Universidade do Minho
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Vasco Sequeira Oliveira

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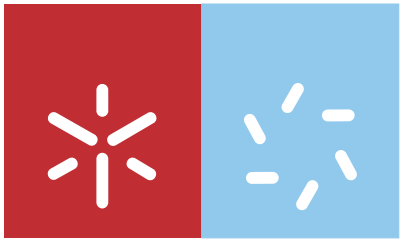

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Role of Wnt signaling in heart disease

Master Thesis - Molecular Genetics

Work performed under both supervision of
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ROLE OF WNT SIGNALING IN HEART DISEASE

Heart failure has a major social-economic impact in our society. Despite major advances in the understanding of this pathology, the mechanisms of its development, as well as its pathophysiology, remain unclear. Therefore, it is our priority to clarify how extra- and intracellular factors are able to modulate heart function. Several pathways and/or factors had already been associated with different phases of heart failure development namely TGF-β, IGF, calcineurin, several GPCRs, MAPK, Akt and GSK-3. More recently, several studies started shedding some light on a putative role of Wnt signaling in heart failure development.

Wnt signaling is a major regulator of cell-fate specification during development, proliferation, survival, migration and adhesion. Several diseases including cancer, diabetes, osteoporosis and psychiatric disorders are the result of deregulation of canonical Wnt signaling, due to either genetic alterations or changes in the levels of its effectors.

The role of canonical Wnt signaling in heart development is well established and it has been shown to be biphasic, in the sense that its activation is initially required for the commitment of cells to a cardiac lineage and in its inhibition, cardiogenesis is triggered. In heart failure development, a possible role for Wnt signaling has only recently been reported, yet, its results are contradictory. Nonetheless, it was not addressed a possible role exerted by extracellular modulators and receptors of the Wnt pathway. Because of its role in the development of other diseases, and since its extracellular and membrane effectors are regarded as potential targets of pharmacological intervention in the treatment of such pathologies, it became imperative the understanding of Wnt signaling regulation in heart disease and how these interventions would affect heart function. Taking these facts into account, our first goal was to perform a detailed gene expression analysis of different Wnt ligands, receptors and co-receptors, during heart disease development in a type 1 diabetes mellitus rat model. Since in other contexts, Wnt signaling interacts with other pathways known to present a role in the development of diabetic heart disease, such as PPARs and FOXO proteins, we also checked their expression levels. With this approach we aimed starting to unveil a possible role for Wnt signaling in heart disease development as well as possible interactions with other pathways, known to be important of this pathology.

FUNÇÃO DA VIA DAS WNTS NA DOENÇA CARDÍACA

A insuficiência cardíaca apresenta um impacto socioeconómico grande na nossa sociedade. Apesar de grandes avanços na compreensão desta patologia, os mecanismos do seu desenvolvimento, assim como a sua fisiopatologia, permanecem obscuros. De tal forma, é nossa prioridade o esclarecimento de como factores extra- e intracelulares são capazes de modular a função cardíaca. Diversas vias e/ou factores já foram associados a diferentes fases do desenvolvimento de insuficiência cardíaca, nomeadamente TGF-β, IGF, calcineurina, várias GPCRs, MAPK, Akt e GSK-3. Mais recentemente, vários estudos sugerem/apontam um potencial papel da via dos Wnts, no desenvolvimento de insuficiência cardíaca.

A via das Wnts é um importante regulador do desenvolvimento, proliferação, sobrevivência e adesão celulares. Várias doenças como cancro, diabetes, osteoporose e disfunções psiquiátricas, são o resultado da desregulação da via canónica das Wnts, devido a alterações genéticas ou alterações a nível celular dos seus factores.

A sua função no desenvolvimento cardíaco é bem conhecida e revelou-se bifásica, já que, inicialmente, a sua activação é necessária para diferenciação numa linhagem cardíaca e posteriormente, a sua inibição activa a cardiogénese. Vários estudos sugerem um potencial envolvimento da via das Wnts na insuficiência cardíaca, no entanto, os seus resultados são contraditórios. Assim, não foi possível identificar o papel desempenhado por moduladores extracelulares e receptores desta via. Devido ao seu papel no evoluir de outras doenças, e porque os seus receptores são potenciais alvos de intervenções farmacológicas no tratamento de tais patologias, tornou-se indispensável o conhecimento da via das Wnts na doença cardíaca e como essas intervenções poderão afectar o coração. Assim, o nosso primeiro objectivo passou por realizar uma análise à expressão genética dos vários ligandos, receptores e co-receptores, durante o desenvolvimento da doença cardíaca num modelo de rato com diabetes tipo 1. Dado que em outros contextos a via das Wnts interagir com outras vias conhecidas por deterem um papel no desenvolvimento da cardiomiopatia diabética, tais como PPARs e FOXOs, também analisamos os seus níveis de expressão. Com esta abordagem, pretendemos revelar o potencial papel da via das Wnts na fisiopatologia da doença cardíaca, assim como, possíveis interacções com outras vias relevantes e associadas a esta patologia.

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LIST OF ABBREVIATIONS

APC	Adenomatus Polyposis Coli
AU	Arbitrary units
BCL9	B-cell lymphoma 9
BNP	Type-B natriuretic peptide
BSA	Body surface area
CAMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CBP	CREB binding protein
CDK2	Cyclin-dependent kinase 2
CK	Casein kinase
CPCs	Cardiac Progenitor Cells
CREB	cAMP response element-binding
CRM1	Chromosomal region maintenance 1
CSCs	Cardiac Stem Cells
CtBP	C-terminal binding protein
CTRL	Control
DKK1	Dickkopf 1
DM	Diabetes mellitus
Drl	Derailed
Dvl	Dishevelled
DYRK1	Dual-specificity tyrosine-phosphorylated and regulated kinase 1
ECs	Endothelial Cells
EF	Ejection Fraction
ERK	Extracellular-signal-regulated kinase
ET-1	Endothelin-1
FOXO	Forkhead box, subclass O
FS	Fractional shortening
FZD	Frizzled
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT4	Glucose transporter 4
GPCRs	G protein-coupled receptors
GRK	G protein-coupled receptor kinases
Gro	Groucho
GSK-3	Glycogen synthase 3
HR	Heart Rate
IGF	Insulin growth factor
IL-1	Interleukin-1
IKK	IkappaB kinase
IP	Intraperitoneal
JNK	C-jun kinase
LDL	Low-density lipoprotein
LEF	Lymphoid enhancer-binding factor
LRP	LDL-receptor related protein
LV	Left Ventricle
MAK	Metastasis-associated kinase
MAPK	Mitogen-activated protein kinase
MMTV	Mouse mammary tumors virus
MSC	Mesenchymal Stem Cell
MST1	Ste20-like kinases
n	Number of experimental units
NCOR	Nuclear receptor corepressor
NFAT	Nuclear factor of activated T Cells
NHR	Nuclear hormone receptor
NPC	Nuclear pore complex
PAR-1	Protease-activated receptor 1
PCP	Planar cell polarity
PtdIns	Phosphatidylinositol
PI3K	PtdIns-3-kinase
PI4KIIa	PtdIns-4-kinase type II
PIP5KI	PtdIns-4-phosphate 5-kinase type I
PIP2	PtdIns(4,5)-biphosphate
PKA	cAMP-dependent protein kinase
PKB/Akt	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PPARs	Peroxisome proliferator-activated receptors
PRMTs	Protein arginine methyltransferases
PTMs	Post-translational modifications
Pygo	Pygopus
p300	CREB binding protein-associated factor
RGS	Regulators of G proteins signaling
ROS	Reactive Oxygen Species
Rspo	R-spondin
RXR	Retinoid X receptor
SCs	Stem Cells
SCR-1	Steroid receptor co-activator-1
sFRPs	Secreted frizzled-related proteins
SGK	Serum- and glucocorticoid-inducible kinase
SMCs	Smooth muscle cells
SMRT	Silencing mediator of retinoid, thyroid hormone receptors
$SCF^{\beta\text{-TrCP}}$	$Skp1/Cul1/F\text{-}box^{\beta\text{-TrCP}}$
SEM	Standard error of the mean
Stbm	Strabismus
STZ	Streptozotocin
TCF	T Cell factor

TNF-α	Tumor necrosis factor α
T1DM	Type 1 diabetes mellitus
Xpo1	Exportin 1
wg	wingless
β-TrCP	β-transducin repeat-containing protein

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INTRODUCTION

"Learning never exhausts the mind."

Leonardo da Vinci

1 | WNT SIGNALING

Wnt signaling is known to play a critical role in a vast array of biological processes, by which regulate cell proliferation, polarity establishment, migration, cell fate differentiation and stem cell self-renewal [1-2]. As a consequence of their participation in a multitude of cellular events, mutations in the Wnt signaling pathway are usually involved in diseases, such as cancer, premature osteoporosis, diabetes and cardiovascular diseases [1-2].

The Wnt pathway is categorically divided in three branches, taking mostly into account, whether they require or not the transcription factor β-catenin: the canonical or Wnt/β-catenin signaling [3], the Planar Cell Polarity (PCP) [4] and the Wnt/Ca^{2+} pathways [5]. Of the three, canonical Wnt signaling is the best understood and acts by regulating the amount of the transcriptional regulator β-catenin, whereas the other two act independent of β-catenin and are termed non-canonical or β-catenin-independent pathways [2-5].

1.1 Wnt/β-catenin signaling (Canonical Wnt pathway)

β-catenin, a homolog of the fly Armadillo, is a key component of the cadherin cell adhesion system and the canonical Wnt signaling [6-7]. In the absence of a Wnt ligand, cytoplasmic β-catenin levels are continuously being kept low, because free β-catenin is being targeted to a proteasome-mediated degradation and only membrane β-catenin complexed with the cadherin cell adhesion system is protected from degradation. β-catenin degradation is achieved through the formation of a rigorous complex, composed of the scaffolding protein Axin, the glycogen synthase kinase 3 (GSK-3), the tumor suppressor Adenomatus Polyposis Coli gene product (APC) and Casein Kinase 1 (CK1) [1, 3, 8]. This protein complex promotes β-catenin sequential phosphorylation exerted by CK1 and GSK-3, with subsequent recognition and binding to $SCF^{\beta\text{-}TrCP}$, an E3 ubiquitin-protein ligase, leading to its ubiquitylation and further proteasomal degradation by the 26S proteasome [9-10]. Removal of cytoplasmic β-catenin prevents it from entering the nucleus and binding to the T cell factor (TCF) and lymphoid enhancer-binding factor (LEF) proteins, which is being repressed by the Groucho (Gro) family of transcription repressors [2]. Thereby, inhibiting the role of β-catenin as a transcriptional co-activator of target genes.

Conversely, upon reception of Wnt ligands, β-catenin levels increase. This is mostly due to stabilization against proteolysis of uncomplexed β-catenin in the cell and is independent of the cadherin cell adhesion system. Wnt ligands are known to interact with cell surface receptors, such as Frizzled (Fzd) receptors and its co-receptor [low-density lipoprotein (LDL)-receptor-related protein] (LRP), leading to the formation of a ternary Fzd-Wnt-LRP complex [1, 3]. Consequently, this complex by the action of the Fzd receptor, activates and recruits the Dishevelled (Dvl) cytoplasmic phosphoproteins, resulting in LRP phosphorylation and activation, with following Axin recruitment to the plasma membrane [2-3]. This results in the disruption of the degradation complex, thence blocking cytoplasmic β-catenin degradation. Stabilized cytoplasmic β-catenin translocates into the nucleus, where displaces Groucho from the co-activators TCF/LEF, activating Wnt target genes [2-3].

Taken together these observations, is noteworthy that instead the traditional cascade of phosphorylation/dephosphorylation events or the production of intracellular second messenger proteins, cells constantly synthesize and degrade β-catenin, unless canonical Wnt pathway is initiated [10].

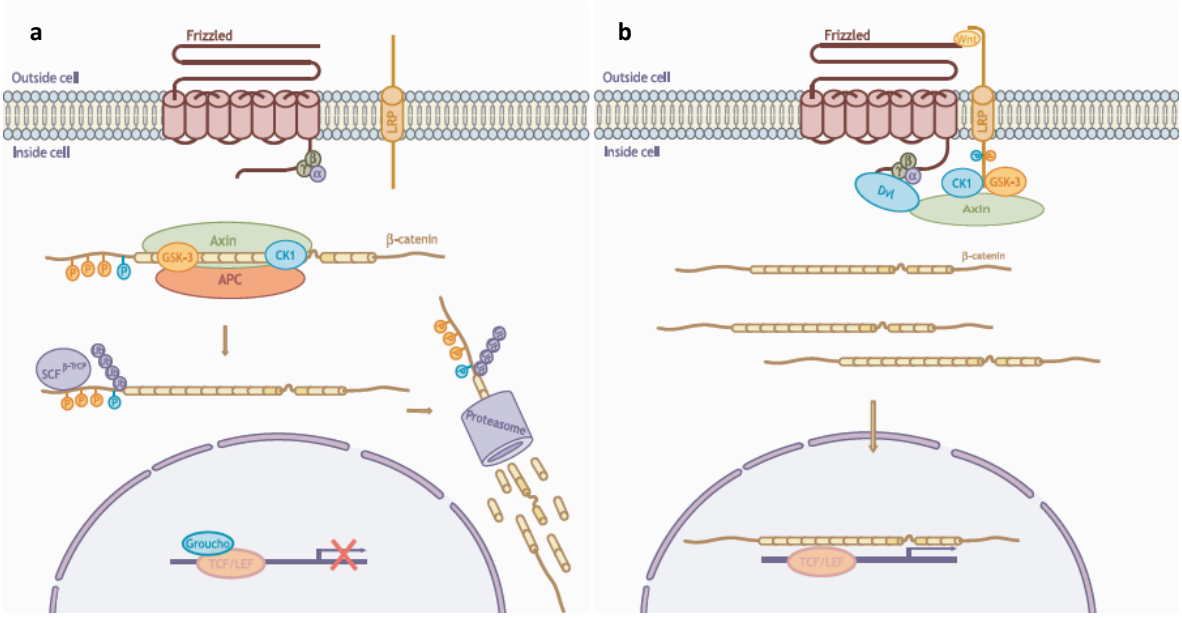

Image 1 | Overview of canonical Wnt signaling.
a) In the absence of Wnt ligands, cytoplasmic β-catenin forms a complex with Axin, APC, GSK-3 and CK1, leading to its phosphorylation, further recognition by the E3 ubiquitin ligase β-TrCP and targeting to proteasomal degradation. Wnt target genes are repressed by TCF/LEF-Groucho association.
b) In the presence of Wnt ligands, a trimeric complex consisted by Fzd-Wnt-LRP is formed. Heteromeric G-proteins and Dvl proteins are activated, leading to the recruitment of Axin to LRPs. This disrupts the degradation complex, subsequent β-catenin stabilization and nuclear translocation, where displaces Groucho from TCF/LEF, activating Wnt target genes.

1.2 Wnt/β-catenin independent pathway (Non-canonical Wnt signaling)

The existence of alternative Wnt signaling pathways, termed non-canonical Wnt pathways, is being supported by accumulating evidences over the last decade. Nonetheless, the precise molecular details are still to be unraveled. Several proteins have been reported to be involved in non-canonical Wnt signal transduction pathways namely Fzd receptors, the transmembrane protein strabismus (Stbm), phospholipase C (PLC), protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII), c-jun kinase (JNK), Rho family GTPases and Dvl proteins [4-5, 11-12].

Various reports suggested that non-canonical Wnt signaling might be separated into two distinct pathways, including the Planar Cell Polarity (PCP) and the Wnt/Ca^{2+} pathways, which may aid to explain the different downstream gene profile observed. In the presence of Wnt ligands, PCP signaling is initiated through Fzd and Stbm (activate Dvl proteins) that in turn activate Rho family of GTPases, Rac and RhoaA, stimulating JNK activity with subsequent JNK-mediated transcriptional regulation [13-14].

On the other hand, Wnt/Ca^{2+} pathways promote intracellular calcium increase due to PLC activation, after G proteins induction (activated by Fzd receptors). Ca^{2+}-sensitive proteins, such as CAMKII and PKC, detect the increased intracellular Ca^{2+}, causing nuclear translocation of nuclear factor of activated T cells (NFAT), a Ca^{2+}-regulated transcription factor [12, 15].

Yet, other models proposed the incorporation of both these pathways into a single non-canonical Wnt pathway or Wnt regulatory network. Of note, is that effectors of the Wnt pathway, such as Fzd and Dvl, appear to function in both canonical and non-canonical pathways, turning the understanding of Wnt signaling an even more complex task, because it is possible that the two could be simultaneously activated and functionally interacting. In addition, another interesting feature about these signaling pathways is the antagonistic regulation observed, where activation of one might even repress activation of the other [16].

2 | CANONICAL WNT SIGNALING: EXTRACELLULAR AND MEMBRANE PLAYERS

2.1 Wnt ligands

In 1973, Sharma *et al.* [17] isolated a *Drosophila melanogaster* mutant gene termed '*wingless*' (*wg*; a fly with no wings), which was subsequent related to cause abnormal wing and mesothorax developments [18]. A decade later, Nusse *et al.* [19] reported a new gene that was responsible for mammary tumors development (mouse mammary tumors virus; MMTV) in mice, called '*INT-1*' and just three years later, Rijsewijk *et al.* [20] identified *INT-1* as the mammalian homologue of *Drosophila* gene *wingless*. The name '*WNT*' (wingless-type MMTV integration site family) thus alludes to the original genes *wingless* and *INT-1*.

Till date, at least, 19 Wnt ligand members had been reported in mammals, conferring a high degree of complexity and Wnt signaling specificity [21]. Wnts are cysteine-rich secreted proteins (presenting up to 23 or 24 highly conserved cysteine residues) of approximately 350-400 amino acids and highly conserved throughout evolution (conserved in all metazoan animals) [19-20]. These proteins are monomeric and share a Wnt signature motif (C-K-C-H-G-[LIVMT]-S-GS-C) [22]. Active Wnts can also be found expressed combinatorially [23], which appears to activate signaling in a distinct manner.

2.2 Wnt receptors

Two distinct receptor families are critical for the activation of canonical Wnt signaling [1]. The first, belongs to the Fzd class of proteins that are essential for both canonical and non-canonical Wnt signaling, and constitute high affinity Wnt receptors. The second, comprise members of a single transmembrane-spanning protein family recognized as the gene *arrow* in *Drosophila* [24] and as *LRP5/6* in vertebrates [25], which function specifically as Fzd co-receptors in canonical Wnt signaling.

2.2.1 Fzd receptors

In the mammalian genome, 10 Fzd protein members had been identified to play a central role in Wnt signaling [26]. Interestingly, because Fzds present seven transmembrane-spanning (7TM) domains that evocates of classical G protein-coupled receptors (GPCRs), they comprise a separate class of GPCRs, the "Class Frizzled" [27].

Fzd proteins expose their large N-terminal domain, that contains a cysteine-rich domain, on the extracellular side and to which, it binds Wnt ligands [28]. Besides one N-terminal region, structural analyses of Fzd receptors predicts, three extra- and three intracellular loops, and an intracellular C-terminal domain [29]. The C-terminal domain contains a PDZ (Postsynaptic density 95, Discs large, Zonula occludens-1)-binding domain, by which cytoplasmic proteins interact with the receptor [29-30]. Amino acid sequence analyses showed that the Fzd sequence is highly rich in putative consensus sites for various serine/threonine and tyrosine kinases [29].

2.2.2 LRP5/6 receptors

The low-density lipoprotein (LDL) receptor (LDLR) family consists of cell surface proteins that are involved in receptor-mediated endocytosis of cognate ligands [31-32]. Two of these members, LRP5 and LRP6 comprise a subfamily of the LDLR family, which mediate diverse steps in metabolism and development.

LRP5 and LRP6 are highly homologous proteins that present high co-expression during embryogenesis and adult tissues remodeling [33-34]. It is likely that LRP6 plays a more dominant role during embryogenesis [25], while LRP5 is critical in adult tissue homeostasis [35]. Most of the LRP amino acid sequence is localized extracellular and consists of YWTD (tyrosine, tryptophan, threonine and aspartic acid) domains, EGF (epidermal growth factor)-like domain and LDL repeats [36-37]. Intriguingly, although being a co-receptor for canonical Wnt signaling, LRPs extracellular domain has a poor affinity to Wnt ligands compared to Fzds, yet, bind with high affinity to its antagonists Dickkopf1 (Dkk1) and Sclerostin [38-39]. The YWTD domain is important for LRPs endoplasmic reticulum maturation and membrane trafficking, which requires a specific chaperone molecule called Boca in *Drosophila* [40] and Mesd in mice [41].

The intracellular domain has proline-, serine- and threonine-rich residues and contains five PPPSP[x]S ('x' denotes any amino acid) repetitive motifs [42]. The phosphorylation of these motifs is a requirement for Axin recruitment and subsequent β-catenin stabilization. Upstream of the PPPSP[x]S repeats exist an S/T cluster that is also a target for phosphorylation [43].

