FoxO1 is crucial for sustaining cardiomyocyte metabolism and cell survival

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Abstract

Diabetic cardiomyopathy is a term used to describe cardiac muscle damage-induced heart failure. Multiple structural and biochemical reasons have been suggested to induce this disorder. The most prominent feature of the diabetic myocardium is attenuated insulin signalling that reduces survival kinases (Akt), potentially switching on protein targets like FoxOs, initiators of cell death. FoxO1, a prominent member of the forkhead box family and subfamily O of transcription factors and produced from the FKHR gene, is involved in regulating metabolism, cell proliferation, oxidative stress response, immune homeostasis, pluripotency in embryonic stem cells, and cell death. In this review we describe distinctive functions of FoxOs, specifically FoxO1 under conditions of nutrient excess, insulin resistance and diabetes, and its manipulation to restore metabolic equilibrium to limit cardiac damage due to cell death. Because FoxO1 helps cardiac tissue to combat a variety of stress stimuli, it could be a major determinant in regulating diabetic cardiomyopathy. In this regard, we highlight studies from our group and others who illustrate how cardiac tissue-specific FoxO1 deletion protects the heart against cardiomyopathy and how its down-regulation in endothelial tissue could prevent against atherosclerotic plaques. In addition, we also describe studies that show FoxO1’s beneficial qualities by highlighting their role in inducing anti-oxidant, autophagic, and anti-apoptotic genes under stress conditions of ischaemia–reperfusion and myocardial infarction. Thus, the aforementioned FoxO1 traits could be useful in curbing cardiac tissue-specific impairment of function following diabetes.

Keywords

FoxO1 • Heart • Metabolism • Cell death • Diabetes

1. Introduction

Type 1 diabetes (T1D) is usually polygenic in origin with genetic and environmental factors playing roles in its development. Dysfunction and β-cell death normally follows an immune attack.1,2 As a result, there is decreased insulin synthesis and secretion.1 Compared to T1D, Type 2 diabetes (T2D) is the major form of diabetes, and is prevalent in 90% of patients suffering from diabetes. The major causes for T2D include a high fat diet, obesity, lack of exercise, increased and prolonged cytokine attacks, glucotoxicity, excessive steroids (both endogenous or exogenous glucocorticoids), and stress.3,4 Whatever the origin, T2D starts with the induction of insulin resistance in adipose tissue and skeletal muscle, followed by a decrease in glucose uptake. As the body compensates by increasing insulin secretion from β-cells, the resulting prolonged activation of these cells cause their exhaustion, decline in function, and eventual death.4 Over time, this leads to the onset of hypoinsulinaemia followed by hyperglycaemia and T2D.5 Key features associated with chronic T1D and T2D include micro- and macrovascular complications; the latter include coronary artery disease, atherosclerosis, and myocardial infarction.5,6 Independent of vascular complications, T1D and T2D may provoke direct injury of cardiomyocytes, eventually leading to cardiac death, termed diabetic cardiomyopathy.6

Diabetic cardiomyopathy describes cardiac-muscle-damage-induced heart failure,7 and multiple structural and biochemical processes have been suggested to induce this disorder.5,6 The most prominent feature of the diabetic myocardium is attenuated insulin signalling. Lower insulin signalling results in reduced levels of survival kinases (Akt), which could increase potential protein targets like FoxO1.8 FoxO1, a prominent member of the forkhead box family and subfamily O of transcription factors9,10 and encoded by the FKHR gene (forkhead domain in rhabdomyosarcoma), is involved in regulating metabolism, cell proliferation, oxidative stress response, immune homeostasis, pluripotency in embryonic stem cells, and cell death.11 In this review, we describe distinctive functions of FoxO proteins, especially FoxO1, under conditions of nutrient excess, insulin resistance,
and diabetes. We also describe how manipulation of this transcriptional factor can restore metabolic equilibrium and limit cardiac damage induced by cell death.

2. Forkhead box (other) transcriptional factor 1 (FoxO1)

The Fox gene was first discovered in 1989 in Drosophila melanogaster. To date, 19 families and their subfamilies have been discovered under the Fox superfamily. FoxO is one of these family members, and includes FoxO1, 3, 4, and 6. Human FoxO is analogous to dFoxO in C. elegans. From the embryo to adulthood, FoxO transcription factors are crucial to heart homeostasis. They play an important role in cell cycle arrest, oxidative stress resistance, cell survival, energy metabolism, and cell death, and among the ‘O’ subfamily, FoxO1 is the most important factor in governing cardiac equilibrium.

2.1 Structure and regulation

FoxOs have a DNA-binding forkhead/winged helix domain (FHDBD), which is highly evolutionarily conserved for all FoxO members, a nuclear localization sequence, a nuclear export sequence, and a C-terminus containing a transactivation domain. The DBD contains 110 amino acids, which is common for most of the Fox family members, and comprises 3\(\alpha\), 3\(\beta\), and 2 winged helices. The \(\alpha\), \(\beta\), and winged helices are aligned and inter-connected in an antiparallel manner to form a 3D structure. The DNA binding of this transcription factor is facilitated by DBD, and specific regions within the N- and C-terminal domains (Figure 1).

