Chapter1

Review of literature & General Introduction

Chaper1

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Overview of cardiac morphogenesis.

One of the earliest signs of organogenesis is the formation of heart within an embryo and this process is conserved from flies to human. The formation of heart is the first sign of an organism's left-right asymmetry and it functions almost from the moment it forms a simple muscular tube which is as early as E8.5 in mouse and CS7 (Carnegie stage 7 in humans) at the gastrulation stage which occurs about 2 weeks after fertilization in humans[49]. The heart functions to pump blood to peripheral organs and maintain their physiology. The process of heart formation begins when a morphogen gradient signaling in the primitive ectoderm or the epiblast causes the formation of cardiac mesodermal cell precursors marked by the expression of MESP1 (Mesoderm Posterior Protein 1), a transcription factor.[50] While migrating towards the anterolateral border of the trilaminar disc and under the influence of WNT/β signaling, these MESP1+ cardiac mesodermal cells become committed to the cardiac lineage and later on gives rise to cardiac crescent (forms at E7.5 in mice and CS8 in humans) and pharyngeal mesoderm. Now, within the MESP+ cells, activation of BMP (Bone morphogenetic protein) signaling induces cardiomyogenesis with Nkx2.5, Isl1 being the earliest cardiac specific markers to be expressed. The zone of expression of transcription factors Nkx2.5, Isl1 marks the cardiac crescent region of the region of heart formation. While still migrating anteriorly, these primitive cardiac mesodermal cells enter a zone of WNT/β inhibition or lineage restriction which is crucial to prevent further differentiation and maintenance of the cardiac fate. [49,51,52]

This early commitment of cardiac precursors gives rise to two distinct pools of cells differing in behavior. The first lineage or the first heart field in the cardiac crescent gives rise to precursors of atria, atrioventricular canal (AVC), regions of inflow and left ventricle (LV). The second lineage or the secondary (also called anterior) heart field in the pharyngeal mesoderm develops medially and caudally to the first lineage and gives rise to right ventricle (RV), outflow tract (OFT) and some regions of atria. A subset of primitive cardiac mesodermal cells undergoes epithelial to mesenchymal transition to give rise to endocardial cells, the myocardial and endocardial cells being separated by cardiac jelly. At this stage myocardial and endocardial transcription factors come into expression. With the ongoing embryo development and steady growth of neural folds at E8.5 and CS9, the heart forming region now acquires a linear muscular tube form resembling an inverted Y shape with a venous pole and an atrial pole (outflow tract). Continuing growth at both the venous and atrial poles in addition to the right/left axial influence, the linear heart tube elongates rightwards spiral to assume an S shaped looped structure (E10.5 and CS10) and the future myocardial chambers become visible as swellings.[49,53] Cardiac protrusions or trabeculae become evident at the endocardial side. Ventricular cardiomyocytes begin to express ANF (Atrial natriuretic factor), Connexin40 (Cx40) and members of Tbx family like Tbx5, 2, 3 and 20. Over the next few days, the ventricles grown tremendously in size due to active proliferation of cardiomyocytes.[49,53,54] Cardiomyocytes make up for about 90% of the cell volume in heart but only about 50% in quantity. Of the non-cardiomyocytes, the majority of the cell type are the endothelial cells followed by fibroblasts and least of all hematopoietic cells (comprising less than 10%) of all cell types. The population of cardiomyocytes is maximum at birth and only declines thereafter; the increase in the size of heart is volumetrically due to hypertrophic response.

A remodeled heart consisting of cardiac chambers, precursors of atrial and ventricular septations, endocardial cushions, precursor of heart valves, formation

of coronary arteries and veins are complete by E14.5 in mice and CS22 in humans. The last stage of embryonic heart development (CS23 in humans) is remodeling of the cardiac valves where the atrioventricular and outflow tract valves mature and the valve leaflets become slender as a result of extracellular matrix remodeling. Any mis-step during the formation of the cardiac crescent to the chamber formation stages leads to congenital heart disease (CHD) and can occur in 1 out of 100 births.

Major Transcription factor families in cardiogenesis

❖ Hippo-Yap pathway in heart development

The Hippo-Yap (Yes associated protein) is involved in regulating the size of an organ. In heart, the Hippo-Yap pathway regulates cardiomyocyte proliferation to maintain size of heart. The Hippo-Yap pathway consists of MST1 (mammalian STE20-like protein kinase 1), MST2, SAV1 (Salvador homologue1), LATS1 (Large tumor suppressor kinase 1), LATS2 and MOB cofactor proteins (MOB 1A and MOB 1B) as core complex. Upon activation of Hippo signaling, MST1, MST2 and SAV1 forms a complex that phosphorylates LATS1 and LATS2. LATS1,2 along with MOB1 phosphorylates co-activators YAP and TAZ. The phosphorylation of transcription factors YAP and TAZ causes their retention in the cytosol and their subsequent degradation preventing its translocation to the nucleus.[55,56] In absence of Hippo signaling the YAP and TAZ transcriptional co-activators translocate to the nucleus and interacts with an array of transcription factors and pathways including TEADs (TEA domain transcription factor family) most notably to induce proliferation and survival pathways. Besides TEADs, YAP and TAZ are also known to interact with KLF5 (Kruppel like factor 5), PAX3 (Paired box protein 3), Forkhead box proteins (FOXO1, FOXM1), ERBB4

