ROLE OF STAT3 FROM EMBRYONIC STEM CELL INTO CARDIAC LINEAGE DIFFERENTIATION AND ITS CARDIOPROTECTIVE ROLE IN ADULT HEART

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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Wong Yu Qian

20 January 2016

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In vitro-derived cardiomyocytes are ideal for use in cell replacement therapy as they constitute a renewable source of wide range of heart cell types that can be produced in large numbers for replacement. However, the transition from bench to bed necessitates a deeper understanding of the regulatory networks of early cardiomyocyte differentiation as it takes place in the early development of embryo. Studies have demonstrated that STAT3 is essential for initial stages of cardiomyogenesis. Despite the important role of STAT3 in cardiomyogenesis, few studies have explored that the role of STAT3 in early cardiomyocyte differentiation because conventional STAT3 KO is embryonic lethal at the time of formation of cardiomyocyte. In this study, we have established an *in vitro* inducible STAT3 knock out and pSTAT3 overexpressed ES cells system in which STAT3 expression can be temporally and specifically deleted or overexpressed by doxycyclin stimulation to allow the study of STAT3 function during early cardiomyocyte differentiation. We show STAT3 expression to be temporal and downregulates in the early phrase of cardiac differentiation but upregulates towards late phrase. We also show an important stage specific role of STAT3 in regulating cardiomyocyte differentiation from ESCs. In addition, STAT3 is found to bind on the promoter region of GATA6 in ESCs. Lastly, we propose a potential regulatory role of STAT3 on promoting cardiomyocyte differentiation through direct transcriptional regulation of GATA6 expression.

Summary II

Cardiovascular disease has become a leading global cause of illness and mortality worldwide. Previously, we have reported that increased apoptosis, inflammation and cardiac fibrosis were observed in aged and also under endotoxin-stress (LPS) STAT3 deficient mice compared to those in wild-type. However, the molecular mechanisms by which STAT3 promotes cardiomyocyte survival and cardiac function remained unclear. Here, we study the possible underlying pathological mechanism regulated by STAT3. Real-time PCR results show that acute LPS treatment which activated STAT3 downregulates Txnip transcript in H9c2 myoblast and wild-type heart in a time-dependent manner. Moreover, western blot assay demonstrates that high expression of Txnip is found in the aged heart of STAT3 deficient mice, indicating that Txnip expression might be regulated by STAT3. Earlier reports of published STAT3 ChIP-sequencing results in non-cardiac cells revealed that Txnip promoter is bound by STAT3. Our present ChIP assays validate the published STAT3 ChIP results in which after LPS treatment, STAT3 binds on Txnip promoter to regulate its gene expression. Furthermore, increased generation of ROS and induction of inflammasome are observed in STAT3 deficient mice as a result of elevated Txnip level which subsequently lead to heart failure. Taken together, our results first address a novel regulatory role of STAT3 in the heart and highlighted the importance of this STAT3-Txnip signaling cascade which seems to have in the physiology and pathogenesis of heart failure and hence to innovate translational seeds for clinical application.

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List of Abbreviations

AMI	Acute myocardial infarction
ANF	Atrial natriuretic factor
ASC	Apoptosis-associated speck-like protein containing a CARD
ASK1	Apoptosis signal regulating kinase
BMP	Bone morphogenetic proteins
BNP	Brain natriuretic peptide
CARD	Caspase recruitment domain
CA-STAT3	Constitutive activated STAT3
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CT-1	Cardiotrophin 1
CVB3	Coxsackievirus B3
DOX	Doxycycline
dpf	Days post-fertilization
EB	Embryoid body
ECM	Extracellular matrix
ESC	Embryonic stem cell
FACS	Fluorescent-activated cell sorting
GFP	Green fluorescent protein
GOI	Gene of interest
gp130	glycoprotein 130
4HT	4-hydroxytamoxifen

Id	inhibitor of differentiation
IL	Interleukin
IP	Ischemia preconditioning
iPSC	induced pluripotent stem cell
I/R	Ischemia-reperfusion
JAK	Janus kinase
KO	Knock out
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
MEF2	Myocyte enhancer factor2
α-MHC	α-myosin heavy chain
MI	Myocardial infarction
Nanog	Nanog homeobox
NLR	Nucleotide-binding domain leucine rich repeat
OCT4	Octamer-binding transcription factor 4
PAMPs	Pathogen-associated molecular patterns
PC	Postconditioning
PCR	Polymerase chain reaction
PHF	primary heart field
pTRE	Tetracycline responsive element
PRRs	Pattern recognition receptors
PYD	Pyrin
qRT-PCR	Quantitative reverse transcriptase-PCR

ROS	Reactive oxygen species
SEM	Standard error of the mean
SH2	Src-homology2
SHF	Secondary heart field
Smad	Mothers against decapentaplegic homolog
SOCS	Suppressor of cytokine signaling
Sox2	SRY-related HMG box
STAT	Signal Transducer and Activator of Transcription
STAT3-CKO	Cardiomyocyte-specific STAT3 KO
STAT3 pY705	Tyrosine 705 phosphorylation
TetR	Tetracycline-control transcriptional activation
Th	T helper
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-α
Trx	Thioredoxin
TrxR	Thioredoxin reductase
TSS	Transcription start site
Txnip	Thioredoxin-interacting protein
VEGF	vascular endothelial growth factor

Chapter 1 Introduction

1.1 Signal Transducer and Activator of Transcription (STAT)

1.1.1 STAT proteins

Our lab has studied STAT (Signal Transducer and Activator of Transcription) proteins, especially STAT3 in different systems for a long time. The STAT proteins are well conserved through evolution (Zhang et al., unpublished data), and are originally described in the interferon-induced regulatory pathways (Darnell, Kerr, & Stark, 1994; Fu, Kessler, Veals, Levy, & Darnell, 1990; Fu, Schindler, Improta, Aebersold, & Darnell, 1992; Schindler, Shuai, Prezioso, & Darnell, 1992). Seven STAT family members exist in mammals, namely STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (Darnell, 1997) and they are characterized by having common functional domains. These include a conserved amino-terminal for tetramerization, a DNA-binding domain, a Src-homology2 (SH2) domain for receptor binding and STAT dimerization and a carboxyl-terminal transactivation domain which consists of phosphorylation site for maximal transcription activity (Booz, Day, & Baker, 2002; Hilfiker-Kleiner, Hilfiker, & Drexler, 2005).

1.1.2 STAT Function and Activation

STAT family proteins are latent cytoplasmic protein but act as transcription factors in the nucleus which play crucial roles in immunity, cell growth, survival differentiation and tumorigenesis (Darnell, 1997; Darnell et al., 1994). It can be stimulated by wide array of ligands, including cytokines, interleukins, interferons, growth factors and hormones (Bromberg, 2001; W. J. Leonard & O'Shea, 1998).

Various members of STATs family display certain distinct and nonetheless some similar functions. STAT1 has been illustrated to play a role in anti-bacterial, anti-virus, apoptotic, growth inhibitory, and tumor suppressive responses (Durbin, Hackenmiller, Simon, & Levy, 1996; Meraz et al., 1996), while STAT3 generally has the opposed function, including anti-apoptosis, cell proliferation, survival, oncogenesis and inflammation. STAT4 and STAT6 are involved in the development of T helper 1 (Th1) and T helper 2 (Th2) cells in the immune system (Kaplan, Schindler, Smiley, & Grusby, 1996; Kaplan, Sun, Hoey, & Grusby, 1996; Shimoda et al., 1996; Takeda et al., 1996; Thierfelder et al., 1996), whereas STAT5a and STAT5b are involved in hormone signaling (Bromberg, 2001; Lim & Cao, 2006).

STAT3 belongs to one of the seven STAT family members, is a well-known transcription factor participating in a wide variety of biological processes such as embryonic stem cells pluripotency maintenance, embryogenesis, cardioprotection, cancer and immunity (Hilfiker-Kleiner et al., 2005; Levy & Darnell, 2002; Takeda et al., 1997; Ying, Nichols, Chambers, & Smith, 2003; Yu, Pardoll, & Jove, 2009). Notably, STAT3 is stimulated by interleukin-6 family of cytokines, include leukemia inhibitory factor (LIF), interleukin-6 (IL-6), oncostatin M (OSM), cardiotrophin-1 (CT-1) and ciliary neurotrophic factor (CNTF) (Heinrich, Behrmann, Muller-Newen, Schaper, & Graeve, 1998). These molecules signal through transmembrane receptor, glycoprotein 130 (gp130) (Heinrich et al., 1998).

In a classical STAT activation pathway, following dimerization of gp130 by cytokines binding, cytoplasmic protein tyrosine kinases in the JAK family (JAK1, JAK2, and TYK2) which are associated with the membrane-proximal portion of gp130 are rapidly activated by autophosphorylation. Activated JAK sequentially phosphorylates the receptor to which it has bound, which enables STAT docking to this complex via binding of SH2 domain of STAT to the phosphotyrosine residue of the receptor. This leads to phosphorylation of STAT and triggers its release and dimerization. Dimerized phosphorylated STAT proteins rapidly translocate to nucleus and bind to certain gene promoters with specific STAT binding sequences and thereby involved in regulating the transcriptional activation of target genes. Therefore, the JAK-STAT pathway denotes an enormously rapid signaling system from the cell surface to the nucleus and elucidates at least part of the basis for the specificity of signals that are induced by different cytokines (W. J. Leonard & O'Shea, 1998; Levy & Darnell, 2002). (Figure 1.1)

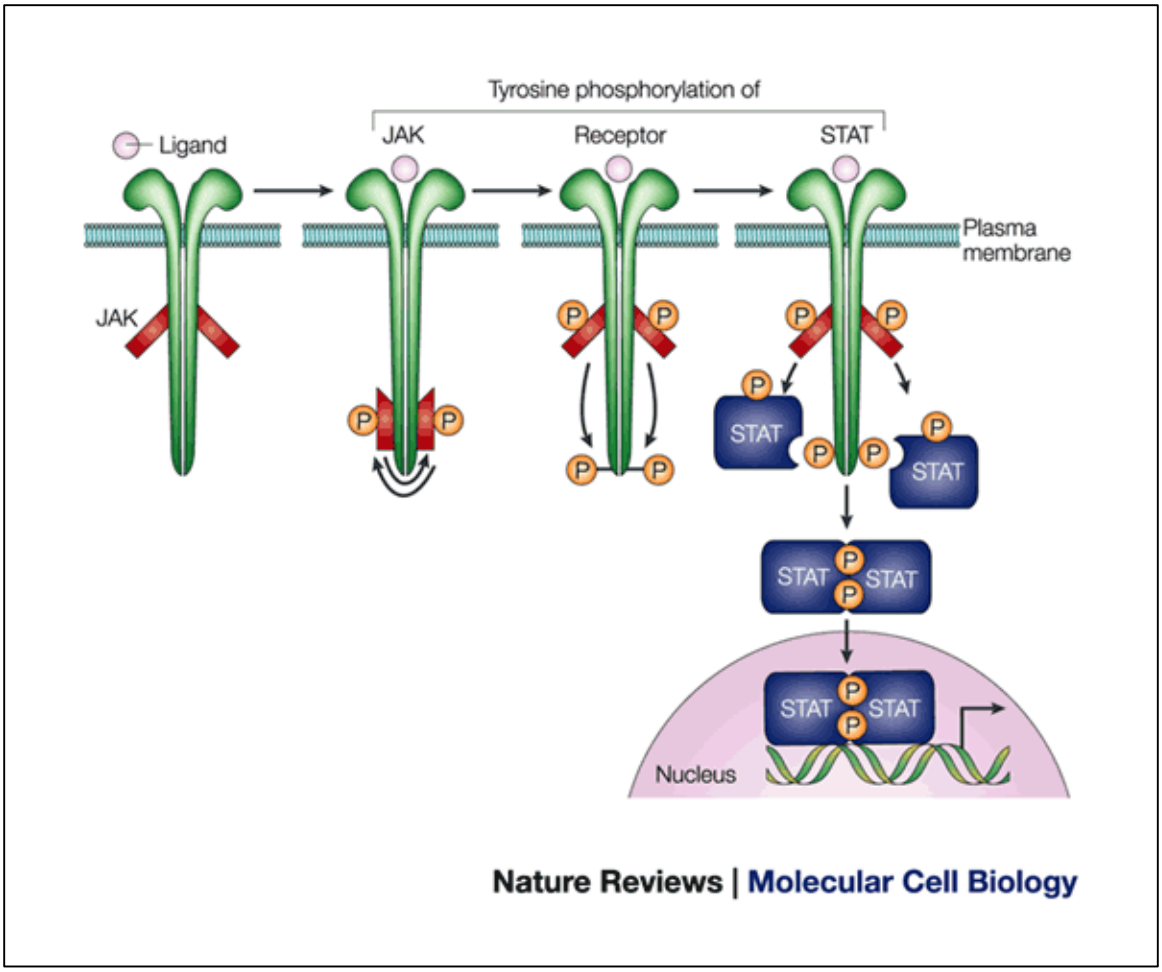


Figure 1.1 JAK-STAT signal transduction pathway.
Cytokine-receptor interaction leads to sequential tyrosine phosphorylation. Once the ligands bind to homodimeric or heterodimeric receptors, Janus kinases (JAK) will undergo transphosphorylation and activated JAK phosphorylates the tyrosine residue of the cytoplasmic domain of the receptors. Phosphorylated sites by JAK kinases serve as docking site for STAT protein. STAT protein will then bind to the phosphorylated site of receptors via SH2 domain. Phosphorylated STAT protein will then homodimerize or heterodimerize before translocation into nucleus and bind to targeted sequence and regulate gene expression.
Figure and caption adapted from (Levy & Darnell, 2002).

1.2 STAT3 in embryonic stem cells pluripotency maintenance

1.2.1 Embryonic stem cells (ESCs)

Embryonic stem cells (ESCs) originate from the inner cell mass of blastocyst, which is an early stage of preimplantation embryo (A. G. Smith, 2001; Thomson et al., 1998). ESCs have high pluripotency to differentiate to all somatic cell types of the adult body and germ cell lineage and they can be cultured indefinitely *in vitro* through self-renewing division (M. J. Evans & Kaufman, 1981; Martin, 1981). Moreover, ESCs will contribute to all tissues formation, including the germ cells in the resulting chimeric mice when reintroduced into blastocyst (Bradley, Evans, Kaufman, & Robertson, 1984).

1.2.2 Self-renewal of ES cells requires STAT3

Self-renewing state of mouse ES cells is maintained in the presence of extrinsic factors such as cytokine leukemia inhibitory factor (LIF) (A. G. Smith et al., 1988; Williams et al., 1988). LIF belongs to the interleukin-6 family of cytokines engages a heterodimeric receptor complex consisting of LIF receptor and gp130, resulting in the activation of JAK and subsequently STAT3 phosphorylation (Boulton, Stahl, & Yancopoulos, 1994). STAT3 activation is essential to maintain ES cells self-renewal (Matsuda et al., 1999; Niwa, Burdon, Chambers, & Smith, 1998; Raz, Lee, Cannizzaro, d'Eustachio, & Levy, 1999), while interruption of STAT3 leads to ES cells differentiation (Ernst, Novak, Nicholson, Layton, & Dunn, 1999; Niwa et al., 1998).

Several investigations have put forth evidence indicating that in the absence of LIF, constitutively active of STAT3 is sufficient to inhibit mouse ES cells differentiation (Matsuda et al., 1999). By using a conditionally active form of STAT3, namely, a fusion protein between STAT3 and estrogen receptor (ER) ligand binding domain, in which STAT3 is activated in response to the synthetic ligand 4-hydroxytamoxifen (4HT), activation of STAT3 is adequate to sustain the self-renewal of ES cells (Matsuda et al., 1999). In contrast, expression of STAT3 dominant-negative mutant using an inducible promoter in ES cells abolishes the self-renewal of ES cells as maintained by LIF and promotes differentiation (Niwa et al., 1998; Raz et al., 1999).

However, it is unclear how downstream targets of LIF/JAK/STAT3 signaling play a preeminent role in the control of ES cells pluripotency maintenance by interacting with the master regulator of pluripotency factors for ES cells; namely, Oct4, Nanog, and Sox2 (Boyer et al., 2005; Loh et al., 2006). Until recently, STAT3 has been reported that it directly regulates Oct4 and Nanog transcription to maintain pluripotency of ES cells and iPSCs. Knockdown of STAT3 expression in ES cells leads to substantial reduction of Oct4. Furthermore, STAT3 binds directly to the distal enhancer of Oct4 and Nanog and positively regulates Oct4 and Nanog to maintain pluripotency of ES cells (Do et al., 2013).

Although LIF-signaling is sufficient to maintain pluripotency of ES cells in serum conditions, LIF alone, however, is inadequate to maintain ES cells in serum-free

conditions, as the presence of fetal calf serum is required for the maintenance. Additional factors present in serum are crucial for the pluripotency maintenance. Recently, it was shown that bone morphogenetic proteins (BMPs) appear to be key serum-derived factors that act in combination with LIF to sustain mouse ES cells self-renewal and pluripotency in serum-free condition (Ying et al., 2003). BMP4 binds to its receptor and triggers the phosphorylation of Smad1 which drives expression of inhibitor of differentiation (Id) genes. As forced expression of Id genes in ES cells can renew in the absence of BMP4, induction of Id expression is therefore critical contribution of the BMP/Smad pathway (Ying et al., 2003). Activated STAT3 and Smad1 form a complex in ES cells following stimulation by LIF plus BMP which inhibit differentiation into mesoderm and endoderm lineages. Thus, LIF/STAT3 and BMP/Smad signaling act in conjunction on distinct target genes and/or commence on common target genes in the maintenance of a stem cell pluripotency phenotype.

1.3 STAT3 is involved in early embryo development and cardiomyogenesis

1.3.1 STAT3 in early embryogenesis

STAT3 plays a crucial function in early mammalian embryogenesis and determination of various cell lineages differentiation. Expression of STAT3 is highly detected in mouse oocytes and becomes phosphorylated and translocates into the nucleus in the four-cell and later stage embryo, indicating that activated STAT3 is present in preimplantation embryos (Do et al., 2013). During mouse embryogenesis, high-level expression of STAT3 mRNA initiates at around E7.5 in

the embryo itself. In addition, STAT3 mRNA was identified in a number of tissues including the yolk sac endoderm, myometrium, cephalic mesenchyme, and blood islands by E9.5 (Duncan, Zhong, Wen, & Darnell, 1997). Detection of STAT3 activity during early postimplantation development in the mouse suggests that STAT3 is crucial during early embryogenesis. In addition, the subsequent differentiation into various cell lineages including the development of many specific organs such as liver development, myeloid cells differentiation, skin remodeling, angiogenesis and astrogenesis by neutral stem cells require STAT3 activation (Chakraborty & Tweardy, 1998; Matsui, Kinoshita, Hirano, Yokota, & Miyajima, 2002; Rajan & McKay, 1998; Sano et al., 1999; Valdembri, Serini, Vacca, Ribatti, & Bussolino, 2002) .

Among the STATs, STAT3 is the only member in which STAT3 null mice were found to be embryonic lethal at E6.5 to E7, suggesting that STAT3 is crucial for early embryo development (Takeda et al., 1997). Subsequently, studies of tissue-specific functions of STAT3 have been carried out using Cre-loxP recombination system driven by discrete promoter in specific tissue. Tissue-specific gene targeting demonstrated that STAT3 functions in would healing in keratinocytes, mammary involution, regeneration of liver, survival of neuron cell, development of Th17 and T cell proliferation and cytoprotection of respiratory epithelium during adenoviral infection (Alonzi et al., 2001; Chapman et al., 1999; Matsuzaki et al., 2006; Murase, Kim, Lin, Hoffman, & McKay, 2012; Sano et al., 1999; Takeda et al., 1998; X. O. Yang et al., 2007).

1.3.2 Early heart development

The heart is the first definitive organ to form and function during mammalian embryonic development (Fishman & Chien, 1997; Olson & Srivastava, 1996). The heart is made up of muscle and non-muscle lineages: atrial/ventricular cardiac myocytes, conduction system cells, smooth muscle/endothelial cells of the coronary arteries and veins, endocardial cells, valvular components and connective cells. Development of heart requires the concurrent differentiation of these cardiovascular cell types that must be organized into a complex organ. There are three major sources of heart cell precursors: cardiogenic mesoderm, cardiac neural crest and the proepicardial organ. Cardiogenic mesoderm forms myocardium, neural crest contributes to vascular smooth muscle of aortic arch and cardiac autonomous nervous system while the proepicardium forms the endothelial, smooth muscle and connective tissues (Kirby, Gale, & Stewart, 1983; Laugwitz, Moretti, Caron, Nakano, & Chien, 2008).

During gastrulation, cardiac myocytes progenitors migrate to the anterior and anterior/lateral ends of the embryo to form heart fields (Rawles, 1943; Solloway & Harvey, 2003). The primary heart field (PHF) is derived from the anterior lateral mesoderm and forms the cardiac crescent (Rosenquist, 1970). In later stages of embryonic development, the cardiac crescent proceeds to fuse and forms a primitive linear heart tube at approximately E7.5 to E8, which eventually forms the left ventricles (Arai, Yamamoto, & Toyama, 1997; Chien, Domian, & Parker, 2008). Distinctive beating of cardiac cells within the developing heart begins at

approximately E7.5 to E8.5 during tube formation (Ji et al., 2003). Shortly thereafter, contractile linear heart undergoes rightward looping to create asymmetrical, curved tube (Brand, 2003; R. G. Kelly & Buckingham, 2002; Yutzey & Kirby, 2002). Chamber maturation and septation occur to give rise to a functional four-chambered heart as the embryo continues to develop (Brand, 2003). Adjacent areas added to the heart at the time of cardiac looping is termed as the secondary heart field (R. G. Kelly, Brown, & Buckingham, 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). The secondary heart field (SHF) is derived from the pharyngeal and splanchnic mesoderm and forms the right ventricle, the outflow tract, the atria and posterior regions of left ventricle (R. G. Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). (Figure 1.2)

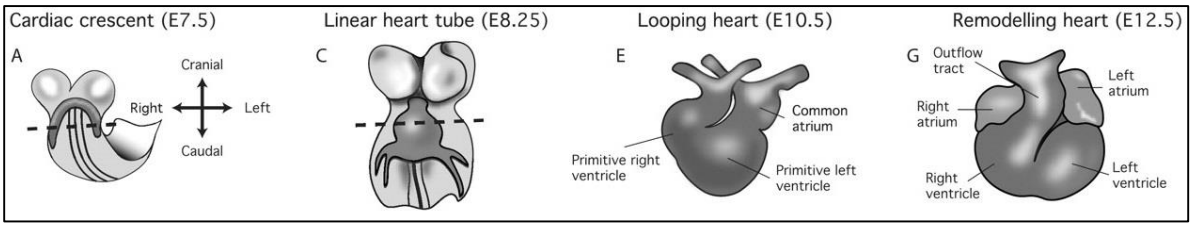


Figure 1.2 Heart development.
The main transitions that occur in early mammalian heart development. Days of embryonic development (E) is staged based on mouse development. Dark shading represents myocardial tissue.
Figure and caption adapted from (Harvey et al., 2002).

1.3.3 STAT3 in early cardiomyogenesis

STAT3 conventional all-tissue knockout mice have previously been found early embryonic lethal at E6.5 to E7, as a result of embryos rapidly degenerated between E6.5 and E7.5 with no obvious mesoderm formation (Takeda et al., 1997). In fact, STAT3 ablation resulted in embryonically lethal less than 1 day before the embryo

would have develop beating cardiomyocyte at E7.5 to E8.5 (Foshay, Rodriguez, Hoel, Narayan, & Gallicano, 2005; Hao et al., 2005; Kotenko et al., 1996; Takeda et al., 1997). Earliest evidence revealed that STAT3 was confined to areas within the embryo, including the presumptive heart field, and was active when cardiomyocyte would be occurring at E7.5 to E8.5 (Duncan et al., 1997). Hence, STAT3 is essential for initial stages of cardiomyogenesis.

The initial regulatory mechanisms which control differentiation of progenitor cells into beating cardiomyocyte is under investigation due to the difficulty of procuring cardiac-committed mesoderm to explore specified signal transduction mechanisms in mammalian development and as well as difficult to analyze the few cardiomyocytes available from early heart tubes. Until recently, a cardiac *in vitro* model system was developed using ES cells. Among the specialized cell types that can form in culture during ES cells differentiation, *in vitro*-derived cardiomyocytes are distinguished by rhythmically contracting structures containing cells that are highly reminiscent of normal heart cells (Boheler et al., 2002; Passier, Denning, & Mummery, 2006). They express cardiac-specific genes including α-myosin heavy chain (α-MHC), cardiac troponin C and the atrial natriuretic peptide (ANP) similar to adult cardiomyocytes (Wobus, Rohwedel, Maltsev, & Hescheler, 1995). Moreover, the electrophysiology characteristics and ion channel expression of these beating cells have been documented and verified as closely mimicking embryonic and post-natal cardiomyocyte electrophysiology (Hescheler et al., 1999; Maltsev, Wobus, Rohwedel, Bader, & Hescheler, 1994; Wobus, Wallukat, & Hescheler,

1991). Because this *in vitro* differentiation of ES cells to cardiomyocyte closely mimics the sequential stages of developing embryonic cardiomyocyte, it is used widely to study early initial stages of cardiac differentiation.

It was illustrated that JAK2 signals through STAT3 to direct cardiomyogenesis in mouse ES cells. Firstly, expression of STAT3 and its activity is higher in cells of beating areas compared with cells on nonbeating areas. While inhibition of STAT3 by dominant negative of STAT3 resulted in significantly fewer beating areas within embryoid body compared with controls. In addition, STAT3 inhibition of ES cells leads to downregulation of key specific cardiac genes (Foshay et al., 2005). Evidently, STAT3 plays a fundamental role in early cardiomyogenesis, in which STAT3 is required during initial remodeling of cardiac progenitor cells to drive the differentiation of cardiomyocytes from ES cells.

1.4 GATA transcription factors and cardiac development

1.4.1 The GATA family of transcription factors

Members of the GATA family are zinc finger-containing transcription factors that play critical roles in cell growth and development, regulation of differentiation including in cell-fate specification and control of cell proliferation and movement. Six different family members of GATA transcription factors (GATA-1-6) are characterized from vertebrate (Laverriere et al., 1994; Yamamoto et al., 1990). They all contain two similar but distinct repeats of a highly conserved zinc fingers required for sequence-specific binding, usually (A/T)GATA(A/G) and for protein-

protein interactions (T. Evans, Reitman, & Felsenfeld, 1988; Omichinski, Clore, et al., 1993; Omichinski, Trainor, et al., 1993).

The first subgroup is composed of GATA-1, -2, and -3 which are expressed in hematopoietic lineages (M. Leonard, Brice, Engel, & Papayannopoulou, 1993) and are essential for normal hematopoiesis (Pandolfi et al., 1995; Pevny et al., 1991; Simon, 1995; Tsai et al., 1994; Zheng & Flavell, 1997). The second subgroup is consisted of GATA-4, -5 and -6, which are expressed in cardiac tissues (Gove et al., 1997) and endoderm-derived tissues (Arceci, King, Simon, Orkin, & Wilson, 1993; Laverriere et al., 1994; Morrisey, Ip, Lu, & Parmacek, 1996; Morrisey, Ip, Tang, Lu, & Parmacek, 1997).

Within the heart, GATA4 appears to be one of the earliest transcription factors expressed in developing murine cardiac cells and its mRNA is presents throughout the developing myocardium and endocardium and also detected at high level in the postnatal heart (Grepin et al., 1994; Heikinheimo, Scandrett, & Wilson, 1994). GATA6 is expressed in the cardiac-committed mesoderm at the late primitive streak stage and also in the developing and postnatal myocardium (Jiang & Evans, 1996; Morrisey et al., 1996). In contrast, GATA5 is restricted to the endocardium (Kelley, Blumberg, Zon, & Evans, 1993).

1.4.2 Roles of GATA-4, -5, -6 in heart development

Over the past decade, emerging evidence has accumulated of different nuclear transcription factors in regulation of cardiac gene expression involved in normal heart development and growth. Three members of the GATA family of DNA-binding transcription factors, GATA-4, -5 and -6 factors are amongst the first to be expressed in the developing heart.

The first evidence which supports that GATA4 is essential for cardiac differentiation derived from *in vitro* studies of pluripotent P19 embryonal carcinoma cells whereby depletion of GATA4 by an antisense strategy prevents terminal cardiac differentiation and triggers massive apoptosis of the precardiac cells, while overexpression of GATA4 increases differentiation of cardiomyocytes (Grepin, Nemer, & Nemer, 1997; Grepin, Robitaille, Antakly, & Nemer, 1995). Hence, GATA4 is well illustrated that it mediates differentiation of cardiomyocyte, cardiomyocyte proliferation and survival. Studies using mouse ES cells with gene-targeted disruption of GATA4 is potentially differentiate into cardiomyocyte *in vitro*, though with reduced efficiency, and are defective in the formation of proper visceral endoderm and definitive endoderm of the foregut (Narita, Bielinska, & Wilson, 1997a, 1997b; Soudais et al., 1995). Subsequently, transgenic mice with gene inactivation of GATA4 is embryo lethal between day 8 and 9 postcoitum due to failure in the formation of heart tube and ventral morphogenesis resulting in cardiac bifida, which suggests that GATA4 is crucial for normal heart development (Kuo et al., 1997; Molkentin, Lin, Duncan, & Olson, 1997). However,

cardiomyocyte differentiation is observed in the GATA4 deficient mice and cardiac atrial natriuretic peptide (ANP) and α-myosin heavy chain (α-MHC) genes are expressed normally, indicating that the function of GATA4 in the developing heart may be compensated by other proteins or signals and observed upregulation of GATA6 in GATA4 deficient mice is potentially compensate for the absence of GATA4 (Kuo et al., 1997; Molkentin et al., 1997).

GATA6 deficient mice die shortly precede heart induction, at E5.5-7.5 before a potential role in cardiac differentiation can be assessed as a result of abnormal visceral endoderm function and defects in extraembryonic tissue (Koutsourakis, Langeveld, Patient, Beddington, & Grosveld, 1999; Morrisey et al., 1998). Hence, it appears that of the mammalian GATA factors, GATA6 is required at the earliest stage of heart development. Nonetheless, GATA6 deficient ES cells are potentially contribute to the heart in chimeric embryos *in vivo* and to differentiate into myocardium *in vitro* which is accompanied by attenuated expression of GATA4 mRNA, indicating that GATA6 may regulate the expression of GATA4 during differentiation (Morrisey et al., 1998). The lack of phenotype in embryoid bodies derived from GATA6 null ES cells could be explained by an important link between GATA6 and BMP4 signaling. The role of GATA6 in BMP4 signaling pathway has been suggested by the phenotypes similarity between inhibition of BMP and GATA6 depletion. It was reported that GATA6 regulates BMP4 expression, through the functional GATA sites in its promoter (Nemer & Nemer, 2003; Peterkin, Gibson, & Patient, 2003). The availability of BMP4 from surrounding wild-type

cells or serum explains the ability of GATA6 deficient ES cells to contribute to heart tissues in chimeric embryo and embryoid bodies.

Taken together, GATA4 appears indispensable for proper endodermal differentiation and ventral morphogenesis and during late heart development such as heart septation and valve formation. Moreover, GATA4 is unable to compensate for the loss of GATA6 in the drive towards terminal differentiation (Peterkin et al., 2003), suggesting that GATA4 might not necessarily be responsible for cardiac myocytes differentiation. Collectively, GATA-4 and -6 are important for mesoderm and endoderm development required for differentiation and proliferation of cardiomyocytes, as well as heart tube formation and morphogenesis.

On the other hand, GATA5 null mice cause no obvious cardiac defects, while GATA5 mutant in zebrafish has reduced cardiac myocytes and develops a similar phenotype, which is cardiac bifida observed in GATA4 null mice (Molkentin, 2000; Reiter et al., 1999). However, GATA5 null mice may express a truncated form of GATA5, which would still be transcriptionally active and a functionally active protein was still produced, and therefore a role for GATA5 involved in cardiogenesis cannot yet be excluded (Nemer, Qureshi, Malo, & Nemer, 1999).

1.4.3 Regulation of cardiac gene expression by GATA family

The temporal and tissues specific expression of cardiac-specific genes during cardiogenesis is tightly regulated by the combinatorial interactions between members of cell-restricted as well as ubiquitous transcription factor families. GATA families are receiving increasing attention because they play essential role for embryonic and postnatal heart development. GATA-4, -5 and -6 have been demonstrated that they have preferential affinity for a subset of GATA elements to activate numerous cardiac-specific genes, as shown for α-MHC, cardiac troponin-C, ANF, BNP and cardiac troponin-I genes regulation (Di Lisi et al., 1998; Grepin et al., 1994; Ip et al., 1994; Murphy, Thompson, Peng, & Jones, 1997; Thuerauf, Hanford, & Glembotski, 1994). These genes contain GATA binding sites at their promoters which are required for cardiac-specific expression.

Moreover, studies in postnatal cardiomyocytes revealed that there is a cooperative interaction of GATA factors among themselves. GATA4 and GATA6 co-localized in postnatal cardiomyocytes and could therefore functionally interact with each other, at a single GATA element, resulting in synergistic activation of ANF and BNP promoters (Charron, Paradis, Bronchain, Nemer, & Nemer, 1999), suggesting that GATA4 and GATA6, both factors sometimes acting in concert to regulate expression of a subset of distinct cardiac genes.

In addition to interactions within GATA families, many of the combinatorial interactions between GATA families and several other cardiac transcription factors

have been well-defined. The best characterized partner for GATA4 is the homeodomain member of the NK family, Nkx2.5. Together, they synergistically activate cardiac-specific genes such as ANF (Durocher, Charron, Warren, Schwartz, & Nemer, 1997; Y. Lee et al., 1998; Sepulveda et al., 1998). Also, Nkx2.5 is crucial for normal heart development as Nkx2.5 gene-targeted disruption in mice results in embryonic lethality due to poor ventricular differentiation and abnormalities in heart tube looping (Lyons et al., 1995). Two separate cardiac-specific enhancers that contain essential GATA-binding sites were reported to control mouse Nkx2.5 gene expression (Lien et al., 1999; Searcy, Vincent, Liberatore, & Yutzey, 1998) . Interestingly, GATA6 contains an Nkx2.5 binding element (NKE), which is crucial for cardiac-specific expression of the promoter during cardiogenesis, indicating a reinforcing regulatory network of GATA factors and Nkx2.5 in developing heart (Molkentin et al., 2000).

1.5 Cardioprotection role of STAT3 in heart failure

1.5.1 Proinflammatory cytokines and inflammation on heart failure

Heart failure (CHF) is one of the global leading causes of mortality and morbidity, remaining as an enormous burden in human health despite the vast amount of research. Heart diseases such as myocardial infarction (MI), hypertension, valvular heart disease, coronary artery disease and dilated cardiomyopathy, eventually lead to heart failure (McMurray & Pfeffer, 2005).