Post-translational modifications are known to regulate the compartmentalization and transcriptional activity of FoxO1. Some of the major post-translational changes include phosphorylation, acetylation, ubiquitination, arginine methylation, and O-glycosylation. Depending on which of these sites are involved and the upstream target, the modifications can either increase or decrease the transcriptional activity of FoxO1. Akt-dependent phosphorylation initiated by insulin is the primary regulator of FoxO1. Insulin, through Akt, increases the phosphorylation of FoxO1 at the Ser256 moiety, making this transcription factor prone to further phosphorylation at Ser319 and Thr24 by Akt and serum glucocorticoid kinase. This is followed by its nuclear export, trafficking that is mediated by 14-3-3s which can bind to FoxO1 only in its phosphorylated state. Binding of 14-3-3 to phosphorylated FoxO weakens its DNA-binding ability and facilitates its nuclear export with subsequent degradation by E3 ubiquitin ligase-mediated ubiquitination. Unlike growth signals, stress signals including AMPK, p38 MAPK, Erk, JNK, and MST-1 are known to phosphorylate FoxO1 at sites different from growth signals, leading to their nuclear import. Phosphorylation of FoxO1 by stress signals is known to disrupt its binding to 14-3-3, thereby promoting nuclear retention of FoxO1 (Figure 2). The DNA-binding ability of FoxO1 is also determined by acetylases and de-acetylases. Acetylases like CBP/p300 bind and acetylate FoxO1, attenuating its DNA binding and transcriptional activity, whereas de-acetylases like Sirtuins increase nuclear retention and transcriptional activity of FoxO1. Recently, O-glycosylation and arginine methylation of FoxO1 has been shown to positively regulate the transcriptional activity of FoxO1 under conditions of chronic hyperglycaemia.

2.2 Biological function of FoxO1

FoxO1 has important roles in systemic homeostasis. In mice, the loss of FoxO1 is embryonically lethal, whereas FoxO3 deletion results in normal birth but the offspring are prone to cardiac hypertrophy and eventual cardiac failure. In different tissues, FoxO1 is involved in regulating the oxidative stress response, cell proliferation, immune homeostasis, pluripotency in embryonic stem cells, cell death, and metabolism. During the oxidative stress response, FoxO1 is known to increase expression of antioxidant genes like superoxide dismutase and GADD45, thereby promoting reactive-oxygen-species-scavenging activity, preventing DNA damage, and fundamentally safeguarding cells from damage. FoxO1 also plays an important role in cell-longevity through the collaborative effect of FoxO1 with Sirt1, which turn on anti-oxidant genes like MnSOD and catalase. In cancer, FoxO1’s ability to regulate genes involved in cell cycle

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**Figure 1** Structure of FoxO1 protein. FoxOs have a DNA-binding Forkhead/winged helix DNA binding domain (FHDBD), a nuclear localization sequence (NLS), a nuclear export sequence (NES), and a C-terminal containing a transactivation domain (TAD). The Akt phosphorylation (P) and 14-3-3-binding sites are dispersed throughout the DNA-binding domain and NLS of FoxO1.

**Figure 2** Mechanisms leading to increased nuclear entry and transcriptional activity of FoxO1. Under conditions of nutrient excess, insulin resistance, and diabetes, when there is impaired insulin receptor signalling accompanied by increased MAPK activation, the ability of Akt to expel FoxO1 from the nucleus is lost, and there is increased MAPK mediated nuclear entry of FoxO1. In the absence of survival kinase and increased stress kinase activation, the ability of 14-3-3 to bind and promote the nuclear exit of FoxO1 is insufficient. As a result, there is decreased nuclear exit, increased nuclear entry, and retention of nuclear FoxO1 leading to its increased transcriptional effect.
progression (cyclin B, PIK), together with their inhibition of cell proliferation (p27kip1) makes FoxO1 a major therapeutic target.17,24 FoxO1 plays an important role in inflammation and sepsis through its ability to increase the activation of TLR4 and NF-kappa B.25 A new role for FoxO1 has emerged in stem cell research due to its ability to maintain the pluripotency of embryonic stem cells, suggested to be due to FoxO's ability to regulate OCT4 and SOX2 genes by a direct effect on their respective promoters.26 FoxOs are also involved in inducing apoptosis and atrophy by up-regulating genes like FasL, Bim, and MURF-1.27 Activation of pro-apoptotic proteins, like Bim and caspases, is known to increase tumour cell death.28 FoxO1 affects metabolism by up-regulating gluconeogenesis. It accomplishes this by increasing hepatic glucose-6-phosphatase, phosphoenolpyruvate carboxykinase mRNA, and protein.17 In skeletal muscle and HepG2 cells, FoxO1 are known to increase pyruvate dehydrogenase kinase (PDK4) protein expression, thereby decreasing glucose oxidation.17 Studies from our laboratory illustrate a role for FoxO1 in cardiac metabolism.18 26 We were the first to describe FoxO1’s function in regulating glucose and fatty acid (FA) metabolism in the heart through its effect on target genes like PDK4 and iNOS, respectively.7

2.3 Overactivation of FoxO1

FoxO1 is over-activated under conditions of fasting, nutrient excess, insulin resistance, diabetes, inflammation, sepsis, and ischaemia.29 During fasting, when Akt signalling is attenuated and stress kinases such as AMPK, JNK, and p38 MAPK are activated, the increase in nuclear FoxO1 is evident, together with its increased transcriptional function. Fasting also increases the expression and activity of Sirt1, resulting in nuclear entrapment and enhancement of FoxO1 activity. During nutrient excess, whether high fat or high glucose, studies have shown that generating excess ROS and reactive nitrogen species results in concomitant activation of MAP kinase pathways and nuclear ingress of FoxO1. Obesity, a consequence of nutrient excess, is also associated with activating the above-mentioned pathways.9 Under conditions of chronic hyperglycaemia, advanced glycated end products and their receptor activation can bring about O-linked β-N-acetylglucosamine modification of FoxO1 at the threonyl moiety, an effect known to enhance the transcriptional function of FoxO1, independent of its phosphorylated state.29 Under conditions of insulin resistance and diabetes, there is a decreased inhibition of FoxO1 phosphorylation and activity by the insulin signalling pathways, which does not allow the 14-3-3 chaperone to bind to FoxO1, making it less prone to ubiquitination-mediated degradation.16 In addition, during diabetes, stress signals are also known to be activated along with the presence of increased ROS and nitrosative stress signalling in the body.30 Following inflammation and sepsis, along with nitrosative stress signalling, toll-like receptor signalling and transcription factors such as NF-kappa B have been shown to either enhance the nuclear presence or transcriptional activity of FoxO1.23 Under conditions of hypoxia and ischaemia, transcription factors like hypoxia inducible factor (HIF-1α) are known to interact with FoxOs and enhance their transcriptional function to combat these situations.31,32

