Role of T-Box 3 in cardiomyocyte apoptosis

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Abstract

Background: T-box 3 (Tbx3) is a transcription factor that plays a key role in the embryonic development of multiple organs, such as mammary glands, limbs and heart. Although Tbx3 is selectively expressed in the cardiac conduction system in healthy hearts, our recent observations suggest that Tbx3 is also expressed in ventricular tissues in response to injury. This study aims to investigate if expression of Tbx3 in ventricular myocytes affects cell survival.

Methods and Results: Adenovirus was used to express Tbx3 in primary culture of neonatal rat ventricular myocytes (NRVMs). Flow cytometry was performed to quantify cells in early-phase apoptosis (i.e., Annexin V positive cells), which started to increase at 48 h after Ad-Tbx3 virus transduction (24.7% vs. 11.8% in control Ad-GFP), and at 72 h the apoptotic cells were increased to 59.1% (vs. 21.3% in Ad-GFP). To further investigate the effect of Tbx3 on apoptosis, TUNEL staining was used to detect DNA cleavage and fragmentation, an event of late-phase apoptosis. TUNEL-positive cells in the Ad-Tbx3 group were 2.5%, 15.0% and 24.9% at 24, 48, and 72 h, respectively, while the control Ad-GFP group had a very low level of TUNEL-positive cells (0.4%, 1.7% and 3.7%, respectively). The *P2x1* gene, encoding an ATP-gated cation channel, was identified by RNA sequencing as a top upregulated gene by Tbx3 in NRVMs. Flow cytometry demonstrated that Tbx3-induced apoptosis was further increased by a P2X1 agonist but was attenuated by a P2X1 antagonist. RNA sequencing revealed that several other apoptosis-related genes, such as *Fas*, were also updated in Tbx3-expressed NRVMs.

Summary: Our study demonstrated a pro-apoptotic role of Tbx3 in ventricular myocytes with P2X1 as one of the mediators.

Keywords: Cardiomyocyte, Tbx3, Apoptosis, P2X1

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List of Abbreviations

Tbx3	T-box transcription factor 3
DBD	DNA binding domain
NLS	Nuclear localization signal
UMS	Ulnar-mammary syndrome
CCS	Cardiac conduction system
AVB	Atrioventricular bundle
SAN	Sinoatrial node
NCDD	Nomenclature Committee on Cell Death
RCD	Regulated cell death
ACD	Accidental cell death
MI	Myocardial infarction
I/R	Ischemia/reperfusion
DISC	Death Inducing Signaling Complex
TNF	Tumor necrosis factor
Bad	Bcl-2 antagonist of cell death protein
Bax	Bcl-2-associated X protein
Atg5	Autophagy-related 5
TAC	Thoracic transverse aortic constriction
SR	Sarcoplasmic reticulum
LTCC	L-type Ca^{2+} channel
RyR	Ryanodine Receptors
CICR	Ca^{2+} induced Ca^{2+} release
NCX	Na^{+}-Ca^{2+} exchanger
SERCA	Sarcoplasmic reticulum Ca^{2+}-ATPase
NRVM	Neonatal rat ventricular myocytes
FBS	Fetal bovine serum
MOI	Multiplicity of infection
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
LCA	Left coronary artery

Introduction

1. Tbx3

The T-box (Tbx) transcription factor family is characterized by an evolutionarily conserved DNA binding domain (DBD) known as T-box (Papaioannou 2001). The T-box domain recognizes and binds to a palindromic sequence called T-element, which either activates or inhibits target gene transcription (Coll, Seidman et al. 2002). In 1927, mutations of a T-box gene locus were first reported to cause truncated tails in mice (Dobrovolskaia-Zavadskaia 1972). Subsequent studies demonstrated the critical roles of T-box genes in both embryonic development and adult biology (Xanthos, Kofron et al. 2001, Bruce, Howley et al. 2003).

The T-box 3 gene (Tbx3) belongs to the Tbx2 subfamily, and the human TBX3 gene is located on the chromosome, consisting of 7 exons (Bamshad, Lin et al. 1997, He, Wen et al. 1999, Carlson, Ota et al. 2001). Tbx3 protein is a 712~740 amino acid protein with a T-box domain, two repression domains, an activation domain, and a nuclear localization signal (NLS) (Carlson, Ota et al. 2001). In 2002, Coll *et al.* demonstrated that T-box domain in Tbx3 binds to the palindromic T-element (5'-TAATTTCACACCTAGGTGTGAAAT-3') or the core 10 bp half T-element (5'-TTTCACACCT-3') (Coll, Seidman et al. 2002). The Tbx3 protein can function as a transcriptional repressor and activator via its repression and activation domains in a context-specific manner (Boogerd, Wong et al. 2008, Lu, Yang et al. 2011) and depending on the presence of co-factors (Willmer, Peres et al. 2015).

Tbx3 is highly expressed in various carcinomas (a type of cancer of the skin tissue or the tissue covering internal organs) and sarcomas (a type of cancer of bone or soft tissue) (Willmer, Cooper et al. 2016, Dong, Dong et al. 2018, Wang 2018). As a tumor-promoting factor, Tbx3 increases tumor cell proliferation, inhibits its apoptosis, promotes tumor formation and migration (Feng, Yao et al. 2018, Krstic, Kolendowski et al. 2019). For example, Tbx3 is upregulated in uracil-induced rat urinary bladder papillomatosis, and inhibition of Tbx3 with antisense oligos induced apoptosis in rat bladder carcinoma cell line (Ito, Asamoto et al. 2005). T Willmer *et al.* found that Tbx3 is increased in chondrosarcoma, promoting tumor cell proliferation by directly inhibiting $p21^{WAF1}$ (Willmer, Hare et al. 2016). The $p21^{WAF1}$, $p16^{INK4a}$ and $p14^{ARF}$ are cell cycle repressors (Brugarolas, Chandrasekaran et al. 1995, Deng, Zhang et al. 1995, Lukas, Parry et al. 1995, Quelle, Zindy et al. 1995), and their transcriptions are inhibited by

Tbx3 either directly or indirectly to promote tumor formation (Lingbeek, Jacobs et al. 2002). In addition, M Rodriguez *et al.* and X Feng *et al.* demonstrated that Tbx3 downregulates the expression of E-cadherin in human melanoma cell lines and human hepatocellular carcinoma cells, which facilitates tumor cell migration and invasion (Rodriguez, Aladowicz et al. 2008, Feng, Yao et al. 2018).

Tbx3 also plays a critical role in embryonic development. In 1997, Bamshad M *et al.* reported that haploinsufficiency of TBX3 is associated with human ulnar-mammary syndrome (UMS), which is characterized by congenital limb malformations, apocrine/mammary gland hypoplasia, and dental and genital anomalies (Bamshad, Lin et al. 1997). Tbx3 is the first gene in the T-Box family found to be associated with abnormal yolk sac: homozygous mutation in Tbx3 results in aberrant yolk sac and embryonic lethality (Davenport, Jerome-Majewska et al. 2003). In addition, Tbx3 is important for heart development. Hoogaars WM *et al.* demonstrated that the expression of Tbx3 is required for the formation of the cardiac conduction system (CCS) (Hoogaars, Tessari et al. 2004). Tbx3 regulates CCS formation by controlling cell division and repressing cell differentiation into chamber myocardium (Christoffels, Hoogaars et al. 2004, Washkowitz, Gavrilov et al. 2012). In the adult heart, Tbx3 is expressed in the sinoatrial node (SAN) and atrioventricular node (AVN) but not in the atrial/ventricular myocardium (Moorman, Soufan et al. 2004). The differential gene expressions between SAN/AVN tissues and atrial/ventricular myocardium are partly due to Tbx3, which represses the expression of atrial/ventricular-specific genes in SAN/AVN, such as *Cx43 (Gja1)*, *Smpx*, *Nppa* and *Cx40 (Gja5)* (Hoogaars, Engel et al. 2007). Bakker ML *et al.* found that ectopic expression of Tbx3 in chamber (atrial/ventricular) myocardium decreased chamber-specific genes and converted cardiomyocytes into pacemaker-like cells (Bakker, Boink et al. 2012). Moreover, they showed that tamoxifen-induced ectopic expression of Tbx3 in chamber cardiomyocytes in adult mice led to the development of heart failure and mice died within 10 days after tamoxifen treatment. In contrast to the pro-proliferative role of Tbx3 in the highly proliferative cancer cells, Tbx3 may play an anti-survival role in the terminally-differentiated, non-proliferative cardiomyocytes.