Proinflammatory cytokine and inflammation have been recently emerged as a major factor in the development and progression of many types of heart failure. Cytokines potently stimulate host immune responses through triggering an inflammatory response by inducing the up-regulation and release of proinflammatory cytokines such as interleukin 1(IL-1), interleukin 6 (IL-6), and Tumor Necrosis Factor-α (TNF-α) (Ulevitch & Tobias, 1995; R. B. Yang et al., 1998). Studies have shown that adult patients with congenital heart disease had significantly elevated levels of circulating IL-6 and TNF-α compared with healthy control subjects. In particular, there are a number of studies have implicated that prolonged expression of proinflammatory cytokines, such as TNF-α may lead to deleterious effects on the heart, contributing to cardiomyopathy and left ventricular dysfunction and remodeling (Bryant et al., 1998; Hamid et al., 2009; Haudek, Taffet, Schneider, & Mann, 2007; Suffredini et al., 1995). Also, significantly elevated levels of circulating cytokines and cytokine receptors, such as IL-6, TNF-α, soluble TNF receptor 1 (sTNFR1), and soluble TNF receptor 2 (sTNFR2) are found in the patients with advanced congenital heart failure (Deswal et al., 2001; Rauchhaus et al., 2000). Taken together, all these observations raise the possibility of a causal relationship of inflammatory cytokine and inflammation in the development and progression of heart failure.

1.5.2 The STAT3 signaling pathway and heart function

All 7 STATs are expressed in the heart and cultured cardiac myocytes (Levy & Darnell, 2002). In the heart, Interleukin-6 cytokine family such as LIF,

cardiotrophin 1 (CT-1), oncostatin M and IL-6 particularly, which is a major cytokine generated during inflammation, transduce their signals via homo- or heterodimers of gp130 predominantly to STAT3 (Fischer & Hilfiker-Kleiner, 2007).

Experimental evidence suggests IL-6-JAK-STAT signaling cascade to be participated in cardioprotection, particularly activation of JAK2-STAT3 signaling in response to hypertrophic stimuli and ischemia (Hilfiker-Kleiner et al., 2004). Induction of cardiac hypertrophy by LIF and gp130 is mediated primarily by STAT3. Silencing of STAT3 blocks LIF-induced hypertrophy, while overexpression of JAK inhibitor SOCS3 abolishes gp130 signaling (Barry, Davidson, & Townsend, 2008; Uozumi et al., 2001).

STAT3 activation following ischemia injury upregulates bcl-2 and downregulates bax, thus reduces cell necrosis through attenuating myocardial infarct size and cardiomyocyte apoptosis (Hattori et al., 2001; Negoro et al., 2000). Mice expressing cardiac-specific, constitutively active STAT3 develop myocardial hypertrophy at early age and are protected from doxorubicin-induced cardiomyopathy (Butler, Huffman, Koch, Hahn, & Gwathmey, 2006). In addition, overexpression of STAT3 in the heart protects from myocardial infarction, increases capillary density and enhances expression of downstream proangiogenic factors, such as vascular endothelial growth factor (VEGF) and VE-cadherin (Booz et al., 2002; Oshima et al., 2005; Osugi et al., 2002).

1.5.3 Role of STAT3 in myocardial infarction and ischemia-reperfusion injury

Conversely, several studies had highlighted the role of STAT3 as a cardioprotective transcription factor using cardiomyocyte-specific STAT3 knockout (STAT3-CKO) mice. The Cre-loxP system is used to ablate STAT3 directed by the α-myosin heavy chain promoter which drives transgene expression specifically to cardiomyocytes.

Since gp130-STAT3 signaling pathway is required for cardiac protection and myocardial ischemia increases the levels of gp130 and IL-6 (Chandrasekar, Mitchell, Colston, & Freeman, 1999), the involvement of JAK-STAT signaling has been investigated in acute myocardial infarction to determine its pathophysiological roles in ischemic heart disease. These STAT3-CKO mice have defects in myocardial capillary angiogenesis, evident from the decreased left ventricular capillary density and enhanced interstitial fibrosis suggesting that STAT3 controls expression of downstream genes in cardiomyocytes that might be involved in regulation of myocardial capillarization or fibrosis. Furthermore, long-term follow-up in STAT3-CKO mice by 12 months of age, these mice showed symptoms of heart failure including dilated heart with massive interstitial fibrosis, increased rate of apoptosis, impaired contractile function and a decreased systolic function overtime (Hilfiker-Kleiner et al., 2004).

Also, STAT3-CKO mice were more susceptible to ischemia-reperfusion induced cardiac injury. Ischemia-reperfusion injury is a pathological condition characterized by an initial restriction of blood supply to an organ followed by the subsequent restoration of blood flow and reoxygenation which surprisingly is often

associated with an exacerbation of tissue injury and a profound inflammatory response (reperfusion injury) (Eltzschig & Eckle, 2011). Results demonstrated that these STAT3-CKO mice experienced larger infarct sizes after 1 hour ischemia and 24 hours reperfusion as compared to wild-type controls, indicating that STAT3-CKO mice were more susceptible to ischemia-reperfusion induced cardiac injury and thus STAT3 mediated cardioprotection is adequate to prevent further injury induced by the inflammatory response following the ischemia-reperfusion injury (Hilfiker-Kleiner et al., 2004).

1.5.4 STAT3 in cardioprotection by ischemia preconditioning and postconditioning

Ischemia preconditioning (IP) describes the administration of small ischemic insults before a subsequent prolonged episode of ischemia-reperfusion, which renders the heart more adaptable or tolerant to an ischemia event and reduces the irreversible tissue injury. In contrast, ischemia postconditioning (PC) refers to reduced infarct size by applying multiple short cycles of ischemia-reperfusion following a sustained ischemia insult (Boengler et al., 2008).

Using cardiomyocyte STAT3 KO mice in which STAT3 was ablated under control of the MLC2v promoter which drives activation of the Cre-recombinase, the important role of STAT3 in mediating the cardioprotection by IP has been revealed. STAT3-CKO and wild-type hearts were subjected to 3 conditions, control conditions, ischemia (30 min of ischemia and 45 min of reperfusion) and ischemia preconditioning just before ischemia (4 cycles of 5 min ischemia and 5 min

reperfusion prior to the ischemia, 30 min of ischemia and 45 min of reperfusion). In STAT3-CKO mice, reduced infarct size by IP was abolished. This finding was further confirmed in cardiomyocytes isolated from both STAT3-CKO and wild-type mice. IP in STAT3-CKO cardiomyocytes showed no increase in cell viability as compared to IP in wild-type cardiomyocytes. In addition, pharmacological preconditioning which utilized there agents (adenosine, diazoxide and TNF-α) known to afford protection failed to rescue/ reduce cell death in STAT3-CKO cardiomyocytes (R. M. Smith et al., 2004). Since the protection conferred by ischemia and pharmacological preconditioning was abolished in STAT3-CKO mice and STAT3-CKO cardiomyocytes, this finding strongly indicates that STAT3 signaling is critical for the preconditioning cell survival program.

More recently, tamoxifen-inducible cardiomyocyte-restricted STAT3 deletion was used to determine the role of STAT3 in the upregulation of cardioprotective proteins by ischemia preconditioning. The mice received vehicle or tamoxifen and were subjected to ischemia preconditioning and the hearts were harvested. Tamoxifen treated mice (STAT3-CKO) showed abrogation of STAT3 tyrosine and serine phosphorylation induced by ischemia preconditioning and STAT1/3 DNA binding activity which examined using EMSA. Furthermore, ischemia preconditioning upregulated cardioprotective protein cyclooxygenase-2 (COX-2) and heme oxygenase-1 (HO-1) in vehicle-treated mice whereas this upregulation was almost completely undetected in tamoxifen-treated mice, demonstrating that

STAT3 is essential for the upregulation of a battery of proteins which are responsible for preconditioning cell survival program (Bolli et al., 2011).

In the case of ischemic postconditioning (PC), studies focus on whether or not STAT3 contributes to cardioprotection by ischemic postconditioning and also to analyze whether or not ischemic postconditioning is effective in aged mice hearts. Young (3 months) and aged (>13 months) mice underwent ischemia-reperfusion without or with ischemia postconditioning [3 cycles of 10 sec ischemia and 10 sec reperfusion (3x10) or 5 cycles of 5 sec ischemia and 5 sec reperfusion (5x5)]. In young mice, ischemia postconditioning by both 3x10 and 5x5 significantly reduced infarct size and this cardioprotective effect was associated with an increase in STAT3 phosphorylation. In contrast, postconditioning by 5x5 only was effective in reduction of infarct size in aged mice. Also, total STAT3 and phosphorylated STAT3 were reduced in aged mice as compared to young mice which might account for the age-related loss of postconditioning. Moreover, the cardioprotection by postconditioning (3x10) was lost in cardiomyocyte-specific STAT3 KO mice, indicating that STAT3 is indispensable not only for ischemia preconditioning but also for postconditioning (Boengler et al., 2008).

1.5.5 Role of STAT3 in bacterial infection

Using STAT3-CKO mice, It was demonstrated that these mice were more susceptible to doxorubicin-induced cardiac injury and development of heart failure (decreased in cardiac systolic function), suggesting that STAT3 has a

cardioprotective function against doxorubicin-induced injury. Also, STAT3-CKO mice treated with lipopolysaccharide (LPS) has revealed a significant increase in apoptosis than their wild-type counterparts (Jacoby et al., 2003). LPS, a major constituent of the outer membrane of gram-negative bacteria, potently stimulates host immune responses through triggering an inflammatory response by inducing the up-regulation and release of proinflammatory cytokines (Ulevitch & Tobias, 1995; R. B. Yang et al., 1998). In addition, cardiomyocyte cultured from STAT3-CKO mice secreted significantly more TNF-α in response to LPS than those in wild-type. This may be the cause for dramatic increase in cardiac fibrosis in aged mice (9 months) despite having no overt signs of heart failure in young cardiac-specific STAT3 null mice (6 months). Overall, these results suggested that cardiomyocyte-restricted knockout of STAT3 results in higher sensitivity to inflammation and that STAT3 pathway is critical in regulation of inflammatory responses of cardiomyocytes, which may contribute to the development of cardiac fibrosis.

1.5.6 Role of STAT3 in viral myocarditis

Viral myocarditis is caused by viral infection by common viral pathogens for instance enteroviruses and adenoviruses that might be directly pathogenic to the myocardium with the resulting cytokine-driven inflammatory response, leading to subsequent destruction of cardiac myocytes. Studies demonstrated that coxsackievirus B3 (CVB3) infection is associated with the activation of JAK-STAT signaling in the heart with an induction of STAT inhibitor suppressor of cytokine

signaling (SOCS) mRNA. SOCS which is a negative feedback of JAK-STAT signaling, when induced, it inhibits tyrosine phosphorylation of the cytokine receptor mediated by JAK, and subsequently leads to inhibition of STAT signaling. In transgenic mice engineered to overexpressed SOCS in the heart, these mice suffered from increased viral infection and this increased susceptibility to viral infection was via inhibition of gp130 signaling. Additionally, cardia-specific knockout of gp130 showed increased susceptibility to viral infection, suggesting that the important role of gp130 signaling via activation of STAT3 in protection from virally induced cardiac damage (Yajima et al., 2006).

1.5.7 STAT3 regulates myocardial apoptosis

STAT3 plays a role in decreasing cardiomyocytes apoptotic cell death. The anti-apoptotic function of STAT3 was demonstrated using acute myocardial infarction (AMI) model in rats. Pretreatment with AG-490 which inhibited STAT3 phosphorylation resulted in significant increase in caspase-3 activity and Bax protein (pro-apoptotic) as well as the number of apoptotic nuclei in AMI hearts (Negoro et al., 2000). Furthermore, mice with cardiomyocyte-restricted STAT3 deletion displayed higher rate of apoptosis (higher number of apoptotic nuclei) induced by ischemia-reperfusion (Hilfiker-Kleiner et al., 2004) and by lipopolysaccharide (Jacoby et al., 2003) than their wild-type counterparts. In addition, during ischemia preconditioning, it has been showed that STAT3 exerted its anti-apoptotic function by upregulation of anti-apoptotic proteins involved both

in the mitochondrial (Bcl-XL and Mcl-1) and death receptor (C-FLIPL and C-FLIPS) pathways (Bolli et al., 2011).

In a nutshell, it is clear that STAT3 has emerged as a central player in cardioprotection in the response of the heart to multiple pathophysiological (myocardial infarction, ischemia-reperfusion injury, bacteria and virus infections) and physiological (aging) forms of stress which cause the development of heart failure. However, the molecular mechanisms underlying STAT3-mediated cardioprotection in both cardiac inflammation and ischemia have not been addressed. Therefore, it is in great potential to uncover the molecular mechanisms of STAT3-regulated cardioprotection and its potential downstream candidate targets that associated with cardiovascular inflammation and ischemia.

1.6 Thioredoxin-interacting protein (Txnip)

1.6.1 Txnip protein

Txnip, as its name suggested, it is an endogenous ubiquitously expressed protein which binds and inhibits thioredoxin (Trx), a major cellular thiol-reducing and antioxidant system, which comprised of thioredoxin reductase (TrxR) and NADPH, and therefore results in oxidative stress induction and modulates intracellular redox state. (Nishiyama et al., 1999; Yamanaka et al., 2000). Also, Txnip promotes apoptosis by abolishing Trx-mediated inhibition of apoptosis-signal-regulating kinase-1 (ASK1), further activating downstream JNK/p38MAPK pathway (Kaimul, Nakamura, Masutani, & Yodoi, 2007). In addition, it can act independently of its

binding to Trx, and contributes to cell growth inhibition, suppression of glucose uptake and metabolic reprogramming (Elgort, O'Shea, Jiang, & Ayer, 2010; Patwari et al., 2009)

1.6.2 Txnip Functions

Roles of Txnip have been studies in several physiology conditions such as glucose metabolism, inflammation, apoptosis, and cardiac viability. Txnip contributes to cell growth inhibition through arrestin domain-mediated suppression of glucose uptake and metabolic reprogramming (Elgort et al., 2010; Patwari et al., 2009). In addition, it acts as tumour suppressor in various malignancies, by suppression of tumour growth and transcription repressor (Jeon et al., 2005), inhibited metastasis and induced apoptosis in cancer cells (Kaimul et al., 2007). Also, Txnip modulates inflammatory responses (Z. Wang et al., 2006; Zhou, Tardivel, Thorens, Choi, & Tschopp, 2010) and vascular cells signaling and apoptosis (Perrone, Devi, Hosoya, Terasaki, & Singh, 2009; Schulze, De Keulenaer, Yoshioka, Kassik, & Lee, 2002; World, Yamawaki, & Berk, 2006; Yamawaki, Pan, Lee, & Berk, 2005).

In the heart, studies have reported that enhanced Txnip expression is associated with increased cell death in diabetic rat heart, in which high expression of Txnip is related to dilated cardiomyopathy (Barth et al., 2006; J. Chen, Cha-Molstad, Szabo, & Shalev, 2009; van Lunteren & Moyer, 2007). Moreover, cardiac Txnip mRNA expression is increased in the rat heart post 48-hour myocardial infarction demonstrated by RT-PCR and Txnip-specific DNA enzyme transfection in H9c2

cell illustrated a reduced in apoptosis in response to cellular stress of H_2O_2 (Xiang et al., 2005). Recently, it has been validated that overexpression of Txnip triggers inflammasome activation and therefore increased ROS production and caspase-1 expression (Zhou et al., 2010; Zhou, Yazdi, Menu, & Tschopp, 2011). In addition, Txnip-KO hearts protect the myocardium from ischemia-reperfusion injury and early phrase of pressure overload (Yoshioka et al., 2012; Yoshioka et al., 2007). However, the mechanism by which Txnip mediates myocardial injury is unclear.

1.7 Txnip and NLRP3 inflammasome

1.7.1 The inflammasome complex

Inflammasome is a large (700kDa) multiprotein cytoplasmic complex, which is sufficient to activate caspase-1 by cleavage of procaspase-1. The inflammasome complex typically comprises of a nucleotide-binding domain leucine rich repeat (NLR) protein, caspase-1 and the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC) (Martinon, Burns, & Tschopp, 2002). Four inflammasome complexes have been identified to date: NLRP1, NLRP3, NLRC4, and AIM2 (Stutz, Golenbock, & Latz, 2009).

1.7.2 NLRP3 inflammasome and activation

The NLRP3 inflammasome consists of the NLRP3 scaffold, ASC, and procaspase-1. Upon activation, NLRP3 oligomerization leads to its pyrin (PYD) domain interacting with the PYD domain of ASC, whose caspase recruitment domain (CARD) interacts and recruits the CARD domain of procaspase-1. Active caspase-

1, a p10/p20 tetramer formed from the auto-catalytic cleavage of procaspase-1 would then process pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18, which are subsequently secreted. IL-1β secretion is the hallmark of NLRP3 inflammasome activation and activation of caspase-1(G. Y. Chen & Nunez, 2010).

While most inflammasomes are triggered by certain stimuli, interestingly the NLRP3 inflammasome is activated by various stimuli of dissimilar physico-chemical nature. These include microbial stimuli (Muruve et al., 2008), crystalline substances (Dostert et al., 2008), pore forming toxins, extracellular ATP and necrotic cell components (Mariathasan et al., 2006). Lately, it was identified that the assembly of NLRP3 inflammasome requires the presence of reactive oxygen species (ROS), which are generated by all known NLRP3 activators. This induces a conformational change in an unidentified protein that subsequently activates the inflammasome (Martinon, Mayor, & Tschopp, 2009). Conversely, ROS inhibitors block inflammasome activation (Dostert et al., 2008; Zhou et al., 2010).

Until recently, Txnip was identified as one of the NLRP3 binding partners, and interaction between Txnip and NLRP3 is essential for activation of the NLRP3 inflammasome (Zhou et al., 2010). In resting cells, Txnip interacts with Trx and hence unavailable to interact with NLRP3. Upon an increase in ROS caused by NLRP3 activators, Txnip is dissociated from oxidized Trx and in turn binds NLRP3 and leads to assembly and activation of NLRP3 inflammasome (Zhou et al., 2010). (Figure 1.3)

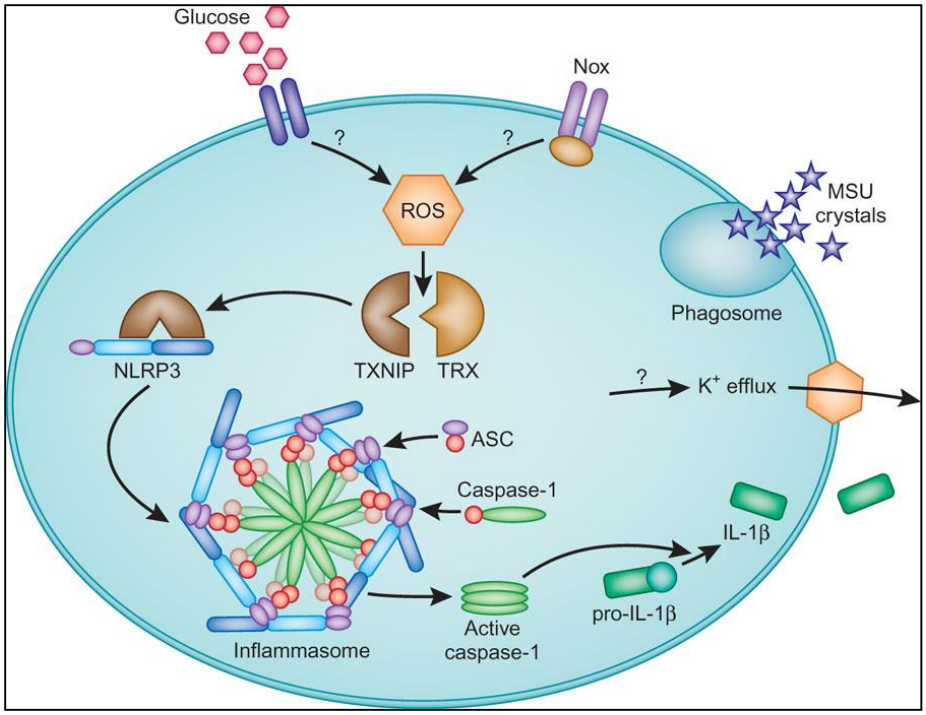


Figure 1.3 Mechanisms of NLRP3 inflammasome activation.
Under normaxic condition, Txnip is bound to Trx and inhibits the reducing activity of Trx. During oxidative stress, ROS causes Txnip to dissociate from Trx and interacts with NLRP3. NLRP3 recruits ASC which in turn interacts with caspase-1 leading to its activation. Once activated, caspase-1 promotes the maturation of the proIL-1B.
Figure adapted from (Davis & Ting, 2010).

1.7.3 NLRP3 inflammasome in the heart

Several groups have independently demonstrated that inflammasome contributes to the pathophysiology of myocardial ischemia-reperfusion (I/R) injury. ASC and caspase-1 were markedly expressed at the site of myocardial I/R injury. Conversely, ASC and caspase-1 deletion experienced reduced inflammatory responses and protect the myocardium from ischemia reperfusion injury with reduced infarct sizes in mice (Kawaguchi et al., 2011). In addition, NLRP3 was upregulated within the myocardium following myocardial I/R injury, particularly in myocardial fibroblast.

Hearts from NLRP3 deletion mice featured smaller infarct size and showed decreased myocardial apoptosis during I/R injury compared with wild-type mice (Sandanger et al., 2013). Another group demonstrated that inflammasome was induced in cardiomyocytes and inflammasome activation in the cardiomyocyte leads to caspase-1 dependent cell death (Mezzaroma et al., 2011). Therefore, targeting the inflammasome formation is a potential strategy for cardioprotection.

1.8 Objectives and significance of study

1.8.1 Objective I

In vitro-derived cardiomyocytes are a prime candidate for the donor cells for use in regenerative medicine as they constitute a renewable source of wide range of cardiovascular cell types including cardiomyocytes. Also, it is an ideal model to understand the process of early development of cardiomyocyte differentiation. Extensive investigations have put forth evidence indicating that STAT3 is important in ES cells pluripotency maintenance, however, STAT3 function during ES cells differentiation is poorly understood. Therefore, the transition from bench to bed necessitates a deeper understanding of the STAT3 function in the regulatory networks of early cardiomyocyte differentiation as it take places in the developing embryo.

Here, we want to establish an *in vitro* inducible STAT3 KO and STAT3 CA ES system because conventional knock out of STAT3 results in early embryonic lethality at the time of cardiomyocyte formation at E7.5-E8.5 and before a potential

functional role in cardiac differentiation can be assessed. Therefore, by using these inducible systems, the function of STAT3 during early cardiac differentiation can be revealed. This project also aims to identify direct downstream targets of STAT3 during course of early cardiomyocyte differentiation in order to understand the transcriptional regulatory mechanisms that underlie early cardiomyocyte differentiation.

1.8.2 Objective II

Cardiovascular disease remains one of the leading causes of human mortality in the developed world. In Singapore alone, approximately 1 out of 3 deaths were attributable to cardiovascular disease, accounting for 29.9% of all deaths in 2014. Moreover, statistics show a pessimistic trend worldwide in which by 2020 cardiovascular disease is expected to be the leading cause of death in the world (B. B. Kelly, Narula, & Fuster, 2012).

Several lines of evidence suggest that STAT3 has function of cardiac protection. However, the underlying molecular mechanism by which STAT3 mediated cardioprotection is not fully elucidated. Therefore, it is crucial to elucidate the changes in genes related to heart pathogenesis and the underlying regulatory role of STAT3 involved in this cardiovascular pathogenesis. In this present study, we would like to examine potential cardiac genes regulated by STAT3, either by upregulating cardioprotective genes or downregulating harmful genes in which together they contribute to the survival of the heart under pathogenesis. Hence, this

study might provide new insight for potential clinic therapy for heart failure and to explore and develop new biomarkers and therapeutic treatments for the disease by developing such STAT3 downstream mediators as novel therapeutic targets.

Chapter 2 Materials and Methods

2.1 Antibodies, chemicals and other reagents

2.1.1 Antibodies

Antibodies against STAT3 (C-20), ASC, α-tubulin, actin were purchased from Santa Cruz; antibodies against STAT3 phospho-Tyr705, STAT3 and IL-1β were purchased from Cell Signaling; antibodies against GATA6 was purchased from Abcam; antibodies against Txnip was purchased from MBL and antibodies against Troponin T, α-Actinin, Flag tag (Flag M2), NLRP3 were purchased from Sigma.

2.1.2 Chemicals

Table 2.1 List of chemicals used in this study

Name	Source
Ascorbic Acid	Sigma
DAPI	Sigma
DMSO	Sigma
Doxycyclin	Sigma
LPS	Sigma

2.1.3 Other reagents

Restriction enzymes and T4 DNA ligase were from New England Biolabs (NEB).

2.2 Plasmids

For recombinant DNA cloning procedures and plasmid DNA amplification, E.coli strain DH5α (Invitrogen) was used as host. Bacteria cells were grown in LB liquid or solid medium with ampicillin (100μg/ml) for selection of positive cells. All plasmids were verified by sequencing.

2.3 Animal Experiments

2.3.1 Generation of Mice with Cardiac-Specific Deletion of STAT3

To study the biological function of STAT3 in the heart, mice express alpha-MHC (Myosin heavy chain) promoter driven Cre recombinase (Agah et al., 1997) was crossed with mice which carry crucial exons of the STAT3 gene flanked by LoxP sites (floxP) to generate cardiomyocyte-specific STAT3 knockout mice. Details of the procedure for generating the mice were described in our previous article (Jacoby et al., 2003). Cardiomyocyte-specific STAT3 KO mice is referred to as STAT3 CKO whereas its control which has both alleles carrying floxP STAT3 gene but without Cre is referred to as $STAT3^{flox/flox}$.

2.3.2 LPS Application Procedures

One dose LPS treatment: Mice of floxP/ floxP ($STAT3^{flox/flox}$) genotype were treated with LPS (0.5mg/kg, i.p), and then sacrificed 2 and 18 hours after treatment. H9c2 myoblast was treated with LPS (100uM) and samples were collected after 0.5, 1, 2, and 18 hours.

2.4 Cell culture

2.4.1 ES cell culture

E14 murine embryonic stem cells were maintained in undifferentiated state with Glasgow Minimum Essential Medium (Gibco, Invitrogen, Carlsbad, CA, USA), supplemented with 15% ES cell-qualified fetal bovine serum (ES-FBS; Gibco), 1mM sodium pyruvate (Gibco), 0.1mM non-essential amino acids (Gibco), 0.1mM 2-mercaptoethanol (Sigma-Aldrich, St Louis, MO, USA), 50U/ml penicillin

(gibco), 50ug/ml streptomycin (gibco) and 1000U/ml murine leukemia inhibitory factor (LIF; Sigma-Aldrich) on gelatin-coated culture dish at 37°C in humidified air with 5% CO_2. Medium was changed every two days and the cells were subcultured when they reached 70-80% confluency. Cells were made to undergo 2 passages after thawing before commencement of cardiomyocyte induction.

2.4.2 H9c2 cell culture

Rat H9c2 cardiac myoblast cell line was cultured in high glucose 1X Dulbecco's modified Eagle's medium (DMEM) with L-Glutamine and D-glucose (Gibco-Invitrogen, CA, USA), supplemented with 10% Fetal Bovine Serum (FBS) (Gibco-Invitrogen, CA, USA) and 1% penicillin (10,000U/mL) and streptomycin (10,000μg/mL) (1% Pen-Strep) (Gibco-Invitrogen, CA, USA) in $25cm^2$ or $75cm^2$ tissue culture flasks (NUNC^{TM}, Roskilde, Denmark). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were passaged by trypsinization upon reaching 80% confluency to prevent them from losing their myoblastic properties at a subcultivation ratio of 1:2 to 1:4. Cells from passages four to 15 were used for experiments.

2.5 Generation of inducible ES cell lines

2.5.1 pTRE3G-IRES Tet-on System

The 3rd generation Tet-on System was purchased from Clontech Laboratories (Cat. no. 631167). These Tet-On 3G Systems are inducible gene expression systems for mammalian cells. The system consists of the Tet-On 3G transactivator, TetR (Supplementary figure 1A) and contains a gene of interest (GOI) under the control

of a TRE3G promoter (pTRE3G) (Supplementary figure 1B, 1C, 1D) which will express high levels of GOI driven by TetR with doxycycline induction.

Full-length of mouse wild-type STAT3 cDNA was cloned from ES cDNA pool by PCR, constitutive activated STAT3 (CA-STAT3) or overexpression of pSTAT3 was obtained from pXJ40-CA (kindly provided by Professor Cao Xinmin), and NlsCre was purchased from commercial entities. mSTAT3, CA-mSTAT3 and NlsCre were cloned into a TetR response vector controlled by the tetracycline responsive element (pTRE-), fused with C-T2ACherry and C-IRESzsGreen respectively.

2.5.2 Generation of Inducible STAT3 KO and overexpressed ES cell lines

The regulatory vector pEF1a-Tet was transfected into mouse $STAT3^{flox/flox}$ ES cells or wild-type ES cell line E14. The G418 resistant cell clones with high luciferase expression and low background were transfected with pTRE3GCret2acherry, pTRE3GSTAT3-IRESzsGreen, pTRE-STAT3CAIRESzsGreen respectively. Selected by puromycin, the transgenic cell clones were obtained. In these clones, the mCherry or GFP, indicating STAT3-KO or pSTAT3-overexpression, could be turned on or off by adding or removing doxycycline (Dox). In conclusion, we have established the ES cell lines in which STAT3 expression can be switched on or off and regulated by Dox induction. (Figure 2.1)

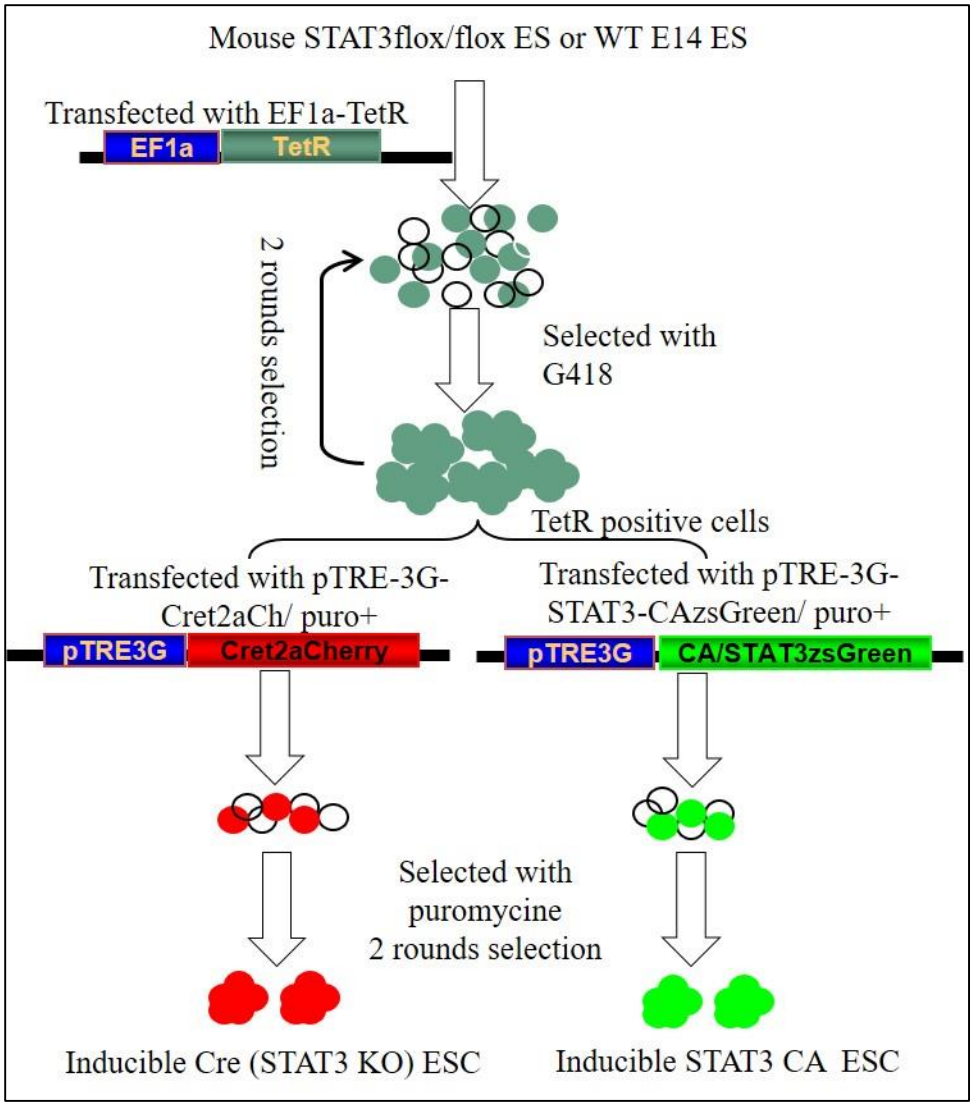


Figure 2.1 Schematic chart for establishing the Inducible STAT3 KO and STAT3 CA ES cell lines.

2.6 ES cells differentiation

2.6.1 Preparation of ES cells for Embryoid Bodies (EB) formation

ES cells were dissociated using 0.1% trypsin-EDTA (Invitrogen) and suspended in Glasgow Minimum Essential Medium (Gibco, Invitrogen) supplemented with 15% ES cell-qualified fetal bovine serum ,1mM sodium pyruvate (Gibco), 0.1mM non-essential amino acids, 0.1mM 2-mercaptoethanol, 50U/ml penicillin, 50ug/ml streptomycin, and 50ug/ml Ascorbic acid (EB medium) (T. Takahashi et al., 2003).

2.6.2 EB formation induced by Hanging Drops method

ES cells were differentiated to form embryoid bodies using the hanging drops method as previously reported (Boheler et al., 2002) (Figure 2.2). Hanging drops each of 25ul, containing 1000 embryonic stem cells were seeded on the lid of $10cm^2$ cell culture dish and the dish was filled with PBS to prevent desiccation of EBs. A total of 50 drops were seeded on each culture dish. To ensure quantifiable measurement of RNA and lysate concentration for RT-PCR and Western blot analysis respectively, an equal number of hanging drops were seeded on each plate. A total of 200 hanging drops were allocated for each differentiation phase. Hanging drops were incubated at 37^0C in humidified air with 5% CO_2 for 3 days. EBs were then seeded on gelatin-coated plate on the 4th day of differentiation. EB medium was changed every two day. The beating cardiomyocytes could be observed as early as day 8.

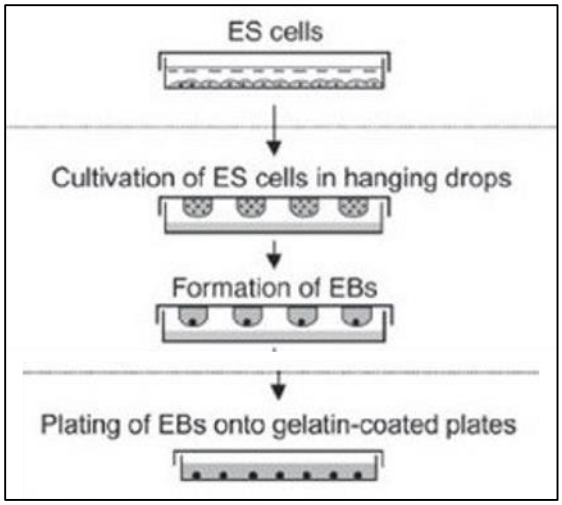


Figure 2.2 Schematic diagram of the experimental protocol used for induction of embryonic stem cells to the cardiac lineage.
Figure adapted from (Boheler et al., 2002).

2.7 Transfection

Transfection was done when cells grown to 70%-80% of confluency. Cells were transfected by Lipofectamine 2000 and the manufacturer's protocol (Life Technologies) was followed. 5µg of plasmid DNA was diluted in Opti-MEM medium for a final volume of 50µl. 2µl of Lipofectamine 2000 was diluted in 48µl Opti-MEM medium followed by a 5 min incubation at room temperature. Diluted DNA was added to diluted Lipofectamine 2000 at 1:1 ratio and incubated for 20 min at room temperature. After incubation, the DNA/lipofectamine mixture was added to each well and gentle swirl to mix. The transfected cells were incubated at 37°C with 5% CO_2. After about 4 to 6 hours, media was removed and replaced with fresh media. Then cells were moved back to 37°C incubator with 5% CO_2 until cells reached full confluency for harvest and the transfection efficiency was checked by viewing in a fluorescent microscopy.