2.4 Consequences of unregulated FoxO1 activation

FoxO1 activation can bring about diverse consequences based upon the type of stimuli. Physiological conditions like fasting provoke FoxO1 to turn on gluconeogenesis and anti-oxidant genes.17 Genetic overexpression of FoxO1 can induce both beneficial and detrimental effects, depending on the duration of the study and the prevailing environment (high glucose/fat and hormones). Pathological conditions such as obesity, insulin resistance, and diabetes lead to unregulated activation of FoxO1, which can result in metabolic disturbances and cell death pathway activation in respective tissues.9,10,17

Following fasting, FoxO1 activation in the liver induces enzymes involved with gluconeogenesis like glucose-6-phosphatase (G6Pase), fructose-1,6-biphosphatase, and phosphoenolpyruvate carboxykinase (PEPCK).33 G6Pase, whose expression pattern is unique to the liver, releases glucose into the system to try and maintain glycemic levels.33 In skeletal muscle, starvation-induced activation of FoxO1 causes up-regulation of atrogin-1 (MAFbx) and MuRF-1 (muscle ring finger-1) genes, which are involved in muscle loss, thereby reducing systemic glucose utilization.34 FoxO1 overexpression in different tissues has various consequences. In adipose tissue, it leads to the suppression of p21 and PPARγ which are mainly involved in adipocyte differentiation.35,36 In the liver, it is known to enhance gluconeogenesis, lipogenesis, and lipid-induced steatosis.33,37 In β-cells, pancreatic and duodenal homeobox factor 1 (Pdx1) is involved with metabolism, function, and survival of β-cells through its ability to regulate genes like insulin, Glut 2 and glucokinase.37,38 Overexpression of FoxO1 in β-cells has been shown to inhibit Pdx1 with resultant deterioration of β-cell function, β-cell death, and ultimately T2D.17 Related to human fetal pancreatic FoxO1 overexpression, it is known to decrease β-cell differentiation through its ability to down-regulate NGN3 and NKKX6 homeobox 1 and insulin gene expressions. In 5V-40 transformed hepatocytes, FoxO1 overexpression can lead to insulin resistance. This effect was suggested to occur due a FoxO1-tuberous sclerosis complex (TSC2) interaction, resulting in TSC2 inhibition and mTOR/p70 S6K pathway activation, leading to a negative feedback effect on tyrosine phosphorylation of IRS2 and Akt.39 Non-alcoholic steatohepatitis is considered one of the characteristic features of hepatic insulin resistance. Studies in human subjects with steatohepatitis showed an increase in both the expression and nuclear presence of FoxO1. In the heart, FoxO1 overexpression causes autophagy through the induction of genes like Gabarap1 and Atg12.40–42

Obesity, cytokines, and steroidal drugs cause insulin resistance and eventually T2D, with a concomitant increase in the nuclear presence of FoxO1. In animal models of T2D, FoxO1 haploinsufficiency has been shown to restore insulin sensitivity.16 FoxO1 haploinsufficiency has also been shown to alleviate high-fat-induced insulin resistance in liver and skeletal muscle.43 Recently, a collaborative role of FoxO1 and notch signalling has been revealed to induce insulin resistance in animal models, which was substantiated by using liver-specific double knock out of both targets.44 In DKO diabetic mice, which have a double knock out of IRS1 and 2 in the liver, FoxO1 deletion normalizes hyperglycaemia.44 An interesting study using insulin-resistant macrophages obtained from either insulin receptor KO or ob/ob mice showed increased macrophage apoptosis, an event mediated by FoxO1, resulting in necrotic plaque core enlargement during atherosclerosis.45 Genetic deletion of FoxO1 in these macrophages made them resistant to apoptosis. In diet-induced obese rats, inhibition of FoxO1 in the hypothalamus using FoxO1 anti-sense oligonucleotide resulted in reduced appetite and body weight, decreased circulating insulin levels, and increased insulin sensitivity.45 A role for FoxO1 in the development of T2D was highlighted by
the study of Kim et al. who demonstrated that a hypothalamic- and pancreas-specific FoxO1 knock in was able to create obesity and glucose intolerance. Unlike other tissues, exposure of adipocytes to free FA leads to insulin resistance with decreased expression of FoxO1. Apart from this, decreased FoxO1 activity resulted in increased expression of adipose PPARγ and its target genes, revealing a new role for this transcription factor in adipose tissue.

T1D is characterized by attenuated survival signalling, and turning on of FoxO1 transcription factors in different tissues, including β-cells, with wide-ranging consequences. A role for FoxO1 in T1D was first reported in NOD mice (non-obese diabetic), a murine model of autoimmune T1D. In these animals, increased nuclear FoxO1 was associated with decreased β-cell proliferation and survival. Interestingly, GLP-1 (glucagon-like peptide-1) treatment of NOD mice for 4 weeks reduced β-cell destruction and increased β-cell proliferation, events that followed the nuclear exit of FoxO1 in β-cells. GLP-1-mediated FoxO1 nuclear omission also correlated to a delay in the onset of autoimmune T1D. In Sprague–Dawley rats treated with streptozotocin (STZ-55 mg/kg) to induce diabetes, maintained for 3 and 6 months, an increase in micro- and macrovascular apoptosis was observed. FoxO1 regulated these effects through its mediation of ROS and caspase-3/7. A role for FoxO1 in decreasing wound healing and increasing bone fracture was suggested in CD-1 (mice lacking cluster domain 1-model of autoimmune disorder) mice injected with 40 mg/kg STZ. These effects were a consequence of a reduction in proliferation and increased apoptosis of fibroblasts, and a decreased generation and increased apoptosis of chondrocytes, respectively.