(receptor tyrosine- protein kinase erbB4), TBX5, RUNX1 (Runt related transcription factor), RUNX2 and SMAD family members.[50]

Sav1 knockout models at birth develop with cardiomegaly due to unregulated cardiomyocyte proliferation. Hippo signaling also has been known to inhibit WNT- β signaling in developing heart as observed in *Sav1* mutant models which showed abundant expression of β-catenin, a readout of the WNT signaling.[50] WNT is associated with pathways leading to cell proliferation and differentiation. YAP also activates cell proliferation pathways via PI3K-mTOR (mammalian target of rapamycin) pathway by activating mTOR. During embryonic development, YAP does not contribute to cardiomyocyte size but only maintains cardiomyocyte proliferation. Postnatal specific deletion of Yap leads to enhanced cardiomyocyte apoptosis. In addition to regulating the proliferation of cardiomyocytes in the developing heart, the Hippo-Yap signaling is also crucial to epicardial growth of the heart. Deletion of both Lats1 and 2 in the epicardial cells resulted in failure of EPDC derived fibroblast population. Furthermore, postnatal over-expression of Yap gene and similarly inhibition of Hippo signaling show improved cardiac functioning following Myocardial Infarction (MI).[50]

❖ Hox and Tale in cardiac development

Hox or homeobox transcription factors are a group of genes originally known for regulating the anterio-posterior axis patterning in *Drosophila*. The hox genes contain a helix-turn-helix DNA binding domain. There are 4 clusters of Hox genes in humans (a-d) and each cluster has an average of 9-11 hox genes. Hoxd-3 gene expression is evident at the heart tube fusion state. Also, Retinoic acid (RA) is an inducer of Hox genes and overexpression of RA in chick explants leads to enhanced expression of a couple of Hox genes.[52,54,57] During the migration of MESP1+ mesodermal cells which are the progenitors of cardiac cells, from the primitive streak to the anterio-lateral plate, simultaneous activation

of Hox genes takes place in the primitive streak throughout the elongation axis. The coordinated expression of WNT, BMP and Hox genes specify the patterning of the mesoderm. Secondary heart field (SHF) cells are characterized by the pronounced expression of transcription factors-Tbx5, Islet 1 (Isl1) and Nkx2.5. The factors determining the pre-patterning of SHF that define the anterior and posterior portions of SHF have recently been identified to be the work of Hoxa1, Hoxa3 and Hoxb1 and that the anterior regions of *Hoxa1*, *Hoxa2*, *Hoxb1* corresponded with the posterior limits of Isl1 and Tbx5 suggesting a coordinated interplay between these transcription factors and the Hox genes in determining the patterning of SHF. Furthermore, any alteration in the SHF during the heart tube elongation stage results in an array of CHD including OFT malformation, tetralogy of Fallot and ventricular septation defects. Interestingly, OFT misalignments are also observed in humans with homozygous Hoxa1 genetic mutations. [57]

TALEs (Three amino acid loop extension) are cofactors associated with Homeobox transcription factors. The role of TALEs is not clearly understood although it is strongly indicated that they function in accordance with anterior Hox genes during heart development.

TALE factors known to interact with Hox genes are PBX and MEIS.[57]

❖ NK genes in cardiac development

Initially, 4 NK genes were discovered in Drosophila, NK1 through 4 and later on the NK homeobox family of transcription factors were categorized into two subfamilies, Nk-1 (and its homologues) and NK-2 (NK2, 3, 4 and its homologues), The NK2 homeodomain genes are vertebrate orthologs of Tinman gene originally discovered in *Drosophila*.[58] The most studied member of the NK family is NK2.5 or NK-2 transcription factor, locus 5. [59]These proteins have a TN domain at the N-terminal region, a homeodomain region and a NK-2 domain

towards the C-terminal region. The homeodomain region of Nkx2.5 has a helix turn helix motif that binds to a consensus DNA sequence 5'-T(C/T)AAGTG-3'. Mouse homozygous null for Nkx2.5 fail to survive beyond the heart tube stage. The expression of Nkx2.5 remains to be elevated in both first and secondary heart fields during heart development in the embryo. Nkx2.5 is believed to have a role in formation of cardiac conduction system during cardiogenesis as the expression of Nkx2.5 has been found to be transiently elevated in specialized cardiomyocytes. The expression of an array of cardiac markers like ANP, BNP (Brain natriuretic peptide), Mef2c (myocyte enhancer factor 2), Cx40 (Connexin 40), MLC2v (Myosin light chain 2v) are found to be downregulated in Nkx2.5 deficient embryos.[52,53,58] Also, the interaction of Nkx2.5 with GATA4, a Zinc figure cardiac transcription factor is well characterized and it is shown that these two acts synergistically to induce ANP.[60] Besides, GATA4, Nkx2.5 also interacts with Tbx5, a T-box transcription factor, via its homeodomain to activate the promoters of ANP and Cx40. Mutations in Nkx2.5 are responsible for an array of CHDs including ASD (Atrial septation defects) and AV conduction defects.[60]