2.8 Histological and Immunofluorescent

Heart tissues were embedded in OCT compound, frozen immediately after excision, and sectioned and mounted on Silane coated slides. Slides were washed with PBS solution, fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS for 10 min. Then slides were blocked with 3% BSA in PBS with 0.1% Triton X-100 for 10 min, followed by incubation with indicated primary antibodies at 4°C overnight. Then slides were washed three times with 0.1% Triton X-100 in PBS, each time for 10 min and incubated with fluorescence-labeled secondary antibodies in dark at room temperature for 1 hour. Nuclei were visualized by DAPI staining. Coverslips were mounted onto slides by VECTASHIELD HardSet Mounting Medium (Vector Laboratories, H-1400). Labeled sections were imaged using Nikon A1R-A1 confocal microscopy system.

2.9 RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was harvested using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, purified by the RNeasy mini kit (Qiagen), and then reverse transcribed using the M-MLV Reverse Transcriptase system (Promega) to obtain cDNA. The cDNA products were subjected to semi-quantitative RT–PCR with KAPA SYBR® FAST Universal 2X qPCR Master Mix (KK4600) using a 7300 Real-Time PCR machine (Applied Biosystems). All gene-specific mRNA expression values were normalized against the internal housekeeping gene GAPDH. The sequences of primers used are in Table 2.2. Triplicate was done for the analysis of each samples.

Table 2.2 List of primers used in this study

Gene (Mouse)	Forward primer (5'- 3')	Reverse primer (5'- 3')
GAPDH	GGTTGTCTCCTGCGACTTCAACAGC	CGAGTTGGGATAGGGCCTCTCTTGC
STAT3	CAACCTTCGAAGAATCAAGCA	TCAGTCAGTGTCTTCTGCACG
GATA6	ATGCGGTCTCTACAGCAAGATGA	CGCCATAAGGTAGTGGTTGTGG
Txnip	CCTAGAAGAGCAGCCTACAGCAGGT	ATGTTCCCAGGGGCCCTTGAGG
ASC	CTTGTCAGGGGATGAACTCAAA	GCCATACGACTCCAGATAGTAGC
NLRP3	ATTACCCGCCCGAGAAAGG	TCGCAGCAAAGATCCACACAG
Caspase-1	ACAAGGCACGGGACCTATG	TCCCAGTCAGTCCTGGAAATG
GATA6 (ChIP-qPCR)	GCCCGCTAAGAACCGTATCA	GGTGTCCAAGGACGCTAGTT
Txnip (ChIP-qPCR)	CAACCATTTTCCCCGCT	CCAGAGGAGGAGAGTGTCAAG

2.10 Illumina Microarray analysis

Inducible STAT3 CA and wild-type TetR ES cells as control and inducible STAT3 KO and STAT3 F/F TetR ES cells as control were treated with Dox for 48 hours. Biological triplicates of induced ES cells were harvested and total RNA was extracted and purified by the RNeasy mini kit with on-column DNase digestion (Qiagen) according to the manufacturer's instructions. The array was done using MouseWG-6 v2 Expression BeadChip Kit (Illumina) according to the manufacturer's protocol. RNA quantity, quality and purity were assessed on an Agilent Bioanalyzer. The microarray analysis was carried out using R language by our bioinformatician.

2.11 Chromatin Immunoprecipitation Assay

ChIP assay was performed following Yang's published ChIP protocol (T. I. Lee, Johnstone, & Young, 2006). Crosslink was performed by addition of formaldehyde at final concentration of 1% for 10 min followed by quenching with Glycine. Cell lysates were fragmented by sonication and pre-cleared with protein-G agarose beads (Invitrogen), and subsequently precipitated with anti-STAT3 antibody (Santa Cruz) or normal rabbit IgG (Santa Cruz) overnight at 4^{o}C with continuous rotation. After washing and elution, cross-linking reversal was carried out by incubating the eluate at 65^{o}C for 8 hours. DNA was isolated with phenol-chloroform and ethanol precipitated with the help of glycogen. The eluted DNA was analyzed by qRT-PCR to inspect possible binding sites of STAT3. The promoter region for GATA6 and

Txnip was analyzed by real-time PCR using the following primers indicated in Table 2.2.

2.12 Protein Extraction and Western Blotting

Cell and tissue were washed twice with ice-cold PBS, and lysed in whole cell extract buffer supplemented with protease inhibitor (Roche, Switzerland). The supernatant was collected by centrifugation, and protein concentrations were measured with BCA assay (Bio-Rad, CA). The absorbance of the proteins in the lysates was then measured using GeneQuant 1300 machine with wavelength set at 595nm. Appropriate amount of lysate samples were mixed with sample buffer, separated by SDS-polyacrylamide gel electrophoresis of a gel percentage dependent on the size of the target protein and transferred onto a polyvinylidene difluoride (PVDF) membrane in cold room. After blocking with 5% nonfat milk in washing buffer, the membranes were incubated with the indicated primary antibodies at 4°C overnight. Following washes, they were incubated in horse-radish peroxidase (HRP) conjugated secondary antibodies. The immunoreactive bands were visualized using SuperSignalR Chemiluminescent Substrate (Pierce) on audiographic film (Thermo Scientific) that was developed by a film cassete. Equal loading the blots was shown by β-Actin level.

2.13 Statistical Analysis

Data are expressed as mean ± SEM. A p value of <0.05 was considered a statistically significant difference.

Chapter 3 Results (Part I)

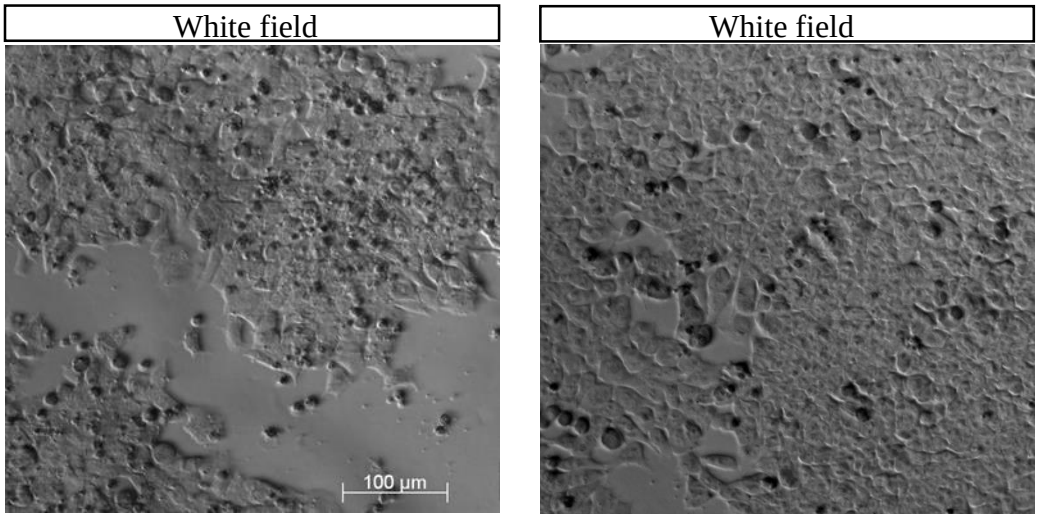
Results (Part I): The role of STAT3 from embryonic stem cells into cardiac lineage differentiation

3.1 Recombinant ES cells allowing Dox inducible expression of a Cre mCherry transgene and STAT3 constitutively expressed GFP transgene.

To determine the biological function of STAT3 in ES cells and *in vitro* cardiomyocyte differentiation of ES cells, we used an inducible Tet-on system to generate mouse ES cell lines harboring a doxycycline (Dox)-inducible Cre and STAT3 constitutively activated (STAT3 CA) transgenes which can be temporally and specifically induced upon Dox addition. Cre and STAT3 CA Tet-on positive ES clones were selected and propagated. Following 24 hours Dox stimulation, mCherry and GFP expression were imaged suggesting that Cre transgene was induced and its recombinase activity function to delete $STAT3^{flox/flox}$ (Figure 3.1A) *in vitro* resulting in a mutant STAT3 protein missing the SH2 domain for STAT3 function whereas STAT3 was constitutively activated (Figure 3.1B) in ES cells.

A **B**

pTRE-Cre **pTRE-STAT3 CA**



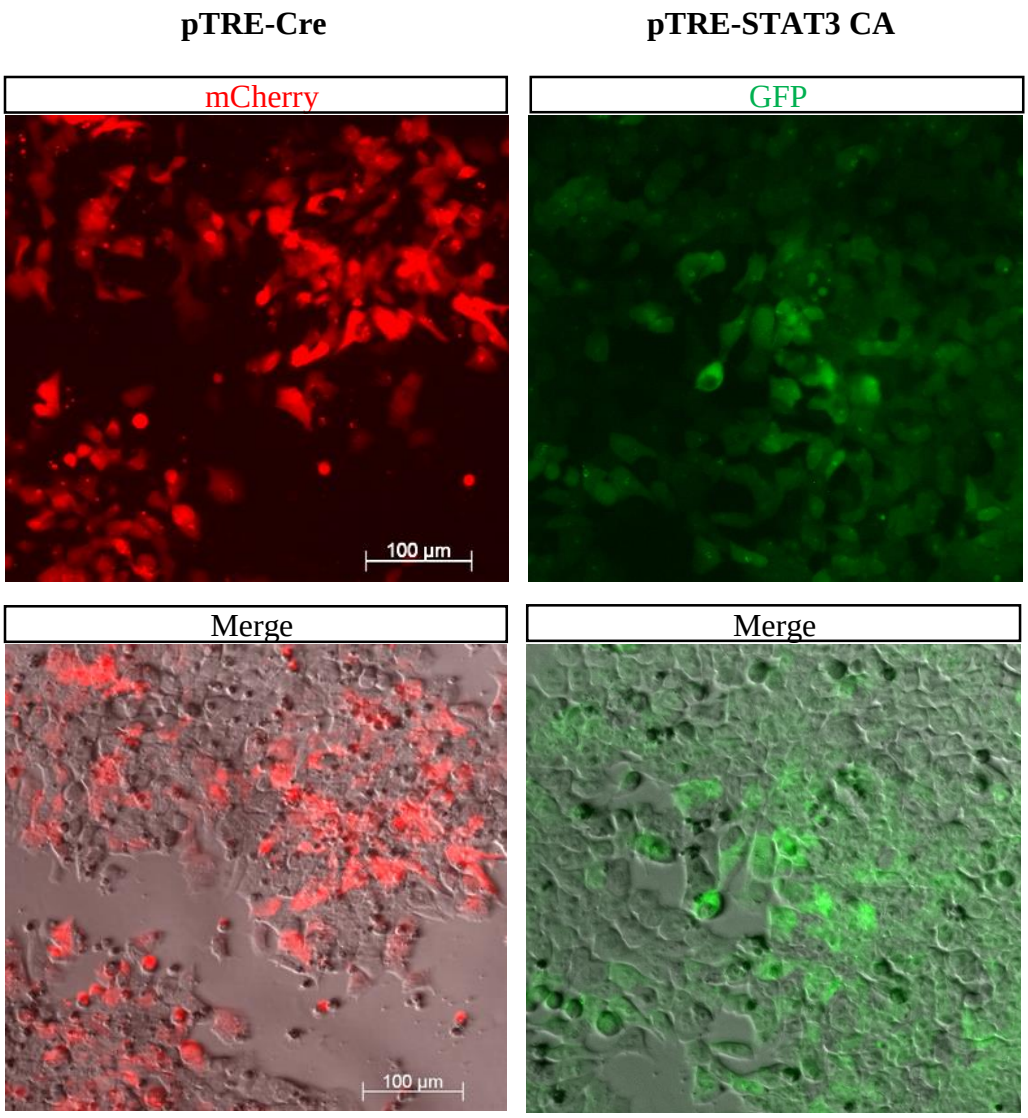


Figure 3.1 Inducible STAT3 KO and STAT3 CA ES clones.
A. In pTRE-CreCh/pEF1aTetR inducible STAT3 KO ESCs, mCherry was observed after 24 hours Dox induction, indicating that Cre was expressed and function to delete STAT3 *in vitro.*
B. In pTRE-STAT3 CA/pEF1aTetR inducible STAT3 CA ESCs, GFP was expressed after 24 hours Dox induction, suggesting that STAT3 CA was expressed.

3.2 STAT3 genomic DNA deletion by genotyping of ES cells after dox induction

Following the previous study described (Jacoby et al., 2003), the primer pair 1 (5′-ATT GGA ACC TGG GAC CAA GTG G) and 3 (5′-GCT GGC TCA TAG GCA AAA ACA C) was used to detect the STAT3 deleted ($STAT3^{D}$) alleles by PCR analysis. The $STAT3^{D}$ was detected as a 480 base pair fragment. Inducible Cre (iCre) ESCs following 24 hours Dox induction, along with mice heart tissue samples as positive control, which included the hearts from STAT3-deficient mice (C/F/D), heterozygous (C/F/+), and $STAT3^{flox/flox}$ mice (F/F) as well as the $STAT3^{flox/flox}$ (F/F) ESCs. The $STAT3^{D}$ allele was detected as a 480-bp fragment in STAT3-deficient heart (C/F/D) and STAT3 KO ESCs (Figure 3.2 lane 1 and 4). With this primer pair, it amplified an approximately 1.5 kb fragment and a 480-bp in heterozygous (C/F/+) heart sample (Figure 3.2 lane 2), whereas the $STAT3^{flox/flox}$ (F/F) heart and F/F ESCs just gave a 1.5 kb fragment (Figure 3.2 lane 3 and 5).

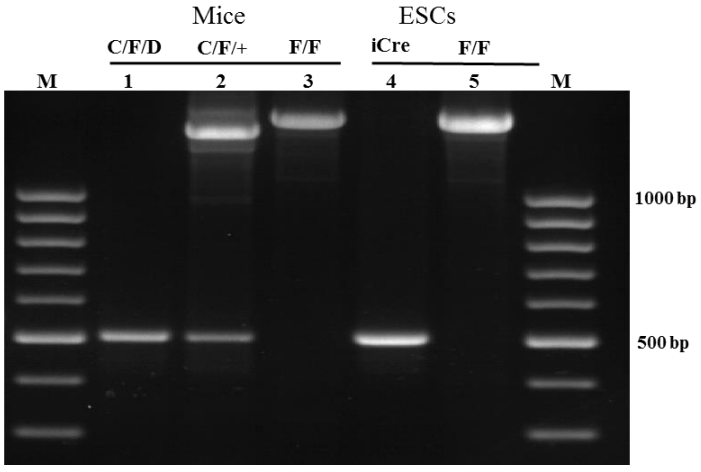


Figure 3.2 Generation of inducible STAT3 KO ES cells.
Ablation of STAT3 genomic DNA in inducible STAT3 KO ES cells was detected as a 480 base pair fragment.

3.3 pSTAT3 and STAT3 protein expressions in inducible STAT3 KO ESCs and inducible STAT3 CA ESCs

Western blot analysis confirmed that pSTAT3 and STAT3 protein levels in these inducible ES cells were deleted or induced by Dox stimulation. Tyrosine 705 (Y705) phosphorylation in STAT3 (pSTAT3) is a prerequisite for increasing STAT3 transcriptional activation (Decker & Kovarik, 1999) and also our inducible STAT3 CA ES cells system functions to induce overexpression of pSTAT3. Therefore pSTAT3 protein expression was detected in these inducible ES cells using an antibody that specifically recognizes STAT3 protein phosphorylated on Y705.

In pTRE-Cre ES cells, pSTAT3 protein level was efficiently ablated and undetected after 48 hours Dox induction while STAT3 protein level was decreased, but still detectable after 48 hours Dox induction (Figure 3.3A). In pTRE-STAT3 CA ES cells, pSTAT3 was rapidly upregulated after 24 hours Dox induction (Figure 3.3B). Since STAT3 antibody can detect total STAT3 which included both pSTAT3 and STAT3 proteins, therefore STAT3 protein level was upregulated slightly upon Dox induction due to the increased expression of pSTAT3 protein level. Our results showed that Clone 1 was a better clone for our study due to its higher level of pSTAT3 induction as compared to clone 2 upon Dox induction (Figure 3.3B). Flagged-STAT3 CA expression was detected using anti-flag antibody. While undetectable in basal condition, STAT3 CA was rapidly upregulated after Dox stimulation (Figure 3.3C).

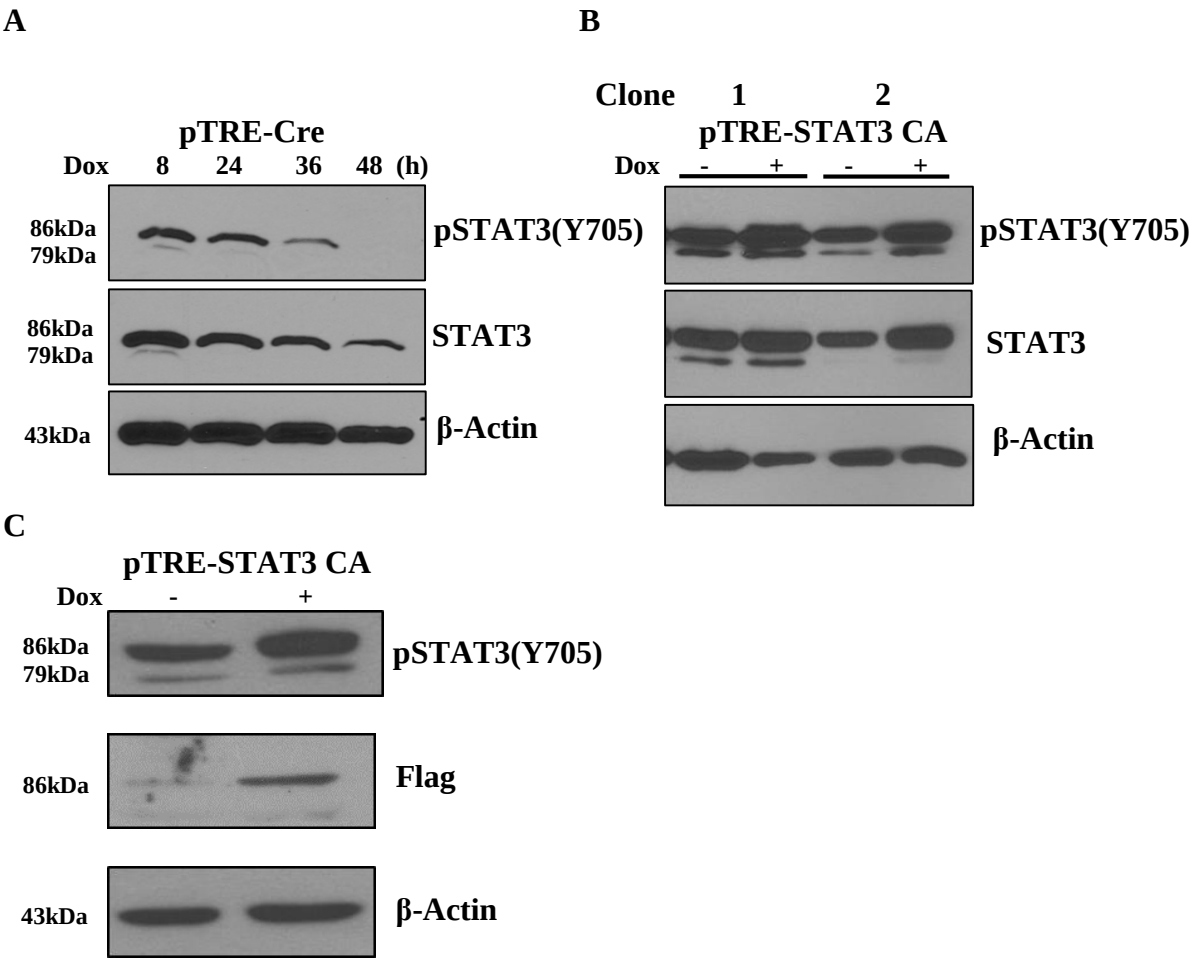


Figure 3.3 pSTAT3 and STAT3 protein levels in inducible ES cell lines after Dox induction.

A. In pTRE-CreCh/pEF1aTetR ESCs, after the Dox induction, pSTAT3 was reduced and undetected after 48 hours induction, while STAT3 was decreased, but still detectable after 48 hours.

B. In pTRE-STAT3 CA/pEF1aTetR ESCs, pSTAT3 and STAT3 were rapidly upregulated after 24 hours Dox induction.

C. In pTRE-STAT3 CA/pEF1aTetR ESCs, after the Dox induction, flagged-STAT3 CA expression was detected, which indicated that CA-STAT3 was expressed in these cells.

3.4 Illumina Microarray Analysis of STAT3 Target Genes

Microarray experiments were conducted in three independent experiments to profile fully the genes activated or repressed by STAT3. STAT3 CA ES cells (Constitutively Activated STAT3 or pSTAT3 overexpression) and STAT3 F/F Cre ES cells (STAT3 KO) were induced with Dox for 2 days and harvested for RNA profiling using illumina microarrays, compared with uninduced wild-type TetR or F/F TetR ES cells as controls.

Heatmaps were generated by using fold-change more than 4-fold, and we found 48 genes differentially expressed by more than 4-fold (38 up and 10 down) between wild-type TetR and STAT3 CA ES using illumina array platform (Figure 3.4A). By using fold-change more than 2-fold, 436 genes were found to be differentially expressed (260 up and 176 down) between wild-type TetR and STAT3 CA ES using illumina array platform. The top elevated and repressed genes (>4-fold) were depicted in Supplementary table 1, arranged according to average fold changes. We found that GATA6, was among the top 2 upregulated genes (11-fold) upon pSTAT3 constitutively overexpressed (Figure 3.4A, indicated by arrow).

To gain an overview of genes modulated when pSTAT3 was overexpressed and STAT3 was knockout, enrichment of functional gene ontology (GO) annotations among genes that are at least 2-fold differentially expressed were searched (Figure 3.4B). 15 biologic functions were significantly associated when pSTAT3 was overexpressed, which included those related to the phosphoprotein, alternative

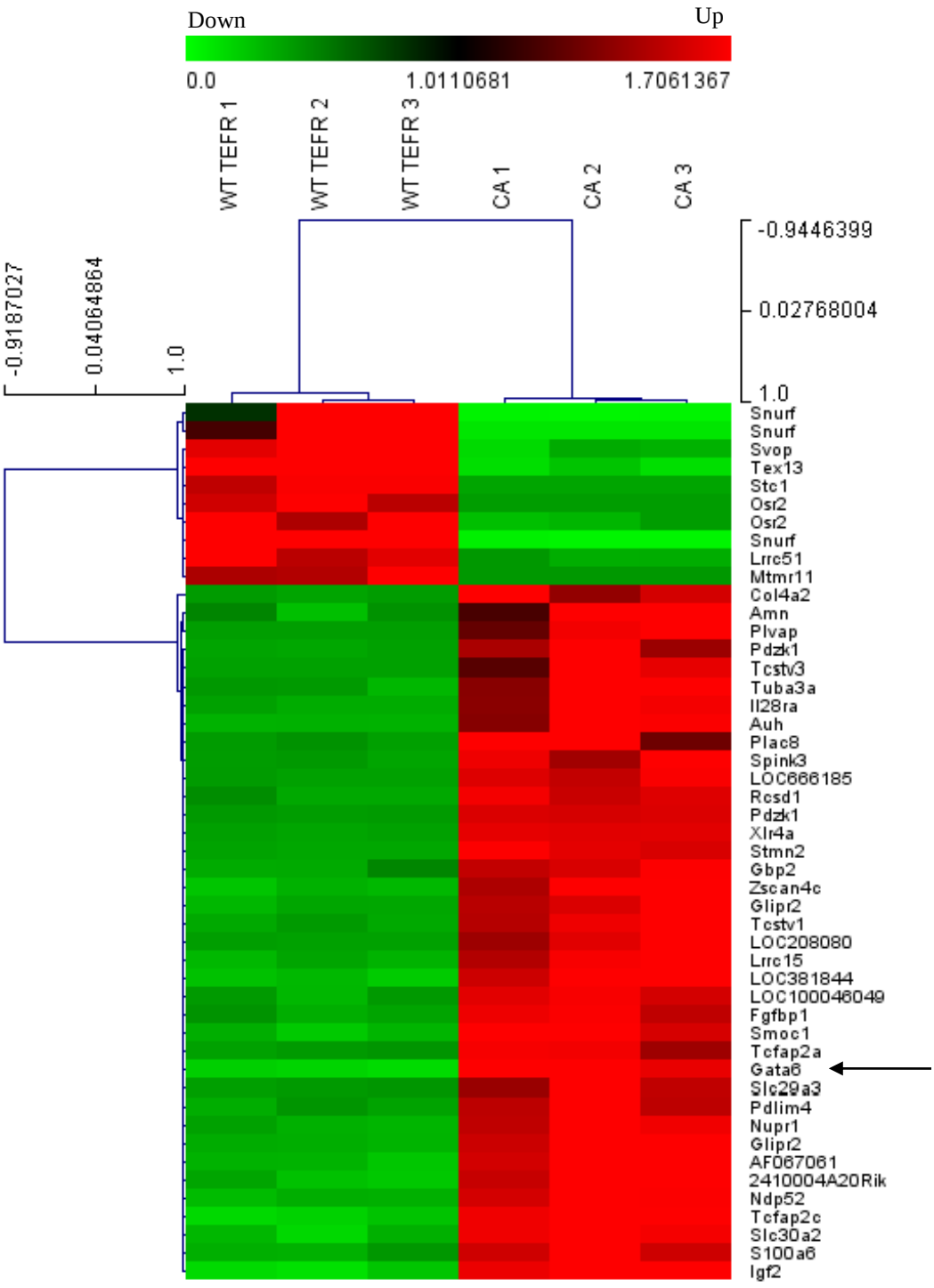
splicing, cytoplasm, development protein, cell fraction, apoptosis, embryonic development ending in birth or egg hatching, epithelium development, vasculature development, heart development, epithelial cell differentiation, and methylation, as well as regulation of transcription, transcription regulator activity and regulation of transcription from RNA polymerase II promoter. GATA6 was involved in 9 biological functions which were epithelium and heart development, embryonic development ending in birth or egg hatching, and epithelial cell differentiation. It was also involved in transcription regulation, transcription regulator activity, and regulation of transcription from RNA polymerase II promoter, apoptosis and phosphoprotein.

Between F/F TetR and STAT3 Cre ES, we found 135 genes differentially expressed by more than 4-fold (102 up and 33 down) using illumina array platform (Figure 3.4C). By using fold-change more than 2-fold, 1244 genes were found to be differentially expressed by more than 2-fold (869 up and 375 down) between F/F TetR and STAT3 Cre ES using illumina array platform. The top 20 elevated and repressed genes were depicted in Supplementary table 2, arranged according to average fold changes. 15 biologic functions were significantly associated when STAT3 is knockout (Figure 3.4D), which included those related to the alternative splicing, cytoplasm, development protein, embryonic development ending in birth or egg hatching, vasculature development, blood vessel development, heart development, embryonic organ development, differentiation, and negative

regulation of differentiation, as well as angiogenesis, vitamin binding, EGF calcium-binding, response to oxidative stress and inorganic substances.

Next, a heatmap was generated using the agree list of genes in expression regulated by STAT3 CA and in STAT3 Cre (Figure 3.4E). There were 209 genes differentially expressed by more than 2-fold (72 upregulated by STAT3 CA while downregulated in STAT3 Cre; 137 downregulated by STAT3 CA while upregulated in STAT3 Cre) between wild-type TetR and STAT3 CA and F/F TetR and Cre ES using illumina array platform. Txnip was one of the genes in which its expression was upregulated when STAT3 is KO (4-fold) while downregulated upon STAT3 constitutively overexpressed (0.48-fold) (Figure 3.4E, indicated by arrow).

A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CA 2
CA 3
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0.04064864
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1.0
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Snurf
Svop
Tex13
Stc1
Osr2
Osr2
Snurf
Lrrc51
Mtmr11
Col4a2
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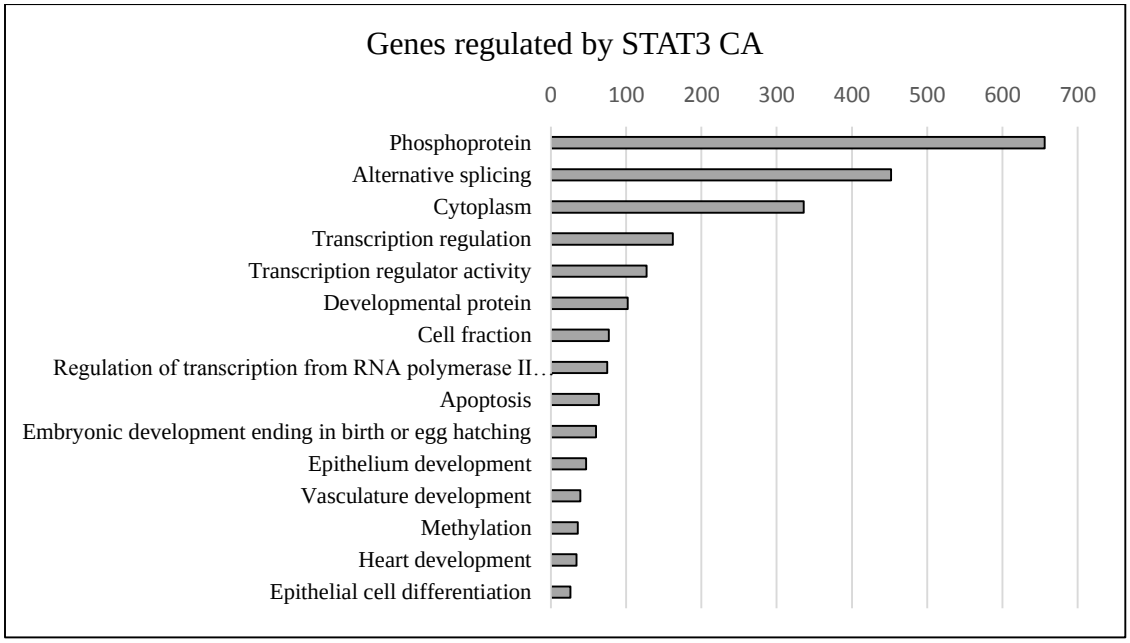
Genes regulated by STAT3 CA
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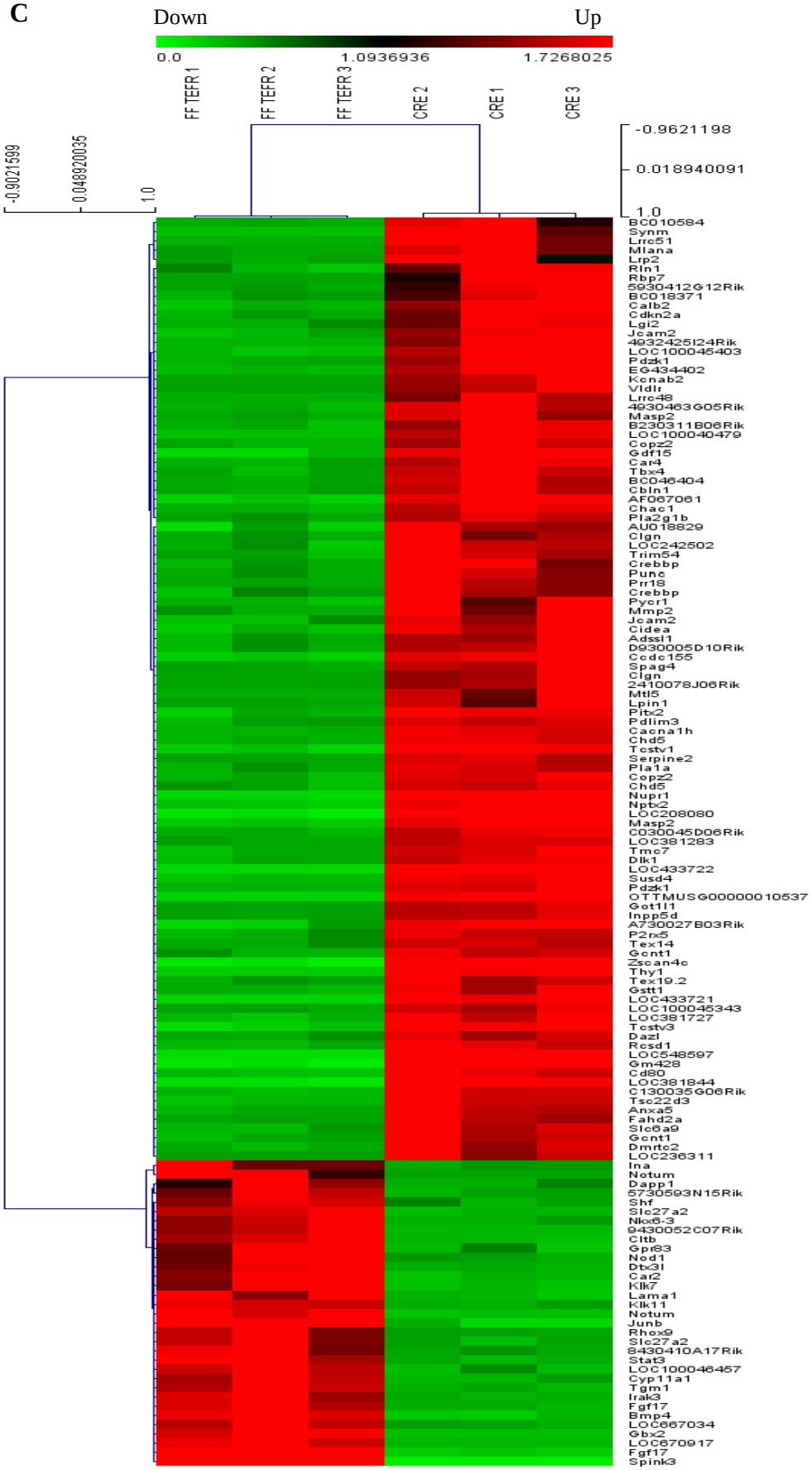
200
300
400
500
600
700
Phosphoprotein
Alternative splicing
Cytoplasm
Transcription regulation
Transcription regulator activity
Developmental protein
Cell fraction
Regulation of transcription from RNA polymerase II…
Apoptosis
Embryonic development ending in birth or egg hatching
Epithelium development
Vasculature development
Methylation
Heart development
Epithelial cell differentiation