To date, a role for FoxO1 in the heart following insulin resistance/ T2D and T1D has not been adequately researched. We were the first to report a role for FoxO1 in excess glucocorticoid-induced cardiac insulin resistance. Glucocorticoids increased the nuclear presence of FoxO1, which was connected to an increase in PDK4 expression and activity, thereby decreasing cardiac glucose oxidation. If prolonged, this effect could lead to cardiac pathologies due to increased lactate formation, thereby decreasing pH, in addition to tilting metabolism towards the use of excess lipids leading to lipotoxicity. In a different study, using in vivo and in vitro models of lipid excess, we showed an increased nuclear presence of FoxO1 in cardiac cells, an effect that contributed to iNOS-mediated nitrosative stress and triglyceride accumulation due to activation of an FoxO1–iNOS–CD36 axis. Apart from this, PP2A expression was also aggravated, a potential cause for increasing cardiac insulin resistance and cell death. Using STZ and diazoxide to induce hypoinsulinaemia and hyperglycaemia (unpublished data), we also reported a nuclear increase in cardiac FoxO1 which increased the expression of iNOS. iNOS by nitrosylating GAPDH caused an increased nitrosative stress in the nucleus, eventually leading to PARP1 activation and turning on of PARP1-AIF cell death signalling.

3. Mechanisms behind FoxO1-induced cardiac injury following insulin resistance and diabetes

Among many insults, alterations in metabolism or activation of cell death signalling are major contributors towards cardiac injury during insulin resistance and diabetes. When activated, FoxO1 alters metabolism with a subsequent impact on cell survival. Activated FoxO1 can also directly turn on the cell death signalling cascade.

3.1 Metabolic role of FoxO1

Under conditions of nutrient excess, insulin resistance, and diabetes, FoxO1 can affect glucose metabolism by directly regulating glucose oxidation through its mediation of PDK4. The metabolism of glucose comprises of a number of processes, which include cellular uptake and glycolysis, with the formed pyruvate undergoing oxidation in the mitochondria to yield ATP. Insulin is a mandatory mediator for all of these glucose catabolic pathways. For example, in the heart, although basal glucose uptake is mediated by GLUT1, the major amount of glucose transported is through GLUT4, which is under the control of insulin. Insulin signalling also plays an important role in enhancing glycolysis through its influence on phosphofructokinase-1, the rate-limiting enzyme for glycolysis. Following glycolysis, glucose undergoes oxidation through the catalytic activity of pyruvate dehydrogenase complex (PDC). PDC is controlled by insulin through its ability to suppress the expression and activity of PDK4. Under conditions of reduced insulin signalling, PDK4 is known to phosphorylate the E1 moiety of pyruvate dehydrogenase of the PDC, thereby preventing the formed pyruvate from undergoing mitochondrial oxidation. In this situation, pyruvate is converted to lactate rather than getting oxidized to acetyl CoA. The heart can utilize formed lactate following its conversion to pyruvate.

T1D is associated with a reduction in circulating insulin and an increased PDK4 gene expression, a down stream target of FoxO1. Evidence of increased PDK4 expression is also documented in high fat and obese models of insulin resistance, and is linked to both inefficient insulin action and FA stimulation of PPARs and their co-activators. Other triggers of insulin resistance that are known to increase PDK4 mRNA and protein expression, both in vivo and in vitro, are stress hormones like glucocorticoids. Our laboratory has shown that in vivo, a single dose of 1 mg/kg of dexamethasone increases PDK4 expression, with an altered metabolism (decreased glucose and increased FA oxidation). Additionally, by using dexamethasone as a means to induce nuclear FoxO1 in cardiomyocytes, we were able to increase PDK4 induction and decrease glucose oxidation. This effect was reversed using insulin, which was able to decrease the nuclear presence of FoxO1. By inhibiting glucose oxidation in conditions of PDK4 activation, the tissue is stimulated to use FA as a preferred substrate, and lactate is the fate of entered glucose. Increased lactate could bring about a decrease in pH, which can initiate certain cell death pathways or affect membrane stability and functional integrity through H+ ions. Prolonged increase in FA oxidation also contributes towards a decrease in pH, along with resultant ROS induction and nitrosative stress. Inhibition of PDK4 has been shown to be a promising target for diabetes and metabolic syndrome treatments, with radicicol and M77976, known PDK4 inhibitors having beneficial effects.

In addition to glucose, FoxO1 also has a significant role in regulating the supply and oxidation of FA. This is important as, on a molar basis, ATRP derived from FA is produced several folds higher than glucose, and cardiac tissue prefers FA to glucose. Exogenous FA is delivered to the heart by lipoprotein lipase-mediated lipolysis of TG-rich lipoproteins and albumin bound FA. After traversing the interstitial space, FA is taken up by the cardiomyocyte using a transporter system, which includes FABPpm, FATP, and FAT/CD36.
these transporters, CD36 or clustered domain 36 plays an important role as a lipid provider to the heart.55,56 It has multiple ligands inside the body, which include free FA, collagen, thrombospondin, anionic phospholipids, and oxidized LDL.57 Other than its role in FA transport, CD36 is involved in the internalization of oxidized lipids, phagocytosis, cell migration, cell adhesion, cholesterol efflux, and anti-angiogenesis.58 To show its function, it is mandatory that CD36 reaches the plasma membrane surface, and there are various mechanisms to achieve this. The most prominent stimuli include insulin, exercise, and oligomycin, which through PI3 kinase, Akt, and AMPK can regulate CD36 plasma membrane translocation acutely.59 Cytoskeletal and vesicular proteins also play a major role in effective translocation of CD36 to the membrane surface. In C2C12 cell lines, it was first shown that nuclear FoxO1 plays an important role in lipid metabolism by increasing the membrane presence of CD36 followed by increased FA oxidation and triglyceride accumulation.60 Interestingly, this occurred in the absence of any change in CD36 mRNA or total protein content, indicating that a signalling mechanism was likely responsible for the membrane increase in CD36.61 Using in vivo and in vitro models of lipid excess, our studies also demonstrated that an increase in nuclear compartmentalization of FoxO1 was accompanied by an increase in membrane CD36 followed by triglyceride accumulation, suggesting a role for FoxO1 in lipid supply and storage.62 We discovered the mechanism behind how nuclear FoxO1 can increase membrane CD36, and reported that these events were a consequence of increased iNOS expression, and iNOS mediated tyrosine nitration of Cdc42, a Rab GTPase involved with shuttling of vesicle protein VAMP2 to the membrane surface, thus increasing membrane CD36.63 Nitrated Cdc42 also mediates cytoskeletal re-arrangement, providing an additional mechanism for this membrane CD36 translocation. In addition to CD36’s function at the plasma membrane, residence of this transporter at the mitochondrial membrane also plays a role in FA provision into the mitochondria favouring their increased oxidation. Whether FoxO1 has any role in increasing mitochondrial membrane CD36 in the heart in conditions like insulin resistance and diabetes has yet to be evaluated.