❖ MADS box in cardiac development

MADS represents a group of evolutionarily conserved proteins ranging from yeasts to humans. MADS takes its name after Minichromosome maintenance factor1 from *S. cerevisiae,* Agamous from *Arabidopsis*, Deficiens from *Antirrhinum majus*, and S for Serum Response Factor (SRF) from *Homo sapiens*. MADS group of proteins perform a diverse range of functions being involved in plant developmental processes to maintaining osmotic balance and stress response in yeast and finally being involved in muscle development and neural transduction.[61] The most well-known MADS members involved in cardiac development are SRF (Serum Response Factor) and Mef2c (Myocyte enhancer factor 2c).

A 67kD protein, SRF contains a DNA binding domain in addition to two other domains that are required for dimerization and co-factor binding, Myocardin being the most notable transcriptional cofactor of SRF.[62] The recognised DNA consensus sequence required for binding of SRF is recognised as 5'-CCWTATAWGG-3'also called the CArG box. SRF is also known to interact with GATA4, Nkx2.5 and ATF6 (Activating transcription factor 6). SRF play key roles in cardiac development during embryogenesis. SRF null mice failed to form a primitive streak and later a mesodermal layer and so the embryo fails to survive. Further, the beating process which initiates at the heart tube fusion and looping stage is absent SRF deficient mice. Thus the role of SRF is critical for the development of primitive streak, proper folding of mesodermal and endodermal layers during cardiogenesis.[52]

Mef2c of Myocyte enhancer factor 2c is a member of MEF2 family of transcription factors. There are 4 Mef2 factors in mammals termed MEF2 A , B, C and D. In addition to the MADS domain, Mef2 proteins also possess a Mef2 domain through which it binds to specific consensus DNA sequence and brings about transcriptional modulations.[63] Mef2c can interact with other transcription factors, co-factors and co-repressors depending on the context of regulation required. Mef2c deficient mice show vasculature defects and the embryo becomes lethal by E9.5.[63,64] Mef2c in addition to Mef2b is expressed during the initial stages of heart formation, its expression being evident at the cardiac crescent stage at E7.5 in mice. The 4 Mef2s are then continually expressed during the later stages of cardiogenesis[52]. Also, Mef2c in the secondary or anterior heart field is shown to be a direct transcriptional target of Isl1 and Gata4.[65] Finally, Mef2c loss of function mutations in humans have been linked with an increased risk of CHD.[66]

❖ bHLH transcription factors in heart development

bHLH or basic Helix-loop-Helix are a group of transcription factors known to be involved in development of many tissues during embryogenesis. bHLH genes are involved in a number of processes like cardiovascular development, neurogenesis and maintenance of stem cells during development. The bHLH superfamily possess a DNA binding domain followed by α helix that precedes a loop , following the loop is another amphipathic α helix. bHLH proteins bind to DNA as dimers and the α helices mediate protein-protein interactions.[52] The bHLH superfamily is divided in 3 classes, Class A bHLH which show ubiquitous expression, Class B bHLH (also called Twist family) which are tissue specific and inhibitory HLH proteins. Common bHLH transcription factors involved in cardiogenesis are Hand factors and Twist factors both belonging to Class B bHLH sub-family.[67]

Hand1 and Hand2 function both as homo and heterodimers and bind to a consensus DNA sequence known as E boxes (CANNTG). The expression of Hand1 gene is observed at the heart tube stage in the OFT and in caudal ventricle whereas the expression of Hand2 is observed at the cardiac crescent stage at E7.5 in mouse embryo. Early insights into the role of Hand in heart development was observed through studies on Drosophila Hand orthologue where perturbation of Hand led to cardiac anomalies.[68] Further, genetic ablation of Hand1 in mice led to embryonic lethality by E9.5. Homozygous Hand1 null mice fail to form a heart tube and lethality occurs by E7.5. Similar results are observed for *Hand2* null mice wherein the embryo fails to survive beyond E10.5 due to vasculature defects and ventricular hypoplasia. Hand2 recently has been found to interact with Gata4 to mediate ventricle maturation as condtional knockout of Gata4 results in repressed expression of Hand2. Also, together with Tbx5 , Mef2c and Gata4, Hand2 is able to drive the reprograming of adult mouse fibroblast to cardiomyocytes.[68]

The appearance of endocardial cushions in the AVC and OFT mark the initiation of heart valve development. The onset of endocardial cushions originate with EMT of the endocardial cells. Twist1 gene is highly expressed in the endocardial cushions and in the early stages of valve formation but its expression declines during the last stages of valve maturation and remodeling. Twist1 promotes cellular proliferation and migration while inhibiting differentiation in the developing valves by influencing the expression of Tbx20, Cdh11, Gadd45a genes. Prolonged expression of Twist1 has been linked to abnormal valve morphogenesis due to unregulated cellular proliferation.[69,70]

