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CALPASTATIN OVER-EXPRESSION REDUCES CARDIAC FIBROSIS IN A MURINE MODEL OF TYPE 2 DIABETES

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CALPASTATIN OVER-EXPRESSION REDUCES CARDIAC FIBROSIS IN A MURINE MODEL OF TYPE 2 DIABETES

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ABSTRACT

Cardiovascular complications are the leading cause of diabetes-related morbidity and mortality. Calpain, a calcium-dependent protease, is increased in diabetic hearts yet its role in diabetic heart disease has not been demonstrated. We hypothesized that inhibition of calpain activity via calpastatin (CAST) over-expression will reduce the onset of myocardial fibrosis, a hallmark of diabetic cardiomyopathy, in the Type 2 murine model, db/db. Histological analysis together with gene expression measurements showed calpastatin over-expression reduced collagen content in db/db-CAST hearts. In order to understand the mechanisms responsible for this change, fibroblasts and known mediators of collagen synthesis and degradation were studied. Results indicated that calpain-induced derangements in MMP activity, stimulation of immune cells and up-regulation of cytokines responsible for promoting fibroblast differentiation and proliferation were reversed by calpastatin over-expression. This study shows that calpain inhibition is an effective means of decreasing collagen accumulation in the diabetic heart.

Keywords: diabetic cardiomyopathy; myocardial fibrosis; collagen; calpain; calpastatin; calpastatin over-expression, fibroblasts; db/db

DEDICATION

I dedicate this thesis to my parents. No words can express how profoundly touched I am each time I realize that every one of your dreams and wishes are to see me happy. My greatest hope is to make you proud.

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I would like to thank my supervisor, Dr. Tianqing Peng first for giving me the opportunity to work in his lab but also for helping me become a better scientist. You encouraged me every time I had a presentation or assignment and provided all the help I required to complete my goals. I will remember how you celebrated my achievements and constantly encouraged my ambitions. I would also like to thank you for giving me the opportunity to hone my skills as a scientific writer, your confidence in my ability to do so was tremendously appreciated. For your advice and guidance, I thank you.

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LIST OF ABBREVIATIONS

AMC	7-amino-4-methylcoumarin
APMA	4-aminophenylmercuric acetate
ATP	Adenosine triphosphate
BM	Basement membrane
CAD	Coronary artery disease
CAPN1	Calpain 1 gene
CAPN2	Calpain 2 gene
CAPN4	Calpain 4 gene
CAST	Calpastatin
COLL	Collagen
DCCT	Diabetes Control and Complications Trial
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT4	Glucose transporter type 4
IκB	Inhibitor of kappa B
IDT	Intensive diabetes therapy

IKK	IκB kinase
LEPR	Leptin receptor
LV	Left Ventricle
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-kappa B
OPN	Osteopontin
PPAR-α	Peroxisome proliferator activated receptor- alpha
RNA	Ribonucleic acid
ROS	Reactive oxygen species
TIMP	Tissue inhibitors of metalloproteinases
TGF-β1	Transforming growth factor-beta 1
TNF-α	Tumour necrosis factor- α
UKPDS	United Kingdom Prospective Diabetes Study
α-SMA	Alpha-smooth muscle actin

CHAPTER 1: INTRODUCTION

Section 1.1: Diabetes Mellitus and its Associated Complications

Diabetes is a chronic, metabolic disorder; the body cannot produce insulin to regulate glucose or fails to use insulin adequately [1]. The result is elevated blood glucose levels which impair physiological functions and lead to damaged organs, nerves and vessels [1]. There are three main types of diabetes including gestational diabetes which occurs early in pregnancy but subsides after delivery [1-4].

Type 1 diabetes is an autoimmune disorder [1-4]. The body's own T-cells attack the β-cells for unknown reason thus destroying pancreatic endocrine function and causing absolute insulin deficiency [5]. Type 1 diabetes affects nearly 10% of the diabetic population and is usually diagnosed in children and young adults [2-4].

Type 2 diabetes is of multifactorial etiology. It is caused by the combined influence of genetic susceptibility and environmental variables such as age, weight, diet and physical inactivity [1, 4-5]. Insulin production may not be sufficient to meet the body's needs. Alternatively, insulin-resistance can develop where the pancreas continues to produce insulin but the body no longer responds to its action [5].

Diabetes is a global pandemic; by 2030, nearly 400 million people will have been diagnosed with it [3]. In Canada alone, 1.2 million people are expected to be diagnosed between 2010 and 2020, bringing the total to 3.7 million [2]. Coping with diabetes is a tremendous struggle in itself. Nonetheless, it can introduce a number of complications including visual impairments and blindness, nerve and kidney damage [1-4]. However, the greatest challenges lie in cardiovascular complications, now the leading cause of diabetes-related morbidity and mortality [3-4]. In Canada, 80% of diabetics die from a

heart attack or a stroke [2]. In 2004 in the United States, heart disease was noted on 68% of diabetes-related death certificates among people aged 65 years or older [1].

Section 1.2: Cardiovascular complications and glycaemic control

Hyperglycaemia may be a risk factor for the development and progression of cardiovascular disease similar to total cholesterol and blood pressure [6]. Epidemiological studies have long indicated a clear connection between the level of hyperglycaemia and the occurrence of cardiovascular disease [7-11]. Surprisingly, glycaemia does not need to rise to diabetic levels to increase the risk of cardiovascular complications as even slight, sustained elevations in blood sugar are sufficient [8, 9, 11, 12].

In the Framingham Study, diabetic men had more than twice the frequency of heart failure than their non-diabetic counterparts [7, 9, 13-15]. Diabetic women are also five times more susceptible to heart failure [7, 9, 13-15]. Additionally, diabetes can also have a detrimental effect on the outcome of cardiovascular disease. Studies suggest that following myocardial infarction, diabetic hearts are more likely to fail through future cardiac events [7].

Clinical trials such as the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) involving patients with Types 1 and 2 diabetes, respectively, have conclusively shown reductions in the risks of diabetes complications after intensive diabetes therapy (IDT) [6, 8, 16]. Unlike conventional therapy which aims only to prevent the symptoms of hyperglycaemia and hypoglycaemia, IDT involved a more stringent regimen of insulin treatment [16].

Intensive control of blood glucose reduced the risk of cardiovascular events by 42% and severe clinical events, defined as non-fatal myocardial infarction, stroke and death from cardiovascular disease by 57% in the DCCT [8, 16]. The UKPDS similarly demonstrated the risk-reducing benefits of glycaemic control in the microvasculature [11].

Section 1.3: Diabetic Cardiomyopathy

Decades ago, Rubler documented the existence of myocardial disease of unknown cause in four diabetic patients [20]. Since then, numerous studies, both epidemiological and clinical, have suggested the existence of a cardiomyopathy distinct to diabetics which is not caused by pre-existing heart conditions such as hypertension or coronary artery disease (CAD) [9, 17, 19, 21-23] and results from numerous alterations both structural and metabolic (Figure 1.1).

Section 1.3.1: Metabolic changes in diabetic cardiomyopathy

Hyperglycaemia can introduce a number of adverse alterations in metabolism, particularly substrate utilization, which could be an underlying mechanism leading to cardiomyopathy [24-26]. Although the normal heart is able to derive energy from multiple substrates including ketones, amino acids, carbohydrates and fatty acids, approximately 70% of ATP generation occurs via fatty acid oxidation and the remaining 30% is provided by glucose and lactate [24, 27, 28]. The diabetic myocardium however, uses fatty acid oxidation almost exclusively for energy production [29].

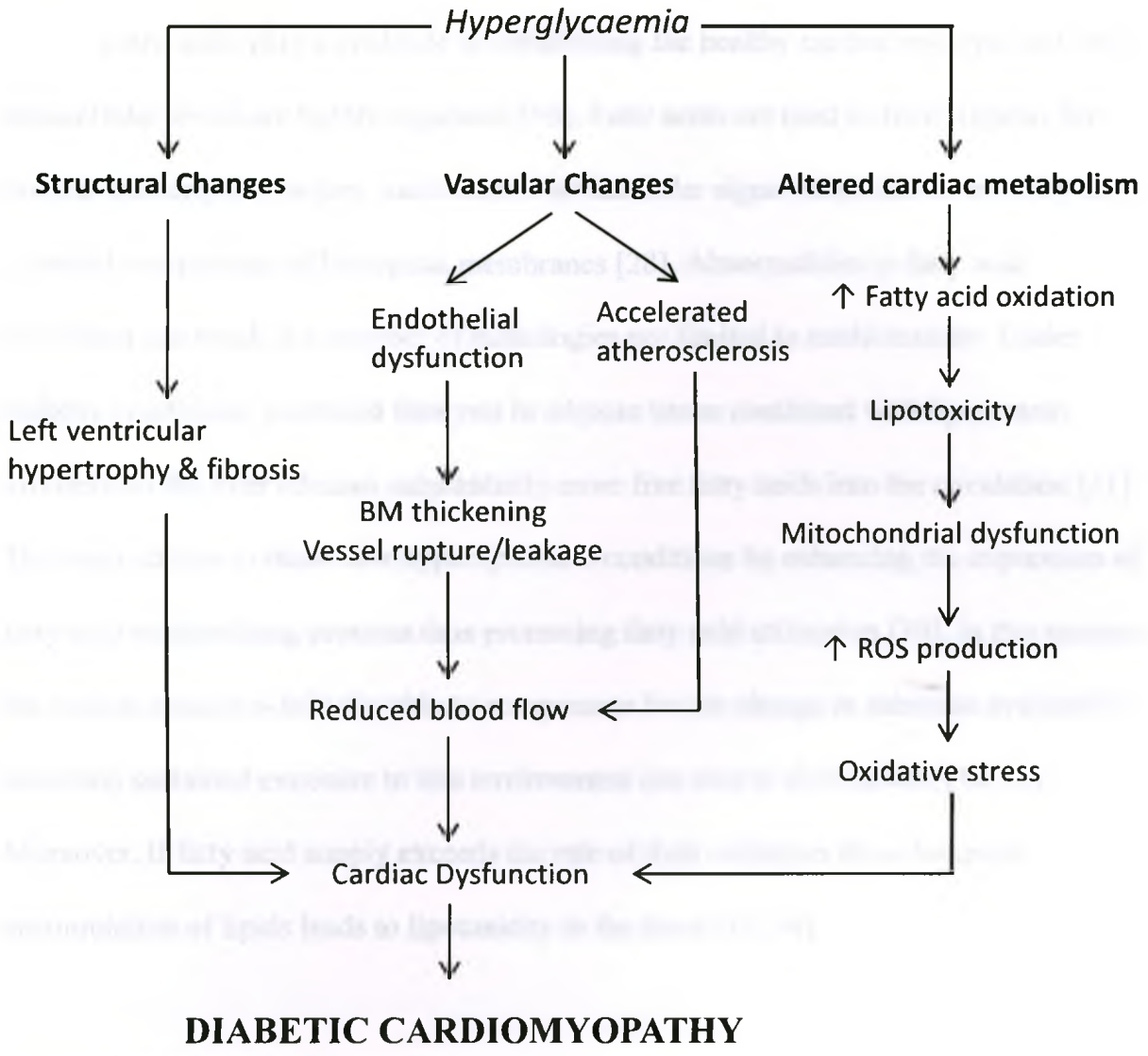

Figure 1.1: Schematic diagram illustrating some of the known mechanisms underlying diabetic cardiomyopathy

Hyperglycaemia can induce a number of changes in the myocardium. Derangements in cardiac metabolism lead to fatty acids becoming the dominant substrate for oxidation. Once the supply of free fatty acids exceeds the demand, lipotoxicity can occur. Fatty acid oxidation can also introduce mitochondrial dysfunction and promote oxidative stress. The coronary arteries are subject to accelerated atherosclerosis and the small vessels undergo microangiopathy. Lastly, owing to hypertrophy and fibrosis, the architecture of the myocardium changes. Diastolic dysfunction, with or without systolic dysfunction, is the end result.

Section 1.3.2: Fatty acid oxidation in the diabetic heart

Fatty acids play a vital role in maintaining the healthy cardiac myocyte and their intracellular levels are tightly regulated [30]. Fatty acids are used as fuels, ligands for nuclear transcription factors, mediators of intracellular signal transduction and they are essential components of biological membranes [28]. Abnormalities in fatty acid regulation can result in a number of pathologies not limited to cardiotoxicity. Under diabetic conditions, increased lipolysis in adipose tissue combined with lipoprotein synthesis in the liver releases substantially more free fatty acids into the circulation [31]. The heart adjusts to these new hyperlipidemic conditions by enhancing the expression of fatty acid metabolizing proteins thus promoting fatty acid utilization [30]. In this manner, the cardiac muscle is initially able to compensate for the change in substrate availability however, sustained exposure to this environment can lead to dysfunction [32, 33]. Moreover, if fatty acid supply exceeds the rate of their oxidation the subsequent accumulation of lipids leads to lipotoxicity in the heart [31, 34].

Section 1.3.3: PPAR-α activation

In the mammalian heart, the expression of proteins for fatty acid oxidation is increased in response to elevations of fatty acids. Gene expression is propagated by peroxisome proliferator-activated receptor (PPAR)-alpha [35, 36]. PPAR-α regulated genes are involved in various steps of fatty acid metabolism. The increased expression of fatty acid metabolizing genes coupled with the increase in fatty acid availability is largely responsible for increased utilization of this substrate for energy [37-39]. PPAR-α activation causes decreased expression of genes involved in glucose uptake, glycolysis,

and pyruvate oxidation [24, 40]. Additionally, glucose uptake is dependent on insulin but impaired insulin functioning combined with reduced glucose transporter type 4 (GLUT4) proteins limit glucose availability in cardiomyocytes [41, 42]. In contrast fatty acids can enter the cardiac cells with ease, as they require no hormones. All these factors serve to further promote the complete dependence of the heart tissue on fatty acids.

Section 1.3.4: Functional and Structural Changes in Diabetic Cardiomyopathy

Cardiac complications typically involve the left ventricle, coronary arteries and the microvasculature [43]. Studies of biopsy specimens reveal morphological changes at the tissue level, chiefly myocyte hypertrophy and perivascular fibrosis. As such, hypertrophy and fibrosis are hallmarks of diabetic cardiomyopathy [44]. Hypertrophy is characterized by increased left ventricular mass, increased wall thickness, stiffness and reduced chamber size [45-47]. Myocardial fibrosis can further lead to ventricular wall stiffness, reduced compliance, and impaired diastolic function thereby exacerbating cardiac injury [48]. Diabetes can also accelerate atherosclerosis leading to coronary and cerebral artery disease [15, 51, 52]. Systemic hyperglycaemia can damage small blood vessels and cause a thickening of the basement membrane (BM) and eventual rupture leading to reduced blood flow and leakage of proteins [53, 54]. Although, many mechanisms of hyperglycaemia-induced cardiac damage have been proposed, including derangement in metabolism, the exact one(s) have not been clearly isolated [55].