C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CRE 1
CRE 3
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1.0
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Chd5
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Dlk1
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Susd4
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P2rx5
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Gcnt1
Zscan4c
Thy1
Tex19.2
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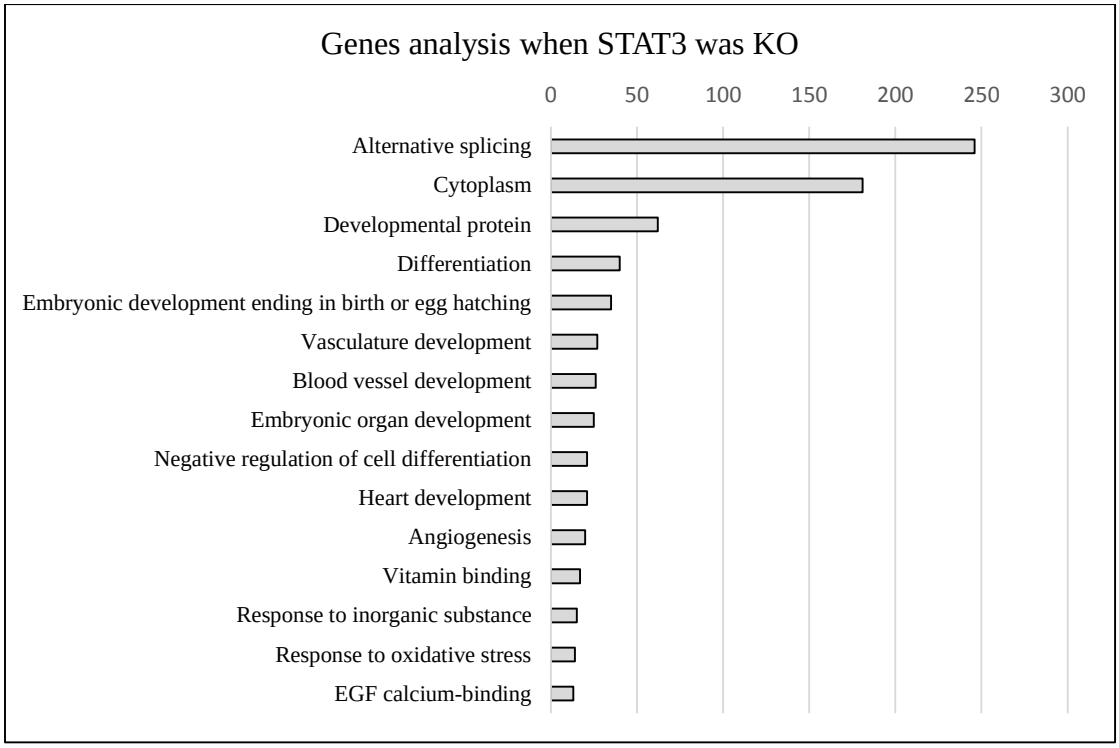
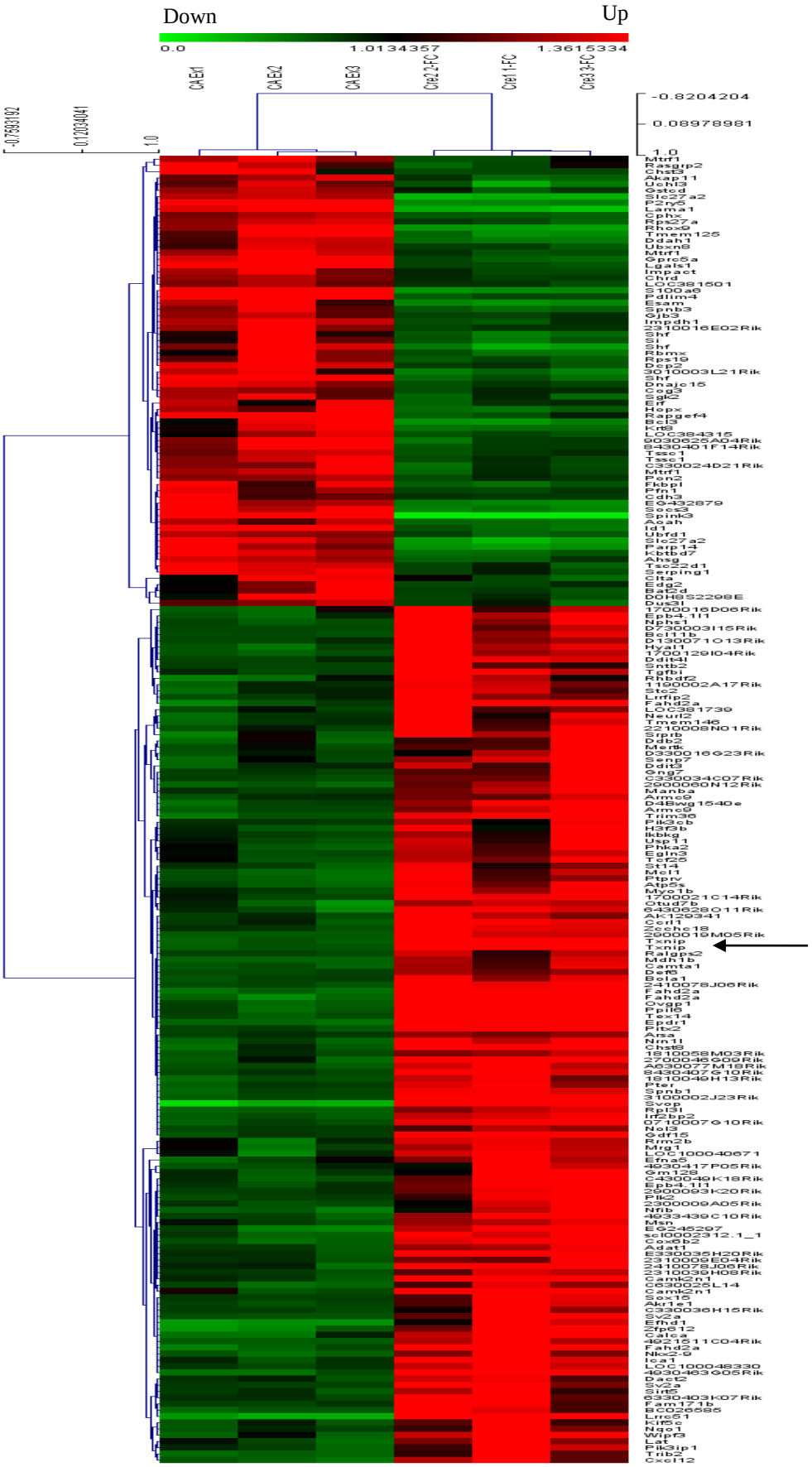

Genes analysis when STAT3 was KO
0
50
100
150
200
250
300
Alternative splicing
Cytoplasm
Developmental protein
Differentiation
Embryonic development ending in birth or egg hatching
Vasculature development
Blood vessel development
Embryonic organ development
Negative regulation of cell differentiation
Heart development
Angiogenesis
Vitamin binding
Response to inorganic substance
Response to oxidative stress
EGF calcium-binding

E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CNEx3
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Cre1-FC
Cre3.1-FC
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0.08978981
1.0
-0.7693192
-0.1203404041
1.0

Figure 3.4 Illumina Microarray Analysis of STAT3 Target Genes.

A. Comparison of the expression of genes regulated by STAT3 CA by heatmap.
B. Gene oncology (GO) analysis of STAT3 CA regulated genes.
C. Comparison of the expression of genes when STAT3 is knockout by heatmap.
D. Gene oncology (GO) analysis of genes when STAT3 is knockout.
E. A heatmap of genes expression which obey STAT3 CA and STAT3 KO agree list.

For heatmaps, red colour indicates upregulation, and green colour indicates downregulation, of gene expression relative to corresponding control, with colour intensity corresponding to the fold-change amplitude (fold-change scale shown at top).

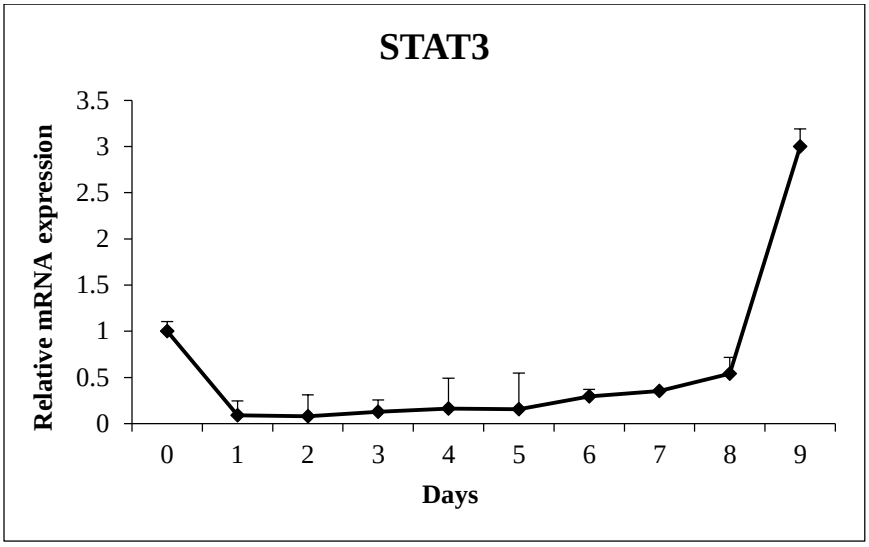
For GO analysis, the bar charts show the number of genes regulated by STAT3 and only top significant categories ($p<0.01$) were selected to show. The same gene may be assigned to more than one category.

3.5 Temporal expression profile of STAT3 during normal ESC differentiation into cardiomyocyte

STAT3 expression profile in ES cells and during normal ES cells differentiation was measured by qRT-PCR and Western blot analysis. STAT3 was highly transcribed at undifferentiated ES cells at day 0 (Figure 3.5A). However, its expression declined once the commencement of ES cells cardiomyocyte differentiation began at day 1 and was decreased further thereafter in the differentiation phrases from day 2 to day 4. STAT3 showed upregulation at late phrase of cardiomyocyte differentiation from day 5 onwards, which increased progressively from day 5 to day 9 (Figure 3.5A).

Western blot was used to examine quantitative protein expressions of STAT3 and pSTAT3. Activation status of STAT3 was revealed by using pSTAT3 and our analysis reaffirmed that STAT3 was active and functional during the course of cardiomyocyte differentiation. Results from Western blot analysis supported that of qRT-PCR. STAT3 and pSTAT3 protein expressions were highly detected at ES undifferentiated state, downregulated at day 1 until day 5 and upregulated towards late cardiomyocyte differentiation process from day 5 onwards and reached its peak on day 8 (Figure 3.5B). These results indicated that STAT3 is a prominent factor in cardiomyocyte differentiation. STAT3 showed temporal expression and it was downregulated during early phrase of cardiac differentiation process but upregulated towards late phrase of differentiation process.

A



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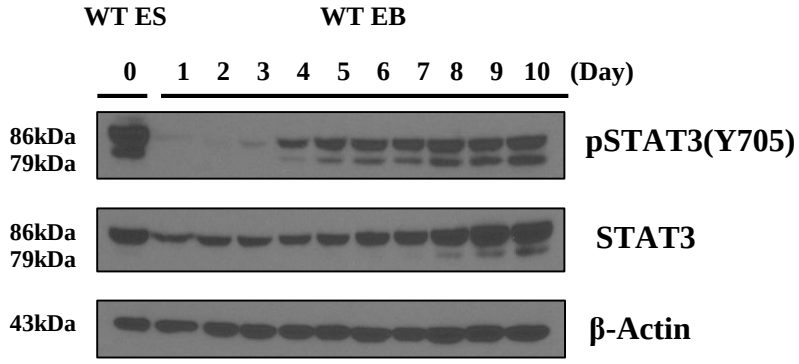


Figure 3.5 STAT3 mRNA and protein dynamic expressions during cardiac differentiation from ESCs.

A. STAT3 mRNA decreased during ESC into mesoderm stage at day 4 and became increased from cardiac progenitor stage at day 5 till cardiomyocyte at day 9.

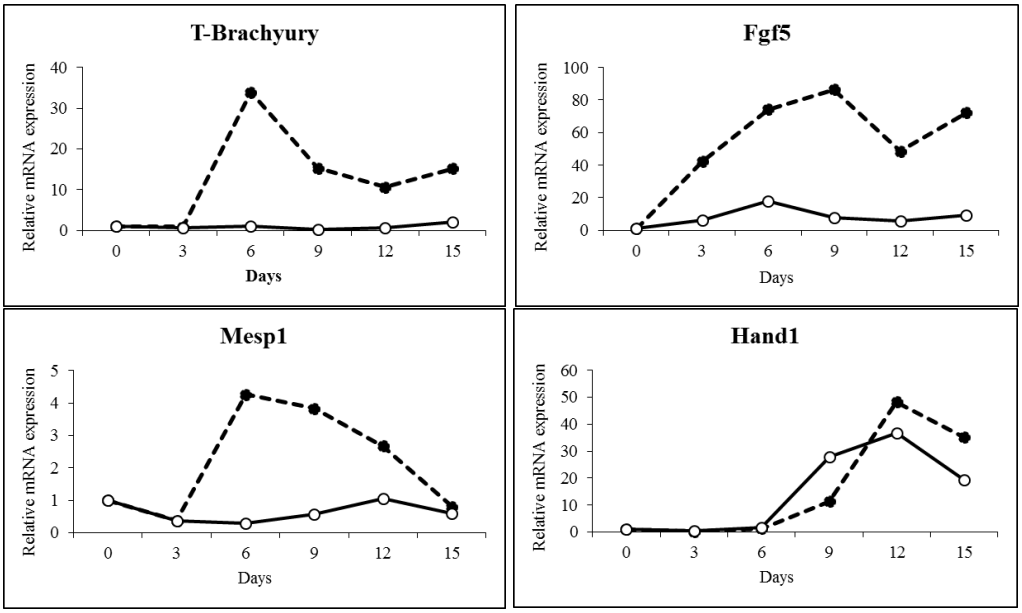
B. pSTAT3 protein decreased during ESC into mesoderm stage and became increased from cardiac progenitor stage at day 5 till cardiomyocyte at day 10.

3.6 STAT3 is required for cardiomyocytes differentiation of ESCs

Next, inducible STAT3 KO system was utilized to identify the role and mechanism of STAT3 in cardiomyocytes differentiation of ESCs. The effect of deletion of STAT3 on cardiomyocytes differentiation of ESCs was first examined. STAT3 was deleted from Day 0 onwards of cardiomyocytes differentiation of ESCs. Undifferentiated ESCs and differentiated EBs were collected at day 0, 3, 6, 9, 12, and day 15 and qRT-PCR was performed to analyze the expression of mesodermal markers and cardiac transcription factors in these STAT3 KO differentiated EBs as compared to uninduced EBs.

Time course qRT-PCR suggested that deletion of STAT3 reduced the expression of mesodermal markers such as T-Brachyury, Mesp1, Fgf5 and Hand1 significantly during differentiation as compared to uninduced EBs (Figure 3.6A). In addition, time course qRT-PCR revealed that deletion of STAT3 led to significantly reduction in the expression of cardiac transcription factors such as GATA4, Mef2c, Nkx2.5 and GATA6 (Figure 3.6B). Together, these results demonstrated that STAT3 is required for ESCs differentiation into mesoderm and cardiac lineage.

A



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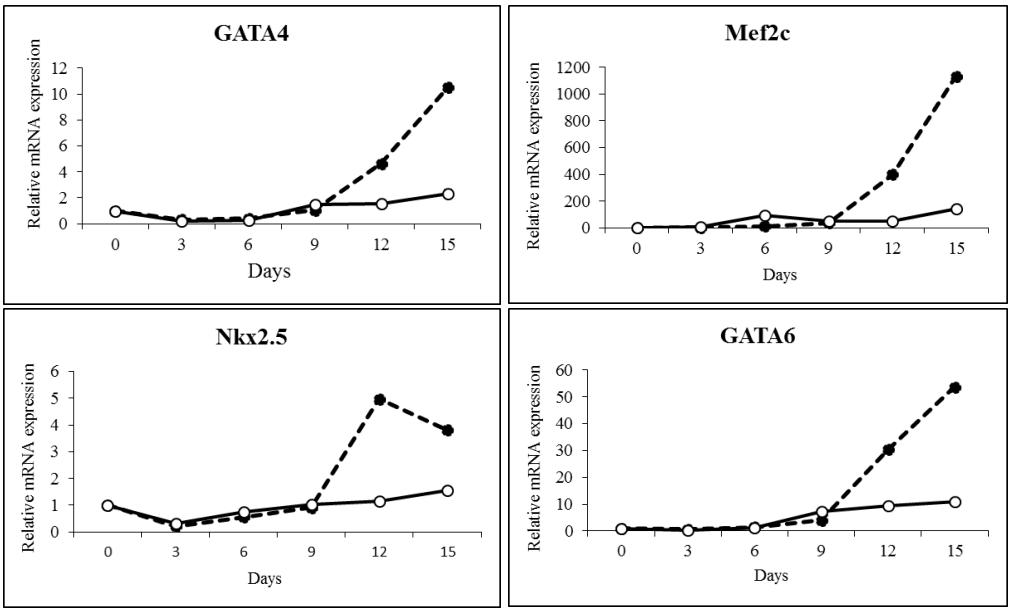


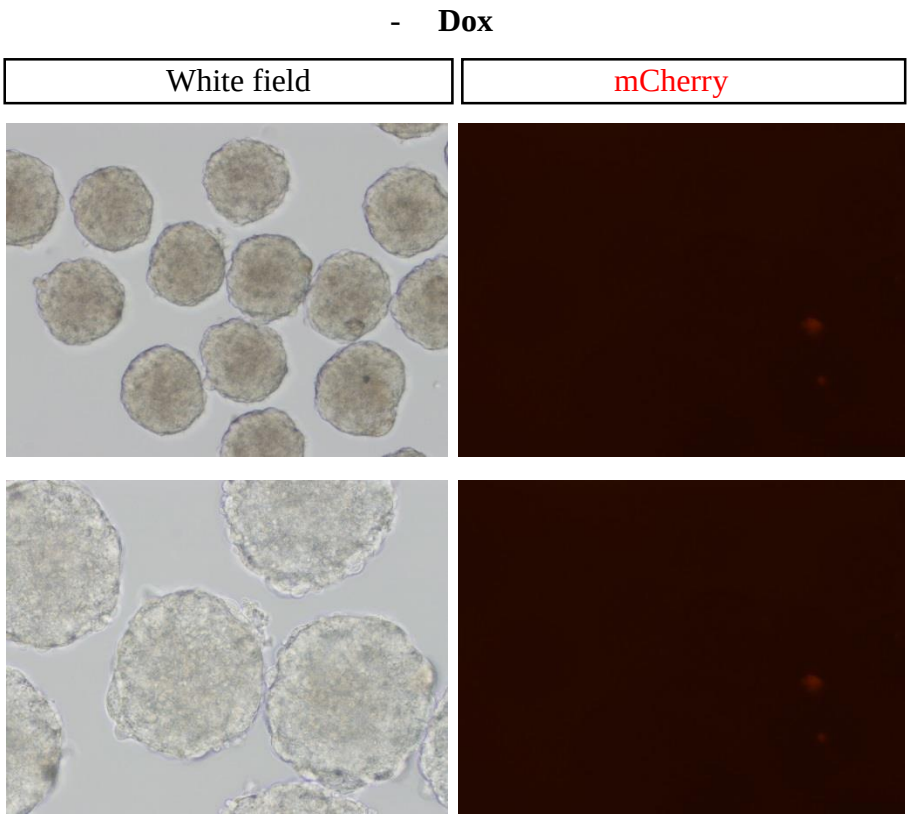
Figure 3.6 Time course qRT-PCR of mesodermal markers and cardiac transcription factors when STAT3 was deleted from D0 of cardiomyocytes differentiation of ESCs.

Deletion of STAT3 led to significantly reduction in the expression of mesodermal markers (A) and cardiac transcription factors (B) when STAT3 was deleted from Day 0 onwards of cardiomyocytes differentiation of ESCs, indicating that STAT3 is required for cardiomyocytes differentiation of ESCs

3.7 Effect of deletion of STAT3 on cell growth

Next, in order to find out whether or not the deletion of STAT3 had any effect on the formation of EBs, the morphology of EBs formed when STAT3 was deleted from Day 0 onwards of cardiomyocyte differentiation from ESCs was first observed. Pictures of Cre inducible embryoid bodies 48 hours following Dox induction showed not much different in the size of STAT3 KO EBs as compared to unstimulated EBs (Figure 3.7 A, B), suggesting that STAT3 has no effect on the cell growth and morphology of EBs formed when STAT3 is deleted.

A



B

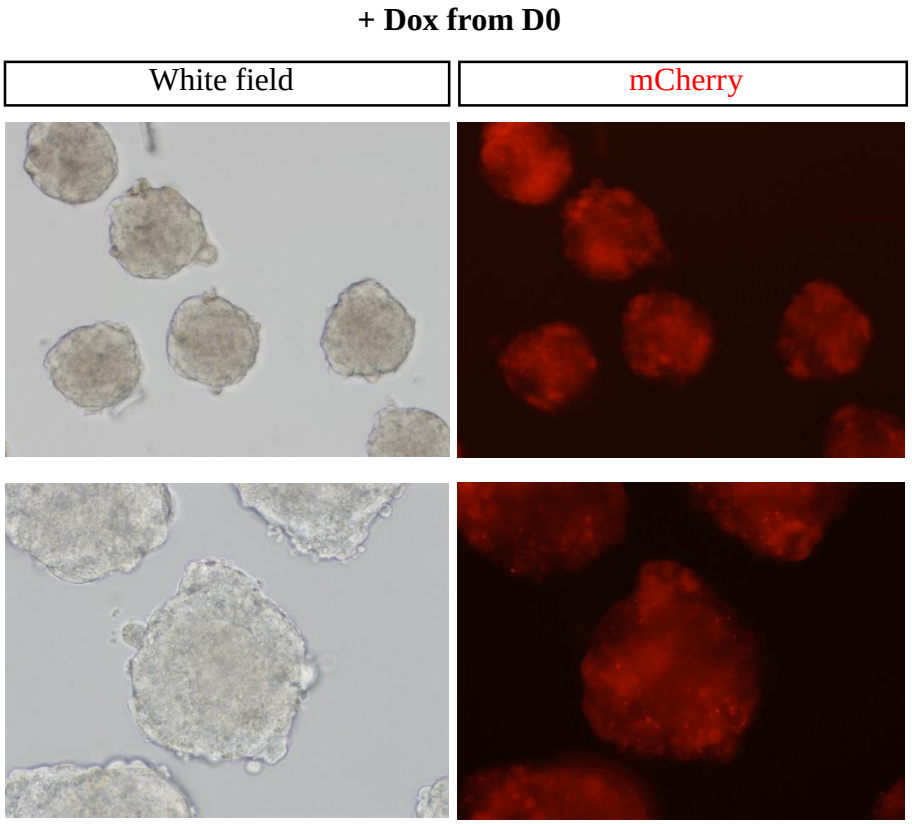


Figure 3.7 Pictures of embryoid bodies 48 hours following Dox stimulation. Not much different in EBs size in STAT3 KO EBs as compared to unstimulated EBs, indicating that STAT3 has no effect on the cell growth and morphology of EBs formed when STAT3 is deleted.

3.8 Inducible deletion of STAT3 delays cardiac differentiation from ESCs

To access how STAT3 function is involved in cardiac lineage differentiation, STAT3 is deleted at different time point prior to the onset of cardiomyocyte precursor throughout the course of cardiomyocyte differentiation from ESCs. Cardiac progenitors are specified at approximately day 4 of culture of differentiating EBs (Kattman, Huber, & Keller, 2006). Therefore, Dox is added at day 0, 3 and 4 to induce deletion of STAT3 and monitor the temporal appearance of beating EBs daily, whether or not the deletion of STAT3 will result in a delay of cardiac differentiation as demonstrated by decreased beating cardiomyocytes or the beating cells occur later than control EBs in which Dox is not added.

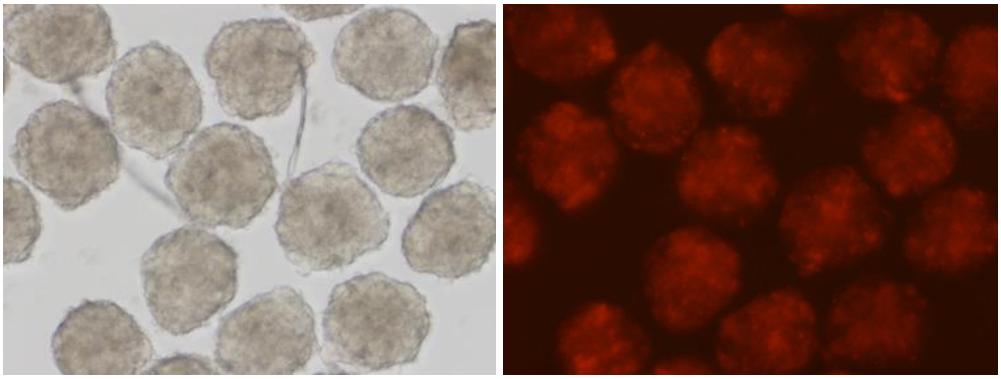
In control EBs (Dox was not added), beating cardiomyocyte was first observed at day 9. However, when STAT3 was deleted at day 0, no beating cardiomyocyte was observed (Figure 3.8B). Therefore, STAT3 might play a crucial role in regulation of cardiomyocyte differentiation. In contrast, when STAT3 was deleted at day 3, beating cardiomyocyte was first observed at day 13, which was 4 days later then the control EB. Interestingly, deletion of STAT3 on day 4 did not affect much on cardiac differentiation because beating cardiomyocyte was observed on day 9 which was the same as control (Figure 3.8B). The beating EBs were maintained thereafter, in good accordance with the contractile phenotype of the cells observed upon microscopic inspection until day 16 of differentiation. Videos of beating cardiomyocyte were recorded at day 9 and day 13.

Day 16 of beating cardiomyocytes were harvested and western blot analysis showed that both pSTAT3 and STAT3 protein levels were decreased when Dox was added at day 0, 3 and 4 (Figure 3.8C). α-Actinin and Troponin T are markers for mature cardiomyocytes and were expressed in the control EBs, indicating that the precursors generated are of the cardiac lineage (Figure 3.8C). α-Actinin and Troponin T were decreased dramatically when STAT3 was deleted at day 0 and 3 as compared to control (Dox was not added) (Figure 3.8C). These results demonstrated that STAT3 deletion may delay cardiac-committed mesoderm development and thus beating cells occurred 4 days later than the control EB when STAT3 was deleted at Day 3. Interestingly, deletion of STAT3 on day 4 of cardiac differentiation did not affect the α-Actinin and Troponin T protein that much as compared with deletion of STAT3 at day 0 and 3 (Figure 3.8C) and therefore, beating cardiomyocyte was first observed at day 9 which was the same as control. In contrast, no sign of beating EBs formed when Dox was administrated constitutively from day 0 of cardiac differentiation, suggesting that deletion of STAT3 from day 0 onwards inhibited cardiac differentiation probably through inhibition on formation of cardiac-committed mesoderm or cardiac progenitor cells.

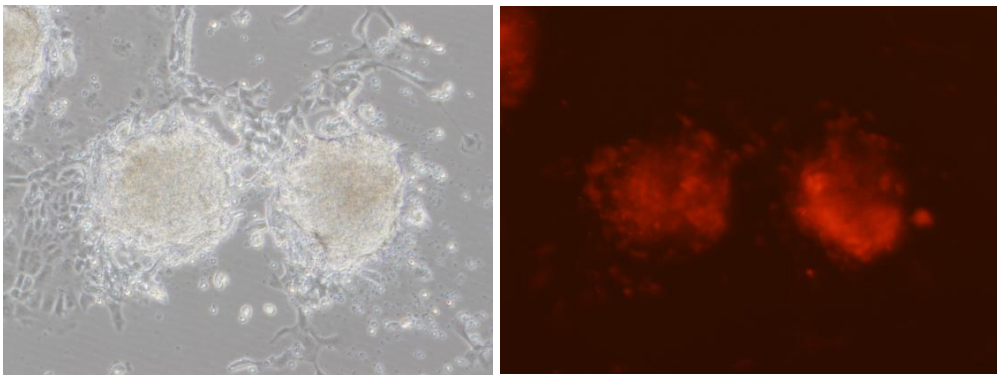
A

White field	mCherry

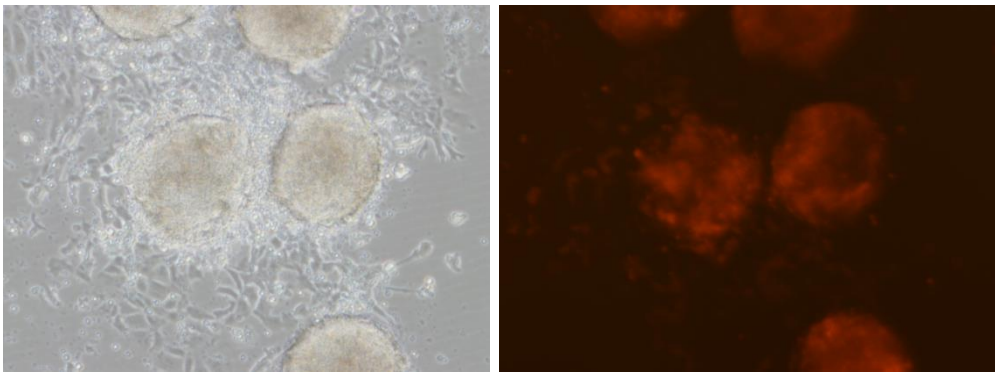
+ Dox from D0



+ Dox from D3



+ Dox from D4



B

Day of Dox being added	Day of beating cardiomyocyte first observed
Control EBs (no Dox added)	Day 9
Day 0	No beating cardiomyocyte was observed
Day 3	Day 13 (4 days later than control)
Day 4	Day 9 (same as control)

C

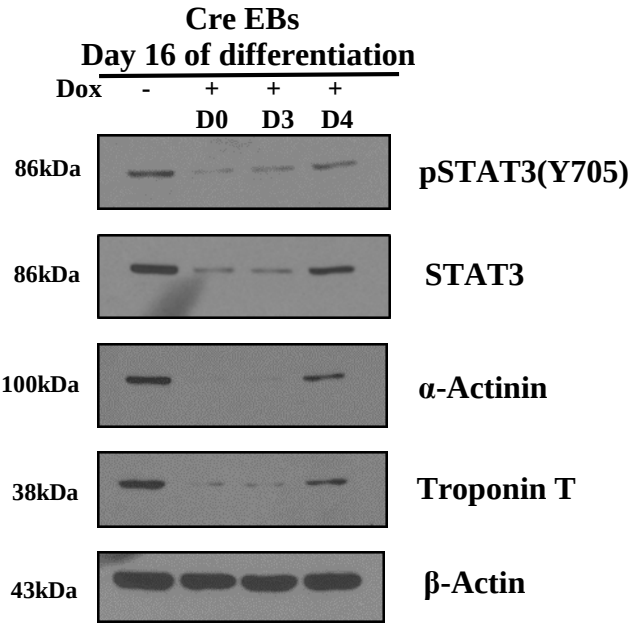


Figure 3.8 Inducible deletion of STAT3 delayed cardiac differentiation from ESCs.

A. Deletion of STAT3 at day 0 inhibited cardiac differentiation as no beating cardiomyocyte was observed as compared to unstimulated EBs in which beating cardiomyocyte was first observed at day 9. In contrast, when STAT3 was deleted at day 3, beating cardiomyocyte was first observed at day 13, which was 4 days later then our control EB.

B. Table summarized the day of beating cardiomyocyte first observed when Dox was added at different time point, at Day 0, 3 and 4 to induce deletion of STAT3.

C. pSTAT3 and STAT3 were rapidly downregulated after Dox induction. α-Actinin and Troponin T, a specific marker of cardiomyocytes were downregulated significantly when STAT3 was deleted at Day 0, 3 and 4.

3.9 STAT3 is essential for cardiomyocyte differentiation from ESCs through positively regulation on GATA6

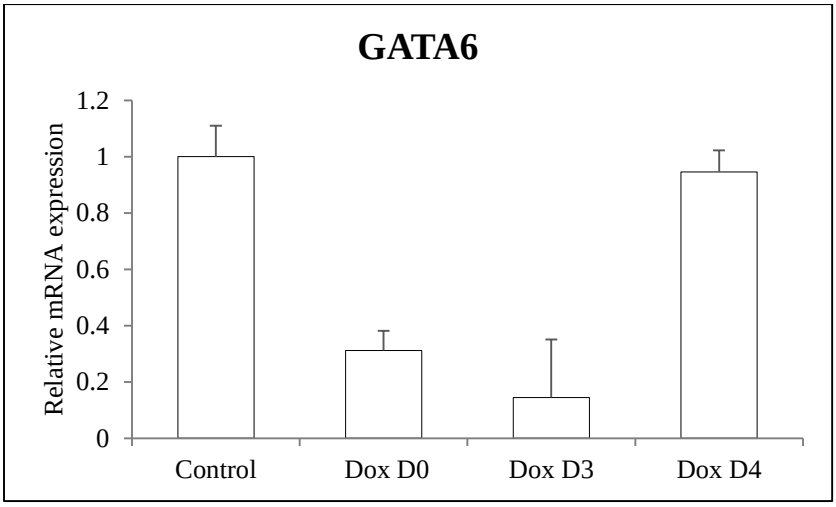
Microarray analysis revealed that GATA6 is one of the top target genes regulated by overexpression of pSTAT3 in ESCs (Figure 3.4A) and our data has demonstrated that STAT3 is essential for cardiomyocyte differentiation (Figure 3.8). Therefore, we next asked whether STAT3 regulates GATA6 expression and thus promoting cardiomyocyte differentiation from ESCs.

We first examined the expression of GATA6 by qRT-PCR and western blot. Deletion of STAT3 on day 0 onwards and day 3 during cardiac differentiation from ESCs led to decreased expression of GATA6 at both mRNA and protein level (Figure 3.9A, B), demonstrating GATA6 is positively regulated by STAT3, and is important in mediating cardiomyocyte differentiation from ESCs. Therefore, no beating cardiomyocyte was observed or beating occurred later when STAT3 was deleted on day 0 and day 3 due to decreased level of GATA6 which is important for cardiac differentiation. In contrast, deletion of STAT3 on day 4 of cardiac differentiation did not affect the GATA6 mRNA and protein levels much (Figure 3.9A, B). This indicated that STAT3 has stage specific function during cardiomyocyte differentiation from ESCs. Deletion of STAT3 during early phrase of cardiomyocyte differentiation (Day 0 and Day 3) will affect or delay the determination of cardiac lineage from ESCs. However, when STAT3 was deleted during later phrase (Day 4), the cardiac lineage has been determined and deletion of STAT3 has no effect anymore on cardiomyocyte differentiation as shown by unaffected level of GATA6 when STAT3 was deleted on day 4.

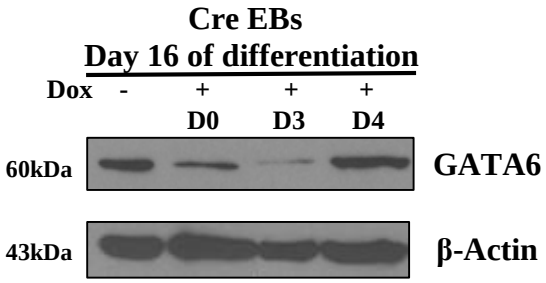
To determine whether GATA6 is a direct target of STAT3, ChIP was performed in E14 ESCs using anti-STAT3 antibody. Immunoprecipitated DNA was analyzed using qRT-PCR with primer sets designed to detect ChIP-enriched DNA fragments. The expression of GATA6 was about 2.6-fold higher in DNA samples immunoprecipated with anti-STAT3 antibody than that of the isotype control IgG antibody (Figure 3.9C), indicating STAT3 enrichment in the GATA6 promoter region. Transcriptional regulation of GATA6 by STAT3 could be a possible mechanism by which STAT3 promotes cardiomyocytes differentiation. This STAT3 binding site is located within 2kb upstream of the TSS of GATA6 genes. Fold enrichment is the relative abundance of DNA fragments at the indicated region over control region as measured by qRT-PCR.

