascular research and also be impactful in positive cardiovascular disease repair/regenerative approaches in near future.