Section 1.4: Fibrosis in the diabetic myocardium

In the heart, myocytes are surrounded by the cardiac extracellular matrix (ECM) which is primarily composed of collagen with smaller amounts of elastin, laminin and

proteoglycans among others [56, 57]. The dominant collagens are type I, accounting anywhere from 50-80%, and type III collagen, comprising around 10% of the ECM; the other collagens are present but to a much lesser degree [56, 57]. Collagen may also be involved in the transmission of force generated by cardiac muscle [58]. Characterized by excess collagen accumulation, myocardial fibrosis is believed to be a maladaptive response occurring independently of organ hypertrophy [48]. Although studies indicate that only 2-4% of the myocardium is collagen, even slight changes in collagen concentration can have substantial effects on the mechanical properties of the heart [56, 57, 59, 60].

Increased ventricular stiffness resulting from elevated levels of collagens can inhibit myocyte recoil during cardiac relaxation resulting in aberrant ventricular filling and consequently stroke volume [61, 62]. Increases in collagen content impair sarcomere extension and compromise the ability of the heart to generate adequate pressure for systemic perfusion [61, 62]. With these factors taken together, it is clear that fibrosis greatly jeopardizes myocardial function and not surprisingly, fibrosis is a leading cause of heart failure (Figure 1.2) [56, 57, 59].

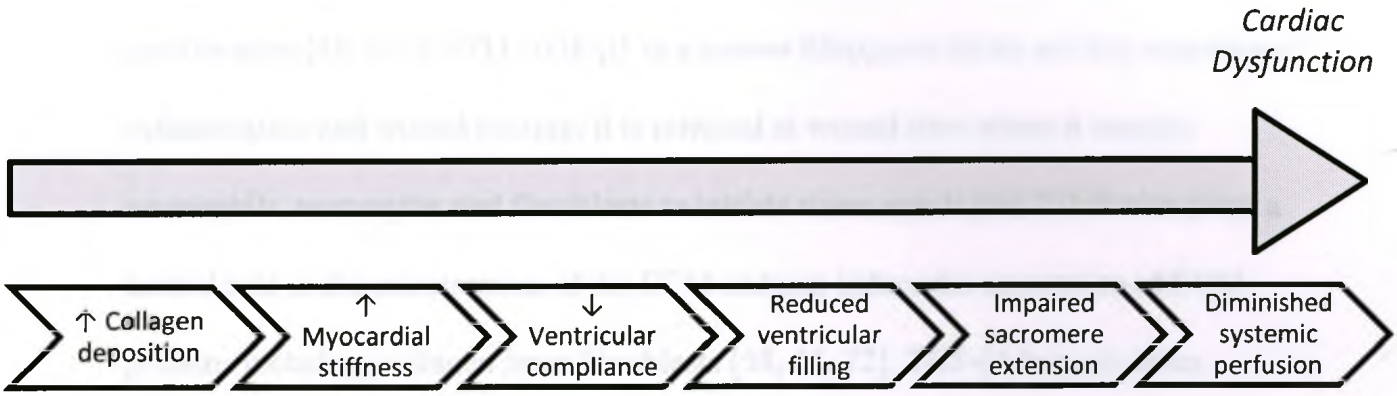

Figure 1.2 Functional consequences of myocardial fibrosis

Fibrosis is a typical feature of diabetic cardiomyopathy. Collagen levels are elevated in the diabetic heart and this starts a cascade event that culminates in cardiac dysfunction that could lead to heart failure. The stiffness and non-compliance of the heart hampers its mechanical abilities by limiting ventricular filling and preventing the complete extension of the sarcomeres. The left ventricle consequently struggles to generate the adequate level of pressure required for systemic perfusion.

Section 1.5: Fibroblast Cell Involvement

Fibrosis is believed to be mediated by fibroblasts [63-67]. The precise manner in which hyperglycaemia stimulates increased collagen deposition by fibroblasts in cardiac tissue is not clearly understood [68]. One possibility is that high glucose up-regulates transforming growth factor (TGF)-β1 expression leading to increased fibroblast proliferation [58, 65, 69-71]. TGF-β1 is a known fibrogenic factor and key regulator of inflammation and wound healing; it is released at wound sites where it recruits neutrophils, monocytes and fibroblasts to initiate tissue repair [69, 72]. It also plays a central role in the maintenance of the ECM and can induce the expression of ECM proteins including collagen from fibroblasts [58, 65, 72]. TGF-β1 has also been demonstrated to induce differentiation of fibroblasts to myofibroblasts [58, 65, 72]. Fibroblasts are quiescent but they migrate and proliferate upon activation [72]. During the reparative process, tissue needs to be contracted and as a result a subpopulation of the fibroblasts will gain contractile properties that resemble those belonging to smooth muscle cells and this modulation allows the cell, now called a myofibroblast, to secrete greater amounts of collagen [69, 73, 74].

Section 1.6: Other mechanisms of fibrosis: MMPs and NF-κB

Maintenance and degradation of the extracellular matrix in the myocardium is regulated by matrix metalloproteinases (MMPs) which are regulated in turn by their inhibitors, tissue inhibitors of metalloproteinases (TIMPs) [75-80]. Modifications in the activities of either MMPs or TIMPs have marked effects on the architecture of the ECM in the myocardium. In the event of impaired MMP regulation an imbalance in collagen

synthesis versus collagen degradation leads to the accumulation of fibrillar collagen and the appearance of myocardial fibrosis [75, 80].

Lastly, a transcription factor implicated in fibrosis is nuclear factor-κB (NF-κB) [81-84]. When inactive, NF-kB is complexed to its inhibitor, inhibitor-κB (IκB) and is thus prevented from entering the nucleus [85, 86]. Activation of IκB kinase (IKK) by extracellular signals initiates the degradation of IκB [85, 86]. Phosphorylation of two serine residues of the IκB structure by IKK leads to ubiquitination and subsequent digestion by the proteasome. NF-κB becomes free to translocate into the nucleus and transcribe its associated genes [85-87]. Specifically, NF-κB is responsible for the activation of pro-inflammatory cytokines some of which, such as tumour necrosis factor-alpha (TNF- α), are elevated in the diabetic heart [81, 82, 85]. Pro-inflammatory cytokines can subsequently induce expression of other cytokines such as TGF-β1. Furthermore, the pro-fibrogenic effect of these cytokines may also include MMP activation [79].

Section 1.7: A Closer Look at Calpain

Calpains are one of three cysteine proteases; caspases and cathepsins are the other two. In response to calcium signaling, calpains direct cellular functioning by cleaving select proteins [88]. Calpain can exist in any one of fifteen different isoforms however; the two most well-characterized members of the calpain family in mammals are μ-calpain (calpain-1) and m-calpain (calpain-2) [88, 89]. They differ only in calcium sensitivity and are ubiquitously distributed but their exact physiological function remains unknown. Calpain exists in the cytosol as an inactive enzyme and translocates to the membrane in

response to increases in cellular Ca^{2+} levels [88-90]. Calpain activity is regulated by calpastatin, an endogenous inhibitor capable of reversible binding of the calpain molecules in the presence of calcium [90].

Section 1.7.1: Structure and Activation

Calpain-1 and calpain-2 are both heterodimers consisting of an 80 kDa catalytic subunit and a regulatory 30 kDa subunit [88]. Although the 30 kDa subunit, encoded by the CAPN4 gene, is identical in both calpains, the catalytic subunit is encoded by different genes: human chromosome 11 for calpain-1 and human chromosome 1 for calpain-2 [88]. Nonetheless, the subunits share 55-65% homology [88]. Calpain activation is triggered by a Ca^{2+} flux which causes inactive calpain to translocate to the plasma membrane where activation occurs in the presence of Ca^{2+} and phospholipids [89].

The crystal structure of calpain reveals four domains (I-IV) in the 80 kDa subunit. Domain II is subdivided into domains IIa and IIb which surround the substrate binding cleft [88, 89]. The 30 kDa subunit has an additional two domains (V-VI) [88, 89]. The first of a two-step mechanism of activation is the release of constraints imposed by interactions between the four domains of the 80 kDa subunit [88]. Upon Ca^{2+} binding to domains IV, VI and III, domain I is released from VI and domain II is released from III resulting in the dissociation of the 80kDa subunit from the 30kDa subunit, a pre-requisite for the formation of the catalytic site in the second stage [88, 89]. In the inactive conformation of calpain, further domain interactions separate subdomains IIa from IIb, a structural constraint that prevents the formation of an active cleft site [88]. The binding of

two additional Ca^{2+} ions in the second step, one to domain IIa and the other to domain IIb, induces a conformational rearrangement vital for the formation of a functional catalytic site [88, 89].

Section 1.7.2: The Biological Role

Calpains constitute a highly conserved superfamily existing ubiquitously from humans to microorganisms and exist in all vertebrate cells examined thus far, suggesting a vital biological role [88]. Mammalian calpains sequenced to date have shown a high degree of homology in their amino acid sequences and there is little indication of alternative splicing in the expression of the calpain gene [88]. By modulating the biological activity of their substrates, calpains influence a number of calcium-regulated cellular processes including signal transduction, cell cycle progression, apoptosis and cytoskeletal remodeling [90, 91].

Calpain is also able to cleave pro-caspase 3, 7, 8, 9 into inactive fragments and as such has been implicated in apoptosis [92]. Calpain has attracted much attention due to the recent discovery of correlations between calpain gene mutations and human diseases. Deregulation of calpain has been implicated in a number of pathologies including secondary degeneration resulting from acute cellular stress, neuronal degeneration, Alzheimer's disease, metastasis, muscular dystrophy, cataracts and diabetes [93-95]. Calpain inhibition has been found to be protective in ischaemia injury models as well [96]. A recent study demonstrated that cardiac over-expression of calpain-1 is sufficient to cause heart failure in transgenic mice [93, 97]. Our lab recently showed that hyperglycaemia-induced calpain-1 activation, mediated through an NADPH oxidase-

dependent pathway, can lead to apoptosis through down-regulation of (Na, K)-ATPase activity in cardiomyocytes and *in vivo* diabetic hearts [92]. Calpain inhibition via calpastatin over-expression imparted an anti-apoptotic effect on cardiomyocytes.

Calpain knockout models have indicated important roles for calpain-4, the regulatory subunit, and also suggest m-calpain may be more important than μ-calpain for embryonic development [98]. Compared to capn4$^{+/-}$ mice which had normal viability and calpain activity, the capn4$^{-/-}$ embryos, generated by disrupting expression of the 80kDa small subunit encoded by the calpain-4 gene (CAPN4), died by 11.5 days of gestation after exhibiting cardiovascular abnormalities at day 10.5 [98]. These embryos also had no detectable calpain activity. Interestingly, in the embryonic stem cells, the RNA levels of the 80kDa subunit of both calpains were normal however, the corresponding polypeptides for each were nearly undetectable [98]. The results point to a role for the regulatory subunit critical to cell survival that is currently unknown.

Transgenic capn1$^{-/-}$ mice had significant decreases in platelet aggregation. It appears that the presence of m-calpain can compensate for the lack of μ-calpain and that m-calpain and μ-calpain may have the same substrates [88]. Previous studies have established a physiological role for calpain in the degradation of polypeptides during platelet activation however in the calpain-1 knockout mice these same polypeptides had normal degradation patterns [96, 99, 100].

On the other hand, knockout of m-calpain has shown to be embryonically lethal [88]. This observation raises a few issues. First that m-calpain, along with calpain-4, must play an essential role in embryonic development, perhaps certain cleavages, that calpain-1 does not. Alternatively, a lack of embryonic lethality after calpain-1 knockout may be

due to the relatively late expression of μ-calpain during development meaning, it would not be involved in calpain-dependent cleavages required for continued growth thus assigning it a non-essential function in embryonic development [88].

Section 1.7.3: Endogenous Regulation of Calpain Activity

Deregulation of calpain can lead to cellular damage and subsequent pathology [95, 99, 101]. Calpain activity is thus tightly regulated by calcium availability as well as an endogenous inhibitor, calpastatin. Calpastatin is an entirely unstructured protein; its polypeptide is in a random coil formation when not bound to calpain [90]. Despite the presence of only a single calpastatin gene in mammals, the use of at least four different promoters and alternative splicing mechanisms allow the production of different calpastatin polypeptides [88-90]. It appears that invertebrates do not possess the calpastatin gene; any regulation of calpain activity by calpastatin is limited to vertebrates [88]. No polypeptide sequenced thus far has homology to the calpastatin amino acid sequence [90, 97]. Calpastatin is the only known inhibitor specific for the calpains [90]. Furthermore, calpastatin does not inhibit any other protease [88-91, 97].

Section 1.8: The db/db Mouse and Diabetic Cardiomyopathy

The db/db mouse was used to model Type 2 diabetes. The mouse has a point mutation in the gene encoding the leptin-receptor (LEPR) [102-105]. Leptin is a hormone that controls satiety and energy expenditure by acting on the hypothalamus through the LEPR. Without the receptor, leptin does not exert its effects and the db/db mouse mimics leptin deficiency and grows massively obese.

As Aasum and colleagues noted, "the metabolic features of db/db mice are similar to the pathogenesis of type 2 diabetes in humans." Specifically, initial insulin resistance is temporarily overcome by enhanced insulin secretion producing a state of hyperinsulinemia [102]. Inevitably, elevated levels of insulin cannot maintain normoglycaemia for long and in concert with dysfunctional changes in pancreatic β-cells, insulin levels fall and severe hyperglycaemia ensues [102, 105]. db/db mice are also reported to exhibit the characteristics of diabetic cardiomyopathy at 10-14 weeks of age [54, 104, 106]. As in the human heart during diabetes, the db/db heart is associated with a dependence on fatty acid oxidation for energy [107]. Derangements in cardiac metabolism progresses with age as do contractile impairments including diastolic dysfunction [106].

Section 1.9: Purpose of Study

Studies suggest that calpain activation may contribute to the progression of heart failure [91]. Calpains are implicated in ischaemia/reperfusion-induced cardiomyocyte apoptosis and cardiac over-expression of calpain-1 was sufficient to cause heart failure in transgenic mice [99]. In contrast, over-expression of calpastatin prevented troponin I degradation and improved contractile function in rat hearts subjected to ischaemia/reperfusion but also inhibited cardiac fibrosis in angiotensin-II induced mice as shown by Manabe and colleagues [100]. Although an association between calpain and cardiac dysfunction is clear, calpain over-activity in the context of diabetes has not been examined nor, is there a type 2 murine model over-expressing the calpastatin gene.

Section 1.10: Hypothesis

Overexpression of calpastatin will impede development of myocardial fibrosis caused by elevations in calpain activity as a result of hyperglycaemia in a type 2 murine model.

Section 1.11: Objectives of Thesis

1. Use a LEPR deficient murine model, db/db, to generate transgenic db/db mice over-expressing the CAST gene (db/db-CAST) by crossing heterozygous $db^{+/-}$ mice with homozygous transgenic mice over-expressing rabbit CAST.
2. Assess heart tissues for indications of fibrosis. Determine if CAST over-expression imparted an anti-fibrotic benefit to db/db-CAST hearts.
3. Understand the action of calpain and CAST in fibroblast cells and specifically their effect on fibroblast proliferation and differentiation to myofibroblasts.
4. Investigate whether CAST over-expression has any effect on impaired collagen turnover and inflammatory responses in the diabetic heart.

