Chapter4

Overall Summary, Impact of the Study & Future direction

Chapter 4

Overall Summary, impact of the study & future direction

To summarize, both the chapters (2 and 3) of this thesis produce novel findings which will help unlock some of the mysteries involved in pathophysiology of cardiovascular diseases and fuel further research of the same. Cardiovascular diseases are a cause of global concern so much that United Nations had developed sustainable development goals that targets to reduce Non-Communicable Diseases (NCDs) including CVDs which accounts for 38% of NCDs. by a third by the year 2030.[82,153] CVDs cause over a million deaths per year in the US. It accounts for 30% of total mortality.[153,154] Even though recent advances in cardiovascular research, hospital care and lifestyle changes have improved the mortality rates, CVDs still are a major cause of mortality and morbidity. In the recent decades, extensive research in the field of cardiac sciences has been done that focuses on both developmental and disease aspect to study and discover unknown factors and molecular networks and signaling hierarchies responsible for cardiogenesis and study the underlying factors and mutations responsible for CHDs. Similarly, research in the field of cardiac diseases aim to identify novel biomarkers, risk factors both at phenotypic and molecular levels so that therapeutics could be developed to target those factors in order to reduce or eliminate the progression to heart failure. Overall, this study is focused on two important and areas of cardiovascular research namely: **the role of Tbx20 in autophagy in heart and cardiac ECM remodeling.**

Autophagy is an evolutionarily conserved phenomena from yeasts to mammals by which cytoplasmic organelles and proteins are degraded intracellularly by fusion with lysosomes and is a de novo phenomenon to maintain quality control. Autophagy can be triggered by various stimuli such as **age**, oxidative stress, **nutrient stress**, ER stress and genotoxic stress.[20,25] Although the function of