❖ Gata transcription factors in cardiogenesis

The Gata family of transcription factors are composed of 6 members termed Gata through 6. The Gata proteins possess a conserved Zinc finger DNA binding domain that recognise a canonical sequence (A/T)GATA(A/T). Gata 4, 5 and 6 are expressed in tissues of mesodermal and endodermal origin including liver, heart and lung. [52] Gata 4, 5 and 6 are predominantly expressed in cardiac cells and have crucial roles in cardiogenesis. Gata4 expression is evident by E7.5 at the cardiac crescent stage and continues expression thereafter till birth and even at 1 week post-natal stage strong expression of Gata4 is observed. Gata4 mediates cardiogenesis. Embryos of mice with Gata4 ablation develop cardiac bifida and are lethal by E8.5. Further, Gata4 genetic deletion in endocardial cells results in embryonic lethality by E12.5 due to abnormalities in EMT.[71] The expression of Gata4 is evident at the linear heart tube stage (E8.5). Gata5 is predominantly expressed in endocardial cells involved in its proliferation and thus believed to be involved in valvulogenesis. Gata6 is expressed in the myocardium, endocardium as well as in the anterior heart field. Gata6 is involved in the formation of OFT as conditional knockout of Gata6 gene led to OFT defects and

embryonic lethality by E18.5. [71,72] Mutations in Gata4 , 5 and 6 are linked with CHDs.

❖ T-box transcription factors in cardiac development

The T box (Tbx) family of transcription factors represents a group of 18 members (in mammals) divided into 5 subfamilies with Brachyury or T being the founding member. The T-box genes are evolutionarily conserved from flies to humans and for many of these T-box genes orthologs have been discovered in flies, worms and fish with high degree of structural and functional similarity. The expression of these genes is generally recorded during development and is tissue and cell specific adding to their critical role in development of those tissues.[73,74] The T box proteins possess a DNA binding element in the T-box domain that binds to a conserved consensus DNA sequence. Tbx factors can function both as transcriptional activators and repressors and can also interact with other transcriptional co-activators and co-repressors.[73,75]

Tbx1,2,3,4, 5,18 and **20** are expressed in heart during embryogenesis.[53] The expression of Tbx5 is evident as early as E7.5 at the cardiac crescent stage and later is evident in the LV and RV. Tbx5 mutation is associated with Holt Oram syndrome and mice knockout for Tbx5 die by E10.5. Similarly, Tbx1 is visible by E8 at the pharyngeal mesoderm, secondary heart field and loss of function Tbx1 genetic mutation is associated with DiGeorge syndrome.[53,74] The expression of *Tbx3* is evident by E8.5 at the heart tube stage in the non-chamber myocardium and later on outline the cardiac conduction system. Tbx3 loss of function mutation is associated with Ulnar-mammary syndrome. The expression of ***Tbx20*****,** a member of the Tbx1 sub-family, is evident at the cardiac crescent stage present in both heart fields, heart tube stage and in the endocardial cushions and also in developing valves. Like other T-box genes both gain and loss of function mutations of Tbx20 are linked with CHDs. While Tbx20 loss of function

mutations are not associated with any recognized human disease or syndrome, Tbx20 is essential for heart looping as Tbx20 knockout mice fail to complete the process and the embryos become lethal by E11.5 [76] and in humans with Tbx20 loss of function mutation develop familial dilated cardiomyopathy while Tbx20 gain of function mutations are associated with atrial septation defects, and patent foramen ovale.[77] The *Drosophila* orthologue of mammalian Tbx20 is Midline and H15[78]. The T-box domain of Tbx20 consists of 180 amino acid residues and Tbx20 has a binding affinity to T/2 site [(**5'**-…AGGTGTGA…-3' or 5'-AGGTGTGA-3', or 5'-AGGTGNTGACAG-3'.[79] Tbx20 promotes proliferation of cardiomyocytes via interaction with BMP2/SMAD1/5/8 and PI3K/AKT/GSK-3β pathways.[80]

Further, it has been known that Tbx20 directly interacts with Nkx2.5 and Gata4 and 5 to regulate cardiogenesis via promoting the expression of other , cell differentiation and proliferation.

Table 1

Gene	**Expression site during embryogenesis relative to heart**	**Associated Human disease**
Tbx1	pharyngeal endoderm, pharyngeal arch, secondary heart lineage mesoderm	DiGeorge and chromosome 22q11 deletion syndromes
Tbx2	Non-chamber myocardium	None identified
Tbx3	Non-chamber myocardium, AV and	Ulnar-mammary syndrome

	interventricular area, delineates cardiac conduction system eventually.	
Tbx4	Atrium	Small patella syndrome
Tbx5	Cardiac crescent, heart tube, common atrium, left ventricle	Holt Oram syndrome
Tbx18	Pro-epicardial organ, epicardium	None identified
Tbx20	Lateral plate mesoderm, cardiac crescent, heart tube, endocardial cushions.	None identified although most recognised phenotype is dilated cardiomyopathy.