Till date, five classes of protein kinases had been identified to phosphorylate LRPs, being divided in two groups. The first, comprises proline-directed kinases which

phosphorylate PPPSP motifs, such as GSK-3 [44], cAMP-dependent protein kinase (PKA) [45], Pftk members [46] and G protein-coupled receptor kinases 5/6 (GRK5/6) [47]. The second contains non-proline-direct kinases like CK1 family members [48] that phosphorylate [x]S sites, the S/T cluster and additional N-terminal regions.

2.3 Unusual Wnt receptors: Drl/Ryk and Ror2

Derailed (Drl), a transmembrane tyrosine kinase receptor from the RYK subfamily, has been shown to be an unusual, yet, essential component of Wnt signaling [49]. *Drosophila* Wnt5 (Dwnt5), is a regulator of axon guidance in the central nervous system, and embryos lacking Dwnt5, share a similar phenotype to those that lack Drl, that is, they display aberrant neuronal projections across the midline. Drl binds to Dwnt5, through its extracellular Wnt inhibitory factor (WIF) domain, indicating that it is a Dwnt5 receptor in the central nervous system [50]. However, how Drl downstream signals are transduced, remain unclear. Unlike Drl in *Drosophila*, the mammalian Ryk homolog functions as a co-receptor along with Fzd [51].

Another tyrosine kinase receptor is known to exist, Ror2, which contains a cysteine-rich domain similar to that of Fzd [52]. Upon Wnt5a binding to Ror2, an inhibitory action over canonical Wnt signaling takes place, although paradoxically, Wnt5a can also induce activation of the canonical pathway by directly engaging Fzd4 [53] or Fzd5 [54].

3 | OTHER EXTRACELLULAR PLAYERS: ANTAGONISTS AND AGONISTS

3.1 The antagonists

Wnt antagonists can be categorically divided according to their model of action and to their ability to inhibit canonical Wnt signaling. Some proteins like Dickkopf (Dkk) [55], Wise [56] and Sclerostin [38], which are capable of inhibiting canonical Wnt signaling by binding to the LRP5/6 receptor, are grouped and classified together. On the other hand, those like secreted frizzled-related proteins (sFRPs) [57], Wnt inhibitory factor 1 (WIF1) [58] and *Xenopus* Cerberus [59], directly bind to and inhibit Wnts and thence, are classified as the sFRP class. In addition, sFRPs are also capable of binding and blocking the access of Wnts to Fzd receptors, thereby, presenting dual inhibitory functions of Wnt signaling [60].

Thus, theoretically, components of the sFRP class will inhibit both canonical and non-canonical pathways, while those binding to LRPs, will specifically inhibit the canonical pathway [61].

3.2 The agonists

Norrin and R-spondin (Rspo) are at least two types of proteins that are unrelated to Wnt ligands and activate the Fzd/LRP receptors [62-63]. Norrin is a secreted protein that is mutated in Norrie disease, which is a developmental disorder defined by vascular abnormalities in the eye. Norrin acts by binding with high affinity to Fzd4 and therefore, activates the canonical Wnt signaling in an LRP5/6-dependent manner [62]. The Rspo proteins constitute a novel class of ligands that induce canonical Wnt signaling, by exhibiting synergy with Wnts, Fzds, and LRP6 [63]. *Rspo2* genes are often co-expressed in a variety of tissues with Wnts, raising the possibility that Rspo2 proteins display a positive feedback role, in order to reinforce canonical Wnt signaling [63]. In line with this notion, it had already been demonstrated that Rspo proteins are capable of physically interacting with the extracellular domains of LRP6 and Fzd8, therefore activating Wnt target genes [64].

4 | INTRACELLULAR PLAYERS

4.1 Dishevelled

Dishevelled (*Drosophila*: dsh; Mammalian: Dvl) is a ubiquitously expressed cytoplasmic scaffolding protein that is known to interact with activated Fzd receptors. Dsh contains 750 amino acids, displaying high homology with three Dvl homologue genes (*DVL1*, *DVL2* and *DVL3*) that had been identified in mice and humans [65]. All Dvl family members possess three conserved regions: an N-terminal DIX (Dishevelled/Axin) domain (also found in the C-terminus of Axin proteins), a central PDZ domain (also found in the C-terminal domain of Fzds) and a C-terminal DEP domain, implicated in membrane targeting [66]. Additionally, other two conserved regions had been implicated to mediate protein interactions and/or phosphorylation, the basic and the proline-rich regions [67].

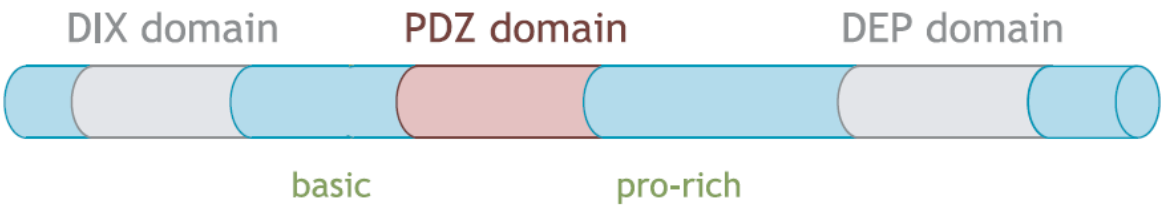

Image 2 | Dvl structure.

It is interesting to mention that, like Wnts and Fzd receptors, also Dvl is a common player between both canonical and non-canonical Wnt pathways and, like β-catenin, it can shuttle between the cytoplasm and the nucleus, yet, it is unclear how the nuclear localization of Dvl is governed.

4.2 β-catenin structure

β-catenin, a protein of 781 amino acids, is arranged in three distinct regions that share a 71% identical amino acid sequence to the fly *Armadillo* gene product [68]. It possesses a large central region (residues 141-664) composed of 12 repeats, known as the Armadillo (Arm) repeats, being each arm disposed in a three α-helix configuration. The arm repeats are structurally very similar, however, presenting some irregularities among the whole arm repeats structure [69].

Image 3 | β-catenin domains structure.

Interestingly, the 12 arm repeat structure forms a stiff scaffold for the binding of many factors, such as TCF, Axin and APC [70]. Contrasting, the N- and C-terminal domains are much smaller and much more flexible [10, 68]. Some authors suggested that these two domains would interact with the central arm domain by a fold-back mechanism, regulating the ability of this region to bind to different co-factors [71-72]. Plus, because these two domains are negatively charged, contrasting to the highly positively charged arm domain, is deductible that the three domains interact in a highly and non-specific, dynamic fashion.

4.3 The 'scaffold' destruction complex of β-catenin: GSK-3, CK1, Axin and APC

As already stated, cytoplasmic β-catenin downregulation is carried out by the formation of a multiprotein destruction complex that targets β-catenin to proteasomal degradation.

GSK-3 is a ubiquitously expressed constitutively active serine/threonine kinase (GSK-3 active form is dephosphorylated), responsible for cellular substrates phosphorylation and is one of the kinases responsible for β-catenin phosphorylation to subsequent proteasome recognition. This kinase specifically recognizes and phosphorylates critical residues in the N-terminal region of β-catenin (Ser33, Ser37 and Thr41), however, by itself, GSK-3 does not efficiently phosphorylate β-catenin [10]. In fact, Liu *et al.* [73] demonstrated that β-catenin phosphorylation by GSK-3, is preceded by a "priming" phosphorylation step governed through a member of the CK1 family of kinases, CK1α, also in the N-terminal region of β-catenin (Ser45).

At least, three CK1s have been implicated in the canonical Wnt signaling namely CK1α, CK1ε and CK1γ, being the role of the first isoform the best characterized of this family [10]. The potential role of this kinase is well illustrated in CK1α deletion experiments, where high concentration levels of β-catenin are observed, through inhibition of β-catenin phosphorylation and further cytoplasmic accumulation [73].

At the 'heart' of the multiprotein destruction complex lays the scaffolding protein Axin, which plays a critical role in bringing GSK-3, CK1α, and β-catenin together to efficiently promote the phosphorylation reaction [10]. Surprisingly, Axin concentration is extremely low when compared to other components, in frog embryos experiments, suggesting that Axin is a rate limiting factor to the assembly of the destruction complex [74]. Moreover, the importance of Axin in β-catenin degradation is highlighted by the increased levels of β-catenin in several human cancers due to mutations in the human *AXIN1* gene [75]. In order to potentiate and exert a more dynamic role in the formation of the β-catenin destruction complex, Axin uses separate domains to interact with GSK-3, β-catenin and CK1α.

A hydrophobic groove in the C-terminal region of GSK-3 binds a central region within the Axin protein, leaving the GSK-3 active site free to phosphorylate β-catenin [76]. C-terminal to the GSK-3 binding site, a short conserved region in Axin, called the β-

catenin-binding domain (CBD), is responsible and sufficient for specific β-catenin-Axin interaction [8, 77]. This interaction occurs in a parallel orientation, where β-catenin through its arm repeats 3 and 4, specifically binds to the Axin-CBD [78]. Thence, the resulting configuration theoretically allows a close proximity between the N-terminal domain of β-catenin with GSK-3, and so, facilitate β-catenin N-terminal phosphorylation [78]. On the other hand, the exact binding site for CK1α on Axin is still not known, yet, deletion analysis demonstrated that it binds C-terminal to the β-catenin-binding site [79].

Axin contains two other conserved domains that are suggested to play a role in signal transduction. The RGS (Regulators of G proteins Signaling) domain near its N-terminal region and a C-terminal DIX domain [80-81]. Through its RGS domain, Axin contacts to another component of the multiprotein destruction complex, the APC protein.

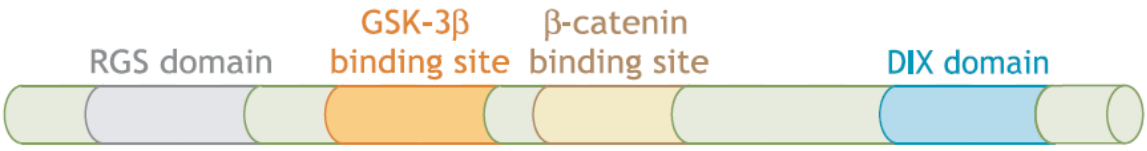

Image 4 | Axin structure.

APC mutations are found in over 80% of colon cancers, making it the most common event for β-catenin stabilization, during oncogenic development [10]. The central region of APC contains two separated domains, both capable of interacting and binding to β-catenin [10, 82]. The first domain comprises three motifs of 15-amino acid repeats (A, B, C), while the second domain contains seven motifs of 20-amino acid repeats [10]. Three serine-alanine-methionine-proline (SAMP) repeats, intercalated among the 20-amino acid APC repeats, are present in the second domain [82]. These SAMP repeats are responsible for mediating APC-Axin binding, through interaction with the Axin-RGS domain [83].

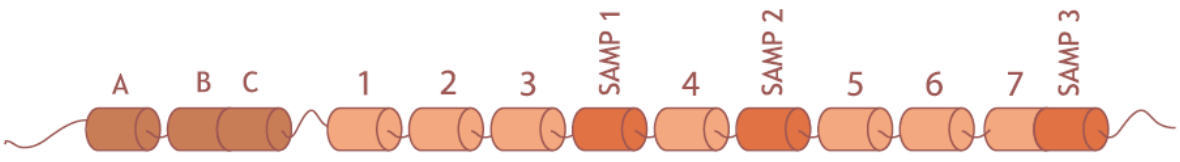

Image 5 | APC domains structure.

5 | CANONICAL WNT SIGNALING ACTIVATION

Binding of a specific Wnt ligand to a Fzd receptor and its co-receptor LRP5/6, rapidly leads to the reconfiguration and subsequent formation of a ternary complex, composed of Fzd-Wnt-LRP. The ternary complex acts as a platform to ensure LRPs proper cytoplasmic domains phosphorylation, which is a key step in receptor activation [37, 84]. Phosphorylation of the LRPs occurs at five PPPSP[x]S repetitive motifs in the cytoplasmic domain, which creates perfect docking sites for the recruitment of the Axin protein. Because Axin is thought to exist at very low concentrations in cells, its sequestration by LRPs would directly compete with its function in the β-catenin destruction complex.

A dual kinase mechanism responsible for the PPPSP[x]S motifs phosphorylation has been predominantly attributed to GSK-3 and CK1 [42-43]. GSK-3 accounts for the most PPPSP phosphorylation and thus, reveal a positive role for GSK-3 in canonical Wnt signaling, which has been in the shadow of the strong negative role it occupies in the destruction complex [2, 42]. At the same time, CK1 has been identified as the responsible for the [x]S phosphorylation motifs observed in LRPs [42]. Intriguingly, these regulatory steps are similar to the β-catenin phosphorylation events, where cytoplasmic Axin brings into close proximity both kinases to functionally co-operate in LRPs phosphorylation [43-44].

In response to Wnt ligands, Dvl is highly phosphorylated through a Fzd-mediated mechanism and further recruited via Fzds-PDZ domain [85]. Various kinases had been reported to phosphorylate Dvl namely CK1 [86], CK2 [87], protease-activated receptor (PAR-1) [88], PKC [67], and metastasis-associated kinase (MAK) [89].

Dvl phosphoproteins assembly serves to create a platform for Axin-GSK-3-CK1 co-localization and thence, is critical for LRP phosphorylation with subsequent Axin recruitment [44, 48]. However, because it is still unclear how the precise steps of receptors activation occur several models had been proposed.

One model stipulates that because activated Dvl interacts with Axin, its recruitment to the plasma membrane triggers LRP6 phosphorylation by GSK-3 [44, 90]. Interestingly, because Axin is required for LRP6 phosphorylation and that in turn, phosphorylated LRP6 recruits Axin, suggests a positive feedforward loop, where it strongly amplifies phosphorylation of all five PPPSP[x]S motifs. Indeed, Baig-Lewis *et al.* [91] proposed a two step activation in which, a signal initiation that is coordinated by Wnt-Fzd-

LRP complexes, leads to the translocation of cytoplasmic Axin to the surface membrane, in a Dishevelled-dependent manner. This event would result in a partial inhibition of the destruction complex that would be sufficient to trigger an initial Wnt signal response. However, in order to generate a stronger signaling cascade, active Dvl might promote LRPs phosphorylation, which ultimately leads to cytoplasmic Axin recruitment, with subsequent signaling amplification [91]. Supporting this model, Mao *et al.* [92] showed that Axin-LRP5 interactions only occur, after Wnt signaling initiation at the surface membrane.

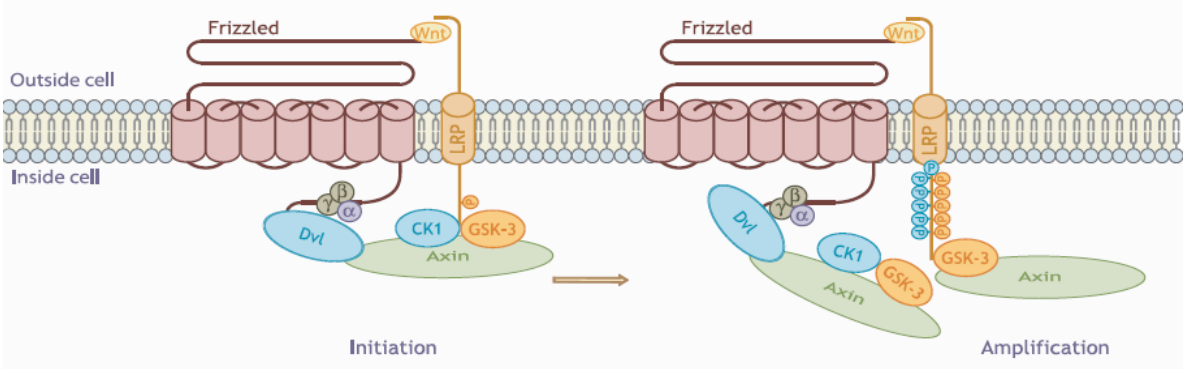

Image 6 | Model of Wnt receptor activation: Initiation and Amplification.
After formation of a ternary complex consisted by Fzd-Wnt-LRP, Dvl recruits Axin that in turn is associated with both GSK-3 and CK1, resulting in the phosphorylation of one or more PPPSP motifs in LRP - Initiation. Presumably, partially phosphorylated LRP might recruit and more efficiently associates with Axin-GSK-3-CK1, promoting more PPPSP motifs phosphorylation – Amplification. *Note:* Although also associated with Axin-GSK-3, one CK1 is omitted in the picture at the right to the ease of understanding the picture.

Another model proposes the formation of LRPs signalsomes, upon Wnt ligand binding [48]. Signalsomes consist in groups of proteins that cluster together to carry out a specific signaling task. Presumably, these multiprotein complexes comprise some sort of endocytic vesicles that have no common vesicular traffic markers, except for occasional co-localization with caveolin [48, 93].

The role of Wnt ligands is therefore highlighted by their capacity to create a bridging point between LRPs and Fzd, which co-polymerize on a Dvl platform. Bilic *et al.* [48] suggested that Dvl proteins cluster together with LRP6 and other components of Wnt signaling namely Fzd, Axin and GSK-3β, in a LRP6-signalsome manner. Consequently, this clustering of LRP6 provides an increase amount of local receptors concentration, with their further phosphorylation triggered by CK1γ and subsequent Axin recruitment [48]. Indeed, CK1γ phosphorylation occurs upstream of the PPPSP[x]S repeats in a S/T cluster region [43]. This region after being phosphorylated, creates a perfect docking site for GSK-3, which presumably aids in the LRP6-GSK-3 interactions [94]. Taken together, this supports a

sequential priming model, where it initiates from the S/T cluster and follows C-terminal [93].

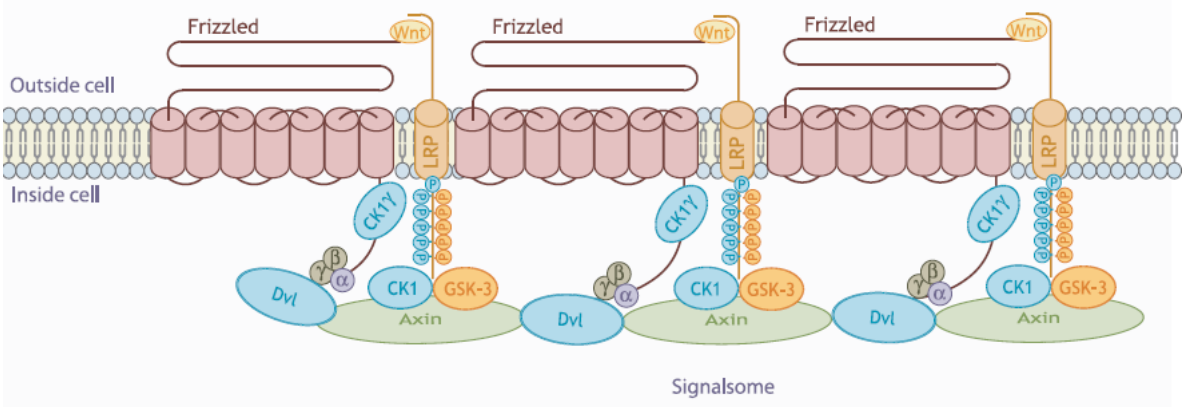

Image 7 | Model of Wnt receptor activation: Signalsome.
Signalsome formation through Dvl polymerization with receptors clustering. Dvl oligomerization induces the aggregation of Fzd-Wnt-LRP complexes, resulting in Axin recruitment and further LRP phosphorylation by GSK-3 and CK1. CK1γ potentiates phosphorylation.

Of note, Dvl itself generates cytoplasmic polymers, which can be found as microscopic punctae and that can be recruited to the plasma membrane, upon Wnt signaling activation [95]. This dynamic polymerization facilitates the aggregation of large Dvl-Axin complexes, which is exerted by both Dvl and Axin DIX domains [93, 95]. However contrasting to the general view, other regions of both proteins may also be involved in this interaction. The ability of Dvl polymerization is thus an important feature to signalosome formation, but at the same time, also requires phospholipids and lipid kinases [93].