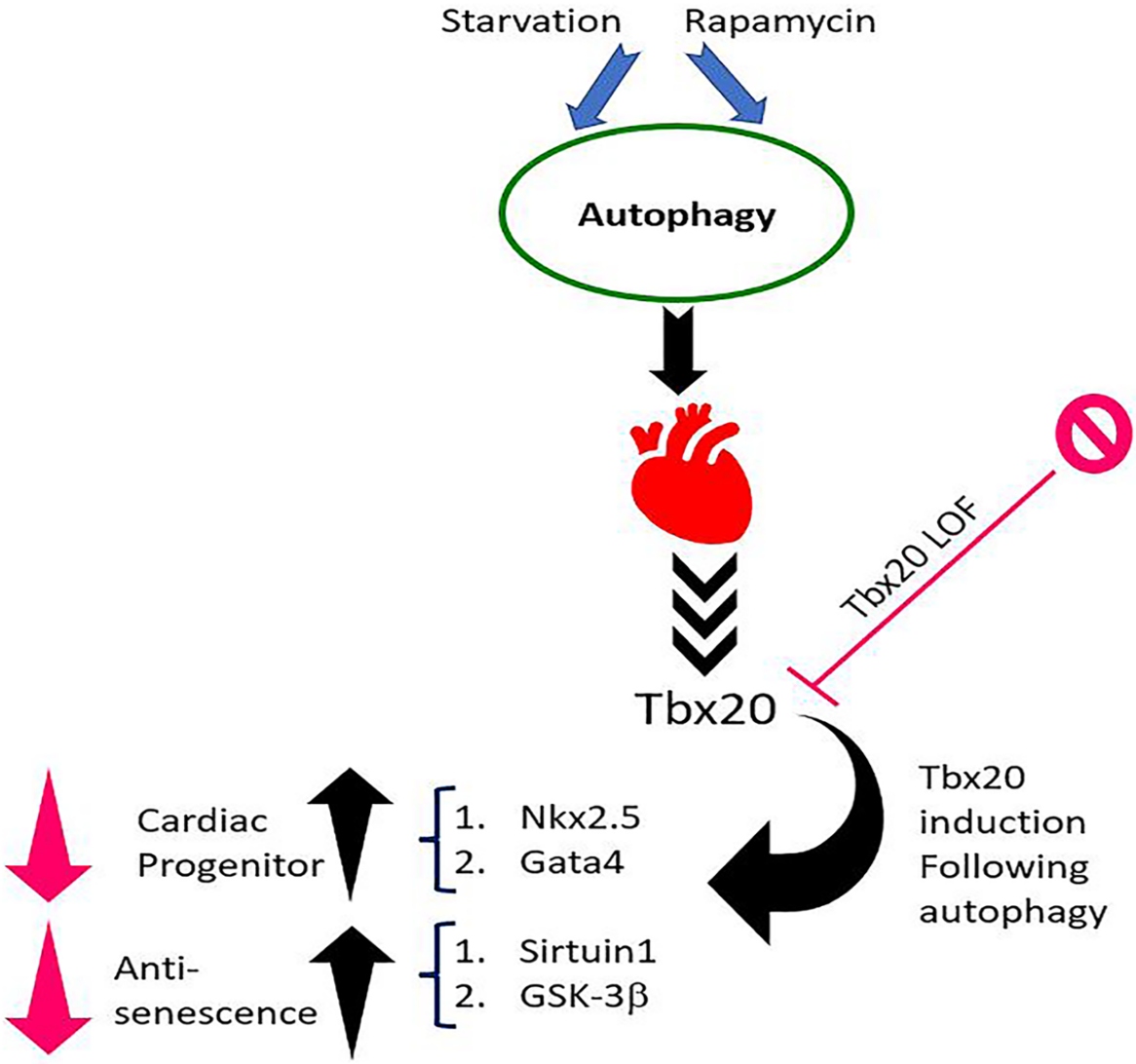
autophagy is adaptive and protective and aims to maintain cellular homeostasis and a pro-survival mechanism under stress conditions, under varying context autophagy can be mal-adaptive or impaired and can lead to cellular apoptosis and necrosis. The importance of autophagy in survival was highlighted when mice deficient for Atg5 or Atg7 failed to survive after birth and die at E1 due to amino acid deficiency and myocardial damage.[12] Atg 5 and 7 are members of Atg (autophagy related) genes required during the initiation and elongation stages of phagophore that later becomes the autophagosome. Under condition of **nutrient stress (starvation)**, autophagy functions to maintain ATP levels intracellularly. Under stress conditions, autophagy functions to limit cellular apoptosis by providing new building substrates in the form of amino acids and fatty acids obtained from degradation of proteins and lipids respectively. Also, these amino acids through TCA (tricarboxylic cycle) provide ATP under stress conditions to restore bioenergetics.[19,20]. Furthermore, autophagy also restricts mitochondrial dysfunction by removing and replacing damaged mitochondria with mitochondrial biogenesis. While, autophagy in its entirety functions as a pro-survival and a protective defense mechanism under stress conditions, irregulated autophagy such as under and over activated autophagy can have deleterious effects. Under performing autophagy machinery would be incompetent to rescue stress grieved cells as damaged / misfolded proteins and inadequate clearance of dysfunctional mitochondria accumulate contributing to enhanced stress susceptibility as is the case with cardiac aging and similarly, over-activated autophagy can often lead to enhanced ROS production, excessive removal of mitochondria causing energy crisis and apoptosis which is pretty much the case with Reperfusion injury (RI).

Aging in itself is a risk factor for CVDs [155] and the hallmarks of cardiac aging include aggregation of misfolded protein, buildup of damaged mitochondria, imbalance between anti-oxidants and pro-oxidants, damaged telomere, ECM remodeling and finally impaired autophagy and elevated apoptosis including

cardiomyocyte cell death. Amongst the current possible therapeutics targeting cardiac aging include calorie restriction and rapamycin administration both of which are closely linked and integral to autophagy.[156] Tbx20, a T-box factor involved in cardiogenesis is a critical transcription factor expressed and required at the cardiac crescent, heart tube and forming of endocardial cushions where its function is to promote proliferation and expression of other key cardiac transcription factors such as ANF. It is known to interact with other cardiac transcription factors such as Gata4 and Nkx2.5 via its T-box domain (DNA binding domain) is also required in adult hearts as conditional ablation in adult cardiomyocytes lead to massive cardiomyocyte death progressing to a heart failure phenotype.[84] Tbx20 is activated under stress conditions to promote proliferation of cardiomyocytes[80,157] and rescue cell cycle arrest[77] in addition to improving cardiac functioning and survival post MI in adult mice.[86]

