Targeting caveolin-3 for the treatment of diabetic cardiomyopathy

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ABSTRACT

Diabetes is a global health problem with more than 550 million people predicted to be diabetic by 2030. A major complication of diabetes is cardiovascular disease, which accounts for over two-thirds of mortality and morbidity in diabetic patients. This increased risk has led to the definition of a diabetic cardiomyopathy phenotype characterised by early left ventricular dysfunction with normal ejection fraction. Here we review the aetiology of diabetic cardiomyopathy and explore the involvement of the protein caveolin-3 (Cav3). Cav3 forms part of a complex mechanism regulating insulin signalling and glucose uptake, processes that are impaired in diabetes. Further, Cav3 is key for stabilisation and trafficking of cardiac ion channels to the plasma membrane and so contributes to the cardiac action potential shape and duration. In addition, Cav3 has direct and indirect interactions with proteins involved in excitation–contraction coupling and so has the potential to influence cardiac contractility. Significantly, both impaired contractility and rhythm disturbances are hallmarks of diabetic cardiomyopathy. We review here how changes to Cav3 expression levels and altered relationships with interacting partners may be contributory factors to several of the pathological features identified in diabetic cardiomyopathy. Finally, the review concludes by considering ways in which levels of Cav3 may be manipulated in order to develop novel therapeutic approaches for treating diabetic cardiomyopathy.

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Abbreviations:
ACCORD, Action to Control Cardiovascular Risk in Diabetes; ADVANCE, Action in Diabetes and Vascular disease: PreterAx and DiamicroN Controlled Evaluation; AGE, advanced glycation end product; Akt/PKB, protein kinase B; Ang II, angiotensin II; AP, antennapedia; APD, action potential duration; AVV, adeno-associated viral vector; BNP, B-type natriuretic peptide; β2AR, β2-adrenergic receptor; CAD, coronary artery disease; CAMKKII, calcium/calmodulin-dependent kinase 2; Cav1, caveolin-1; Cav2, caveolin-2; Cav3, caveolin-3; CIBM, caveolin binding motif; CICR, calcium induced calcium release; CIRI, cardiac-ischaemia reperfusion injury; C-MAD, C-terminal membrane attachment domain; CMR, cardiac magnetic resonance imaging; CCR4, cholesterol recognition/interaction amino acid sequence and consensus; CSD, caveolin scaffolding domain; CLUPID, Calcium Uprgulation by Percutaneous Administration of Gene Therapy in Cardiac Disease; DCM, diabetic cardiomyopathy; DPP4, dipeptidyl peptidase-4; DRM, detergent-resistant membrane; EC, excitation; ECG, electrocardiogram; ECM, extracellular matrix; EF, ejection fraction; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; FTA, free fatty acid; FRET, fluorescence resonance energy transfer; GADPH, glyceraldehyde phosphate dehydrogenase; GLP-1, glucagon-like peptide-1; GLUT-1, glucose transporter-1; GLUT-4, glucose transporter-4; HbA1c, glycosylated haemoglobin; HF, heart failure; IIDD, insulin-dependent diabetes mellitus; IDDM, insulin-independent diabetes mellitus; iNOS, inducible nitric oxide synthase; IPC, ischaemic preconditioning; IRS1, insulin receptor substrate 1; LDL, low-density lipoprotein; LQTS, long QT syndrome; LTCC, L-type voltage-gated calcium channel; LV, left ventricular; MAPK, mitogen-activated protein kinase; MI, myocardial infarction; mRNA, micro RNA; MRI, magnetic resonance imaging; NADPH, nicotinamide adenine dinucleotide phosphate; ncRNA, non-coding RNA; NCX, sodium-calcium exchanger; N-MAD, N-terminal membrane attachment domain; NOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; ox-CaMKII, oxidised calcium/calmodulin-dependent kinase 2; PARP, poly ADP ribose polymerase; PCEK, phosphoinosistide-3 kinase; PKA, protein kinase A; PKC, protein kinase C; PIB, phospholamban; PPARα, peroxisome proliferator-activated receptor alpha; PPM, post-translational modification; RAAS, renin–angiotensin–aldosterone system; RISK, reperfusion injury salvage kinetic; ROCK, RhoA/Rho kinase; ROS, reactive oxygen species; RyR, ryanodine receptor; SERCA, sarcoplasmic/endoplasmic reticulum Ca2+ ATPase; SIDS, sudden infant death syndrome; SQD, superoxide dismutase; SR, sarcoplasmic reticulum; SRL, stress hormone imaging; STZ, streptozotocin; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; VD, tissue Doppler imaging; TGF-β1, transforming growth factor-beta 1; TNRα, tumour necrosis factor alpha; TTE, transthoracic echocardiography; T2D, thiazolidinedione; UCP, uncoupling protein; UKPDS, United Kingdom Prospective Diabetes Study; VADT, Veterans Affairs Diabetes Trial.

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1. Introduction

Diabetes encompasses a group of chronic, metabolic diseases where-by metabolic dysregulation and oxidative stress trigger the development of further complications including, cardiomyopathy, retinopathy, nep-hropathy and nephropathy. Diabetes is approaching pandemic proportions with a worldwide prevalence of 366 million individuals in 2011. It is estimated that by 2030 more than 550 million people will be diabetic (Diabetes, 2012). Diabetes is characterised by hyperglycaemia (>7.0 mmol/L, fasting glucose) as a result of insufficient insulin secretion and/or activity (American Diabetes Association, 2010). There are two main types of diabetes, type I and type II (T1DM and T2DM), both of which are characterised by abnormal glucose metabolism. T1DM or insulin-dependent diabetes mellitus (IDDM) is caused by the autoimmune destruction of pancreatic β cells leading to insulin deficiency (American Diabetes Association, 2010). T1DM usually presents at an early age with symptoms including polyuria, polydipsia, weight loss and fatigue. In comparison, T2DM or insulin-independent diabetes mellitus (IDDM) develops due to insufficient insulin secretion or insulin resistance (American Diabetes Association, 2010). T2DM accounts for approximately 90% of cases of diabetes. Unlike T1DM, the risk of developing T2DM can be reduced by changes to diet and exercise (Diabetes, 2012).

1.1. Diabetic cardiac complications

As mentioned previously, diabetes is associated with an array of systemic conditions. For example, more than two thirds of diabetic patients develop cardiovascular complications (Boudina & Abell, 2010) with cardiovascular disease being attributed to approximately 44% and 52% of fatalities in patients with T1DM and T2DM respectively (Diabetes, 2012). Moreover, diabetes is associated with an increased cardiovascular risk, with women having a greater risk than men (5-fold and 2-fold respectively) (Kannel et al., 1974). It is currently not known why diabetes poses a greater threat to the health of the female cardiovascular system compared to that of the male although a link to oestrogen-female risk, with women having a greater risk than men (5-fold and 2-fold respectively) (Kannel et al., 1974). It is currently not known why diabetes poses a greater threat to the health of the female cardiovascular system compared to that of the male although a link to oestrogen (Armoni et al., 2005). Data from both animal and human studies have led to the identification of the key features of DCM; left ventricular (LV) dysfunction, myocardial hypertrophy and fibrosis, metabolic dysregulation and defects in myocardial contractile properties. From a clinical perspective, these structural and functional disturbances have been divided into three stages; early, middle and late (Fang et al., 2004). Early stage DCM is recognised by mild LV dysfunction in the presence of a normal ejection fraction (EF) (Boyer et al., 2004). Molecular level abnormalities include cardiac steatosis and increased free fatty acids (FFA) (Maisch et al., 2011), impaired Ca²⁺ homeostasis and depleted levels of glucose transporter proteins, GLUT-1 and GLUT-4 (Rodrigues et al., 1998; Belke et al., 2000). The middle stage is characterised by diastolic and systolic dysfunction with a reduced EF (~50%) alongside hypertrophy, fibrosis and dilation (Maisch et al., 2011). Insulin resistance increases, leading to the formation of advanced glycation end products (AGEs) (Cooper, 2004) and over-activation of the renin–angiotensin–aldosterone system (RAAS) (Fang et al., 2004). There are also changes to the levels of transforming growth factor (TGF)-β1 and insulin growth factor 1 as well as increased apoptosis and necrosis (Miki et al., 2013). Finally, late stage DCM patients exhibit systolic and diastolic dysfunction in association with hypertrophy and microvascular changes, leading to HF (Miki et al., 2013). Recently, DCM has been classified into 4 different stages, with stage 4 reflecting late stage DCM but with the addition of coronary artery disease (CAD) and macroangiopathy (Maisch et al., 2011), as shown in Fig. 1.

1.1.2. Is diabetic cardiomyopathy a distinct phenotype?

There is evidence to suggest that the pathogenic mechanism of DCM may differ during the early stages of T1DM and T2DM. In T1DM, there is a reduction in glucose uptake in the heart (Avogaro et al., 1990; Doria et al., 1991; Herrero et al., 2006). This may be linked to the down-regulation of the glucose transporter, GLUT-4 (see Section 2.1) (Camps et al., 1992). As a consequence, the heart relies on FFA as an alternative energy source, increasing FFA uptake and utilisation (Avogaro et al., 1990; Doria et al., 1991; Herrero et al., 2006). This dependence on FFA utilisation may be further exacerbated by increased levels of systemic FFA, due to increased lipolysis in the absence of insulin inhibition (Heptulla et al., 2003). However, it must be noted that insulin treatment has been shown to restore short-term cardiac metabolism (Avogaro et al., 1990). On the other hand, studies have shown the development of insulin resistance in T1DM, concurrent with increased cardiac lipid content and cardiac dysfunction (Perseghin et al., 2003; Nadeau et al., 2010).

Similarly, there is a down-regulation of GLUT-4 in T2DM, reducing cardiac glucose uptake (Armoni et al., 2005). However, cardiac responsiveness to insulin remains intact in patients with T2DM (Utriainen et al., 1998). Instead, T2DM is associated with increased systemic FFA, most likely due to obesity and the inability of adipocytes to cope with the excess calories associated with the Western diet. This increased FFA promotes FFA uptake and utilisation in the heart, outcompeting glucose as a metabolic substrate (Randle et al., 1964; Coort et al., 2004; Peterson et al., 2004). The increased use of FFA in turn further inhibits glucose utilisation, promoting the metabolic switch to FFA oxidation and metabolic inflexibility (Nuutila et al., 1992). This metabolic switch has been shown to occur before the onset of hyperglycaemia and insulin resistance (Buchanan et al., 2005). The metabolic shift from glucose to FFA usage in T1DM and T2DM marks the beginning of the pathogenic journey to DCM. Therefore, although the initial stages of disease manifestation may differ between T1DM and T2DM, the development of cardiovascular complications and end-points is closely paralleled.
long-term prospective study of T1DM found no significant changes to myocardium structure suggesting that diabetes may not have direct effects on the heart (Genuth et al., 2013; Litwin, 2013). However, it has recently been suggested that there are four distinct stages of DCM. Initially, DCM presents as mild LV dysfunction with a normal ejection fraction (EF) in the presence of increased free fatty acids (FFA) and hyperglycaemia (early/stage 1). As the disease progresses, heart function (red line) decreases with a concomitant increase in structural changes (blue line). The middle stage (stages 1–3) is typified by diastolic and systolic dysfunction with a reduced EF alongside structural changes including dilation and fibrosis. On the molecular level, insulin resistance ensues along with advanced glycation end-product (AGE) formation. Late stage (stages 3–4) DCM is characterised by systolic and diastolic dysfunction in association with hypertrophy and microvascular changes. The addition of stage 4 is to aid the definition of DCM in the presence of coronary artery disease (CAD) and macroangiopathy. CAN, cardiovascular autonomic neuropathy; GLUT4, glucose transporter-4; RAAS, renin–angiotensin–aldosterone system; TGF-β1, transforming growth factor-beta 1.