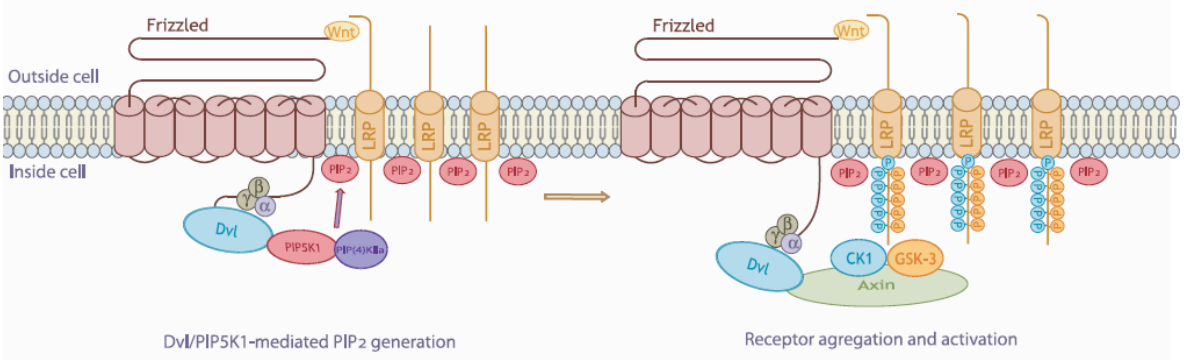

Image 8 | Model of Wnt receptor activation: Receptor endocytosis.
PIP_2-mediated formation, promoted by PPI4KIIα and PIP5KI kinases. PIP5KI binds directly to Dvl and induces PIP_2 formation, with subsequent receptor clustering/phosphorylation.

Under Wnt3a stimulation, Dvl induces the formation of phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P2; PIP2) by sequential regulatory steps of PtdIns-4-kinase type II (PI4KIIa) and PtdIns-4-phosphate 5-kinase type I (PIP5KI). In fact, PI4KIIa was observed to be regulated by both PDZ and DEP domains of Dvl, while the DIX domain was identified to bind and activate PIP5KI [96]. This results in the formation of a ternary complex composed of Dvl-PI4KIIa-PIP5KI, leading to PIP2 formation [96-97], which is required for Wnt3a-induced clustering and phosphorylation of LRP6 [67]. Because PIP2 is well known to induce general receptors endocytosis, it is normal to speculate that LRPs internalization might in fact be a key step in Wnt signaling.

6 | NUCLEAR EVENTS

Upon cytoplasmic stabilization, β-catenin enters the nucleus to further induce a Wnt genetic program, however, shuttles through an unclear mechanism.

Over the years, an emergent body of evidence has shown that the transcriptional activity of β-catenin is modulated by a variety of interacting partners. Despite presenting potent transcriptional activator domains at the N- and C-terminus, β-catenin-DNA interactions are very weak, thus it must depend on interactions with DNA-binding factors to regulate gene expression [98].

TCF/LEF members consist in a subfamily of the HMG-box-containing superfamily of transcription factors that are involved in β-catenin nuclear translocation, with further DNA association [99]. Via their HMG domain, TCF/LEFs ensure the binding to a conserved sequence on DNA, the Wnt-response element (WRE: C/T-C-T-T-T-G-[A/T]-[A/T]), leading to β-catenin-mediated gene transcription [100].

The binding of β-catenin to an N-terminal region on TCF/LEFs assists the assembly of multimeric complexes, consisting in transcriptional activators like CBP/p300 [cAMP response element-binding (CREB) binding protein/CREB binding protein-associated factor] [101] and B-cell lymphoma 9 (BCL9) and its nuclear associator Pygopus (Pygo) [102], which are capable of activating target genes.

In the absence of Wnt signaling, TCF represses gene expression through interacting with Groucho (Gro; TLE in human), which is capable of promoting histone deacetylation and chromatin condensation [103]. Thus, TCF/LEFs not only function as transcriptional

activators, but also as transcriptional repressors, because in the absence of β-catenin they assemble complexes with transcriptional co-repressors, such as C-terminal Binding Protein (CtBP) [104] and Gro, forming multimeric transcriptional repressor complexes.

Under Wnt signaling activation, β-catenin physically interacts and displaces Gro from TCF/LEFs, with subsequent recruitment of others transcriptional co-activators [105].

7 | THE ROLE OF WNT SIGNALING: EMBRYONIC AND ADULT HEART

7.1 Embryonic heart

Despite its many roles in many cell types, including the development of the embryonic heart, the role of canonical Wnt signaling in the adult and/or disease heart remains still elusive [106-107].

The actual paradigm of embryonic cardiac development ensures that canonical Wnt signaling is initially required for the commitment of a cell to a cardiac lineage [106-108] and that in its inhibition cardiogenesis is triggered [109-110]. Apparently, canonical Wnt signaling acts early during development to enhance cardiac specification, through primarily induction of a special group of cells called, cardiac progenitor cells (CPCs). Nonetheless, in later stages, silencing of canonical Wnt effectors by specific antagonists, promotes specification of cardiac precursors, leading to the formation of the heart cellular content, such as cardiomyocytes, smooth muscle cells (SMCs) and endothelial cells (ECs). As a matter of fact, it has been demonstrated that later activation of canonical Wnt signaling, during cardiac stem cell differentiation, blocks cardiac induction and differentiation [111]. By contrast, non-canonical Wnt signaling activation, as observed by Wnt11, is required for the induction of cardiac tissues [112].

7.2 Adult heart

On the other hand, the role of Wnt signaling in the adult heart and/or disease heart is still undergoing its first steps. It was widely accepted that the heart consisted of a post-mitotic organ with a fixed number of terminal differentiated myocytes, which could not reenter cell cycle [113]. This way, in the absence of cardiac diseases, myocytes would maintain throughout life till the death of the organism. Fortunately, in the last decade

some reports challenged this notion and supported evidences in favor of the regeneration of the young, adult and aged myocardium [114-115]. These results highlighted a novel route of understanding about the growth and aging heart that is attributed to a resident niche of stem cells (SCs) located in the apex, the atria and ventricular myocardium, called cardiac stem cells (CSCs) [116-117]. Thus, CSCs would presumably be responsible for the biology of the heart namely formation of myocytes, SMCs and ECs [118]. This view of the heart as a self-renewing organ, whereby myocytes regenerate throughout the lifespan of the organism, contrasts to the general/old view of the terminal differentiated heart.

Anversa *et al.* [119] suggested a classification of cardiac immature cells, in the adult heart, into 4 types: 1) CSCs, which give rise to 2) cardiac progenitor cells (CPCs); thereafter, CPCs would differentiate into 3) precursors cells; lately, these precursors cells may originate 4) amplifying cells, resulting in SMCs, ECs and myocytes; expressing the first 3 cell types the molecular markers, c-kit, MDR1 and Sca-1; the second, the third and the fourth express also GATA4, while the last no longer express these markers.

The adult heart can react to distinct stimuli, such as exercise (physiological) or pressure overload (pathological), through cardiac enlargement. Cardiac enlargement is defined by an enlargement of cells, hypertrophy, which in pathological conditions results in irreversible life-threatening heart failure [120]. Several reports identified a role for GSK-3β [121-122] and β-catenin [123-124] in the development of hypertrophic responses. It has been shown that GSK-3β inhibits cardiac hypertrophy [121-122], whereas β-catenin stabilization results indirectly in hypertrophic responses increase [124]. Nonetheless, these authors reported that β-catenin stabilization occurred by a Wnt-independent mechanism (involving Akt/protein kinase B (PKB)) and CyclinD1, a Wnt/β-catenin target gene, did not express. However, contrary to this result, Masuelli *et al.* [123] reported in hypertrophic cardiomyopathic hearts an accumulation of β-catenin regulated by an increased Wnt expression, with subsequent GSK-3β decrease. In accordance, canonical Wnt signaling had also been reported to be associated to skeletal muscle hypertrophy, reinforcing a possible parallel role in the adult and disease heart [125]. Of interest, it was demonstrated that stabilized β-catenin *in* isolated cardiomyoctes or *in vivo*, results in increased myocytes growth, with or even without a hypertrophic stimulus [124, 126]. Nonetheless, this stabilization is thought to occur through Akt activation, rather than a Wnt dependent mechanism.

Although these studies demonstrate that activation of GSK-3β/β-catenin is of great importance for normal myocytes growth, they however do not directly implicate, *per se*, Wnt signaling activation. At the same time, there is still scarce or no information on the function of several Wnt effectors (ligands, receptors, co-receptors) in heart disease and to that extent the elucidation of such factors has to be defined.

8 | DIABETES MELLITUS AND HEART DISEASE

Cardiovascular diseases are the leading cause of morbidity and mortality in patients suffering from diabetes mellitus [127]. Diabetes, which represents a deficiency or a resistance to insulin, promotes great alterations at cardiac levels, such as cell hypertrophy, metabolic abnormalities, extracellular matrix alterations, oxidative stress and apoptosis, that may impair myocardial function, thereby contributing to diabetic cardiomyopathy development [128-129]. Diabetic cardiomyopathy has been characterized as a ventricular dysfunction that occurs in diabetic patients, independent of coronary artery diseases or hypertension [130].

It is commonly accepted that diabetic patients present an oxygen toxicity enhancement due to an increase generation of reactive oxygen species (ROS). The excessive release of ROS induces oxidative stress that subsequent leads to abnormal gene expression, impaired signal transduction and cardiomyocytes apoptosis [131-133]. Taking these evidences, it was hypothesized that oxidative damage could alter the structure and function of CPCs that are thought to be recruited during heart disease development, resulting in defects of myocyte formation.

In response to cardiac damage, it has been reported that CPCs are activated by growth factors, leading to enhanced cell division and repair [208-209]. It is now believed that in the first stages of cardiac disease, CSCs and CPCs are recruited and induced to differentiate in order to renew the cardiac tissue content.

Rota *et al.* [134] reported an altered CPCs function in diabetic animals, which present high levels of ROS. At the same time, because ROS are a considerable threat to cellular constituents/organelles, such as injury to lipids and membranes, proteins and nucleic acids, these authors measured the telomeric length in CPCs and mycotes. They observed a reduced length on both cells suggesting that diabetes simulates myocardial aging and

cellular senescence. In addition, these authors also evaluated the expression of the tumor suppressor proteins, p53 and $p16^{INK4a}$, and determined an increased expression of both proteins, with subsequent observation of apoptosis and necrosis activation [134].

In addition, Gonzalez *et al.* [135] observed that chronological aging contributes to the shortening of telomeres in CPCs, which generates progeny that rapidly acquires the senescent phenotype and thence, ventricular dysfunction. However, they have also found that the senescence heart presents fully functional CPCs (containing long telomeres), which right after activation, migrate to the damaged regions, generating a young population of cardiomyocytes, partially reverting the aging myopathy. This could suggest that the aging heart like other organs is the result of stem cells dysfunction. Thence, since the senescent heart contains functional competent CPCs there is at least, a potential to correct cardiac dysfunction and extend myocardium lifespan. Of note, functional CPCs are found after myocardial infarction, yet, these CPCs do not repair the damage [136]. A possible explanation could be the fact that CPCs may not sense the presence of damage around or that their activation, growth and migration could be defective.

Hence, because CPCs seem to be highly affected in cardiac aging and disease, a clear understanding of the CPCs role in both cardiac pathophysiology and physiological aging may expand frontiers and rise new strategies to improve and prolong life.

9 | THE ROUTE FROM DIABETES MELLITUS TO OXIDATIVE STRESS AND VICE-VERSA/BACK

As already mention, diabetic cardiomyopathy interferes in CPCs regulation, presumably due to ROS toxicity enhancement, which is well associated to the aging heart. This way, diabetic cardiomyopathic patients turned out as useful systems for the study of Wnt signaling pathways and therefore, comprehension of all molecular mechanisms therein involved.

In order to defend itself from stress conditions, such as diabetes, the heart detains a cardioprotective system, which is responsible for the activation of mechanisms that evolve into an initial adaptive hypertrophic response [120]. Nevertheless, despite starting as a natural cellular response to counteract the harmful injuries, the accompanying remodeling of the heart tissue through fibrosis and dilation might progressively result in

irreversible life-threatening heart failure [120]. Hence, the heart detains several cardioprotective systems that aid protecting against its metabolic malfunctioning and impaired molecular mechanisms.

One of such mechanisms of defense consists in the activation of specific transcription factors, of which the subclass of transcription factors family members Forkhead box O (FOXO) proteins, play a central role. FOXOs are able to confer oxidative stress resistance, by trans-activation of antioxidant enzymes (i.e. MnSOD and catalase) [137-138] and also regulation of cell cycle arrest [139], apoptosis [140], DNA damage repair events [141] and even cell metabolism [142].

9.1 'Forkhead box, subclass O' (FOXO)

The Forkhead family of transcription factors comprise a family of near 100 different members, which share a highly conserved "winged-helix" structural motif that comprises the DNA-binding domain, known as the Forkhead box/Fox box (Fox) [143]. In mammals, the subclass O ('other') contains four 'Forkhead box, subclass O' (FOXO) family members that are known as FOXO1 (FKHR), FOXO3 (FKHRL1), FOXO4 (AFX/Mllt7) and FOXO6, being all related to the *Caenorhabditis elegans* ortholog Daf16 and the *Drosophila* ortholog dFoxo [143-144]. FOXOs are traditional transcription factors because they contain a DNA-binding domain located N-terminal and a trans-activation domain in the C-terminal end.

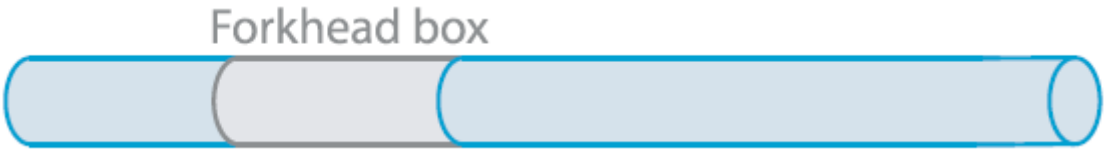

Image 9 | FOXOs structure.

Of the four FOXO members, FOXO6 appears to be more elusive in the developing brain due to a specific temporal and spatial expression [145]. This apparently contrasts with the other three members that possess a ubiquitously expression.

FOXO1, FOXO3 and FOXO4 exhibit a high versatility gene regulation, where post-translational modifications, such as phosphorylation, acetylation, methylation, O-linked glycosylation and ubiquitylation, change FOXOs intracellular localization, turnover and trans-activation properties [146]. This way, FOXO members are capable of regulating a wide range of genes, through association with other transcription activators/factors, such as β-

catenin [147-148], PPAR-α [149], PPAR-γ [150], Androgen receptor (AR) [151], Progesterone receptor (PR) [152], Smad3 and Smad4 [153], among others.

Mutational analysis of *FOXO* genes have been implicated in embryonic heart development, since mutant forms of its members' exhibit deficient vascular and cardiac growth [154-155]. Moreover, because FOXOs negatively regulate cell cycle progression, overexpression of its members results in impaired cardiomyocyte proliferation, decreased myocardium thickness and heart size, with subsequent heart failure [156]. Thus, because FOXOs are transcription factors that govern the steady state of cells, it is of great significance the understanding of their transcriptional activity regulation.

9.1.1 FOXO members regulation: Post-translational modifications (PTMs)

FOXOs transcriptional regulatory functions require their translocation into the nucleus, where they activate several gene expression programs. Stimulation of cell growth by insulin or growth factors determines FOXOs nuclear-exclusion and inhibition of FOXO-dependent transcriptional regulation [157-158]. It is interesting to mention that specific PTMs govern the nuclear-cytoplasmic shuttling of FOXO proteins and that the outcome of this two-way dynamic process controls the homeostasis of the cells.

9.1.2 FOXOs nuclear exclusion/negative regulation

Nuclear exclusion occurs upon FOXOs-phosphorylation by a particular set of kinases that phosphorylate specific sites on FOXOs. Some of these kinases include the Akt/PKB [158-159], a close-related Akt-family member called, serum- and glucocorticoid-inducible kinase (SGK) [160], CK1 [161], a member of the dual-specificity tyrosine-phosphorylated and regulated kinase group (DYRK1) [162], the inhibitor of nuclear factor kB kinase (IkappaB kinase; IKK) [163] and, cyclin-dependent kinase 2 (CDK2) or the Mitogen-activated protein kinase/Extracellular-signal-regulated kinase (MAPK/ERK) that phosphorylate FOXO1 [164] and FOXO3 [165], respectively.

Survival factors, such as insulin and insulin-like growth factors (IGFs), bind to their cell surface receptors and further trigger the activation of the phosphatidylinositol-3-kinase (PI3K) pathway [166]. In turn, this leads to phosphorylation/activation of a serine/threonine kinase called Akt/PKB that it is known to play a major role in cell survival [167]. FOXOs dependent-Akt/SGK phosphorylation promotes their nuclear exclusion,

probably by alterations in its conformational status through permanently exposure of the NES (nuclear export signal) over the NLS (nuclear localization signal), which also aids to further block nuclear import [159, 161]. Consequently, these phosphorylated sites also disrupt the DNA-binding activity of FOXOs, by affecting the interaction of their DNA-binding domain with the transcriptional co-activators p300/CBP and facilitate their binding to the 14-3-3 adaptor protein [168-169]. This leads to inhibition of FOXOs' trans-activation functions, subsequent cytoplasmic translocation and accumulation, resulting in proteasome degradation.

CK1, is also able to phosphorylate two residues on FOXO proteins, however, requires to be first primed for phosphorylation by Akt [161]. On the other hand, DYRK1 phosphorylates a single residue on FOXOs and contrasting to CK1, is independent of both Akt or CK1 activity, and being simultaneously independent of growth signals [162]. Apparently, all of these specific phosphorylations promote FOXOs association within a complex, comprising of nuclear-export molecules, such as Ran and the exportin CRM1, along with the adaptor 14-3-3 protein that lead to FOXOs nuclear exclusion [161].

9.1.3 FOXOs nuclear import/positive regulation

Conversely, FOXOs inactivation can be balanced by several signaling pathways that positively regulate FOXOs nuclear stabilization. While FOXOs nuclear exclusion follows a response to growth signals, nuclear import is defined by a response to stress signals [170].

Recent studies demonstrate that oxidative stress triggers the induction of protein arginine methyltransferases (PRMTs). These proteins were reported to cause the methylation of two arginine residues in FOXO1 and thereby, directly inhibiting Akt-mediated phosphorylation with subsequent nuclear localization increase and apoptosis triggering [171]. Oxidative stress can also induce phosphorylation of FOXO3 through the Ste20-like kinases (MST1) on the cytoplasm that impairs interactions with the 14-3-3 adaptor protein, and further contributes to FOXO3 nuclear translocation [172]. Moreover, alternative phosphorylation can even balance against FOXOs nuclear exclusion, as FOXOs-mediated phosphorylation by c-Jun N-terminal kinase (JNK) promotes FOXOs' nuclear import, in opposition to Akt-mediated nuclear exclusion of FOXO4 [170, 173]. In agreement, it was recently shown that JNK signaling increases stress resistance and lifespan in both *Caenorhabditis elegans* and *Drosophila* [173-174]. These authors observed that Daf16/dFoxo

is the downstream target of JNK signaling, reinforcing the notion that ROS-JNK-FOXO pathway governs stress resistance and extends lifespan.

9.2 FOXO and canonical Wnt signaling: β-catenin, the Libra

It was recently discovered an evolutionary conserved interaction between β-catenin and FOXO members in both *Caenorhabditis elegans* and mammals, where FOXOs compete with TCF transcription factors for a free β-catenin limited pool [147-148, 175]. Presumably, β-catenin plays a dual role in the regulation of cell cycle progression, since acts as a positive control via TCF and on the other side, as a negative regulator when associated to FOXOs.