Table 1: *T-box transcription factors in cardiac development.*

❖ **Tbx20 in adult heart under stress conditions.**

It is known that post-birth, the proliferation of cardiomyocytes significantly drops with the cells withdrawing from the cell cycle and adult cardiomyocytes binucleated as these cells do not undergo cytokinesis. The growth of heart after birth is majorly contributed by hyperplastic growth. Heart failure is a cause of global concern and Cardiovascular diseases are the major cause of morbidity and mortality.[81,82] Myocardial Infarction (MI) is the leading cause of death worldwide. MI is also is a common Ischemic disease. MI occurs when blood flow to the coronary artery is blocked generally due to rupture of an atherosclerotic plaque. Following obstruction of blood flow to a portion of the heart, a condition

of hypoxia and starvation prevails in that particular zone (known as the infarct zone) due to lack of Oxygen (O_2) and nutrients respectively.[83] The general therapy that follows Ischemia is Reperfusion where the sudden restoration of blood flow and O_2 causes a huge surge in the ROS levels leading to myocardial damage. This phenomenon of Ischemia followed by Reperfusion is otherwise known as Ischemia / Reperfusion (IR) injury[83] and accounts for massive death of cardiomyocytes. Recent studies have revoked the cell cycle withdrawal status of cardiomyocytes in light of MI or IR injury showing that following MI or IR stress conditions *in-vivo* or mimicking those conditions with either / and ROS stress induction , **starvation** or hypoxic stress inductions *in-vitro*, cardiomyocytes can re-enter cell cycle as a protective defence mechanism but this population is not enough to restore cardiac homeostasis following an MI. Strategies to induce proliferation of cardiomyocytes and reprogram fibroblasts into cardiomyocytes following MI are a hot topic to prevent further worsening and avoid an ultimate heart failure that follows. One of them is reliance on **Tbx20**.

Most of the studies relating to the T-box family members including Tbx20 are in the context of cardiac development and during embryogenesis but Tbx20 is a critical requirement in adult heart as well. Studies with Tbx20 gene conditional silencing in adult murine cardiomyocytes led to onset of heart failure.[84] Of late, studies has shown the cardioprotective effects of Tbx20 following cardiac stress both *in-vitro* and *in-vivo*. Tbx20 overexpression has been shown to improve cardiomyocyte proliferation and conversely reduce ROS mediated cardiomyocyte apoptosis in cardiomyocyte cells exposed to ROS and hypoxia injuries.[6,85] Furthermore, Tbx20 overexpression in adult murine model subjected to MI led to improved cardiac functioning, cardiomyocyte proliferation and survival post MI via interacting with BMP/Smad1/5/8, PI3K-AKT and YAP-TAZ pathways.[86] Tbx20 also known to also act as transcriptional repressor supresses cell cycle inhibitory markers like Meis, p21 and Btg2 to inhibit cell

cycle arrest and similarly Tbx20 ablation *in-vitro* showed G2/M cell cycle arrest.[77]

AIM 1: Determine the function of Tbx20 in autophagy regulation in heart

Rationale for Aim1:

Autophagy is an evolutionarily conserved catabolic phenomenon that recycles cellular components to provide for bioenergetics and fuel for cellular survival under stress conditions. At the basal level, the goal of the autophagic machinery is to maintain cellular and organ homeostasis. This is achieved by providing catabolites like fatty acids and amino acids which in turn serve as substrates for many metabolic processes. The cargo (can be organelles, protein aggregates, lipids, cellular proteins) to be degraded is sent to lysosomes via autophagosomes whereby fusion of lysosomes with the latter forms autolysosomes and the cargo is thereafter degraded by lysosomal hydrolases. Autophagy can also be activated under stress conditions like nutrient scarcity/ caloric restriction, ROS (Reactive Oxygen Species) accumulation, ER (Endoplasmic Reticulum) stress, and mitochondrial damage where autophagy serves as a substrate recycling machinery remove protein aggregates and provides much-needed ATP for the survival of cells. [9–11]Autophagy has been known to be a critical factor in the survival of neonates postpartum wherein it was found that Atg5 knockout mice models failed to survive after birth. Furthermore, in mice that survived the brief period of nutrient deprivation postpartum (which is the usual scenario), massive upregulation of cardiac autophagy was observed giving away the importance of autophagy in pro-survival while also bringing cardiac autophagy to the centre.[12] Impaired and altered autophagy has been an underlying cause of cardiac diseases like AMI, Ischemic heart disease (IHD) and cardiomyopathy. A similar role of autophagy has been found in acute myocardial infarction (AMI) wherein inhibition of autophagy enlarges the infarct zone and decreases the ATP content of cardiomyocytes and there have been theories that augmenting autophagy

would be a therapeutic approach in restoring the cellular integrity and cardiac functioning in these case[13–17] Aging is perhaps one of the greatest risk factors responsible for failing hearts. In fact, aged individuals with no underlying cardiac condition show poor cardiac functionality, diastolic function and left ventricular dilatation. [18–20]Accumulation of protein aggregates, misfolded proteins, a poor balance between ROS and anti-oxidants, mitochondrial derangements, attenuated expression of Sirtuins (a class of NAD^+ dependent deacetylating enzymes) especially Sirtuin 1, 3 (Sirt 1,3), GSk-3β contribute to cardiac aging.[21–23] As such, impaired and poor levels of autophagy are prevalent in aging hearts while apoptotic levels are on a surge. Augmenting autophagy by either calorific restriction or Rapamycin (Rap) administration has shown improvement in cardiac functioning and improved life longevity.[24–26]