2. Cell death

Cell death is a critical physiological process in the multicellular organism, which maintains the balance between “old” and “new” cells. In physiological conditions, cell

death removes unnecessary cells or tissue. For instance, programmed cell death removes tissue between fingers and toes during embryonic development (Zakeri and Ahuja 1997). However, dysregulation of cell death results in a variety of diseases. Failure of cell death and accumulation of damaged cells in multicellular organisms may lead to cancer and give cancer cells the ability to resist chemotherapeutic drugs (Hanahan and Weinberg 2011, Alfarouk, Stock et al. 2015), while excessive cell death induces neurodegenerative disease (Bredesen, Rao et al. 2006). Extensive efforts have been made to understand the mechanisms behind different types of cell death with the hope of developing new therapeutic strategies for human diseases. Programmed cell death was first described in the 1960s (Kerr 1965, Lockshin and Williams 1965). Cell death was traditionally classified as (1) Type I cell death - apoptosis. Apoptosis is a highly regulated process in which cells exhibit cell shrinkage, membrane blebbing, nuclear condensation, and later apoptotic body formation. The inflammatory response is not induced as apoptotic bodies can be removed by neighboring cells or phagocytes (D'Arcy 2019). (2) Type II cell death - autophagy. Cell contents that need to be degraded are surrounded by double-membrane vesicles, known as autophagosomes, and then transport to lysosomes where proteases are present. Some cellular organelles, such as mitochondria, directly fuse with lysosomes (Levine and Kroemer 2008). (3) Type III cell death - necrosis. Necrosis is associated with cell swelling and membrane rupture followed by spilling of cell contents into the extracellular space (Kung, Konstantinidis et al. 2011), inducing inflammatory responses (Whelan, Kaplinskiy et al. 2010). More recently, a large body of scientific evidence suggested that cell death has more complex forms, and the classification based on cell morphology has limitations. Accordingly, the Nomenclature Committee on Cell Death (NCDD) has proposed to define cell death in terms of morphology, biochemical basis and functions (Galluzzi, Bravo-San Pedro et al. 2015, Galluzzi, Vitale et al. 2018). Apoptosis, autophagy, regulated necrosis/necroptosis, ferroptosis, and pyroptosis, which are controlled by a series of molecular machineries and can be intervened by drugs or genetic modifications, are defined as regulated cell death (RCD). Programmed cell death, a type of RCD, specifically refers to the physiological process in embryonic development and mature tissues. In contrast, accidental cell death (ACD) refers to death happening under extreme conditions, such as high temperatures/pressure, extreme pH changes, or shear force, and cannot be intervened (Galluzzi, Vitale et al. 2012).

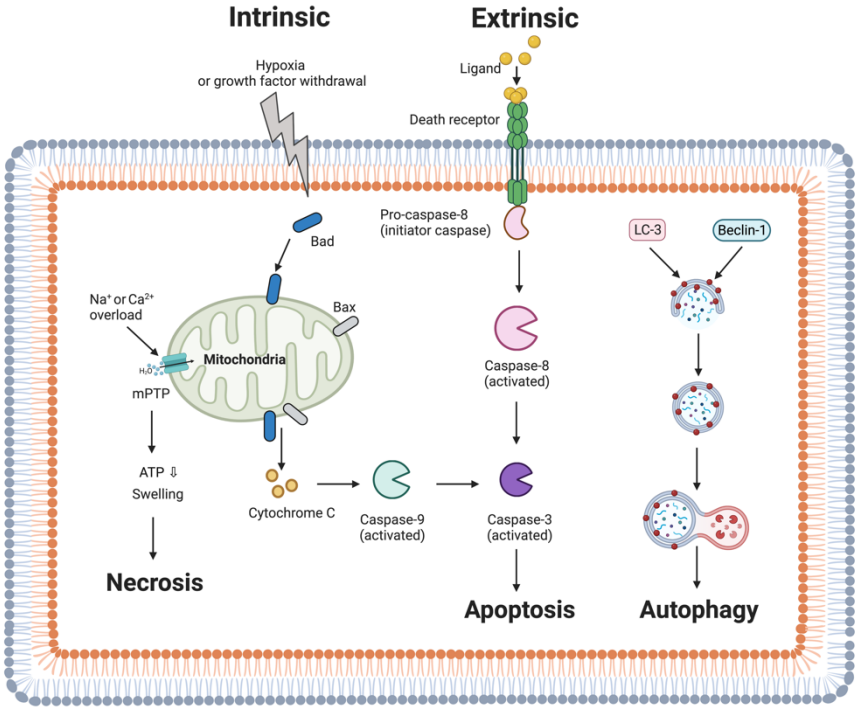

Figure 1. Cell Death Pathways. Apoptosis: With the binding of ligands, Fas or TNF receptors activate caspase-8, and cleaved caspase-8 activates the effector caspase-3. In the intrinsic pathway, mitochondrial injury leads to the insertion of Bad and Bax on the mitochondrial outer membrane and subsequent cytochrome c release. Cytochrome c activates caspase-9. Then, activated caspase-9 activates caspase-3. Necrosis: Na^+ and Ca^{2+} overload leads to the opening of the mitochondrial permeability transition pore (mPTP). The consequences of mPTP are ATP synthesis cessation and mitochondria swelling. Autophagy: Autophagosome formation is the key event regulated by Beclin 1 and Light Chain 3 (LC3). (Figure created with BioRender.com with permission)

2.1 Cell death and heart diseases

Cell death plays a critical role in the pathogenesis of myocardial infarction (MI) and the development of heart failure. MI occurs when a major coronary artery branch is blocked (Foo, Mani et al. 2005). When coronary flow is reduced or stopped, the myocardium forms the ischemic zone, where cardiomyocytes are deprived of oxygen, nutrients, and survival factors, causing myocardial apoptosis and necrosis (Del Re, Amgalan et al. 2019). Although both apoptosis and necrosis are involved in ischemic injuries, studies have shown that apoptotic cardiomyocyte death predominates in the early stage of MI in both human patients (Saraste, Pulkki et al. 1997, Veinot, Gattinger

et al. 1997) and animal studies (Palojoki, Saraste et al. 2001). Since the adult mammalian heart lacks a significant endogenous regenerative mechanism, excessive loss of cardiomyocytes causes increased load on the non-infarcted myocardium leading to ventricular hypertrophy (Bhatt, Ambrosy et al. 2017). The late-stage cardiac remodeling after MI causes chamber dilation and wall thinning (Pfeffer and Braunwald 1990).

Increased myocyte apoptosis has been reported in the myocardium of patients with MI, arrhythmogenic right ventricular cardiomyopathy and congestive heart failure (Mallat, Tedgui et al. 1996, Olivetti, Abbi et al. 1997). Ischemia/reperfusion (I/R), mechanical stretching and doxorubicin treatment also induce apoptosis in cardiomyocytes (Cheng, Li et al. 1995, Narula, Haider et al. 1996, Kumar, Kirshenbaum et al. 1999). Under these cardiac conditions, apoptosis is mediated by two different pathways (Kang and Izumo 2003, Whelan, Kaplinskiy et al. 2010) (Fig.1A): extrinsic pathway involves extracellular ligands and their cognate surface receptors, e.g., TNF-α/TNF receptor or Fas Ligand/Fas receptor. The binding of ligands to their receptors leads to the formation of Death Inducing Signaling Complex (DISC), and subsequent activation of initiator caspase (caspase-8). Caspase-8 activates the effector caspase-3/7 (Marin-Garcia and Akhmedov 2016, Moe and Marin-Garcia 2016), which initiates key apoptotic events, such as phosphatidylserine exposure on the surface of the bilayer lipid plasma membrane and DNA cleavage in the nucleus. The intrinsic pathway involves damaged mitochondria within cells. Cytotoxic stimuli, such as hypoxia, heat, and radiation, cause mitochondrial injury leading to cytochrome c release from the mitochondria, which induces apoptosis (Konstantinidis, Whelan et al. 2012). For example, cytoplasmic Ca^{2+} overload activates Bad (a pro-cell death protein of the Bcl-2 family) and Bax (Bcl-2-associated X protein) via Ca^{2+}/calmodulin-dependent calcineurin, and the subsequent insertion of Bad and Bax on mitochondrial outer membrane that forms pores for cytochrome c release into the cytosol (Takemura and Fujiwara 2004).