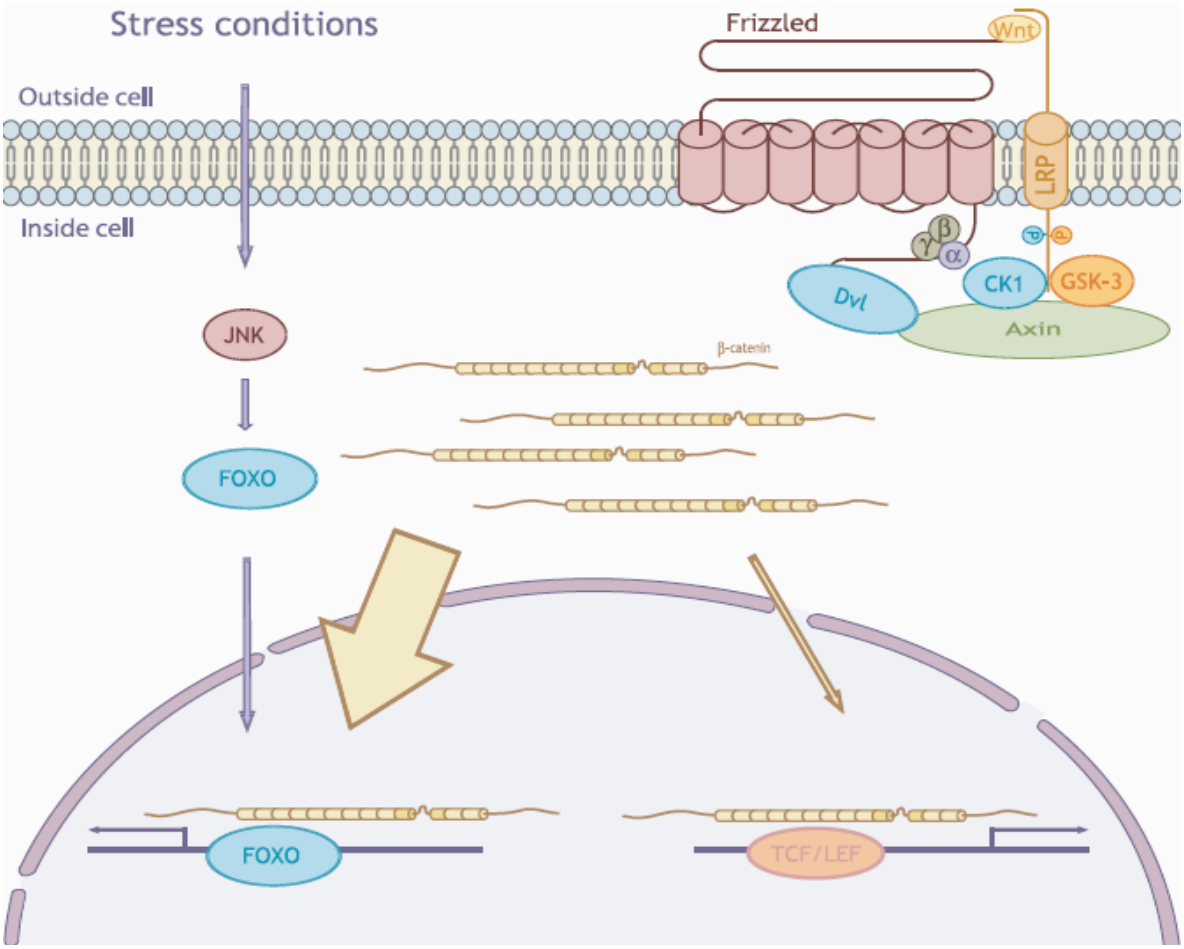

Image 10 | β-catenin, the Libra.
Increased stress conditions antagonize the effects of canonical Wnt signaling. Activation of Fzd-LRP receptor complex by Wnt ligands results in GSK-3 recruitment to the plasma membrane, consequent β-catenin stabilization and accumulation in the cytoplasm. JNK-mediated activation of FOXO proteins, upon stress signals, diverts the majority of β-catenin pool to FOXO-mediated gene transcription over TCF/LEF transcription factors. Thick arrow represents high amount of β-catenin, whereas thin arrow, low amount.

It appears that β-catenin only shifts from one "branch" to the other, under certain conditions of oxidative stress [147, 175]. In addition, it has been suggested a pathophysiological role for β-catenin-FOXOs interaction in bone diseases, where a reduced β-catenin-TCF-mediated gene expression was observed [148].

Canonical Wnt signaling is known to play an important role in bone morphogenesis, since it increases bone mass formation through osteoblasts-mediated production [176]. Conversely, oxidative stress may lead to a decrease in the osteoblast number and bone mass formation rate, because β-catenin is required for stimulation of FOXOs target genes and thence, shifting it away from the canonical Wnt pathway [148].

It has become apparent that β-catenin is an important cell cycle controller, by rapidly changing the cells spatial and temporal gene expression demands, "giving" the highest chances of survival upon diverse signals. Of note, cells avoid simultaneous and competing signals, such as cell cycle progression and cell cycle arrest, by temporally inhibiting a signaling pathway over other.

10 | LIPID HOMEOSTASIS

Lipids are major important to organisms, as they regulate energy homeostasis and organ physiology, and are simultaneous emerging as vital mechanisms to the triggering of cell signaling cascades [177-178]. The homeostasis of energy and lipid compounds is tightly regulated by a lipoprotein transport system that delivers some of its subcomponents, such as cholesterol and fatty acids to the different types of tissues [179].

The normal/healthy heart consumes more energy per gram of tissue than any other organ, which is mainly due to fatty acid catabolism for ATP generation [180]. Nevertheless, under some specific physiological and pathological situations the heart has the ability to switch its substrates requirements towards glucose utilization. This way, members of the nuclear hormone receptor (NHR) superfamily called, Peroxisome proliferator-activated receptors (PPARs) [181], had been implicated in the regulation of several of these metabolic demands, including fatty acid transport across the plasma membrane and uptake by cells [182], intracellular fatty acid binding [183] and activation [184] and also, β- [185] and ω-oxidation [186] or even storage [187].

However, growing evidences support a new role for PPARs in the heart, beyond the well-characterized mediation of metabolic processes.

10.1 Peroxisome Proliferator-Activated Receptors (PPARs)

PPARs are classified as nuclear ligand-activated transcription factors that function as sensors for fatty acids and their derivates and thereby, govern important metabolic signaling pathways involved in lipid metabolism. As ligand-dependent receptors, PPARs form heterodimers with the Retinoid X Receptor (RXR) to promote the formation of an active complex [183, 188]. In the absence of a PPAR ligand, PPARs/RXR heterodimers can still form, yet, recruiting a corepressor protein complex, such as the silencing mediator of retinoid, thyroid hormone receptors (SMRT) and nuclear receptor corepressor (NCoR), inhibiting downstream gene transcription [189-190]. The binding of agonists promotes dissociation of the corepressor complex from the PPARs/RXR heterodimer and leads to the recruitment of a coactivator protein, such as the steroid receptor co-activator-1 (SRC-1) or CBP to create a complex that binds to PPAR response elements (PPRE) in target genes [189-192].

Three isoforms of PPARs had been identified in vertebrates, PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3) that present distinct tissue distributions and physiological roles [181].

PPARs share common structural characteristics and functional organization with other members of the NHR superfamily namely five distinguishable domains [193] termed ‘A/B’, ‘C’, ‘D’, ‘E’ and ‘F’ domains. The N-terminal ‘A/B’ is an hypervariable domain that presents an activation function-1 (AF-1) for the regulation of PPAR activity, which is ligand-independent and has low levels of basal transcriptional activity [194]. Additionally, the N-terminal domain also contains multiple Ser/Thr phosphorylation sites, under various protein kinases regulation [195]. These phosphorylation sites are presumably involved in mediating crosstalk signaling mechanisms and in modulating AF-1 activity and coactivators interaction [195-196]. Following the A/B domain is the DNA-binding domain (DBD) of the ‘C’ domain, a region that contains two very highly conserved zinc finger motifs, responsible for targeting the receptor to the PPRE after dimerization with the RXR [183, 187]. Next to the ‘C’ domain, is a flexible hinge domain ‘D’ capable of connecting the DBD and the ligand-binding domain (LBD) of the ‘E’ domain [187, 193]. This hinge region promotes protein

flexibility, because it allows for simultaneous receptor dimerization and DNA-binding [197], and it also contains a corepressor binding site [190]. The LBD is ligand-dependent and harbors a hormone-dependent activation function-2 (AF-2) that it is necessary for the interaction and heterodimerization with the RXR [187, 198]. Upon ligand-binding, a conformational shift in the AF-2 domain occurs, allowing PPARs/RXR heterodimers to successfully bind and recruit transcriptional coactivators to responsive promoters [182, 198]. Concerning the F-domain, no function has been discovered to date.

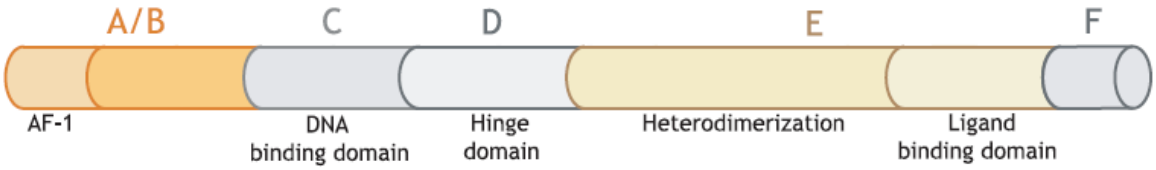

Image 11 | PPARs structure.

10.2 PPARs in the heart

The lipid and energy homeostasis in the cardiovascular system relies on the coordinated regulation of a lipid transport system. Hence, perturbation of this system plays a major role in the pathogenesis of cardiac disease.

It has long been accepted the importance of PPAR family of nuclear receptors in the regulation of the cardiovascular function and metabolism, as they are key transcriptional determinants of myocardial energy and lipid metabolism. In addition to their metabolic roles, PPARs are also responsible for various extra-metabolic roles, such as cardiac inflammation [199-200], cardiac remodeling [201-202], oxidative stress [200, 203] and cardiac hypertrophy regulation [201-202, 204]. Of the three isoforms, PPARα is the only form that has been characterized to occur in all of these extra-metabolic processes, presumably because it is the isoform mostly studied in the heart.

It was recently suggested that PPARα synthetic agonists might detain a role to counteract the cardiovascular inflammatory response. Surprisingly, activation of PPARα decreases inflammatory mediators like tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) [199]. At the same time, because these inflammatory cytokines are induced by macrophages in response to low-density lipoprotein (LDL) levels, PPARα also directly regulates the inflammatory response by lipid-induced catabolism [199]. Moreover, it was demonstrated that PPARα regulates cardiac remodeling during cardiac hypertrophy

development [201-202]. Through mutational analysis, Smeets *et al.* [201] demonstrated a decrease in mice cardiac hypertrophy, which is due to PPARα and PPARβ/δ regulation. Furthermore, the role of PPARα in cardiac hypertrophy inhibition has already been well documented in cell cultured cardiomyocytes. Endothelin-1 (ET-1) is a potent vasoconstrictor peptide that functions as cardiac hypertrophic stimuli. It has been reported that when neonatal rat cardiomyoctes are treated with ET-1, co-administration of a PPARα agonist decreases cardiac hypertrophy [204].

Additionally, it was also demonstrated the involvement of PPARα in oxidative stress resistance [200, 203]. Mutant animals null for PPARα, were observed to present an oxygen toxicity enhancement, which was correlated with a decreased expression of the MnSOD antioxidant enzyme.

The role of the other PPARs in the heart is still elusive, yet, we cannot rule out their roles in cardiac disease. *PPARγ* has been extensively reported to be highly expressed and remarkably important to adipocyte development, opposing to the low concentrations found in the heart [205]. However, despite its low levels in cardiac muscle, PPARγ agonists had been demonstrated to have a cardioprotective role against ischemic insults [206] and to inhibit cardiac hypertrophy [207]. Recently it was even shown that cardiomyoctes overexpressing *PPARγ* were resistant to oxidative stress-induced apoptosis and at the same time, mitochondrial function was preserved [208]. Conversely to *PPARγ*, *PPARβ/δ* expression levels appear extremely high in the heart, yet, almost nothing it is known about its activity and function [209]. Nevertheless, *PPARβ/δ* high expression levels should at least suggest a possible role in the regulation of cardiac genes.

10.3 PPARs convergence with canonical Wnt signaling and FOXOs

Interaction of PPARs and Wnt signaling has been demonstrated in different mechanisms. A crosstalk between canonical Wnt and PPARγ signaling was reported in osteoblastogenesis [210], adipogenesis [211], and cancer [212].

Because osteoblasts and adipocytes originate from a common pluripotent precursor, the mesenchymal stem cell (MSC), it was hypothesized that inhibition of a lineage could stimulate the differentiation over the other. As a matter of fact, several studies demonstrated an inverse co-relationship between adipogenesis and osteoblastogenesis, reinforcing the believed concept [213-214].

Wnt5a, a Wnt ligand more associated to non-canonical Wnt signaling cascades, was demonstrated to suppress PPARγ-mediated adipogenesis, through directly counteracting PPARγ and inducing a osteoblastogenic program [215]. Additionally, the specific canonical Wnt signaling receptor LRP5 was suggested to stimulate osteoblastogenesis *in* humans [210] and, *in vitro* assays demonstrated that canonical Wnt signaling disruption causes myoblasts trans-differentiation into adipocytes and ultimately PPARγ activation [216].

Other studies using colon cancer cells, demonstrated PPARγ-β-catenin interactions, where PPARγ is apparently stabilized in epithelial cells [212]. These authors observed that PPARγ is able to form complexes with β-catenin and TCF-4 and, overexpression of stabilized β-catenin results in enhanced transcriptional activity of PPARγ. Saez *et al.* [217] found that in mammary gland tumor developments PPARγ activation leads to a similar phenotype, resulting from overactivation of canonical Wnt signaling as well as to an upregulation of endogenous Wnt target genes. These authors observed a downregulation of *Wnt5a* in result of induced PPARγ activation, suggesting a possible PPARγ-Wnt signaling interaction. These observations thus demonstrate an interaction between the Wnt and PPAR pathways, in different contexts.

Similarly, an interaction between PPARs and FOXOs had already been made [150]. Due to the fact that both PPARγ and FOXO1 are the most abundant isoforms expressed in insulin-responsive tissues, such as hepatic, adipose and pancreatic cells, it was plausible to assume these two transcription factors would functionally interact [218-219]. PPARγ agonists have a key role in the regulation of lipid catabolism and glucose homeostasis. Glucose is uptaken by cells through the insulin-responsive glucose transporter 4 (GLUT4), which in turn is negatively regulated by PPARγ [220]. In an unliganded state (absence of ligand), PPARγ binds certain regions on the *GLUT4* promoter, keeping it in a repressed stage. Once synthetic ligands, such as Rosiglitazone and Pioglitazone, two hypoglycemic drugs from the thiazolidinedione (TZD) family are present, they bind to PPARγ, causing detachment of corepressors and subsequent association of coactivators, which result in PPARγ detachament from the *GLUT4* gene promoter. Ultimately, this leads to alleviation of the repression, increased *GLUT4* transcription and subsequent, insulin-responsiveness [220]. Yet, this mechanism has only been proved in adypocyte tissues and thence it remains to be resolved in the muscle.

Moreover, it was described in a two-hybrid screen functional interaction of FOXO1 with PPARγ. Dowell *et al.* [150] reported that FOXO1-PPARγ interactions antagonize each other functions, in a reciprocal manner. These authors discovered that FOXO1 disrupts the DNA-binding activity of PPARγ/RXRα heterodimers and thus, suppress PPARγ activity. As a matter of fact and in accordance, it has been recently reported that FOXO1 upregulates *GLUT4* gene expression at both the transcriptional and post-transcriptional levels and in simultaneous, by both directly binding to the *GLUT4* promoter and indirectly, via repressing PPARγ transcription [221]. Nonetheless, it was also demonstrated a negative role for FOXO1 in *GLUT4* transcription for other tissues, where it was demonstrated to repress *GLUT4* expression [222]. Yet, it is not known the reason for such duality in the regulation of glucose metabolism.

Thus, as with Wnt signaling, a cross-talk between PPAR and FOXO pathways can be traced. Of interest, an interaction between pathways is mostly observed in common mechanisms/cells namely adipogenesis, adipocyte cells and oxidative stress.

11 | AIM

Contrasting to the well documented role of Wnt signaling in heart development, its role in the adult heart and/or heart failure state remains to be fully elucidated as well as to its possible interactions with the PPAR and FOXO pathways.

In order to its understanding, we started by analyzing the expression of the various effectors of the Wnt pathway in heart disease, more concretely in type 1 induced diabetic cardiomyopathy, as well as *PPAR* and *FOXO* genes. We used this model because it is easily obtained, and since this pathology mostly evolves due to an increase in oxidative stress, in which the three pathways are known to play a determinant role.

MATERIAL AND METHODS

12 | MATERIAL AND METHODS

All animal experiments were conducted in conformity with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the Portuguese law on animal welfare.

Adult male Wistar Han rats (Charles River Laboratories, Spain) were housed in groups of 5 per cage in a controlled environment under a 12:12h light/dark cycle at a room temperature (RT) of 22ºC.

12.1 Streptozotocin (STZ)-induced diabetes

Streptozotocin (STZ) is an antibiotic that causes pancreatic β-cell destruction, thus it is used as an agent capable of inducing insulin-dependent diabetes mellitus (IDDM), also known as type 1 diabetes mellitus (T1DM) [223].

STZ (no. S0130; 65 mg/Kg; Sigma, USA) was dissolved in a 50 mM sodium citrate buffer (pH = 4.5) to a final concentration of 10 mg/mL. Upon 6 hours of fasting, rats weighing 250-300 g received a onetime intraperitoneally (IP) injection of 0.1 mL of STZ/100 g, within 15 min after STZ solution preparation. Control animals received an equal volume of vehicle (no. NC9521441; citrate buffer, pH = 4.5; Fisher). On the first day after injection, 10% sucrose was administered to the animals, to overcome the initial hyperinsulinemia induced by the STZ. Animals had free supply of food and water. Blood glucose levels were assessed one week later (> 300 mg/dL), using a 'One Touch Basic' blood glucose monitoring system to confirm STZ injection-induced hyperglycemia.

This procedure resulted in two temporal separated experimental groups, defined by STZ-exposure time: four-week control animals (Ctrl, n = 7) and four-week diabetic animals (DM, n = 10); six-week control animals (Ctrl, n = 18) and six-week diabetic animals (DM, n = 19). Hemodynamic studies, collection of samples for morphometric analysis and left ventricle (LV) samples for molecular studies was carried out, only for Ctrl and DM six-week animals, after six weeks of protocol initiation. For four-week animals, only LV samples were collected.

12.2 Echocardiography assessment

Ecochardiographic evaluation was performed before hemodynamic assessment (only for six-week animals), using a 7.5 MHz transducer. Animals were anaesthetized with ketamine (75 mg/kg; IP) and xylazine (5 mg/kg; IP) and allowed to stabilize for 15 min. From the left parasternal short-axis view, two-dimensional guided M-mode (MM) tracings were made just below the mitral valve at the level of the papillary muscles for measurements of the interventricular septum thickness (IVS, mm), LV internal diameter (LVD, mm), posterior wall thickness (LVPW, mm), fractional shortening (FS, %), ejection fraction (EF, %) and heart rate (HR, bpm), using Vivid 7 system (VingMed Ultrasound, GE) equipped with a 10S ecocariographic probe (GE-Medical Systems). FS was calculated from measurements for the LVD in systole and diastole: FS (%) = [(LVDd-LVDs/LVDd] x 100. LV EF was determined by M-mode echocardiography applying the formula [(LV-end-diastolic^3 – LV-end-systolic^3)/LV-end-diastolic^3] x 100 (%). IVS, LVD and LVPW were normalized for body surface area. All the measurements and images were obtained with regular sinus rhythm and stored in the system for off-line analysis (EchoPAC work station version 3.2 system, VingMed Ultrasound, GE).

12.3 Myocardial function

Hemodynamic studies: Animals from the Ctrl and DM (six-week diabetic animals) groups were anesthetized by inhalation of a mixture of sevoflurane (4%) and oxygen, intubated for mechanical ventilation (respiratory frequency 100/min and weight-adjusted tidal-volume; Harvard Small Animal Ventilator - Model 683) and placed over a heating pad (37ºC). The right jugular vein was cannulated for fluid administration (prewarmed 0.9% NaCl solution) to compensate for perioperative fluid losses. The heart was exposed through a median sternotomy, the pericardium widely opened, and a 2F-high-fidelity tip pressure micromanometer (SPR-1035; Millar Instruments, USA) was inserted through an apical puncture into the LV cavity. After 15 min of stabilization, hemodynamic recordings were made with respiration suspended at end expiration. Parameters were converted online to digital data with a sampling frequency of 1000 Hz. LV pressures were measured

at end-diastole (EDP) and end-systole (P_{max}). Peak rates of LV pressure rise (dP/dt_{max}) and decline (dP/dt_{min}) were also measured.

At the end of the hemodynamic study, animals were euthanized by exsanguination, the LV cavity was carefully dissected and LV free-wall samples were collected for molecular studies, immediately immersed in liquid nitrogen and stored at -80 ºC.

12.4 Molecular Studies

Real-Time RT-PCR analysis: Total mRNA was extracted from the LV samples of both temporal separated groups (Ctrl and DM, at four- and six-week treatment), using TriPure (no. 11667165001, Roche, USA) isolation reagent according to the manufacturer's instructions. Concentration and purity were assayed by spectrophotometry (BioPhotometer, Eppendorf; Germany). All mRNA samples were normalized to a final concentration of 15 ng/µL and subsequently reverse transcribed to complementary Deoxyribonucleic acid (cDNA), through a Reverse Transcription-Polymerase Chain Reaction (RT-PCR) step. To obtain an inside negative control (RT^-) a sample mix of all extracted samples was used to perform a LV RT^- control. Reverse transcription (30 µL of final volume; 10 min at 22 ºC; 50 min at 50 ºC; 10 min at 95 ºC) was performed in a standard Thermocycler Machine (Whatman Biometra, Germany), using SuperScript® II Reverse Transcriptase (no. 18064-014; Invitrogen, USA) 200 U/µL, 5x First Strand Buffer (no. 1130449; Invitrogen, USA), RNAse inhibitor 40 U/µL (no. 27838811; Promega, Madison, USA), DTT 0.1 mM (no. 1350135; Invitrogen, USA), random primers 30 ng/mL (no. 767205; Invitrogen, USA) and dNTPs 5 mM (no. 7901; Fermentas, USA). The newly transcribed cDNA quantities were relative quantified by Real Time-Polymerase Chain Reaction (Real Time-PCR; LightCycler II, Roche, Swiss) using SYBR green (no. 204143; Qiagen, Germany) as a marker and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Results were normalized for GAPDH and presented in arbitrary units (AU).