Tbx20, a T-box transcription factor, is known to be crucial for cardiogenesis and murine models with Tbx20 knockout fail to survive beyond E10.5 Tbx20 promotes cardiomyocyte proliferation via the Bmp2/pSmad1/5/8 and PI3K/AKT/GSK3β/β-catenin signaling pathways.[4] Tbx20 has also been found to act as a cardio-protectant against oxidative stress and downregulation of Tbx20 has been linked to increased apoptosis in cultured rat cardiomyocytes.[5] Further, the cardio-protective role of Tbx20 under ROS and hypoxic conditions in the H9c2 cell line was reported. [6] Tbx20 is known to interact with and induce other transcription factors like Nkx2.5 and Gata4 [7,8] which are also important for cardiogenesis, maintaining cardiac homeostasis and promoting the expression of Troponin-I and myosin heavy chain protein but its role in autophagy in heart is unknown. Therefore the specific **aim1** of this study is :

Determine the function of Tbx20 in autophagy regulation in heart

The function and role of Tbx20 under the influence of autophagy is studied through *in-vivo* system in adult mice model as well as in-vitro system using H9c2 cells, discussed in elaboration in Chapter 2 (of this thesis). Tbx20 as a potential candidate to induce anti-senescence-like characteristics in the aging mice

population is studied in both in-vivo. Autophagy induced expression of Tbx20 activates GSK-3β and transcription factors Nkx2.5, Gata4 and Sirt1 after subjection to starvation (Strv) and rapamycin treatment in both *in-vivo* (BALB/c mice) and *in-vitro* (H9c2 cell line) model systems. The upregulation of Nkx2.5 and Gata4 following autophagy induction is indicative of progression towards progenitor like cardiomyocyte characteristics, while activation of Sirt1 and GSK3β suggests an anti-aging/ senescence since Sirtuin1 is closely linked with aging, is known to be a mediator of caloric restriction and Sirt1 transgenic mice prevent early mortality. With pre-existing knowledge of the expression of Sirt1 and GSK-3β, under autophagy conditions along with the cardioprotective roles they play, these two were chosen as possible candidates that could interact with Tbx20. Further, Tbx20 loss of function (LOF) assay in the H9c2 cell line validated Tbx20-dependent expression of Sirt1, GSK-3β, Nkx2.5 and Gata4.

❖ ECM in heart development and disease

The ECM (Extracellular matrix) plays an important role during embryogenesis in general and cardiogenesis in particular. In addition to providing a scaffold and maintaining structural integrity, it provides a spatiotemporal regulation of an array of cellular events. Cellular adherence to the matrix via integrins reduces the chances of cellular apoptosis. The major ECM molecules found in heart include proteoglycans, hyaluronan, Collagens, Periostin, fibrillin and fibronectin. Disruption of genes involved in matrix formation during embryogenesis can lead to cardiac malformations.

Hyaluronic acid (HA) is found in the cardiac jelly at the heart tube stage and later in OFT cushions and around the cardiomyocytes in the interstitial space.[87] Major proteoglycans found in developing heart are versican and aggrecan. Versican is a type of chondroitin sulphate proteoglycan. There are 4 isoforms of versican termed V0 through V4. The isoforms V0 and V1 are abundantly expressed in the

cardiac jelly, AV valves , OFT cushions while V2 is expressed predominantly in the myocardium and cardiac neural crest. Mice embryos homozygous null for Versican die by E10.5. Similar to versican, aggrecan also is a chondroitin sulphate proteoglycan and is also expressed in the cardiac jelly, in the epicardium and the AV valves.[87,88] As, the OFT and AV valves mature the expression of aggrecan significantly declines. Inhibited expression of both Versican and Aggrecan during the later stages of valve maturation and remodeling are attributed to Adamts, a matrix metalloproteinase that cleaves aggrecan and versican.