Necrosis is also controlled by a series of signal pathways, including extrinsic and intrinsic pathways. Unlike apoptosis, necrosis evokes the inflammatory response and induces fibrosis in the heart (Whelan, Kaplinskiy et al. 2010). The features of necrosis are cell membrane ruptures and mitochondria swelling. In cardiomyocytes, diverse stimuli, such as ischemia and ischemia/reperfusion, trigger necrotic cell death. Opening of the mitochondrial permeability transition pore (mPTP) is a major event in

necrosis (Nakagawa, Shimizu et al. 2005) (Fig.1B). During ischemia, deprivation of oxygen induces anaerobic metabolism and H^+ accumulation (acidosis) in cardiomyocytes (Cohen, Yang et al. 2007). In order to remove excess H^+, Na^+-H^+ exchanger (NHE) is stimulated, which results in redundant Na^+ influx (Murphy, Perlman et al. 1991). In response, Na^+ overload in ischemic cardiomyocytes stimulates Na^+-Ca^{2+} exchanger (NCX) and leads to intracellular Ca^{2+} overload (Griffiths, Ocampo et al. 1998). Excess cytosolic Ca^{2+} is transported into mitochondria and Ca^{2+} overload in mitochondrial matrix promotes opening of mPTP (Nakagawa, Shimizu et al. 2005). The consequences of mPTP are ATP synthesis cessation and mitochondria swelling (Briston, Roberts et al. 2017). On the other hand, it is now recognized that a new type of necrotic cell death, termed necroptosis, can be initiated by the death receptors, such as Fas, but with caspase inhibition (Vercammen, Brouckaert et al. 1998, Degterev, Huang et al. 2005, Hitomi, Christofferson et al. 2008, Shan, Pan et al. 2018). The necroptotic and apoptotic pathways share some of the upstream components, and the downstream signaling for necroptosis involves the RIP1-RIP3-MLKL pathway, as well as other less-characterized pathways. In human end-stage heart failure, the protein levels of RIP1, pSer227-RIP3 and pThr357- MLKL are increased, indicating activation of the necroptotic pathways (Szobi, Goncalvesova et al. 2017, Corsetti, Chen-Scarabelli et al. 2019). Inhibition of RIP1 by Necrostatin-1 attenuated adverse cardiac remodeling in the ischemia-reperfusion animal model (Oerlemans, Liu et al. 2012). Thus, necroptosis is an emerging mechanism of cell death in heart disease, but the role of necroptosis in MI is not clear.

Cardiomyocytes are terminally differentiated cells and lack the ability to divide (Kikuchi and Poss 2012), and many therapeutic efforts for heart disease patients aimed to reduce cardiomyocyte death. However, many recent studies targeting cell death pathways have been unsuccessful in the clinic. For example, the strategy of neutralizing circulating TNF-α with a recombinant soluble TNF receptor failed to improve the heart function of patients and even aggravated heart failure, although it showed promise in animal studies and small pilot clinical trials (Kadokami, Frye et al. 2001, Balakumar and Singh 2006). This may reflect an incomplete understanding of the mechanisms of cardiomyocyte death in heart disease. Therefore, it is critical to further investigate the mechanism of cardiomyocyte deaths in heart disease, which would guide the designing of effective therapeutic strategies for patients.

2.2 Other types of regulated cell deaths in heart diseases

Autophagy promotes cell survival during cellular starvation. Degradation of lipids and proteins within lysosomes can provide materials used for ATP generation and new protein synthesis (Levine and Yuan 2005). It also plays an important role in recycling cellular components, creating new cellular structures and organelles and removing senescent cells from aged tissues (Mizushima, Levine et al. 2008). In the heart, autophagy activation is found in ischemia either with or without reperfusion and in heart failure (Stutz, Kolbe et al. 2017). Beclin 1 is one of the critical factors for early autophagosome formation (Fig.1C), and single Beclin 1 allele knockout attenuated the cardiac dysfunction after pressure overload, while Beclin 1 overexpression aggravated cardiac dysfunction (Zhu, Tannous et al. 2007). This suggests that autophagy is detrimental in heart disease. However, cardiac-specific knockout of autophagy-related 5 (Atg5), which is critical for autophagosome formation, induced left ventricular dilatation, systolic dysfunction and heart failure, and was accompanied by increased cardiomyocyte apoptosis (indicated by TUNEL assay). In addition, thoracic transverse aortic constriction (TAC)-induced pressure overload in Atg5 knockout mice resulted in greater cardiac dysfunction and left ventricular dilatation than in control wild-type mice within 1 week (Nakai, Yamaguchi et al. 2007). These observations suggest that autophagy may play either a detrimental or protective role in cardiac remodeling after injury and further investigations are needed to better understand its role in different cardiac diseases.

Ferroptosis is a form of regulated cell death mediated by iron-dependent lipid peroxidation (Dixon, Lemberg et al. 2012). Fe^{3+} induces reactive oxygen species (ROS) generation and oxidative damage to cellular membranes, and iron chelator or anti-lipid peroxidation can protect cells from ferroptosis. The morphological characteristics of ferroptosis include mitochondrial shrinkage and cristae disappearing, which are distinct from apoptosis and necrosis (Dixon, Lemberg et al. 2012). Baba *et al.* found iron overload in both cardiomyocytes and non-cardiomyocytes in the I/R animal model (Baba, Higa et al. 2018). In the primary culture of adult mouse cardiomyocytes, Fe^{3+} or ferroptosis activators induced ferroptosis, while antioxidant ferrostatin-1 rescued cells (Baba, Higa et al. 2018). *Fang et al.* demonstrated that ferrostatin-1 or the iron chelator reduced scar size and serum cardiac biomarkers during ischemia/reperfusion (I/R) *in vivo* (Fang, Wang et al. 2019). Another study also indicated that ferrostatin-1

decreases cardiomyocyte death in heart transplantation (Li, Feng et al. 2019). These findings suggest that ferroptosis plays a role in cardiomyocyte death in heart diseases.

Pyroptosis is a form of regulated cell death that is also involved in heart diseases. It is closely associated with immune response, characterized by pore formation on the plasma membrane and the release of inflammatory cytokines (D'Souza and Heitman 2001). Pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) promote inflammasome assembly and the procaspase-1 activation. Caspase-1 (activated procaspase-1) cleaves gasdermin D (GSDMD), which forms pores on the plasma membrane (He, Wan et al. 2015, Shi, Zhao et al. 2015). During ischemia either with or without reperfusion, inflammasome components, activated caspase-1 and pro-inflammatory cytokines were increased in both non-cardiomyocytes and cardiomyocytes, and the inhibition of inflammasome function was reported to be cardioprotective (Kawaguchi, Takahashi et al. 2011, Mezzaroma, Toldo et al. 2011). On the other hand, caspase-1 plays a role in the crosstalk between pyroptosis and apoptosis in ischemic heart disease. Syed *et al.* investigated I/R injury in mice with cardiomyocyte-specific overexpression of procaspase-1, and found that the increased caspase-1 led to caspase-3 activation, which is a pro-apoptotic factor (Syed, Hahn et al. 2005). Another study showed that caspase-1 overexpression induces cardiomyocyte apoptosis and cardiac failure in transgenic mice (Merkle, Frantz et al. 2007). These studies suggest that proptosis play a role in ischemic heart disease.

3. Ca^{2+} homeostasis in heart

The contraction of cardiomyocytes is triggered by the increases in cytosolic, typically to ~100μmolL^{-1}, primarily due to Ca^{2+} release from intercellular sarcoplasmic reticulum (SR) (Fabiato and Fabiato 1975). During systole, the opening of L-type calcium channels (LTCC) allows Ca^{2+} influx that increases the Ca^{2+} level near the Ryanodine Receptors (RyR) on SR (Bassani, Yuan et al. 1995). Activation of RyR causes the release of Ca^{2+} stored inside the SR to the cytosol. This mechanism is called Ca^{2+} induced Ca^{2+} release (CICR). Increased cytosolic Ca^{2+} induces cardiomyocyte contraction by activating the cross-bridge cycling of the thick and thin myofilaments. During diastole, ~80% of the cytosolic Ca^{2+} is removed and transported to SR via sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) (Bhogal and Colyer 1998), while the

rest of cytosolic Ca^{2+} is transported to the extracellular space by Na^{+}-Ca^{2+} exchanger (NCX) located on the sarcolemma (Trafford, Diaz et al. 1997, Trafford, Diaz et al. 2001). Thus, Ca^{2+} homeostasis is tightly controlled by ion channels and transporters in cardiomyocytes.