FoxO1’s regulation of FA uptake makes it a significant player in inducing cardiac dysfunction. Increased FA flux into the cell can enhance FA oxidation with resultant generation of ROS.64 Increased ROS activates stress signalling and cell death signal activation, thereby causing catastrophic events like necrosis and apoptosis.65 ROS can also decrease the anti-oxidant capacity of cells, either directly or through their ability to form reactive nitrogen species.66 In situations where FA delivery exceeds the capacity for FA oxidation, this uncoupling results in intracellular triglyceride accumulation, and diacylglycerol and ceramide generation, which are toxic to the cell.67 Ceramides augment the expression of PP2A, a causative factor for induction of apoptosis through dephosphorylation of BAD and survival kinases.68 They have also been shown to promote caspase-independent cell death and even necrosis.69 With increasing accumulation of cardiac triglycerides and ceramides, contractile force and frequency are hampered due to their respective actions on structural and contractile proteins. Thus, by regulating the FoxO1–CD36 axis, we could reduce the damage associated with excess lipid-induced cell death, especially during insulin resistance and diabetes.

### 3.2 Role of FoxO1 in cell death

Apart from its metabolic role, FoxO1 has an important function in mediating cell death.37 Cell death can be classified broadly into two categories; programmed and non-programmed. Apoptosis, autophagy, and apoptosis-like programmed cell death are some examples of programmed death processes.64,65 In apoptosis and apoptosis-like programmed cell death, there is chromatin condensation whereas autophagy involves a sequential process where cell organelles are sequestered into autophagic vesicles followed by lysosomal degradation.66 Necrosis is an example of non-programmed cell death and is characterized by organelle swelling, especially in the cytosol, followed by plasma membrane rupture.67,68 FoxO1 participates in the regulation of all types of cell death seen during insulin resistance and diabetes.

With apoptosis, FoxO1 has been reported to induce expression of proteins involved in both the intrinsic and extrinsic pathways of this programmed cell death.69,70 The intrinsic cascade starts with a destabilized mitochondrial membrane pore opening, and subsequent release of cytochrome c to the cytosol.71,72 This released cytochrome c forms a complex with apoptosis activating factor-1 (Apaf-1), and procaspase-9, to create an active complex called apoptosisosome. This active apoptosisosome complex then cleaves and activates the executional caspases like caspase-3 and 7.73 These caspases activate caspase-activated DNase and inactivate PARP1 and bring about DNA damage.74 The extrinsic pathway starts with the activation of receptors by Fas ligand or TNFx. This results in the activation of procaspase-8 into an active dimerized caspase-8 through mediation of the death-induced signalling cascade, which involves adapter proteins like FADD and TRADD.75 Activated caspase-8 can result in activation of executional caspases like caspase-3, which can bring about DNA damage as described above. FoxO1 is suggested to bring about cell death through its ability to increase the expression of caspases and cell death receptors, both of which are reported to be augmented during diabetes. In addition to these traditional death pathways, apoptosis is also under the control of BH-domain-containing family members and these include pro-apoptotic (Bax, Bak, Bid, Bim, Bad, Noxa, and Puma) and anti-apoptotic (Bcl-2, Bcl-xL, and Mcl-1) proteins.76,77 FoxO1 has been shown to directly increase the expression of some pro-apoptotic proteins like Bim and Puma. We also suggest a role for FoxO1 in regulating pro-apoptotic BAD, a mechanism mediated through PP2A.78 BAD activity is regulated by phosphorylation-dependent inhibition (through insulin signalling) and de-phosphorylation-mediated activation (via PP2A/B).79,80 In conditions like insulin resistance and diabetes, when there is a decreased survival kinase signalling followed by an increased presence of nuclear FoxO1 and PP2A induction, there is an activation of the pro-apoptotic protein BAD. It should be noted that in cardiomyocytes in which FoxO1 is overexpressed, there is suppression in activity of PP2A/B.81 One explanation for the discrepancy between these in vivo (diabetes) and in vitro (overexpression) studies regarding the effect of FoxO1 on PP2A could be the presence of excess lipids observed with diabetes. These excess lipids brought in by the FoxO1–CD36 pathway could turn on PP2A, there by de-phosphorylating and activating the BAD induced apoptotic pathway. Thus, although FoxO1 emerges as a potential participant in regulating cell death both directly and indirectly, its role during conditions of insulin resistance and diabetes has yet to be explored.