Next, D13 EBs were co-stained with STAT3 and cardiac specific marker, Actn2 to examine the expression of STAT3 when Dox was added on D0 of cardiomyocytes differentiation of ESCs. Immunofluorescent staining demonstrated that decreased expression of STAT3 and cardiac specific marker, Actn2 following STAT3 deletion on D0 as compared to uninduced EBs (Figure 3.9D). Similarly, D13 EBs were co-stained with GATA6 and cardiac specific marker, Actn2 and the immunostaining suggested that decreased expression of GATA6 and cardiac specific marker, Actn2 following STAT3 deletion on D0 as compared to uninduced EBs (Figure 3.9E). Together, these results indicated that STAT3 is essential for cardiomyocyte differentiation of ESCs through positive regulation on GATA6.

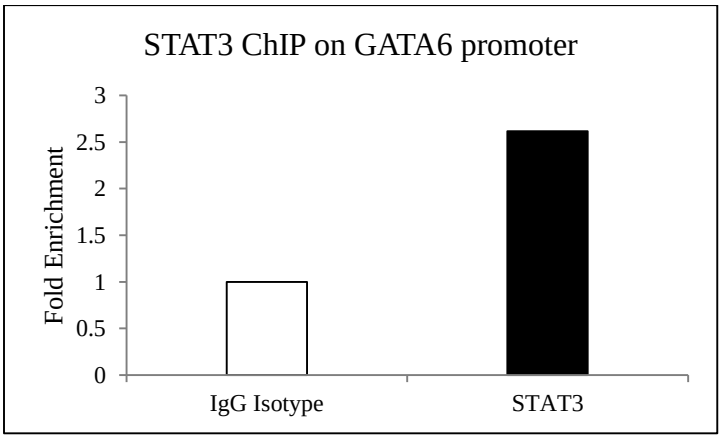
A


GATA6
Relative mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
Control
Dox D0
Dox D3
Dox D4

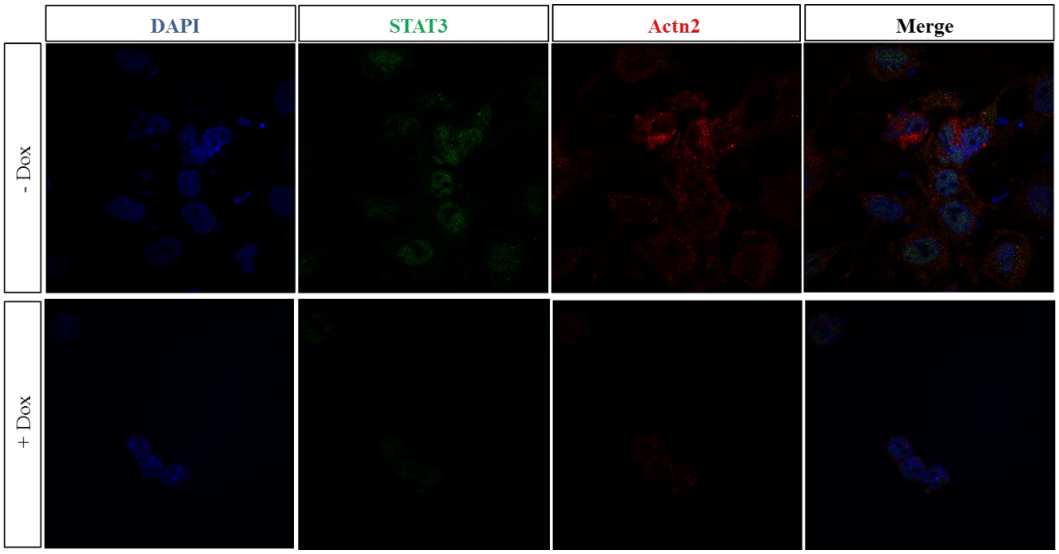
B


Cre EBs
Day 16 of differentiation
Dox - + + +
D0 D3 D4
60kDa
GATA6
43kDa
β-Actin

C


STAT3 ChIP on GATA6 promoter
Fold Enrichment
3
2.5
2
1.5
1
0.5
0
IgG Isotype
STAT3

D



E

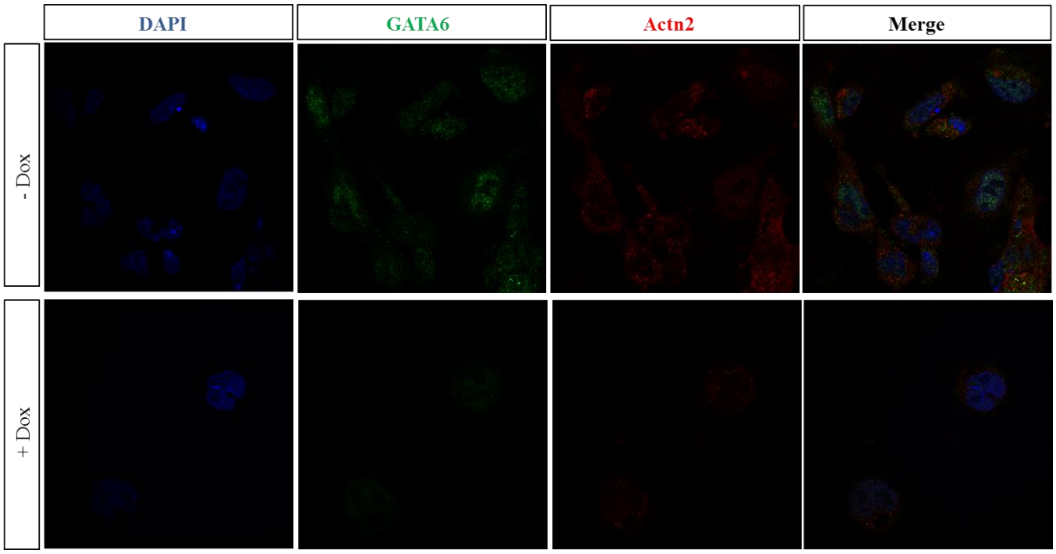


Figure 3.9 STAT3 is essential for cardiomyocyte differentiation from ESCs through positively regulation on GATA6.

A. Deletion of STAT3 from Day 0 onwards and Day 3 during cardiac differentiation downregulated GATA6 mRNA levels as compared to control. Deletion of STAT3 on Day 4 of cardiac differentiation did not affect the GATA6 level much.

B. Consistent with its mRNA levels, GATA6 protein levels decreased significantly when STAT3 was deleted on Day 0 and Day 3 during cardiac differentiation. Deletion of STAT3 on Day 4 of cardiac differentiation did not affect the protein level of GATA6.

C. STAT3 binds GATA6 promoter in mouse E14 ESCs.

D. Decreased expression of STAT3 and Actn2 following STAT3 deletion on D0.

E. Decreased expression of GATA6 and Actn2 following STAT3 deletion on D0.

Chapter 4 Results (Part II)

Results (Part II): Cardioprotection role of STAT3 in adult heart

4.1 Data Extraction of STAT3-ChIP publication

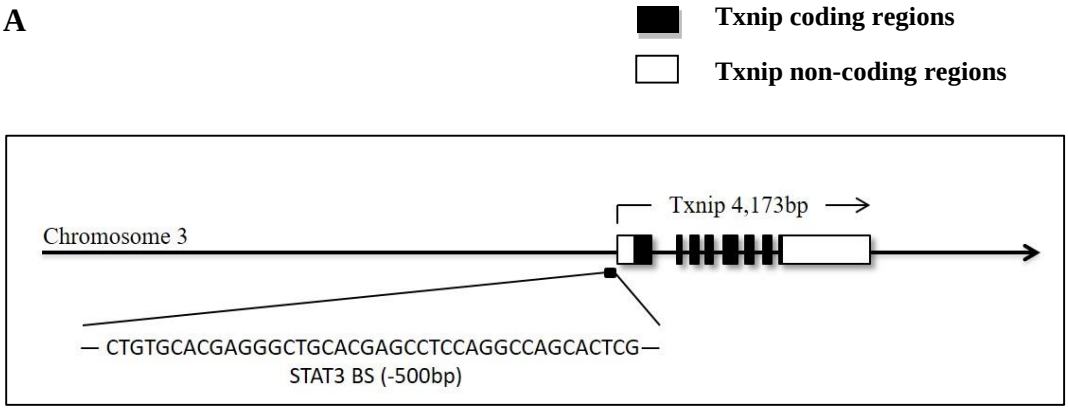
The protection role of STAT3 in the heart has been clearly illustrated in the introduction. Next, we would like to address the underlying cardioprotection mechanism played by STAT3, to find out potential downstream target genes of STAT3 in which together they contribute to the survival of the heart under pathogenesis. Supplementary table 3 revealed the potential list of cardiac genes which may involve in the pathophysiology of cardiovascular diseases and might also contain STAT3 binding sites in 10kb distance from its TSS. Location of STAT3 binding site was extracted from published STAT3 ChIP-sequencing datasets (Durant et al., 2010; Ho et al., 2011; Hutchins, Poulain, & Miranda-Saavedra, 2012). From this list of cardiac genes, we had chosen Txnip, thioredoxin interacting protein, also known as thioredoxin binding protein 2 or vitamin D3 upregulated protein for our subsequent studies.

4.2 Enrichment of STAT3 binding on Txnip promoter

To determine whether Txnip is a direct target of STAT3 and to validate the published STAT3-ChIP datasets (Figure 4.2A), we performed STAT3-ChIP using anti-STAT3 antibody to access the binding of STAT3 to the Txnip promoter in H9c2 myoblast. Expression of Txnip was about 15-fold higher in DNA samples immunoprecipitated with anti-STAT3 antibody than that of the isotype control IgG antibody, indicating that elevated binding of STAT3 on Txnip promoter region at

18hr after LPS treatment (Figure 4.2B). This implies that Txnip is one of the potential target genes regulated by STAT3, and therefore contributes to cardioprotection under pathogenesis. SOCS3 gene which is a downstream targeting gene regulated by STAT3 (Hilton, 1999) was used as a positive control in this ChIP experiment. STAT3 binding site on SOCS3 promoter was examined and results showed upregulation of STAT3 binding on SOCS3 promoter at both 2hr (24-fold) and 18hr (12-fold) after LPS treatment (Results not shown). Further studies are needed to elucidate the detailed mechanism of STAT3 involvement in Txnip expression during pathogenesis of heart failure.

A



B

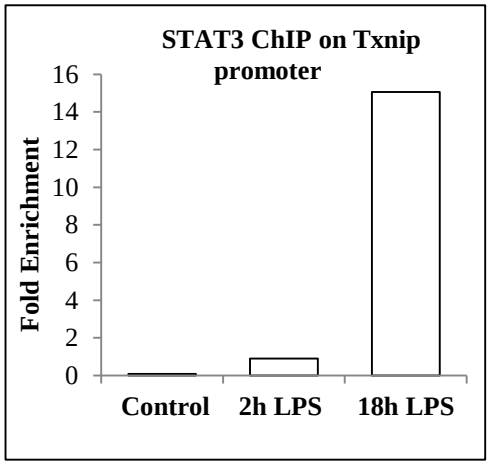


Figure 4.2 STAT3 binds Txnip promoter.
A. The map of partial mouse chromosome 3 harboring Txnip locus. Along the Txnip locus, the small black box indicated one STAT3 binding site located 6.6 kb upstream. Binding site for STAT3 was identified by extraction of different ChIP-Sequencing datasets, Promo genome sequence analysis and Sabioscience websites. B. STAT3-ChIP assay showed elevated binding of STAT3 on Txnip promoter at 18hr after LPS treatment.

4.3 Reduced expression of Txnip immediately in the wild-type heart and H9c2 myoblast after LPS treatment

Initial studies were performed to screen whether Txnip expression is directly regulated by STAT3 in cardiomyocytes. STAT3 was activated in both wild-type mice and H9c2 myoblast by injection of LPS into mice for 2 hours and 18 hours while applying LPS treatment for 30 minutes, 1 hour, 2 hours and 18 hours in H9c2 myoblast. qRT-PCR result suggested that Txnip mRNA expression was reduced after 2 hours LPS treatment in wild-type mice as compared to untreated mice. Txnip mRNA expression was also reduced after 18 hours of LPS treatment, in which its mRNA expression was lower than those of untreated mice, but slightly higher than 2 hours LPS treatment (Figure 4.3A). Consistence with its mRNA expression, western blot assay demonstrated that reduced Txnip protein expression was observed after 2 hours LPS treatment. Protein expression of Txnip was reduced after 18 hours of LPS treatment as compared to untreated mice, but still slightly higher than those of 2 hours LPS treatment (Figure 4.3B). Also, H9c2 myoblast treated with LPS suggested that Txnip protein level decreased in a time-dependent manner, decreased after 30 minutes, 1 hour and 2 hours LPS treatment and then retained close to control level of Txnip protein at 18 hours (Figure 4.3C). Thus, this suggested that STAT3 may play a role in the regulation of Txnip in this LPS-induced heart inflammation.

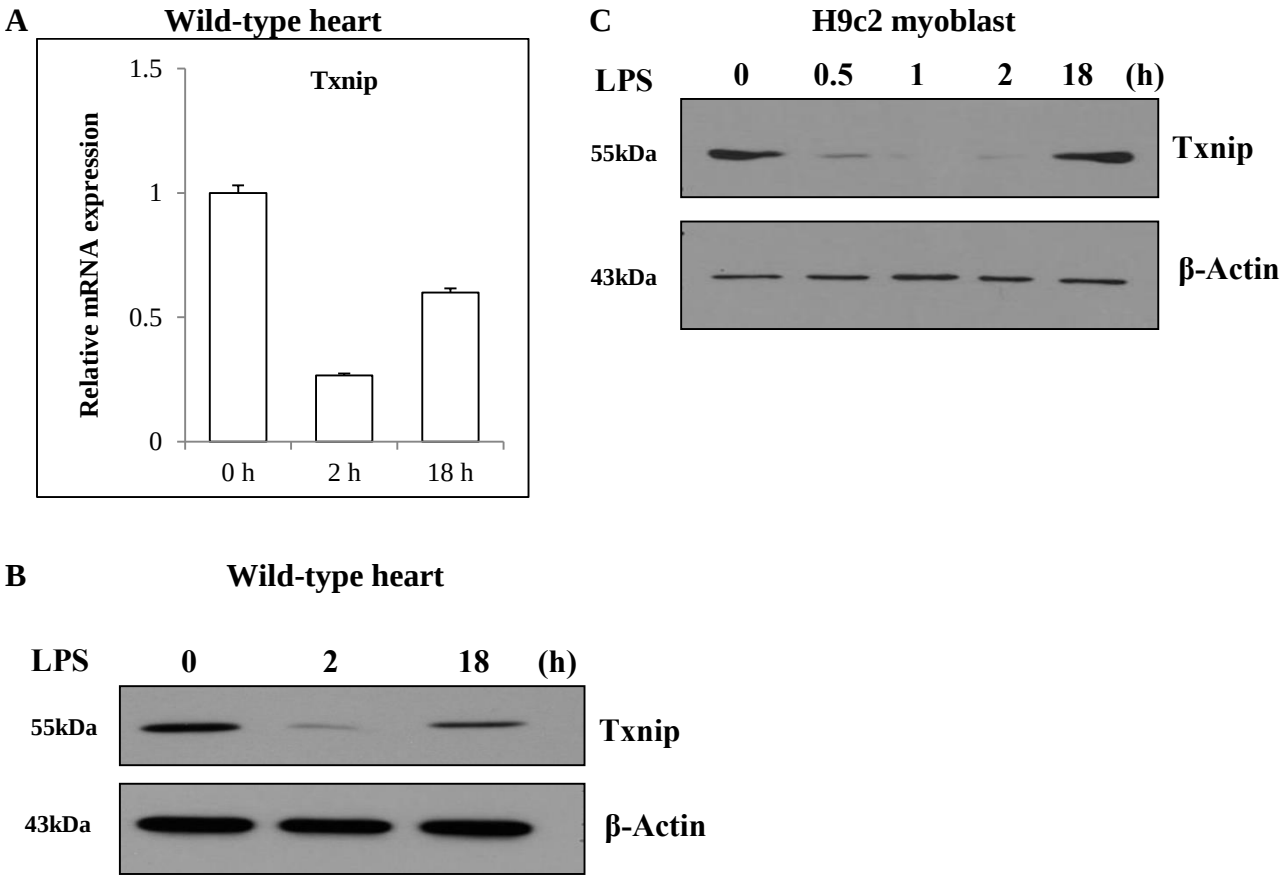
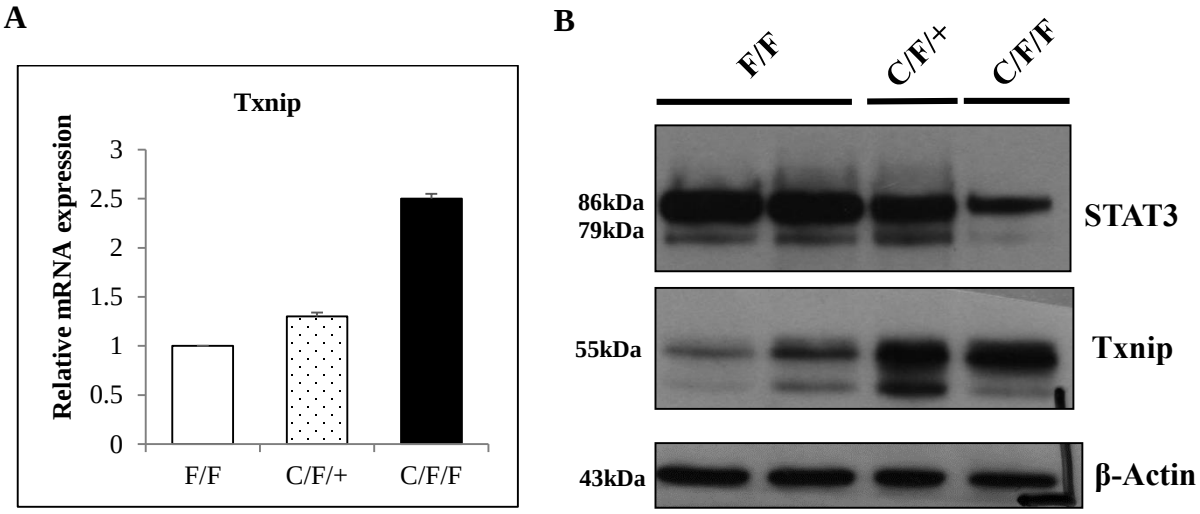


Figure 4.3 STAT3 activation by LPS treatment decreased Txnip expression in wild-type mice and H9c2 myoblast.
A. Txnip mRNA level was reduced after 2 hours and 18 hours of LPS treatment in wild-type heart.
B. Protein expression of Txnip was reduced after 2 hours and 18 hours of LPS treatment in wild-type heart which was consistence with its mRNA expression.
C. H9c2 myoblast treated with LPS and Txnip protein level decreased in a time-dependent manner and then retained close to control level at 18 hours.

4.4 Increased Txnip expression in hearts of STAT3 deficient mice

Knowing that Txnip is a direct target of STAT3 and its expression was reduced after STAT3 activation by LPS, these results suggested that Txnip is negatively regulated by STAT3. Studies have demonstrated that deletion of Txnip in mice protects the myocardium from ischemia-reperfusion (I/R) injury (Yoshioka et al., 2012). Therefore, we hypothesized that Txnip activation might be the mechanism

involved in causing STAT3 CKO heart failure. Hence, we want to address whether deletion of STAT3 will result in an increase of Txnip expression, further validating that STAT3 negatively regulated Txnip expression. The whole hearts were harvested from wild-type mice, STAT3 heterozygous (C/F/+) and STAT3 CKO (C/F/F) mice. High level of Txnip expression was detected in STAT3 deficient heart. Decreased in STAT3 increased the expression of Txnip at both the mRNA and protein levels (Figure 4.4A, B). This implied that increased Txnip expression may cause the STAT3 CKO heart failure and one of the STAT3 mediated cardioprotection pathways may involve downregulation of Txnip. Therefore, modulation of Txnip expression can be an effective strategy for treating cardiovascular diseases in which ROS accumulation and cardiomyocyte apoptosis occur (Jacoby et al., 2003).



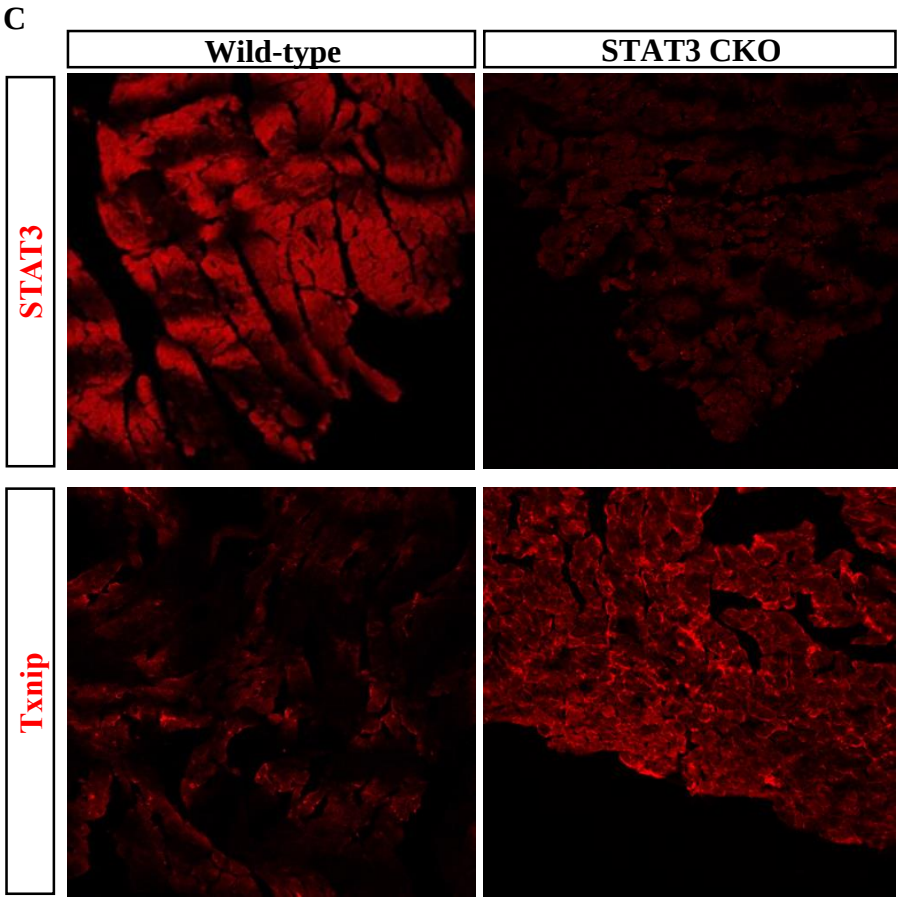


Figure 4.4 Deletion of STAT3 increased Txnip expression in STAT3 deficient heart.

A. Txnip mRNA expression was increased in STAT3 deficient heart.

B. In consistence with its mRNA expression, Txnip protein expression was greatly upregulated in STAT3 deficient heart.

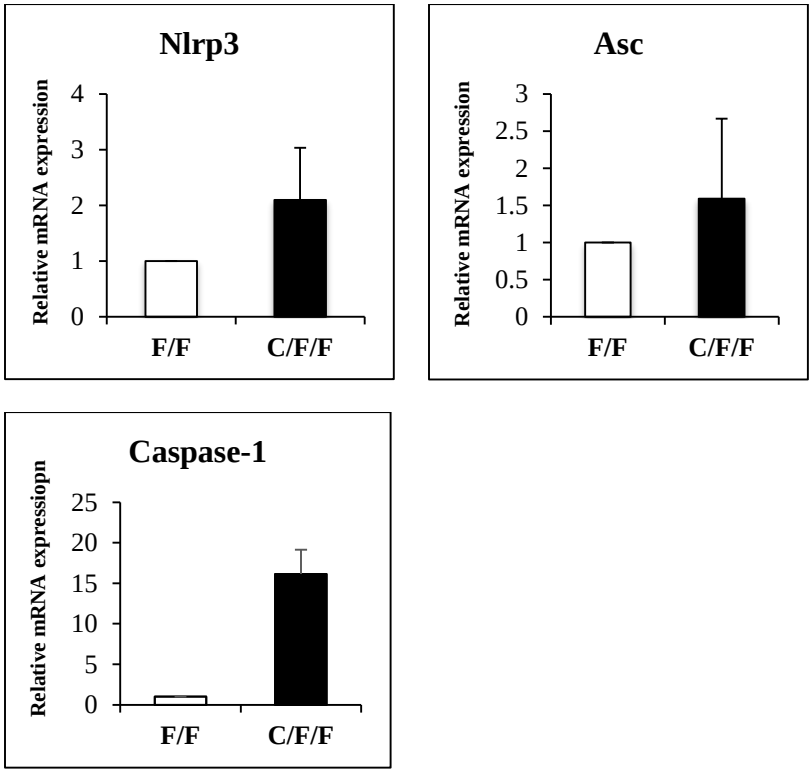
C. Immunofluorescent staining showed increased expression of Txnip in the STAT3 deficient heart.

4.5 Expression of the components of the inflammasome in the STAT3 deficient heart

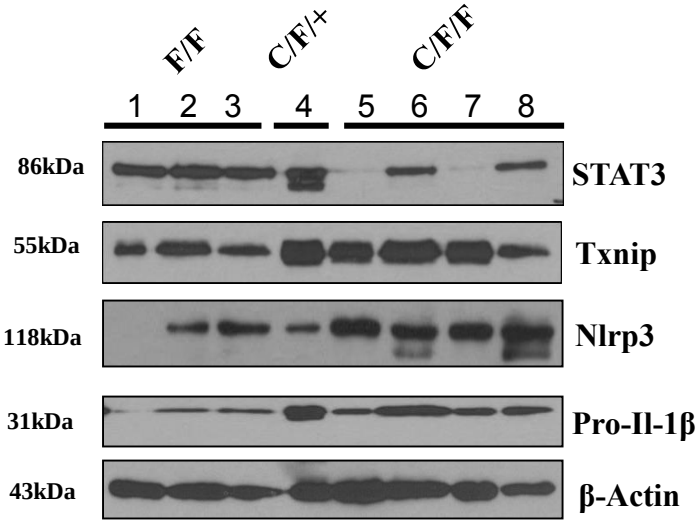
Knowing that Txnip was increased in STAT3 deficient heart and inhibition of Txnip is suggested as a potential cardioprotective approach, we would like to investigate multiple downstream mechanisms by which Txnip can cause heart pathogenesis in the absence of STAT3. Also, knowing that Txnip is essential for activation of NLRP3 inflammasome in macrophages (Zhou et al., 2010), therefore, we next asked whether increased in Txnip expression will result in activation of inflammasome in the heart.

We proposed that the involvement of Txnip in activating the NLRP3 inflammasome and thereby accounting for the increased inflammation and apoptosis observed in STAT3 CKO heart. Expression of the components of the inflammasome was quantified by three different investigators. Firstly, NLRP3, ASC and Caspase-1 mRNA levels were upregulated in STAT3 deficient heart compared with wild-type heart (Figure 4.5A). Next, protein expressions of Txnip, NLRP3, and pro IL-1β were markedly increased in STAT3 deficient heart compared with wild-type heart (Figure 4.5B). Immunofluorescent staining of key structural components of the inflammasome, NLRP3 and ASC revealed that increased expression of NLRP3 and ASC when STAT3 was deleted in the heart (Figure 4.5C). Hence, deletion of STAT3 in the heart increased Txnip expression, which subsequently resulted in induction of inflammasome and elevated oxidative stress which may account for the heart pathogenesis observed in STAT3 CKO heart.

A


Nlrp3
Relative mRNA expression
4
3
2
1
0
F/F
C/F/F
Asc
Relative mRNA expression
3
2.5
2
1.5
1
0.5
0
F/F
C/F/F
Caspase-1
Relative mRNA expressiopn
25
20
15
10
5
0
F/F
C/F/F

B


F/F
C/F/+
C/F/F
1 2 3 4 5 6 7 8
86kDa
STAT3
55kDa
Txnip
118kDa
Nlrp3
31kDa
Pro-Il-1β
43kDa
β-Actin

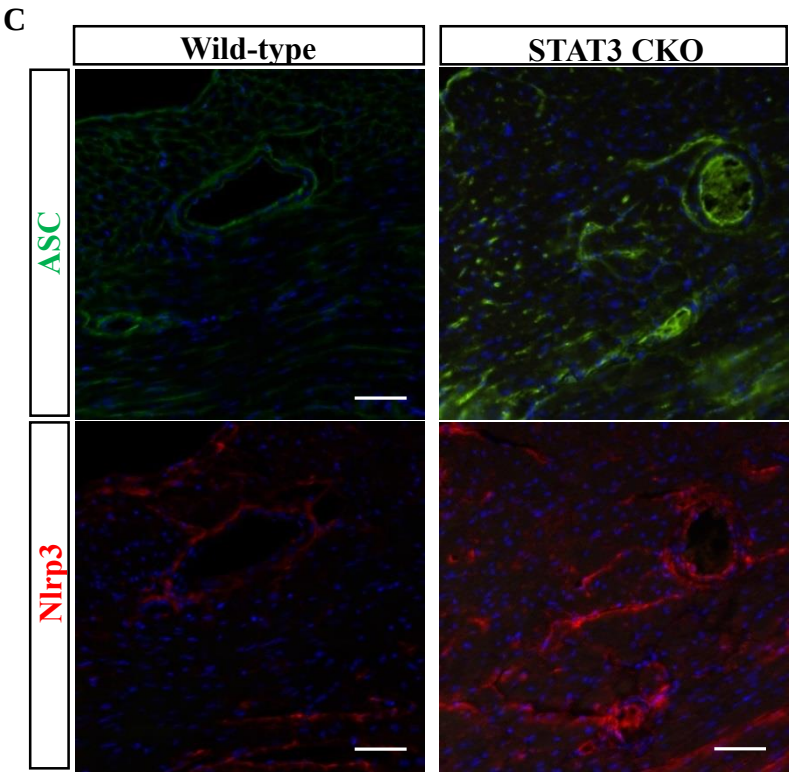


Figure 4.5 Deletion of STAT3 increased Txnip, which resulted in induction of inflammasome in aged STAT3 CKO hearts.

A. Important structural components of the inflammasome, ASC, NLRP3 and Caspase-1 mRNA were upregulated in STAT3 deficient heart.

B. Western blot demonstrated that upregulation of Txnip, NLRP3 and IL-1β protein expressions in the C/F/F heart which were consistent with their mRNA expression.

C. Immunostaining fluorescent showed increased expression of inflammasome markers, ASC and NLRP3 in STAT3 deficient heart.

Chapter 5 Discussion

Establishing of *in vitro* inducible STAT3 KO and STAT3 CA ES cell lines

Several investigations have demonstrated that STAT3 signaling is essential for early mammalian embryogenesis and determination of various cell lineages differentiation. However, analysis of STAT3 function during cardiomyogenesis was under investigation primarily due to the limitation of the model systems that were used. In mammalian development, it is difficult to procure cardiac-committed mesoderm to explore specified signal transduction mechanisms and as well as difficult to analyze the few cardiomyocytes available from early heart tubes. Additionally, the difficulty in analyzing STAT3 during early cardiomyogenesis was exemplified because STAT3 knockout resulted in early embryonically lethal at the time of cardiomyocyte formation at E7.5-E8.5 (Duncan et al., 1997; Takeda et al., 1997). STAT3 knockout is embryonic lethal and it failed to provide a potential functional role for STAT3 in cardiac differentiation. Moreover, it is noteworthy that cardiomyocyte specific KO of STAT3 generated by conventional Cre/loxP technology (Jacoby et al., 2003) did not address the function of STAT3 during early cardiomyocyte differentiation because the Cre driven by α-myosin heavy chain (α-MHC) promoter is weakly expressed before birth, reaching high levels long after cardiomyocyte differentiation. Taken together, it was difficult to elucidate very early molecular events in mammalian cardiomyogenesis using *in vivo* mammalian systems.

Consequently, in this study, we have established an *in vitro* inducible STAT3 knock out and pSTAT3 overexpressed ES cells system in which STAT3 expression can be temporally and specifically deleted or overexpressed by doxycyclin stimulation to allow the study of STAT3 function during early cardiomyocyte differentiation (Figure 3.1). Genotyping of inducible STAT3 KO ES cells following 24 hours Dox stimulation showed deletion of STAT3 alleles as detected as a 480bp fragment (Figure 3.2). Western blot further confirmed that pSTAT3 and STAT3 protein levels were significantly reduced in inducible STAT3 KO ES cells while tremendously upregulated in inducible STAT3 CA ES cells following Dox stimulation (Figure 3.3). Here, we have established a new tool to study function of STAT3 during ES cell differentiation into multicellular aggregates of ES cells, called embryoid bodies (EBs) which consist of derivatives of all three germ layers and in our project, we focus on cardiac lineage differentiation.

Illumina Microarray Analysis of STAT3 Target Genes

Firstly, SOCS3 gene which is a downstream targeting gene regulated by STAT3 (Hilton, 1999) was used as a positive control in this microarray experiment. SOCS3 expression was upregulated upon overexpression of pSTAT3 (>2-fold) while downregulated when STAT3 is KO (<0.34-fold). In addition, inhibitor of DNA-binding 1 (Id1) is a direct target gene transcriptionally induces by STAT3 (Yu et al., 2014). Its expression was upregulated upon overexpression of pSTAT3 (>2.5-fold) while downregulated when STAT3 is KO (<0.43-fold). Studies have demonstrated that Id1 is important in development, stem cell self-renewal activity, differentiation,

senescence, angiogenesis and migration (Fong, Debs, & Desprez, 2004; Romero-Lanman, Pavlovic, Amlani, Chin, & Benezra, 2012; Ying et al., 2003). Also, Our GO analysis was consistent with the known function of Id1 and demonstrated that it is involved in alternative splicing, phosphoprotein, developmental protein as well as vasculature development, epithelium development, heart development and epithelial cell differentiation, cell morphogenesis involved in differentiation.

Among the genes which contain STAT3 binding site, insulin like growth factor-2 (Igf2) and SPARC Related Modular Calcium Binding 1 (Smoc1) were among the top 10 upregulated genes by pSTAT3 overexpression, while Odd-skipped related 2 (Osr2) and Stanniocalcin-1 (Stc1) were those of the top 10 downregulated genes when pSTAT3 was overexpressed (Supplementary table 1). Igf2 works in cooperation with LIF to sustain ES cells self-renewal (A. Takahashi, Takahashi, Matsumoto, & Miyata, 1995) while Smoc1 is crucial for development of ocular and limb in mice and humans (Okada et al., 2011). Osr2 functions as a transcription factor for craniofacial, limbs, and kidney development (Lan, Kingsley, Cho, & Jiang, 2001) whereas Stc1 is protein hormone that regulates calcium/phosphate homeostasis and is upregulated by IL-6 in response to hypoxia (Westberg et al., 2007).

In addition, Laminin subunit alpha-1 (Lama1) and JunB proto-oncogene (JunB) were among the top 10 downregulated genes when STAT3 is KO which also contain STAT3 binding site (Supplementary table 2). Lama1 is expressed early during

embryo development at two cell-stage and widely expressed during organogenesis (Dziadek & Timpl, 1985; Ekblom, Falk, Salmivirta, Durbeej, & Ekblom, 1998) but its expression is highly restricted in adults (Falk, Ferletta, Forsberg, & Ekblom, 1999). It mediates cell adhesion, migration, and organization of cells into tissues during embryonic development by interacting with other extracellular matrix components and these interactions are important for epithelial morphogenesis (Ekblom et al., 1998). In addition, it promotes angiogenesis by directly regulates the expression profile of genes and protein in endothelial cells (Dixelius et al., 2004; Malinda et al., 1999).