Dysregulation of the SR calcium cycling can lead to heart disease. Studies showed that reduced SERCA2a function impaired cardiomyocyte contractility and relaxation. For example, the SERCA2a knockout mice had slower cytosolic Ca^{2+} removal during diastole and reduced SR Ca^{2+} content, and these transgenic mice developed end-stage heart failure (Andersson, Birkeland et al. 2009, Louch, Hougen et al. 2010, Li, Louch et al. 2012). On the other hand, overexpression of SERCA2a restored intracellular Ca^{2+} handling and improved cardiac function in mice with heart failure (He, Giordano et al. 1997, Muller, Lange et al. 2003, Chen, Escoubet et al. 2004). Clinical trials suggested that adenoviral overexpression of SERCA2a had beneficial effects on heart failure patients with improved cardiac contractility and calcium handling (Jaski, Jessup et al. 2009).

4. P2X1

P2 receptors are a group of purinergic receptors, which are found in all human organ systems and participate in various physiological activities (Burnstock and Knight 2004, Burnstock 2018). The P2 receptors are grouped into two families: P2X receptors which are ligand-gated ion channels, and P2Y receptors which are G-protein-coupled receptors (Abbracchio and Burnstock 1994). P2X1, a member of the P2X purinergic receptor-cation channel family, is an ATP-activated ion channel with high Ca^{2+} permeability (Burnstock 2017). Micromolar nucleotides are able to activate P2X1, resulting in Ca^{2+} influx (Valera, Hussy et al. 1994). Studies have revealed unique features of P2X1 as compared to other P2X receptors. For example, P2X1 possesses higher fractional calcium currents and calcium permeability among other ligand-gated ion channels (Egan and Khakh 2004). P2X1 receptors can be quickly internalized (movement from plasma membrane to cytoplasm), and return back to the plasma membrane, which regulates their responsiveness to repeated ATP stimulation (Dutton, Poronnik et al. 2000, Ennion and Evans 2001, Lalo, Allsopp et al. 2010).

P2X1 plays a role in cardiovascular physiology and disease. Previous studies demonstrated that P2X1 is mainly located on arterial smooth muscle (Vulchanova, Arvidsson et al. 1996, Glass, Townsend-Nicholson et al. 2000), and knockout of P2X1

induces a slight increase in blood pressure in rats (Gonzalez-Montelongo and Fountain 2021). Immunohistochemistry showed a low expression level of P2X1 in cardiomyocytes, primarily located in the intercalated discs (Jiang, Bardini et al. 2005). However, P2X1 is upregulated in heart diseases. *Hou et al.* demonstrated that a 2.7-fold increase in mRNA of P2X1 was found in rat heart failure (Hou, Malmsjo et al. 1999). In human failing hearts, western blot showed the protein level of P2X1 was increased in the atria (Berry, Barden et al. 1999), and the authors hypothesized that upregulation of P2X1 expression is a potential mechanism for increased cell death observed in dilated cardiomyopathy patients due to Ca^{2+} cytotoxicity. Cardiac injury is associated with increased ATP release (Vassort 2001), which may provide an endogenous agonist for P2X receptors. For example, stretching of rat atrial myocytes leads to ATP lease, which acts on P2X7 stimulating cardiomyocyte apoptosis (Pustovit, Kuzmin et al. 2016). Since P2X1 is sensitive to ATP and has a high permeability to Ca^{2+}, it is likely that increased P2X1 in cardiomyocytes induces intercellular Ca^{2+} overload and cell death after cardiac injuries. In the immune system, ATP-binding P2X1 activation has been demonstrated to mediate thymocyte apoptosis (Chvatchko, Valera et al. 1996). Future studies are needed to investigate the mechanisms for increased P2X1 expression in failing hearts and how it contributes to the pathogenesis of heart diseases.

Objectives and Hypothesis

Although Tbx3 expression is restricted to the cardiac conduction system in healthy adult hearts, our recent studies suggest that its expression is induced in ventricular myocytes in response to cardiac injuries. The objective of this project is to investigate the effects of Tbx3 on cardiomyocyte survival and the underlying mechanisms. We will test the hypothesis that Tbx3 induces apoptosis in ventricular myocytes.

Methods

1. Primary culture of neonatal rat ventricular myocytes

The lower one-third part of the cardiac ventricles of 2-day-old neonatal rats (Sprague-Dawley, Harlan, Charles River, Montreal) was collected, cut into small strips, and digested in Hanks' Balanced Salt Solution (HBSS, calcium-free, ThermoFisher, 14170112) containing 0.25% trypsin (ThermoFisher, Catalogue No.:15090046) overnight. The next day, ventricular tissues were digested with collagenase (type II, Worthington Biochemical, Catalogue No.: LS004177) at 1 mg/ml in HBSS. Isolated cells were incubated in M-199 medium (Life Technologies, Catalogue No.: 11150067) supplemented with 10% fetal bovine serum (FBS), 19.4 mM glucose, 2 mM-glutamine, 2 unit/mL penicillin, 0.8 µg/mL vitamin B12, 10 mM HEPES, and 1x MEM nonessential amino acids. Cells were pre-plated twice for 1-hour to remove non-cardiomyocytes, and then plated at 2 million per well in 6-well plates that were pre-coated with 0.1% gelatin (StemCell Technologies, Catalogue No.: 07903).

2. Adenoviral gene transfer in NRVMs

Isolated NRVMs were transduced with adenovirus that expresses mouse *Tbx3* and GFP (Ad- Tbx3, Vector Biolabs) or control adenovirus that only expresses GFP (Ad-GFP, Vector Biolabs) at an MOI (multiplicity of infection) of 1-5. At 24 h after virus treatment, ~95% of NRVMs were GFP-positive suggesting successful transduction. Cells were collected at 24, 48, and 72 h after transduction, and used for flow cytometry and TUNEL assay as described below.

3. RNA isolation and Real-time Quantitative PCR

Total RNA was isolated from NRVMs using RNeasy mini kit (Qiagen, Catalogue No.: 74104) and genomic DNA was removed by on-column digestion with RNase-Free DNase (Qiagen, Catalogue No.: 79254) according to the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, Catalogue No.: 4368814). Real-time qPCR was performed with iTaq Universal SYBR green Kit (Bio-Rad) on a CFX Connect Real-Time PCR Detection System (BioRad). The relative transcript levels of the tested genes were analyzed with the $2^{-\Delta\Delta C(t)}$ method.

4. Western Blot

NRVMs cultured in 6-well plates were washed with ice-cold PBS and then collected in RIPA buffer (ThermoFisher, Catalogue No.: 89900) that was supplemented with Halt Protease Inhibitor Cocktail (ThermoFisher, Catalogue No.: 78430). The protein concentration of the NRVM samples was determined using Pierce Rapid Gold BCA Protein Assay Kit (ThermoFisher, Catalogue No.: A53227). Equal amounts of proteins were run on 4%-12% SDS-polyacrylamide and transferred to a PVDF membrane. The transferred membrane was incubated with primary antibodies overnight at 4°C and followed by a 2-hour incubation with a peroxidase-conjugated secondary antibody (Cell Signaling, Catalogue No.: 7074, 1:2000). Immunoreactivity was detected by chemiluminescence (ECL Western Blotting analysis system, Amersham Biosciences). Primary antibodies include anti-Tbx3 antibody (Abcam, Catalogue No.: ab99302, 1:1000), anti-GAPDH (Cell Signaling Technology, Catalogue No.: 3683s, 1:10000), and anti-P2X1(Alomone Labs, Catalogue No.: APR-001, 1:1000).

5. TUNEL assay

NRVMs cultured on 8-chamber culture slides were used for TUNEL assay (In Situ Cell Death Detection Kit, Fluorescein and TMR red, Roche, Catalogue No.:11684795910 and 12156792910). Cells were fixed with freshly made 4% paraformaldehyde in PBS for 30 min, and permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate for 2 min. The TUNEL reaction mixture was added to the cells and incubated for 1 h at 37°C. Apoptotic cells were identified by TUNEL assay with the help of DAPI counterstaining of cell nuclei. The images of cells were obtained using a Leica DMi8 fluorescent microscope at 200x magnification, and the number of $TUNEL^+$ cells was analyzed by ImageJ.