Table 1 | Specific PCR primer pairs used in the work.

Primer	Sequence Forward (Fwd) 5'→3'	Sequence Reverse (Rev) 5'→3'
LRP5	CTCTCAGTTCCCCTGTGCTC	GCTCATCAGATCCATCAGCA
LRP6	TGGCTTAGCCCTGGATTATG	CCTCCCAGTGCCATCAGTAT
Fzd1	CCTGCGGACTGTAGAGGAAG	CTGAAGGAATTGACCCTGGA

Fzd2	ACATCGCCTACAACCAGACC	CGGGTAGAATTGATGCACCT
Fzd3	GAAGCAAAGCAGGGAGTGTC	GAGTGATCTGTCAGCCGTGA
Fzd4	AACCTCGGCTACAACGTGAC	TGGCACATAAACCGAACAAA
Fzd5	GCGACCTTCCTCATTGACAT	TCCCAGTGACACACACAGGT
Fzd6	GAAAAGCAGCGTATCGGAAG	CGAACAAGCAGAGATGTGGA
Fzd7	ACACCGGAGCTCAACCATAC	TAGGCAATATCCGTGCACAA
Wnt2	CAACAGAGCTGGAAGGAAGG	AGCCAGCATGTCCTCAGAGT
Wnt2b/13	ACTGGGGTGGCTGTAGTGAC	GGGCATCCTTAAGCCTCTTC
Wnt3	CAGGAGTGCATTCGCATCTA	TCCAGCCGTACAATCTACCC
Wnt4	CTGGAGAAGTGTGGCTGTGA	GGACTGTGAGAAGGCTACGC
Wnt5a	GCAGCACAGTGGACAACACT	GGCTCATGGCATTTACCACT
Wnt5b	TGACTACTGCCTGCGAAATG	CTCTTGAAGCGGTCATAGCC
Wnt6	CGTGGAGATATCCGTGCTTT	AAAGCCCATGGCACTTACAC
Wnt7a	CCCGAACCCTCATGAACTTA	GCCTAGCTCTCGGAATTGTG
Wnt7b	CAGGCAGAAAGGTTCTGGAG	GCACAGCTGCGTTGTACTTC
Wnt9a/14	GAGACACTGGTGGAGGCTGT	AGATGGCGTAGAGGAAAGCA
Wnt10a	CCTGGAGACTCGGAACAAAG	AACCGCAAGCCTTCAGTTTA
Wnt11	CAGTGCAACAAGACCTCCAA	ACCACTCTGTCCGTGTAGGG
CyclinD1	AGGGGATTCAGGACGACTCT	GGGCAACCTTCCCAATAAAT
Foxo1	AACCAGTCCAACTCGACCAC	TGCTCATAAAGTCGGTGCTG
Foxo3	GGGGAACTTCACTGGTGCTA	GAGAGCAGATTTGGCAAAGG
Foxo4	CAGTGACCTCATGGATGGTG	CTCTGAAGCAGGGGACAAAG
Axin2	AGTCAGCAGAGGGACAGGAA	CTTGGAGTGCGTGGACACTA
PPARα	TTCCAGCCCCTCCTCAGTCAG	AGCCCTTGCAGCCTTCACAT
PPAR-γ	GCGAGGGCGATCTTGACAG	ATGCGGATGGCCACCTCTTT
PPAR-β/δ	AGGCCCGGGAAGAGGAGAAA	GCAGGGAGGAAGGGGAGGAA
Rspo2	GCTGCTTTGATGAATGTCCA	TGCGGTTGTTTCTGCTACAC
Norrin	TGCATGAGGCACCATTATGT	GACAGTGCTGAAGGACACCA
Ryk	ACATCGACCCCTTTGAGATG	GAAACTTAGGCCGCTCCTCT
Ror2	ATGTGGACTCCCTCCAGATG	AGGAAAGACGAAGTGGCAGA
GRK5	TGGCACTCAATGAAAAGCAG	ACAAGGCTCGTTCTTCCTCA
GRK6	GCCAGTGACCAAAAACACCT	CCCCTTCCGCTTCTTTATTC
GSK-3α	ATTTGCTTGTGGACCCTGAC	CTGACCACACATCGATGGAC
GSK-3β	TCCGATTGCGGTATTTCTTC	TCACAGGGAGTGTCTGCTTG

GAPDH	TGCCACTCAGAAGACTGTGG	GGATGCAGGGATGATGTTCT
GATA4	CCCTCTGTGTGGGAACAACT	GTGCTCCACCTGGAAAGGTA

Two additional primer pairs, commonly used for heart hypertrophy were used for the evaluation of disease: type-B natriuretic factor (BNP**)** and endothelin-1 (ET-1**)**.

Table 2 | Specific PCR primer pairs used in the work.

Gene	Primer Forward (Fwd) 5'→ 3'	Primer Reverse (Rev) 5'→3'
BNP	CAGAGCTGGGGAAAGAAGAG	GGACCAAGGCCCTACAAAAGA
ET-1	CGGGGCTCTGTAGTCAATGTG	CCATGCAGAAAGGCGTAAAAC

12.5 Statistical analysis

Values are presented as means ± standard error of mean (SEM) and '*n*' represents the number of experiments. Differences between groups were analyzed using Student's t-test.

RESULTS

13 | NOTE:

Due to health problems in our animal facility, some animals died before we were able to perform echocardiographic and hemodynamic evaluation. This included mostly animals with four-weeks STZ-treatment. We thus established a collaboration with another department and were able to collect samples at this time point which we used for expression analyzes experiments. However we were not able to perform the above mentioned evaluations in the animals from our collaborators due to the protocols they were going to perform subsequently.

Echocardiographic and hemodynamic evaluation was only able to be performed in six-weeks STZ-treated animals (before the animal facility problem) and, taking the results obtained in these animals, we inferred possible results at four-weeks of treatment.

13.1 Echocardiographic evaluation

The use of this technique allowed us to non-invasively monitor the progression of cardiac dysfunction, in order to estimate ventricular morphofunctional alterations during heart failure development and evaluate differences among experimental groups. All echocardiographic parameters of six-week animals are presented in **Table 3**.

Table 3 | Doppler echocardiographic measurements.

Parameter	Ctrl	DM
IVSId (mm/cm^2)	35.2 ± 1.9	52.5 ± 2.2 ***
IVSIs (mm/cm^2)	63.6 ± 2.5	82.4 ± 5.8 **
LVDId (mm/cm^2)	175 ± 6	216 ± 7 ***
LVDIs (mm/cm^2)	94.8 ± 5.8	109 ± 6
LVPWId (mm/cm^2)	34.5 ± 1.7	48.5 ± 3 ***
LVPWIs (mm/cm^2)	55.6 ± 2.2	70.6 ± 3.9 **
EF (%)	82.2 ± 1.8	85.0 ± 1.7
FS (%)	46.3 ± 1.8	49.8 ± 1.8
HR (bpm)	271 ± 8	232 ± 8 **

Ctrl, control; DM, diabetic; IVSId and IVSIs, interventricular septum thickness index in diastole and systole; LVDId and LVDIs, left ventricle internal diameter index in diastole and in systole; LVPWId and LVPWIs, left ventricle posterior wall thickness index in diastole and systole; EF, ejection fraction; FS, fractional shortening; HR, heart rate. (**p < 0.01 versus Ctrl; ***p < 0.001 versus Ctrl; Data are mean ± SEM).

Our echocardiographic data supports the progression of the disease state in our DM model (**Table 3**). IVSId/s and LVPWId/s parameters were significantly increased in diabetic animals, indicating left ventricle (LV) hypertrophy both during diastole (relaxation + filling) and systole (contraction + ejection). Moreover, the presence of diabetes promoted a significant dilatation of the LV (diastolic LVDI increased) and decreased HR.

EF is defined as the fraction of end diastolic volume that is ejected out of the ventricle during each contraction, whilst, FS is defined as the changes in the diameter of LV, between the contracted and relaxed state of the heart. Both parameters were similar between groups.

13.2 General features

Somatic and cardiac growth of six-week animals of both groups is represented in **Table 4**. At the end of the hemodynamic protocol, several morphometric parameters were measured, including body weight, body surface area (BSA), gastrocnemius-muscle-to-tibial-length and plasma glucose levels.

Table 4 | General features of Ctrl and DM animals.

Parameter	Ctrl	DM
Body weight (g)	390 ± 19.5	280.5 ± 8.37 ***
BSA (cm^2)	0.040 ± 0.001	0.030 ± 0.001 ***
GW/TL (mg/mm)	56.63 ± 1.94	36.39 ± 2.1 ***
Plasma glucose (mg/dL)	238.75 ± 9.4	443 ± 7 ***
LV + septum (g)	0.69 ± 0.028	0.54 ± 0.021 **
LV/BSA (g/cm^2)	17.24 ± 0.437	16.59 ± 0.616
Heart/BSA (g/cm^2)	24.88 ± 0.876	26.93 ± 0.842

Ctrl, control; DM, Diabetic; BSA, body surface area; GW/TL, gastrocnemius-muscle-weight-to-tibial-length (**p < 0.01 versus Ctrl; ***p < 0.001 versus Ctrl; Data are mean ± SEM).

Body weight and surface area were significantly smaller in diabetics when compared to controls. Tibial length was similar among the two, whereas the ratio of gastrocnemius-muscle-to-tibial-length was significantly lower in the DM group, a suggestive finding of cachexia (syndrome defined by loss of weight and muscle atrophy). Plasma glucose levels were significantly higher in the DM group, a confirmation of the

diabetic state of our models. In accordance to hypertrophy development observed in the echocardiographic analysis, LV + septum mass was increased when compared to the control group and we observe a tendency to both LV/BSA heart/BSA increase.

13.3 Myocardial function

For six-week animals, hemodynamic analysis allowed to evaluate several parameters.

Figure 1 represents the LV peak rate of pressure rise (**a**) and decline (**b**), while **Figure 2** describes the maximal pressure that can be developed by the ventricle at any given LV volume (**a**), in opposition to the passive filling properties of the myocardium (**b**).

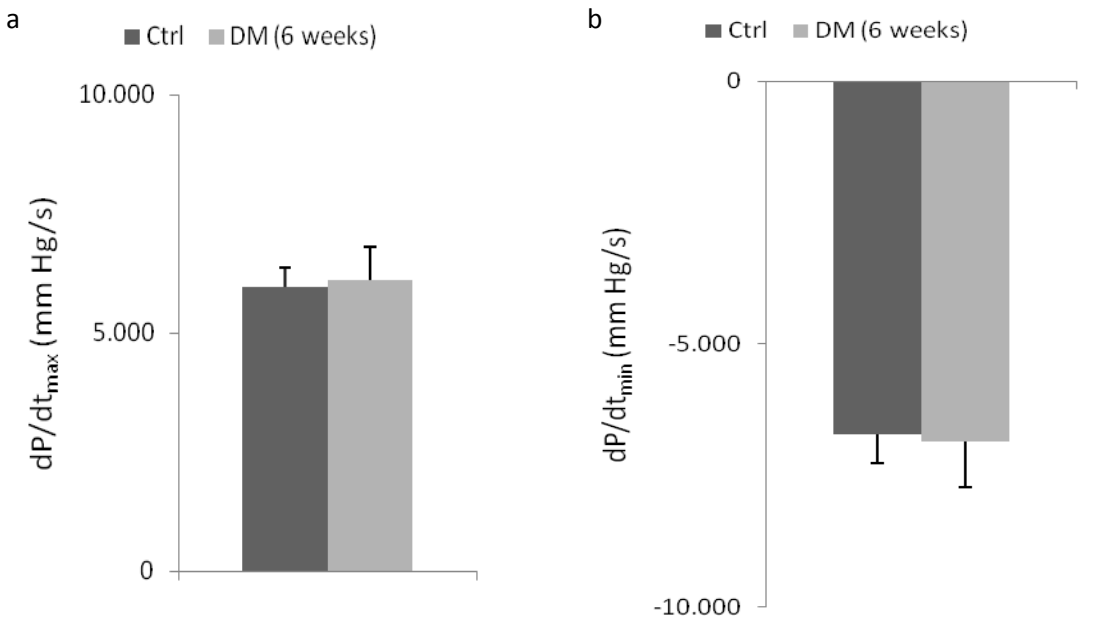

Figure 1 | Baseline hemodynamic assessment of left ventricle function. (**a**) peak rates of pressure rise (dP/dt_{max}, mm Hg/s) and (**b**) decline (dP/dt_{min}, mm Hg/s).

dP/dt_{max} and dP/dt_{min} (respectively, **Figure 1 | a** and **Figure 1 | b**) are used as indexes of ventricular performance. From our data we did not detect significant alterations between diabetics and controls, suggesting that heart's contractility and relaxation functions remained unaltered.

Regarding the maximal pressure, P_{max} (**Figure 2 | a**), we did not detect any alterations for diabetics, suggesting that LV hypertrophy previously described, was able to normalize the increased overload. Despite no significant differences were found in end-diastolic pressure (EDP) (**Figure 2 | b**), we observe a tendency to its increase, which might

be due to LV compliance (wall elastic properties) decrease, such as a result to ventricular hypertrophy and other extracellular matrix changes previously described in this model.

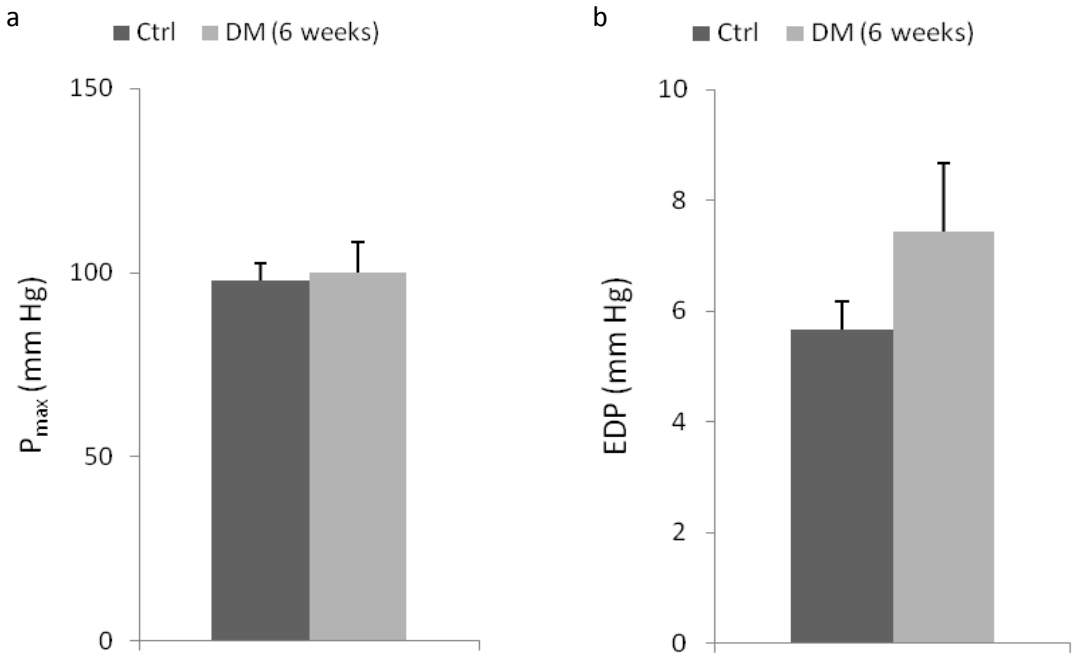

Figure 2 | Baseline hemodynamic assessment of left ventricle function. (**a**) end-systolic (P_{max}, mm Hg) and (**b**) end-diastolic (EDP mm Hg) pressures.

We conclude from these results that by six-weeks of diabetic state in this rat model, although not presenting alterations in its performance, it does already present some alterations in its morphology (hypertrophy and dilation), indicative of an adaptation to a stress condition.

At the same time, we inferred that by four-weeks upon treatment, DM animals will show only mild, if any, morphological and heart performance alterations, and thence we assume that this stage represents a very early stage of diabetic cardiomyopathy development.

13.4 Gene expression

Type-B natriuretic peptide (BNP) is a marker of cardiac dysfunction that correlates with the severity of chronic pressure overload and left ventricular hypertrophy [224]. Similarly, the expression of endothelin-1 (ET-1) is directly correlated to disease severity and prognosis of heart failure [225]. For this reason we determined the expression of these two markers to confirm if their expression was correlated with progression of heart disease. We analyzed their gene expression at four-weeks upon diabetes induction (**Figure 3** and **Table 5**).

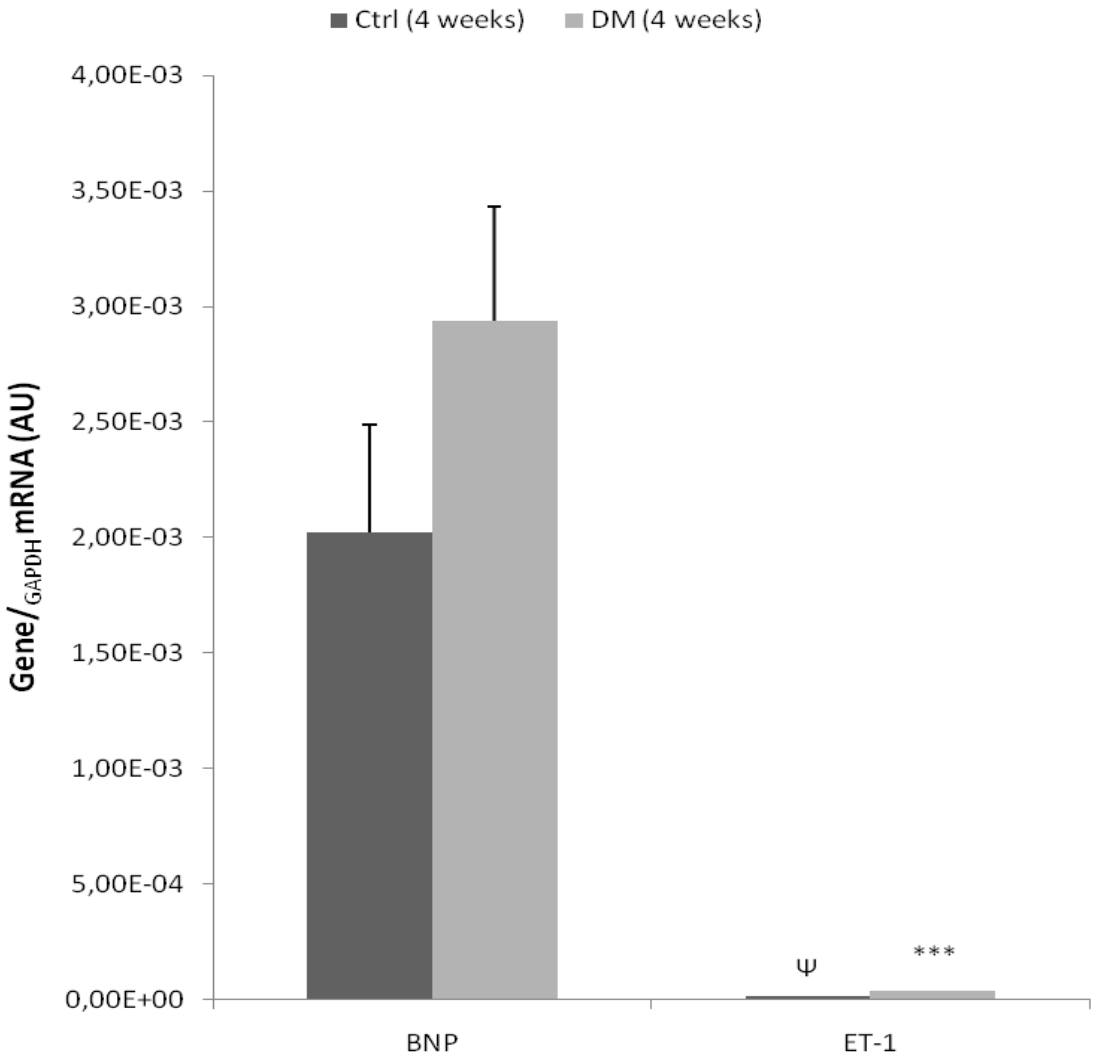

Figure 3 | Expression of *BNP* and *ET-1* genes, in left ventricle heart samples of four-week animals, collected after treatment. Results are normalized for *GAPDH* and expressed in arbitrary units (AU). (***p < 0.001 versus Ctrl; Data are mean ± SEM; Ψ: *ET-1* graph in supplements).