There is still no consensus on the definition of DCM and the diagnostic criteria fail to differentiate between other cardiomyopathies. As the presentation of DCM can vary and the early stages may be asymptomatic for long periods, it is difficult to examine the pathogenesis of DCM. However, hyperglycaemia, hyperinsulinemia and hyperlipidaemia are distinct insults to the diabetic heart that are otherwise absent in non-diabetic patients. Given the 200–500% increase in risk for developing cardiovascular complications compared to patients without diabetes, DCM clearly poses a significant health problem. Irrespective of whether DCM is a distinct phenotype future work should focus upon the mechanisms that lead to the development of cardiac pathologies in the diabetic myocardium in order to help establish early diagnosis and subsequent treatment.

1.3. Animal models for the study of diabetic cardiomyopathy

Ideally, long-term, large-scale human studies need be carried out to characterise DCM, especially as current knowledge is largely based on small animal models. However, patient studies are complicated by ethical issues coupled with genetic heterogeneity between individuals and exposure to a range of different environmental factors, leading to varied progression and severity of the disease and the presence of other comorbidities. Therefore, animal models have tremendous value for investigating and dissecting the complex mechanisms underlying the pathogenesis of diabetes of both T1DM and T2DM (King, 2012). The development of rodent models has been central to diabetes research as they can be manipulated to develop diabetes in the absence of atherosclerosis. T1DM is typified by the loss of pancreatic β cells and so the use of chemical induction to destroy the islets e.g. using streptozotocin (STZ) or Alloxan, has been widely exploited. However, as can be seen from Table 1 and elsewhere data are not always consistent. Furthermore, in terms of cardiac function the STZ rat is reported to exhibit reduced diastolic function which progresses to systolic dysfunction.

![Clinical stages of diabetic cardiomyopathy (DCM).](image-url)
The table provides an overview of studies that have examined the E-C coupling protein profiles in T1DM and T2DM models.

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The table provides an overview of studies that have examined the E-C coupling protein profiles in T1DM and T2DM models.

⇑ indicates that the parameter is elevated, ⇑ depressed, = no change, n.r., not reported.

(Weinstein et al., 2006) with other investigators reporting preserved systolic function (Wei et al., 2003). The study by Loganathan used MRI to evaluate the cardiac hemodynamics of the STZ myocardium and thus the discrepancies in characterising cardiac function may be due to the method of detection with MRI providing greater sensitivity compared to echocardiography methods and allowing for the identification of subtle changes to systolic function. Some of the inconsistencies associated with for example protein expression levels may be associated with the size and frequency of the STZ dose, route of administration and the interval after injection that the animals are examined. Interestingly, a recent report has also shown that the development of cardiomyopathy, in the STZ female rat is more rapid than in the aged-matched male (Moore et al., 2014); mirroring the human diabetic female predisposition to a higher mortality as described in the introduction. In addition to the STZ/alloxan models there are several genetically derived T1DM mouse models such as the Akita mouse (Ins2Akita) and the OVE26 mouse (Song et al., 2007). The Akita mouse phenotype is characterised by only diastolic dysfunction (LaRocca et al., 2012) whereas the OVE26 mouse exhibits impaired diastolic and systolic dysfunction. The majority of T2DM rodent models are genetically derived (monogenic and polygenic) and include the db/db mouse (Aasum et al., 2003), New Zealand Obese (NZO) mouse (Kluge et al., 2012) the ob/ob mouse (Christoffersen et al., 2003; Boudina et al., 2005), the Zucker Diabetic Fatty (ZDF) rat (Paradise et al., 1985) and the Goto-Kakizaki rat (Santos et al., 2003) (non-obese). All are characterised by decreased systolic and diastolic function and exhibit Ca²⁺ handling abnormalities (Huynh et al., 2014). Despite the value of animal models for providing instructive insights into the many facets of diabetes pathophysiology, exhibiting both systemic and organ-specific effects, it is clear that there is no one single model that can be directly translated to human T1DM or T2DM. Therefore, a caveat to any investigation using an animal model of disease is that data should always be considered within the context of the species and the model. For example, the heart rate in a mouse is typically more
than four times that of man, a factor that will impact upon the cardiac contractile cycle and the relative contribution of the proteins that regulate Ca^{2+}-handling/excitation–contraction (E–C) coupling. As such large animal models of diabetes with a closer cardiac phenotype to man e.g. porcine have been developed (Bellinger et al., 2006) but these are much more expensive and more difficult to manipulate and maintain compared to murine models. The establishment of human tissue biobanking facilities also offers researchers routes to study biopsy samples and biofluids to help translate findings from animal models into man. However, there is no single diagnostic test to assess the presence of DCM and due to the insidious nature of diabetes many asymptomatic DCM patients go undetected for years. As such the true prevalence of DCM is not known. Therefore, opportunities to gain access to samples from biorepositories to investigate the early stages and development of the disease are likely to be limited.

1.4. Mechanisms of diabetic cardiomyopathy

The current therapies for treating diabetes are limited in terms of efficacy and side effects. Therefore, it is important to understand the pathogenesis of cardiovascular disease in diabetes for the development of novel therapeutics to efficiently treat and manage DCM. As mentioned previously, LV diastolic dysfunction is a hallmark of early DCM; this is when there is impaired refilling of the atria/ventricles with blood, preventing the heart to relax normally between beats (see Section 1.4.3). As the disease progresses, systolic dysfunction develops. Alongside this cardiac dysfunction, major anatomical changes have been identified in diabetic patients, including LV hypertrophy and myocardial fibrosis (Di Bello et al., 1995). These changes are further complicated and compounded by systemic metabolic dysregulation and the excessive formation and accumulation of reactive oxygen species (ROS) within cardiac myocytes. Ultimately, this leads to adversely modified proteins involved in regulating cellular processes in the heart. An overview of the pathological processes underlying diabetes is presented in Fig. 2. There are numerous reviews of DCM providing overviews of the principal mechanisms of disease (e.g. Huynh et al., 2014). Therefore, this article will focus mainly upon the effects of hyperglycaemia and oxidative stress upon the myocardium at the molecular level and explore the putative involvement of the protein caveolin-3 (Cav3) in the pathology of diabetic cardiomyopathy. Changes to expression levels of Cav3 have been demonstrated as a causative agent of a wide range of cardiovascular pathologies, including contractile dysfunction, arrhythmias, cardiac hypertrophy, heart failure (HF) and myocardial ischemia (Park et al., 2002; Woodman et al., 2002; Aravamudan et al., 2003; Cohen et al., 2003b; Koga et al., 2003; Hayashi et al., 2004; Fujita et al., 2006; Vatta et al., 2006; Horikawa et al., 2008; Tsutsumi et al., 2008). To explore whether modulation of this protein may be a viable therapeutic target for treating DCM we will first review the cellular pathways mediated by Cav3 within the context of diabetes.

2. Impaired insulin signalling

The diabetic heart displays an altered metabolic phenotype, typified by reduced glucose uptake and utilisation with increased FFA uptake and oxidation. Physiologically, a consequence of reduced uptake and utilisation of glucose causes the reliance on FFA oxidation for energy production. Normally, healthy hearts use FFAs as their primary source of energy, reproducing ~70% of total ATP under resting conditions (Neely & Morgan, 1974; Lopaschuk et al., 1994, 2010). In contrast, the diabetic heart depends solely on FFA oxidation. The increased FFA levels cause the activation of the transcription factor, peroxisome proliferator-activated receptor alpha (PPARα). This causes the upregulation of genes associated with the uptake of FFA and β oxidation (Ferrer, 2007). Additionally, PPARα inhibits the utilisation of glucose, reinforcing the metabolic switch to FFA oxidation (Bayeva et al., 2013). Similarly, the accumulation of FFA antagonises insulin signalling by inhibiting the phosphorylation and activation of the insulin receptor substrate 1 (IRS1) (Dresner et al., 1999; Griffin et al., 1999; Yu et al., 2002; Solinas et al., 2006).

Hyperglycaemia is a hallmark of diabetes and is associated with hyperinsulinaemia and insulin resistance (Shanik et al., 2008) typified by the loss of the glucose transporter, GLUT-4, impairing insulin signalling and reducing glucose entry into the cell (Goldin et al., 2006) resulting in a reduction in ATP production (Nikolaidis et al., 2004). GLUT-4 is tissue specific, localised mainly to skeletal and cardiac muscle and adipose tissue. Glucose uptake occurs at the plasma membrane via GLUT-4 and is subsequently phosphorylated so that it is retained within the cells. When insulin levels drop GLUT-4 is trafficked back into the intracellular membrane compartments (Watson & Pessin, 2001). In support of the role of insulin signalling in GLUT-4 localisation, studies of fat cells isolated from STZ treated rats (T1DM model) have shown that dosing with insulin restores GLUT-4 levels with glucose uptake comparable to that of control animals (Berger et al., 1989). Thus glucose diffusion into cells is regulated by the continuous shutting of GLUT-4 within the cell, and the number and duration of GLUT-4 molecules at the surface membrane (Bryant et al., 2002). However, the mechanisms governing GLUT-4 trafficking are complex with extensive investigations indicating that this process is not only stimulated by insulin (Karlsson et al., 2002) but is also influenced by stress, anoxia, ischemia and catecholamines in the heart (Sun et al., 1994; Wheeler et al., 1994, Eger et al., 1999). Our understanding of the mechanism of GLUT-4 translocation was significantly advanced by data showing that GLUT-4 is targeted to particular regions of the plasma membrane, termed caveolae, a specialised type of lipid raft (Ros-Baro et al., 2001; Chamberlain & Gould, 2002).