Collagen (Col) is produced particularly by fibroblasts and developing mesenchyme. Collagen types I, III and V are found in the leaflets of AV valves, atrial wall and in vasculature of mice. Col-I ablated mice are lethal between E12 and E14 sue to defects in microvessel formation. E1Collagen III knockouts show post-natal death due to defects in cardiovascular and brain development. Col V deficient mice show early embryonic lethality by E10 due to defects in fibril and valve formation defects. Similarly Col-XI deficient mice die at birth possibly due to thickening of AV valves and interventricular septum.[89] Periostin , a secretory ECM molecule is a member of fasciclin gene family. In avian developing hearts, the expression of Periostin is observed at HH (Hamburger Hamilton) 17 in the developing myocardium at the endothelial side and later spreads to the OFT cushions by HH21.[87] Periostin null mice have shortened mitral and tricuspid valve leaflets. The expression of Collagen also is repressed in the valve leaflets. Fibrillin is an elastin type ECM molecule. Fibrillin is expressed in the developing valve leaflets and continues to express till the last stages of valve maturation. Mutations in Fibrillin1 are associated with Marfan' syndrome, a connective tissue disorder. A common manifestation of Marfan's is thickened AV valves and aortic aneurysm. Fibronectin another ECM molecule known to interact with integrins, proteoglycans and Collagens is expressed in the pharyngeal arch arteries (PAA), endocardial cushions and is required during EMT regulated development.

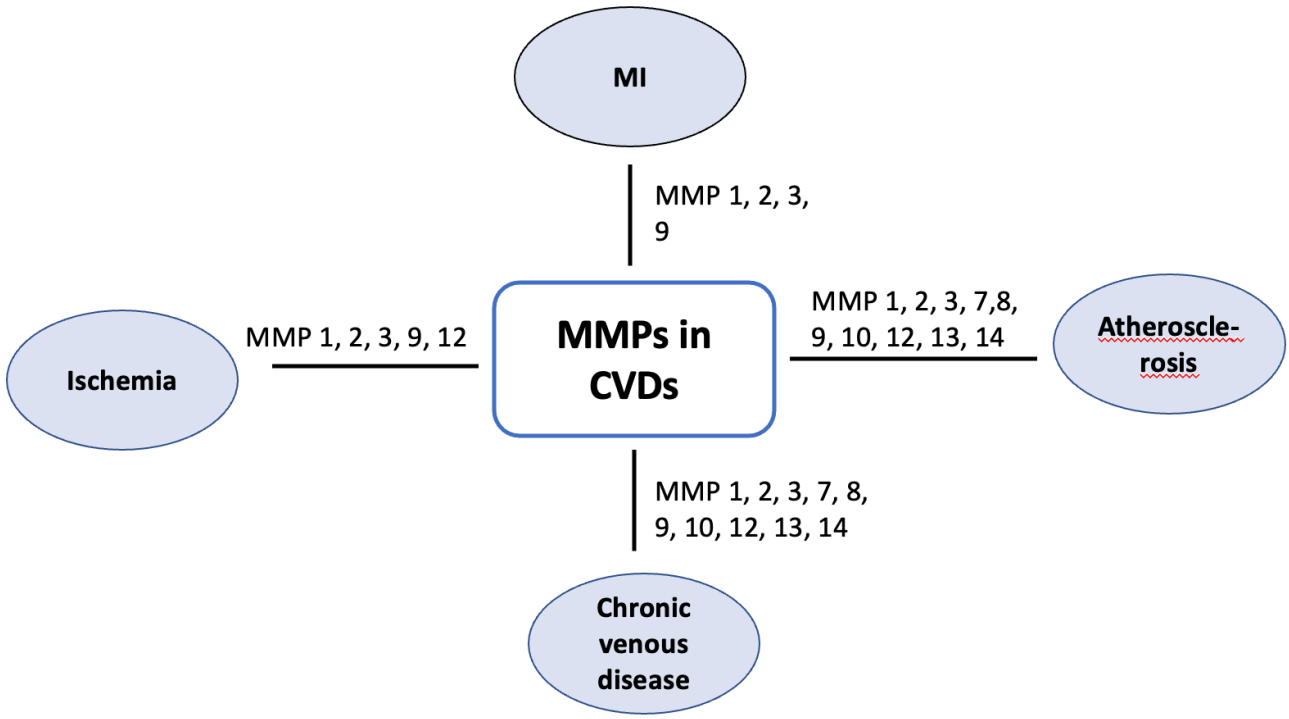
Fibronectin deficient mice embryos fail to fuse into the linear heart tube and thus do not survive beyond E10.[87]

While the ECM does provide an important 3D scaffold maintaining the structural integrity and homeostasis of heart by orchestrating cellular responses, the changing profile, composition and ratio of its constituents have been heavily implicated in pathogenesis of cardiovascular diseases. The ECM takes prime stage during pathophysiology of myocardial disease. The significant changes in the composition and biochemistry that led to a dysregulated remodeled ECM is of particular importance to tissue and cellular functioning. MI and cases of IR are generally found associated with expansion of fibrotic network as a result of ECM remodeling.