Autophagy is another form of cell death under the programmed category. Beclin-1 is the major protein involved with autophagocytic cell death.82 Some of the typical features of autophagy include increased lysosomal formation, increased lysosomal hydrolase like cathepsin-D activation, acidic pH induction, and lysosomal leakage with formation of autophagosomes.80,82 During fasting, FoxO1 has...
shown to increase the genes responsible for inducing autophagy like Gabarapl1 and Atg12 in the heart.\textsuperscript{3} \textsuperscript{3} \textsuperscript{3} Although FoxO1 has a well-defined role in induction of autophagy, whether this mode of programmed cell death is prevalent in diabetes is debated.

Necrosis leads to ATP depletion and cellular content release into neighbouring regions, which promote the non-programmed form of cell death.\textsuperscript{84} Conditions like an increase in cytosolic calcium and formation of excess reactive oxygen/nitrogen species initiate this necrotic pathway, a prominent feature observed during long-term obesity and diabetes, and their associated cardiovascular dysfunctions.\textsuperscript{84} Receptor interacting protein-1 (RIP-1) is one of the major upstream regulators of necrotic cell death. RIP-1 is protected by the chaperone Hsp-90 and inhibited by caspases.\textsuperscript{85} Interestingly, nitric oxide has been shown to inhibit caspases by nitrosative modification, thus preventing caspase-mediated RIP inhibition, tilting the equilibrium from caspase-mediated apoptosis to necrotic cell death.\textsuperscript{85} Indeed, in neuronal cells, iNOS-induced NO has been shown to inactivate caspase-3.\textsuperscript{86} Apart from RIP-1, activated PARP1 can also induce necrosis-like cell death, and PARP1 is known to be inactivated by caspase-3.\textsuperscript{85} In STZ diabetic hearts, we have shown that the increase in nuclear FoxO1 was accompanied by an increased induction of iNOS followed by caspase-3 S-nitrosylation (unpublished data). Although this nitrosylation reduced caspase-3’s ability to inactivate PARP1 in cardiac tissue, its role in regulating RIP-1 is still unknown. Overall, FoxO1-induced iNOS could play an important role in determining this mode of cardiac cell death during diabetes.

Apart from a role as a mediator in necrotic cell death, PARP1 can initiate a novel cell death pathway which is independent of apoptosis and necrosis, called parthanatos.\textsuperscript{87} If activated, PARP1 can stimulate initiation of a novel cell death pathway which is independent of apoptosis and necrosis, called parthanatos.\textsuperscript{87} In STZ diabetic hearts, FOXO1 was shown to increase the genes responsible for inducing autophagy like Gabarapl1 and Atg12. Although FoxO1 has a well-defined role in induction of autophagy, whether this mode of programmed cell death is prevalent in diabetes is debated.

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Necrosis leads to ATP depletion and cellular content release into neighbouring regions, which promote the non-programmed form of cell death.\textsuperscript{84} Conditions like an increase in cytosolic calcium and formation of excess reactive oxygen/nitrogen species initiate this necrotic pathway, a prominent feature observed during long-term obesity and diabetes, and their associated cardiovascular dysfunctions.\textsuperscript{84} Receptor interacting protein-1 (RIP-1) is one of the major upstream regulators of necrotic cell death. RIP-1 is protected by the chaperone Hsp-90 and inhibited by caspases.\textsuperscript{85} Interestingly, nitric oxide has been shown to inhibit caspases by nitrosative modification, thus preventing caspase-mediated RIP inhibition, tilting the equilibrium from caspase-mediated apoptosis to necrotic cell death.\textsuperscript{85} Indeed, in neuronal cells, iNOS-induced NO has been shown to inactivate caspase-3.\textsuperscript{86} Apart from RIP-1, activated PARP1 can also induce necrosis-like cell death, and PARP1 is known to be inactivated by caspase-3.\textsuperscript{85} In STZ diabetic hearts, we have shown that the increase in nuclear FoxO1 was accompanied by an increased induction of iNOS followed by caspase-3 S-nitrosylation (unpublished data). Although this nitrosylation reduced caspase-3’s ability to inactivate PARP1 in cardiac tissue, its role in regulating RIP-1 is still unknown. Overall, FoxO1-induced iNOS could play an important role in determining this mode of cardiac cell death during diabetes.

Apart from a role as a mediator in necrotic cell death, PARP1 can initiate a novel cell death pathway which is independent of apoptosis and necrosis, called parthanatos.\textsuperscript{87} If activated, PARP1 can stimulate formation of poly-ADP ribose, which, when formed in excess, can lead to a mitochondrial membrane leak. This results in mitochondrial to nuclear translocation of proteins like apoptosis-inducing factor (AIF) and endonuclease-G, which can lyse DNA and cause a caspase-independent apoptosis like cell death.\textsuperscript{87} In STZ diabetes, we showed that increased nuclear FoxO1 resulted in increased iNOS induction and nitrosative stress. As a result, GAPDH was nitrosylated followed by its association with Siah-1 and their combined nuclear entry, increasing nitrosative stress inside the nucleus.\textsuperscript{88} This combined with increased nitrosylation of caspase-3 accompanied by PARP1 activation turned on PARP1-AIF-dependent parthanatos. Thus, parthanatos could be considered as an emerging candidate to explain cardiac cell death during diabetes.

### 3.3 FoxO1-induced iNOS bridges metabolism to cell death

Changes in cardiac metabolism and cell death pathway stimulations are an unavoidable consequence of diabetes. As indicated, FoxO1 has a significant impact in altering metabolism. For instance, the metabolic consequences of FoxO1 up-regulation have been reported in BAECs, which demonstrated an increase in LDL oxidation.\textsuperscript{89} In cardiomyocytes, we have described how lipid excess increases nuclear FoxO1 content, with increased delivery and myocardial accumulation of lipids.\textsuperscript{9} In both these studies, FoxO1-induced iNOS was the primary candidate for bringing about these metabolic changes. As iNOS is also known to activate cell death pathways per se, we believe that the FoxO1–iNOS axis bridges metabolism to cell death during diabetes.