6. Immunofluorescence

NRVMs were fixed with fresh 4% paraformaldehyde for 10 min at room temperature, and then blocked in 0.5% Triton X-100/1% BSA/10% goat serum/PBS at room temperature for 30 min and followed by incubation with primary antibody diluted in 0.5% Triton X-100/1% BSA/3% goat serum/PBS for overnight. Cells were washed with 1% BSA/PBS and incubated with secondary antibodies at room temperature for 1 h. Cells were then mounted with ProLong gold antifade reagent containing DAPI (Life

Technologies, Catalogue No.: P36941). Primary antibodies used include goat anti-Tbx3 (Santa Cruz, Catalogue No.: sc-166623, 1:50), mouse anti-α-sarcomeric activin (α-SA, Sigma, Catalogue No.: A7811, 1:100), goat anti-cardiac troponin C (Abcam, Catalogue No.: ab30807, 1:100). Secondary antibodies include anti-mouse IgG (Alexa Fluro-568, 1:300), anti-goat IgG (Alexa Fluro-647, 1:300).

7. Flow cytometry

At 24, 48 or 72 h after transduction with Ad-Tbx3 or Ad-GFP, NRVMs were lifted with trypsin (TrypLE, Gibco, Catalogue No.:12604021) and dispersed into single cells. Annexin V conjugated to Phycoerythrin (PE) (PE Annexin V Apoptosis Detection Kit I, BD, Catalogue No.: 559763) was used to identify apoptotic cells at an earlier stage, and 7-Amino-Actinomycin (7-AAD), which is permeable in cells with damaged plasma membrane, was used to detect cells with necrosis or late apoptosis. Cells were resuspended in 1x binding buffer at a concentration of 1 x 10^6 cells/ml, and then 100 µl of the cell solution was transferred to a 1.5 ml culture tube. After adding 5 µl of PE-Annexin V and 5 µl 7-AAD, the cell solution was incubated for 15 min at RT (25°C) in the dark, and then analyzed by FACS Flow Cytometer (BD FACS Aria III) within 1 h.

8. Statistics

Statistical analyses were performed using SPSS software (version 23; IBM, Armonk, NY, USA). Data were presented as the mean ± standard deviation (SD) with $p<0.05$ considered significant. Differences between two groups were analyzed with the two-tailed *t*-test. Differences between three or more groups were analyzed by one-way ANOVA and, if significant differences were detected, followed by Bonferroni *post-hoc* comparisons.

Results

1. Adenoviral expression of Tbx3 in neonatal rat ventricular myocytes (NRVMs)

Primary culture of neonatal rat ventricular myocytes (NRVMs) was chosen as the cell model in this project because of their ease of isolation and maintenance in cell culture, making them an excellent system for mechanistic studies of cardiomyocyte biology (Kapoor, Liang et al. 2013, Liang, Cho et al. 2015, Lu, Kamkar et al. 2020). Recombinant adenovirus confers high-efficient gene transfer in NRVMs and was chosen for *Tbx3* gene transfer in this project. Adenovirus expressing the mouse *Tbx3* and GFP under two independent CMV promoters (Ad-Tbx3) or control adenovirus expressing GFP only under one CMV promoter (Ad-GFP) was used to transduce NRVMs. At 48 h after viral transduction, immunocytostaining with anti-Tbx3 and anti-cTnC (cardiac troponin C) antibodies demonstrated that Tbx3 was not expressed in control Ad-GFP-transduced NRVMs, but is robustly expressed in Ad-Tbx3-transduced NRVMs (Fig. 2). The Tbx3 fluorescence in Ad-Tbx3 group overlapped with DAPI, suggesting localization of Tbx3 protein at the nuclei consistent with its role as a transcription factor (Fig. 2).

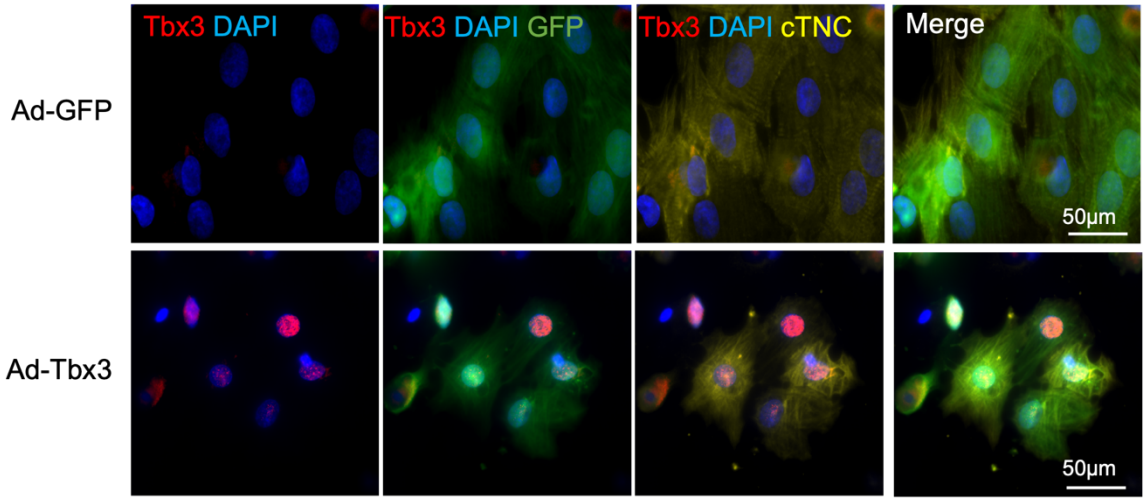

Figure 2. Adenoviral expression of Tbx3 in neonatal rat ventricular myocytes (NRVMs). Primary culture of neonatal rat ventricular myocytes (NRVMs) was transduced with adenovirus expressing Tbx3 and GFP (Ad-Tbx3 group) or control adenovirus expressing GFP only (Ad-GFP group). Cells were fixed with 4% paraformaldehyde at 48h after transduction, and stained with anti-Tbx3 (red), anti-cTnC (cardiomyocyte marker, yellow) and DAPI (blue).

2. Expression of Tbx3 induces apoptosis in neonatal rat ventricular myocytes (NRVMs)

Because Tbx3 is expressed in the sinoatrial node (the primary pacemaker of the heart) but not in healthy ventricular myocytes, our lab has been interested to see if artificial expression of Tbx3 in ventricular myocytes will confer a pacemaker-like phenotype. In our previous studies, we observed the suppression of ventricular genes, such as *Scn5a*/$Na_v1.5$ (Lu, Kamkar et al. 2020) in NRVMs at 48 h after Ad-Tbx3 transduction. We also noticed that, to our surprise, many NRVMs had rounded up and became detached by day 3-4 after Ad-Tbx3 transduction, suggesting that they may have undergone cell death. In contrast, the control Ad-GFP-transduced NRVMs maintained a normal morphology. As this phenomenon was consistently observed in multiple independent experiments, it is important to investigate how Tbx3 expression leads to cell death in NRVMs. In this project, we measured cell apoptosis in Tbx3-expressing NRVMs with two approaches.

We first performed flow cytometry to quantify cells in early phase apoptosis (Godard, Deslandes et al. 1999), which showed that the percentages of apoptotic cells (defined as Annexin V^+/7-AAD^- cells) in the Ad-Tbx3 group increased to 24.7% (vs. 11.8% in Ad-GFP) at 48 h and to 59.1% (vs. 21.3% in Ad-GFP) at 72 h (Fig. 3A). The relatively high level of Annexin V^+/7-AAD^- cells in the Ad-GFP group at 48 and 72 h may be due to damages to the cells during the chemical and physical detachment of the cells from culture plates.

The increased apoptosis was also confirmed by TUNEL staining, which detects apoptotic DNA fragmentation. $TUNEL^+$ cells in Ad-Tbx3 started to increase at 24 h, and further increased to 15% at 48h and 24.9% at 72 h (Fig. 3B), while the percentage of $TUNEL^+$ cells in Ad-GFP was very low (0.4%, 1.7% and 3.7%). These results suggest that forced expression of Tbx3 in healthy ventricular myocytes promotes cell apoptosis.