In addition to those genes that contain STAT3 binding site, our microarray analysis also revealed some interesting genes those are important during embryonic development and regulation of STAT3 of these genes are yet to be discovered. Serine protease inhibitor Kazal type 3 (Spink3), one of the top 10 downregulated genes when STAT3 was KO while upregulated when pSTAT3 was overexpressed (Supplementary table 1 and 2), has been shown that it is expressed during early development and this suggests that it may have important physiological roles in differentiation and proliferation of numerous cell types during development (J. Wang et al., 2008). Another interesting gene is Leucine rich repeat containing protein 51 (LRRC51), one of the top 10 downregulated genes when pSTAT3 was overexpressed but upregulated when STAT3 was KO (Supplementary table 1). However, the exact function of LRRC51 remains unknown.

Moreover, a heatmap was generated using the agree list of genes in expression regulated by STAT3 CA and in STAT3 KO ES cells (Figure 3.4E). We found long length of agreement in gene list regulated by STAT3 CA and in STAT3 KO. Txnip was one of the genes in which its expression was upregulated when STAT3 was KO while downregulated upon STAT3 constitutively overexpressed. Nonetheless, the exact function of Txnip in ES cells pluripotency and differentiation remains unclear.

Collectively, our microarray results were consistent with known changes in gene expression of STAT3 targeted genes. Also, it revealed some potentially novel genes that might be regulated by STAT3 during embryonic development, heart development and differentiation. Therefore, further functional analysis on identified genes is needed to facilitate a better understanding of the underlying molecular mechanisms by which STAT3 is involved in maintaining the ES cells.

In vitro Cardiomyocytes differentiation derived from ESCs

A differentiation program is established to generate cardiomyocyte precursors from embryonic stem cells (ESCs). The differentiation program adopts the hanging drops approach to generate embryoid bodies (EB) of equivalent size and contains the same number of ESCs as was previously reported (Boheler et al., 2002). In our present differentiation program, in regard to cardiac differentiation, ascorbic acid is added which enhances spontaneous differentiation of ESCs into cardiomyocytes. Ascorbic acid has been demonstrated to specifically induce cardiac-specific genes including GATA4, Nkx2.5, α-MHC, β-MHC, and ANF in mouse ES cells derived

cardiomyocytes and they also showed spontaneous and rhythmic contraction (T. Takahashi et al., 2003). This is in agreement with the findings that ascorbic acid increased the number of beating areas in human ES cells (Passier et al., 2005). In addition, mesenchymal stem cells from adult human bone marrow developed features of cardiomyocytes when differentiated in a medium containing ascorbic acid (Shim et al., 2004). Consistent with these reports, it has been reported that by using a stable form of ascorbic acid, namely, L-ascorbic acid 2-phosphate (A2-P), it significantly enhanced cardiac differentiation of mouse ES cells when added during the early phrase of cardiac differentiation (Sato et al., 2006).

Since the effects of ascorbic acid are always attributed to its antioxidant properties, the effect of alternative antioxidant agents were examined to assess the involvement of the antioxidant properties of ascorbic acid in the promotion of cardiac differentiation. Treatment with alternative antioxidant agents was unable to mimic the effect of ascorbic acid on cardiac differentiation. These results indicate that the promoting effect of ascorbic acid on cardiac differentiation is independent of its antioxidant properties, or that its antioxidant properties is insufficient to induce cardiac differentiation of ESCs (Sato et al., 2006; T. Takahashi et al., 2003). Then, Sato's group demonstrated that ascorbic acid increases collagen synthesis, leading to the promotion of cardiac differentiation from ES cells (Sato et al., 2006). This finding was supported by another group who reported that ES cell-derived cardiomyocytes cultured on extracellular matrix (ECM) secreted from cardiac fibroblast matured more rapidly, suggesting that the matrix components are

involved in the cardiac differentiation and the growth characteristics of cardiomyocytes (Baharvand, Azarnia, Parivar, & Ashtiani, 2005). Collagen and other ECM components may constitute the *in vitro* microenvironment that supports cardiac differentiation which is generally exhibited *in vivo*. In the heart, cardiomyocytes are surrounded by a basement membrane consisting of many other molecules, the most predominant molecules are interstitial collagen type I and III (Eghbali et al., 1989). However, further studies are required to better understanding the mechanisms underlying this enhancing effect of ascorbic acid.

Despite the use of ascorbic acid to direct ESCs to the cardiac lineage, ascorbic acid does not partake in the signaling of STAT3, at least for now no study reported on that of ascorbic acid signals through STAT3. Therefore, this approach increases cardiomyocyte precursors yield but eliminates confounding factors that might partake in STAT3 signaling pathway.

Temporal activation of STAT3 is required for ESCs derived cardiomyocytes

Having established a differentiation program that directs ESCs to the cardiac lineage, and little is known of the very earliest targets of STAT3 regulation in differentiating cardiomyocytes, therefore, we question the expression of activated pSTAT3 and STAT3 during this differentiation process to assess whether STAT3 is directly involved in initial stages of cardiac differentiation.

In our *in vitro* model of cardiomyocyte differentiation, STAT3 and pSTAT3 were detected throughout differentiation, levels of pSTAT3 and STAT3 that were high

in undifferentiated ES cells decreased dramatically as soon as the commencement of cardiomyocyte differentiation that was day 1 of the differentiation process. pSTAT3 and STAT3 levels were steadily increased again in EBs from day 5 and reached its peak on day 8, which is approximately 1 to 3 days before the average onset of beating within EBs (Figure 3.5). This observation fits nicely with the known temporal activation of STAT3 compared with the onset of beating *in vivo* (Duncan et al., 1997; Ji et al., 2003), that is correlation of phosphorylation of STAT3 with these timelines suggested that STAT3 activation is involved in cardiomyocyte differentiation. In our study, we showed that downregulation of STAT3 during early cardiac differentiation, followed by upregulation again during late cardiac differentiation implies a temporal expression of STAT3 in cardiomyocyte differentiation. The correlation of STAT3 phosphorylation with these timelines suggests that phosphorylation of STAT3 is corresponds with cardiomyocyte derivation and might be involved in cardiomyocyte differentiation. Our experimental evidence complemented what has been reported that STAT3 activity is elevated at the undifferentiated stage (day 0), as demonstrated by its phosphorylation, as well as during cardiomyocyte differentiation process (day 5 onwards). It is interesting to note that STAT3 is required in both undifferentiated ESCs to maintain its self-renewal division and in lineage differentiation. STAT3 functions as a regulator of cardiomyocyte differentiation and proliferation during development.

STAT3 is considered to be the most important transcription factor in the STATs family during mouse embryo development because only the loss of STAT3 results in embryonic fatality (Takeda et al., 1997). Among many diverse roles of STAT3, its most prominent role is in cell proliferation. In ES cells, STAT3 is an essential mediator downstream of LIF in maintaining ES cells self-renewal and pluripotency (Matsuda et al., 1999; Niwa et al., 1998). Often, STAT3 function is associated with proliferation rather than differentiation. Nonetheless, the results of the present study suggest that temporal activation of STAT3 is required for ES cells derived-cardiomyocyte. In addition to embryonic stem cells, role of STAT3 is also implemented in proliferation and differentiation of adult muscle stem cell-derived myoblast. Differentiation of muscle stem cell-derived myoblast is mainly responsible for postnatal muscle growth and injury-induced muscle regeneration. Several groups have independently detected activated STAT3 in LIF-treated proliferating myoblasts and regenerating muscles (Kami & Senba, 2002; Spangenburg & Booth, 2002). This is further validated by reduction in myoblast proliferation in the absence of a functional STAT3 gene despite LIF induction (Sun et al., 2007). Wang's group (K. Wang, Wang, Xiao, Wang, & Wu, 2008) showed STAT3 to be functioning downstream of JAK2 and activates expression of myocyte enhancer factor 2 (MEF2) genes, which are essential regulators of cardiac myogenesis and right ventricular development. Targeted deletion of the MEF2 gene results in a small left ventricle and complete loss of the right ventricle (Lin, Schwarz, Bucana, & Olson, 1997). The role of STAT3 in proliferation and pluripotency in previous publications is consistent with our study that reveals STAT3 to be

activated at ES undifferentiated state. Upon removal of LIF and activation of cardiac differentiation, STAT3 is downregulated during early cardiac differentiation, followed by upregulation again during late cardiac differentiation implies a temporal expression of STAT3 in cardiomyocyte differentiation.

Inducible deletion of STAT3 delays cardiac differentiation from ESCs

Next, the morphology of EBs formed when STAT3 is deleted from Day 0 onwards of cardiomyocyte differentiation from ESCs is first observed in order to find out whether or not the deletion of STAT3 has any effect on the formation of EBs. STAT3 KO EBs 48 hours following Dox induction showed not much different in the size as compared to unstimulated EBs, suggesting that STAT3 has no effect on the cell growth and morphology of EBs formed (Figure 3.7).

The beauty of the inducible STAT3 KO ES system that we have established is that STAT3 can be specifically deleted at different time point during the commencement of cardiomyocyte differentiation from ESCs. Here, in order to understand function of STAT3 and its temporal activity during cardiomyocyte differentiation, 3 time points were chosen to delete STAT3 by Dox stimulation at Day 0 onwards, Day 3 and Day 4 of cardiomyocyte differentiation. Knowing that cardiac progenitors are specified at approximately day 4 of EBs differentiation and cardiac-committed mesoderm has to be formed prior to cardiac progenitors (Kattman et al., 2006), therefore, we decided to delete STAT3 before and at Day 4 in order to determine whether STAT3 is directly involved in initial stages of cardiac

differentiation by examine the effect of STAT3 deletion on formation of cardiac-committed mesoderm and later differentiation into cardiac lineage.

Interestingly, inducible deletion of STAT3 EBs from day 0 onwards and day 3 resulted in significantly downregulation of cardiac-specific genes, Actinin and Troponin T protein expression as revealed by western blot (Figure 3.8C). The absence of differentiated cardiac myocytes could result from a failure in formation of cardiac-committed mesoderm or cardiac progenitor cells due to deletion of STAT3 from day 0 onwards of cardiomyocyte differentiation. In contrast, deletion of STAT3 from day 3 resulted in delay formation of beating cardiomyocyte first observed on day 13 which was 4 days later than the control EBs, indicating that STAT3 impacts development around the time when mesoderm progenitors commit to a cardiac fate. While inducible deletion of STAT3 EBs from day 4 resulted in lesser downregulation of cardiac-specific genes as compared to those inducible STAT3 deletion EB from day 0 onward and day 3 (Figure 3.8C). This result indicated that inducible STAT3 deletion on Day 4 after cardiac-committed mesoderm has become committed to the cardiac lineage at approximately day 3 did not show any difference compared with unstimulated EBs, that is, beating cardiomyocyte was first observed at Day 9 (Figure 3.8B). Therefore, alteration of STAT3 activity is an important event in the initialization of ES cells differentiation and the orientation of cardiac lineage differentiation.

It has been reported that STAT3 has an essential role in the promotion of ESCs differentiation into cardiomyocyte. By using specific dose of LIF and BMP2, it could efficiently differentiate mESCs into cardiomyocyte via their synergistic activation of STAT3 signal transduction pathway, indicated by upregulation of cardiac-specific genes and protein expression. Nonetheless, neither of the LIF nor BMP2 by themselves were sufficient for cardiomyocyte differentiation. In addition, synergistic effect of LIF and BMP2 induced expression of cardiac-specific transcripts and STAT3 tyrosine and serine phosphorylation was attenuated by STAT3 inhibitors (Rajasingh et al., 2007). In relation, another study demonstrated that the dominant negative form of STAT3 ESCs resulted in significantly reduced beating areas within EBs and also downregulation of cardiac-specific genes (Foshay et al., 2005). One of these genes, Nkx2.5 was not expressed and it has been reported that its promoter contains STAT3 binding site (Reecy et al., 1999), suggesting that a potential regulatory role of STAT3 on Nkx2.5, and lack of Nkx2.5 could prevent complete cardiomyocyte differentiation via downstream effect. Nkx2.5 is expressed specifically within cardiac progenitor cells of the heart-forming region precedes cardiogenic differentiation and throughout adult life in the mouse (Lints, Parsons, Hartley, Lyons, & Harvey, 1993).

STAT3 is essential for cardiomyocyte differentiation from ESCs through positive regulation on GATA6

The formation of beating heart is a multistep process which requires the activation of a cascade of transcription factors under tight temporal and spatial control defining each development stage of the heart. We have here demonstrated that the

important role of STAT3 in promoting the cardiomyocyte differentiation through regulation of GATA6 transcription factor. Both mRNA and protein level of GATA6 expression were decreased dramatically when STAT3 was deleted on Day 0 onwards and Day 3 during cardiac differentiation from ESCs (Figure 3.9A, B), indicating that GATA6 is positively regulated by STAT3, and is important in mediating cardiomyocyte differentiation from ESCs. Therefore, no beating cardiomyocyte was observed or beating occurred later when STAT3 was deleted on Day 0 and Day 3 due to decreased level of GATA6 which is essential for cardiac differentiation. In contrast, deletion of STAT3 on Day 4 of cardiac differentiation did not affect the GATA6 mRNA and protein levels much (Figure 3.9A, B). This suggested that STAT3 has stage specific function during cardiomyocyte differentiation from ESCs. Deletion of STAT3 during early phrase of cardiomyocyte differentiation (Day 0 and Day 3) will affect or delay the determination of cardiac lineage from ESCs. However, when STAT3 was deleted during later phrase (Day 4), the cardiac lineage has been determined and deletion of STAT3 has no effect anymore on cardiomyocyte differentiation as shown by unaffected level of GATA6 when STAT3 was deleted on day 4.

GATA6 downregulation by STAT3 inducible deletion in differentiating EBs is mediated by the direct binding of STAT3 to the promoter region of GATA6 factor (Figure 3.9C), indicating that GATA6 is an immediate downstream target of STAT3 regulation during early cardiomyocyte differentiation. This is in agreement with previous findings that ChIP-Seq data verifies a STAT3 binding site on GATA6

promoter (Kidder, Yang, & Palmer, 2008) in ESCs. It has been reported that STAT3 binds both active and inactive genes in ESCs. Also, STAT3 is associated with genes highly expressed in undifferentiated ESCs and differentiated EBs (Kidder et al., 2008). Thus, regulation of GATA6 by STAT3 provides a pathway in which STAT3 deletion could result in the loss of or a delay in beating cardiomyocyte formed due to decreased GATA6 expression and therefore incomplete differentiation of cardiomyocyte.

A number of studies have linked GATA6 to heart formation. Based on studies of function of GATA6 using knock down models in zebrafish and xenopus embryos, it results in either heartless embryos or embryos in which cardiogenesis is severely perturbed. Also, GATA6 is required for the maintenance and differentiation of cardiac progenitors in zebrafish and xenopus embryos (Peterkin et al., 2003). Then again, another group reported that morpholino knock down of GATA6 in zebrafish causes defects in heart development, characterized by a lack of circulation by 3-4 dpf (days post-fertilization) and formation of a non-looped heart tube (Holtzinger & Evans, 2005).

The important of GATA6 during early cardiogenic differentiation is further validated by other studies that reveal coexpression of GATA transcription factors with the homeoprotein Nkx2.5 in the cardiac-committed mesoderm during the earliest stages of its specification and are known to be important determinants of cardiac gene expression. In that study, by using P19 CL6 model system of

cardiomyocyte differentiation, it has been reported that upregulation of GATA4 and GATA6 expression occurs before the transcriptional activation of Nkx2.5 in these differentiating cardiomyocyte. Furthermore, overexpression of GATA4 and GATA6 at the time just prior to that of normal Nkx2.5 induction results in a significant upregulation of endogenous Nkx2.5 expression, suggesting that GATA4 and GATA6 are capable of acting upstream of Nkx2.5 in the transcriptional cascade that leads to cardiac differentiation (Brewer et al., 2005). Regulation of Nkx2.5 expression by a GATA-dependent enhancer has been reported by others as well (Lien et al., 1999; Searcy et al., 1998). However, the onset of GATA6 expression observed during cardiac differentiation is not addressed yet. Therefore, the regulatory mechanisms that activate expression of GATA factors within cardiac committed mesoderm are of particular interest because they may represent the initial steps in the cardiac differentiation program. In our study, we propose that initial transcriptional activation of GATA6 by STAT3 is essential for the onset of cardiogenic commitment and have thus established a link between two families of genes known to have major roles in early cardiomyocyte differentiation.

From the discussion described earlier, our study revealed that STAT3 expression was downregulated during early cardiac differentiation, followed by upregulation again during late cardiac differentiation. We here propose that decline in level of STAT3 expression during early cardiac differentiation is essential for the completion of the cardiomyocyte differentiation programme. Studies in xenopus and chick revealed that a reduction in the level of GATA6 expression prior to the

terminal differentiation of cardiomyocytes (Gove et al., 1997; Laverriere et al., 1994). Injection of GATA6 mRNA to maintain level of GATA6 in gastrulating embryos results in transient block of differentiation of heart precursors. When the injected GATA6 mRNA decays, cardiomyocytes resumed differentiation, giving rise to normal, beating hearts but increased in myocardial tissues by holding the cells in the precursor state for a longer period would increase the proliferation of cardiac progenitor cells (Gove et al., 1997). This suggests that GATA6 might regulate proliferation of cardiac progenitor cells. These observations are in agreement with our findings that STAT3 expression falls during early cardiac differentiation, leading to decreased level of GATA6 since GATA6 is a potential cardiac differentiation gene directly regulated by STAT3 positively and decline in levels of STAT3 and GATA6 during early differentiation is associated with complete differentiation of cardiomyocyte. Then, STAT3 expression is upregulated again during late cardiac differentiation, which is approximately 1 to 3 days before the average onset of beating within EBs. Therefore, we propose that STAT3 is at the upstream of the cardiac differentiation cascade by regulating expression of GATA6 which is essential for cardiomyocyte differentiation and temporal activation of STAT3 and GATA6 is important for the completion of the cardiomyocyte differentiation.

Collectively, our *in vitro* data obtained from ESCs-derived cardiomyocyte point towards role of STAT3 activation is necessitated in both undifferentiated and differentiated stages. STAT3 is positioned at a critical juncture upstream of the ES

cells differentiation, distinctly regulating the cardiomyocyte differentiation pathway by controlling the expression of GATA6 which is essential for cardiac differentiation. Moreover, we have provided another new tool to study function of STAT3 during ES cells differentiation into cardiac lineage.

Transcriptional regulation of Txnip expression by STAT3

Several studies demonstrated that STATs family proteins mediate gene transcription through chromatin modification. Chromatin immunoprecipitation (ChIP)-sequencing technology revealed that STATs family proteins bind to their target gene locus and act as transcription activator on a global scale (Durant et al., 2010; Good et al., 2009; Hutchins et al., 2012; Li et al., 2012; Wei et al., 2010). On the other hand, in T cells, STAT5 is required for binding on the target gene promoter to suppress transcription through chromatin modification (Mandal et al., 2011; X. P. Yang et al., 2011).

There are several criteria for identifying a STAT3 target gene. The first one is the presence of an evolutionarily conserved STAT3 binding consensus motif (Ehret et al., 2001) for the activating or repressing STAT3 transcription factor in the promoter or enhancer regions of a gene of interest. This requirement was met in Txnip as shown by both gene promoter analysis and ChIP assay (Figure 4.2). A second commonly presented form of evidence is that constitutively active mutant form of STAT3 induces or suppresses the expression of the given gene, which in our case we used LPS treatment instead to activate STAT3, which could indeed suppress Txnip expression (Figure 4.3). Another way of identifying a role for

STAT3 in the transcriptional regulation of a particular gene is to show that acute genetic deletion of STAT3 gene results in the increase or loss of target gene expression. Txnip mRNA and protein levels are increased in STAT3 CKO as compared to wild-type heart (Figure 4.4), suggesting that role of STAT3 in regulating Txnip.

Regulation of Thioredoxin-interacting protein by STAT3 is crucial for heart protection

Research has established the importance of STAT3 in development as global STAT3 deletion results in embryonic lethality (Takeda et al., 1997), yet cardiac specific knock-out of STAT3 mice survive into adulthood and do not show symptoms of dysfunction up to age of 6 months. Nonetheless, with advanced age at 9 months, these STAT3-CKO mice exhibited severe fibrosis, and experienced higher sensitivity to inflammation and apoptosis (Jacoby et al., 2003). This postulates a post-natal role of STAT3 in cardiac protective function. However, the molecular mechanism by which STAT3 contributes to heart protection and function is not well understood.

In this study, we first reported that the possible pathological mechanism of cardiac inflammation and fibrosis in cardiomyocyte-restricted STAT3 knock out mice. Firstly, after LPS endotoxin challenge, STAT3 wild-type heart and rat H9c2 myoblast showed significant decreased in the expression of Txnip (Figure 4.3). Endotoxin stress has been proposed that it is a potential trigger for cytokine

signaling during heart failure in humans. Endotoxin levels are greatly increased in adult patients with congenital heart disease (Sharma et al., 2003). Recent studies suggest that the JAK-STAT signaling pathway participates in negative regulation of LPS responses. Loss of STAT3 in mouse cardiomyocytes leads to increased TNF-α production after LPS stress *in vitro*, suggesting that STAT3-mediated signaling is required to regulate innate immunity in cardioprotection from endotoxin stress (Jacoby et al., 2003). In the present study, after acute LPS challenge (2 hours to 18 hours) *in vivo* and *in vitro*, Txnip mRNA and protein levels were decreased in both STAT3 wild-type heart and rat H9c2 myoblast, suggesting that STAT3 negatively regulates LPS responses and at the same time, it also negatively regulates Txnip expression after LPS stress.

In addition, we observed that STAT3-CKO heart displayed higher expression of Txnip as seen in the increased of both Txnip mRNA and protein levels in comparison with its controls (Figure 4.4). Deletion of STAT3 increased Txnip expression, which also experienced significantly more induction of inflammasome activation, as indicated by the significantly more NLRP3 inflammasome3 components observed in STAT3-CKO heart (Figure 4.5). Since the STAT3-CKO heart develops increased activation of NLRP3 inflammasome and upregulation expression of Txnip, we suspect that STAT3 plays a crucial role in the regulation of innate immunity of NLRP3 inflammasome activation at the molecular level through regulating other intermediate proteins involved in the NLRP3 inflammasome assembly. Here, we propose the intermediary protein to be Txnip.

Hence, increased Txnip expression and induction of NLRP3 inflammasome activation are account for the higher sensitivity of inflammation, apoptosis and severe fibrosis which eventually lead to heart failure in STAT3-CKO heart.

This is further validated in systemic deletion of Txnip in mice. Mice with deletion of Txnip exhibit impaired mitochondrial function, but provide protection to the myocardium from ischemia-reperfusion injury. Protection from myocardial ischemia is enhanced as result of a coordinated shift to increased anaerobic metabolism, which provides an energy source outside of mitochondria (Yoshioka et al., 2012; Yoshioka et al., 2007). In addition, microarray analysis demonstrated that higher expression of Txnip was found in the heart of myocardial infarction mice as compared to wild-type mice (Lachtermacher et al., 2010).

Fittingly, it was reported that overexpression of Txnip has been shown to increase cardiomyocytes sensitivity to oxidative stress-induced apoptosis, suggesting that regulation of Txnip may protect against apoptosis from mechanical overload or oxidative stress. While the Txnip natural antagonist, thioredoxin (Trx), has function of cardioprotection. Elevated Trx levels attenuate myocardial damage induced by ischemia-reperfusion injury (Y. Wang, De Keulenaer, & Lee, 2002). Moreover, Thioredoxin overexpressed mice promote neovascularization by increasing capillary and arteriolar density during ischemia stress and prevent the post-ischemic ventricular remodeling. Also, Trx overexpression reduced Txnip expression, while increased angiogenic protein VEGF, anti-apoptotic proteins BCL-2 and survivin (Adluri et al., 2011).

Inflammation response has been implicated in myocardial infarction and heart failure. Inflammation is an innate immune response that prevents the spread of infection and promotes tissue healing after the clearance of pathogens but also causes excessive damage and maladaptive ventricular remodeling leading to impaired myocardial function and heart failure (Frangogiannis, Smith, & Entman, 2002). Inflammation is an innate immune response regulated by several families of pattern recognition receptors (PRRs). Toll-like receptors (TLRs) is the best studies PRR family and it is triggered by pathogen-associated molecular patterns (PAMPs) and initiates a signaling cascade leading to enhanced synthesis of inflammatory cytokine (Ionita, Arslan, de Kleijn, & Pasterkamp, 2010; Takeuchi & Akira, 2010). IL-1β is the first upstream inflammatory cytokines produced, and it is potent to induce secretion of other cytokines (Dinarello, 2009) . Release of IL-1β is tightly regulated and inflammasomes are important regulators of activation of inflammatory caspases and IL-1β (Martinon et al., 2002).

Our present study revealed that deletion of STAT3 in the heart significantly increases the mRNA of key components of inflammasome, NLRP3, ASC and Caspase-1 and Txnip when compared to wild-type mice (Figure 4.5). Protein expression levels of NLRP3, pro-IL-1β and Txnip were increased in STAT3 deficient heart (Figure 4.4 and 4.5). In view of that, this data suggests that STAT3 may exert its effects indirectly by negatively regulate other proteins involved in the NLRP3 assembly which in this study is Txnip, thereby deletion of STAT3 in the

heart accounts for increased inflammation mediated by NLRP3 inflammasome activation and apoptosis as observed. This is in agreement with previous findings that demonstrated Txnip mediates NLRP3 inflammasome activation (Zhou et al., 2010). Fittingly, it was reported that intramyocardial NLRP3 siRNA significantly decreased the activation of NLRP3 inflammasome and infarct size and restored heart function during I/R injury (Liu et al., 2014). However in our study, cleaved IL-1β was not observed but could be reexamined to further confirm this. Nonetheless, we indeed showed that, an initial signal leads to increased pro-IL-1β synthesis as revealed by increased protein level of pro-IL-1β because pro-IL-1β is not constitutively expressed and requires transcriptional and translational induction. Here, the increased of pro-IL-1β as activated by NLRP3 inflammasome suggesting that increased signaling cascade leading to enhanced synthesis of inflammatory cytokine that might account for the increased inflammation, apoptosis and fibrosis as observed in STAT3 deficient heart.

Here, it is hypothesized that the increased inflammasome activation would stimulate a more enhanced inflammatory cytokine signaling and subsequently leading to increased apoptosis and fibrosis as observed in aged STAT3 CKO heart. Several recent key publications based on studies of myocardial I/R injury showed NLRP3, ASC and caspase-1 were upregulated at the site of myocardial I/R injury whereas knock out mice model of various inflammasome components revealed reduced inflammatory responses, reduced infarct sizes and myocardial fibrosis (Kawaguchi et al., 2011; Mezzaroma et al., 2011; Sandanger et al., 2013).

Inflammation responses driven by NLRP3 inflammasome has been implicated in the pathophysiology of heart failure, particularly in myocardial I/R injury. Neutralization of IL-1β reduces I/R injury suggesting that IL-1β is the key mediator in the pathophysiology of I/R injury (Hwang et al., 2001; Pomerantz, Reznikov, Harken, & Dinarello, 2001). Moreover, it has been reported that IL-1β and TNF-α promote tissue fibrosis *in vivo* (Neumann, Lane, Allen, Herskowitz, & Rose, 1993; Ono, Matsumori, Shioi, Furukawa, & Sasayama, 1998; Yue, Massie, Simpson, & Long, 1998). Levels of IL-1β are related to the degree of interstitial fibrosis (Ono et al., 1998). In line with these recent reports, our data show that targeting inflammasome activation may have a cardioprotective role against heart failure.

As a whole, we illustrate clear molecular evidence of the role of key transcription factor, STAT3, exerts its patho-physiological in heart disease and confers its cardioprotection role in adult heart by negatively regulating of the NLRP3 inflammation activation through mediating Txnip expression.

Even though our data and other studies have been identified involvement of Txnip in NLRP3 activation (Zhou et al., 2010), nonetheless, opposing results were found by another research group using bone marrow-derived macrophages (BMDM) model (Masters et al., 2010). IL-1β secretion and caspase-1 activation were directly compared by using Txnip-deficient macrophages and NLRP3-deficient macrophages and found that these parameters were lower only in NLRP3-deficient macrophages. Hence, this study reported that they did not identify Txnip in

regulating inflammasome or IL-1β production by the inflammasome, in BMDM. However, this difference may be attributed to the distinct roles of Txnip in activation of NLRP3 inflammasome in different systems, and in this case, in cardiovascular system and hematopoietic lineage cells especially of the myeloid lineage such as macrophages. In addition, another group first revealed that STAT3 Tyrosine phosphorylation is critical for pro-IL-1β production by using in murine bone marrow-derived dendritic cells and this implies that STAT3 is probably required for activation of NLRP3 inflammasome assembly (Samavati et al., 2009). This is at first sight contradictory to data presented here and to other studies indicating a pro-inflammatory role for STAT3. Nonetheless, it appears that pro- and anti-inflammatory roles of STAT3 are mostly cell- and stimulation-specific, and also multiple post-translational modifications, such as phosphorylation plays a major role in this process. Here, we propose that STAT3 confers cardioprotection in the adult heart by its anti-inflammatory role in which it negatively regulates activation of NLRP3 inflammasome possibly through mediating Txnip expression.

Chapter 6 Conclusion, limitation of the study, future work

6.1 Conclusions

6.1.1 Conclusion from part I

ESCs is pluripotency cell that can potentially proliferates indefinitely as well as differentiates into almost all the cell types in our body. Hence, it is regarded that ES-derived cardiomyocytes are ideal for use in regenerative medicine as they represent a renewable source of multiple heart cell types that are participating in the process of myocardial regeneration on the repair of major heart injuries. However, the transition from bench to bed necessitates a deeper understanding of the STAT3 function in the molecular control of early cardiomyocyte differentiation as studies have revealed that STAT3 is crucial for early cardiomyocyte differentiation. Conventional STAT3 KO is embryonic lethal before the function of STAT3 in early cardiomyocyte differentiation can be assessed. Therefore, we need a tool which can temporally and specifically delete or overexpress STAT3 expression in ES cells. In this study, we have established an *in vitro* inducible STAT3 knock out and pSTAT3 overexpressed ES cells system in which STAT3 expression can be deleted or overexpressed by doxycyclin stimulation to allow the study of STAT3 function during early cardiomyocyte differentiation. Conclusively, our present study has illustrated an efficient cardiomyocyte differentiation program in generating cardiomyocyte from ESCs. STAT3 showed temporal expression during cardiomyocyte differentiation from ESCs and plays stage specific function in promoting cardiomyocyte differentiation from ESCs. We identified a molecular mechanism by which STAT3 and GATA6 may interact functionally and a

promoting role of STAT3 in cardiac differentiation may be mediated through upregulation of GATA6 expression.

6.1.2 Conclusion from part II

Finding a cure for cardiovascular disease remains a major unmet medical need. Therefore, elucidating the molecular mechanism of heart pathogenesis is of great clinical significance for devising new treatments. Studies have revealed that STAT3-deficient hearts are more sensitive to endotoxin-induced cell death and inflammation, possibly due to elevated production of TNF-α, which eventually leads to severe cardiac pathogenesis of myocardial fibrosis with advanced age. Therefore, STAT3 plays a crucial role to promote cardiomyocyte survival and cardiac function. Hence, it is essential to elucidate the changes in genes related to heart pathogenesis and the underlying regulatory role of STAT3 involved in this cardiovascular pathogenesis. Given the increased Txnip expression and inflammation as demonstrated by increased activation of inflammasome3 we observed in STAT3-deficient heart, this supports the notion that some of these deteriorate effects might be mediated by Txnip induction in the absence of STAT3. Therefore, our findings now reveal for the first time that STAT3 signaling pathway might regulate Txnip expression and hence contributes to cardioprotection under pathogenesis condition. Hence, this study supports the potential role of Txnip as a therapeutic target for cardioprotection, be it through STAT3 signaling pathway or by novel compounds designed to specifically inhibit cardiac Txnip expression, and

hence providing new insight for potential clinic therapy for heart failure and to explore and develop new biomarkers and therapeutic treatments for the disease.

6.2 Limitation of the study and future work

6.2.1 Limitation of the study and future work for part I

More studies can be done to find out the phenotype of STAT3 knock out ES cells derived cardiomyocyte. Electrophysiology patch clamp studies is useful for further characterization of cardiomyocytes. Also, more expression profiles of mesodermal and cardiac genes can be examined to better understand the consequence of STAT3 KO in ES cells derived cardiomyocyte. This will aid in elucidating a more prominent role that STAT3 plays in cardiogenesis, particularly during early stages of cardiomyocyte differentiation. In our study, STAT3 is downregulated during early differentiation process, thus the next question to elucidate is the array of genes that are regulated by STAT3 prior designation to a cardiac fate.

Our preliminary data are not conclusive in elucidating STAT3 signaling acts through GATA6 to regulate ES cell derived cardiomyocyte. Future works intending to pursue STAT3 and GATA6 in cardiomyocyte precursors using suggested cardiac differentiation program should first seek to isolate a pure sample of cardiac precursors by means of FACS sorting. It is critical to carry out more studies at characterizing the precursors. Co-immunoprecipitation analysis using STAT3 and GATA6 will be convincing to deduce physical interactions among these transcription factors. Epigenetic studies is suggested to verify the mode of action

of STAT3 on GATA6 expression. Given that ChIP-Seq data verifies a STAT3 binding site on GATA6 promoter (Kidder et al., 2008), STAT3 may be involved in epigenetic modifications of GATA6 gene transcription. To prove this, H3K27 tri-methylation and H3K9 acetylation ChIP assay can be carried out using E14 ES cells and STAT3 inducible KO ES cells.

6.2.2 Limitation of the study and future work for part II

Our present study has proved that upregulation of Txnip in STAT3-deficient heart experienced higher inflammation as demonstrated by higher activation of inflammasome3 in the STAT3-deficient heart and thereby eventually lead to fibrosis and heart failure. However, the causal role and molecular events by which increased Txnip is responsible for the increased activation of inflammasome3 in STAT3-deficient heart have not been fully elucidated.

In future, we would like to examine whether inhibiting Txnip expression in STAT3-deficient heart can rescue or reverse the phenotype and thus provide cardioprotection. Pharmacological tool such as calcium channel blockers, verapamil (J. Chen et al., 2009) which specifically inhibit Txnip expression can be used *in vivo* by feeding the mice, both wild-type and STAT3 CKO mice with or without verapamil in their drinking water (1mg/ml) for 3 weeks at different ages such as 2 month old, 4 month old and 6 month old mice and the mice will be sacrificed at age 3 month old, 6 month old and 9 month old. Real-time RT-PCR and western blot can be carried out to reveal a significant decrease in the mRNA and

protein expression of Txnip. Next, inflammatory mediators such as TNF-α and IL-1B, and antioxidant status such as Thioredoxin (Trx), thioredoxin reductase 1 (TrxR1), and nitrotyrosine formation can be tested. Also, fibronectic markers such as Tissue inhibitor of metalloproteinase (Timp1), Fibroblast specific protein 1 (FSP1) and collagen genes can be examined to test whether or not these markers will reduce in reduced Txnip expression in STAT3-deficient heart. Furthermore, histological examination can be carried out. Sections can be stained with hematoxylin/eosin to view the overall phenotype. Masson's trichrome can be used to detect fibrosis in heart sections while in situ labelling of apoptotic cells can be performed on tissue sections. All these results will suggest that whether down-regulation of cardiac Txnip expression *in vivo* in STAT3-deficient heart might reduce apoptosis, cardiac fibrosis and heart failure.