Table 5 | Comparison between groups of the mean relative expression for *BNP* and *ET-1* genes. Results are normalized for *GAPDH* (left ventricle heart samples of Ctrl and DM groups)

	BNP	*ET-1*
DM/Ctrl	1.46	2.56

At this stage, no statistical differences for *BNP* expression were still observed, although there seems to be a tendency for an increased of its expression, whilst, *ET-1* shows a clear upregulation. It is thus clear that at this time point there is already some neurohumoral regulation, indicative of installment and progression of heart disease.

In order to clarify the role of Wnt signaling in the heart, a wide gene expression of approximately all known Wnt ligands, Fzds, LRPs, intervening kinases and downstream targets, was performed, using Real Time-PCR.

WNTS, THE LIGANDS

In the mammalian genome, 19 Wnt ligands have already been reported. After database search, we found rat homologues for 12 Wnt ligands and analyzed their gene expression in control and DM animals at four-weeks of treatment (**Figure 4**).

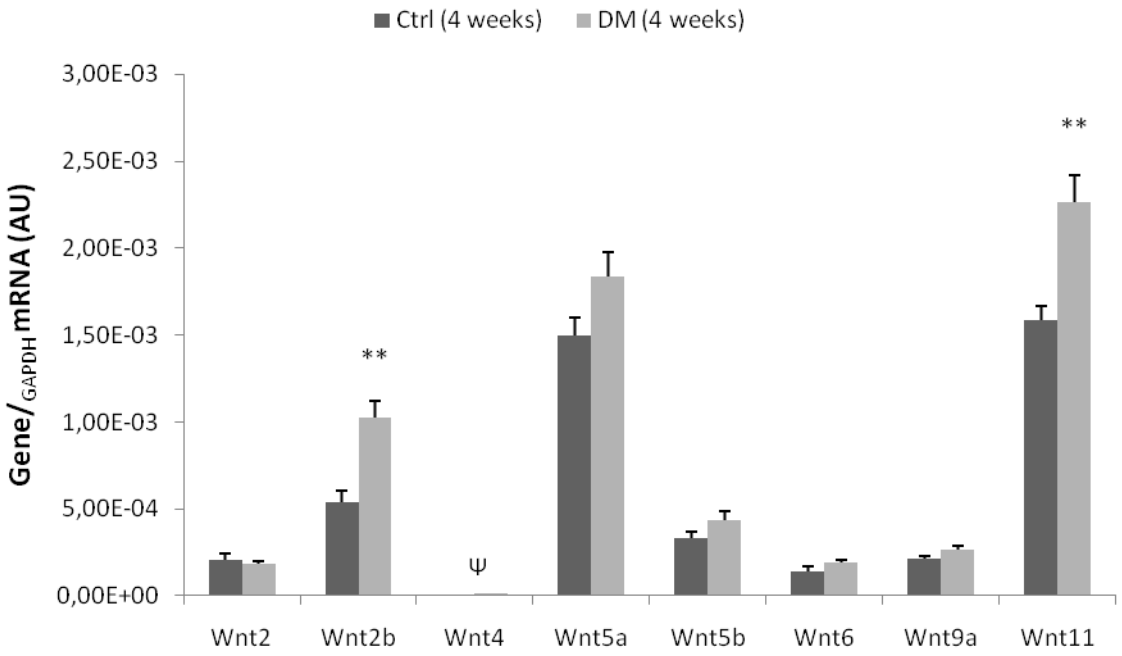

Figure 4 | Expression of *Wnt* genes, in left ventricle heart samples of four-week animals, collected after treatment. Results are normalized for *GAPDH* and expressed in arbitrary units (AU). (**p < 0.01 versus Ctrl; Data are mean ± SEM; Ψ: *Wnt4* graph in supplements).

Wnt3, *Wnt7a*, *Wnt7b* and *Wnt10a* expressions were not detected in both control and DM animals.

Wnt2, Wnt2b, *Wnt4*, *Wnt5a, Wnt5b, Wnt6, Wnt9a* and *Wnt11* expression was detected in both control and DM groups. Of these, *Wnt2b* and *Wnt11* showed a statistical relevant increase in expression (1.89 and 1.43 fold increase, respectively) (**Table 6**) at four-weeks upon treatment.

We conclude from these results that of all Wnt genes, by us analyzed, only *Wnt2, Wnt2b, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt9a and Wnt11* seem to be expressed in normal and diabetic hearts, and that only the expression of *Wnt2b* and *Wnt11* is significantly upregulated in the heart of diabetic animals at early stages of disease.

Table 6 | Comparison between groups of the mean relative expression of the highest expressed *Wnt* genes. Results are normalized for *GAPDH* (left ventricle heart samples of Ctrl and DM groups).

	Wnt2b	*Wnt5a*	*Wnt11*
DM/Ctrl	1.89	1.23	1.43

THE RECEPTORS:

Because 10 Fzd and 2 LRP receptors are found in mammals, we analyzed their expression levels (**Figure 5** and **Table 7,** and **Figure 6**, and **Table 8**, respectively). Again after database search, we found rat homologues for 7 Fzds (*Fzd1* to *-7*) and the 2 LRPs (*LRP5* and *LRP6*).

FZDS

Expression analyses of control and DM animals at four-weeks upon STZ-treatment revealed that although expression of all Fzds can be seen in both groups, only *Fzd3* and *Fzd5* showed a significant upregulation in DM animals when compared to control animals (1.89 and 1.42 fold increase, respectively).

Table 7 | Comparison between groups of the mean relative expression for *Fzd* genes. Results are normalized for *GAPDH* (left ventricle heart samples of Ctrl and DM groups).

	Fzd1	*Fzd2*	*Fzd3*	*Fzd4*	*Fzd5*	*Fzd6*	*Fzd7*
DM/Ctrl	1.41	1.10	1.89	1.21	1.42	0.975	1.06

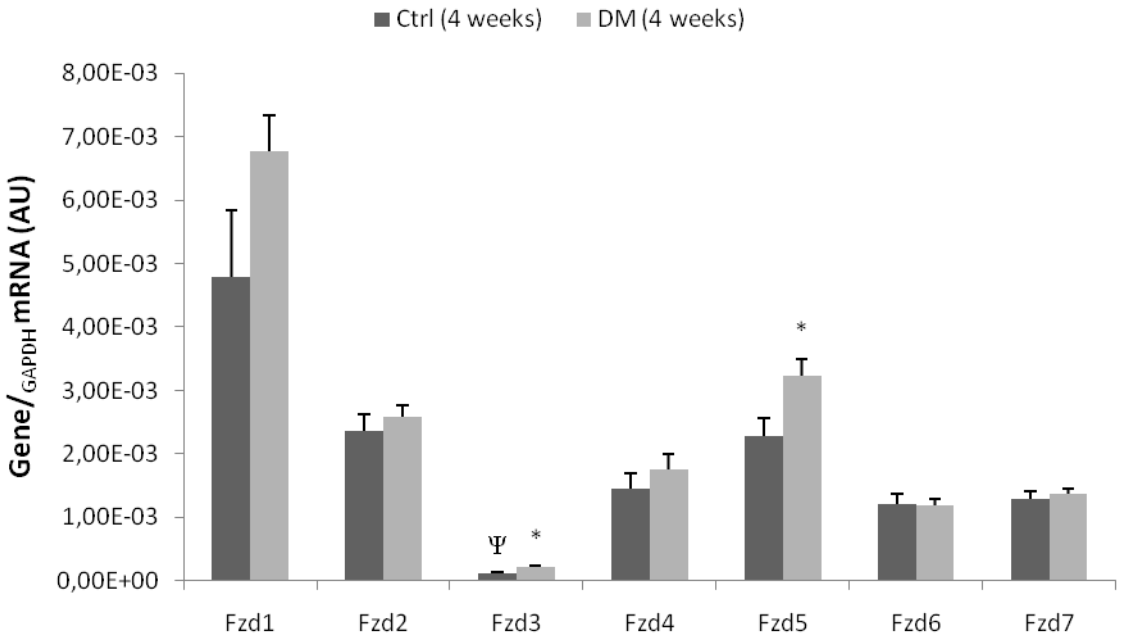

Figure 5 | Expression of *Fzd* genes, in left ventricle heart samples of four-week animals, collected after treatment. Results are normalized for *GAPDH* and expressed in arbitrary units (AU). (*p < 0.05 versus Ctrl; Data are mean ± SEM; Ψ: *Fzd3* graph in supplements).

LRPs

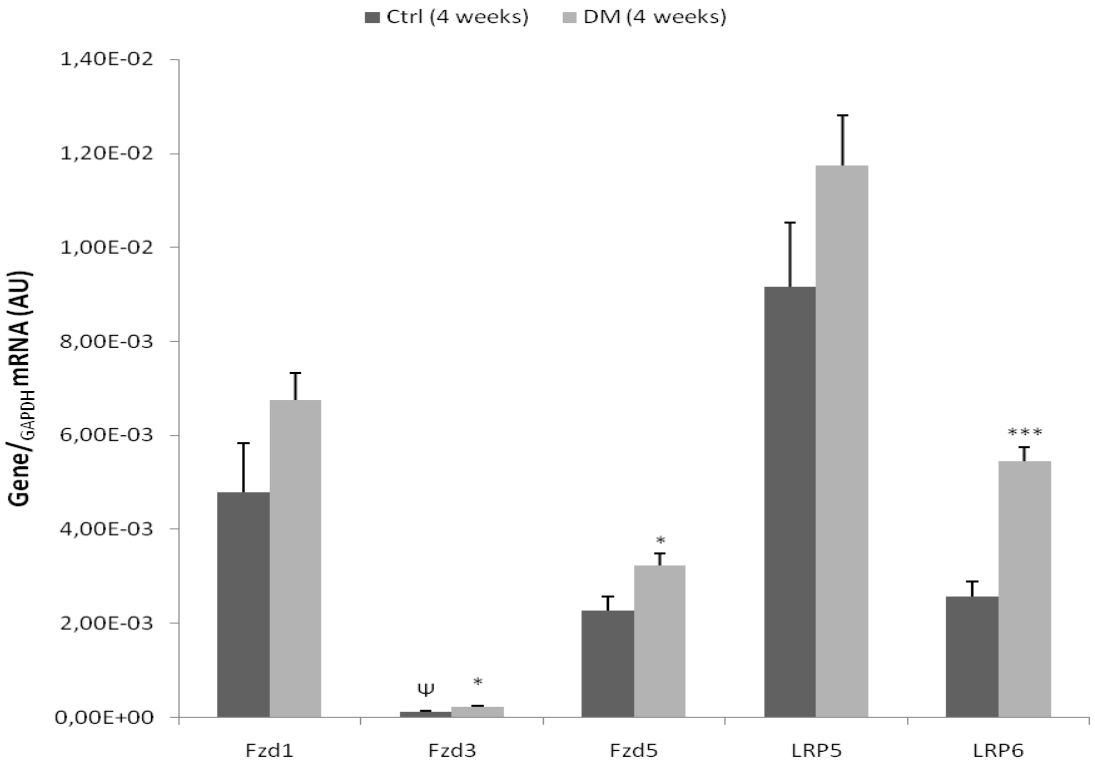

Figure 6 | Expression of relevant *Fzd* and *LRP* genes, in left ventricle heart samples of four-week animals, collected after treatment. Results are normalized for *GAPDH* and expressed in arbitrary units (AU). (*p < 0.05 versus Ctrl; ***p < 0.001 versus Ctrl; Data are mean ± SEM; Ψ: *Fzd3* graph in supplements).

Table 8 | Comparison between groups of the mean relative expression for *Fzd* and *LRP* genes. Results are normalized for *GAPDH* (left ventricle heart samples of Ctrl and DM groups).

	Fzd1	*Fzd3*	*Fzd5*	*LRP5*	*LRP6*
DM/Ctrl	1.41	1.89	1.42	1.28	2.13

Expression analyzes for Fzd co-receptors, *LRP5* and *LRP6* revealed that, like Fzds, both are expressed in four-week control and DM animals, yet, only *LRP6* presents significant alterations, a suggestive finding of canonical Wnt signaling upregulation.

CANONICAL WNT AGONISTS AND UNUSUAL RECEPTORS

Although not usually regarded as fundamental players in canonical Wnt pathway, we also looked at the expression of two of its agonists', *Rspo* and *Norrin,* and two of its unusual receptors, *Ryk* and *Ror2*. Results are depicted in **Figures 7 and 8,** and in **Tables 9** and **10**.

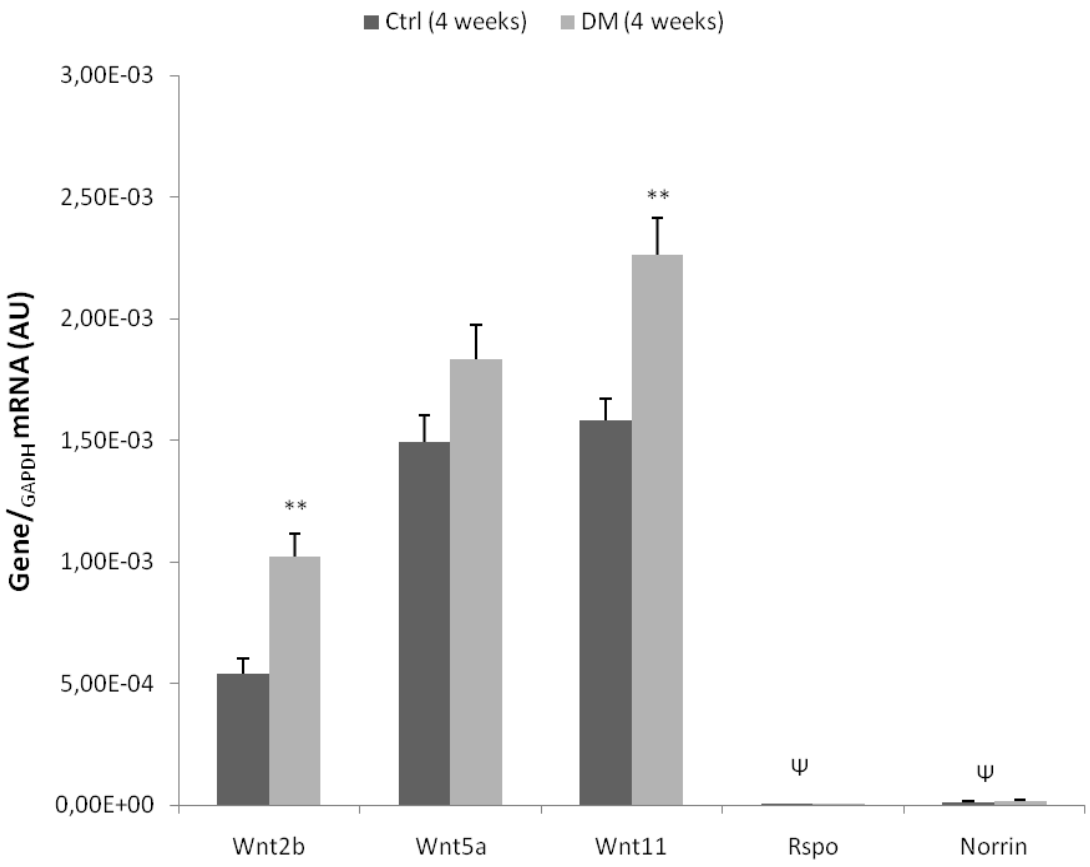

Figure 7 | Expression of relevant *Wnt* and unrelated *Wnt* genes, in left ventricle heart samples of four-week animals, collected after treatment. Results are normalized for *GAPDH* and expressed in arbitrary units (AU). (**p < 0.01 versus Ctrl; Data are mean ± SEM; Ψ: *Rspo* and *Norrin* graphs in supplements).

Rspo expression was practically unchanged in diabetic animals at four-weeks of treatment and although off statistical relevance, we detected a tendency for *Norrin* increased (**Figure 7** and **Table 9**). The expression of the Ryk and Ror2 did not change (**Figure 8** and **Table 10**).

Table 9 | Comparison between groups of the mean relative expression for *Wnt* and unrelated *Wnt* genes. Results are normalized for *GAPDH* (left ventricle heart samples of Ctrl and DM groups).

	Wnt2b	*Wnt5a*	*Wnt11*	*Rspo*	*Norrin*
DM/Ctrl	1.89	1.23	1.43	1.10	1.70

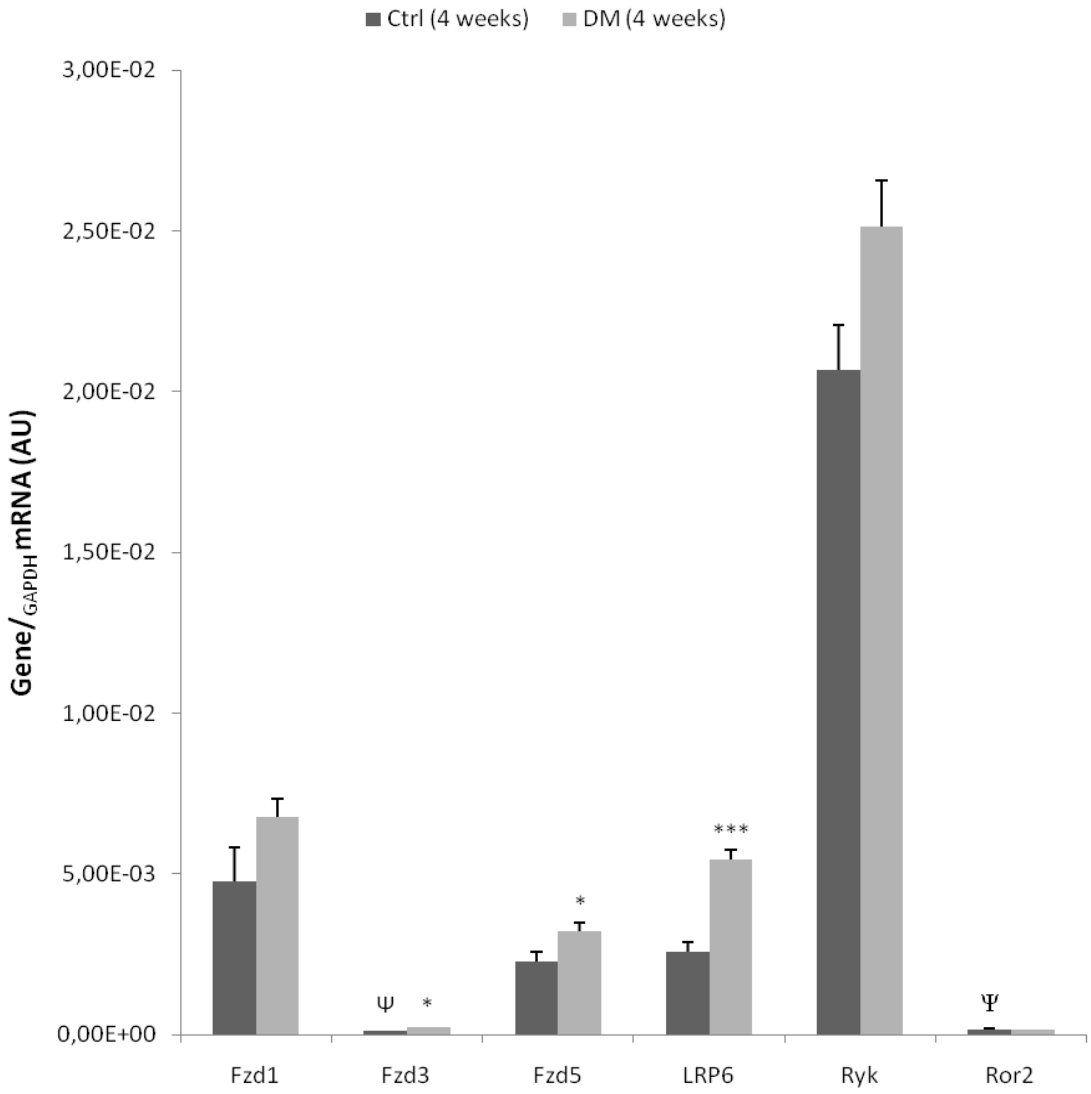

Figure 8 | Expression of *Fzd*, *LRP6* and unusual receptor genes, in left ventricle heart samples of four-week animals, collected after treatment. Results are normalized for *GAPDH* and expressed in arbitrary units (AU). (*p < 0.05 versus Ctrl; ***p < 0.001 versus Ctrl; Data are mean ± SEM; Ψ: *Fzd3* and *Ror2* graphs in supplements)

These results showed that the expression of Wnt signaling "unusual" players is not changed in the early stages of diabetic cardiomyopathy.

Table 10 | Comparison between groups of the mean relative expression for *Fzd*, *LRP6* and unusual receptor genes. Results are normalized for *GAPD*H (left ventricle heart samples of Ctrl and DM groups).