2.1. The role of caveolae for insulin signalling

Caveolae are cave-shaped invaginations of the cell membrane that are generally described as 50–100 nm structures, although caveolae up to 2000 nm in diameter have been reported (Sheets et al., 1997; Pralle et al., 2000; Edidin, 2001; Kirkham et al., 2008). There is now a large body of data within the literature that implicate caveolae as specialised microdomains that spatially regulate (i) vesicular transport, including transcytosis and endocytosis (Kurzhalia et al., 1992; Schnitzer, 2001); (ii) cholesterol homeostasis (Murata et al., 2005); (iii) mechanosensing (Yagi et al., 1988; Radel & Rizzo, 2005; Parton & Simons, 2007; Sinha et al., 2011) and (iv) signal transduction (Harvey & Calaghan, 2012). Studies spanning more than 20 years have established the caveolae environment as essential for insulin signalling. For example disruption of caveolae using the reagent β-methyl-cyclodextrin, which depletes cholesterol from the membrane, was shown to lead to impaired glucose uptake with experiments replacing the cholesterol resulting in regeneration of the caveolae structures and restoration of insulin signalling (Gustavsson et al., 1999; Parpal et al., 2001). Insulin signalling pathways were first shown to result in the phosphorylation of caveolin-1 (Cav1), the main structural protein component of caveolae (Mastick et al., 1995). Later studies showed that Cav1 has a role in stabilising the insulin receptor activity allowing for an increase in the phosphorylation of the receptor itself, and conferring protection from proteasomal degradation pathways (Cohen et al., 2003a, 2003c). Significantly, transgenic mice with a global Cav1 knock-out fed a high fat diet were found to develop elevated levels of FFAs, hypertriglyceridaemia and hyperinsulinaemia, with those on a normal chow diet exhibiting insulin resistance (Cohen et al., 2003c). The N-terminal region of Cav1, termed the caveolin scaffold domain (CSD) (Yamamoto et al., 1998; Nystrom et al., 1999) was shown to interact with the insulin-receptor in adipose tissue, compounding the importance of caveolins for modulating insulin signalling.
2.2. The role of caveolin-1 and caveolin-3 for glucose uptake

In addition to Cav1, two other isoforms of caveolin have been identified termed caveolin-2 (Cav2) and caveolin-3 (Cav3). Cav1 and Cav2 are predominantly expressed in adipocytes, fibroblasts and endothelial cells (Rothberg et al., 1992; Lisanti et al., 1994; Scherer et al., 1994). Cav3 is muscle specific and is found in both striated muscle (skeletal and cardiac), as well as smooth muscle (Song et al., 1996; Tang et al., 1996; Way & Parton, 1996). Thus, in terms of the cardiac setting, Cav3 is found exclusively within the cardiac myocyte and Cav1 and Cav2 are localised to the endothelium and endocardium regions of the heart; though there is evidence to suggest that there may be some degree of overlap (Rybin et al., 2003; Robene et al., 2008; Cho et al., 2010) with evidence of a Cav1 population within the myocardium (Head et al., 2006; Patel et al., 2007; Kozera et al., 2009). A central role for caveolins in caveolae formation has been demonstrated by genetic ablation of Cav1 or Cav3 resulting in the loss of caveolae (Drab et al., 2001; Galbiati et al., 2001b; Park et al., 2002; Woodman et al., 2002). Likewise, the addition of caveolin to cells, which ordinarily would not form caveolae can result in de novo caveolae formation (FRA et al., 1995; Whiteley et al., 2012).

The relationship of Cav3 and insulin signalling was established by genetic ablation, with the Cav3 null mice exhibiting impaired insulin signalling and glucose homeostasis with increased adiposity and body weight (Oshikawa et al., 2004). However, loss of Cav3 leads to perturbation of insulin-stimulated glucose uptake in all other organs except the skeletal muscle, which may suggest that compensatory mechanisms are activated by the ablation of Cav3, such as GLUT-4 overexpression. Both the Cav1 and Cav3 knockout mouse exhibit elevated levels of both GLUT-4 and Akt, suggesting that some of the downstream molecular pathways are common to the two isoforms but are dependent upon the expression levels of the tissue specific caveolin. However, downstream phosphorylation substrates e.g. GSK-3β and IRS-1 shown to be disturbed in fat pads of the Cav1 deficient mouse remained unchanged in the skeletal muscle of Cav3 knock-out mice, although the phosphorylation was 3-fold greater compared to controls. Alignment of Cav1 and Cav3 shows a high degree of sequence similarity with 75% identical residues (95% similarity) (Razani et al., 2002). Specifically, the CSD is fully conserved mapping on to residues 55–74 in the Cav3 primary sequence. Given this level of homology between the two isoforms it was not surprising that Cav3 was also reported to be a positive regulator of the insulin receptor in striated muscle (Yamamoto et al., 1998). Further analysis supported these findings by showing that although the density of the insulin receptor was unchanged in the Cav3 knockout mouse, Cav3 has a protective/stabilisation role against hyperphosphorylation and degradation (Capozza et al., 2005).

In the diabetic heart, Penumathsa and Maulik (Penumathsa et al., 2008, 2009) identified increased levels of Cav1 but decreased levels of Cav3. Further experimentation using immunoprecipitation methods identified an association between GLUT-4 and Cav3 but that Cav1 bound to endothelial nitric oxide (eNOS); both Cav1 and Cav3 are well characterised inhibitors of eNOS (see Section 4.1). Other investigators...
had previously described that increased phosphorylation of AMP-activated protein kinase (AMPK) enhances GLUT-4 translocation to the plasma membrane (Russell et al., 1999). Building upon these studies, work from Penumathsa and colleagues (Penumathsa et al., 2009) proposed a mechanism whereby increased levels of Cav1 leads to depressed phosphorylation of Akt/ PKB and AMPK through the inhibition of eNOS, which impairs expression and translocation of GLUT-4 to the caveolae, whereas loss of Cav3 protein impacts upon GLUT-4 stabilisation once it is within the caveolae. In conclusion, it is the relative levels of Cav1 with respect to Cav3 and their relationship and interactions with eNOS and GLUT-4 that are critical factors that regulate glucose uptake.

2.2.1. The effect of hyperglycaemia upon caveolae and caveolin expression

In addition, to playing a crucial role in glucose uptake there is also evidence to indicate that hyperglycaemia exerts effects upon expression levels of Cav1 and Cav3. One study focussing upon the myocardium found that culturing H9C2 cardiomyocytes, a rat heart cell line, in high glucose (25 mM) led to depressed levels of Cav3 when compared to low glucose (5 mM) and mammotrophic controls, but only after prolonged exposure (36–48 h) (Lei et al., 2013). The same group also revealed a reduction to Cav3 expression in the STZ rat heart. Similarly, macrophages exposed to high glucose concentration were shown to have a reduced number of caveolae, with those remaining having smaller dimensions. Also, there was a reduction in Cav1 levels accompanied by an increase in NO synthase activity due to elevated activation of NADPH oxidase (Hayashi et al., 2007). Caveolae formation at the plasma membrane is intimately associated to the free cholesterol content of the cell, which is regulated by the levels of LDL. Experiments targeting cholesterol synthesis in Madin–Darby canine kidney (MDCK) epithelial cells using simvastatin, so that cholesterol levels were lowered by ~50%, showed that not only was there a reduced number of caveolae but also that Cav1 expression was depressed at both the protein and transcription level (Hailstones et al., 1998). However, increasing LDL concentrations have been reported to upregulate both caveola formation and Cav1 mRNA expression in fibroblasts (Fielding et al., 1997), an effect that can be reversed by the application of oxysterols. Whilst diabetes is not associated with a greater propensity for elevated LDL levels when matched for sex, age and weight with non-diabetics (National Cholesterol Education Program Expert Panel on Detection & Treatment of High Blood Cholesterol in, 2002), the circulating LDLs may be more atherogenic in the diabetic as they are more susceptible to oxidation and glycation, which leads to greater adherent properties and facilitates penetration into the vessel wall (Krentz, 2003). Hyperlipidaemia is a characteristic of T2DM, and as such treatments aimed at lowering cholesterol synthesis (e.g. Zocor) are proven to reduce the risk of developing new-onset diabetes. These studies serve to reinforce the complex relationship between GLUT-4 translocation and caveolae, Cav1, Cav3 and LDL levels and cellular glucose uptake. However, the current understanding of these mechanisms remains incomplete especially within the heart.

In summary, caveolae and caveolins have a dual role for regulating glucose homeostasis (i) through a direct interaction of caveolins with the insulin receptor to stabilise activity and (ii) to provide a microenvironment for localisation and stabilisation of GLUT-4 by the differential modulation of the Akt/AMPK/eNOS signalling pathway.

3. Impaired excitation–contraction coupling is a hallmark of diabetic cardiomyopathy

Cardiac contractility is impaired in T1DM and T2DM in both animal models and humans (Huynh et al., 2014). At the molecular level, excitation–contraction (E–C) coupling is regulated by the action of several proteins within the t-tubule, sarcosome and sarcoplasmic reticulum (SR). Depolarization of the plasma membrane leads to the flow of extracellular calcium (Ca^{2+}) into the cytoplasm through the L-type voltage-gated calcium channels, LTCCs, (I_{CaL}) localised to the t-tubules and surface membrane. This influx of Ca^{2+} initiates the opening of the ryanodine receptors (RyR2) within the junctional SR, that release a bolus of Ca^{2+} from the SR into the cytosol, generating the systolic Ca^{2+} transient, formed by the summation of the Ca^{2+} sparks from the RyRs (Cheng et al., 1993), which in the healthy heart are synchronous across the ventricle. This finely tuned process is termed calcium induced calcium release (CICR) (Bers, 2001). Elevated levels of intracellular Ca^{2+} bind to the myofilament protein troponin C triggering the sliding of thin and thick filaments initiating the mechanical contraction of heart (Bers, 2002). Relaxation requires [Ca^{2+}], to return to diastolic levels, which occurs by two mechanisms (i) the transfer of Ca^{2+} back into SR through the action of SERCA2a and (ii) the extrusion of Ca^{2+} from the cell by the sodium–calcium exchanger, NCX1, located within the t-tubules and plasma membrane. Each of these channels and receptors is in turn regulated by the association of an array of accessory proteins (Bers, 2004).

The modulation of E–C coupling proteins in T1DM or T2DM, especially in human subjects, remains patchy and for some of the Ca^{2+} cycling proteins the data is incongruent (see Table 1 for summary). Depression of I_{CaL}, through the LTCC and reduction of the channel expression levels have been identified in two different mouse models of T2DM, the ZDF rat (Howarth et al., 2011) and db/db obese mouse (Pereira et al., 2006). However, LTCC expression levels and activity in models of T1DM are less consistent. For example, in both a rodent and canine model of T1DM (induced by STZ and alloxan respectively) no change to the L-type calcium current density was found (Lacombe et al., 2007; Lengyel et al., 2007). In contrast, another investigation reported a reduction in I_{CaL} density in the T1DM Akita mouse (Ins2Akita mutation) concomitant with a reduction of cell surface LTCCs (Lu et al., 2007). The data are more consistent for SERCA2a with an overall consensus for depressed activity in both T1DM and T2DM e.g. Zhao et al. (2006) and Belke et al. (2004). There is less agreement between protein level changes (see Table 1) but this may relate to the levels of phospholamban (PLB) and its phosphorylation status. The dynamic balance between the unphosphorylated and phosphorylated states of PLB regulates the activity of the Ca^{2+} pump. Several studies have reported a change to the relative levels of total PLB and phosphorylated form in the diabetic heart (Kim et al., 2001; Choi et al., 2002). A reduction in SERCA2a pump function will limit Ca^{2+} transfer back into the SR and so impair the ability of the cardiac myocytes to relax (observed clinically as diastolic dysfunction). The inability to pump Ca^{2+} back into the SR leads to Ca^{2+} overload which has been suggested to result in a reduced Ca^{2+} sensitivity of the contractile proteins (Jweied et al., 2005).