CHAPTER 2: METHODS AND MATERIALS

Section 2.1: Generation of the CAST Transgenic Model

Mice that are homozygous for the LEPR mutation are sterile [108]. Therefore, to generate the desired db/db-CAST model, heterozygous mice of the BKS strain were crossed with homozygous transgenic mice over-expressing rabbit CAST of a C57BL/6 background. This cross can yield four possible genotypes: $Lepr^{db/-}$ $CAST^{+/-}$, $Lepr^{db/-}$ $CAST^{-/-}$, $Lepr^{-/-}$ $CAST^{+/-}$, $Lepr^{-/-}$ $CAST^{-/-}$, each with a 25% probability of occurrence (Figure 2.1). Mice with the first two genotypes, identified qualitatively by black fur colour, are crossed to yield the second filial generation. In addition to the F1 genotypes, this cross can yield another four genotypes each with a 12.5% chance of occurrence: $Lepr^{-/-}$ $CAST^{+/-}$, $Lepr^{-/-}$ $CAST^{-/-}$, $Lepr^{db/db}$ $CAST^{-/-}$, $Lepr^{db/db}$ $CAST^{+/-}$. From this final cross, the db/db ($Lepr^{db/db}$ $CAST^{-/-}$), db/db-CAST ($Lepr^{db/db}$ $CAST^{+/-}$) and WT ($Lepr^{-/-}$ $CAST^{-/-}$) mice used in this study are produced (Figure 2.2).

Note: *$Lepr^{db/db}$, $Lepr^{db/-}$, and $Lepr^{-/-}$ denote homozygous (db/db) mice, heterozygous (db+/-) mice and mice lacking the mutation, respectively.*

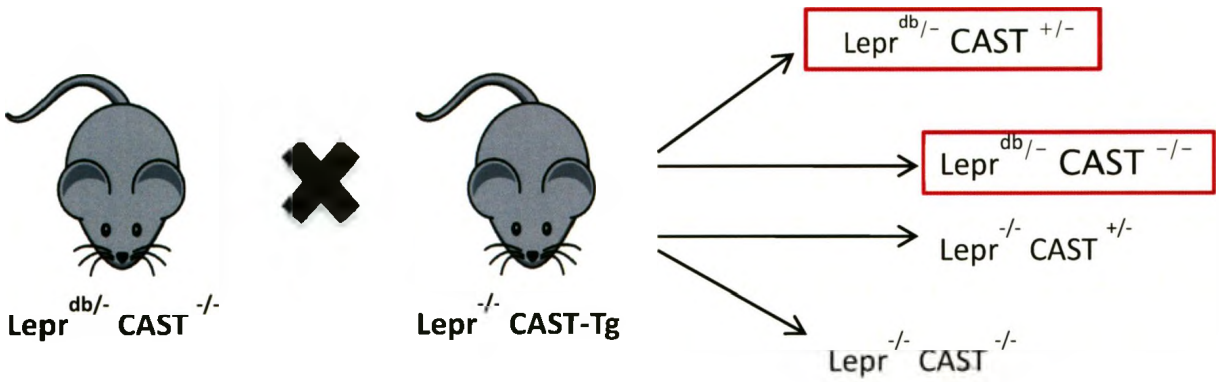

Figure 2.1: Schematic illustration representing the first filial cross

The db/db mouse is sterile. In order to produce db/db and db/db mice over-expressing the CAST gene (db/db-CAST), heterozygous $db^{+/-}$ mice will be crossed with $db^{+/-}$ CAST mice to yield four possible genotypes. Genotypes outlined in red were used in the second filial cross.

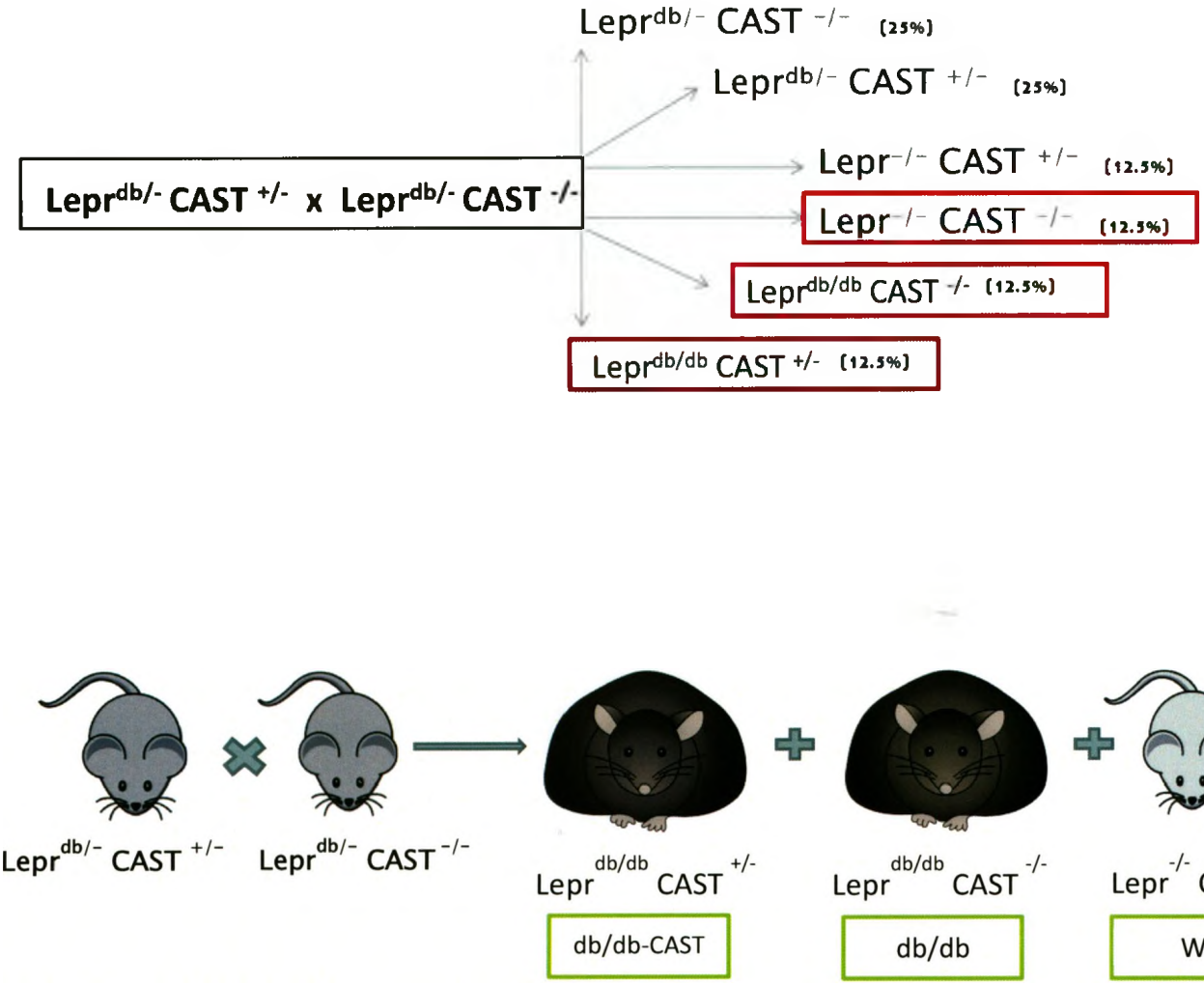

Figure 2.2: Schematic illustration representing the second filial cross and offspring

CAST transgenic offspring heterozygous for the LEPR mutation were crossed with heterozygous offspring without the CAST gene from the first generation. Four unique genotypes are produced with a 12.5% chance of occurrence in addition to the two F1 genotypes, with are each produced with a 25% chance of occurrence. Genotypes outlined in red were used. Outlined in green are the common names for each genotype as they will be referred to in this study.

Section 2.2: Genotyping

To determine transgenic CAST over-expression, 1-2mm of tail was obtained from three week old mice and digested overnight at 37°C in a solution of Proteinase K (0.222mg/mL, Sigma-Aldrich, MO, USA) and 1 mL PBND [50 mM KCl, 10mM Tris-HCl (pH8.3), 2.5mM $MgCl_2$-$6H_2O$, 0.1 mg/mL gelatin, 0.45% NP40, 0.45% Tween-20]. Proteinase K digests unwanted proteins, including nucleases, and keratin. Samples are then heated first at 50°C for 1.5 hours then at 100°C for 5 minutes to inactivate Proteinase K. Undigested material is pelleted after centrifugation (Eppendorf Centrifuge 5702, NY, USA) for 10 minutes at 10008xg and 2μL of lysate is used for PCR.

A pair of primers specific for rabbit CAST mRNA, not for the mouse CAST gene, were used to amplify the transgenic rabbit CAST gene for 35 cycles (Px2 Thermal Cycler, Thermo Electron Corporation, OH, USA):

Forward CAST primer: 5' GTTGGCTTAGGCTGCTTTTCGT 3'

Reverse CAST primer: 5' CCAGACTCCGTGACACCCCTT 3'

The PCR reaction was set as follows:

12.5 μL 2X PCR Mastermix (Fermentas),

0.4 μL (25μM) forward and reverse CAST primer

10.1 μL DEPC-treated water

2 μL lysates

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25 μL Total Volume

Samples were run on a 2% agarose gel (containing ethidium bromide (EtBr)) at 80 volts for 30 minutes and the signal was visualized under UV light.

Section 2.3: Calpain Activity

The calpain activity assay measures the fluorescence intensity of AMC (7-amino-4-methylcoumarin) cleaved from the peptide substrate, N-succinyl-LLVY-AMC (Cedarlane Laboratories, Burlington, Ontario). Heart tissue was placed in 250µL of lysis buffer [1M HEPES (pH 7.4), 50 mM CHAPS (5 mL), 1M 0.1% DTT (0.5mL), 5mM EDTA (20µL)] homogenized and centrifuged at 22680xg for 15 minutes. Supernatant was removed and protein concentration was measured using a DC Protein Kit (Bio-Rad) and the concentration of all samples was equalized by adding appropriate amounts of reaction buffer (63 mM imidazole–HCl, pH 7.3, 10 mM β-mercaptoethanol, 1 mM EDTA, and 10 mM EGTA). The samples were incubated for two hours at 37 °C in either reaction buffer with or without calcium chloride (5 µM or 5 mM) or reaction buffer with or without calcium chloride (5 µM or 5 mM) in addition to 5 µM of calpain inhibitor, PD150606 (Li et al., 2009). The fluorescence intensity of cleaved AMC was quantified with a multilabel reader (excitation, 360 nm; emission, 460 nm) (Victor^3 1420 Multilabel Counter, PerkinElmer, MA, USA) and calpain activity was determined as the difference between calcium-dependent and calcium-independent fluorescence. (Li et al., 2009).

Section 2.4: Insulin measurement

Blood samples were collected and centrifuged at 907xg for 15 minutes at 4°C. Plasma samples were transferred and stored at -85 °C until use. An ELISA-based rat/mouse insulin kit was purchased from Millipore (St. Charles, MO) and used to conduct insulin measurements. Each well of the microtiter assay plate provided was washed three times with 300µL of wash buffer (50 mM Tris Buffered Saline with Tween-20) that was diluted 10 fold using distilled water. Diluted wash buffer was decanted after each wash.

To designated standard and control wells: 10µL rat insulin standards were added in the following order of concentration: 0.2 ng/mL, 0.5 ng/mL, 1 ng/mL, 2 ng/mL, 5ng/mL and 10 ng/mL. Additionally, 10 µL matrix solution (charcoal stripped pooled mouse serum) was also added.

To sample wells: 10 µL of the plasma samples were added.

Subsequent additions to both control and sample wells included:

- 10 µL assay buffer (0.05 M phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08% sodium azide, and 1% BSA)
- 80 µL detection antibody (pre-titered biotinylated anti-insulin antibody)

The plate was then sealed and incubated for two hours at room temperature after which the solutions were decanted from the plate and each well was again washed three times with diluted wash buffer, 300 µL per wash. Subsequently, 100 µL of enzyme solution (pre-titered streptavidin-horseradish peroxidase conjugate in buffer) was added to each well and the plate was sealed and incubated for 30 minutes. The enzyme solution was

emptied out and each well was washed 6 times with 300μL of the diluted wash buffer per wash. Then 100 μL of substrate solution (3, 3', 5, 5'-tetramethylbenzidine in buffer) was added to each well, the plate was covered until a blue colour formed in wells at which point 100 μL of the stop solution (0.3 M HCl) was added. Absorbance was read at 450 nm and 590 nm in a microplate reader (Model 680 Microplate Reader, Bio-Rad, Hercules, CA, USA) and insulin was measured as the difference between the two.

Section 2.5: RNA Isolation and cDNA synthesis

Heart tissue was homogenized in 1 mL TRIzol reagent (Invitrogen, Burlington, Ontario) and chloroform (EMD Chemicals Inc., Gibbs Town, NJ, USA) was used to separate aqueous and organic phases before centrifugation at 14490xg for 15 minutes. Isopropyl alcohol (EMD Chemicals Inc., Gibbs Town, NJ, USA) was added to the aqueous phase then decanted to precipitate the RNA. Reagent alcohol (VWR International, Westchester, PA, USA) was added then decanted and the pellet was dissolved in DEPC-treated water. UV absorbance at 260 nm was used to measure total RNA and at 260:280 nm to measure and standardize sample purity using a spectrophotometer. To synthesize cDNA, 1 μL of oligo (dT) (0.5 μg/mL, Sigma Genosys, Oakville, Ontario) was added to 3 μL total RNA (3 μg), and denatured at 65°C for 10 minutes. A mixture of dNTP (Sigma Aldrich, MO, USA), reverse transcriptase (Sigma Aldrich, MO, USA) and reverse transcription buffer (Sigma Aldrich, MO, USA) were added to the samples followed by a 50 minute incubation at 37°C. The reaction was stopped by heating the sample at 65°C for 5

minutes. DEPC-treated water was added to 20µL of cDNA to bring the total volume to 180µL. cDNA samples were stored at -20°C.

Section 2.6: Qualitative Real Time RT-PCR

The reaction mixture was as follows:

10 µL	2X SYBR Green Taq MasterMix (Fermentas, CA, USA)
5 µL	DEPC-treated water
0.5 µL	Forward primer (50 µmol/L)
0.5 µL	Reverse primer (50 µmol/L)
4 µL	cDNA
20 µL	Total volume

GAPDH was the housekeeping gene used to normalize the data. Real-time RT-PCR was performed in the MiniOpticon Thermocycler (Bio-Rad Laboratories, Hercules, CA, USA). The primers (Sigma GenoSys, Oakville, ON, Canada) and temperature profiles used are found in Table 2.1.