Next, Txnip knockdown can be done using Txnip siRNA and scramble siRNA as control in STAT3-deleted cardiomyocyte *in vitro*. In addition, double KO of Txnip and STAT3 CKO mice could be generated in order to examine whether or not KO of Txnip can rescue the increased inflammation and fibrosis in STAT3 CKO heart. Immunostaining and similar experiments as discussed above can be performed such as to examine the levels of inflammatory mediators, antioxidant status and cardiac fibrosis. Apoptosis markers such as cleaved caspase-3 and Tunel assay can be performed in reduced Txnip expression in STAT3-deficient cardiomyocyte. This might suggest that Txnip may be involved in the pathogenesis of heart failure in

which cardiomyocyte apoptosis represents one of the key mechanism and it is a potential downstream gene regulated by STAT3.

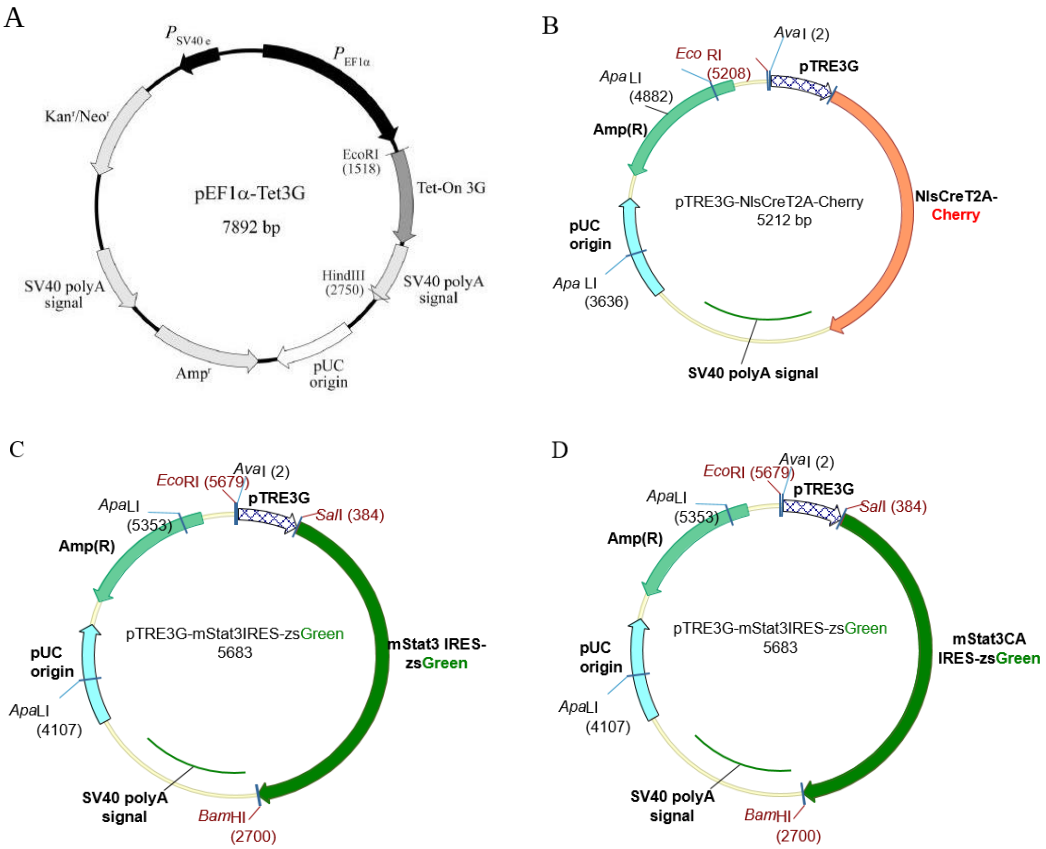
Knowing that inhibition of Txnip is suggested as a potential cardioprotective approach on the basis of knockdown experiment, we would like to investigate multiple downstream mechanisms by which Txnip can cause heart pathogenesis in the absence of STAT3 in addition to activation of inflammasome3. Enhanced Txnip expression might contribute to heart pathogenesis by three possible mechanisms, involving induced release of inflammatory mediators such as TNF-α, altered antioxidant status, and disrupted Trx-ASK-1 inhibitory complex leading to activation of the p38 MAPK/JNK apoptotic pathway (C. L. Chen et al., 2008; Ichijo et al., 1997; Nishiyama et al., 1999; Yamawaki et al., 2005).

Inflammatory mediators such as TNF-α, IL-1B, and IL-6 and oxidative markers such as 4-HNE and nitrotyrosine formation can be examined in reduced Txnip expression in STAT3-deficient heart and cardiomyocyte. In addition, the increased expression of antioxidant (MnSOD and HO-1) and anti-apoptosis (Bcl-xL) proteins, as well as decreased levels of pro-apoptotic (Bad and Bax) proteins will be examined. Also, the association between Txnip and Trx can be examined using immunoprecipitation and Trx activity assay can be measured before and after Txnip expression being inhibited *in vitro* and *in vivo*. In addition, p38 MAPK/JNK apoptotic pathway can be further confirmed by the expression of cleaved caspase-3 and cleaved PARP.

Nonetheless, we cannot exclude the possibility that some of the protective effects observed in STAT3 wild-type mice as compared to STAT3-deficient mice are independent of elevated Txnip levels and based on the regulation of other potential genes by STAT3 involved in apoptosis and inflammation signaling pathway. Besides Txnip, we are also interested in looking for other potential cardiac genes regulated by STAT3 to unravel this critical signaling network in cardioprotection. Microarray experiment is chosen to unravel this network and identify critical STAT3 target genes. Total RNA is harvested from wild-type and STAT3 CKO heart at age 3 month old, 6 month old and 9 month old and send for microarray analysis. Gene expression between wild-type and STAT3 CKO can be compared followed by RT-PCR and ChIP to further confirm the microarray analysis.

Supplemental Data

Figure S1. pTRE3G-IRES Tet-on System and major constructs of pTRE3G-IRES Tet-on System



Supplemental Table

Table S1. Differentially expressed genes more than 4-fold in STAT3 CA ES cells.

Gene Symbol	Fold Change	Regulation
Igf2	11.34	up
GATA6	11.08	up
Tcfap2c	9.69	up
LOC381844	7.24	up
Slc30a2	7.02	up
Zscan4c	6.37	up
Khdc3 2410004A20Rik	6.36	up
Smoc1	6.27	up
AF067061	6.25	up
Ndp52	5.66	up
Auh	5.56	up
Lrrc15	5.43	up
Glipr2	5.37	up
Nupr1	5.17	up
Glipr2	5.11	up
Il28ra	4.86	up
S100a6	4.82	up
Stmn2	4.68	up
Tcstv1	4.58	up
Pdzk1	4.56	up
LOC100046049	4.51	up
Tuba3a	4.50	up
Xlr4a	4.46	up
Fgfbp1	4.45	up
Pdlim4	4.44	up
Tcstv3	4.39	up
Ubap1I LOC208080	4.32	up
Rcsd1	4.26	up
Gm7969 LOC666185	4.26	up
Gbp2	4.24	up
Col4a2	4.23	up
Spink3	4.22	up
Plvap	4.21	up
Amn	4.15	up
Pdzk1	4.07	up
Tcfap2a	4.06	up
Slc29a3	4.04	up
Plac8	4.01	up
Snurf	48.51	down
Snurf	43.99	down

Snurf	19.91	down
Tex13	11.32	down
Svop	6.68	down
Osr2	5.48	down
Lrrc51	4.70	down
Stc1	4.60	down
Osr2	4.19	down
Mtmr11	4.00	down

Table S2. Differentially expressed genes more than 4-fold in STAT3 KO ES cells.

Gene Symbol	Fold Change	Regulation
Zscan4c	20.77	up
Gm428	17.77	up
Ubap1l LOC208080	16.82	up
LOC381844	15.95	up
LOC548597	13.81	up
LOC433722	11.14	up
Nupr1	11.02	up
LOC433721	10.30	up
Gm13119 OTTMUSG00000010537	9.90	up
Tcstv3	8.96	up
Gdf15	8.52	up
Tcstv1	8.37	up
Ccdc155	8.22	up
Thy1	8.21	up
AF067061	8.19	up
Nptx2	7.54	up
Masp2	7.33	up
Gm16513 AU018829	7.05	up
A730027B03Rik	6.78	up
Synm	6.49	up
Spink3	24.61	down
Fgf17	8.11	down
Junb	7.94	down
Bmp4	6.83	down
Notum	6.48	down
Gbx2	6.37	down
Klk7	6.32	down
Lama1	5.92	down
Car2	5.71	down
Cltb	5.67	down
RIKEN cDNA 9430052C07Rik	5.61	down
Slc27a2	5.57	down
LOC670917	5.48	down
Fgf17	5.01	down
STAT3	5.00	down
Tgm1	4.93	down
Cyp11a1	4.83	down
Dtx3l	4.80	down
Irak3	4.80	down
Slc27a2	4.69	down

Table S3. STAT3 binding sites on selected cardiac genes were extracted from published datasets (Durant et al., 2010; Ho et al., 2011; Hutchins et al., 2012).

Datasets	ADRA1A	ADRA1B	ADRA1D	ADRA2A	ADRA2C	ADRB1	ADRB2	ADRB3	AGTRAP	AGTR1A	AT2AR	CACNA1A	CACNA1D	CACNA1E	CACNA2D1	CCL8	COL1A1	COL1A2	COL3A1	CRHR1	CRHR2	ENDRA	ENDRB	ICAM1	KCNE1	KCNH1	KCNH2	KCNJ10	KCNJ11	KCNJ2	KCNK5
ESCs									+			+												+				+			
ESCs									+			+												+							
T Cells		+					+	+				+	+	+	+									+		+	+	+			+
MΦs			+	+							+		+	+								+	+						+	+

KCNQ1	MMP1a	MMP1b	MMP12	MMP2	MMP9	MYH6	MYH7	NPPA (ANP)	NPPB (BNP)	NPPC (CNP)	S100A8	S100A9	SAA3	SCN4B	SCN5A	SERPINA3A	SOD1	TAB2	TIMP1	TIMP2	TIMP3	TIMP4	TXNIP	UST2R	References:
																							+		Ho et al., 2011
+																									Chen et al., 2008
			+							+			+	+		+			+	+			+		Durant et al., 2010
+				+													+	+		+			+		Hutchins et al., 2012

Bibliography

Adluri, R. S., Thirunavukkarasu, M., Zhan, L., Akita, Y., Samuel, S. M., Otani, H., . . . Maulik, N. (2011). Thioredoxin 1 enhances neovascularization and reduces ventricular remodeling during chronic myocardial infarction: a study using thioredoxin 1 transgenic mice. *J Mol Cell Cardiol, 50*(1), 239-247. doi: 10.1016/j.yjmcc.2010.11.002

Agah, R., Frenkel, P. A., French, B. A., Michael, L. H., Overbeek, P. A., & Schneider, M. D. (1997). Gene recombination in postmitotic cells. Targeted expression of Cre recombinase provokes cardiac-restricted, site-specific rearrangement in adult ventricular muscle in vivo. *J Clin Invest, 100*(1), 169-179. doi: 10.1172/jci119509

Alonzi, T., Maritano, D., Gorgoni, B., Rizzuto, G., Libert, C., & Poli, V. (2001). Essential role of STAT3 in the control of the acute-phase response as revealed by inducible gene inactivation [correction of activation] in the liver. *Mol Cell Biol, 21*(5), 1621-1632. doi: 10.1128/mcb.21.5.1621-1632.2001

Arai, A., Yamamoto, K., & Toyama, J. (1997). Murine cardiac progenitor cells require visceral embryonic endoderm and primitive streak for terminal differentiation. *Dev Dyn, 210*(3), 344-353. doi: 10.1002/(sici)1097-0177(199711)210:3<344::aid-aja13>3.0.co;2-a

Arceci, R. J., King, A. A., Simon, M. C., Orkin, S. H., & Wilson, D. B. (1993). Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Mol Cell Biol, 13*(4), 2235-2246.

Baharvand, H., Azarnia, M., Parivar, K., & Ashtiani, S. K. (2005). The effect of extracellular matrix on embryonic stem cell-derived cardiomyocytes. *J Mol Cell Cardiol, 38*(3), 495-503. doi: 10.1016/j.yjmcc.2004.12.011

Barry, S. P., Davidson, S. M., & Townsend, P. A. (2008). Molecular regulation of cardiac hypertrophy. *Int J Biochem Cell Biol, 40*(10), 2023-2039. doi: 10.1016/j.biocel.2008.02.020

Barth, A. S., Kuner, R., Buness, A., Ruschhaupt, M., Merk, S., Zwermann, L., . . . Sultmann, H. (2006). Identification of a common gene expression signature in dilated cardiomyopathy across independent microarray studies. *J Am Coll Cardiol, 48*(8), 1610-1617. doi: 10.1016/j.jacc.2006.07.026

Boengler, K., Buechert, A., Heinen, Y., Roeskes, C., Hilfiker-Kleiner, D., Heusch, G., & Schulz, R. (2008). Cardioprotection by ischemic postconditioning is lost in aged and STAT3-deficient mice. *Circ Res, 102*(1), 131-135. doi: 10.1161/circresaha.107.164699

Boheler, K. R., Czyz, J., Tweedie, D., Yang, H. T., Anisimov, S. V., & Wobus, A. M. (2002). Differentiation of pluripotent embryonic stem cells into cardiomyocytes. *Circ Res, 91*(3), 189-201.

Bolli, R., Stein, A. B., Guo, Y., Wang, O. L., Rokosh, G., Dawn, B., . . . Xuan, Y. T. (2011). A murine model of inducible, cardiac-specific deletion of STAT3: its use to determine the role of STAT3 in the upregulation of cardioprotective proteins by ischemic preconditioning. *J Mol Cell Cardiol, 50*(4), 589-597. doi: 10.1016/j.yjmcc.2011.01.002

Booz, G. W., Day, J. N., & Baker, K. M. (2002). Interplay between the cardiac renin angiotensin system and JAK-STAT signaling: role in cardiac hypertrophy,

ischemia/reperfusion dysfunction, and heart failure. *J Mol Cell Cardiol, 34*(11), 1443-1453.

Boulton, T. G., Stahl, N., & Yancopoulos, G. D. (1994). Ciliary neurotrophic factor/leukemia inhibitory factor/interleukin 6/oncostatin M family of cytokines induces tyrosine phosphorylation of a common set of proteins overlapping those induced by other cytokines and growth factors. *J Biol Chem, 269*(15), 11648-11655.

Boyer, L. A., Lee, T. I., Cole, M. F., Johnstone, S. E., Levine, S. S., Zucker, J. P., . . . Young, R. A. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell, 122*(6), 947-956. doi: 10.1016/j.cell.2005.08.020

Bradley, A., Evans, M., Kaufman, M. H., & Robertson, E. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature, 309*(5965), 255-256.

Brand, T. (2003). Heart development: molecular insights into cardiac specification and early morphogenesis. *Dev Biol, 258*(1), 1-19.

Brewer, A. C., Alexandrovich, A., Mjaatvedt, C. H., Shah, A. M., Patient, R. K., & Pizzey, J. A. (2005). GATA factors lie upstream of Nkx 2.5 in the transcriptional regulatory cascade that effects cardiogenesis. *Stem Cells Dev, 14*(4), 425-439. doi: 10.1089/scd.2005.14.425

Bromberg, J. F. (2001). Activation of STAT proteins and growth control. *Bioessays, 23*(2), 161-169. doi: 10.1002/1521-1878(200102)23:2<161::aid-bies1023>3.0.co;2-0

Bryant, D., Becker, L., Richardson, J., Shelton, J., Franco, F., Peshock, R., . . . Giroir, B. (1998). Cardiac failure in transgenic mice with myocardial expression of tumor necrosis factor-alpha. *Circulation, 97*(14), 1375-1381.

Butler, K. L., Huffman, L. C., Koch, S. E., Hahn, H. S., & Gwathmey, J. K. (2006). STAT-3 activation is necessary for ischemic preconditioning in hypertrophied myocardium. *Am J Physiol Heart Circ Physiol, 291*(2), H797-803. doi: 10.1152/ajpheart.01334.2005

Chakraborty, A., & Tweardy, D. J. (1998). Stat3 and G-CSF-induced myeloid differentiation. *Leuk Lymphoma, 30*(5-6), 433-442. doi: 10.3109/10428199809057555

Chandrasekar, B., Mitchell, D. H., Colston, J. T., & Freeman, G. L. (1999). Regulation of CCAAT/Enhancer binding protein, interleukin-6, interleukin-6 receptor, and gp130 expression during myocardial ischemia/reperfusion. *Circulation, 99*(3), 427-433.

Chapman, R. S., Lourenco, P. C., Tonner, E., Flint, D. J., Selbert, S., Takeda, K., . . . Watson, C. J. (1999). Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. *Genes Dev, 13*(19), 2604-2616.

Charron, F., Paradis, P., Bronchain, O., Nemer, G., & Nemer, M. (1999). Cooperative interaction between GATA-4 and GATA-6 regulates myocardial gene expression. *Mol Cell Biol, 19*(6), 4355-4365.

Chen, C. L., Lin, C. F., Chang, W. T., Huang, W. C., Teng, C. F., & Lin, Y. S. (2008). Ceramide induces p38 MAPK and JNK activation through a mechanism involving a thioredoxin-interacting protein-mediated pathway. *Blood, 111*(8), 4365-4374. doi: 10.1182/blood-2007-08-106336

Chen, G. Y., & Nunez, G. (2010). Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol, 10*(12), 826-837. doi: 10.1038/nri2873

Chen, J., Cha-Molstad, H., Szabo, A., & Shalev, A. (2009). Diabetes induces and calcium channel blockers prevent cardiac expression of proapoptotic thioredoxin-

interacting protein. *Am J Physiol Endocrinol Metab, 296*(5), E1133-1139. doi: 10.1152/ajpendo.90944.2008

Chien, K. R., Domian, I. J., & Parker, K. K. (2008). Cardiogenesis and the complex biology of regenerative cardiovascular medicine. *Science, 322*(5907), 1494-1497. doi: 10.1126/science.1163267

Darnell, J. E., Jr. (1997). STATs and gene regulation. *Science, 277*(5332), 1630-1635.

Darnell, J. E., Jr., Kerr, I. M., & Stark, G. R. (1994). Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science, 264*(5164), 1415-1421.

Davis, B. K., & Ting, J. P. (2010). NLRP3 has a sweet tooth. *Nature immunology, 11*(2), 105-106. doi: 10.1038/ni0210-105

Decker, T., & Kovarik, P. (1999). Transcription factor activity of STAT proteins: structural requirements and regulation by phosphorylation and interacting proteins. *Cell Mol Life Sci, 55*(12), 1535-1546.

Deswal, A., Petersen, N. J., Feldman, A. M., Young, J. B., White, B. G., & Mann, D. L. (2001). Cytokines and cytokine receptors in advanced heart failure: an analysis of the cytokine database from the Vesnarinone trial (VEST). *Circulation, 103*(16), 2055-2059.

Di Lisi, R., Millino, C., Calabria, E., Altruda, F., Schiaffino, S., & Ausoni, S. (1998). Combinatorial cis-acting elements control tissue-specific activation of the cardiac troponin I gene in vitro and in vivo. *J Biol Chem, 273*(39), 25371-25380.

Dinarello, C. A. (2009). Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol, 27*, 519-550. doi: 10.1146/annurev.immunol.021908.132612

Dixelius, J., Jakobsson, L., Genersch, E., Bohman, S., Ekblom, P., & Claesson-Welsh, L. (2004). Laminin-1 promotes angiogenesis in synergy with fibroblast growth factor by distinct regulation of the gene and protein expression profile in endothelial cells. *J Biol Chem, 279*(22), 23766-23772. doi: 10.1074/jbc.M311675200

Do, D. V., Ueda, J., Messerschmidt, D. M., Lorthongpanich, C., Zhou, Y., Feng, B., . . . Fu, X. Y. (2013). A genetic and developmental pathway from STAT3 to the OCT4-NANOG circuit is essential for maintenance of ICM lineages in vivo. *Genes Dev, 27*(12), 1378-1390. doi: 10.1101/gad.221176.113

Dostert, C., Petrilli, V., Van Bruggen, R., Steele, C., Mossman, B. T., & Tschopp, J. (2008). Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science, 320*(5876), 674-677. doi: 10.1126/science.1156995

Duncan, S. A., Zhong, Z., Wen, Z., & Darnell, J. E., Jr. (1997). STAT signaling is active during early mammalian development. *Dev Dyn, 208*(2), 190-198. doi: 10.1002/(sici)1097-0177(199702)208:2<190::aid-aja6>3.0.co;2-d

Durant, L., Watford, W. T., Ramos, H. L., Laurence, A., Vahedi, G., Wei, L., . . . O'Shea, J. J. (2010). Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity, 32*(5), 605-615. doi: 10.1016/j.immuni.2010.05.003

Durbin, J. E., Hackenmiller, R., Simon, M. C., & Levy, D. E. (1996). Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell, 84*(3), 443-450.

Durocher, D., Charron, F., Warren, R., Schwartz, R. J., & Nemer, M. (1997). The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *EMBO J, 16*(18), 5687-5696. doi: 10.1093/emboj/16.18.5687

Dziadek, M., & Timpl, R. (1985). Expression of nidogen and laminin in basement membranes during mouse embryogenesis and in teratocarcinoma cells. *Dev Biol, 111*(2), 372-382.

Eghbali, M., Blumenfeld, O. O., Seifter, S., Buttrick, P. M., Leinwand, L. A., Robinson, T. F., . . . Giambrone, M. A. (1989). Localization of types I, III and IV collagen mRNAs in rat heart cells by in situ hybridization. *J Mol Cell Cardiol, 21*(1), 103-113.

Ehret, G. B., Reichenbach, P., Schindler, U., Horvath, C. M., Fritz, S., Nabholz, M., & Bucher, P. (2001). DNA binding specificity of different STAT proteins. Comparison of in vitro specificity with natural target sites. *J Biol Chem, 276*(9), 6675-6688. doi: 10.1074/jbc.M001748200

Ekblom, M., Falk, M., Salmivirta, K., Durbeej, M., & Ekblom, P. (1998). Laminin isoforms and epithelial development. *Ann N Y Acad Sci, 857*, 194-211.

Elgort, M. G., O'Shea, J. M., Jiang, Y., & Ayer, D. E. (2010). Transcriptional and Translational Downregulation of Thioredoxin Interacting Protein Is Required for Metabolic Reprogramming during G(1). *Genes Cancer, 1*(9), 893-907. doi: 10.1177/1947601910389604

Eltzschig, H. K., & Eckle, T. (2011). Ischemia and reperfusion[mdash]from mechanism to translation. *Nat Med, 17*(11), 1391-1401.

Ernst, M., Novak, U., Nicholson, S. E., Layton, J. E., & Dunn, A. R. (1999). The carboxyl-terminal domains of gp130-related cytokine receptors are necessary for suppressing embryonic stem cell differentiation. Involvement of STAT3. *J Biol Chem, 274*(14), 9729-9737.

Evans, M. J., & Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature, 292*(5819), 154-156.

Evans, T., Reitman, M., & Felsenfeld, G. (1988). An erythrocyte-specific DNA-binding factor recognizes a regulatory sequence common to all chicken globin genes. *Proc Natl Acad Sci U S A, 85*(16), 5976-5980.

Falk, M., Ferletta, M., Forsberg, E., & Ekblom, P. (1999). Restricted distribution of laminin alpha1 chain in normal adult mouse tissues. *Matrix Biol, 18*(6), 557-568.

Fischer, P., & Hilfiker-Kleiner, D. (2007). Survival pathways in hypertrophy and heart failure: the gp130-STAT3 axis. *Basic Res Cardiol, 102*(4), 279-297. doi: 10.1007/s00395-007-0658-z

Fishman, M. C., & Chien, K. R. (1997). Fashioning the vertebrate heart: earliest embryonic decisions. *Development, 124*(11), 2099-2117.

Fong, S., Debs, R. J., & Desprez, P. Y. (2004). Id genes and proteins as promising targets in cancer therapy. *Trends Mol Med, 10*(8), 387-392. doi: 10.1016/j.molmed.2004.06.008

Foshay, K., Rodriguez, G., Hoel, B., Narayan, J., & Gallicano, G. I. (2005). JAK2/STAT3 directs cardiomyogenesis within murine embryonic stem cells in vitro. *Stem Cells, 23*(4), 530-543. doi: 10.1634/stemcells.2004-0293

Frangogiannis, N. G., Smith, C. W., & Entman, M. L. (2002). The inflammatory response in myocardial infarction. *Cardiovasc Res, 53*(1), 31-47.

Fu, X. Y., Kessler, D. S., Veals, S. A., Levy, D. E., & Darnell, J. E., Jr. (1990). ISGF3, the transcriptional activator induced by interferon alpha, consists of multiple interacting polypeptide chains. *Proc Natl Acad Sci U S A, 87*(21), 8555-8559.

Fu, X. Y., Schindler, C., Improta, T., Aebersold, R., & Darnell, J. E., Jr. (1992). The proteins of ISGF-3, the interferon alpha-induced transcriptional activator, define a gene family involved in signal transduction. *Proc Natl Acad Sci U S A, 89*(16), 7840-7843.

Good, S. R., Thieu, V. T., Mathur, A. N., Yu, Q., Stritesky, G. L., Yeh, N., . . . Kaplan, M. H. (2009). Temporal induction pattern of STAT4 target genes defines potential for Th1 lineage-specific programming. *J Immunol, 183*(6), 3839-3847. doi: 10.4049/jimmunol.0901411

Gove, C., Walmsley, M., Nijjar, S., Bertwistle, D., Guille, M., Partington, G., . . . Patient, R. (1997). Over-expression of GATA-6 in Xenopus embryos blocks differentiation of heart precursors. *EMBO J, 16*(2), 355-368. doi: 10.1093/emboj/16.2.355

Grepin, C., Dagnino, L., Robitaille, L., Haberstroh, L., Antakly, T., & Nemer, M. (1994). A hormone-encoding gene identifies a pathway for cardiac but not skeletal muscle gene transcription. *Mol Cell Biol, 14*(5), 3115-3129.

Grepin, C., Nemer, G., & Nemer, M. (1997). Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor. *Development, 124*(12), 2387-2395.

Grepin, C., Robitaille, L., Antakly, T., & Nemer, M. (1995). Inhibition of transcription factor GATA-4 expression blocks in vitro cardiac muscle differentiation. *Mol Cell Biol, 15*(8), 4095-4102.

Hamid, T., Gu, Y., Ortines, R. V., Bhattacharya, C., Wang, G., Xuan, Y. T., & Prabhu, S. D. (2009). Divergent tumor necrosis factor receptor-related remodeling responses in heart failure: role of nuclear factor-kappaB and inflammatory activation. *Circulation, 119*(10), 1386-1397. doi: 10.1161/circulationaha.108.802918

Hao, A., Novotny-Diermayr, V., Bian, W., Lin, B., Lim, C. P., Jing, N., & Cao, X. (2005). The LIM/homeodomain protein Islet1 recruits Janus tyrosine kinases and signal transducer and activator of transcription 3 and stimulates their activities. *Mol Biol Cell, 16*(4), 1569-1583. doi: 10.1091/mbc.E04-08-0664

Harvey, R. P., Lai, D., Elliott, D., Biben, C., Solloway, M., Prall, O., . . . Kirk, E. (2002). Homeodomain factor Nkx2-5 in heart development and disease. *Cold Spring Harb Symp Quant Biol, 67*, 107-114.

Hattori, R., Maulik, N., Otani, H., Zhu, L., Cordis, G., Engelman, R. M., . . . Das, D. K. (2001). Role of STAT3 in ischemic preconditioning. *J Mol Cell Cardiol, 33*(11), 1929-1936. doi: 10.1006/jmcc.2001.1456

Haudek, S. B., Taffet, G. E., Schneider, M. D., & Mann, D. L. (2007). TNF provokes cardiomyocyte apoptosis and cardiac remodeling through activation of multiple cell death pathways. *J Clin Invest, 117*(9), 2692-2701. doi: 10.1172/jci29134

Heikinheimo, M., Scandrett, J. M., & Wilson, D. B. (1994). Localization of transcription factor GATA-4 to regions of the mouse embryo involved in cardiac development. *Dev Biol, 164*(2), 361-373. doi: 10.1006/dbio.1994.1206

Heinrich, P. C., Behrmann, I., Muller-Newen, G., Schaper, F., & Graeve, L. (1998). Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem J, 334 (Pt 2)*, 297-314.

Hescheler, J., Fleischmann, B. K., Wartenberg, M., Bloch, W., Kolossov, E., Ji, G., . . . Sauer, H. (1999). Establishment of ionic channels and signalling cascades in the embryonic stem cell-derived primitive endoderm and cardiovascular system. *Cells Tissues Organs, 165*(3-4), 153-164. doi: 16695

Hilfiker-Kleiner, D., Hilfiker, A., & Drexler, H. (2005). Many good reasons to have STAT3 in the heart. *Pharmacol Ther, 107*(1), 131-137. doi: 10.1016/j.pharmthera.2005.02.003

Hilfiker-Kleiner, D., Hilfiker, A., Fuchs, M., Kaminski, K., Schaefer, A., Schieffer, B., . . . Drexler, H. (2004). Signal transducer and activator of transcription 3 is required

for myocardial capillary growth, control of interstitial matrix deposition, and heart protection from ischemic injury. *Circ Res, 95*(2), 187-195. doi: 10.1161/01.res.0000134921.50377.61

Hilton, D. J. (1999). Negative regulators of cytokine signal transduction. *Cell Mol Life Sci, 55*(12), 1568-1577.

Ho, L., Miller, E. L., Ronan, J. L., Ho, W. Q., Jothi, R., & Crabtree, G. R. (2011). esBAF facilitates pluripotency by conditioning the genome for LIF/STAT3 signalling and by regulating polycomb function. *Nat Cell Biol, 13*(8), 903-913. doi: 10.1038/ncb2285

Holtzinger, A., & Evans, T. (2005). Gata4 regulates the formation of multiple organs. *Development, 132*(17), 4005-4014. doi: 10.1242/dev.01978

Hutchins, A. P., Poulain, S., & Miranda-Saavedra, D. (2012). Genome-wide analysis of STAT3 binding in vivo predicts effectors of the anti-inflammatory response in macrophages. *Blood, 119*(13), e110-119. doi: 10.1182/blood-2011-09-381483

Hwang, M. W., Matsumori, A., Furukawa, Y., Ono, K., Okada, M., Iwasaki, A., . . . Sasayama, S. (2001). Neutralization of interleukin-1beta in the acute phase of myocardial infarction promotes the progression of left ventricular remodeling. *J Am Coll Cardiol, 38*(5), 1546-1553.

Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., . . . Gotoh, Y. (1997). Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science, 275*(5296), 90-94.

Ionita, M. G., Arslan, F., de Kleijn, D. P., & Pasterkamp, G. (2010). Endogenous inflammatory molecules engage Toll-like receptors in cardiovascular disease. *J Innate Immun, 2*(4), 307-315. doi: 10.1159/000314270

Ip, H. S., Wilson, D. B., Heikinheimo, M., Tang, Z., Ting, C. N., Simon, M. C., . . . Parmacek, M. S. (1994). The GATA-4 transcription factor transactivates the cardiac muscle-specific troponin C promoter-enhancer in nonmuscle cells. *Mol Cell Biol, 14*(11), 7517-7526.

Jacoby, J. J., Kalinowski, A., Liu, M. G., Zhang, S. S., Gao, Q., Chai, G. X., . . . Fu, X. Y. (2003). Cardiomyocyte-restricted knockout of STAT3 results in higher sensitivity to inflammation, cardiac fibrosis, and heart failure with advanced age. *Proc Natl Acad Sci U S A, 100*(22), 12929-12934. doi: 10.1073/pnas.2134694100

Jeon, J. H., Lee, K. N., Hwang, C. Y., Kwon, K. S., You, K. H., & Choi, I. (2005). Tumor suppressor VDUP1 increases p27(kip1) stability by inhibiting JAB1. *Cancer Res, 65*(11), 4485-4489. doi: 10.1158/0008-5472.can-04-2271

Ji, R. P., Phoon, C. K., Aristizabal, O., McGrath, K. E., Palis, J., & Turnbull, D. H. (2003). Onset of cardiac function during early mouse embryogenesis coincides with entry of primitive erythroblasts into the embryo proper. *Circ Res, 92*(2), 133-135.

Jiang, Y., & Evans, T. (1996). The Xenopus GATA-4/5/6 genes are associated with cardiac specification and can regulate cardiac-specific transcription during embryogenesis. *Dev Biol, 174*(2), 258-270. doi: 10.1006/dbio.1996.0071

Kaimul, A. M., Nakamura, H., Masutani, H., & Yodoi, J. (2007). Thioredoxin and thioredoxin-binding protein-2 in cancer and metabolic syndrome. *Free Radic Biol Med, 43*(6), 861-868. doi: 10.1016/j.freeradbiomed.2007.05.032

Kami, K., & Senba, E. (2002). In vivo activation of STAT3 signaling in satellite cells and myofibers in regenerating rat skeletal muscles. *J Histochem Cytochem, 50*(12), 1579-1589.

Kaplan, M. H., Schindler, U., Smiley, S. T., & Grusby, M. J. (1996). Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity, 4*(3), 313-319.

Kaplan, M. H., Sun, Y. L., Hoey, T., & Grusby, M. J. (1996). Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature, 382*(6587), 174-177. doi: 10.1038/382174a0

Kattman, S. J., Huber, T. L., & Keller, G. M. (2006). Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Dev Cell, 11*(5), 723-732. doi: 10.1016/j.devcel.2006.10.002

Kawaguchi, M., Takahashi, M., Hata, T., Kashima, Y., Usui, F., Morimoto, H., . . . Ikeda, U. (2011). Inflammasome activation of cardiac fibroblasts is essential for myocardial ischemia/reperfusion injury. *Circulation, 123*(6), 594-604. doi: 10.1161/circulationaha.110.982777

Kelley, C., Blumberg, H., Zon, L. I., & Evans, T. (1993). GATA-4 is a novel transcription factor expressed in endocardium of the developing heart. *Development, 118*(3), 817-827.

Kelly, B. B., Narula, J., & Fuster, V. (2012). Recognizing global burden of cardiovascular disease and related chronic diseases. *Mt Sinai J Med, 79*(6), 632-640. doi: 10.1002/msj.21345

Kelly, R. G., Brown, N. A., & Buckingham, M. E. (2001). The arterial pole of the mouse heart forms from Fgf10-expressing cells in pharyngeal mesoderm. *Dev Cell, 1*(3), 435-440.

Kelly, R. G., & Buckingham, M. E. (2002). The anterior heart-forming field: voyage to the arterial pole of the heart. *Trends Genet, 18*(4), 210-216.

Kidder, B. L., Yang, J., & Palmer, S. (2008). Stat3 and c-Myc genome-wide promoter occupancy in embryonic stem cells. *PLoS One, 3*(12), e3932. doi: 10.1371/journal.pone.0003932

Kirby, M. L., Gale, T. F., & Stewart, D. E. (1983). Neural crest cells contribute to normal aorticopulmonary septation. *Science, 220*(4601), 1059-1061.

Kotenko, S. V., Izotova, L. S., Pollack, B. P., Muthukumaran, G., Paukku, K., Silvennoinen, O., . . . Pestka, S. (1996). Other kinases can substitute for Jak2 in signal transduction by interferon-gamma. *J Biol Chem, 271*(29), 17174-17182.