But the role of Tbx20 under nutrient stress (starvation) in specific and autophagy in general in heart and additionally with focus to aging has not been studied which is the highlight of Chapter 2 of this thesis. The novel role of Tbx20 under autophagy influence was studied. Aged BALB/c mice served as the in-vivo part of the study and H9c2, a rat cardio-myoblast cell line formed the in-vitro part of the study. In both the systems autophagy was induced by starvation or rapamycin treatment. The study highlights the multifaceted role of Tbx20 under autophagy influence wherein Tbx20 promotes stimulation of Gata4 and Nkx2.5 to induce fetal like cardiomyocyte characteristics and additionally and most notably interacts with Sirt1 (a well-known longevity associated gene) to attenuate senescence phenotype.

Figure 1: Proposed model



[*Figure 1*: *Hypothetical proposed model depicting the role of Tbx20 under autophagic influence*. *Tbx20 functions as a master regulator under the autophagic influence to promote a cardiac progenitor like pool and alleviate the senescence environment. Starvation and Rapamycin mediated autophagy induction drives the expression of Tbx20. Tbx20 being a transcription factor with DNA binding domain, interacts with Nkx2.5, Gata4, Sirt1 and GSK-3β. These markers then drive a two-way pathway to promote cardiac health under*

autophagy scenario wherein Transcription factors Nkx2.5 and Gata4 promote the generation of cardiac progenitor-like pool in heart, markers GSK-3β and Sirt1 are responsible for delaying senescence and improving cardiac aging as also especially seen in our aged murine model systems where the expression of Sirt1 and GSK-3β significantly declines in the aged control group. **]**

Impaired and low autophagy levels have been implicated in an array of pathological diseases including those related to age. High levels of ROS, low levels of antioxidants, low levels of NAD^+, poor mitochondrial integrity and mismanaged protein aggregates topped with underperforming autophagy machinery led to clinical pathophysiology and these are hallmarks of an aging system.[119,120] Our findings, for the first time, proposes a two-way regulatory role of Tbx20 to promote cardiac homeostasis and improve cardiac aging under the influence of autophagy. In one direction the autophagy induced elevated Tbx20 interacts with and promotes the generation of cardiac progenitor like pool by inducing cardiac transcription factors Nkx2.5 and Gata4.[8,59,129] On the other hand, it induces the expression of Sirt1 and GSK-3β. The latter two genes especially Sirt1 is a well-known longevity promoter.[19,22,130] Sirt1 also has been implicated in its cardioprotective role. [21,23] Also, low levels of active GSK-3β and higher levels of pGSK-3β contribute to aging in the heart.[115] Impaired autophagy with age leads to a cellular catastrophe and while autophagy induction either through caloric restriction or by rapamycin treatment, senescence-related effects could be improved,[18]. Overall, the work highlights a key underlying unexplored role played by Tbx20 in this scenario to provide cardio protection and improve cardiac aging by upregulating anti-senescence markers. This could lead to a futuristic approach to treating cardiac aging through the targeted expression of Tbx20.