RyR2 expression profiles were reported unchanged in a db/db mouse model of diabetes (T2DM) and in an STZ T1DM (Bidasee et al., 2001; Belke et al., 2004). However, both models reported a change to RyR2 function, with further experimentation finding a reduction in the open probability in the STZ rat. Interestingly, dosing the rats with insulin restored RyR2 activity to that measured in control animals. The measured SR Ca^{2+} imbalance identified in the db/db mouse was shown to be associated with a gain of function of the receptor due to down-regulation of the FKBP12.6 binding partner. In another study, a reduction in levels of both RyR2 and FKBP12.6 proteins has been reported in the STZ T1DM rat model also finding that the RyR2 was highly
phosphorylated compared to control animals (Yaras et al., 2005). The association and dissociation of FKBP12.6 from RyR2 in response to hyperphosphorylation of the receptor are a highly contentious and well-studied area, especially since an established feature of HF and arrhythmogenesis is leaky RyR2s and aberrant SR Ca\(^{2+}\) release. Controversy exists as to whether FKBP12.6 release from RyR2 is a result of phosphorylation through the action of protein kinase A (PKA) and/or by Ca\(^{2+}\)/calmodulin-dependent kinase 2 (CaMKII) (Camors & Valdivia, 2014). To our knowledge no such detailed studies of this mechanism have been undertaken in the diabetic heart. In a mouse model of T2DM, reduced RyR2 expression has also been observed with a reduction in SR Ca\(^{2+}\) content and increased Ca\(^{2+}\) efflux via the sodium/calcium exchanger (NCX1) (Pereira et al., 2006); the phosphorylation state of RyR2, or kinase activities were not examined in this study. There are fewer investigations regarding NCX1 expression and activity but in general an increase in protein levels accompanied by elevated activity (I\(_{\text{ncx}}\)) has been associated as a compensatory mechanism in several models of HF (Benitah et al., 2002). At the protein level NCX1 has been reported to be upregulated in the Akita mouse T1DM model but the increase in protein was not mirrored by a change in function (LaRocca et al., 2012). In contrast a reduction in NCX1 expression was observed in the myocardium of the STZ rat (Choi et al., 2002). However, if modulation of NCX1 is a compensatory mechanism then changes to both expression and function will depend upon the severity of the DCM.

The inconsistencies between data from the different models likely represent the differences in diabetes progression, insulin and glucose levels and extent of cardiac dysfunction flagging that the data must be considered in the context of the parameters of the animal model as discussed in Section 1.3. In addition, one of the studies employed both male and female animals e.g. Malhotra et al. (1981), which may be a contributing factor leading to inconsistencies between reports, especially given the recent study showing that the onset and progression of DCM is more rapid in the female rats (Moore et al., 2014).

Inspection of Table 1 finds a picture emerging of both expression level and functional changes to the key proteins regulating E–C coupling in both T1DM and T2DM. Together, impaired SERCA2a activity and RyR leak will combine to result in Ca\(^{2+}\)-release abnormalities with a reduction in the SR Ca\(^{2+}\) load and delay the decay of the Ca\(^{2+}\) transient. In addition, to a delicate balance and interplay between the E–C coupling proteins there is also evidence to suggest that Cav3 plays a role that may impact upon contractile function by directly, or indirectly, associating with both RyR2 and the LTCCs.

### 3.1. Populations of E–C coupling proteins are regulated by caveolin-3

Caveolae-based populations of the LTCC within Cav3 rich domains in cardiac myocytes have been described (Baligepalli et al., 2006a). However, rather than a direct interaction between Cav3 and Ca,1.2 (the ion channel subunit of LTCCs) it is thought that Cav3 serves to sequester PKA bringing it in close proximity to the ion channel and a number of proteins involved in the β2AR/cAMP pathway to form a macromolecular signalling complex. Building upon this study, Orchard and colleagues (Bryant et al., 2014) have recently shown that disruption of Cav3 leads to a decrease in the amplitude of the calcium current in response to β2-adrenergic stimulation, linking Cav3 to PKA activity. However, the depression of I\(_{\text{Ca,L}}\) was not consistent with perturbation of only a small sub-population of LTCCs, suggesting that this signalling pathway may have implications for LTCCs within the t-tubules and hence E–C coupling. However, it still remains unknown as to whether there is a direct interaction between Cav3 and PKA or if other intermediary proteins are involved. The influence of Cav3 and caveolae upon I\(_{\text{Ca,L}}\) has also been demonstrated by Timofeyev and co-workers (Timofeyev et al., 2013) with a direct interaction between adenyl cyclase, a key component of the β2-AR cascade, and Cav3 indicated as a possible mechanism. It is intriguing that I\(_{\text{Ca,L}}\) is depressed in several models of DCM (Table 1); given the evidence that Cav3 is a regulator of the LTCC, then there may well be a correlation between changes to Cav3 expression levels, or localisation within the cell, with depression of LTCC function in DCM.

Cav3 has also been shown to co-localise with RyR2 (Head et al., 2005). Further studies from Scriven and colleagues (Scriven et al., 2005) determined that the co-localised RyR2 and Cav3 were not part of the dyad architecture (where CICR occurs), formed between the t-tubules and junctional SR, but instead form part of a non-dyadic subpopulation of RyRs that were predominantly found within, but near the mouth of t-tubules. Murphy and co-workers also reported that Cav3 expression is highest in or close to the t-tubule (Murphy et al., 2009). Vassilopoulos and co-workers (Vassilopoulos et al., 2010) have since reported that Cav3 directly interacts with the transmembrane region of RyR. Li and co-workers (Li et al., 2006) had previously identified a population of Cav3 within isolated rabbit skeletal SR membranes which would lend support for an interaction between Cav3 and RyR via the transmembrane domains. Our group has subsequently described a direct interaction between Cav3 and RyRs and additionally showed evidence of a Cav3 population within the SR (Whiteley et al., 2012). Bioinformatics analysis identified up to nine putative caveolin binding motifs (CBM) within the RyR2 primary sequence, many of which are within the putative transmembrane region of the receptor. To evaluate whether the CBMs were sterically accessible within the RyR tetramer we undertook a bioinformatics analysis of the RyR secondary structure and further analysed the position of the motifs within the putative positions of the transmembrane helices defined within the electron cryomicroscopy three-dimensional (3D) structure available at the time (~10 Å) (Samso et al., 2005). This approach allowed us to discount several of the CBMs and suggest those potentially accessible to facilitate Cav3 binding. Significantly, a direct interaction was confirmed experimentally between RyR1 and Cav3, although whether the putative CBMs are involved remains unclear.

It should be noted that recently, doubts have been cast over the validity of the CBM as a site for interaction with caveolins (Byrne et al., 2012; Collins et al., 2012). Both of these groups conducted a rigorous bioinformatics study finding that this hydrophobic motif may not be sterically accessible in many proteins. Curiously, the CBM motif was found to be enriched in yeast proteins which was surprising since yeast does not contain caveolae or caveolins, leading to the investigators to suggest that this linear amino acid sequence may have an alternative function. Furthermore, analysis of the position of the CBM within currently available crystal structures, found them to be buried within the tertiary and quaternary structures, adding further credence to this supposition. Of particular relevance to this review article is the association between Cav1 and Cav3 with NOS (see Section 4.1), one of the most well characterised protein–protein interactions, as the CBM motif was mapped to within the hydrophobic core and so would be inaccessible, casting doubts on whether it is the interaction site. However, conformations changes to NOS leading to exposure of this domain cannot be ruled out based upon the current data available.

In summary, the functional significance of an interaction between the cardiac RyR2 and the skeletal muscle RyR1 with Cav3 in healthy cardiac myocytes is not yet known. Pertinently, none of the mechanisms involving Cav3, LTCCs and RyR2s have been explored specifically within the diabetic heart and so it is not yet known whether there is disruption of these interaction pathways under pathological conditions or DCM. However, as discussed earlier there are now several reports that indicate that Cav3 expression levels are depressed in response to diabetes and specifically in the myocardium (Penumathsa et al., 2009; Lei et al., 2013). Therefore, given the intimate relationship between Cav3 and the E–C coupling proteins it would seem reasonable to speculate that changes to expression profiles and function of E–C coupling proteins may be intrinsically linked to modulation of Cav3.

### 3.2. Myocardial remodelling is a feature of diabetic cardiomyopathy

The metabolic shift in energy source usage leads to the development of not only functional changes but also structural remodelling of the
diabetic heart. Anatomical adaptations include myocardial stiffness due to fibrosis and increase in the LV mass, hypertrophy, factors that contribute towards impaired cardiac compliance. Here we shall specifically concentrate on the morphological adaptations to cardiac myocytes that have been related to Cav3.

3.2.1. T-tubule morphology and the role of caveolin-3

Central to CICR is the spatial arrangement of the LTCCs and RyR2s within the dyadic cleft; formed by the juxtaposition of the t-tubules, invaginations of the surface membranes into the interior of the cell and the junctional portion of the SR (JSR). It is now established that for optimal CICR the geometric spacing between the t-tubules and the JSR should be between 12 and 15 nm (Forbes & Sperelakis, 1982; Langer & Peskoff, 1996; Franzini-Armstrong et al., 1999). Remodelling of the t-tubular network is a key feature of both small and large animal models of HF (Wei et al., 2010; Pinalli et al., 2013) and importantly, is a feature of the human failing heart (Lyon et al., 2009; Crossman et al., 2011). Our group also recently revealed that in addition to remodelling of the t-tubular network the SR also undergoes structural rearrangement (Pinalli et al., 2013). To-date there are few studies examining the morphology of the t-tubular system in the diabetic heart with one finding only modest changes in a STZ T1DM rat model after 8 weeks, with evidence of an irregular organisation and depressed contraction (Ward & Crossman, 2014).

Studies of the STZ rat by S. Zhang et al. (2014) reported that the t-tubules were disordered by labelling the high-affinity copper transporter (CTR1) purported to be localised to the t-tubules. A comparison of the protocols found that the Zhang study examined the cardiac myocyte morphology 16 weeks after injection, compared to 8 weeks post-injection, which is the norm for most STZ studies including that of Ward and Crossman, suggesting that the loss of the t-tubules is progressive. Intriguingly, administration of a novel treatment, a Cu (II)-selective chelator triethylenetetramine (TETA) led to restoration of the t-tubule organisation. The mechanisms by which the t-tubular network is regenerated are not yet known. However, these studies stained for the t-tubules only and therefore, there are currently no studies to-date that have explored whether the SR is remodelled.

Cav3 was first shown to play a role in t-tubule formation during cardiac development (Parnot et al., 1997) and is localised to both the sarcolemmal membrane and the t-tubules in adult rat cardiac myocytes (Head et al., 2005). Furthermore, it is now established that Cav3 along with several other proteins including BIN1 (Hong et al., 2014) and telethonin (Tcap) (Ibrahim et al., 2013) are essential for t-tubule structure along with junctophilin-2 (Wei et al., 2010; Bennett et al., 2013), an SR protein that is important for maintaining the spatial relationship between the t-tubule network and the SR. For a review of t-tubule morphology in health and disease see Ibrahim et al. (2011). Cav3 Knock-out models exhibit disruption of the t-tubular network (Galbiati et al., 2001a) and developmental studies show Cav3 as a marker of t-tubule biogenesis (Pavlovic et al., 2010). Furthermore, co-localisation of junctophilin-2 and Cav3 has also been reported, (Oshikawa et al., 2004), although it is not known whether there is a direct physical association between Cav3 and junctophilin-2.