Table 2.1 Primer Sequences and Temperature Profiles for Real Time RT-PCR

Primer	Sequence (5'→3')	PCR Conditions	
		Phase	*Temperature-Time*
TGF-β1	(+) TTGCTTCAGCTCCACAGAGA (-) TGGTTGTAGAGGGCAAGGAC		
Osteopontin	(+) GACGACGATGATGACGATGA (-) CCTCAGTCCATAAGCCAAGC		
α-SMA	(+) GTCCCAGACATCAGGGAGTAA (-) TCGGATACTTCAGCGTCAGGA		
CAST	(+) GTTGGCTTAGGCTGCTTTTCG (-) CCAGACTCCGTGACACCCCTT		
Collagen I	(+) ACGGCTGCACGAGTCACAC (-) GGCAGGCGGGAGGTCTT	Initial Denaturation Denaturation Annealing Extension	95°C- 10 minutes 95°C- 15 seconds 60°C- 30 seconds 72°- 30 seconds
Collagen III	(+) GTTCTAGAGGATGGCTGTACT (-) TTGCCTTGCGTGTTTGATATT		
TIMP1	(+) ATTCAAGGCTGTGGGAAATG (-) CTCAGAGTACGCCAGGGAAC		
TIMP2	(+) CACAGACTTCAGCGAATGGA (-) CTTGGGAAGCTTGAGAGTGG		
TIMP3	(+) AGCTGGCAAAGGCTTAAACA (-) CAAGCTTCCAGCCAAACTTC		

Section 2.7: Collagen Staining

Tissue was fixed in 10% formalin and then embedded in paraffin and 7 μm sections were cut and transferred to slides which were de-paraffinized in two changes of xylene and hydrated in two changes of 100% ethanol for 10 minutes, 95% ethanol for 3 minutes, 85% ethanol for 3 minutes and finally in 75% ethanol for 2 minutes. Slides were washed and placed in Picrosirius red solution for one hour then subsequently dehydrated in ethanol and cleared in xylene before being mounted in a resinous medium. Picrosirius red solution was comprised of 0.5g Sirius red (Cat#365548, Sigma-Aldrich, MO, USA) and 500mL picric acid solution (Cat# P6744-1GA, Sigma-Aldrich, MO, USA). Sigma-Scan Pro software was used to quantify levels of collagen deposition. A total of 10 fields from each slide were analyzed with a 20X objective lens.

Section 2.8: Echocardiography

Mice were anaesthetized using a ketamine/xylazine (100/10 mg per kg body weight) combination injected intraperitoneally. Echocardiographic measurements were conducted using tissue Doppler imaging at Robarts Research Institute (London, Ontario). M-mode images were obtained and analyzed [106].

Section 2.9: Cardiac Fibroblast Cell Culture

Three month old mice were injected with 40μL heparin in a 1:40 dilution in distilled water. Thirty-minutes later, heart tissue was removed from mice, minced and washed three times with 1X D-Hanks solution then incubated at 37°C with an enzyme mixture

prepared from 0.01g collagenase type 2 (Worthington Biochemical Corp., Lakewood, NJ) and 0.006 g dispase (Roche Diagnostics, Mannheim, Germany) in 10 mL D-Hanks solution (Sigma-Aldrich, MO, USA) for forty-five minutes to digest tissue and liberate fibroblast cells. The enzyme/sample mixture was centrifuged for 5 min at 8xg (Eppendorf Centrifuge 5180R, NY, USA) after which the supernatant was discarded and the pelleted fibroblast cells were suspended in 3mL Dulbecco's modified eagle medium (DMEM) (HyClone Laboratories, ThermoFisher Scientific, Utah, USA) supplemented with 10% FBS (Gibco, Invitrogen, Grand Island, NY, USA) and 100 µg/ml penicillin-streptomyocin (Invitrogen, Burlington, ON, Canada) and seeded in Falcon plates. After two hours of incubation at 37°C, the medium was replaced with 3mL of fresh medium.

Section 2.10: Cardiac Fibroblast Digestion and High Glucose Stimulation

Cardiac fibroblast cells were cultured for three days at which point the culture medium was removed and the cells were suspended in 2 mL of trypsin for 2 minutes in order to re-suspend cells that have adhered to the plate surfaces. The solution was then centrifuged for 5min at 8xg. The supernatant was discarded and the pellet was suspended in 2mL of culture medium. An equal number of cells was measured into the appropriate wells in a 96-well plate and incubated at 37°C. The next day, culture media is replaced with 100µL of either normal or high glucose media and incubated for one additional day at 37°C. Normal glucose (5.5 mM) media is comprised of 50mL DMEM supplemented with 2% FBS and 100 µg/ml penicillin-streptomyocin. High glucose media, 33 mM, is prepared the same way except for the addition of 0.243g of D-glucose (dextrose) (Invitrogen, Burlington, ON, Canada).

Section 2.11: MTT Proliferation Assay

After high glucose stimulation, 15µL of MTT solution is added to all wells. MTT solution is prepared by mixing 5mg of MTT (Thiazolyl blue: 98% TLC, Sigma) and 1mL of distilled water. The solution is stored at -20°C. Three to four hours after incubation with MTT solution at 37°C, the wells are decanted and 150µL dimethyl sulfoxide (DMSO) is added to each well. A multilabel reader is used to measure absorbance (540nm). Proliferation is assessed using the averaged ratios of high to normal glucose for each group.

Section 2.12: MMP activity measurement

Fibroblasts were cultured and stimulated as described in Section 2.9 and 2.10. The supernatant of cell culture media was collected, centrifuged at 1000rpm for 15 minutes and stored at -70°C until use. The fluorimetric SensoLyte® 390 Generic MMP Assay Kit (Anaspec, CA, Cat #72202) was used to measure MMP activity. As per the manufacturer's instructions the samples were incubated with 1 mM (APMA) for two hours at 37°C. MMP substrate was diluted 1:100 in assay buffer and 50µL were added to samples and control wells. Additional assay buffer was added in order to bring the total volume to 50µL in control wells only. The samples were incubated at room temperature for 30 minutes and fluorescence intensity was measured at Ex/Em=330 nm/390 nm.

Section 2.13: Toluidine Blue Staining for Mast Cells

Tissue was fixed in 10 % formalin and embedded in paraffin. Sections of 5 micron thickness were transferred to slides which were de-paraffinized in two changes of xylene and hydrated in two changes of 100% ethanol for 10 minutes, 95% ethanol for 3 minutes, 85% ethanol for 3 minutes and finally in 75% ethanol for 2 minutes before being washed and placed in acidic toluidine blue solution. After three minutes, the slides were subsequently dehydrated in ethanol and cleared in xylene before being mounted in a resinous medium. Mast cell numbers were visually quantified using a light microscope at 20x magnification.

Section 2.14: Statistical Analysis

The Student's t-test was used to compare differences between two treatment groups. ANOVA followed by the Tukey test was performed to examine statistical differences across multiple groups. A value of $p < 0.05$ was considered statistically significant and data are given as mean ± SD.

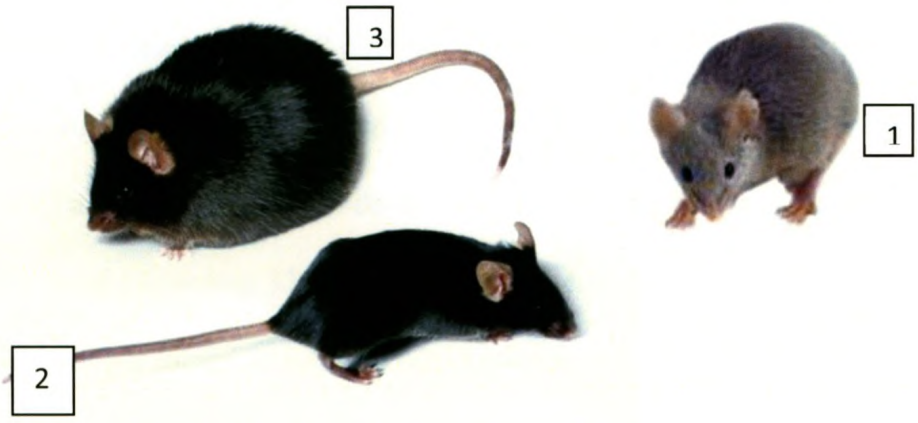
CHAPTER 3: RESULTS

Section A: Characterization of the mouse model

3.1 Generation of CAST Transgenic Mice

Mice homozygous for the LEPR mutation are sterile [108]. To produce the desired db/db-CAST model, homozygous transgenic mice over-expressing rabbit CAST are bred with mice heterozygous for the LEPR mutation in two successive crosses. Homozygous (db/db) and heterozygous (db+/-) offspring are separated on account of body size. As demonstrated by Figure 3.1A, a db/db mouse is twice as large as its heterozygous littermate. Among the db/db group, mice are screened for CAST overexpression by genotyping tissue obtained from mice tails upon weaning at three weeks of age (Figure 3.1B).

A



B

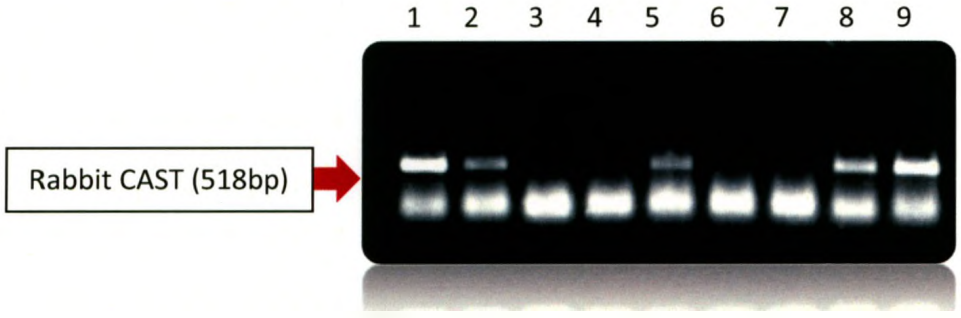


Figure 3.1: Characterization of WT, db/db and db/db-CAST mice

A) The picture depicts the qualitative differences between the three types of mice used in this study. Wildtype mice are grey (1). Heterozygous mice are black (2) and lean, db/db, and db/db-CAST mice are black and obese (3).

B) Genotyping. Tail samples were used to detect the presence of the rabbit CAST gene by PCR. A representative agarose gel for rabbit CAST gene from 9 different mice shows that lanes #1, 2, 5, 8 and 9 are positive and lane #3, 4, 6 and 7 are negative.

3.2: Insulin Measurement and General Parameters

The db/db mouse is a type 2 diabetic model that exhibits many consequences of diabetes including hyperglycaemia, hyperinsulinaemia and hyperlipidaemia [102]. At three months of age all db/db and db/db-CAST mice had high blood glucose defined as a blood glucose level exceeding 33mmol/L and was measured using a OneTouch glucose monitor. Body weight measurements do not show any significant difference in weight between db/db and db/db-CAST mice. Nor is there any significant difference in the weight of the heart (Table 3.1). To ensure differences in plasma insulin levels are not contributing to the results, insulin measurements were taken and found to be similar between db/db and db/db CAST groups (Figure 3.2).

Table 3.1: Weight and Blood Glucose

	Body Weight (g)	Heart Weight (g)	Blood Glucose
WT	35.06 ± 1.03	0.14 ± 0.03	Normal
db/db	68.52 ± 3.55	0.17 ± 0.01	High (>33mmol)
db/db-CAST	67.81 ± 3.91	0.16 ± 0.01	High (>33mmol)

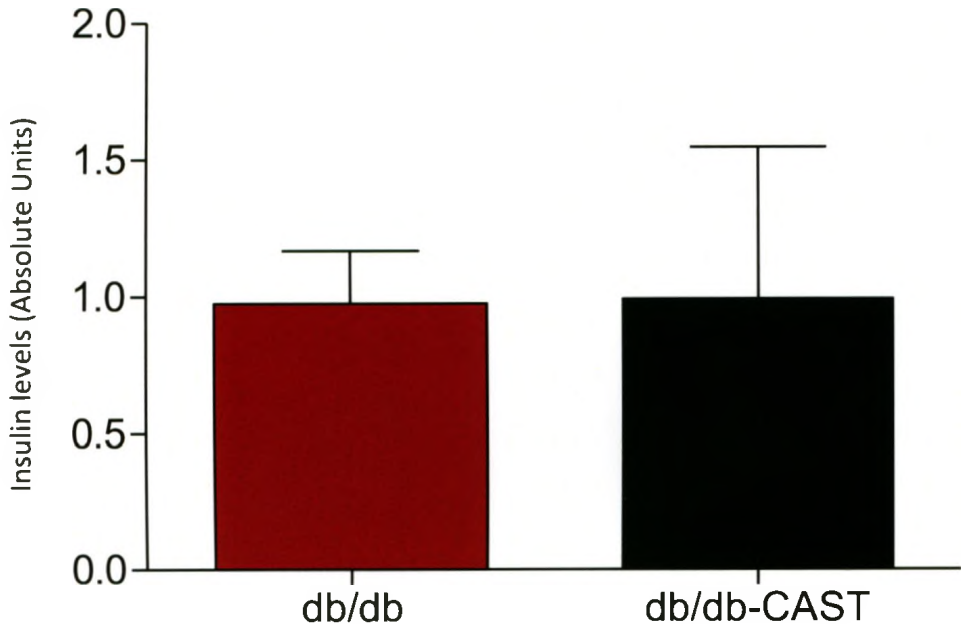


Figure 3.2: Insulin levels are similar in db/db and db/db CAST mice.

Measurements of insulin from both db/db and db/db CAST mice did not show any difference. Data are mean ± SD, n=5.

3.3: Calpain activity is elevated in diabetic mice

An activity assay measuring calpain activity in heart tissues from WT and db/db mice showed a significant increase in calpain activity in db/db mice compared to control mice (Figure 3.3). This ensured the db/db mouse model would be appropriate in which to test our hypothesis regarding calpain hyperactivity.

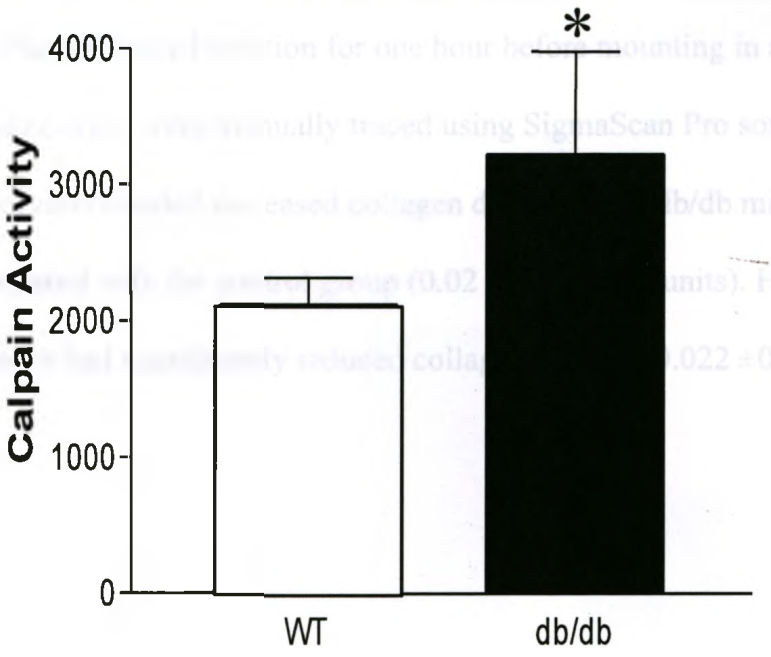


Figure 3.3: Diabetic hearts have elevated calpain activity.