The mechanisms by which iNOS regulates metabolism is through the induction of NO-mediated cellular signalling or protein modifications.\textsuperscript{90} NO produced from iNOS can generate reactive nitrogen species which include nitrosyl moieties, nitrosothiols, and peroxynitrite. The conversion of NO to peroxynitrite occurs in the presence of superoxides, free radicals which are augmented during insulin resistance and diabetes. In the cardiovascular system, increased NO with resultant peroxynitrite stimulates PKCζ. Activated PKCζ increases the transcriptional activity of proteins like Erk, HIF-1, and NF-kappa B, which bring about multiple alterations in metabolism of both glucose and FA. They also cause a further induction of iNOS, creating a positive feedback loop that generates copious amounts of NO.\textsuperscript{90} Apart from alterations in metabolism brought about by NO signalling, NO is known to influence metabolism by bringing about direct protein modifications that include nitration and nitrosylation, with either loss or gain of function of the target proteins.\textsuperscript{90,91} We have shown that under conditions of lipid excess in the heart, FoxO1-induced iNOS caused tyrosine nitration of Cdc42, a Rab GTPase, activating and increasing its association to VAMP2 in addition to re-arranging the cytoskeleton, thus increasing lipid influx through CD36 and promoting lipotoxicity.\textsuperscript{9} Nitration/nitrosylation modifications can also affect proteins indirectly, by (a) altering their ability to undergo phosphorylation and (b) provoking compartmentalization of proteins from cytosol to mitochondria/nucleus.\textsuperscript{92} Examples of proteins undergoing compartmentalization following these modifications include aldolase-A and GAPDH.\textsuperscript{93} Overall, preventing excess NO or superoxide generation could limit the metabolic complications seen during nutrient excess, insulin resistance, and diabetes.\textsuperscript{94} Indeed, systemic scavengers like SOD prevent the conversion of NO to peroxynitrite, thereby protecting the cells from reactive nitrogen species attack.\textsuperscript{9} Additionally, NOS inhibitors like L-NIL and L-NIO and highly selective 1400W, GW273629 and GW274150, have been found to be useful in the above-mentioned pathological conditions.\textsuperscript{95}

Other than metabolism, NO signalling can affect cell survival. This effect is either a direct consequence of overactivating PKCζ, ERK, and NF-kappa B-induced apoptosis, or indirectly through ROS-induced cell death.\textsuperscript{96,97} Protein modifications induced by NO can also contribute towards the cell death process.\textsuperscript{97} In this regard, nitration modification has been shown to have an impact on organelles like mitochondria. For example, nitration of major mitochondrial proteins like cytochrome c, MnSOD, aconitase, voltage-gated anion channel, succinate dehydrogenase, ATP synthase, ATPase, and voltage-gated K⁺ channels weakens mitochondrial function and contributes towards cell death.\textsuperscript{97,98} Apart from mitochondria, nitration modification also affects protein pumps and cytoskeletal proteins including Na⁺–K⁺–ATPase, SERCA2A, actinin, synuclein, desmin, myosin heavy chain, profilin, and tubulin, which can result in decreased cellular function and eventually cell death.\textsuperscript{97,98} Following lipid excess, we showed that FoxO1-induced iNOS directed FA towards triglyceride accumulation, a key player in lipotoxicity-induced cell death. In these hearts, although decreased OXPHOS expression could explain the reduced mitochondrial efficiency for oxidizing FA, the contribution of nitration modification of mitochondrial proteins was not explored.\textsuperscript{9} In a different study, using STZ diabetes, we found an increase in cytochrome c release to the cytosol followed by an increased caspase-3 cleavage, likely brought about by FoxO1-induced iNOS mediated tyrosine nitration of cytochrome c.\textsuperscript{9} Like nitration modification, S-nitrosylation is another major nitrosative stress-mediated protein modification, which can regulate cell death. There are more than...
100 substrates that can be S-nitrosylation modified, which include caspase (1–8), haemoglobin, myoglobin, GAPDH, NM2A receptor complex, aquaporin-1, glutathione, ferritin, homocysteine, serum albumin, aldehyde dehydrogenase, and hexokinase.1–5,99,100 In HEK293 (Human Embryonic Kidney 293—cell models that are easy to transfect) cells, iNOS induction promoted S-nitrosylation of GAPDH followed by its association to Siah1 and their combined nuclear ingress with degradation of nuclear proteins.104 In the STZ diabetic hearts, we showed that following FoxO1 induction of iNOS, GAPDH S-nitrosylation and its association with Siah1 was increased followed by their nuclear translocation and increased nitrosative stress in the nucleus. Irrespective of NO signalling and nitration/nitrosylation modifications, nitrosative stress induced alterations in metabolism and cell death have a significant role in affecting cardiovascular function.9,106 From our studies, we show that FoxO1 is a major inducer of iNOS in the heart under conditions of lipid excess, insulin resistance, and diabetes, and could be playing a role in nitrosative stress-mediated complications, which can alter metabolism, and regulate cell death, thus leading to cardiomyopathy.