Koutsourakis, M., Langeveld, A., Patient, R., Beddington, R., & Grosveld, F. (1999). The transcription factor GATA6 is essential for early extraembryonic development. *Development, 126*(9), 723-732.

Kuo, C. T., Morrisey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M. S., . . . Leiden, J. M. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev, 11*(8), 1048-1060.

Lachtermacher, S., Esporcatte, B. L., Montalvao, F., Costa, P. C., Rodrigues, D. C., Belem, L., . . . Campos-de-Carvalho, A. C. (2010). Cardiac gene expression and systemic cytokine profile are complementary in a murine model of post-ischemic heart failure. *Braz J Med Biol Res, 43*(4), 377-389.

Lan, Y., Kingsley, P. D., Cho, E. S., & Jiang, R. (2001). Osr2, a new mouse gene related to Drosophila odd-skipped, exhibits dynamic expression patterns during craniofacial, limb, and kidney development. *Mech Dev, 107*(1-2), 175-179.

Laugwitz, K. L., Moretti, A., Caron, L., Nakano, A., & Chien, K. R. (2008). Islet1 cardiovascular progenitors: a single source for heart lineages? *Development, 135*(2), 193-205. doi: 10.1242/dev.001883

Laverriere, A. C., MacNeill, C., Mueller, C., Poelmann, R. E., Burch, J. B., & Evans, T. (1994). GATA-4/5/6, a subfamily of three transcription factors transcribed in developing heart and gut. *J Biol Chem, 269*(37), 23177-23184.

Lee, T. I., Johnstone, S. E., & Young, R. A. (2006). Chromatin immunoprecipitation and microarray-based analysis of protein location. *Nat Protoc, 1*(2), 729-748. doi: 10.1038/nprot.2006.98

Lee, Y., Shioi, T., Kasahara, H., Jobe, S. M., Wiese, R. J., Markham, B. E., & Izumo, S. (1998). The cardiac tissue-restricted homeobox protein Csx/Nkx2.5 physically associates with the zinc finger protein GATA4 and cooperatively activates atrial natriuretic factor gene expression. *Mol Cell Biol, 18*(6), 3120-3129.

Leonard, M., Brice, M., Engel, J. D., & Papayannopoulou, T. (1993). Dynamics of GATA transcription factor expression during erythroid differentiation. *Blood, 82*(4), 1071-1079.

Leonard, W. J., & O'Shea, J. J. (1998). Jaks and STATs: biological implications. *Annu Rev Immunol, 16*, 293-322. doi: 10.1146/annurev.immunol.16.1.293

Levy, D. E., & Darnell, J. E., Jr. (2002). Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol, 3*(9), 651-662. doi: 10.1038/nrm909

Li, P., Spolski, R., Liao, W., Wang, L., Murphy, T. L., Murphy, K. M., & Leonard, W. J. (2012). BATF-JUN is critical for IRF4-mediated transcription in T cells. *Nature, 490*(7421), 543-546. doi: 10.1038/nature11530

Lien, C. L., Wu, C., Mercer, B., Webb, R., Richardson, J. A., & Olson, E. N. (1999). Control of early cardiac-specific transcription of Nkx2-5 by a GATA-dependent enhancer. *Development, 126*(1), 75-84.

Lim, C. P., & Cao, X. (2006). Structure, function, and regulation of STAT proteins. *Mol Biosyst, 2*(11), 536-550. doi: 10.1039/b606246f

Lin, Q., Schwarz, J., Bucana, C., & Olson, E. N. (1997). Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science, 276*(5317), 1404-1407.

Lints, T. J., Parsons, L. M., Hartley, L., Lyons, I., & Harvey, R. P. (1993). Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. *Development, 119*(3), 969.

Liu, Y., Lian, K., Zhang, L., Wang, R., Yi, F., Gao, C., . . . Tao, L. (2014). TXNIP mediates NLRP3 inflammasome activation in cardiac microvascular endothelial cells as a novel mechanism in myocardial ischemia/reperfusion injury. *Basic Res Cardiol, 109*(5), 415. doi: 10.1007/s00395-014-0415-z

Loh, Y. H., Wu, Q., Chew, J. L., Vega, V. B., Zhang, W., Chen, X., . . . Ng, H. H. (2006). The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet, 38*(4), 431-440. doi: 10.1038/ng1760

Lyons, I., Parsons, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, L., & Harvey, R. P. (1995). Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. *Genes Dev, 9*(13), 1654-1666.

Malinda, K. M., Nomizu, M., Chung, M., Delgado, M., Kuratomi, Y., Yamada, Y., . . . Ponce, M. L. (1999). Identification of laminin alpha1 and beta1 chain peptides active for endothelial cell adhesion, tube formation, and aortic sprouting. *FASEB J, 13*(1), 53-62.

Maltsev, V. A., Wobus, A. M., Rohwedel, J., Bader, M., & Hescheler, J. (1994). Cardiomyocytes differentiated in vitro from embryonic stem cells

developmentally express cardiac-specific genes and ionic currents. *Circ Res, 75*(2), 233-244.

Mandal, M., Powers, S. E., Maienschein-Cline, M., Bartom, E. T., Hamel, K. M., Kee, B. L., . . . Clark, M. R. (2011). Epigenetic repression of the Igk locus by STAT5-mediated recruitment of the histone methyltransferase Ezh2. *Nature immunology, 12*(12), 1212-1220. doi: 10.1038/ni.2136

Mariathasan, S., Weiss, D. S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., . . . Dixit, V. M. (2006). Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature, 440*(7081), 228-232. doi: 10.1038/nature04515

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A, 78*(12), 7634-7638.

Martinon, F., Burns, K., & Tschopp, J. (2002). The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell, 10*(2), 417-426.

Martinon, F., Mayor, A., & Tschopp, J. (2009). The inflammasomes: guardians of the body. *Annu Rev Immunol, 27*, 229-265. doi: 10.1146/annurev.immunol.021908.132715

Masters, S. L., Dunne, A., Subramanian, S. L., Hull, R. L., Tannahill, G. M., Sharp, F. A., . . . O'Neill, L. A. (2010). Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1beta in type 2 diabetes. *Nat Immunol, 11*(10), 897-904. doi: 10.1038/ni.1935

Matsuda, T., Nakamura, T., Nakao, K., Arai, T., Katsuki, M., Heike, T., & Yokota, T. (1999). STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *EMBO J, 18*(15), 4261-4269. doi: 10.1093/emboj/18.15.4261

Matsui, T., Kinoshita, T., Hirano, T., Yokota, T., & Miyajima, A. (2002). STAT3 down-regulates the expression of cyclin D during liver development. *J Biol Chem, 277*(39), 36167-36173. doi: 10.1074/jbc.M203184200

Matsuzaki, Y., Xu, Y., Ikegami, M., Besnard, V., Park, K. S., Hull, W. M., . . . Whitsett, J. A. (2006). Stat3 is required for cytoprotection of the respiratory epithelium during adenoviral infection. *J Immunol, 177*(1), 527-537.

McMurray, J. J., & Pfeffer, M. A. (2005). Heart failure. *Lancet, 365*(9474), 1877-1889. doi: 10.1016/s0140-6736(05)66621-4

Meraz, M. A., White, J. M., Sheehan, K. C., Bach, E. A., Rodig, S. J., Dighe, A. S., . . . Schreiber, R. D. (1996). Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell, 84*(3), 431-442.

Mezzaroma, E., Toldo, S., Farkas, D., Seropian, I. M., Van Tassell, B. W., Salloum, F. N., . . . Abbate, A. (2011). The inflammasome promotes adverse cardiac remodeling following acute myocardial infarction in the mouse. *Proc Natl Acad Sci U S A, 108*(49), 19725-19730. doi: 10.1073/pnas.1108586108

Mjaatvedt, C. H., Nakaoka, T., Moreno-Rodriguez, R., Norris, R. A., Kern, M. J., Eisenberg, C. A., . . . Markwald, R. R. (2001). The outflow tract of the heart is recruited from a novel heart-forming field. *Dev Biol, 238*(1), 97-109. doi: 10.1006/dbio.2001.0409

Molkentin, J. D. (2000). The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. *J Biol Chem, 275*(50), 38949-38952. doi: 10.1074/jbc.R000029200

Molkentin, J. D., Antos, C., Mercer, B., Taigen, T., Miano, J. M., & Olson, E. N. (2000). Direct activation of a GATA6 cardiac enhancer by Nkx2.5: evidence for a reinforcing regulatory network of Nkx2.5 and GATA transcription factors in the developing heart. *Dev Biol, 217*(2), 301-309. doi: 10.1006/dbio.1999.9544

Molkentin, J. D., Lin, Q., Duncan, S. A., & Olson, E. N. (1997). Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev, 11*(8), 1061-1072.

Morrisey, E. E., Ip, H. S., Lu, M. M., & Parmacek, M. S. (1996). GATA-6: a zinc finger transcription factor that is expressed in multiple cell lineages derived from lateral mesoderm. *Dev Biol, 177*(1), 309-322. doi: 10.1006/dbio.1996.0165

Morrisey, E. E., Ip, H. S., Tang, Z., Lu, M. M., & Parmacek, M. S. (1997). GATA-5: a transcriptional activator expressed in a novel temporally and spatially-restricted pattern during embryonic development. *Dev Biol, 183*(1), 21-36. doi: 10.1006/dbio.1996.8485

Morrisey, E. E., Tang, Z., Sigrist, K., Lu, M. M., Jiang, F., Ip, H. S., & Parmacek, M. S. (1998). GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes Dev, 12*(22), 3579-3590.

Murase, S., Kim, E., Lin, L., Hoffman, D. A., & McKay, R. D. (2012). Loss of signal transducer and activator of transcription 3 (STAT3) signaling during elevated activity causes vulnerability in hippocampal neurons. *J Neurosci, 32*(44), 15511-15520. doi: 10.1523/jneurosci.2940-12.2012

Murphy, A. M., Thompson, W. R., Peng, L. F., & Jones, L., 2nd. (1997). Regulation of the rat cardiac troponin I gene by the transcription factor GATA-4. *Biochem J, 322 (Pt 2)*, 393-401.

Muruve, D. A., Petrilli, V., Zaiss, A. K., White, L. R., Clark, S. A., Ross, P. J., . . . Tschopp, J. (2008). The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. *Nature, 452*(7183), 103-107. doi: 10.1038/nature06664

Narita, N., Bielinska, M., & Wilson, D. B. (1997a). Cardiomyocyte differentiation by GATA-4-deficient embryonic stem cells. *Development, 124*(19), 3755-3764.

Narita, N., Bielinska, M., & Wilson, D. B. (1997b). Wild-type endoderm abrogates the ventral developmental defects associated with GATA-4 deficiency in the mouse. *Dev Biol, 189*(2), 270-274. doi: 10.1006/dbio.1997.8684

Negoro, S., Kunisada, K., Tone, E., Funamoto, M., Oh, H., Kishimoto, T., & Yamauchi-Takihara, K. (2000). Activation of JAK/STAT pathway transduces cytoprotective signal in rat acute myocardial infarction. *Cardiovasc Res, 47*(4), 797-805.

Nemer, G., & Nemer, M. (2003). Transcriptional activation of BMP-4 and regulation of mammalian organogenesis by GATA-4 and -6. *Dev Biol, 254*(1), 131-148.

Nemer, G., Qureshi, S. T., Malo, D., & Nemer, M. (1999). Functional analysis and chromosomal mapping of Gata5, a gene encoding a zinc finger DNA-binding protein. *Mamm Genome, 10*(10), 993-999.

Neumann, D. A., Lane, J. R., Allen, G. S., Herskowitz, A., & Rose, N. R. (1993). Viral myocarditis leading to cardiomyopathy: do cytokines contribute to pathogenesis? *Clin Immunol Immunopathol, 68*(2), 181-190.

Nishiyama, A., Matsui, M., Iwata, S., Hirota, K., Masutani, H., Nakamura, H., . . . Yodoi, J. (1999). Identification of thioredoxin-binding protein-2/vitamin D(3) up-regulated protein 1 as a negative regulator of thioredoxin function and expression. *J Biol Chem, 274*(31), 21645-21650.

Niwa, H., Burdon, T., Chambers, I., & Smith, A. (1998). Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev, 12*(13), 2048-2060.

Okada, I., Hamanoue, H., Terada, K., Tohma, T., Megarbane, A., Chouery, E., . . . Saitsu, H. (2011). SMOC1 is essential for ocular and limb development in humans and mice. *Am J Hum Genet, 88*(1), 30-41. doi: 10.1016/j.ajhg.2010.11.012

Olson, E. N., & Srivastava, D. (1996). Molecular pathways controlling heart development. *Science, 272*(5262), 671-676.

Omichinski, J. G., Clore, G. M., Schaad, O., Felsenfeld, G., Trainor, C., Appella, E., . . . Gronenborn, A. M. (1993). NMR structure of a specific DNA complex of Zn-containing DNA binding domain of GATA-1. *Science, 261*(5120), 438-446.

Omichinski, J. G., Trainor, C., Evans, T., Gronenborn, A. M., Clore, G. M., & Felsenfeld, G. (1993). A small single-"finger" peptide from the erythroid transcription factor GATA-1 binds specifically to DNA as a zinc or iron complex. *Proc Natl Acad Sci U S A, 90*(5), 1676-1680.

Ono, K., Matsumori, A., Shioi, T., Furukawa, Y., & Sasayama, S. (1998). Cytokine gene expression after myocardial infarction in rat hearts: possible implication in left ventricular remodeling. *Circulation, 98*(2), 149-156.

Oshima, Y., Fujio, Y., Nakanishi, T., Itoh, N., Yamamoto, Y., Negoro, S., . . . Azuma, J. (2005). STAT3 mediates cardioprotection against ischemia/reperfusion injury through metallothionein induction in the heart. *Cardiovasc Res, 65*(2), 428-435. doi: 10.1016/j.cardiores.2004.10.021

Osugi, T., Oshima, Y., Fujio, Y., Funamoto, M., Yamashita, A., Negoro, S., . . . Kishimoto, T. (2002). Cardiac-specific activation of signal transducer and activator of transcription 3 promotes vascular formation in the heart. *J Biol Chem, 277*(8), 6676-6681. doi: 10.1074/jbc.M108246200

Pandolfi, P. P., Roth, M. E., Karis, A., Leonard, M. W., Dzierzak, E., Grosveld, F. G., . . . Lindenbaum, M. H. (1995). Targeted disruption of the GATA3 gene causes severe abnormalities in the nervous system and in fetal liver haematopoiesis. *Nat Genet, 11*(1), 40-44. doi: 10.1038/ng0995-40

Passier, R., Denning, C., & Mummery, C. (2006). Cardiomyocytes from Human Embryonic Stem Cells. In A. Wobus & K. Boheler (Eds.), *Stem Cells* (Vol. 174, pp. 101-122): Springer Berlin Heidelberg.

Passier, R., Oostwaard, D. W., Snapper, J., Kloots, J., Hassink, R. J., Kuijk, E., . . . Mummery, C. (2005). Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. *Stem Cells, 23*(6), 772-780. doi: 10.1634/stemcells.2004-0184

Patwari, P., Chutkow, W. A., Cummings, K., Verstraeten, V. L., Lammerding, J., Schreiter, E. R., & Lee, R. T. (2009). Thioredoxin-independent regulation of metabolism by the alpha-arrestin proteins. *J Biol Chem, 284*(37), 24996-25003. doi: 10.1074/jbc.M109.018093

Perrone, L., Devi, T. S., Hosoya, K., Terasaki, T., & Singh, L. P. (2009). Thioredoxin interacting protein (TXNIP) induces inflammation through chromatin modification in retinal capillary endothelial cells under diabetic conditions. *J Cell Physiol, 221*(1), 262-272. doi: 10.1002/jcp.21852

Peterkin, T., Gibson, A., & Patient, R. (2003). GATA-6 maintains BMP-4 and Nkx2 expression during cardiomyocyte precursor maturation. *EMBO J, 22*(16), 4260-4273. doi: 10.1093/emboj/cdg400

Pevny, L., Simon, M. C., Robertson, E., Klein, W. H., Tsai, S. F., D'Agati, V., . . . Costantini, F. (1991). Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature, 349*(6306), 257-260. doi: 10.1038/349257a0

Pomerantz, B. J., Reznikov, L. L., Harken, A. H., & Dinarello, C. A. (2001). Inhibition of caspase 1 reduces human myocardial ischemic dysfunction via inhibition of IL-18 and IL-1beta. *Proc Natl Acad Sci U S A, 98*(5), 2871-2876. doi: 10.1073/pnas.041611398

Rajan, P., & McKay, R. D. (1998). Multiple routes to astrocytic differentiation in the CNS. *J Neurosci, 18*(10), 3620-3629.

Rajasingh, J., Bord, E., Hamada, H., Lambers, E., Qin, G., Losordo, D. W., & Kishore, R. (2007). STAT3-dependent mouse embryonic stem cell differentiation into cardiomyocytes: analysis of molecular signaling and therapeutic efficacy of cardiomyocyte precommitted mES transplantation in a mouse model of myocardial infarction. *Circ Res, 101*(9), 910-918. doi: 10.1161/circresaha.107.156786

Rauchhaus, M., Doehner, W., Francis, D. P., Davos, C., Kemp, M., Liebenthal, C., . . . Anker, S. D. (2000). Plasma cytokine parameters and mortality in patients with chronic heart failure. *Circulation, 102*(25), 3060-3067.

Rawles, M. E. (1943). The Heart-Forming Areas of the Early Chick Blastoderm. *Physiological Zoology, 16*(1), 22-43. doi: 10.2307/30151667

Raz, R., Lee, C. K., Cannizzaro, L. A., d'Eustachio, P., & Levy, D. E. (1999). Essential role of STAT3 for embryonic stem cell pluripotency. *Proc Natl Acad Sci U S A, 96*(6), 2846-2851.

Reecy, J. M., Li, X., Yamada, M., DeMayo, F. J., Newman, C. S., Harvey, R. P., & Schwartz, R. J. (1999). Identification of upstream regulatory regions in the heart-expressed homeobox gene Nkx2-5. *Development, 126*(4), 839-849.

Reiter, J. F., Alexander, J., Rodaway, A., Yelon, D., Patient, R., Holder, N., & Stainier, D. Y. (1999). Gata5 is required for the development of the heart and endoderm in zebrafish. *Genes Dev, 13*(22), 2983-2995.

Romero-Lanman, E. E., Pavlovic, S., Amlani, B., Chin, Y., & Benezra, R. (2012). Id1 maintains embryonic stem cell self-renewal by up-regulation of Nanog and repression of Brachyury expression. *Stem Cells Dev, 21*(3), 384-393. doi: 10.1089/scd.2011.0428

Rosenquist, G. C. (1970). Location and movements of cardiogenic cells in the chick embryo: the heart-forming portion of the primitive streak. *Dev Biol, 22*(3), 461-475.

Samavati, L., Rastogi, R., Du, W., Huttemann, M., Fite, A., & Franchi, L. (2009). STAT3 tyrosine phosphorylation is critical for interleukin 1 beta and interleukin-6 production in response to lipopolysaccharide and live bacteria. *Mol Immunol, 46*(8-9), 1867-1877. doi: 10.1016/j.molimm.2009.02.018

Sandanger, O., Ranheim, T., Vinge, L. E., Bliksoen, M., Alfsnes, K., Finsen, A. V., . . . Yndestad, A. (2013). The NLRP3 inflammasome is up-regulated in cardiac fibroblasts and mediates myocardial ischaemia-reperfusion injury. *Cardiovasc Res, 99*(1), 164-174. doi: 10.1093/cvr/cvt091

Sano, S., Itami, S., Takeda, K., Tarutani, M., Yamaguchi, Y., Miura, H., . . . Takeda, J. (1999). Keratinocyte-specific ablation of Stat3 exhibits impaired skin remodeling, but does not affect skin morphogenesis. *EMBO J, 18*(17), 4657-4668. doi: 10.1093/emboj/18.17.4657

Sato, H., Takahashi, M., Ise, H., Yamada, A., Hirose, S., Tagawa, Y., . . . Ikeda, U. (2006). Collagen synthesis is required for ascorbic acid-enhanced differentiation of mouse embryonic stem cells into cardiomyocytes. *Biochem Biophys Res Commun, 342*(1), 107-112. doi: 10.1016/j.bbrc.2006.01.116

Schindler, C., Shuai, K., Prezioso, V. R., & Darnell, J. E., Jr. (1992). Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. *Science, 257*(5071), 809-813.

Schulze, P. C., De Keulenaer, G. W., Yoshioka, J., Kassik, K. A., & Lee, R. T. (2002). Vitamin D3-upregulated protein-1 (VDUP-1) regulates redox-dependent vascular smooth muscle cell proliferation through interaction with thioredoxin. *Circ Res, 91*(8), 689-695.

Searcy, R. D., Vincent, E. B., Liberatore, C. M., & Yutzey, K. E. (1998). A GATA-dependent nkx-2.5 regulatory element activates early cardiac gene expression in transgenic mice. *Development, 125*(22), 4461-4470.

Sepulveda, J. L., Belaguli, N., Nigam, V., Chen, C. Y., Nemer, M., & Schwartz, R. J. (1998). GATA-4 and Nkx-2.5 coactivate Nkx-2 DNA binding targets: role for regulating early cardiac gene expression. *Mol Cell Biol, 18*(6), 3405-3415.

Sharma, R., Bolger, A. P., Li, W., Davlouros, P. A., Volk, H. D., Poole-Wilson, P. A., . . . Anker, S. D. (2003). Elevated circulating levels of inflammatory cytokines and bacterial endotoxin in adults with congenital heart disease. *Am J Cardiol, 92*(2), 188-193.

Shim, W. S., Jiang, S., Wong, P., Tan, J., Chua, Y. L., Tan, Y. S., . . . Sim, E. (2004). Ex vivo differentiation of human adult bone marrow stem cells into cardiomyocyte-like cells. *Biochem Biophys Res Commun, 324*(2), 481-488. doi: 10.1016/j.bbrc.2004.09.087

Shimoda, K., van Deursen, J., Sangster, M. Y., Sarawar, S. R., Carson, R. T., Tripp, R. A., . . . Ihle, J. N. (1996). Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature, 380*(6575), 630-633. doi: 10.1038/380630a0

Simon, M. C. (1995). Gotta have GATA. *Nat Genet, 11*(1), 9-11. doi: 10.1038/ng0995-9

Smith, A. G. (2001). Embryo-derived stem cells: of mice and men. *Annu Rev Cell Dev Biol, 17*, 435-462. doi: 10.1146/annurev.cellbio.17.1.435

Smith, A. G., Heath, J. K., Donaldson, D. D., Wong, G. G., Moreau, J., Stahl, M., & Rogers, D. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature, 336*(6200), 688-690. doi: 10.1038/336688a0

Smith, R. M., Suleman, N., Lacerda, L., Opie, L. H., Akira, S., Chien, K. R., & Sack, M. N. (2004). Genetic depletion of cardiac myocyte STAT-3 abolishes classical preconditioning. *Cardiovasc Res, 63*(4), 611-616. doi: 10.1016/j.cardiores.2004.06.019

Solloway, M. J., & Harvey, R. P. (2003). Molecular pathways in myocardial development: a stem cell perspective. *Cardiovasc Res, 58*(2), 264-277.

Soudais, C., Bielinska, M., Heikinheimo, M., MacArthur, C. A., Narita, N., Saffitz, J. E., . . . Wilson, D. B. (1995). Targeted mutagenesis of the transcription factor GATA-4 gene in mouse embryonic stem cells disrupts visceral endoderm differentiation in vitro. *Development, 121*(11), 3877-3888.

Spangenburg, E. E., & Booth, F. W. (2002). Multiple signaling pathways mediate LIF-induced skeletal muscle satellite cell proliferation. *Am J Physiol Cell Physiol, 283*(1), C204-211. doi: 10.1152/ajpcell.00574.2001

Stutz, A., Golenbock, D. T., & Latz, E. (2009). Inflammasomes: too big to miss. *J Clin Invest, 119*(12), 3502-3511. doi: 10.1172/jci40599

Suffredini, A. F., Reda, D., Banks, S. M., Tropea, M., Agosti, J. M., & Miller, R. (1995). Effects of recombinant dimeric TNF receptor on human inflammatory responses following intravenous endotoxin administration. *J Immunol, 155*(10), 5038-5045.

Sun, L., Ma, K., Wang, H., Xiao, F., Gao, Y., Zhang, W., . . . Wu, Z. (2007). JAK1-STAT1-STAT3, a key pathway promoting proliferation and preventing premature differentiation of myoblasts. *J Cell Biol, 179*(1), 129-138. doi: 10.1083/jcb.200703184

Takahashi, A., Takahashi, Y., Matsumoto, K., & Miyata, K. (1995). Synergistic effects of insulin-like growth factor II (IGF-II) with leukemia inhibiting factor (LIF) on establishment of rat pluripotential cell lines. *J Vet Med Sci, 57*(3), 553-556.

Takahashi, T., Lord, B., Schulze, P. C., Fryer, R. M., Sarang, S. S., Gullans, S. R., & Lee, R. T. (2003). Ascorbic acid enhances differentiation of embryonic stem cells into cardiac myocytes. *Circulation, 107*(14), 1912-1916. doi: 10.1161/01.cir.0000064899.53876.a3

Takeda, K., Kaisho, T., Yoshida, N., Takeda, J., Kishimoto, T., & Akira, S. (1998). Stat3 activation is responsible for IL-6-dependent T cell proliferation through preventing apoptosis: generation and characterization of T cell-specific Stat3-deficient mice. *J Immunol, 161*(9), 4652-4660.

Takeda, K., Noguchi, K., Shi, W., Tanaka, T., Matsumoto, M., Yoshida, N., . . . Akira, S. (1997). Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc Natl Acad Sci U S A, 94*(8), 3801-3804.

Takeda, K., Tanaka, T., Shi, W., Matsumoto, M., Minami, M., Kashiwamura, S., . . . Akira, S. (1996). Essential role of Stat6 in IL-4 signalling. *Nature, 380*(6575), 627-630. doi: 10.1038/380627a0

Takeuchi, O., & Akira, S. (2010). Pattern recognition receptors and inflammation. *Cell, 140*(6), 805-820. doi: 10.1016/j.cell.2010.01.022

Thierfelder, W. E., van Deursen, J. M., Yamamoto, K., Tripp, R. A., Sarawar, S. R., Carson, R. T., . . . Ihle, J. N. (1996). Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature, 382*(6587), 171-174. doi: 10.1038/382171a0

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., & Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science, 282*(5391), 1145-1147.

Thuerauf, D. J., Hanford, D. S., & Glembotski, C. C. (1994). Regulation of rat brain natriuretic peptide transcription. A potential role for GATA-related transcription factors in myocardial cell gene expression. *J Biol Chem, 269*(27), 17772-17775.

Tsai, F. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., . . . Orkin, S. H. (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature, 371*(6494), 221-226. doi: 10.1038/371221a0

Ulevitch, R. J., & Tobias, P. S. (1995). Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu Rev Immunol, 13*, 437-457. doi: 10.1146/annurev.iy.13.040195.002253

Uozumi, H., Hiroi, Y., Zou, Y., Takimoto, E., Toko, H., Niu, P., . . . Komuro, I. (2001). gp130 plays a critical role in pressure overload-induced cardiac hypertrophy. *J Biol Chem, 276*(25), 23115-23119. doi: 10.1074/jbc.M100814200

Valdembri, D., Serini, G., Vacca, A., Ribatti, D., & Bussolino, F. (2002). In vivo activation of JAK2/STAT-3 pathway during angiogenesis induced by GM-CSF. *FASEB J, 16*(2), 225-227. doi: 10.1096/fj.01-0633fje

van Lunteren, E., & Moyer, M. (2007). Oxidoreductase, morphogenesis, extracellular matrix, and calcium ion-binding gene expression in streptozotocin-induced diabetic rat heart. *Am J Physiol Endocrinol Metab, 293*(3), E759-768. doi: 10.1152/ajpendo.00191.2007

Waldo, K. L., Kumiski, D. H., Wallis, K. T., Stadt, H. A., Hutson, M. R., Platt, D. H., & Kirby, M. L. (2001). Conotruncal myocardium arises from a secondary heart field. *Development, 128*(16), 3179-3188.

Wang, J., Ohmuraya, M., Hirota, M., Baba, H., Zhao, G., Takeya, M., . . . Yamamura, K. (2008). Expression pattern of serine protease inhibitor kazal type 3 (Spink3) during mouse embryonic development. *Histochem Cell Biol, 130*(2), 387-397. doi: 10.1007/s00418-008-0425-8

Wang, K., Wang, C., Xiao, F., Wang, H., & Wu, Z. (2008). JAK2/STAT2/STAT3 are required for myogenic differentiation. *J Biol Chem, 283*(49), 34029-34036. doi: 10.1074/jbc.M803012200

Wang, Y., De Keulenaer, G. W., & Lee, R. T. (2002). Vitamin D(3)-up-regulated protein-1 is a stress-responsive gene that regulates cardiomyocyte viability through interaction with thioredoxin. *J Biol Chem, 277*(29), 26496-26500. doi: 10.1074/jbc.M202133200

Wang, Z., Rong, Y. P., Malone, M. H., Davis, M. C., Zhong, F., & Distelhorst, C. W. (2006). Thioredoxin-interacting protein (txnip) is a glucocorticoid-regulated primary response gene involved in mediating glucocorticoid-induced apoptosis. *Oncogene, 25*(13), 1903-1913. doi: 10.1038/sj.onc.1209218

Wei, L., Vahedi, G., Sun, H. W., Watford, W. T., Takatori, H., Ramos, H. L., . . . Kanno, Y. (2010). Discrete roles of STAT4 and STAT6 transcription factors in tuning epigenetic modifications and transcription during T helper cell differentiation. *Immunity, 32*(6), 840-851. doi: 10.1016/j.immuni.2010.06.003

Westberg, J. A., Serlachius, M., Lankila, P., Penkowa, M., Hidalgo, J., & Andersson, L. C. (2007). Hypoxic preconditioning induces neuroprotective stanniocalcin-1 in brain via IL-6 signaling. *Stroke, 38*(3), 1025-1030. doi: 10.1161/01.STR.0000258113.67252.fa

Williams, R. L., Hilton, D. J., Pease, S., Willson, T. A., Stewart, C. L., Gearing, D. P., . . . Gough, N. M. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature, 336*(6200), 684-687. doi: 10.1038/336684a0

Wobus, A. M., Rohwedel, J., Maltsev, V., & Hescheler, J. (1995). Development of cardiomyocytes expressing cardiac-specific genes, action potentials, and ionic channels during embryonic stem cell-derived cardiogenesis. *Ann N Y Acad Sci, 752*, 460-469.

Wobus, A. M., Wallukat, G., & Hescheler, J. (1991). Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca2+ channel blockers. *Differentiation, 48*(3), 173-182.

World, C. J., Yamawaki, H., & Berk, B. C. (2006). Thioredoxin in the cardiovascular system. *J Mol Med (Berl), 84*(12), 997-1003. doi: 10.1007/s00109-006-0109-6

Xiang, G., Seki, T., Schuster, M. D., Witkowski, P., Boyle, A. J., See, F., . . . Itescu, S. (2005). Catalytic degradation of vitamin D up-regulated protein 1 mRNA enhances cardiomyocyte survival and prevents left ventricular remodeling after myocardial ischemia. *J Biol Chem, 280*(47), 39394-39402. doi: 10.1074/jbc.M502966200

Yajima, T., Yasukawa, H., Jeon, E. S., Xiong, D., Dorner, A., Iwatate, M., . . . Knowlton, K. U. (2006). Innate defense mechanism against virus infection within the cardiac myocyte requiring gp130-STAT3 signaling. *Circulation, 114*(22), 2364-2373. doi: 10.1161/circulationaha.106.642454

Yamamoto, M., Ko, L. J., Leonard, M. W., Beug, H., Orkin, S. H., & Engel, J. D. (1990). Activity and tissue-specific expression of the transcription factor NF-E1 multigene family. *Genes Dev, 4*(10), 1650-1662.

Yamanaka, H., Maehira, F., Oshiro, M., Asato, T., Yanagawa, Y., Takei, H., & Nakashima, Y. (2000). A possible interaction of thioredoxin with VDUP1 in HeLa cells detected in a yeast two-hybrid system. *Biochem Biophys Res Commun, 271*(3), 796-800. doi: 10.1006/bbrc.2000.2699

Yamawaki, H., Pan, S., Lee, R. T., & Berk, B. C. (2005). Fluid shear stress inhibits vascular inflammation by decreasing thioredoxin-interacting protein in endothelial cells. *J Clin Invest, 115*(3), 733-738. doi: 10.1172/jci23001

Yang, R. B., Mark, M. R., Gray, A., Huang, A., Xie, M. H., Zhang, M., . . . Godowski, P. J. (1998). Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature, 395*(6699), 284-288. doi: 10.1038/26239

Yang, X. O., Panopoulos, A. D., Nurieva, R., Chang, S. H., Wang, D., Watowich, S. S., & Dong, C. (2007). STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem, 282*(13), 9358-9363. doi: 10.1074/jbc.C600321200

Yang, X. P., Ghoreschi, K., Steward-Tharp, S. M., Rodriguez-Canales, J., Zhu, J., Grainger, J. R., . . . Laurence, A. (2011). Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. *Nature immunology, 12*(3), 247-254. doi: 10.1038/ni.1995

Ying, Q. L., Nichols, J., Chambers, I., & Smith, A. (2003). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell, 115*(3), 281-292.

Yoshioka, J., Chutkow, W. A., Lee, S., Kim, J. B., Yan, J., Tian, R., . . . Lee, R. T. (2012). Deletion of thioredoxin-interacting protein in mice impairs mitochondrial function but protects the myocardium from ischemia-reperfusion injury. *J Clin Invest, 122*(1), 267-279. doi: 10.1172/jci44927

Yoshioka, J., Imahashi, K., Gabel, S. A., Chutkow, W. A., Burds, A. A., Gannon, J., . . . Lee, R. T. (2007). Targeted deletion of thioredoxin-interacting protein regulates cardiac dysfunction in response to pressure overload. *Circ Res, 101*(12), 1328-1338. doi: 10.1161/circresaha.106.160515

Yu, H., Pardoll, D., & Jove, R. (2009). STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer, 9*(11), 798-809. doi: 10.1038/nrc2734

Yu, H., Yue, X., Zhao, Y., Li, X., Wu, L., Zhang, C., . . . Hu, W. (2014). LIF negatively regulates tumour-suppressor p53 through Stat3/ID1/MDM2 in colorectal cancers. *Nat Commun, 5*, 5218. doi: 10.1038/ncomms6218

Yue, P., Massie, B. M., Simpson, P. C., & Long, C. S. (1998). Cytokine expression increases in nonmyocytes from rats with postinfarction heart failure. *Am J Physiol, 275*(1 Pt 2), H250-258.

Yutzey, K. E., & Kirby, M. L. (2002). Wherefore heart thou? Embryonic origins of cardiogenic mesoderm. *Dev Dyn, 223*(3), 307-320. doi: 10.1002/dvdy.10068

Zheng, W., & Flavell, R. A. (1997). The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell, 89*(4), 587-596.

Zhou, R., Tardivel, A., Thorens, B., Choi, I., & Tschopp, J. (2010). Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nature immunology, 11*(2), 136-140. doi: 10.1038/ni.1831

Zhou, R., Yazdi, A. S., Menu, P., & Tschopp, J. (2011). A role for mitochondria in NLRP3 inflammasome activation. *Nature, 469*(7329), 221-225. doi: 10.1038/nature09663