Calpain activity assays confirm increased calpain activity in diabetic myocardia. Data are mean ± SD, n=5. **P* <0.05 *vs.* WT.

Section B: Myocardial Remodelling

3.4: Collagen Deposition

Myocardial fibrosis is a typical feature of cardiomyopathy in the diabetic heart and is characterized by excessive production and accumulation of extracellular collagen [9, 10, 19, 22, 51]. Tissue was formalin-fixed and embedded in paraffin. Sections of 7 micron thickness were de-paraffinized in xylene and rehydrated through graded alcohols then placed in Picrosirius red solution for one hour before mounting in a resinous medium. Collagen areas were manually traced using SigmaScan Pro software. The histological analysis revealed increased collagen deposition in db/db mice (0.035 ± 0.003 area units) compared with the control group (0.02 ± 0.004 area units). Heart sections of db/db-CAST mice had significantly reduced collagen content (0.022 ± 0.004 area units) (Figure 3.4).

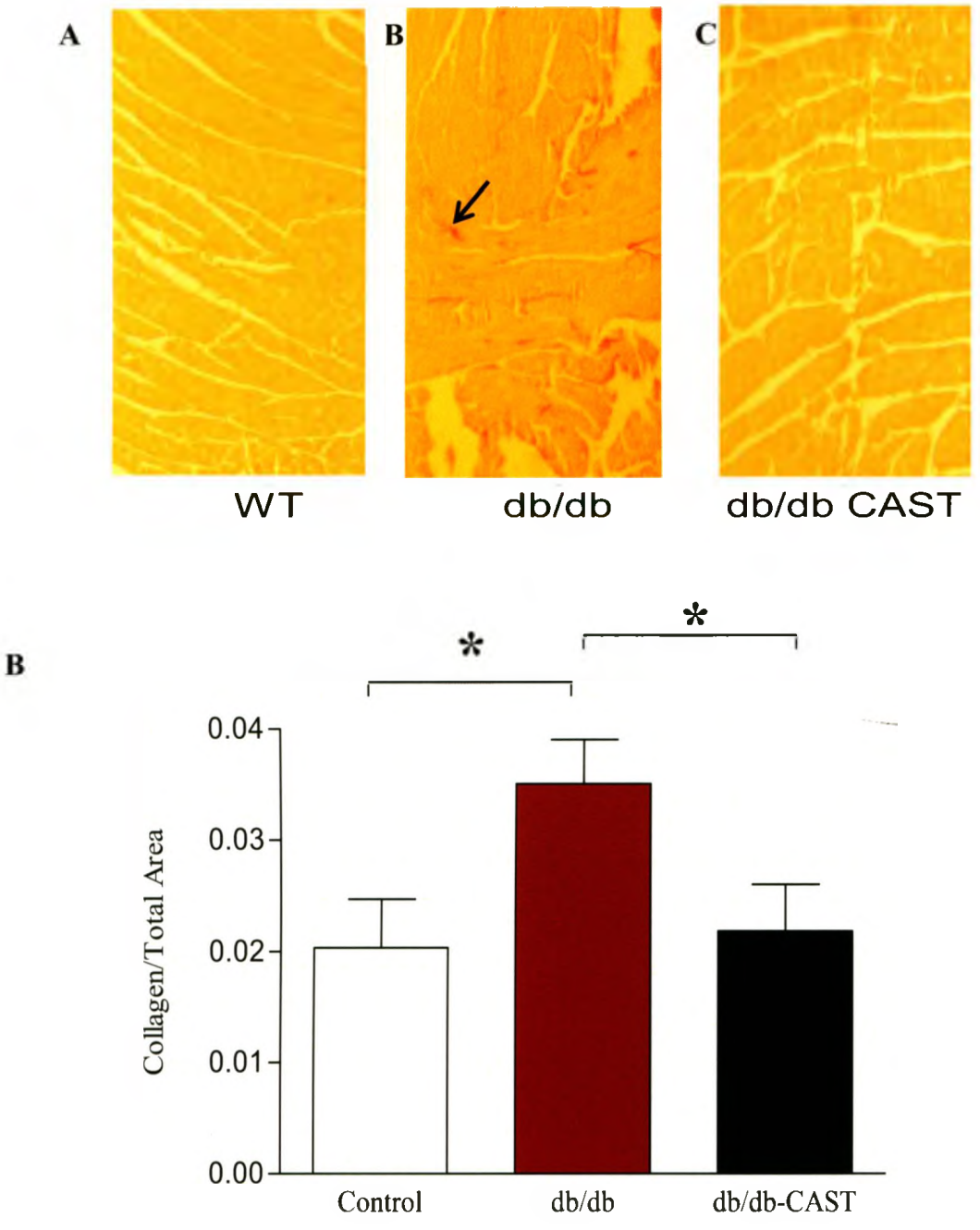


Figure 3.4: Collagen deposition in the diabetic heart is reduced by CAST over-expression

A) Representative micro-pictures for collagen staining from one out of five hearts in each group are presented. Collagen is highlighted by red staining (arrow, original magnification x20). B) Quantification of collagen content. The amount of collagen was assessed manually in 10 fields per tissue section and shown as the ratio between collagen quantified in the field and total area of the field. Data are mean ± SD, n=5/group. *$p<0.05$.

3.5: Gene Expression of Collagens I & III

In order to assess the impact of calpain hyperactivity on levels of collagens I and III, the major collagen types in the myocardium [56], heart tissues were analyzed using real time RT-PCR. Collagens I and III, expressed as a ratio of collagen I or III to the house-keeping gene, GAPDH, were up-regulated in db/db hearts but over-expression of CAST lowered their levels by 53% and 64%, respectively (Figure 3.5).

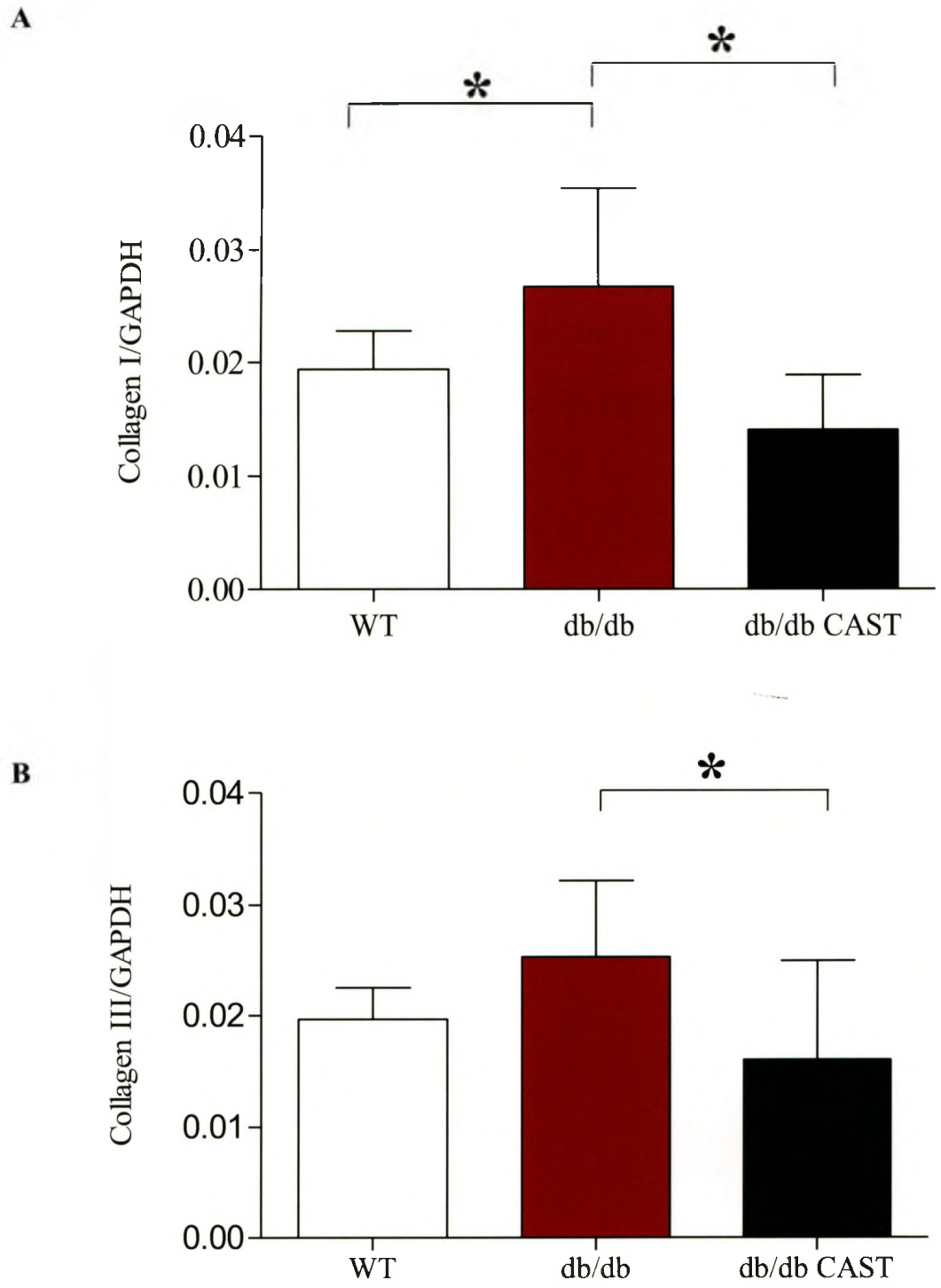


Figure 3.5: CAST downregulates collagen I and III mRNA expression.

The mRNA levels of collagen I (A) and collagen III (B) in the myocardium were quantified by real time RT-PCR. Data are expressed as the ratio of Collagen I or III to GAPDH. Data are mean ± SD, n=11-13/group, *$p<0.05$.

3.6: Collagen Ratios

The ratio of collagen I to collagen III at the mRNA level has also been reported as an indicator of fibrosis in the myocardium [109, 110]. In this study, there was a clear shift in the collagen ratio from db/db mice to db/db-CAST mice whose collagen ratio was equivalent to the control (Figure 3.6). Taken together, the results suggest calpain inhibition is important in reducing increases in absolute amounts of collagens I and III induced by hyperglycaemia.

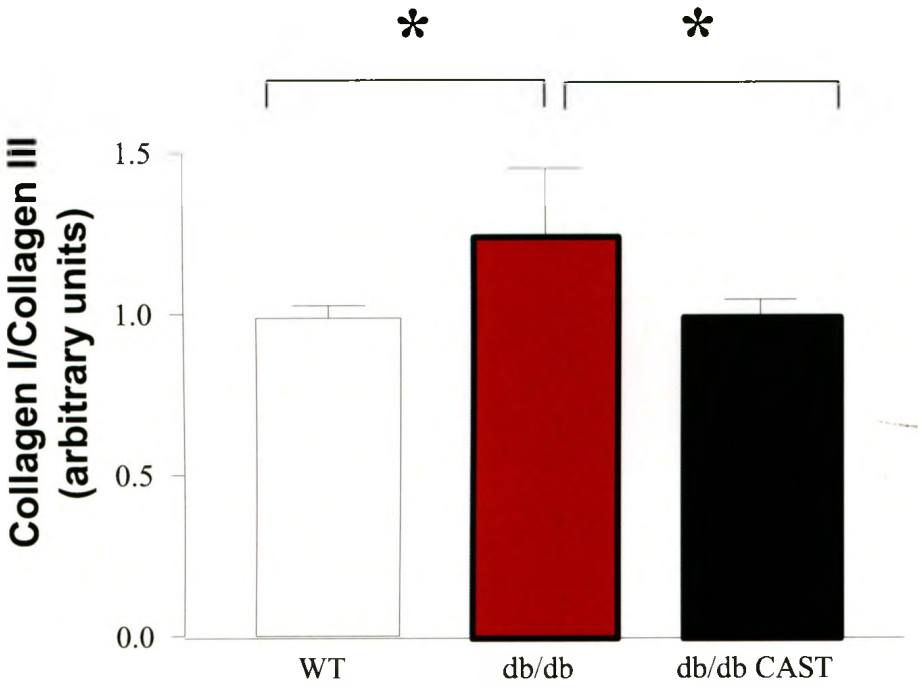


Figure 3.6: Collagen Ratios in the diabetic heart

Ratio of collagen I to collagen III at the mRNA level show that CAST maintains the necessary equilibrium between the two collagen types. Data are mean ± SD, n=11-13/group, *p<0.05.

3.7: Left Ventricular (LV) Wall Thickness

Structural changes at the microscopic level can translate to macroscopic alterations of the entire organ [51]. Echocardiography was used to assess left ventricular wall thickness. Although not statistically significant, the results show greater wall thickness in db/db diabetic mice compared to both control and db/db-CAST mice (Figure 3.7).

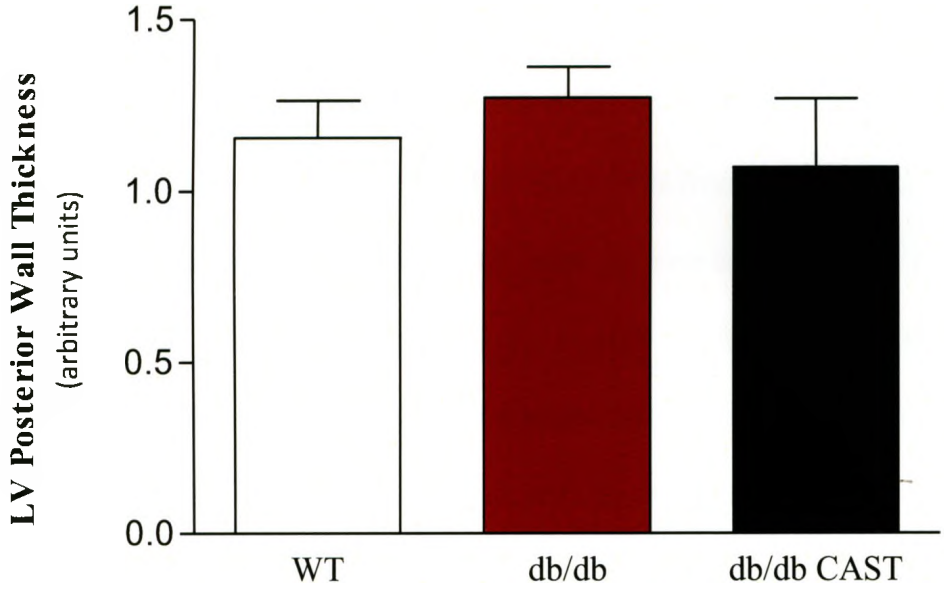


Figure 3.7: Diabetic hearts exhibit increased left ventricular wall thickness.

The LV wall thickness was assessed at the papillary level by echocardiography. Data are mean ± SD, n=6/group.