4. Diabetic cardiomyopathy
Data from the Framingham study has revealed that cardiovascular disease is the major cause of diabetes-associated mortality, with vascular disease like hypertension and atherosclerosis playing a significant role in its aetiology.102–104 Independent of vascular complications, T1D and T2D can provoke direct cardiomyocyte injury. Diabetic cardiomyopathy portrays the condition where heart failure is induced due to a number of intrinsic biochemical and morphological changes occurring in the cardiac muscle, and includes abnormalities in intracellular calcium handling, altered contractile ability, fibrosis, changes in metabolism, and cell death.105–108

Changes in metabolism are a key event for inducing diabetic cardiomyopathy.105,106 For example, an increased serine phosphorylation of IRS-1 can bring about a deficit in glucose processing by cells, and by default, unregulated lipid utilization and storage.109–111 Excess glucose and lipids in the system can induce ROS in cardiac cells, bringing about a change in calcium homeostasis.105 Apart from this, hyperglycaemia and hyperlipidaemia can increase the formation of glycated end products. Glycation of collagen, SERCA2a and RyR2, can magnify the damage to cardiac muscle.112–114 Diabetes decreases glucose oxidation by an induction of PDK4, thereby increasing lactate formation and decreasing the pH of myocardial tissue.6 This altered pH can bring about changes in cardiac ionic balance (by altering H4 and Ca2+ ions), which can then result in structural and functional changes.6 Diabetes also increases FA delivery and oxidation, with the excess unmetabolized lipids accumulating as triglycerides.6 This, along with the formation of ceramides in the heart, can cause cell death through PP2A mediated.6 We have shown that FoxO1-mediated induction of PDK4 leads to a reduction in glucose oxidation and increased lactate accumulation.10 Regarding lipids, FoxO1, by inducing iNOS, brought about protein modification of trafficking proteins followed by increased membrane translocation of CD36.6 This led to an unregulated lipid flux followed by increased triglyceride accumulation and PP2A induction, features that contribute towards cardiac dysfunction and death.6,8,9

Cell death is another leading cause of diabetic cardiomyopathy.115 An external attack by the surrounding cells due to several assaults, followed by their release of cytokines can accelerate the death process.116–119 An intrinsic mechanism due to growth imbalance and stress signals within the cardiac cells can also bring about cell death during diabetes.120–122 This occurs due to decreased IRS–Akt signalling, increased reactive oxygen/nitrogen species generation, reactive oxygen species-mediated MAPK stimulation, reduced antioxidant capability, and decreased mitochondrial function.123–125 In the STZ diabetic hearts, we showed that FoxO1, through the mediation of iNOS, could modify protein activity by nitration/nitrosylation and induce cell death in a caspase dependent and independent manner. Recent findings by Battiprolu et al. have shown that a high-fat diet induces a hypertrophic response in addition to decreasing left ventricular function, an effect which was not apparent in FoxO1-deficient mice following a high-fat diet.8 Thus, FoxO1, either directly or indirectly through the regulation of iNOS, can bring about alterations in metabolism, induce cell death, and promote diabetic cardiomyopathy.

5. Conclusion and perspectives
FoxO1 is a major transcription factor involved in regulating metabolism and cell death. Its role in the heart during pathological conditions like insulin resistance and diabetes has not been fully explored.8,10 During insulin resistance and diabetes, there is a decrease in growth and an increase in stress signalling. With attenuated growth signals, the mechanisms responsible for exporting nuclear FoxO1 are weakened, thereby promoting its nuclear retention.9,10 Increased nuclear presence of FoxO1 increases its transcriptional activity, with up-regulation or repression of target genes.8,110 Studies from our laboratory have shown that FoxO1 up-regulates the expression of PDK4 and iNOS, thus controlling glucose and FA metabolism during insulin resistance and diabetes.10 By inducing PDK4 and inhibiting glucose oxidation, FoxO1 compels the heart to use more FA.10 This is achieved through the FA transporter, CD36, and is mediated by FoxO1’s induction of iNOS.9 Chronically, increased FA influx increases oxidation with the resultant generation of ROS. If FA oxidation is uncoupled to FA delivery, triglyceride accumulation and ceramide synthesis ensue with resultant cardiac lipotoxicity.8 The decreased glucose oxidation increases the production of lactate, leading to a decrease in pH with debilitating effects on the heart.6 Independent of its metabolic effects, FoxO1 can also bring about cell death through multiple mechanisms which include transcription effects directly and NO signalling or nitration/S-nitrosylation protein modifications indirectly.7 It should be noted that the alterations in metabolism brought about by FoxO1 can also cause cell death through glucolipotoxicity.8–10 Altogether, these different mechanisms create a vicious cycle of events that eventually facilitate the development of cardiomyopathy seen during diabetes (Figure 3).

During the early stages of diabetes, metabolic regulators like AMPK are involved with energy homeostasis to compensate for the decrease in glucose utilization.126 More chronically, the progression of diabetes with its associated increase in glucose and lipid metabolites turns off AMPK stimulation.124 Under these conditions, excess FA entering the cells turn on nuclear receptors like PPARs. PPARs are associated with increased lipid utilization, ROS generation, and aggravation of lipotoxicity.127,128 Unlike AMPK (acutely) and PPARs (chronically), FoxO1 is a transcription factor that is activated immediately on attenuation in growth signals, and remains activated for the duration of diabetes.129,130 Knocking out of this transcription factor has been shown to be lethal, which makes it a vital player for harmonious cardiac function. However, its over-activation has also proved to be...
detrimental. FoxO1 regulation is of crucial importance as both its up- or down-regulation can lead to serious consequences. This protein has prominent expression in the cardiovascular system, specifically in vascular and endothelial cells, and has a significant function right from the embryonic stage. In cardiomyocytes, it is involved in combating stress by up-regulating antioxidant, anti-apoptotic, and autophagy genes. In fact, a cardiomyocyte-specific down-regulation of FoxO1 has been shown to decrease cardiac function, and increase scar formation, and apoptosis under conditions of stress like ischaemia or myocardial infarction. In contrast, under conditions of genetic or high fat induced insulin resistance, deletion of FoxO1 has been shown to rescue heart from not only diabetic cardiomyopathy but also from tissue-specific insulin resistance. In addition, animals with FoxO1-specific deletion show decreased atherosclerotic plaque formation due to decreased formation of ICAM1 and TNFα gene induction in aortic endothelial cells. Thus, maintaining FoxO1’s equilibrium, both in activity and in expression, is mandatory for healthy cardiac function.

Gaining more insight into the mechanism(s) by which cardiac forkhead proteins are regulated may assist in devising new therapeutic strategies to restore cardiac function during insulin resistance and diabetes.

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