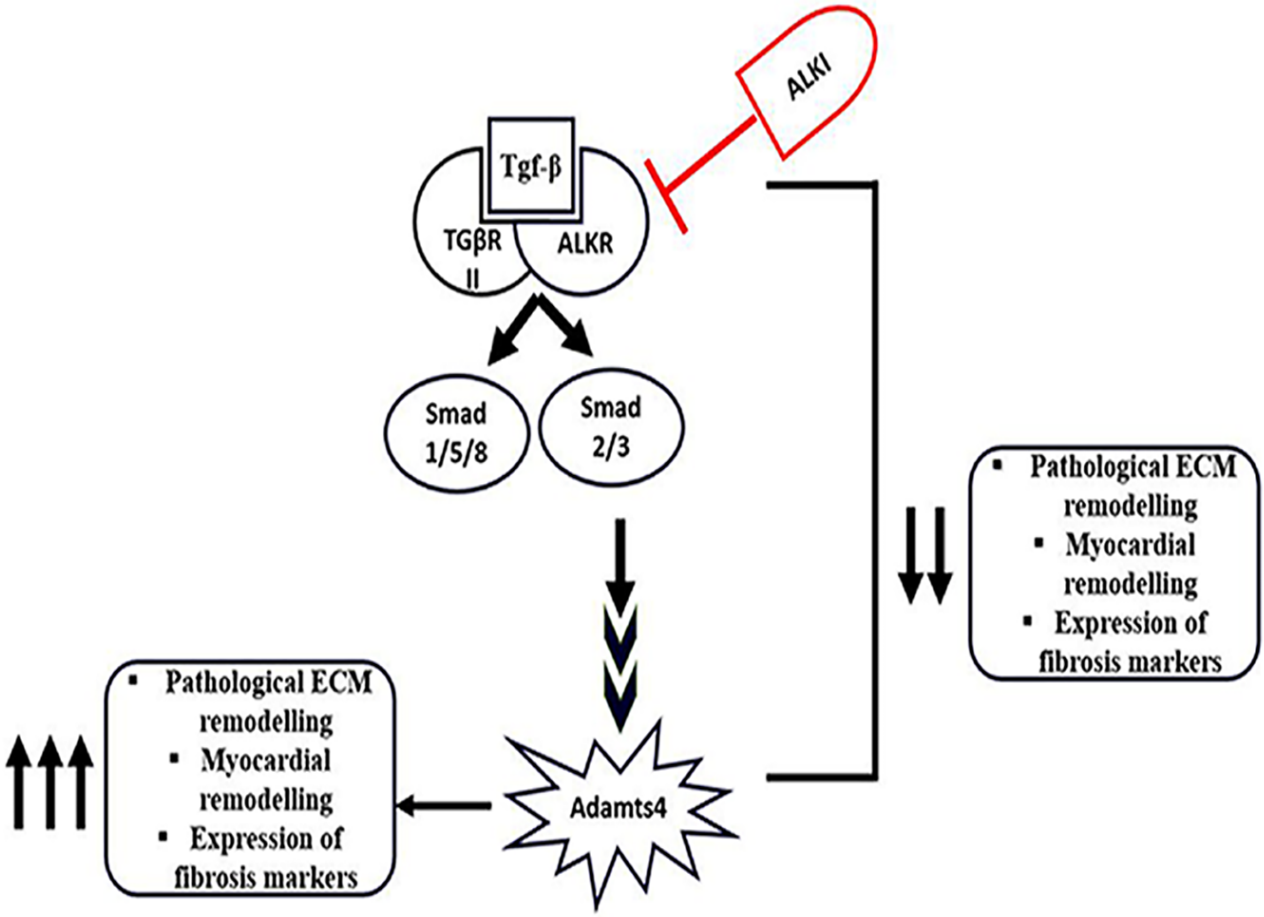
Cardiac ECM remodeling is another area that has drawn attention of lot of researchers. The function of intact normal ECM besides providing a 3D scaffold in which the cells are embedded and maintenance of structural integrity is to help in signal transduction and migration. The ECM consists of structural and non-structural proteins that preserve cellular plasticity. ECM remodeling in heart occurs in response to Ischemia, MI, pressure and volume overload, myocarditis and diabetic cardiomyopathy.[132,147] The composition and biochemistry of a remodeled ECM varies greatly from an intact healthy one. There are 3 stages of ECM remodeling that follows a MI injury namely- inflammatory, proliferative and maturation phases in an attempt for reparative healing. [90] During the inflammatory phase, degradation of matricellular protein and synthesis of MMPs occur. The MMPs which can be Collagenases, Gelatinases or stromelysins chemically, are synthesized by fibroblasts and endothelial cells then drive the infiltration and recruitment of inflammatory cells. A provisional matrix is formed which further aids in migration of phagocytes to the site of injury in an attempt to clear dead cells as a part of the healing process. During the proliferative phase, myofibroblasts (are activated fibroblasts expressing α-SMA) are imposed with the task of repairing the infarct which it does by synthesizing large amounts of matricellular proteins and their cross linkage. The matrix proteins synthesized by myofibroblasts differ from those synthesized by fibroblasts in a normal heart. Maturation which is the final phase in the process of remodeling results in a collagen-based scar at the infarct region.[90,147]. Cardiac ECM remodeling is a critical contributor in the progression of Left Ventricular (LV) remodeling where under-expression or breakdown of Collagen is responsible for LV dilatation whereas over deposition of Collagen leads to fibrosis.[92] Although the aim of ECM remodeling is reparative following an injury such as MI or Ischemic stroke, often the exacerbated replacement fibrosis that occurs during remodeling causes more damage than reparation and ultimately heart failure. MMPs are heavily implicated in a variety of CVDs including Coronary heart disease, MI, atherosclerosis,