Section C: Fibroblast Proliferation and Differentiation

3.8: Hyperglycaemia induces fibroblast proliferation

Fibroblasts are believed to be key mediators of fibrosis in the injured or failing heart [63, 64, 111]. In the diabetic, cardiac fibroblasts are exposed to sustained hyperglycaemia [67]. To understand the effect this would have on the fibroblast population in the myocardium, cardiac fibroblast cells from wildtype mice were cultured and subjected to either normal (5.5 mM) or high glucose treatments (30 mM). The use of cells isolated from wildtype mice ensures the results will not be complicated by the effects of pre-existing hyperglycaemia. Exposure to high glucose for 24 hours was sufficient to cause an increase in fibroblast proliferation. However, under the same hyperglycaemic conditions, proliferation was reduced by pharmacological calpain inhibition (Figure 3.8A). Cardiac fibroblasts from db/db mice were also cultured. Compared to the control group, the db/db group had higher proliferation regardless of glucose exposure, in vitro (Figure 3.8B). Increases in fibroblast proliferation became more pronounced upon 24 hour high glucose treatment, once again confirming hyperglycaemic conditions are sufficient to induce a proliferative response in cardiac fibroblast cells. These results suggest that fibroblast cells are more prone to proliferation in db/db mice whether in normal or high glucose medium implying greater fibroblast activity in the diabetic myocardium.

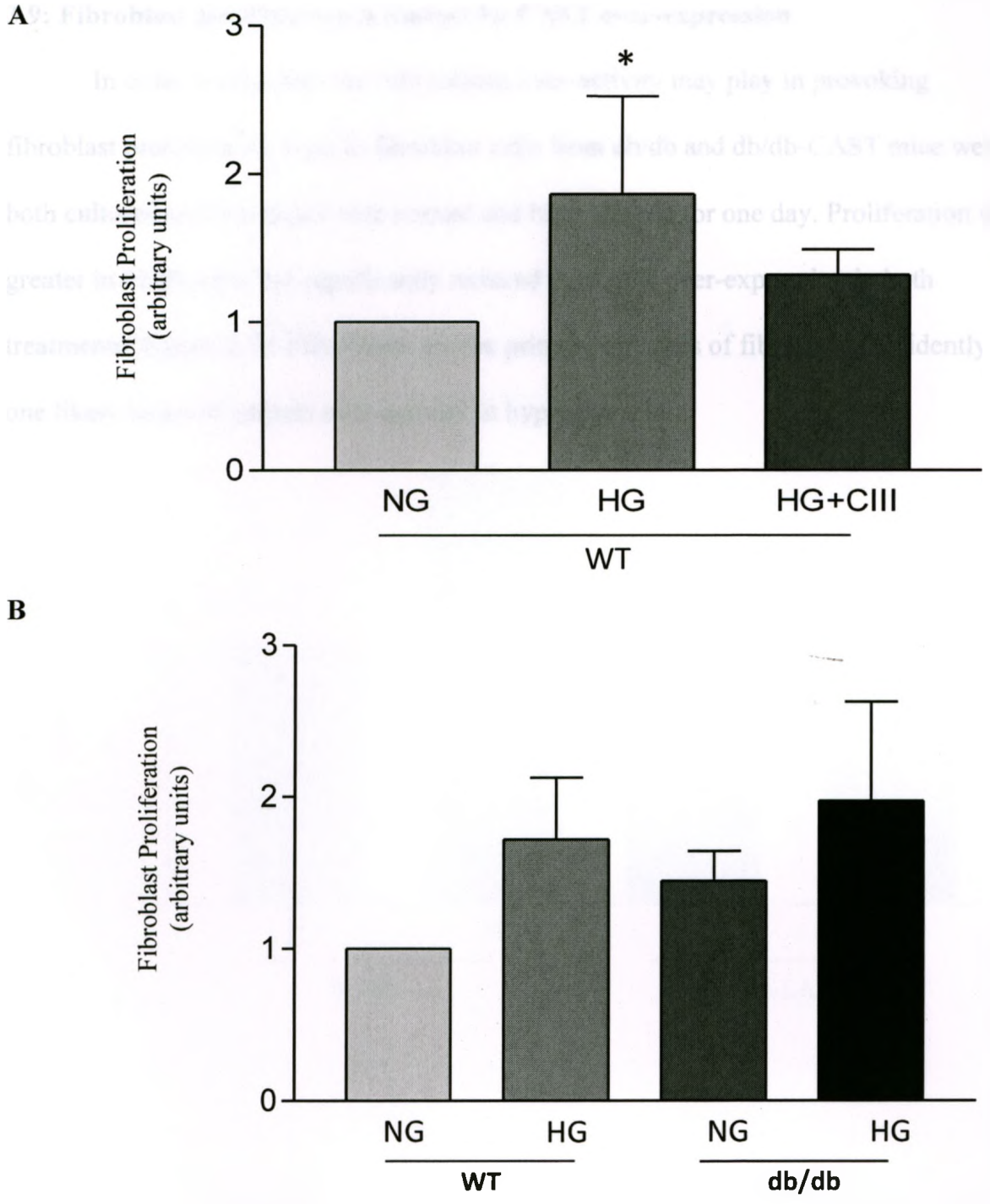


Figure 3.8: Proliferation of cardiac fibroblast cells from WT and db/db hearts.

Cardiac fibroblast cells were isolated from WT and db/db mouse hearts. Twenty-four hours after passaging, fibroblast cells were subjected to normal or high glucose for one day. An MTT assay was performed to assess cell proliferation. A) High glucose increased fibroblast proliferation which was reversed by calpain inhibitor-III (10 μM). Data are mean ± SD, n=4 *$p<0.05$. B) db/db cardiac fibroblasts were more proliferative than WT cardiac fibroblasts regardless of glucose treatment. Proliferation is expressed using arbitrary units. Data are mean ± SD, n=4.

3.9: Fibroblast proliferation is limited by CAST over-expression

In order to elucidate the role calpain over-activity may play in provoking fibroblast proliferation, cardiac fibroblast cells from db/db and db/db-CAST mice were both cultured and incubated with normal and high glucose for one day. Proliferation was greater in db/db cells but significantly reduced by CAST over-expression in both treatments (Figure 3.9). Fibroblasts are the primary effectors of fibrosis and evidently, one likely target of calpain over-activity in hyperglycaemia.

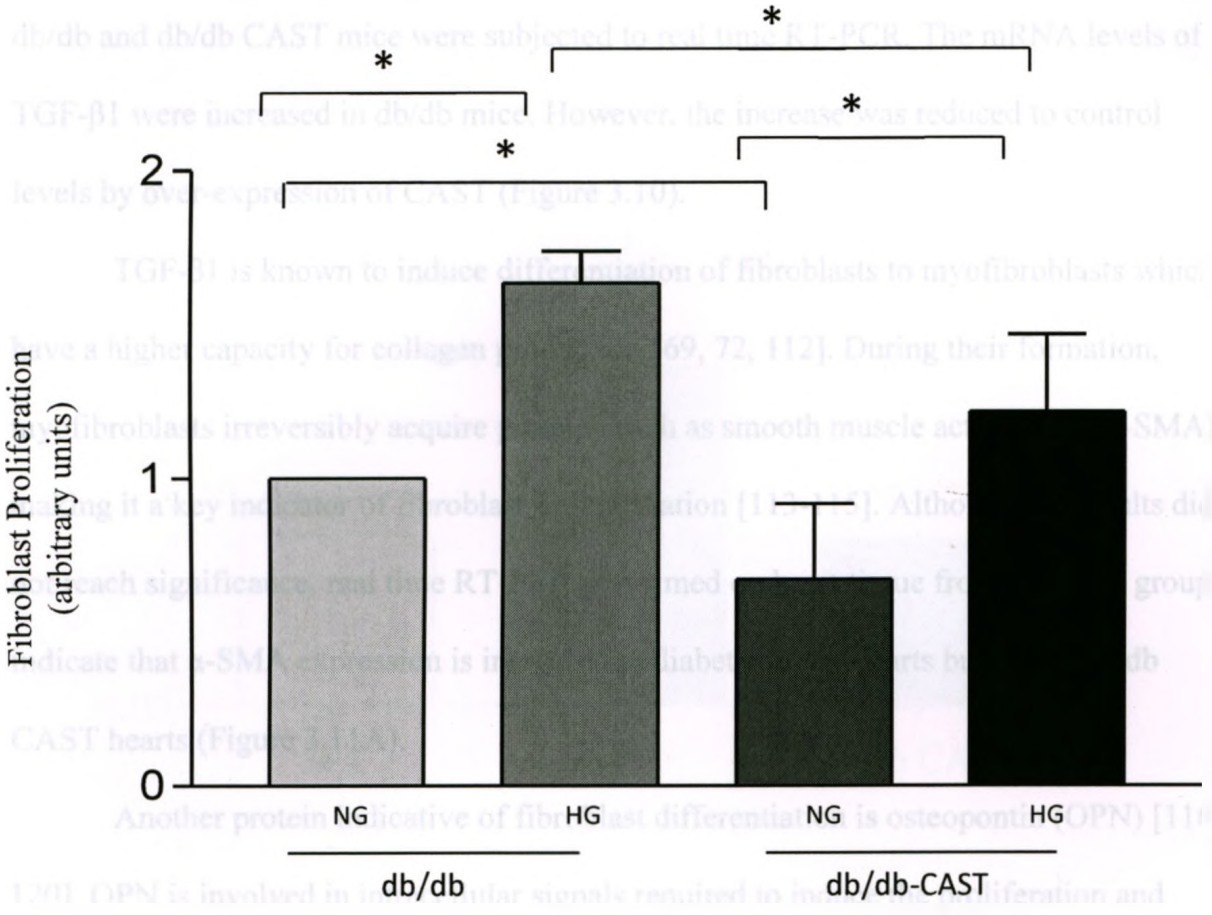


Figure 3.9: CAST over-expression limits fibroblast cell proliferation.

MTT assays of cultured cardiac fibroblast cells from db/db and db/db CAST mice shows CAST over-expression impedes proliferation of fibroblasts in diabetic murine hearts. Proliferation is calculated as a ratio of average high glucose to average normal glucose wells and expressed using arbitrary units. Data are mean ± SD, n=4, *$p<0.05$.

3.10: TGF-β1 promotes fibroblast differentiation in db/db hearts

TGF-β1 is a known fibrogenic cytokine [58, 65]. To determine if TGF-β1 is differentially expressed among the three experimental groups, heart tissues from WT, db/db and db/db CAST mice were subjected to real time RT-PCR. The mRNA levels of TGF-β1 were increased in db/db mice. However, the increase was reduced to control levels by over-expression of CAST (Figure 3.10).

TGF-β1 is known to induce differentiation of fibroblasts to myofibroblasts which have a higher capacity for collagen production [69, 72, 112]. During their formation, myofibroblasts irreversibly acquire proteins such as smooth muscle actin-alpha (α-SMA) making it a key indicator of fibroblast differentiation [113-115]. Although the results did not reach significance, real time RT-PCR performed on heart tissue from the three groups indicate that α-SMA expression is increased in diabetic db/db hearts but not in db/db CAST hearts (Figure 3.11A).

Another protein indicative of fibroblast differentiation is osteopontin (OPN) [116-120]. OPN is involved in intracellular signals required to induce the proliferation and migration of Myofibroblasts [116, 120, 121]. As expected, OPN levels were highest in diabetic hearts but CAST reduced OPN expression (Figure 3.11B). Taken together the results suggest calpain inhibition prevents fibroblast differentiation.

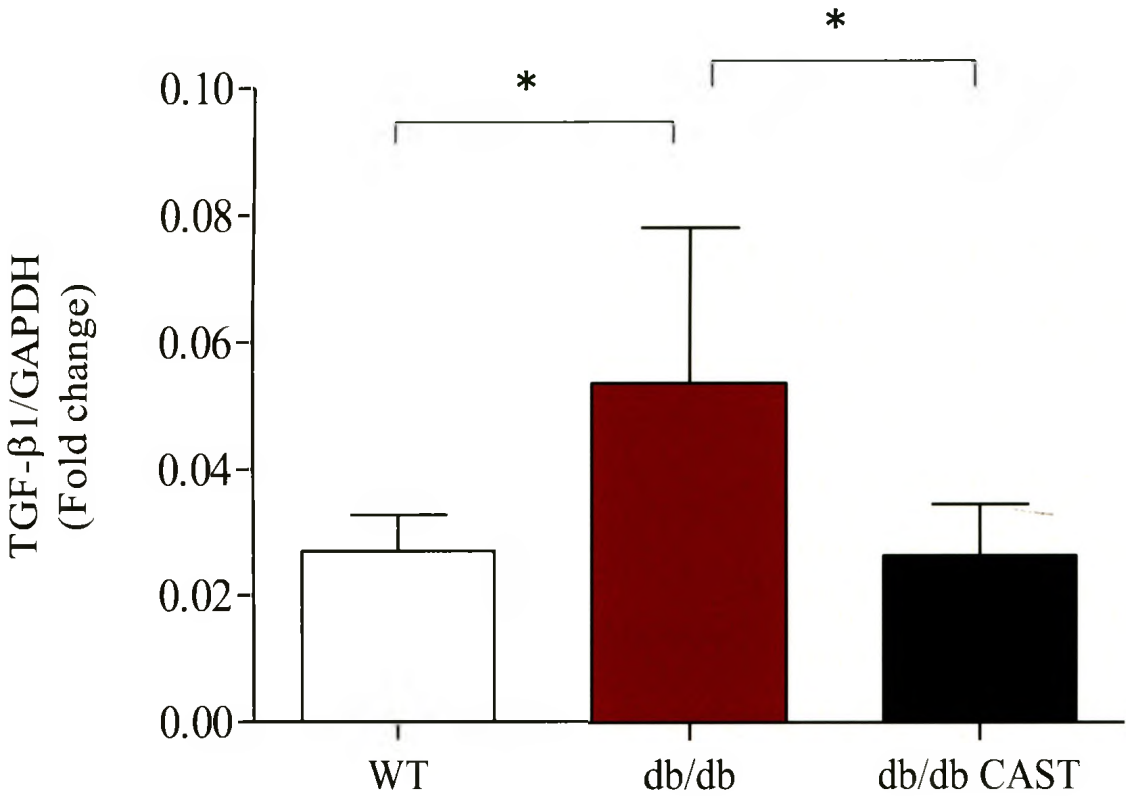


Figure 3.10: TGF-β1 mRNA expression is up-regulated in the diabetic heart

The mRNA levels of TGF-β1 in the diabetic heart were determined by real-time RT-PCR. Up-regulation of TGF-β1 is seen in diabetic hearts however, CAST over-expression leads to reduced TGF-β1 levels. TGF-β1 mRNA levels are expressed relative to GAPDH. Data are mean ± SD, n=3-7, *$p<0.05$.