While Cav3 has been reported to be down-regulated in the myocardium of the STZ T1DM rat (Penumathsa et al., 2009; Lei et al., 2013) no investigations of the impact of Cav3 depletion upon the t-tubular network were undertaken. To our knowledge, to-date, there have been no other studies of the t-tubular morphology in patients with T1DM or T2DM or in any animal models of T2DM. However, it is not unreasonable to suggest that changes to Cav3 expression levels, in addition to impacting upon the targeting and function of ion channels, may also have the potential to alter the integrity of the t-tubular network.

3.2.2. Hypertrophy and the role of caveolin-3

Hypertrophic cardiomyopathy is feature of DCM, characterised by the thickening of the myocardium, and in most cases affects the left ventricular myocardium. Significantly, mice global with global ablation of Cav3 exhibit severe cardiac hypertrophy and dilation (Woodman et al., 2002). This increased cardiac mass was proposed to be due to hyper-activation of the MAPK pathway, leading to the concept of Cav3 as a negative regulator of p42/44 MAPK (ERK 1/2) pathway. Further support for a role of Cav3 as a regulator of cardiac hypertrophy via MAPK inhibition comes from experiments in which over-expression of Cav3 in rat cardiac myocytes has been shown to inhibit the hypertrophic response, and suppress the MAPK pathway (Koga et al., 2003). Significantly, both ERK and MEK are co-localised to caveolae (Engelman et al., 1998). Clinical support for a role of Cav3 in cardiomyopathy also comes from patients with familial hypertrophic cardiomyopathy shown to carry Cav3 mutations (T63S) (Hayashi et al., 2004). The same study linked the point mutation with incorrect targeting of Cav3 finding reduced cell surface expression of Cav3, thus indicating a possible mechanism by which the disease manifests. These data and others indicate a cardioprotective role for Cav3 against hypertrophy. In addition, LTCCs co-localising with Cav3 within caveolae have also recently been suggested as a source of pathological Ca2+ that promotes cardiac hypertrophy, and represent a population distinct from those LTCCs orchestrating E–C coupling (Makarewich et al., 2012).

3.3. Diabetic cardiomyopathy is characterised by rhythm disturbances

In addition to the modification of Ca2+ handling proteins, diabetes also results in cardiac electrical remodelling with patients exhibiting an abnormal electrocardiogram (ECG) characterised by a prolonged QT, reflective of dysfunctional ventricular repolarisation (Kahn et al., 1987). This correlates to a prolongation of the action potential duration (APD). The shape and duration of the action potential is regulated by the concerted action of a series of ion channels. In brief, the action potential can be separated into an inward and outward component. The inward current is formed by the sodium channel, Nav1.5 (INa) and the LTCC, Ca1.2 (Ica/l). The outward element is formed by a combination of potassium channels K4.3/K4.2 (Iko), K1.5 (IKur), K11.1 or HERG (Iko), K7.1 (Iks), Kir2.1–2.3 (IK1), Kir3.1-3.4 (Ica/Kir) and Kir6.2 (Ica/KATP). The STZ rat model of T1DM showed a 50% reduction of the transient outward potassium current Ito and Iks with changes to the Ikα current density also exhibiting regional variation across the different chambers and regions of the heart (Casisi et al., 2000). In addition to a reduction in the potassium current density, a depression of Ica/l has also been reported (Wang et al., 1995). A prolonged APD, associated with reduction of Ito and Ikα, during repolarization, has since been demonstrated in various animal models of diabetes (Gallego et al., 2009). A recent study of a mouse model of T2DM has also shown heterogeneity in ion channel remodelling across the heart wall and also linked the reduction of the potassium current amplitude with the down-regulation of K4.2 and its accessory β-subunit KCNH2 (Sato et al., 2014). KCNH2 is an auxiliary protein that facilitates the trafficking of the Kv channels to the sarcolemma as well as regulating ion channel inactivation. A reduction in protein levels of K4.2 and KCNH2 was linked to hyper-activation of the peroxisome proliferator-activated receptor-α (PPARα) protein in the diabetic heart as a consequence of elevated levels of FFA (Marionneau et al., 2008). A link between β2-adrenoreceptor (β2-AR) stimulation and Iko amplitude has also been demonstrated as a property of cardiac myocytes isolated from the STZ rat model of T1DM (Setien et al., 2013). The mechanisms responsible for the cardiac ion channel remodelling and electrical instability observed in diabetes remain incompletely understood. Significantly, many of the ion channels that regulate the cardiac action potential have been identified within caveolae; these include HCN4 (Ye et al., 2008), K1.5 (Martens et al., 2001), Kir6.1 (Garj et al., 2009a, 2009b; Davies et al., 2010), Ca3.2 (Markandeya et al., 2011) and K11.1 (Bailijepalli et al., 2007). In addition, several ion channels localised to the caveolae have also been shown to be a molecular partner with Cav3. Therefore, the next section will consider the role of Cav3 in influencing the cardiac action potential.
3.3.1. Caveolin-3 is a regulator of ion channels forming the cardiac action potential

Cav3 has been reported in a number of studies to regulate the inward rectifying potassium channel Kir2.1 that is responsible for the generation of $I_{Kr}$, maintaining the resting potential and shapes the final repolarization phase of the action potential (Vaidyanathan et al., 2013). Furthermore, the introduction of three Cav3 mutations previously identified in patients with rhythm disorders, Long QT syndrome (LQTS), was each shown, to varying extents, to reduce the potassium current ($I_{Kr}$) density and also surface membrane expression of Kir2.1, suggesting that this molecular partnership is necessary for channel trafficking since all resulted in loss of Kir2.1 function. Co-immunoprecipitation and FRET studies indicated a physical association between Cav3 and Kir2.1 but the mechanisms and structural domains involved remain unresolved; although there is some evidence to suggest that the basis for Cav3 regulation of Kir2.1 involved channel nitrosylation (discussed in Section 4.3). Pertinently, levels of Kir2.1 have been shown to be depressed in rat models of both MI and T2DM with MI (Wang et al., 2011) although the investigators did not examine Cav3 levels. Also Cav3 has also been shown to play a central role in the rhythmic disturbance the LQTS, which is characterised by a pronounced QT propagation resulting in impaired repolarisation (Vatta et al., 2006). A particular form of QTs, QTq, has been shown to be linked to a number of mutations in Cav3, as identified in patients with this disease; many of which locate to the putative transmembrane region of Cav3. Functional studies have supported a role for the mutation of both MI and T2DM with MI (Wang et al., 2011) although the investigators did not examine Cav3 levels. Also Cav3 has also been shown to play a central role in the rhythmic disturbance the LQTS, which is characterised by a pronounced QT propagation resulting in impaired repolarisation (Vatta et al., 2006). A particular form of QTs, QTq, has been shown to be linked to a number of mutations in Cav3, as identified in patients with this disease; many of which locate to the putative transmembrane region of Cav3. Functional studies have supported a role for the mutation at residue 97 of Cav3 (from F to C), where a gain of function (late $I_{Na}$) of the Na,1,5 sodium channel was observed (Vatta et al., 2006). Interestingly, the Na,1,5 sodium channel is both located to caveolae and has been shown to co-immunoprecipitate with Cav3 in cardiac myocytes (Yarbrough et al., 2002). However, the mutant Cav3 F97C was shown to remain associated with Nav1.5, suggesting that a lack of association between the two proteins was not the cause of the observed increase in late sodium current (Vatta et al., 2006).

Clearly, Cav3 has a multitude of binding partners influencing multiple cellular processes (see Table 2). Insights into how Cav3 can (i) bind multiple proteins in close proximity and yet (ii) maintain spatial segregation of a range of diverse signalling pathways can be gleaned from its three-dimensional structure as shown in Fig. 3 (Whiteley et al., 2012). The structure shows how Cav3 oligomersize to form a disc-shaped arrangement with protein densities extending into the cytoplasm. Labeling studies identified the cytoplasmic densities as the N-terminal region of each contributing monomer. Thus this region of each Cav3 monomer forming the homo-oligomer is highly exposed, which prevents multiple binding sites for protein partners to interact with, potentially simultaneously and independently, to orchestrate a variety of cell signalling processes.

3.4. Chronic hyperglycaemia leads to protein post-translational modification

In Section 2.1 we considered how changes to GLUT-4 cell surface numbers contributes towards elevated circulating levels of glucose and so an extension is to consider the effects of glucose imbalance at the protein level. Increased concentrations of circulating glucose leads to the generation of advanced glycation end products (AGEs), resulting in the non-enzymatic glycation and oxidation of proteins or lipids (Goldin et al., 2006). Clinically, these modifications result in cardiac stiffness and hinder cardiac relaxation, potentially contributing to the development of diastolic dysfunction (Bidasee et al., 2004; Montagnani, 2008; Huyhn et al., 2014). At the molecular level, SERCa2a is glycosylated by AGEs formation in the STZ T1DM rat model and was proposed to be a contributory factor connecting towards depressed pump activity (Brownlee, 1995; Bidasee et al., 2004). As discussed in Section 3, CaMKII is a regulator of RyR2 function and thus it is significant that oxidised CaMKII (ox-CaMKII) has been detected in human pacemaker tissue from diabetic patients after an MI, a finding mirrored in nodal tissue in an STZ T1DM model (Luo et al., 2013). However, the effect of CaMKII oxidation in the context of RyR2 function has not been explored. In addition, O-linked glycosylation of CaMKII has been identified in both the heart and brain in response to hyperglycaemia (Erickson et al., 2013). This post-translational modification increases the enzyme activity with studies of cardiac myocytes showing that O-GlcNAcylation resulted in increased Ca$^{2+}$ sparks culminating in arrhythmogenic Ca$^{2+}$ wave formation (Erickson et al., 2013). Currently, to our knowledge, there are no data as to whether these post-translational modifications impact upon the relationship between Cav3 and the E-C coupling proteins.

4. Oxidative stress and cardiac contractile function

Both hyperglycaemia and elevated FFA promote oxidative stress by stimulating the production of ROS. ROS production is a by-product of mitochondrial respiration. Normally, antioxidants such as superoxide dismutase (SOD), glutathione peroxidase and catalase degrade superoxide to water. However, in diabetes ROS production is increased (Boudina et al., 2007). ROS production in the mitochondrial matrix can also stimulate ROS production in the cytosol. Hydrogen peroxide ($H_2O_2$) can cross the mitochondrial membrane and activate protein kinase C (PKC) in the cytosol which simultaneously activates NADPH oxidase (Isfort et al., 2014). Unlike other sources of ROS, NADPH oxidase generates ROS as a defensive mechanism to destroy invading pathogens (Bedard & Krause, 2007). NADPH oxidase activity can be stimulated by PKC, as mentioned previously as well as angiotensin II (AngII), endothelin 1 (ET-1) and tumour necrosis factor α (TNFα) which are all upregulated in diabetes (Koya & King, 1998; Schneider et al., 2002; Singh et al., 2008; Mirza et al., 2012). Interestingly, NADPH oxidase expression and activity has been shown to be upregulated in hypertrophy, HF and T1DM suggesting that NADPH oxidase may be a major source of ROS production and oxidative stress in DCM (Li et al., 2002, 2010; Heymes et al., 2003).