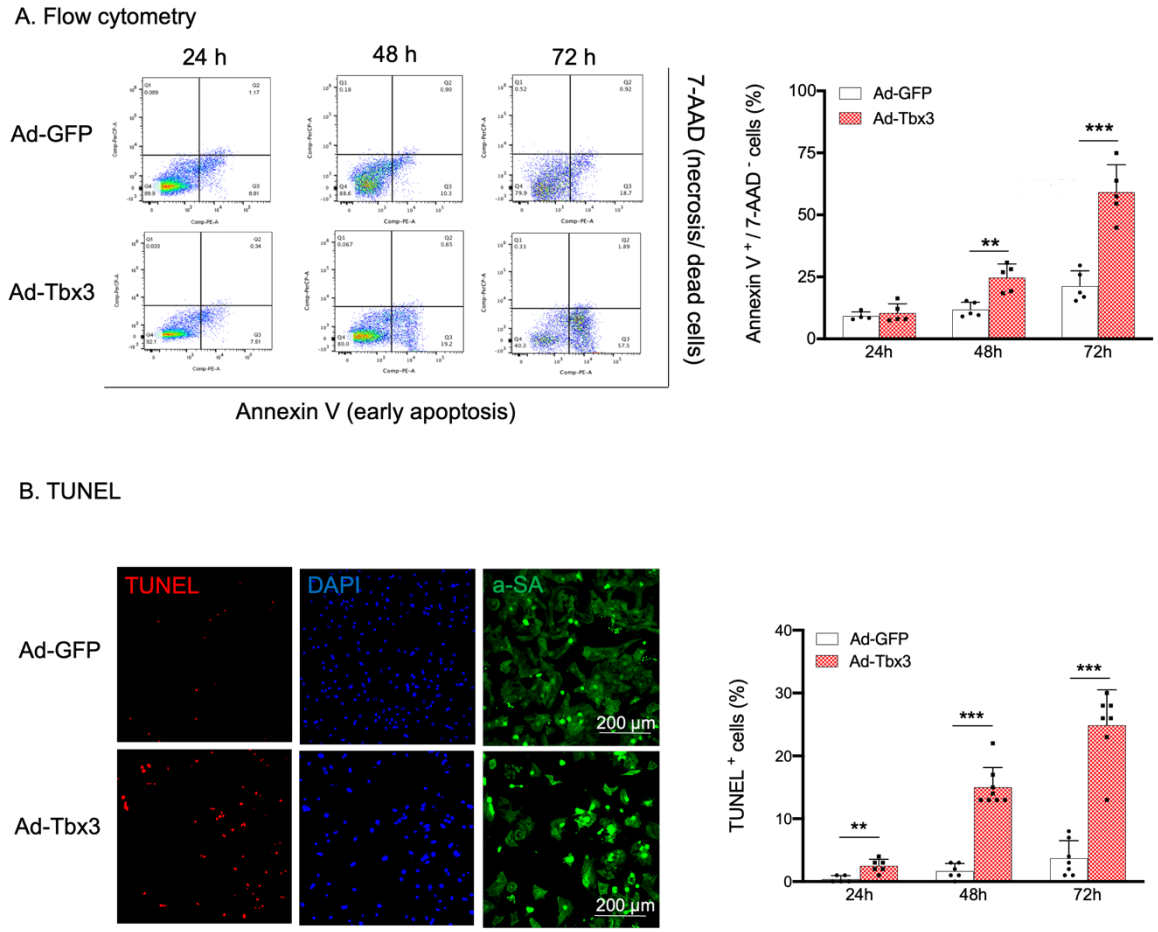

Figure 3. Adenoviral expression of Tbx3 induces apoptosis in neonatal rat ventricular myocytes (NRVMs). (A) Cells treated with staurosporine (0.5 µM, 24 h) were the positive control (gating control). **p<0.01, ***p<0.001, n=5 per group. Data are presented as the mean ± standard deviation of two individual experiments. **(B)** Cells were fixed with 4% paraformaldehyde at 72h after transduction with Ad-Tbx3 or Ad-GFP, and stained with TUNEL (red), anti-alpha-sarcomeric actinin (alpha-SA, cardiomyocyte marker, green) and DAPI (blue) (imaging data at 24 h and 48 h are not shown). The bar graph (right panel) summarizes the percentage of apoptotic cells. **p<0.001, ***p<0.0001. Data are presented as the mean ± standard deviation of two individual experiments.

3. Tbx3-induced apoptosis is likely mediated by upregulation of P2X1, an ATP-gated Ca^{2+} channel.

To investigate the underlying mechanism of Tbx3-induced myocyte apoptosis, our lab has performed RNA sequencing studies. RNA sequencing of NRVMs at 48 h after Ad-Tbx3 transduction suggested that Tbx3 overexpression decreases transcripts of multiple calcium handling genes, such as *Atp2a2* (encoding SERCA2a, the calcium pump responsible for transporting Ca^{2+} back into sarcoplasmic reticulum) and *Slc8a1* (encoding the Na^{+}/Ca^{2+} exchanger responsible for transporting Ca^{2+} to the outside of the cells) (Fig. 4A). This suggests that Tbx3-expressing NRVMs likely have impaired handling of Ca^{2+} during diastole. In addition, we also found that Tbx3 increased the mRNA level of *P2X1* among the P2X family (Fig. 4B).

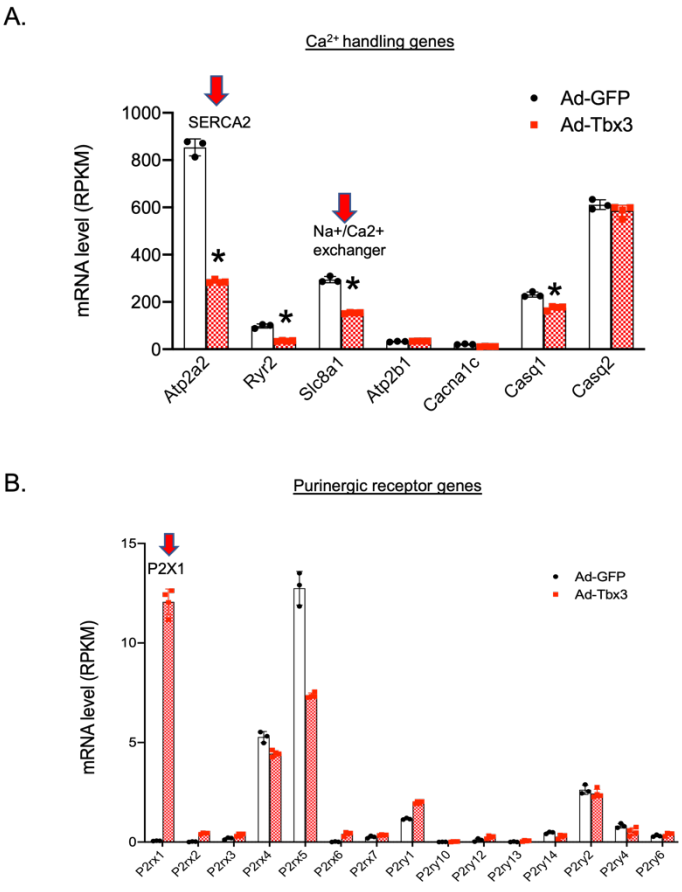

Figure 4. Tbx3 induces upregulation of P2X1 (Ca^{2+} channel) and downregulation of Ca^{2+} handling protein genes in cardiomyocytes. (A) and **(B)** NRVMs were collected at 48 h after transduction with Ad-Tbx3 and Ad-GFP and analyzed by RNA sequencing (n=4). Data are presented as the mean ± standard deviation.

The upregulation of P2X1 protein in the Ad-Tbx3 group was confirmed by western blot (Fig. 5A). Flow cytometry showed that ATP treatment (an agonist of P2X receptors) further increased the apoptosis in the Ad-Tbx3 group, but had no effects in Ad-GFP cells (Fig. 5B). In addition, treatment of NRVMs with Evans Blue (an antagonist of P2X receptor) attenuated Ad-Tbx3-induced apoptosis, but had no effects in Ad-GFP cells (Fig. 5C). These observations suggest that the P2X1 receptor is likely one of the mediators for Tbx3-induced apoptosis in NRVMs.