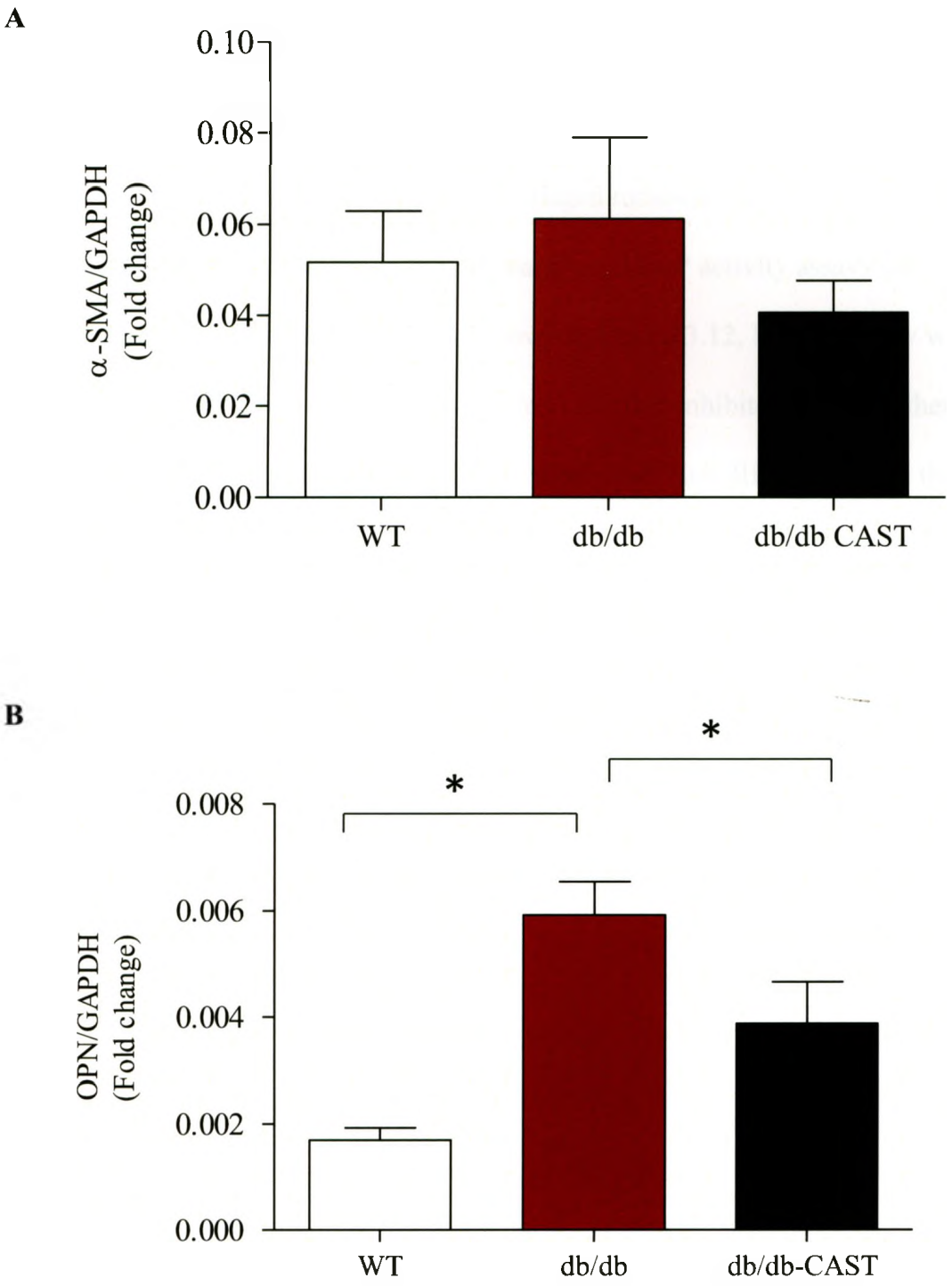


Figure 3.11: CAST inhibits fibroblast differentiation as indicated byα-SMA and OPN expression.

The mRNA levels of α-SMA (A) and OPN (B) in the heart were determined by real-time RT-PCR. mRNA expression is shown as a ratio of α-SMA or OPN to GAPDH. Data are mean ± SD, n= 8-11, *$p<0.05$.

Section D: Collagen Turnover and Inflammation

3.11: CAST reverses MMP and TIMP imbalance

In order to determine if impaired collagen turnover may be a perpetuating factor in the development of fibrosis in the myocardium, MMP activity assays were performed on cultured cardiac fibroblast cells. As shown in Figure 3.12, MMP activity was consistently lower in the high glucose treated cells that inhibited calpain either transgenically or pharmacologically using calpain-inhibitor III than in cells that were exposed only to a hyperglycaemic medium suggesting calpain and hyperglycaemia may work synergistically to increase MMP activity. The mRNA levels of TIMP1 and TIMP2 were measured in heart tissues collected from the three groups. As shown in Figure 3.13, the levels of both TIMP1 and TIMP2 mRNA were elevated in the diabetic group. However, CAST over-expression reduced TIMP1 and TIMP2 mRNA expression in the diabetic heart.

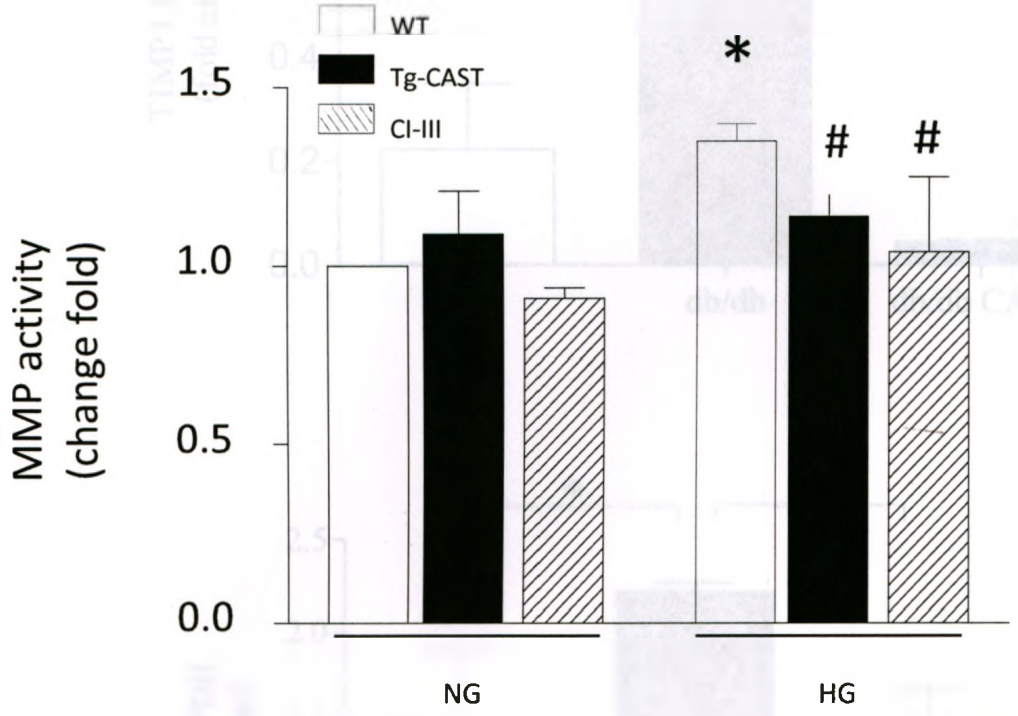


Figure 3.12: High glucose mediates increased MMP activity in cultured cardiac fibroblast cells.

The activity level of MMPs were decreased in fibroblast cells cultured from the myocardium of transgenic mice over-expressing CAST and wildtype mice whose cells were treated with calpain-inhibitor III. Data is mean ± SD, n=3, *$p<0.05$.

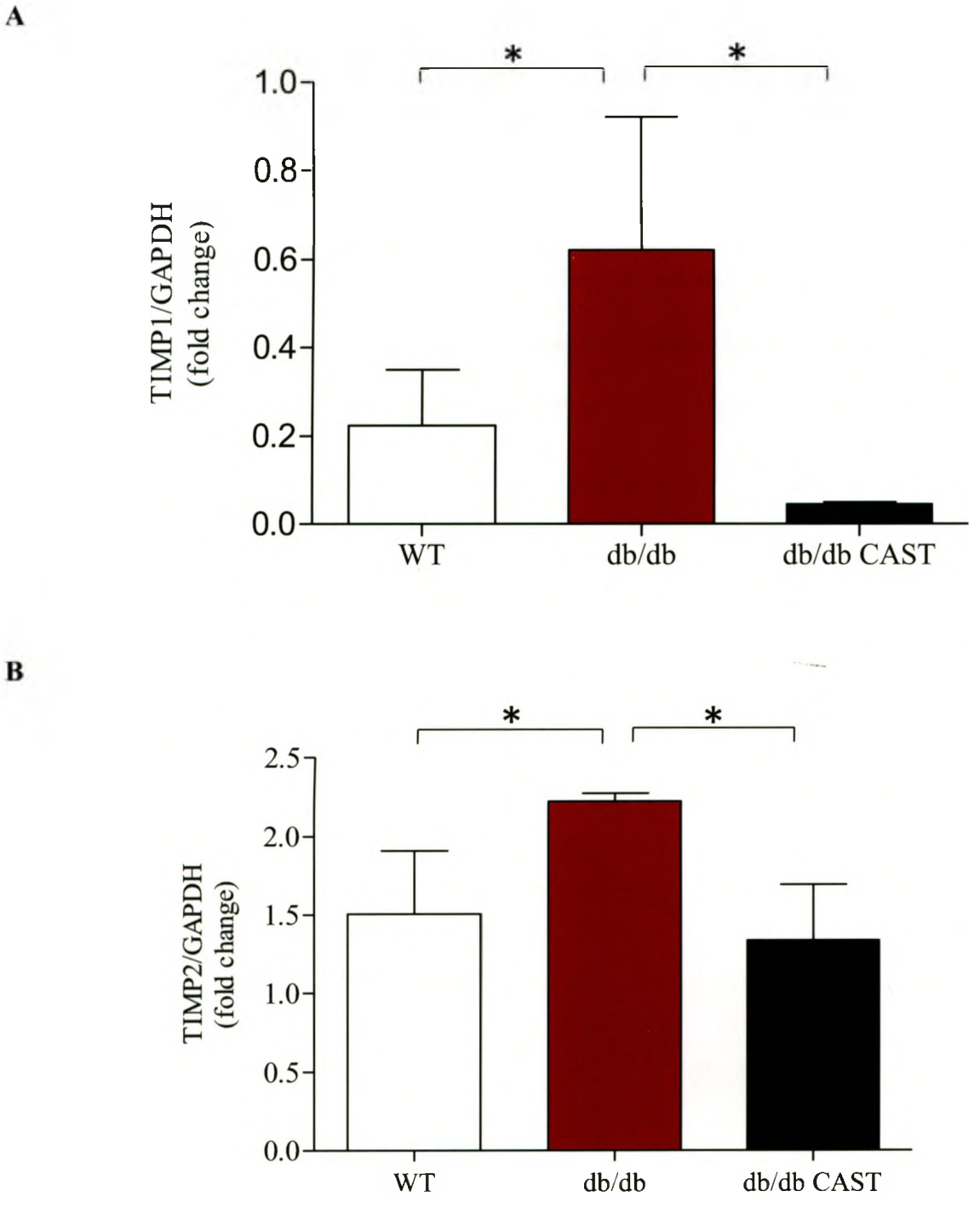


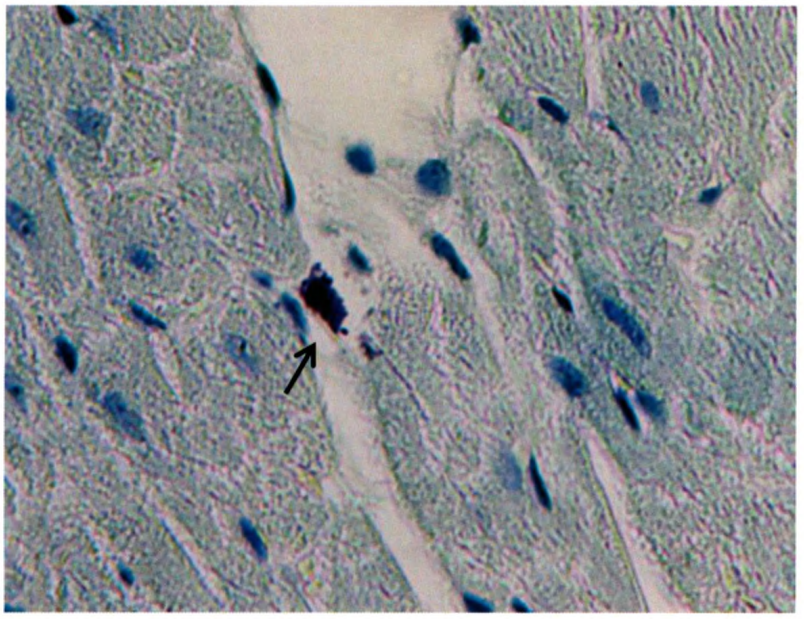
Figure 3.13: TIMP expression in diabetic db/db hearts

The mRNA levels of TIMP1 and TIMP2 were quantified by real-time RT-PCR in the hearts of diabetic mice. Their mRNA expression is shown relative to GAPDH. Data is mean ± SD, n=3-6, *$p<0.05$.

3.12: Mast cells in the diabetic heart

Mast cell infiltration is correlated with fibrosis in numerous organ systems [122, 123]. It has been suggested that mast cells may potentiate fibroblast proliferation and may thus promote the progression to myocardial fibrosis in diabetes [123-126] . In this study, heart sections of 5 micron thickness were stained in toluidine blue and mast cell numbers were visually quantified at 20x magnification. The db/db mice had greater mast cell density than wildtype mice. However, as shown in Figure 3.14, CAST over-expression prevented mast cell infiltration in the diabetic heart.

A



B

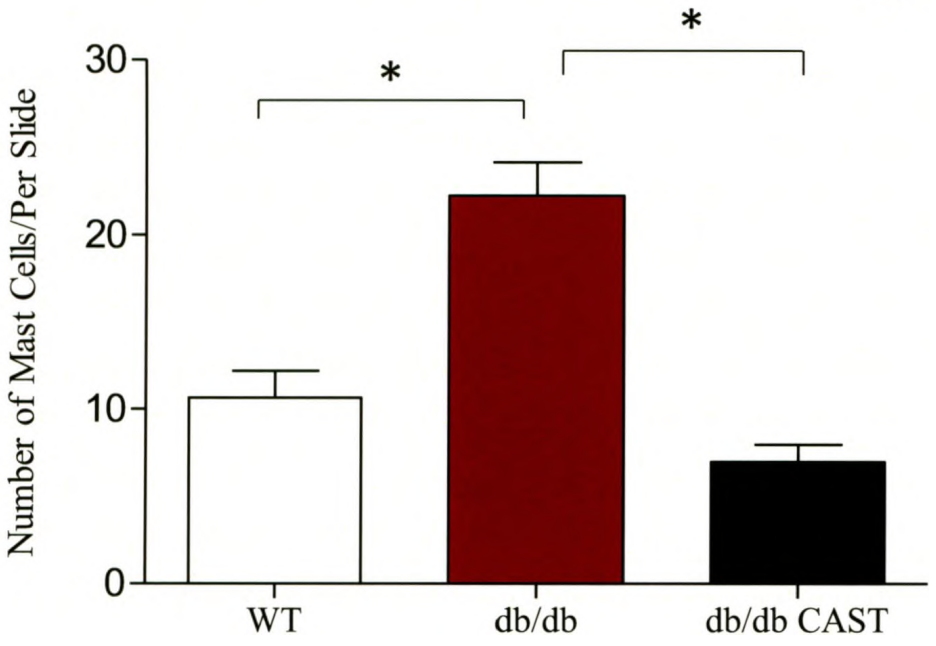


Figure 3.14 Mast cell hyperplasia is minimized by CAST over-expression.