Another source of ROS is endothelial NOS (eNOS). In healthy hearts, eNOS produces nitric oxide (NO) from L-arginine. However, uncoupling of eNOS can lead to the production of superoxide and may be associated with endothelial dysfunction in diabetes (Thum et al., 2007). Similarly, elevated ROS levels lead to oxidative stress and have detrimental effects to the structure and function of the heart. ROS and oxidative stress promote DNA damage, which activates poly ADP ribose polymerase (PARP) (Du et al., 2003). PARP subsequently inhibits glycerolaldehyde phosphate dehydrogenase (GAPDH), resulting in the increase in AGEs and PKC (Brownlee, 1995; Guo et al., 2003). AGEs, PKC and ROS itself stimulate altered cell signalling that ultimately leads to the development of further diabetic complications including inflammation, hypertrophy, fibrosis and apoptosis (Huyhn et al., 2014). ROS can also oxidise proteins and lipid membranes e.g. thiol oxidation, carboxylation and nitration (Adachi et al., 2004; Lokuta et al., 2005; Shao et al., 2011). Given the numerous sources of ROS and the complex cellular effects the next section will specifically focus upon the role of NOS and Cav3 in terms of contractile function and dysfunction.

4.1. Nitric oxide synthase and role in cardiac function

Nitric oxide synthases (NOS) catalyse the production of nitric oxide (NO) and are regulated by calmodulin binding. NOS catalyses the conversion of L-arginine and oxygen to L-citrulline and nitric oxide (NO). There are three NOS isoforms, endothelial (eNOS/NOS3), neuronal (nNOS/NOS1) and inducible (iNOS/NOS2) that are confined to various subcellular locations (Barouch et al., 2002). The activity of nNOS and eNOS is dependent upon calcium and caveolin binding. The direct interaction of NOS with caveolins results in negative regulation of the enzyme. Studies have demonstrated a direct correlation between the activity of NOS and Cav3 expression in the heart (Aravamudan et al., 2003). Ablation of caveolins has demonstrated their involvement in the regulation of the NOS isoforms, eNOS and nNOS, where the reduction or abolition of either Cav1 or Cav3 leads to up-regulation or
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Table 2
A list of the Caveolin-3 binding partners identified in the heart.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Tissue</th>
<th>Experiment</th>
<th>Site</th>
<th>Disease implications</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>Neonatal mouse cardiac myocytes</td>
<td>Co-IP</td>
<td>CSD?</td>
<td>–</td>
<td>Feron et al. (1998a)</td>
</tr>
<tr>
<td>nNOS</td>
<td>Rat left ventricular cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>HF</td>
<td>Bendall et al. (2004)</td>
</tr>
<tr>
<td>Cα,1,2</td>
<td>Mouse neonatal and adult ventricular myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cα,3,1</td>
<td>Neonatal mouse ventricular cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>Hypertrophy? HF?</td>
<td>Balijepalli et al. (2006b)</td>
</tr>
<tr>
<td>Cα,3,2</td>
<td>Neonatal mouse ventricular cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>Hypertrophy? HF?</td>
<td>Markandeya et al. (2011)</td>
</tr>
<tr>
<td>HCN4</td>
<td>Mouse cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>Arrhythmia?</td>
<td>Ye et al. (2008); Bosman et al. (2013)</td>
</tr>
<tr>
<td>hERG</td>
<td>Rat and rabbit ventricular cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>Arrhythmia?</td>
<td>Guo et al. (2012)</td>
</tr>
<tr>
<td>KIR2.1</td>
<td>Human ventricular cardiac myocytes</td>
<td>Co-localisation</td>
<td>–</td>
<td>Arrhythmia formation in LQT9</td>
<td>Vaidyanathan et al. (2013)</td>
</tr>
<tr>
<td>KIR6.2</td>
<td>Adult and neonatal rat ventricular cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>–</td>
<td>Garg et al. (2009a)</td>
</tr>
<tr>
<td>Na1,5</td>
<td>Adult rat cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>Arrhythmia?</td>
<td>Yarbrough et al. (2002)</td>
</tr>
<tr>
<td>Ncx1</td>
<td>Bovine cardiac sarcolemmal vesicles</td>
<td>Co-IP</td>
<td>–</td>
<td>Hypertrophy?</td>
<td>Bossert et al. (2002)</td>
</tr>
<tr>
<td>Cav1</td>
<td>Neonatal rat atrial cardiac myocytes</td>
<td>Co-localisation</td>
<td>–</td>
<td>Hypertrophy?</td>
<td>Volonte et al. (2008)</td>
</tr>
<tr>
<td>Cav2</td>
<td>Neonatal rat cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>–</td>
<td>Rybin et al. (2001)</td>
</tr>
<tr>
<td>Cx43</td>
<td>Mouse cardiac myocytes</td>
<td>Yeast two-hybrid</td>
<td>Co-localisation</td>
<td>Arrhythmia?</td>
<td>Liu et al. (2010)</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Adult ventricular cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>Cardiac protein during ischaemia</td>
<td>Kono et al. (2007)</td>
</tr>
<tr>
<td>A2AR</td>
<td>Adult and neonatal rat ventricular cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>–</td>
<td>Garg et al. (2009a)</td>
</tr>
<tr>
<td>AT1R</td>
<td>Rat cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>–</td>
<td>Ricchiuti et al. (2011)</td>
</tr>
<tr>
<td>α1aAR</td>
<td>Adult rat cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>–</td>
<td>Head et al. (2005)</td>
</tr>
<tr>
<td>α1bAR</td>
<td>Mouse neonatal and adult ventricular cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>Hypertrophy? HF?</td>
<td>Xiang et al. (2002); Head et al. (2005); Balijepalli et al. (2006b)</td>
</tr>
<tr>
<td>Eκα</td>
<td>Neonatal and adult rat cardiac myocytes</td>
<td>Co-localisation</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>M4-mACHR</td>
<td>Adult rat cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>–</td>
<td>Head et al. (2005)</td>
</tr>
<tr>
<td>MR</td>
<td>Rat cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>–</td>
<td>Ricchiuti et al. (2011)</td>
</tr>
<tr>
<td>VDR</td>
<td>Rat cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>–</td>
<td>Zhao &amp; Simpson (2010)</td>
</tr>
<tr>
<td>AC</td>
<td>Mouse neonatal and adult ventricular cardiac myocytes</td>
<td>Co-IP</td>
<td>CSD?</td>
<td>Hypertrophy? HF?</td>
<td>Balijepalli et al. (2006b)</td>
</tr>
<tr>
<td>Gα(s)</td>
<td>Mouse neonatal and adult ventricular cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>Hypertrophy? HF?</td>
<td>Head et al. (2005); Yarbrough et al. (2002); Head et al. (2005); Balijepalli et al. (2006b)</td>
</tr>
<tr>
<td>p38MAPKα</td>
<td>Adult rat cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>↑ Association in IR injury</td>
<td>Das et al. (2007)</td>
</tr>
<tr>
<td>PKA</td>
<td>Mouse neonatal and adult ventricular cardiac myocytes</td>
<td>Co-IP</td>
<td>CSD?</td>
<td>Hypertrophy? HF?</td>
<td>Balijepalli et al. (2006b)</td>
</tr>
<tr>
<td>PKG1α</td>
<td>Rat ventricular cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>↑ Association in diabetes</td>
<td>Balijepalli et al. (2006b)</td>
</tr>
<tr>
<td>PP2a</td>
<td>Rat ventricular cardiac myocytes</td>
<td>Co-IP</td>
<td>–</td>
<td>Hypertrophy? HF?</td>
<td>Balijepalli et al. (2006b)</td>
</tr>
</tbody>
</table>

hyper-activation of NOS (Razani et al., 2001; Sunada et al., 2001; Barouch et al., 2002; Ohsawa et al., 2004), which, in turn increases bioavailable levels of NO. Conversely, over-expression of Cav3 has been shown to down-regulate NOS activity (Aravamudan et al., 2003). A direct interaction between NOS and Cav1 or Cav3 negatively regulates the enzyme activity. This inhibitory effect is reversed with the addition of excess calcium/calmodulin (Feron et al., 1998b) and Akt-induced phosphorylation of eNOS (Garcia-Cardena et al., 1996).

In cardiac cells, it is well established that NOS and eNOS are spatially segregated between the sarcolemma (Feron et al., 1996) and the SR, and are both regulators of contractile function. In contrast, expression of iNOS is associated with response mechanisms to pathological stresses and is distributed within the cytoplasm. A redistribution and migration of eNOS and nNOS between cellular locations have been reported in a number of cardiac pathologies e.g. (Bendall et al., 2004). For example, in a congenital eNOS deficient mouse LV hypertrophy, with diastolic dysfunction, was induced by pressure overload (Ichinohe et al., 2004), which would imply that eNOS has a cardioprotective effect. In the heart, NOS activity influences cardiac contractility by inhibiting the L-type voltage gated calcium channels, LTCCs (I_{Ca,L}) but has a stimulatory effect upon SR Ca^{2+} release via the RyR2s (Mery et al., 1993). Therefore, NOS has a dual role as both a negative and positive inotropic regulator by the effects of the spatially segregated isoforms. Regulation of the LTCCs is via a macromolecular complex formed between nNOS and the α1aAR clustered within caveolae. However, the relationship between NOS/NO and contractile function is more complex as factors such as the local concentration of NO and the activity of PKA have been shown to influence the open probability of the RyR2s (Lim et al., 2008).
Independent of \([\mathrm{Ca}^{2+}]\). Akt/PKB has been shown to phosphorylate Ser1177 and activate eNOS (Bauer et al., 2003); Akt/PKB is activated by the action of phosphoinositide-3 kinase (PI3K) (Vanhaesebroeck et al., 1997). Phosphorylation of Thr495 by PKC is proposed to be inhibitory by preventing calmodulin binding (Fleming et al., 2001). In contrast, inhibition of iNOS activity, repression of Cav3 at the protein level (Xia et al., 2007).