Ischemia and pressure overload hearts. MMPs are endopeptidases that require Zinc as its cofactor. There are over 25 MMPs identified in humans and are secretory part of the ECM. A disbalance between the levels of MMPs and TIMPs are also heavily indicted in CVDs. TIMPs or Tissue inhibitors of metalloproteinases are one of the ongoing therapeutics to target MMPs. An important MMP is the Adamts family which apart from inhabiting the functions of an MMP also acts as disintegrins. Adamts4 (also called aggrecanase-I) is one of members of the Adamts subgroup. To date 19 members of mammalian ADAMTS proteinases have been identified with functions ranging from ECM degradation, cell adhesion and proteolysis. It is a metalloproteinase and a disintegrin with thrombospondin like motifs.[33,34] Adamts4 has been known to bind to the ECM proteins and executes cleavage of ECM proteoglycans like aggrecan, versican and brevican apart from regulating collagen turnover.[35,36] Elevated levels of MMP-2, ADAMTS-1, and ADAMTS-7 is associated with initial stages of chronic venous disease, whereas the serum elevation of MMP-1, 2,3, 7, 8, 9, 10, 12, 13 and 14 are found elevated in atherosclerosis while additionally MMP 7 and MMP 9 are biomarkers of plaque instability in coronary heart disease.[158] The role of Adamts4 in the pathophysiology of osteoarthritis is well documented but its role in cardiac remodeling is less known. Only recent studies have shown in Adamts4 null mice fed with high fat diet, loss of Adamts4 leads to reduction of atherosclerotic plaque.[41]

Thus, the study on ECM remodeling with focus to Adamts4 as a potential biomarker of adult cardiac injury was studied and is elaborated in Chapter 3 (of this thesis). The novel finding of this study is that Adamts4 is regulating the expression the expression of other ECM markers involved in pathophysiology of cardiac fibrosis. H_2O_2 and hypoxia and hyperglycemic shock injury inductions in H9c2 cells showed increased expression of Adamts4 along with a couple of other fibrosis-related markers like α-SMA, Tgf-β1, Collagen-III and Periostin were found to be elevated implying that an injury mediated ECM remodeling could be

a cause for the elevation of these markers. Additionally, pre-treating the cells with SB431542, an inhibitor of ALK4 and 5 (one of the two binding receptors of Tgf-β1) receptor that eventually leads to inhibition of Tgf-β1 also suppressed the expression of Adamts4, this inhibition was further extrapolated to the expression of α -SMA, Collagen-III and Periostin proteins as these were also found to be downregulated after ALKI pre-treatment. To better understand the hierarchy between Adamts4 and Tgf-β1, Adamts4 LOF by Adamts4 siRNA transfection was performed. In groups where Adamts4 knockdown did not affect the pattern of Tgf-β1 expression but the expression of the other three mentioned markers-Collagen-III, α -SMA, and Periostin along with Adamts4 were found to significantly decline to somewhat similar levels when ALKI pre-treatment with stress induction was done. These findings indicate that Adamts4 expression is under Tgf-β1 regulation and also that other ECM and fibrosis related markers including Col-III, α -SMA, and Periostin seem to be governed by Adamts4 since loss of function of Adamts4 significantly inhibited the expression of these markers following injury to H9c2 cells. Moreover, not only our marker of interest, Adamts4 but also α -SMA showed significantly enhanced expression in patients with MI and DCM injury indicating a cardiac fibrosis like condition following cardiac injury in adults. Additionally in patients suffered from DCM with T2D (DCM&D), ADAMTS4 was also found to be significantly upregulated even more than in patients with DCM alone and the same was hinted by in-vitro studies with glucose shock treatment. Our findings are highly indicative of the potential role of Adamts4 in the pathophysiology of ECM remodeling in heart thus proposing it as a novel cardiac injury biomarker.