	Fzd1	*Fzd3*	*Fzd5*	*LRP6*	*Ryk*	*Ror2*
DM/Ctrl	1.41	1.89	1.42	2.13	1.21	0.96

WNT SIGNALING: DOWNSTREAM TARGETS

The observation that canonical Wnt ligand, *Wnt2b*, as well as the co-receptor LRP6 are of great significance in DM animals, led us to assume that this pathway is upregulated in diabetics hearts. For this reason, we assessed the expression of two downstream targets of canonical Wnt signaling, *Axin2* (an axin-related protein that presumably plays the same role as Axin in the destruction complex) and *CyclinD1*. At four-weeks of treatment, none of these genes showed changes with statistical relevance, nonetheless, we observe a tendency for *Axin2* increase (1.49 fold increase), contrasting to the mild increase of *CyclinD1* (1.29 fold increase) (**Figure 9** and **Table 11**).

Wnt11, a ligand more associated to non-canonical Wnt signaling and one of the *Wnts* upregulated in our model, has been shown to be crucial for embryonic cardiogenesis, and *GATA4* a member of the family of GATA transcription factors, seems to be required for the expression of *Wnt11* during this process. In addition, *GATA4* has been reported to be upregulated in hypertrophied hearts [226]. For this reason, we looked at the expression of *GATA4* to observe if any increase is also detected in DM. At four-weeks upon treatment, *GATA4* showed already an increase (1.41 fold increase), even if off statistical relevance, in DM animals compared to controls at this early stage of the disease (**Figure 9** and **Table 11)**.

Table 11 | Comparison between groups of the mean relative expression for *GATA4, Axin2* and *CyclinD1* genes. Results are normalized for *GAPDH* (left ventricle heart samples of Ctrl and DM groups).

	GATA4	*Axin2*	*CyclinD1*
DM/Ctrl	1.41	1.49	1.27

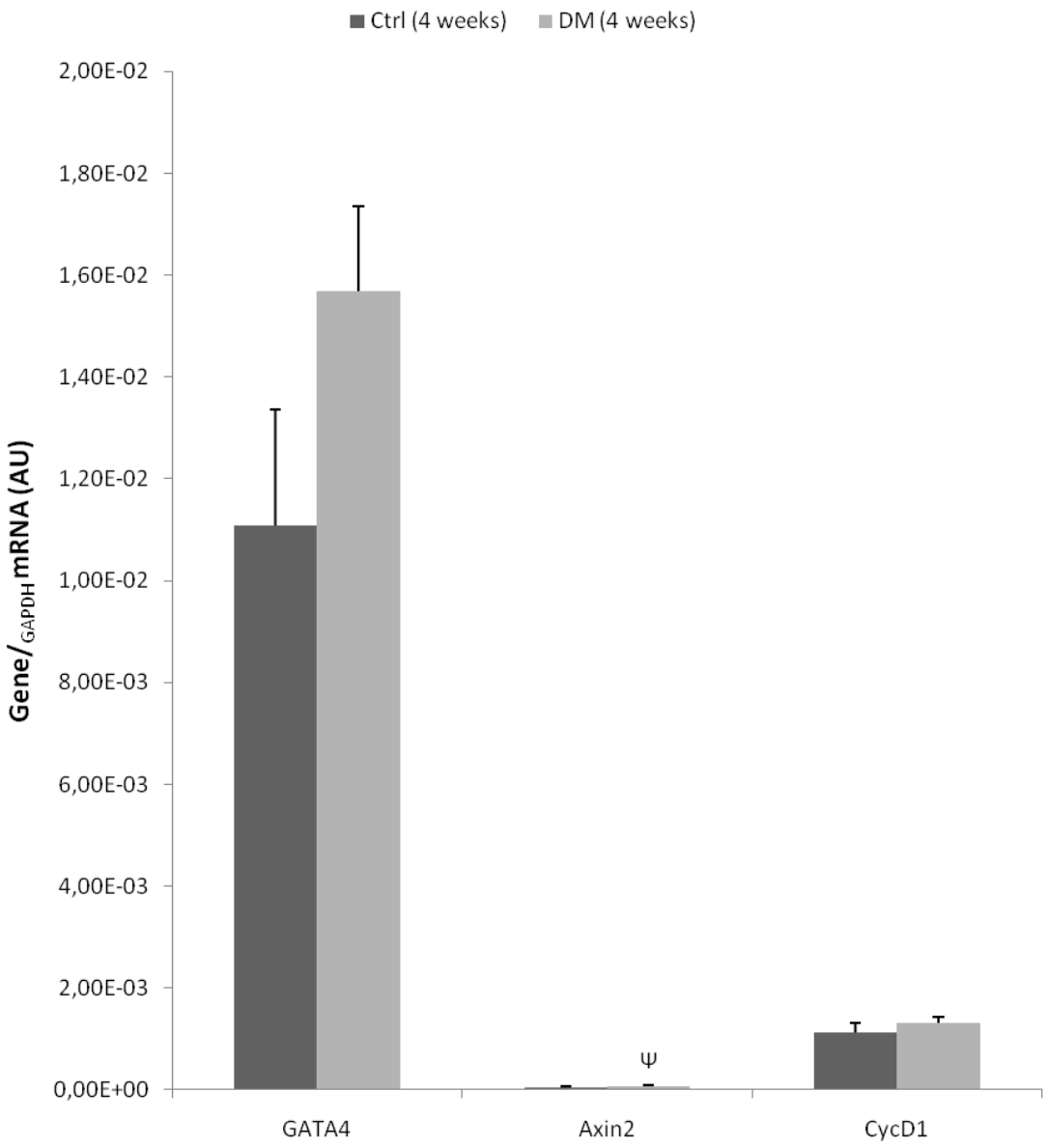

Figure 9 | Expression of *GATA4*, *Axin2* and *CyclinD1* genes, in left ventricle heart samples of four-week animals, collected after treatment. Results are normalized for *GAPDH* and expressed in arbitrary units (AU). (Data are mean ± SEM; Ψ: *Axin2* graph in supplements).

Overall, these results supported that in early stages of diabetic cardiomyopathy, there is upregulation of canonical and non-canonical Wnt ligands, as well as Fzd and LRP6 receptors. This led us to hypothesize a possible role for both branches in this pathology, which is rather interesting, since the canonical branch, through *Wnt2b*, is associated to anti-cardiogenic pathways in the developing embryo, while, non-canonical signaling by *Wnt11* has the opposite function. Thence is of major importance to unroll the specific function of each branch in cardiac disease.

INTRACELLULAR WNT SIGNALING REGULATORS: THE KINASES

Several intracellular factors present a very important role in the regulation of Wnt signaling. One major event in the transduction of extracellular events is the phosphorylation of LRP6 and to that purpose, several kinases namely GSK-3, GRK5 and GRK6, are responsible for this key regulatory step. For this reason, we decided to check the expression levels of these genes to determine how their expression varied in four-week DM animals, when compared to controls. Results are presented in **Figure 10** and **Table 12**.

As in human, rat GSK-3 exists in two isoforms, *GSK-3α* and *GSK-3β* and although both isoforms had been described to have expression in the heart [227], virtually all studies conducted so far, only examined the role of the GSK-3β isoform.

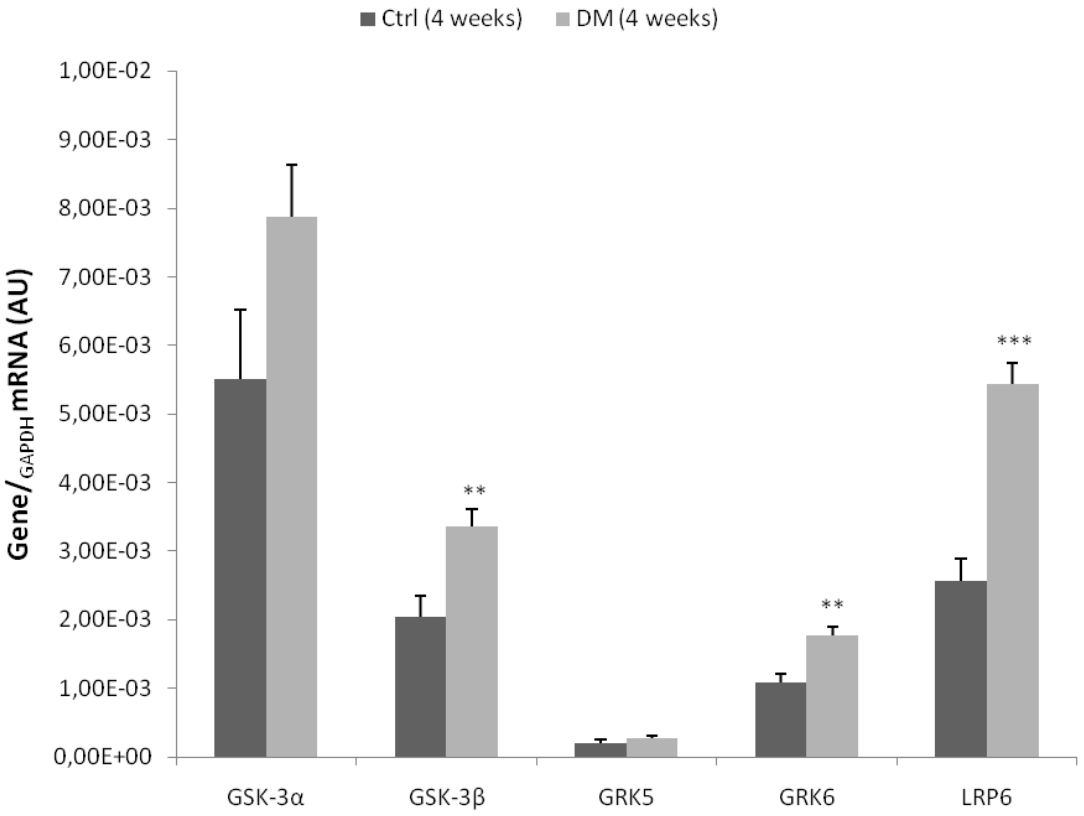

Figure 10 | Expression of relevant kinases and *LRP6* genes, in left ventricle heart samples of four-week animals, collected after treatment. Results are normalized for *GAPDH* and expressed in arbitrary units (AU). (**p < 0.01 versus Ctrl; ***p < 0.001 versus Ctrl; Data are mean ± SEM).

In our model, a marked upregulation on both GSK-3 isoforms was observed at four-weeks. Despite the fact that the baseline expression of *GSK-3α* is higher than *GSK-3β,* the

α isoform showed a lower increase in DM animals (1.43 fold increase), which is still of statistical significance, when compared to the β isoform (1.64 fold increase).

Similarly, analyzes of the G Protein-coupled receptor kinases *GRK-5* and *-6* expression levels revealed an increased expression of these two genes, being *GRK6* significantly upregulated (1.62 fold increase), when compared to *GRK5* (1.36 fold increase).

These results showed that the expression of several kinases, involved in LRP6 phosphorylation and activation, are upregulated in the heart of DM animals in the early stages of diabetic cardiomyopathy.

Table 12 | Comparison between groups of the mean relative expression for kinases and *LRP6* genes. Results are normalized for *GAPDH* (left ventricle heart samples of Ctrl and DM groups).

	GSK-3α	*GSK-3β*	*GRK5*	*GRK6*	*LRP6*
DM/Ctrl	1.43	1.64	1.36	1.62	2.13

ENERGY METABOLISM AND OXIDATIVE STRESS:

PPARS

The heart obtains most of its energy requirements to function properly from fatty acids and glucose oxidation. In T1DM, as cells are unable to use glucose as a source of energy, they turn exclusively to fatty acids oxidation. PPARs, the natural sensors of lipids, play a preponderant role in this process, as their activation induces the expression of genes that encode for proteins involved in fatty acids oxidation. At the same time, as PPARγ agonists had been found to have a cardioprotective role against ischemic insults, inhibit cardiac hypertrophy and aptoptosis we looked at the expression of the different PPAR isoforms in both groups at the early stages (four-weeks) of the disease. Results are presented in **Figure 11** and **Table 13**.

Table 13 | Comparison between groups of the mean relative expression for *PPAR* genes. Results are normalized for *GAPDH* (left ventricle heart samples of Ctrl and DM groups).

	PPARα	*PPARβ/δ*	*PPARγ*
DM/Ctrl	1.00	1.00	1.66

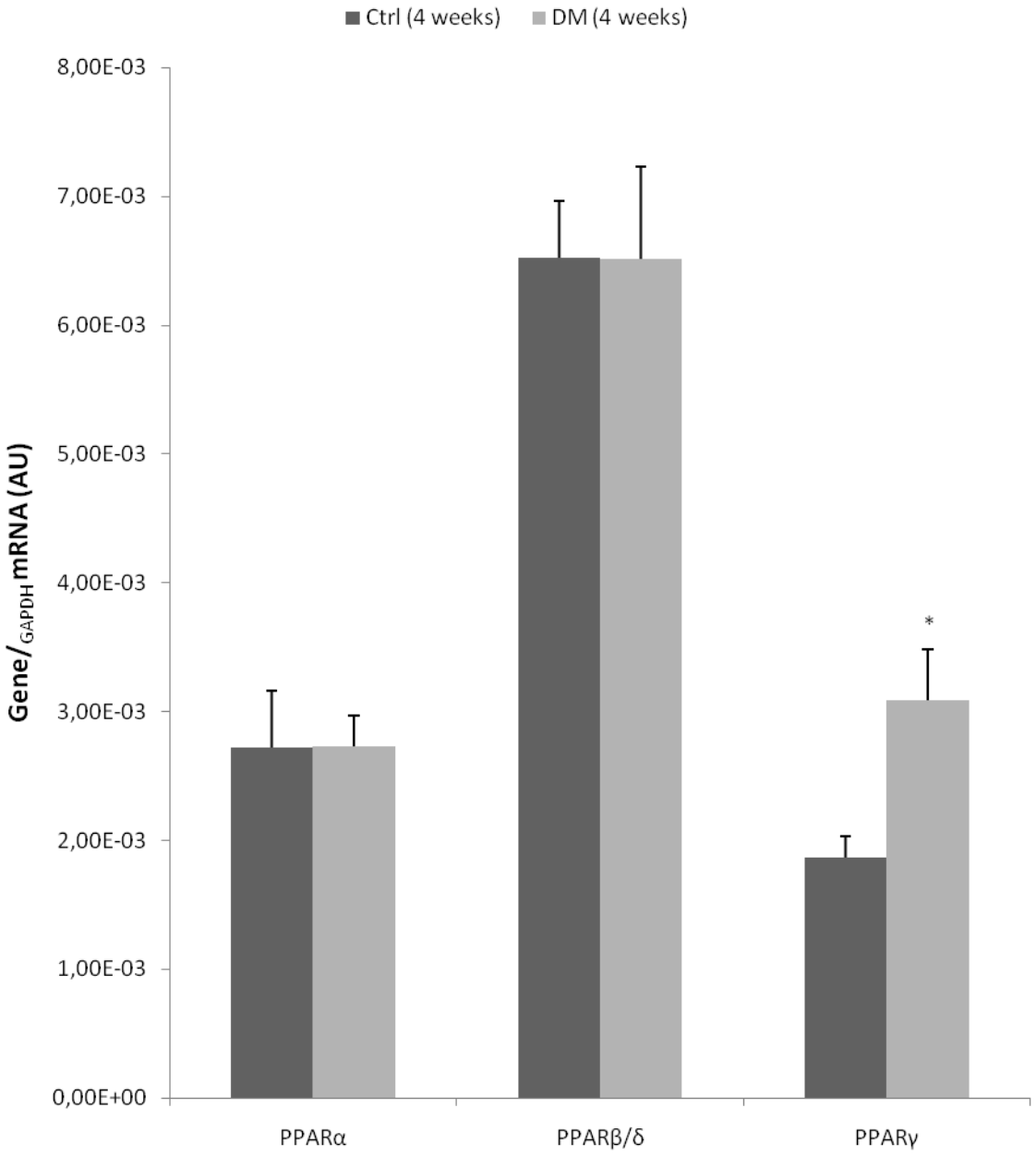

Figure 11 | Expression of *PPAR* genes, in left ventricle heart samples of four-week animals, collected after treatment. Results are normalized for *GAPDH* and expressed in arbitrary units (AU). (*p < 0.05 versus Ctrl; Data are mean ± SEM).

Of the three *PPAR* genes (α, β/δ and γ) expressed in the heart, *PPARβ/δ* has the highest expression levels in control animals (**Figure 11**). At four-weeks upon STZ treatment only the expression of *PPARγ* is upregulated (1.66 fold increase) in DM animals (**Figure 11** and **Table 13**).

FOXOs

Increased fatty acids oxidation, as well as hyperglycemia, observed in the diabetic state, results in increased ROS production, leading to enhance oxidative stress in the cells. To that purpose, we analyzed the levels of expression of FOXO transcription factors, as they regulate, amongst other processes, oxidative stress resistance, through governing the transcription of antioxidant enzymes. Results are presented in **Figure 12** and **Table 14**.

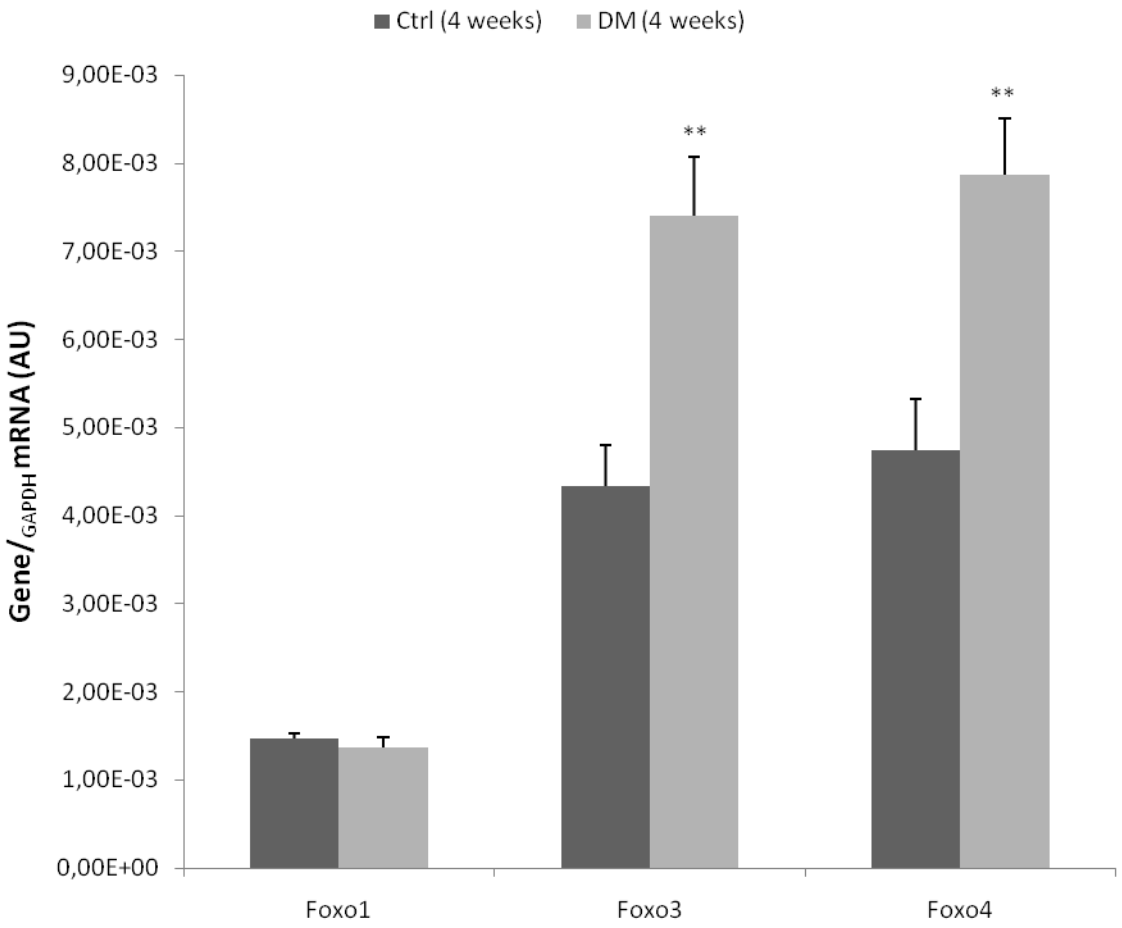

Figure 12 | Expression of *Foxo* genes, in left ventricle heart samples of four-week animals, collected after treatment. Results are normalized for *GAPDH* and expressed in arbitrary units (AU). (**p < 0.01 versus Ctrl; Data are mean ± SEM).

Expression analysis at four-weeks of treatment showed a significant increased expression of *Foxo3* and *-4* (1.71 and 1.66 fold increase, respectively) but no changes were observed in *Foxo1* levels.

We conclude from these results that in early stages of diabetic cardiomyopathy there is already a significant increase in the expression of genes involved in fatty acid

oxidation, which is accompanied by an increase in the expression of genes involved in regulation of oxidative stress.

Table 14 | Comparison between groups of the mean relative expression for *Foxo* genes. Results are normalized for *GAPDH* (left ventricle heart samples of Ctrl and DM groups).