5. Targeting caveolin-3 for treatment of diabetic cardiomyopathy

There are currently no specific treatments for DCM. Instead, management for patients with DCM generally involve (i) lifestyle changes, (ii) glycaemic and lipid control and (iii) treatment of hypertension/coronary artery disease — if present (although, DCM is usually diagnosed as cardiovascular disease in the absence of both). There are numerous detailed reviews of current treatments, recently reviewed by Huynh and colleagues (Huynh et al., 2014), and there are currently no specific treatments for DCM.
proteins assembling to form a macromolecular signalling complex and (iv) is an indirect regulator of protein post-translational modification. The previous sections have considered how Cav3 is linked to (i) glucose imbalance (ii) modulation of E–C coupling proteins (and hence contractile function) (iii) ion channels forming the cardiac action potential and (iv) sources of ROS. Although animals with global ablation of Cav3 do not develop diabetes per se they do exhibit features associated with diabetes including hyperlipidemia, defective insulin signalling and increased body weight. Similarly, evidence from studies of HF show that they share common pathologies to DCM such as impaired contractile function and structural remodelling, with data to suggest that suggest Cav3 has a cardioprotective role, indicating that strategies for maintaining Cav3 levels may well be beneficial for developing novel approaches to explore new treatments for DCM. Further, as reviewed in the earlier sections there is substantive data to indicate that expression levels of Cav3 are down-regulated in diabetes and so it is not unreasonable to propose that modulation of Cav3 levels warrants further exploration. However, exploiting Cav3 as a therapeutic target poses a significant challenge since the protein cannot be modulated using pharmacological reagents, as with for example enzymes and ion channels.

5.1. Treatment with the caveolin-3 scaffold domain

The N-terminal scaffold domain (CSD) has been indicated as the main site for binding a multitude of proteins with diverse functions within the cell (Schlegel et al., 1999; Schlegel & Lisanti, 2000). Most of the work on the scaffolding domain has involved Cav1, but alignment of Cav1 and Cav3 show a high degree of sequence similarity, as mentioned in Section 2.2 (Razani et al., 2002; Okamoto et al., 1998; Schlegel & Lisanti, 2000). There is also a substantive body of experimental data for the Cav3 CSD as a key regulatory and interaction region (Kamishima et al., 2007; Garg et al., 2009b). Injection of the Cav1 CSD mimetic peptide into permeabilised rat cardiac myocytes has been shown to be cardioprotective in post-ischemic reperfusion (Young et al., 2001). In addition, systemic application of the CSD into the blood stream of a mouse with acute inflammation had anti-inflammatory effects and inhibited vascular leak (Bucci et al., 2000). Given that several studies of the STZ rat model of T1DM converge to indicate depressed levels of Cav3 then therapeutically a Cav3 CSD mimicry peptide may have applications.

As with any therapeutic agent, a delivery system to target the appropriate system is required. There are several studies describing the administration of a cell permeable Cav1 CSD peptide through the coupling of the domain to antennapedia (AP), a homeodomain of the Drosophila transcription factor, to form a fusion protein Cav1-AP. It has been demonstrated that conjugation of the CSD with a 16 amino acid residue AP peptide facilitates uptake into cultured mammalian cells with studies also showing that this approach has applications in vivo (Bucci et al., 2000). The Cav1-AP linked peptide has been used effectively in animal models for reducing inflammation (Young et al., 2001) and also tumour development in mouse models of disease (Gratton et al., 2003). Loading of permeabilised rat neonatal cardiac myocytes with the Cav3 CSD revealed that the Cav3/eNOS interaction is a mediator of cholinergic modulation of cardiac myocyte function (Feron et al., 1998a). However, since Cav3 is expressed in skeletal muscle systemic administration of the CSD may lead to undesirable off-target effects. For example, a transgenic mouse with a global overexpression of Cav3 was shown to exhibit muscular dystrophy (Galbiati et al., 2000). Furthermore, as shown in Table 2 a multitude of other cardiac proteins have been shown to be Cav3 binding partners, which may involve the CSD domain. Therefore, exogenous application of a mimicry peptide may influence a number of cell pathways. In addition, since the interacting region of Cav3 with many target proteins has not yet been characterised, and may involve regions other than the CSD, a more robust strategy may be required to deliver intact Cav3 in a site-directed approach.

5.2. Gene therapy for up-regulation of caveolin-3

Gene therapy offers many benefits such as directed and controlled expression of the target protein to specific cell types. There has been much study of both viral and non-viral vector systems for gene therapy targeting of cardiovascular diseases (for review see Williams et al., 2010). One of the most well-known gene therapy clinical trials (Phase I/II) has targeted the E–C coupling protein SERCA2a. Numerous animal studies had reported the decline of SERCA2a levels in late/end-stage HF with data showing that overexpression of the protein resulted in improved cardiac function. This led to the project ‘calcium up-regulation by percutaneous administration of gene therapy in cardiac disease’ (CUPID) (Greenberg et al., 2014; Zsebo et al., 2014). CUPID has now entered phase II trials and is a randomised, double-blind, placebo-controlled study delivering recombinant adeno-associated viral vector serotype 1 (AVV1) with SERCA2a complementary DNA to patients with advanced HF (Class III/IV) (n = 39). The results from the study have been promising reporting improvement of HF symptoms and signs, restoring diastolic and systolic function. Extensive studies have established the efficiency of transgene expression using the AVV vectors for infection of dividing and non-dividing cells and sustained, persistent expression of the transgene, an important criterion for treating a chronic illness such as HF or diabetes. Crucially, AVVs have an established clinical safety profile, as they are non-pathogenic and cannot replicate since almost all of the viral genome has been removed, leaving a delivery vehicle. This system has the additional advantage of having reduced immunogenicity.

There are multiple AVV serotypes that exhibit different tropism towards different organs with 12 serotypes now identified. For cardiac gene therapy serotype 1 appears to be more optimal for larger mammals (Williams et al., 2010). Other considerations with gene therapy are the number and duration of each administration. The CUPID trial reported, from a three year follow-up, that the best results were from three high doses with an 82% reduction in cardiovascular events and trend towards better survival compared to the placebo group, although the low and mid-dose groups also showed a delay in mortality rates (Zsebo et al., 2014). Studies are now underway with larger patient cohorts. The adenovirus can be delivered by intra-coronary infusion (catheter based) and also surgically during a bypass operation. Significantly, a global transgenic mouse over-expressing SERCA2a was found to be protective against STZ non-insulin dependent induced cardiac dysfunction (Trost et al., 2002), which would suggest that targeting SERCA2a may also be a rationale target. Equally, the success with the CUPID trial both in terms of optimising vehicle delivery and efficacy of the treatment has provided a proof of concept for gene therapy for other myocardial proteins including Cav3 which is also a membrane protein.

5.3. miRNA and antagonisirs for targeting caveolin-3

It is now known that a large fraction of the genome is comprised of regions of non-coding RNAs (ncRNA), classified according to the length of the non-coding region. MicroRNAs (miRNA) are a subset of ncRNAs, corresponding to small segments that are typically between 21 and 23 nucleotides in length. It has emerged over the last decade that miRNAs are key regulators of the translational efficiency of mRNAs encoding for approximately 60% of proteins in humans (Friedman et al., 2009). Profiling of miRNAs has shown they regulate, through RNA silencing and gene-expression, a host of cellular processes in both health and disease (Sayed & Abdellatif, 2011) and for example, are essential for growth and development (Krichevsky et al., 2003). Investigations of altered expression of miRNAs in the pathogenesis of cardiovascular diseases are an intense area of research (Small et al., 2010) and there has been a lot of excitement and expectations for exploiting these small ncRNAs therapeutically.

There are several studies that have investigated the role of miRNAs in insulin dysregulation and in diabetes. For example, miRNAs 103 and 107 have been shown to be upregulated in the livers of an obese
mouse model (ob/ob) and in the Goto–Kakizaki rat, T2DM models (Trajkovski et al., 2011). Importantly, human liver biopsy taken from patients with hepatic disorders such as non-alcoholic fatty acid disease, commonly associated with diabetes, also mirrored the findings of the animal study with elevated levels of miR103/107. Administration of an antagonist (miRNA inhibitors), complementary to both miRNAs, improved insulin-stimulated glucose uptake (Trajkovski et al., 2011). Interestingly, inhibition of miR103/107 resulted in upregulation of Cav1 in adipose tissue. Given the imbalance between Cav3 and Cav1 with respect to GLUT-4 translocation (Section 2.2) studies assessing levels of miR103/107 in the diabetic myocardium may be valuable. Current knowledge of expression patterns of miRNA in the diabetic heart is quite sparse; for a recent review see Zhou et al. (2014). Profiling of the myocardium from STZ animal models of T1DM has shown increased levels of miRNA for gene-regulation of hypertrophy and fibrosis (Diao et al., 2011). Patient studies have identified 41 dysregulated miRNAs associated with HF (non-diabetic and diabetic heart groups) of which 10 were specific to the diabetic cohort (Greco et al., 2012). Unsurprisingly, the study highlighted that many altered miRNAs were common to both the diabetic/HF and non-diabetic/HF patients, consistent with the similar pathologies e.g. hypertrophy and fibrosis. The miRNAs linked specifically to the diabetic/HF patients were associated with oxidative stress pathways and hyperglycemia. Significantly, in a study of an STZ T1DM mouse myocardium exhibiting fibrosis decreased levels of miR133 were identified (Chen et al., 2014). Furthermore, mice with cardiac-overexpression of miR133, similarly dosed with STZ, had significantly reduced fibrosis in comparison with evidence to suggest a mechanism involving suppression of transforming growth factor β1 and reduced ERK1/2 activation. This study illustrates that strategies aimed at increasing levels of specific miRNAs can be beneficial. A recent study of embryonic stem cells identified miR-22 as a regulator of Cav3, further showing that the administration of the compound puerarin (a traditional Chinese medicine) led to enhanced Cav3 expression, through suppression of miR-22, promoting t-tubule development (Wang et al., 2014). The underlying mechanisms by which puerarin antagonises the miRNA are not known.

Therapeutically, two main approaches have been developed to increase levels of miRNAs: (i) a mimetic miRNA with a “guide strand” (a copy of the miRNA) that is conjugated to a “passenger strand”. The passenger strand is the component that is required for cellular uptake and can be a molecule such as cholesterol or (ii) the use of AVV mediated delivery of the miRNA. Systemic delivery of a synthetic miRNA linked to cell permeable moieties has the disadvantage that there is no discrimination between cell-types and thus off-target effects may result since miRNAs like proteins can be widely distributed. However, if the aim were to up-regulate a protein then a strategy would be the development of anti-miRNA, an antagonist to reduce endogenous levels of the relevant miRNA. The development of anti-miRNAs as pharmacological agents is still in its relative infancy but the strategies behind delivery are as for any cell based therapy i.e. they must exhibit a high affinity for the target miRNA, be cell permeable, conjoined to a delivery vehicle and can be site-directed, stable in-vivo and not rapidly metabolised. One of the most advanced clinical trials (Phase II) for treatment of hepatitis C is targeting miRNAs with an antagonist, miravirsen, to miR-122, which regulates replication of the hepatitis C virus (Nana-Sinkam & Croce, 2013).

In addition to representing therapeutic targets the identification of expression level changes to miRNAs that are selective for DCM, also holds promise for identifying specific proteins and pathways that define the aetiology of cardiac dysfunction in the diabetic heart.