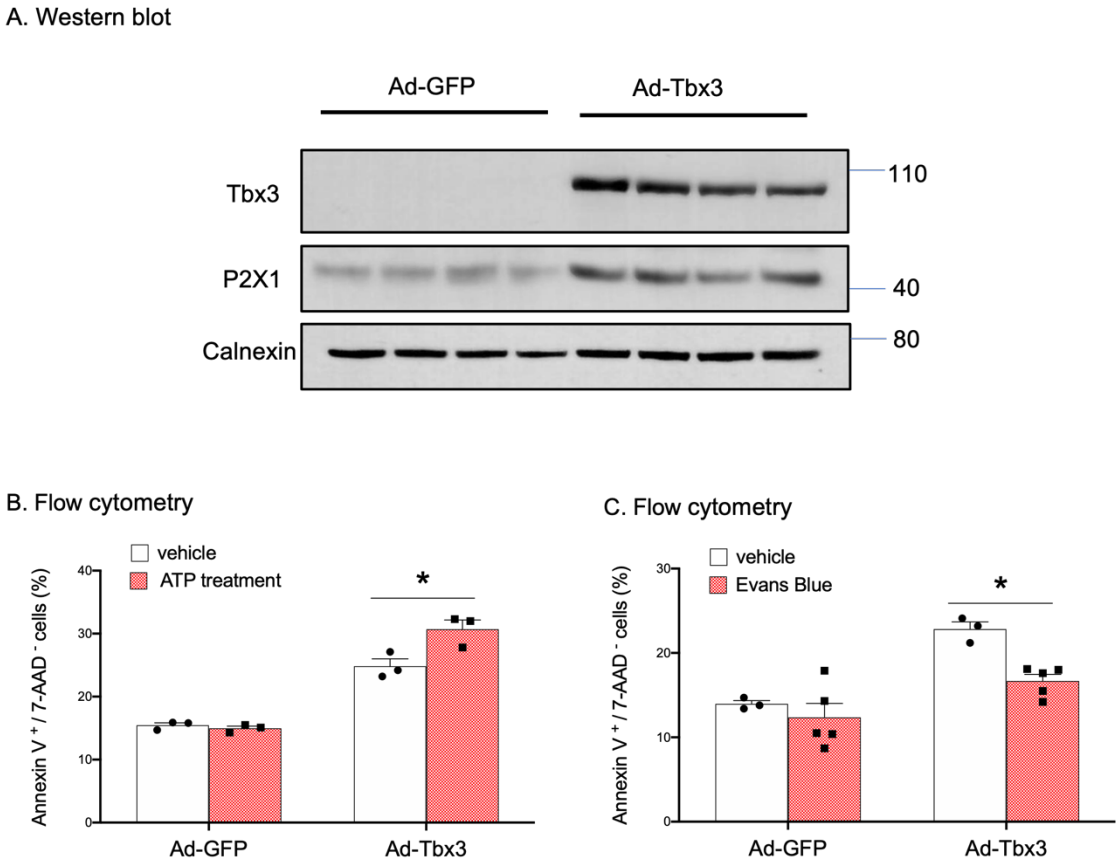

Figure 5. Tbx3 induces upregulation of P2X1 (a Ca^{2+} channel) and downregulation of Ca^{2+} handling protein genes in cardiomyocytes. (A) NRVMs were collected at 48 h after transduction with Ad-Tbx3 and Ad-GFP and analyzed by western blot (n=4). **(B)** and **(C)** Flow cytometry showed the percentage of apoptotic cells treated with P2X1 agonist (ATP) and antagonist (Evans blue) after Tbx3 overexpression. n=3 or 4 per group, *p<0.05. Data are presented as the mean ± standard deviation.

4. Tbx3-induced apoptosis in NRVMs is also associated with upregulation of Fas

Our RNA sequencing indicated that, in addition to *P2X1*, other apoptosis-associated genes, such as *Fas*, were also upregulated in Ad-Tbx3-transduced NRVMs. Specifically, overexpression of Tbx3 increased the mRNA of *Fas* by ~15 fold at 48 h as compared to control Ad-GFP NRVMs (Fig. 6A). qPCR analyses confirmed that the mRNA level of *Fas* was significantly increased in the Ad-Tbx3 group by 4-fold (Fig. 6B), and western blot also confirmed the upregulation of Fas protein by Tbx3 (Fig. 6C). This suggests that the Fas/Fas ligand system is another potential mediator of Tbx3-induced apoptosis in NRVMs.

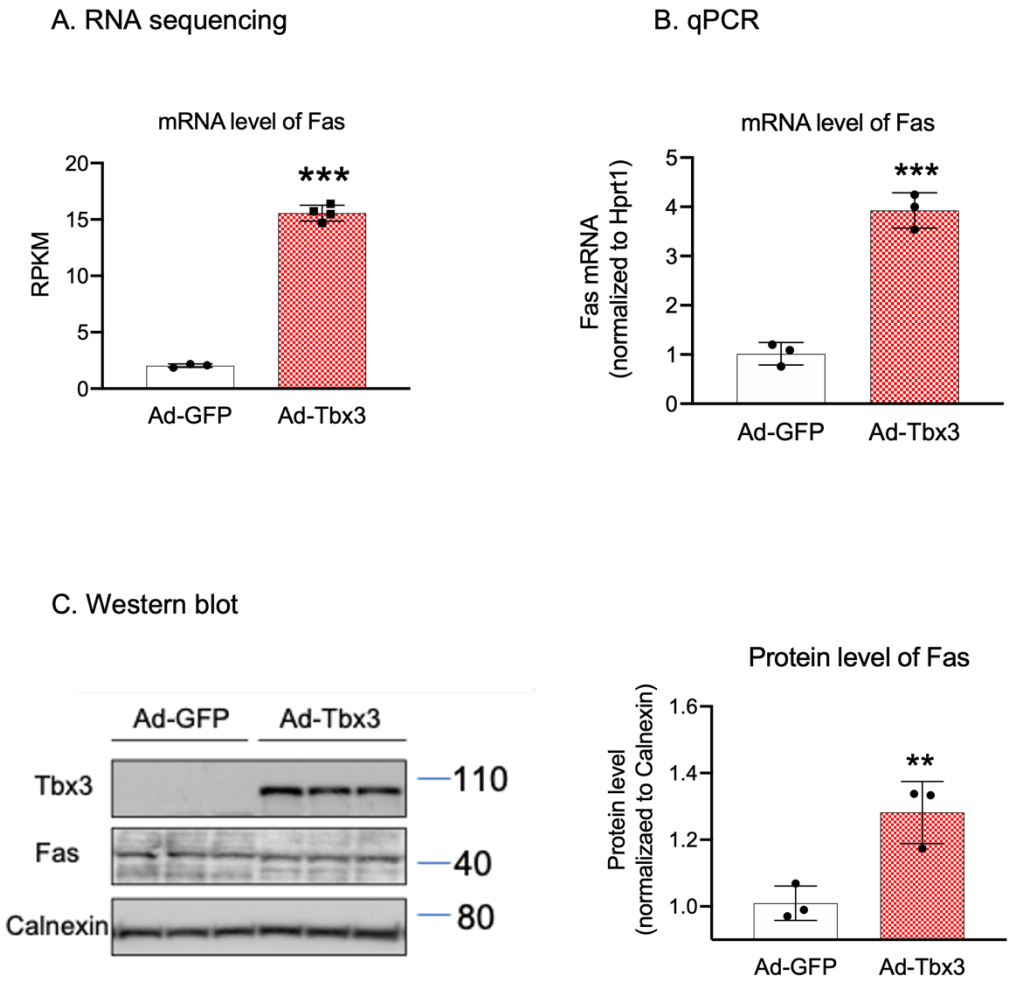

Figure 6. Tbx3-induced upregulation of Fas in cardiomyocytes. (A) and **(B)** NRVMs were collected at 48 h after transduction with Ad-Tbx3 and Ad-GFP and analyzed by RNA sequencing, qPCR and western blot (n=4). *p<0.05, **p<0.001, ***p<0.001. Ad-GFP, n=3; Ad-Tbx3, n=3 or n=4. Data are presented as the mean ± standard deviation.

Discussion

Although Tbx3 has been shown to promote proliferation (Willmer, Hare et al. 2016) and inhibit apoptosis (Carlson, Ota et al. 2002) in highly proliferative cancer cells, the present study demonstrates that Tbx3 promotes apoptosis in ventricular cardiomyocytes as demonstrated by both Annexin V flow cytometry and TUNEL staining. In this study, we further showed that Tbx3 upregulates P2X1, which is one of the downstream effectors of Tbx3-induced apoptosis in ventricular cardiomyocytes.