Figure 2



[***Figure 2: Hypothetical Proposed model depicting hierarchy and inter-relationship between Tgf- β1 and Adamts4****. ALKI acting as an inhibitor of Tgf-β1 inhibits the stimulation of Adamts4 by Tgf-β1 which normally is activated under stress or injury conditions and activates downstream molecule Adamts4 and Adamts4 then proceeds with pathological ECM remodeling to restore the damaged physiology of the cells and tissues.* **]**

Both the studies highlight novel findings. Investigation of Tbx20 and its interplay with Sirt1 under autophagy influence opens new doors of future investigation. Current possible therapeutics rely on caloric restriction and rapamycin administration to improve aging in general and cardiac aging in particular as some promising results has been outcomes of such studies. [156] Calorie restriction attenuates cardiac hypertrophy and fibrosis in murine models. Calorie restriction is linked with upregulation of Sirt1 and autophagy via inhibition of mTORC1 pathway. Similarly, Rapamycin, a macrolide identified as a direct target of mTORC1has been linked with longevity. Rapamycin treatment was originally shown to improve lifespan of *Drosophila*. Since then, studies demonstrating the impact of rapamycin on longevity have been conduction on rodents. Rapamycin has also been reported to improve cardiac functioning and myocardial stiffness.[156] Identification of Adamts4 as a biomarker of cardiac injury opens doors for further research and development of therapeutics and drugs that can target Adamts4 to prevent exacerbation of fibrosis following an insult to heart. Current therapeutics to reduce reactive fibrosis rely on ACE1 (Angiotensin Converting Enzyme 1) inhibitor drugs, targeting Tgf-β and inhibition of the same and administration of non-selective MMP inhibitor with doxycycline improved cardiac function and prevented progression of fibrosis.[30,44].

Recent therapeutics for CVDs have incorporated the implementation of Cardiac Targeting Peptides (CTPs) as a potential molecular therapy. CTPs are 5 to 30 amino acid long peptides that can be natural like ANP and BNP or synthetic such as the peptide sequence NH2-APWHLSSQYSRT-COOH. [159] NH2-APWHLSSQYSRT-COOH has been shown capable of targeting cardiomyocytes. CTPs can be used to deliver a range of cargos from peptides, whole proteins, nucleic acids such as siRNA, RNA, DNA to nanoparticles and viral particles specifically to heart thus preventing non specific uptake by other cells and tissues which reduce the bioavailability of the therapeutic agent. The delivery is swift with peak levels of the administered cargo achieved within 15

minutes of administration.[159,160] With the implementation of CTPs and identifying the role of Tbx20 in aged hearts, CTP mediated overexpression of Tbx20 may be a possibility to improve fetal like characteristics in adult and aged hearts and more importantly Tbx20 mediated expression of Sirt1 can prevent early senescence in heart. Similarly, identification of Adamts4 specific inhibitor or een targeting CTPs loaded with Adamts4 siRNA soon enough after an assault to heart can prevent reactive fibrosis and LV dilatation.

However, much investigation is required before such therapeutics become clinically approved and identifying the role of Tbx20 as a multitasking factor responsible for improved cardiac homeostasis and Adamts4 as a biomarker of cardiac injury is one of the many steps required to achieve the goal of understanding and treating CVDs to reduce the mortality and morbidity rates in the long run.