The expansion of fibrotic network otherwise known as replacement fibrosis[90] is the end result of reparative process following a MI or IR injury since adult cardiomyocytes are unable to proliferate and even if a small population does (<1%)[86] it is hugely ineffective to fill up the infarct space and accommodate for the massive death of cardiomyocyte that ensues after a MI, so the ECM compensates for this by promoting synthesis of Collagens and synthesis, proliferation of fibroblasts and even switching of fibroblasts to myofibroblasts to compose a fibrotic scare in order to prevent ventricular dilation. Myofibroblasts produce ECM that differs from the one fibroblasts synthesise in order to promote fibrosis. One of the key components of ECM, the MMPs (matrix metalloproteinase) also known as matrixins have been emphasised heavily in myocardial diseases. The MMPs are a family of at least 25 members recognised in mammals.[91] Activation of MMPs can be detected as early as 10 minutes into coronary occlusion.[90] MMPs can be collagenases, gelatinases, stromelysins and matrilysins in biochemical nature. The surge in cardiomyocyte necrosis that ensues in an Ischemic response triggers the matrix degrading action and huge amounts of MMPs are synthesised by fibroblasts, endothelial cells and leucocytes These MMPs further help in the migration of inflammatory cells like interleukins

and cytokines by digesting the matrix and clearing the way. MMPs heve been found to be significantly upregulated in a number of cardiac diseases such as MI, pressure and volume overload systems, atherosclerosis and coronary artery disease.[36,91,92] The reparative process of healing following a cardiac injury often lead to uncontrolled fibrosis as a result of extensive Collagen inter-linkage leading to myocardial stiffness which adversely impacts systolic functioning. Further, remodeled deposition of ECM deprives cardiomyocytes of pro-survival signaling mechanisms that an intact normal ECM would help transduce.[93] Inhibition of MMPs by TIMPs (tissue inhibitors of matrix metalloproteinases) have some implications in
improvement of cardiac functioning following a cardiac injury.[94]

Figure 1



[Figure 1: MMPs linked with Cardiovascular diseases (CVDs). **]**

AIM2: **Study the differential ECM remodeling and affected associated signaling pathway through induction of ROS, hypoxia and high glucose mediated injury inductions in cardiac cell line.**

Rationale for Aim2:

ECM remodeling in heart or cardiac remodeling remains an important factor in the pathophysiology of cardiovascular diseases.[27,28] Collagen-I forms the major component of the matrix interstitium of the myocardium in addition to Collagen-III, fibronectin, proteoglycans, tissue inhibitors of matrix metalloproteinases (TIMPs) and matrix metalloproteinases (MMPs). There are three stages of cardiac remodeling following cardiac injury namely- inflammatory, proliferative and maturation phases leading to a mature scar formation.[29] The preliminary stages of ECM remodeling are necessary as it prevents rupture of the ventricular wall, however, exacerbated ECM remodeling leads to progressive fibrosis in the heart and cardiac disfunctioning.[30,31] The MMPs (zinc-dependent proteases) are involved in the turnover of matrix proteins like Collagen.[32] **Adamts4**, a member of Adamts family is an important MMP. Adamts4, also is a disintegrin with thrombospondin like motifs.[33,34] The mode of action of Adamts4 is by binding to ECM proteins and thereafter cleaving ECM proteoglycans like aggrecan, versican, brevican in addition to regulating Collagen turnover.[35,36] Adamts4 modulates the pathophysiology of osteoarthritis through degradation of matrix proteoglycans and eventually lead to cartilage degradation which manifests as degenerative osteoarthritis.[37,38] Besides osteoarthritis, Adamts4, has also been linked with cancer and angiogenesis where its role remains controversial. Some studies report it to be an indicator of early-stage cancer like in cases of colorectal cancer, others findings suggest that its mutated and truncated fragments may suppress tumour growth through inhibition of angiogenesis. [39,40] However, the involvement of Adamts4 in cardiac remodeling is relatively less known. Only a few studies have shown the involvement of Adamts4 in atherosclerotic plaque

development.[41] and recent studies have shown elevated expression of both Adamts4 and Adamts1 in patients with acute aortic dissection and coronary artery disease.[42,43]

Therefore, **Aim 2** of this study is to study the differential ECM remodeling and affected associated signaling pathway through induction of ROS, hypoxia and high glucose mediated injury inductions which is elaborated and discussed in Chapter 3 of this thesis. To better understand the molecular insights of Adamts4 induction and associated affected signaling pathway activation, we have used H9c2, a rat ventricular myoblast cell line for several *in vitro* assays. Likewise, Adamts4 expression was induced in H9c2 cells, subjected to hypoxia (Hyp) and ROS injury inductions *in vitro*. Moreover, the expression of Adamts4 was manipulated with siRNA-mediated loss of function and TGF- β inhibitor studies in H9c2 cells to evaluate its regulation and dependency on TGF- β signaling since TGF-β has been long known to be a characteristic marker for inflammatory and fibrotic responses following pathological stress including MI, ischemia and reperfusion (I/R) injury.[44–48] Finally and most importantly, we also validated our hypothesis in human clinical samples and demonstrated the induced expression of ADAMTS4 in patients with indicated cardiac ailments. To study the role of Adamts4 In diabetic cardiomyopathy H9c2 were pre-conditioned and grown at low concentration (5mM) supplemented glucose as the control group and the cells to be treated were grown in media supplemented with 20mM of glucose for 48 hours following which the expression of Glut1, Collagen-III and Adamts4 was studied with the help of qPCR and / or Western Blot. An enhanced expression of Adamts4 indicated the involvement of Adamts4 in ECM remodeling in the context of diabetic cardiomyopathy also.