A previous study has reported the effects of tamoxifen-induced Tbx3 expression in adult mouse atrial and ventricular myocytes.(Bakker, Boink et al. 2012) While the authors focused on the effects of Tbx3 on cardiac electrophysiology, they also noticed that these mice rapidly developed heart failure and died at 8-10 days after the start of daily tamoxifen injections (i.e. they died at 4-5 days after the last tamoxifen injection). Although the mechanism for Tbx3-induced rapid heart failure was not investigated by the authors, their observations are consistent with our findings that ventricular myocytes die at day 3-4 after Tbx3 expression.

In the present study, both Annexin V and TUNEL assays were used to detect apoptotic cardiomyocytes. While Annexin V staining showed no difference between Ad-Tbx3 and Ad-GFP groups at 24 h after virus transduction, TUNEL staining demonstrated increased apoptosis in Ad-Tbx3 group at this early timepoint. This observation may be explained by the different sensitivities of two approaches. *Gatti et al.* (Gatti, Belletti et al. 1998) reported that only cells with significant changes in adhesion surface were Annexin V positive, while early apoptotic cells with few changes in adhesion surface were Annexin V negative. In addition, the flow cytometric analysis of Annexin V-stained cells required that samples were prepared in the cell suspension. Some cells were likely damaged during the chemical and physical detachment of cells from their culture(van Engeland, Ramaekers et al. 1996, Micoud, Mandrand et al. 2001). This likely caused a higher background apoptosis in both Ad-Tbx3 and Ad-GFP groups, which masked the small difference in apoptosis between these two groups at 24 h. By contrast, both TUNEL and Annexin V analysis demonstrated increased apoptosis in Ad-Tbx3 group at 48 h and 72 h when the difference between Ad-Tbx3 and Ad-GFP was greater.

Intracellular Ca^{2+} is tightly regulated in cardiomyocytes and is the key mediator of excitation-contraction coupling (Bers 2002, Aronsen, Louch et al. 2016). In addition, Ca^{2+} homeostasis also plays important roles in many cellular processes, including cell death. Previous studies have suggested a role of Ca^{2+} in cardiomyocyte apoptosis and necrosis (Foo, Mani et al. 2005, Kwong and Molkentin 2015). For example, Ca^{2+}-activated calpain induces caspase-12 cleavage and triggers apoptosis (Nakagawa and Yuan 2000). A key step in cardiomyocyte necrosis is the opening of mPTP due to Ca^{2+} overload (Santulli, Xie et al. 2015, Luongo, Lambert et al. 2017). Therefore, in healthy cardiomyocytes, Ca^{2+} influx equals its efflux to maintain a relatively constant level of intracellular Ca^{2+} (Eisner, Bode et al. 2013). SERCA and NCX are two major mechanisms that remove cytosolic calcium to allow muscle relaxation during diastole. (Andersson, Birkeland et al. 2009, Eisner, Bode et al. 2013). Our RNA-sequencing study showed that both SERC2A and NCX gene transcripts were reduced by Tbx3 in NRVMs. This suggests that the calcium removal mechanism is likely impaired in Tbx3-expressing NVRMs. Future studies to measure SERCA and NCX protein levels and functional changes are required to test this hypothesis.

The present study also demonstrated that Tbx3 upregulates the transcript and protein of P2X1 in ventricular cardiomyocytes. P2X1 is a member of the P2X purinergic receptor-cation channel family. The P2X1 channel is activated by ATP and is permeable to Ca^{2+}(Erlinge and Burnstock 2008). Musa.H *et al.* demonstrated that the mRNA level of P2X1 is higher in SAN than in left ventricle and right atrium of healthy mouse hearts (Musa, Tellez et al. 2009). Western blot and immunohistochemistry studies by other groups showed a low level of P2X1 expression in intercalated discs of ventricular cardiomyocytes, but their functional role is not clear (Vulchanova, Arvidsson et al. 1996, Hansen, Bennett et al. 1999). Because Tbx3 is expressed in SAN but not in atrial or ventricular tissues, the expression pattern of P2X1 mirrors that of Tbx3. During myocardial ischemia, ATP is released from multiple cell types in the heart, such as sympathetic nerve terminals, vascular endothelial cells, red blood cells and cardiomyocytes (Bodin and Burnstock 2001, Cinar, Zhou et al. 2015, Dong, Yang et al. 2016, Burnstock 2017). ATP-induced activation of P2X receptors, such as P2X4 and P2X7, have been shown to induce apoptosis (Ferrari, Los et al. 1999, Solini, Santini et al. 2007, Yang, Elner et al. 2011). Several studies have shown that P2X1 was increased in left ventricular tissues in both animal and human failing hearts (Hou, Malmsjo et al. 1999, Jiang, Bardini et al. 2005). Consistent with the role of P2X1 in

Tbx3-induced apoptosis in NRVMs, our flow cytometry analysis demonstrated that a P2X1 agonist further increased the percentage of Annexin V^+ cardiomyocytes in the Ad-Tbx3 group, while a P2X1 inhibitor attenuated it. Although the downstream pathways were not investigated in the present study, it is speculated that activation of P2X1 enhances the Ca^{2+} influx in Tbx3-expressing cardiomyocytes. The increased Ca^{2+} influx, together with the impaired Ca^{2+} removal mechanisms (SERCA2a and NCX), may cause intracellular Ca^{2+} overload and trigger apoptosis in ventricular cardiomyocytes.

Our studies also showed that Ad-Tbx3 increased the mRNA and protein level of Fas, which is the cell death receptor in apoptotic pathway (He, Zhu et al. 2016, Zhao and Zhang 2017, Lin, Tsai et al. 2021, Song, Tian et al. 2021). Under physiological conditions, the expression level of Fas is low in cardiomyocytes (Feng, Li et al. 2003). However, ischemia (Jeremias, Kupatt et al. 2000), hypoxia (Tanaka, Ito et al. 1994), volume overload (Wollert, Heineke et al. 2000, Setsuta, Seino et al. 2004) and cardiotoxicity (Nakamura, Ueda et al. 2000) have been shown to induce Fas expression and cardiomyocyte apoptosis. Future studies are warranted to investigate a potential role of Fas in Tbx3-induced apoptosis in ventricular cardiomyocytes.

One limitation of the present study is that NRVMs are relatively immature, and do not fully represent the characteristics of adult cardiomyocytes. Further studies are needed to verify our key findings using adult cardiomyocytes.

Conclusions

Our results are consistent with the conclusion that Tbx3 is pro-apoptotic in ventricular cardiomyocytes with P2X1 as one of the mediators.

Future Directions

The present study demonstrated a role of Tbx3 in ventricular cardiomyocyte apoptosis. Further work will investigate if Tbx3 is involved in cardiomyocyte death in heart disease, as well to further investigate the mechanisms for Tbx3-induced cell apoptosis.

Both animal and human studies suggested that the Wnt/β-catenin signaling is activated in the myocardium after myocardial infarction (Malekar, Hagenmueller et al. 2010, Dawson, Aflaki et al. 2013, Hou, Ye et al. 2016). Our recent study has demonstrated that Wnt/β-catenin signaling directly activates the transcription of Tbx3 in ventricular myocytes (Lu, Kamkar et al. 2020). Therefore, we are interested to see if Tbx3 expression is activated in post-MI hearts. Our preliminary data showed that Tbx3 was induced in both the infarct area and border zone at 12 h and 24 h after MI as compared with the sham group. Co-staining of TUNEL, Tbx3 and cTNC (cardiac troponin C, cardiomyocyte marker) on cryosections of rat hearts in the sham and MI groups showed that a subset of $TUNEL^+$ cells (indicating apoptosis) colocalized with $Tbx3^+$ cardiomyocytes, indicating that Tbx3 is likely involved in MI-induced apoptosis in the rat hearts. Moreover, we also cultured NRVMs in a hypoxic environment to mimic the condition of *in vivo* MI. Western blot showed that lowering the O_2 level from 20% (air) to 5% caused upregulation of Tbx3 and activation of caspase-3 (cleaved form, indicating activation of apoptosis).

These preliminary data suggest that Tbx3 is increased in ventricular cardiomyocytes with hypoxia as a potential upstream mediator. Therefore, future studies are required to answer the following important questions: What are the time courses of Tbx3 expression and apoptosis in ventricular myocytes after MI? How does Tbx3 regulate apoptotic genes in leading to apoptosis in ventricular myocytes? Does inhibition of Tbx3 reduce cardiomyocyte apoptosis and improve heart function after myocardial infarction? Answers to these questions may identify an important role of Tbx3 in heart disease.

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