Heart sections were stained using toluidine blue. A) A representative micro-picture for mast cell staining (arrow pointing to mast cells, purple-red colour signal) from 3-6 heart tissues in each group B) Mast cells were visually quantified at 20x magnification and expressed as the number of mast cells in each section. Data is mean ± SD, n=3-6, *$p<0.05$.

CHAPTER 4: DISCUSSION

Elevated calpain activity is a feature of the failing myocardium [91, 96, 101] . However, it has been relatively understudied as a potential mechanism underlying the maladaptive changes seen in the diabetic heart. This study demonstrated first that a successful animal model of type II diabetes over-expressing calpastatin could be generated in order to study the influence of calpain in diabetic heart failure. Second, collagen deposition is limited by calpastatin over-expression and may consequently hinder progression to heart dysfunction and eventual failure. Lastly, the fibroblast cell is one of the targets of calpain over-activity as calpastatin over-expression clearly prevented fibroblast activity including proliferation (Figure 4.1).

The db/db mouse exhibits the characteristic features of type 2 diabetes early in its life cycle [102, 103]. They become identifiably obese around three to four weeks of age. Elevation of blood glucose begins at four to eight weeks [102]. Initially, hyperglycaemia leads to insulin resistance and hyperinsulinemia [102, 103, 105]. Lipolysis and enhanced lipoprotein synthesis in the liver results in a greater supply of free fatty acids which eventually hijack cardiac metabolism [24-26]. Cardiac dysfunction appears soon after and becomes progressively more pronounced with age [106]. The db/db and db/db-CAST mice had the same metabolic features. Both grew obese within three weeks and were hyperglycaemic at the time of sacrifice. There was no difference in body weight and heart weight between the two and no modified behaviour in db/db-CAST mice was observed. Previous studies in our lab and others have shown calpastatin over-expression does not interfere with calpain activity in normal physiologic conditions [90, 95, 97]. Thus, the systemic effect of calpastatin-over-expression on metabolism, if any, may be negligible in db/db mice.

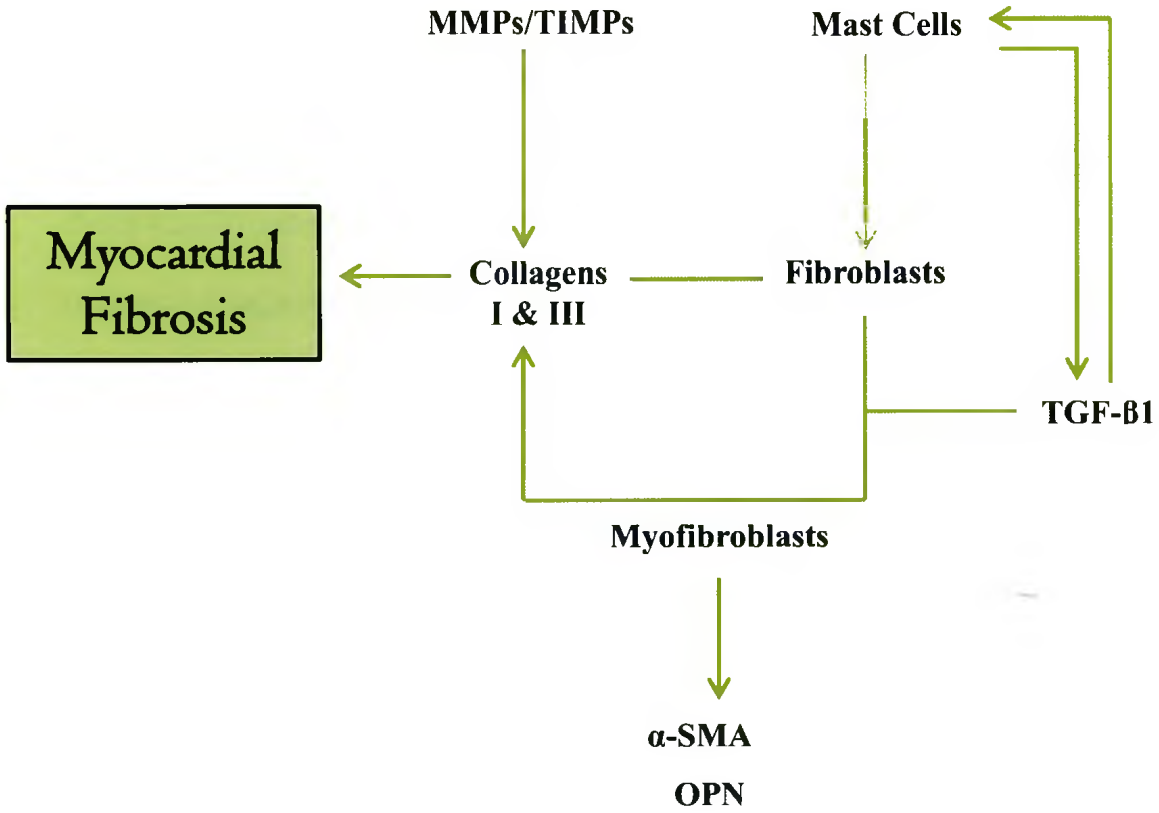


Figure 4.1: Schematic diagram representing the different players influenced by calpain inhibition.

This study showed calpain inhibition reduced the amount of absolute collagen deposited in the ECM. A mechanistic study points to the involvement of mast cells, TGF-β in stimulating fibroblast activity. Modifications in collagen turnover may also be involved.

In this study, we demonstrated that inhibition of calpain activity via calpastatin over-expression was successfully able to inhibit the increase in collagen deposition seen in db/db hearts. Collagen is vital to the maintenance of cardiac architecture. Yet, slight increases in collagen content can translate into mechanical impairments [57, 127]. An echocardiograph also showed the thickness of the left ventricular wall to be decreased in db/db-CAST mice suggesting microscopic improvements were sufficient enough to cause structural changes in the whole organ. Furthermore, the decrease in collagen accumulation in the treatment group resulted in a collagen ratio equivalent to the one in the wildtype control group. Collagen I is characterized by tensile strength whereas collagen III has greater elastic potential [56]. Thus, ratios between collagen types are significant as they may have a substantial impact on the diastolic and systolic function of the heart [110, 127]. Clearly as calpain over-activity is restrained, the amount of total collagen accumulation in the hyperglycaemic heart is diminished and the relative amounts of collagens I and III are brought back to homeostatic levels.

Having established a correlation between calpain activity and collagen content in the myocardium, we isolated and cultured cardiac fibroblast cells from all three groups of mice and subjected each set to high and normal glucose treatments. Many biological mechanisms thought to contribute to or cause myocardial fibrosis point in some way to the involvement of the fibroblast cell [63, 65, 74, 128]. The fibroblast is hard to ignore; it is the cell type largely responsible for maintaining the extracellular matrix [56, 57]. The study aimed to identify fibroblasts as targets of calpain hyperactivity and examine the mechanisms that relate calpain to fibroblast stimulation. Results showed a significant increase in proliferation under the influence of hyperglycaemia, not surprising since they

are a significant cell population in organs commonly affected by diabetes including the kidney and liver [63, 129]. The novel finding proved to be the effect of calpastatin on fibroblast proliferation. In comparison, cultured fibroblast cells from db/db-CAST mice had consistently reduced levels of fibroblast proliferation than db/db mice. Furthermore, when wildtype fibroblast cultures were subjected to high glucose in addition to a pharmacological calpain inhibitor, they mirrored the results from db/db-CAST cultures with a drop in proliferation compared to wildtype fibroblast cells that had been treated only with high glucose. Because the calpain inhibitor used is not entirely specific for calpain but also a number of other proteases, the results have their limitations. However, combined, the effects of calpastatin over-expression and pharmacological calpain inhibition make a compelling case for an association between fibroblast activation and calpain activity.

TGF-β1 has gained notoriety as a potent fibrogenic cytokine [65]. Upon tissue injury, it can spur fibroblasts into action by stimulating the release of ECM proteins, particularly collagen, and initiate wound healing and repair [58] . But further research into the mechanisms behind fibrosis has highlighted its ability to promote excess and secretion of collagen from fibroblasts [58, 65, 71, 72]. Among its pro-fibrotic abilities, TGF-β can induce the differentiation of fibroblasts to specialized contractile fibroblasts called myofibroblasts [70, 72, 74]. Over the course of their development, myofibroblasts irreversibly acquire proteins, one of which is α-SMA, a well-accepted marker of myofibroblast differentiation [113-115]. Another marker is OPN which coordinates intercellular signals required to integrate myofibroblast proliferation, migration and ECM deposition [117-120, 130-133]. OPN may require its secretion by myofibroblasts, and

may promote differentiation [120, 133]. This study showed an up-regulation of TGF-β in db/db mice as expected but in the calpastatin over-expression group the expression of TGF-β was reduced. The expression of α-SMA and OPN showed the same trend suggesting calpastatin prevented TGF-β stimulation and consequently prevents the cytokine from stimulating fibroblast differentiation.

Being a key pro-inflammatory cytokine, TGF-β1 can induce mast cell chemotaxis [82, 134-136]. Mast cell infiltration is a common observation in tissue repair processes regardless of the underlying diagnosis [137, 138]. Hearts from db/db mice showed increased mast cell density in tissue sections than db/db CAST mice indicating the involvement of calpain at some point in the inflammatory process. Previous studies, *in vitro*, have showed that mast cells may enhance fibroblast viability when co-cultured [137]. Interestingly, mast cells are a major source of TGF-βI and TNF-α for example, and may stimulate fibroblast activity through these secretion products [137, 139]. Yet, the exact relationship between mast cells and fibroblasts is still under examination.

Collagen synthesis is driven by fibrogenic factors, especially TGF-β, acting at the transcriptional level. However, collagen degradation is regulated by the MMP superfamily and impaired collagen turnover is a perpetuating factor in the development of myocardial fibrosis [76-80]. This study shows that stimulation by calpain is not limited only to the fibroblast, whether directly or indirectly through fibrogenic factors, but also to the remodelling machinery in the ECM. MMP levels were increased in the fibroblast cells of wildtype mice exposed to high glucose but not in the fibroblast cells of calpastatin transgenic mice. Wildtype fibroblast cells treated with calpain inhibitor III did not demonstrate an increase in MMP activity either thus highlighting the multidimensional

role of calpain in promoting the fibrotic phenotype. MMP production is regulated by TIMPs. In agreement with the results of our study, elevations in TIMP expression are a confirmed occurrence in diabetes [75, 140]. Yet, we showed that with calpastatin over-expression levels of TIMPs were reduced (TIMP1) or returned to normal levels (TIMP2).

This study has shown calpain inhibition as an effective means of decreasing total collagen accumulation in the diabetic heart. Experiments designed to understand the mechanisms responsible for the ability of calpain to promote fibrosis under hyperglycaemic conditions have pointed to over-activation of fibroblasts and the signalling pathways affecting fibroblast function. Derangements in MMP activity and stimulation of the immune response may also play a concerted role in the development of fibrosis in diabetic cardiomyopathy [134].

There are limitations in using mice to model human heart disease, a major one being the much higher heart rate and basal systolic contraction levels in the murine heart and the difference in resident cell populations in the myocardium across species [102]. But although the cardiac phenotype present in the mouse cannot be extrapolated to the human diabetic in its entirety, the fact that rodents are not susceptible to atherosclerosis is a major boon in their use because a diabetic model without any complicating coronary artery disease provides a suitable backdrop to examine the evidence of a cardiomyopathy unique to diabetics [102]].

CHAPTER 5: FUTURE STUDIES

Collagen deposition was consistently decreased in db/db CAST mice. Undoubtedly, this should restore some measure of function to the myocardium. The most obvious next step should consist of a functional study using echocardiography or perfusion techniques to determine if the myocardia from mice over-expressing calpastatin have improved mechanical properties compared to db/db mice.

In the course of cellular metabolism, mitochondria generate numerous reactive oxygen species such as superoxide; highly reactive and capable of damaging nucleic acids and protein structure [44, 141-144]. The cell has numerous antioxidant defense mechanisms however, should ROS production exceed ROS elimination, oxidative stress, another hallmark event in the course of diabetic cardiomyopathy, will ensue [145-149]. A transcription factor, NF-κB, is implicated in promoting fibrosis by enhancing ROS production [81, 150]. NF-κB is also an important mediator of the inflammatory response and activates numerous cytokines including TGF-β and TNF-α and participates in the recruitment of immune cells, including mast cells [151, 152]. Its activation is initiated by the degradation of its inhibitor, IκB which can be accomplished by calpain [153]. Thus, it could prove worthwhile to measure NF-kB activity by extracting nuclei from all three groups of mice to determine if NF-kB activity is down-regulated by calpastatin. Further, in previous studies in our lab we demonstrated that hyperglycaemia is associated with increased activity of calpain in mitochondria in a type 1 diabetes model. The same approach can be used to determine if increased mitochondrial calpain plays a causal role in raising whole cell ROS production in a type 2 model.

Arguably, the greatest leap in understanding the calpain/calpastatin system would come with a precise account of the biological roles calpain performs and upon which

substrates it acts. Unfortunately, this task is complicated for a number of reasons. Calpain is distributed throughout the cytosol and as result has no association with a specific organelle that would hint to its purpose [88, 89]. Nor is the amino acid sequence of any calpain isoform indicative of its substrate because calpain functions conformationally [88, 89]. The protease is also difficult to purify in an undegraded form [75]. Another significant obstacle in determining calpain function experimentally is that the calcium requirement for calpain activation in experiments is much higher than physiological concentrations found in living cells rendering laboratory studies difficult [88, 89]. However, continued research on the calpain molecule itself and indirect studies on the consequences of altered calpain activity in various pathologies, such as this study on diabetic cardiomyopathy, should provide a clearer picture of this protease's elusive properties.

CHAPTER 6: SIGNIFICANCE OF STUDY

Cases of diabetes continue to rise despite awareness initiatives and extensive research into its cause and treatment [1-4]. Rising obesity rates, sedentary lifestyles and an aging population are all contributing factors. Though the economic cost is enormous, the cost to patients is greater [4]. The ultimate goal is to find ways in which diabetes can be cured. However, this goal is complicated by the introduction of numerous related illnesses and pathologies especially diabetic cardiomyopathy[2-4]. This study demonstrated, for the first time, that hyperglycaemia-induced fibrosis in the myocardium can be reversed or inhibited by calpain inhibition. Yet, this only scratches the surface as calpain activation has been shown to have key importance in the promotion and development of the fibrotic phenotype not only in the diabetic but all patients of heart disease [95, 97, 99, 101, 154]. Additionally, understanding the calpain-calpastatin system may lead to a deeper understanding of cardiovascular biology in general, both in normal and diseased states.

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