5.4. Indirect targeting of caveolin-3

Cav3 orchestrates a plethora of signalling and thus systemic off-target effects need to be considered. Therefore, an alternative approach to modulating Cav3 expression levels may be to target molecules involved in specific pathways. Several groups have identified elevated expression of PKCβ3 in the myocardium of STZ T1DM rats (Xia et al., 2007; Liu et al., 2012) with a more recent study linking amplified PKCβ3 activity with depressed levels of Cav3 concomitant with alteration of the Akt/eNOS signalling pathway (Lei et al., 2013). Significantly, STZ rats treated with a PKCβ3 inhibitor restored Cav3 expression to levels comparable to control animals. Further, the dosed rats exhibited improved cardiac diastolic function compared to the non-treated diabetic group. The exact mechanisms that led to increased levels of PKCβ3 and activity are not known, nor are the mechanisms that link these molecular alterations to Cav3 expression, although increased levels of ROS are thought to be a contributory factor (Xia et al., 2007). However, what is apparent is that PKCβ3 inhibitors may represent a novel therapeutic target for treating DCM by restoring Cav3 levels. Given the varied and number of Cav3 interacting partners then this strategy may also have benefits that extend beyond maintaining the integrity of the Akt/eNOS signalling pathway. Significantly, randomised clinical trials using the PKC inhibitor, ruboxistaurin, have shown some beneficial effects for treating diabetic neuropathy, although further studies are required (Bansal et al., 2013). Furthermore, the application of PKCβ3 inhibitors to rat cardiac myocytes exposed to methylglyoxal-AGE showed improved contractile properties compared to untreated cells (L. Zhang et al., 2014).

As discussed in Section 3.1, Cav3 is reported to not only directly interact with RyR2, but also form non-dyadic microdomains and participates in a novel mechanism that contributes towards RyR2 dysfunction via nitrosylation. However, the NO signalling pathways are complex both in the myocardium and throughout the vasculature. Moreover, factors influencing these pathways will depend not only upon expression levels of the NOS isoforms but also cellular location. For example, NOS isoforms are known to migrate between the SR and sarclemma in response to stress and although this spatial rearrangement has been characterised, in part, in HF models (Bendall et al., 2004; Damy et al., 2004) there are no equivalent studies in models of DCM. Furthermore, NOS activity will also be influenced by the availability of cofactors (flavins, heme, NADPH, tetrahydrobiopterin (BH4) and calmodulin) but NOS patterns are not well resolved in DCM. Currently, there also remains an incomplete understanding of NO targets and thus a strategy directed at manipulating NO bioavailability may have extensive, undesirable, off target effects even within a specific tissue. Therefore, although NOS inhibitors may represent a therapeutic target for DCM a greater knowledge of NO signalling pathways is required.

An alternative strategy may represent targeting RyR2 binding partners, although an understanding of how Cav3 influences RyR2 function still needs to be answered. The 1,4 benzothiazepine derivative, known as K201 (formerly referred to as JTV519), has been shown to have cardioprotective effects by binding to, and stabilising, the quaternary structure of the RyR2, resulting in a conformation that enhances FKBP12.6 binding (Kohno et al., 2003). While there are multiple studies, both in vivo and in vitro, to substantiate this mechanism there are some reports that suggest that the actions of K201 to suppress aberrant Ca2+ release is independent of FKBP12.6 binding (Hunt et al., 2007; Xiao et al., 2007). Indeed, there is now a body of evidence to suggest that Ca2+ leak is a result of redox imbalance due to increased levels of NO (Section 4.3). Post-translational modification of the cysteine thiols are suggested to result in conformational changes to RyR2 that promote the dissociation of calmodulin, which leads to diastolic SR Ca2+ leak, yet the conformational change induced by K201 binding is protective (Tateishi et al., 2009; Ono et al., 2010). However, as discussed earlier there is also the proposal that due to elevated levels of ROS thiol groups are oxidised and there is hyponitrosylation and it is this nitroso-redox imbalance that promotes channel leak (Gonzalez et al., 2010). Gonzalez and co-workers also showed that inhibitors of xanthine oxidase improved Ca2+ handling in failing myocytes that had been exposed to oxidative stress in a rat model of HF. Allopurinol (a XOR inhibitor), has been used for years for hyperuricemia, to treat conditions such as gout and is currently in clinical trials in patients
with T1DM to test whether kidney function is improved (Maahs et al., 2013). Given the molecular interplay between NOS and XOR activity and RyR2 function based on work of for example Cutler et al. (2012) then a direction for future studies may be to investigate whether XOR inhibitors have beneficial effects for treating contractile dysfunction in the context of the nitroso-redox imbalance in DCM.

Small molecules such as tocotrienols, a member of the vitamin E family of nutrients, are attracting attention as antioxidants for use in cardiovascular diseases as well as cancers (Das et al., 2005; Srivastava & Gupta, 2006). Tocotrienols have been shown to help decrease blood glucose and HbA1c levels and improve parameters associated with metabolic syndrome (Weng-Yew & Brown, 2011). High-fat fed rats given tocotrienols showed improved glucose and insulin tolerance and improved ventricular function (Wong et al., 2012). Tocotrienols in cell culture also led to an upregulation of MAPK and PI3-K activity (Numakawa et al., 2006). Further studies in cardiac myocytes identified two tocotrienol isoforms (α and δ) that resulted in reduced association of p38MAPKβ and eNOS with Cav3. The tocotrienol treated group exhibited better outcomes in reperfusion compared with Cav3 modulation of p38MAPKβ and eNOS activities (Das et al., 2005). Data from clinical trials as to the efficacy of oral supplements of vitamin E are mixed with some evidence for it preventing atherosclerosis (Munteanu et al., 2004). Epidemiological studies have revealed a correlation between high dose vitamin E intake and reduced risk coronary heart disease in both males and females (Rimm et al., 1993; Stampfer et al., 1993). The administration of vitamin E to STZ T1DM rats has shown in several studies to have cardioprotective effects against oxidative stress and apoptosis with restoration of the QT interval (Shirpour et al., 2009). However, the mechanisms by which vitamin E confers protection are unknown and doubts have been cast as to whether the beneficial effects are due to the compound's properties as an anti-oxidant or due to its interaction with specific cell signalling pathways. An intriguing possibility is that the molecular actions of tocotrienols involve Cav3 and eNOS and maybe an area worth further exploration within this context.

Cardiac specific ablation of PI3K (both α and β subunits separately and a double KO) results in a reduction of phosphorylated Akt/PKB with an overall increase in Akt/PKB expression (Wu et al., 2011). The KO animals exhibit deranged t-tubules and misaligned LTCCs and RyR2s. Further, the double knockout animals went into decompensated HF thereby, implicating both subunits as fundamental for contractile function. As discussed previously PI3K and Akt/PKB co-localise with Cav3 within caveolae, with changes to expression levels of any of the components would be predicted to influence the PI3K signalling pathway. Indeed, it has been shown that depression of Cav3 switches the PI3K pathway from cell survival mechanisms to apoptosis (Smythe & Rando, 2006). PI3K inhibitors are being evaluated for cancer treatment (Marone et al., 2008) and thus there maybe merit in investigating whether these PI3-K based therapies have value for the treatment of DCM.

Life-style changes are the first line of treatment for T2DM patients, with the significant benefits of exercise training for preservation of myocardial contractility well established (Powers et al., 2002). It is therefore pertinent that exercise training of spontaneously hypertensive mice has shown that there are elevated levels of Cav3 compared to the sedentary group (Lee et al., 2006). Likewise, in the db/db mouse model of obesity and T2DM, aerobic interval training resulted in improved cardiac contractile performance through restoration of SR Ca2+ release and protein levels of SERCA2a and NCX1 (Stolen et al., 2009). Furthermore, STZ T1DM rats put on an exercise regimen exhibited altered RyR2 activity (Ca2+ sensitivity) by modulating the phosphorylation state (Shao et al., 2009). These data compound the value of exercise and provide insights into the molecular level remodelling that occurs and contributes towards preserving/delaying dysfunctional contractility.

6. Conclusion

Diabetics are a high-risk population exhibiting both macrovascular and microvascular complications. Although there remains some

Fig. 4. Caveolin-3 (Cav3) has multiple cellular roles that may be implicated in the pathogenesis of DCM. In models of type 1 diabetes there is a loss of Cav3 in the myocardium. The cartoons summarise the possible ramifications of depressed levels of Cav3 in cardiac myocytes. (A) Loss of Cav3 may lead to the increased nitrosylation of the ryanodine receptor (RyR2) via the loss of inhibition on nitric oxide synthases (NOS). Similarly, the increased action of NOS can lead to the nitrosylation of the sodium channel Na1.5, prolonging the action potential. (B) Also, reduction in Cav3 results in altered cell signalling; the p42/44 MAPK (ERK 1/2) pathway is no longer inhibited, resulting in hypertrophy. Likewise, phosphoinoside-3 kinase (PI3K) and protein kinase B (PKB/Akt) are no longer sequestered to the caveolae, stimulating apoptotic pathways. (C) The relative levels of Cav1 and Cav3 regulate the translocation of the GLUT-4 transporter to the caveolae. Cav1 and Cav3 also have a role in stabilisation of the insulin receptor. A change to the expression levels of Cav1 and Cav3 contributes towards impaired glucose uptake into the cell. (D) Lastly, a loss of Cav3 in t-tubules disrupts the formation of macromolecular signalling complexes, dampening the calcium current (I_Ca,M) and promoting the formation of disordered t-tubules. Moreover, the loss of inward rectifying potassium channel (Kir2.1) causes a reduction in the potassium current (I_KiT).
controversy as to whether DCM is a distinct phenotype there are two unique insults, hyperglycaemia and metabolic abnormalities that additionally contribute towards decreased compliance and contractility. Moreover, the increased probability of further heart complications in diabetic patients remains despite current treatments; thus whether this risk derives from diabetes or a combination of additional pathogenic cardiovascular insults, they undoubtedly remain a high-risk patient group. Furthermore, there are currently no therapies specifically for treating DCM and the majority of medications are directed at systolic dysfunction whereas a hallmark of early DCM is diastolic dysfunction. Here we have considered how Cav3 is not only critical for glucose uptake, with a diverse role in multiple cell signalling pathways, but also how it influences the structural integrity of cardiac myocytes by influencing t-tubule morphology which is essential for maintaining contractile function. The Ca\textsuperscript{2+} cycling mechanisms that are perturbed in the diabetic heart are numerous but a key role for Cav3 as a regulating factor is emerging. However, there still remains a large void in our knowledge and further focus is now required to disentangle the complexities of the various pathways. The data overall, indicates that Cav3 plays a cardioprotective role and that down-regulation is a feature of numerous cardiovascular pathologies as summarised in Fig. 4. We have reviewed here the strategies and approaches for up-regulating Cav3 and considered how this strategy may be beneficial in a clinical setting. In conclusion, modulation of Cav3 levels, whether through exercise or pharmacological manipulation, to target the heart represents a new approach to improving contractility and/or rhythmicity and promote pro-survival mechanisms. Although, it should be noted that in addition to the restoration of Cav3 protein levels the expression of Cav1 should also be considered as in some pathways (e.g. GLUT4 translocation) it is the maintenance of the isoform balance that is also important. In conclusion, we suggest that given the role of in multiple cellular processes, with a general trend to depletion of protein under pathological conditions that a Cav3-targeted therapy aimed at preservation and maintenance of physiological levels warrants further investigation and may have applications not only for DCM but also for treating other forms of HF.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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