	Foxo1	*Foxo3*	*Foxo4*
DM/Ctrl	0.932	1.71	1.66

GENE REGULATION IN ADAPTIVE HYPERTROPHY, IN DIABETIC CARDIOMYOPATHY

Since the aim of this project was to study regulation of gene expression, with special emphasis on Wnt signaling effectors, we went on to analyze the expression of some of the genes, which showed altered expression at four-weeks upon diabetes induction.

By six-weeks upon diabetes induction the ecocardiographic and hemodynamic results show that there is already some hypertrophy of the LV, without many alterations in heart function. This indicates that, by this stage, the heart already started to "respond" to the stress signals and an adaptative response ensues.

As in the previous analyzes we started at analyzing the expression of the *bona fide* heart failure markers: *ET-1* and *BNP* and since *GATA4* and *GSK-3β,* analyzed in the mean time, point out as regulators of the Wnt pathway that are also considered by others as "hypertrophy markers", we also analyzed the expression of these two genes, together with *ET-1* and *BNP,* at four- and six-weeks of a diabetic state (**Figure 13** and **Table 15**).

Table 15 | Comparison between groups of the mean relative expression for *GSK-3β, ET-1, GATA4* and *BNP* genes at four- and six-weeks of treatment. Results are normalized for *GAPDH* (left ventricle heart samples of Ctrl and DM groups).

	GSK-3β	*ET-1*	*GATA4*	*BNP*
DM/Ctrl (4-weeks)	1.64	2.56	1.41	1.46
DM/Ctrl (6-weeks)	1.50	1.68	1.49	1.63

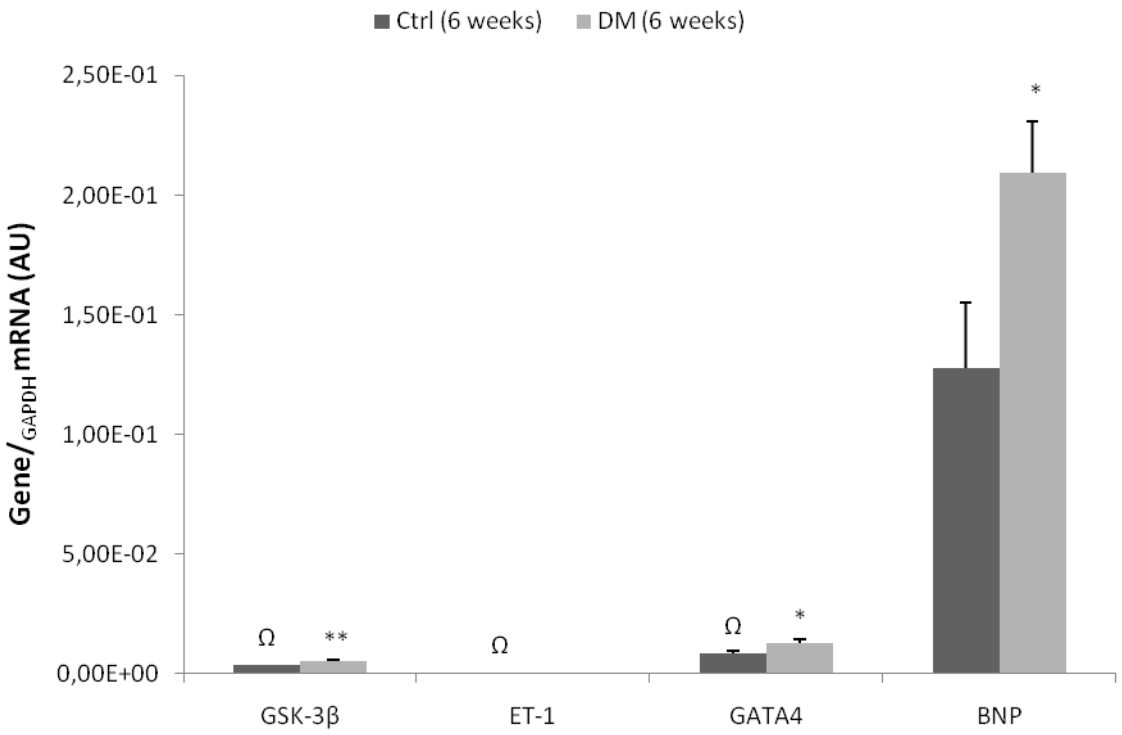

Figure 13 | Expression of *GSK-3β*, *ET-1, GATA4* and *BNP* genes, in left ventricle heart samples of six-week animals, collected after treatment. Results are normalized for *GAPDH* and expressed in arbitrary units (AU). (*p < 0.05 versus Ctrl; **p < 0.01 versus Ctrl; Data are mean ± SEM; Ω: *GSK-3β*, *ET-1* and *GATA4* graphs in supplements).

As expected, by this stage we also observed an increased expression of all the markers analyzed in the diabetic group. However, when compared to the earliest time point analyzed, *GSK-3β* and *ET-1* showed a reduction of their expression levels in the diabetic group, having *ET-1* a marked downregulation. On the other hand, both *BNP* and *GATA4* expressions showed a slightly increase.

We conclude that, as expected, hypertrophy markers are upregulated in diabetic animals during an adaptative response, but slight differences seem already to be observed in the expression of these markers in relation to an earliest time point of the disease.

WNT SIGNALING IN ADAPTIVE HYPERTROPHY

From the many Wnt effectors analyzed at four-weeks diabetic state, only a few showed a marked upregulation in diabetic animals, namely, *Wnt2b, Wnt11, Fzd3 and Fzd5* and the co-receptor *LRP6.*

We thus decided to look at the expression of these genes at six-weeks upon STZ-treatment.

By six-weeks upon diabetes induction one can still observe increased expression of *LRP6*, but, surprisingly, the fold increase in the expression levels in DM animals compared to control animals is much lower (1.49 fold increase) than the ones observed at four-weeks (2.13 fold increase). The other genes, at this time point, are all downregulated and off statistical significance between groups (**Figure 14** and **Table 16**).

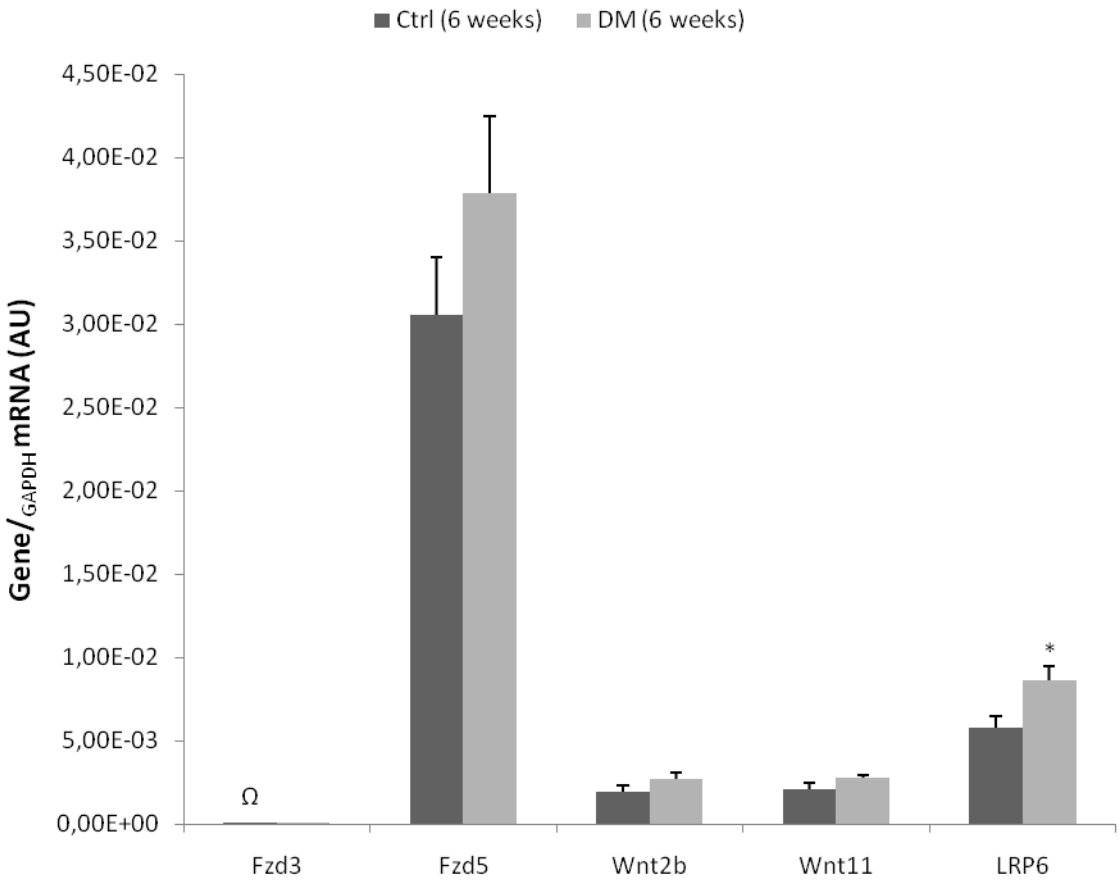

Figure 14 | Expression of *Fzd3, Fzd5*, *Wnt2b*, *Wnt11* and *LRP6* genes, in left ventricle heart samples of six-week animals, collected after treatment. Results are normalized for *GAPDH* and expressed in arbitrary units (AU). (*p < 0.05 versus Ctrl; Data are mean ± SEM; Ω: *Fzd3* graph in supplements).

Table 16 | Comparison between groups of the mean relative expression for *Fzd3, Fzd5*, *Wnt2b, Wnt11* and *LRP6* genes at four- and six-weeks of treatment. Results are normalized for *GAPDH* (left ventricle heart samples of Ctrl and DM groups).

	Fzd3	*Fzd5*	*Wnt2b*	*Wnt11*	*LRP6*
DM/Ctrl (4-weeks)	1.89	1.42	1.89	1.43	2.13
DM/Ctrl (6-weeks)	1.45	1.24	1.40	1.30	1.49

METABOLISM AND OXIDATIVE STRESS IN ADAPTIVE HYPERTROPHY

By six-weeks upon STZ-treatment and as expected, one still observes a high upregulation in *PPAR* and *Foxos* (**Figure 15** and **Table 17**).

Interestingly, the expression of *PPARγ* and *Foxo3* increase in comparison to the levels observed in diabetic animals at four-weeks of treatment (**Table 17**), with *PPARγ* showing a very marked increase.

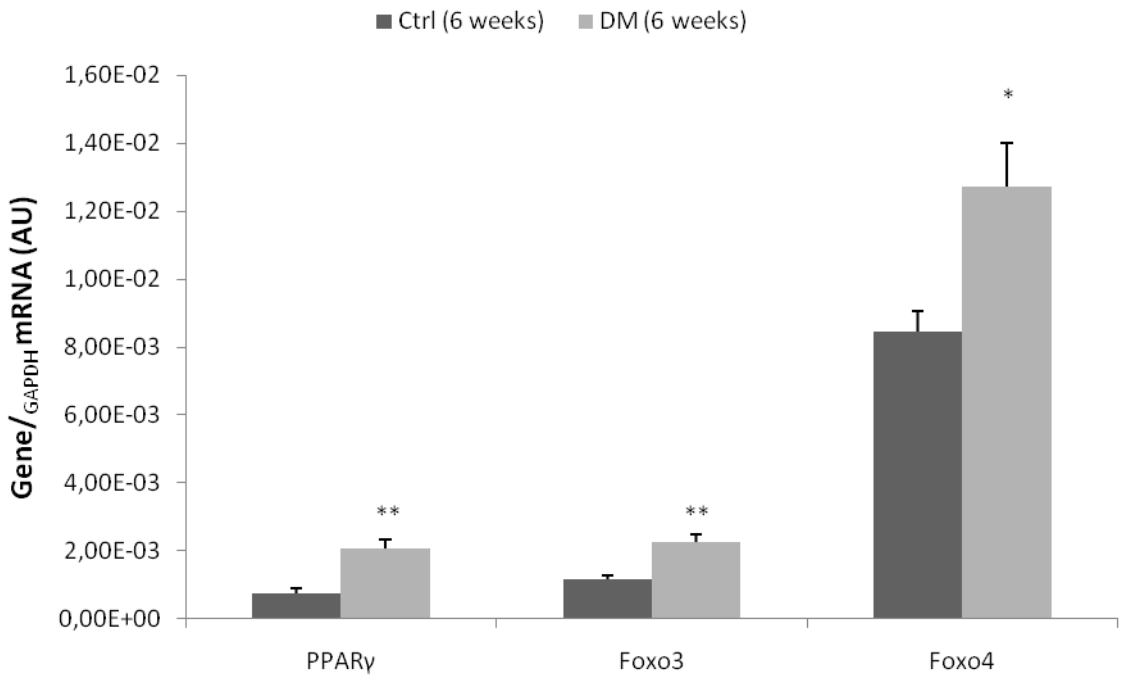

Figure 15| Expression of *PPARγ, Foxo3* and *Foxo4* genes, in left ventricle heart samples of six-week animals, collected after treatment. Results are normalized for *GAPDH* and expressed in arbitrary units (AU). (*p < 0.05 versus Ctrl; **p < 0.01 versus Ctrl; Data are mean ± SEM).

Table 17| Comparison between groups of the mean relative expression for *PPARγ, Foxo3* and *Foxo4* genes at four- and six-weeks of treatment. Results are normalized for *GAPDH* (left ventricle heart samples of Ctrl and DM groups)

	PPARγ	*Foxo3*	*Foxo4*
DM/Ctrl (four-weeks)	1.66	1.71	1.66
DM/Ctrl (six-weeks)	2.75	1.94	1.50

Our results demonstrate that in an adaptive stage of diabetic cardiomyopathy genes related to metabolic and oxidative stress are markedly increased, a result somehow expected due the metabolic needs and stress that diabetic hearts support. The importance of this result is discussed below.

DISCUSSION

14 | DISCUSSION

14.1 Effects of diabetes mellitus: myocardial structure

We adopted the STZ-induced rat model of diabetes due to its simplicity, high reproducibility and mainly because alterations in cardiac functions are primarily due to diabetic cardiomyopathy [228]. These features turn it out as one of the most widely used hyperglycemic models of type 1 diabetes mellitus.

Diabetic cardiomyopathy develops as a result of an hyperglycemic state and, as observed in other models of heart failure, there is an initial adaptive stage of the heart to stress conditions, but as stress conditions endure the heart loses the capacity to adapt to the stress factors and heart failure (with systolic and diastolic dysfuntion) ensues. As previously mentioned, several pathways are associated with the different stages of disease progression, but the role of Wnt signaling in heart disease is still not very clear. In our study we started by analyzing heart function and gene expression in the early stages (adaptive stage) of diabetic cardiomyopathy. By the end of six-weeks upon diabetes induction the heart morphology and function already showed some hypertrophy of the LV wall and some dilation of the LV chamber, but these morphological alterations did not correlate with altered heart function. We assumed then that by four-weeks these changes should be still very conspicuous and, for that reason, in the time window between the four- and six-weeks upon diabetes induction an adaptive stage in diabetic cardiomyopathy can be considered. Moreover, by six-weeks the morphometric alterations point out to development of cachexia, a result already predicted, since insulin-mediated stimulation of PPARγ is a key regulatory factor of adipocyte differentiation [200].

14.2 Gene expression

REGULATION AT THE SURFACE: LIGANDS VERSUS RECEPTORS

The existence of several Wnt signaling pathways is nowadays well accepted. Because 19 Wnt ligand members, 10 Fzd receptors and 2 LRP receptors were found in the mammalian genome, a great degree of complexity has arisen, turning the understanding of Wnt signaling cascades a very difficult task.

Since additional levels of complexity to Wnt signaling are linked to the divergence of functional activities among Wnt proteins, a broad classification of Wnts into two separate groups had already been made established. A first group termed 'Wnt1 group' containing Wnt1, Wnt2, Wnt2b/13, Wnt3 and Wnt3a appears to be capable of exclusively activating canonical Wnt signaling [1, 229]. A second group termed 'Wnt5a group' containing Wnt4, Wnt5a and Wnt11 possesses more elaborate signaling properties and has been associated to non-canonical Wnt signaling [230-231]. Simultaneously, it has been reported that the 'Wnt5a group' may suppress β-catenin-mediated signaling and thus, serve as dominant-negative forms of 'Wnt1 group' [232].

Our results showed that in the early stages of diabetic cardiomyopathy there is an upregulation of *Wnt2b* and *Wnt11*. This is a very interesting result in the sense that not only we observe upregulation of Wnt ligands, which work on distinct Wnt pathway branches, but also because Wnt11 has been shown to be important during embryonic cardiogenesis [233] having an important role in the differentiation of cardiac cells. In addition, it was recently shown by Afouda *et al.* [234] and Flaherty *et al.* [235] that Wnt11 is required for mediating the cardiogenesis-induced function of GATA4 and other members. Although not presenting significant differences at an early stage of diabetes, there is already upregulation of GATA4, which is more marked during myocardial adaptive hypertrophic stage.

In parallel to an upregulation of some Wnt ligands, we also observed upregulation of some Fzd receptors and the LRP6 co-receptor. As not all Fzd and LRPs normally expressed in the heart are upregulated in DM hearts, we assumed that the ones upregulated have a specific function in diabetic cardiomyopathy. Regarding the Fzd receptors, one cannot conclude that they are specifically working together with Wnt2b or Wnt11, since they activate both Wnt pathways (canonical and non-canonical), in a

complex manner. An increase of *Wnt2b*, a ligand more associated to canonical Wnt signaling and the canonical specific receptor, *LRP6*, strongly supports an involvement of the canonical Wnt pathway, together with a possible activation of the non-canonical JNK-pathway by *Wnt11*, in the early stages of this disease.

CANONICAL WNT SIGNALING IN HEART DISEASE

The importance of canonical Wnt signaling activation in diabetic cardiomyopathy can possibly be divided in two processes: CPCs population maintenance and proliferation, and β-catenin stabilization for oxidative stress resistance responses through FOXO proteins.

As previously mentioned, it is nowadays accepted by some that the heart possesses a certain degree of regeneration, which contrasts to the old view of a terminal differentiated organ. This is mostly due to a special population of cells, the CSCs and CPCs present in the heart [148-149].

Canonical Wnt signaling has been shown, in other contexts, to be important for the maintenance and proliferation of a stem cell like profile [236]. Our expression analyzes show that several Wnt ligands, receptors and co-receptors are expressed in the normal heart, suggesting a possible role in the maintenance of the CSC/CPC population. Interestingly, in the diabetic heart only one canonical Wnt ligand and one canonical Wnt signaling co-receptor are upregulated. This specific upregulation could aid, in the early stages of disease, to enhance the proliferative capacity of CSC/CPC population, so the pool of these cells increase, being possibly directed to differentiation by Wnt11.

In addition, an upregulation of canonical Wnt signaling could represent a mechanism, not yet demonstrated in heart disease, whereby β-catenin is stabilized and further recruited by FOXOs to respond to increased ROS levels. Although β-catenin stabilization can be achieved by indirect mechanisms namely GSK-3 inhibition, some consider that stabilization of β-catenin by the Wnt pathway is absolutely required for FOXOs activity [148]. This shifting to another pathway could also aid to explain, why we observe such a modest increased expression of two important downstream targets genes of canonical Wnt signaling, *Axin2* and *CyclinD1*. It is interesting to note that as disease progresses the expression of all Wnt effectors initiate to decrease, although still being higher in diabetics when compared to controls. The highest reduction observed between

the four- and six-weeks animals is for the Wnt co-receptor LRP6. This result might suggest that as disease progresses, canonical Wnt signaling starts to be downregulated, although not totally. The maintenance of a certain level of activity is most likely still required for β-catenin-FOXO interactions, because as we demonstrate, and as expected, high expression of *Foxo* genes is maintained at six-weeks, supporting the role of these proteins in diabetic cardiomyopathy.

NON-CANONICAL WNT SIGNALING IN HEART DISEASE

As already mentioned, one of the Wnt ligands specifically upregulated in the heart of DM animals is *Wnt11*. This finding is rather interesting, taking into account the role of this ligand in cardiogenesis during embryonic development due to its ability to induce a cardiac phenotype in progenitor cells [112]. Several authors have already demonstrated during embryonic cardiogenesis that cardiac specification depends mostly on the inhibition of canonical Wnt signaling [106, 112, 237-238]. This can be achieved by specific canonical Wnt inhibitors and/or by activation of a non-canonical Wnt pathway by Wnt11, and/or by other pathways (e.g. bone morphogenetic proteins, Smads, etc). To further support a role for Wnt11 in the induction of a cardiac phenotype, other authors have also demonstrated that Wnt11 is able to induce *in vitro* differentiation of endothelial circulating progenitor cells [239], as well as in other adult progenitors [235]. Despite all these observations, so far a role for this ligand in heart disease has not been demonstrated. We show for the first time that during diabetic cardiomyopathy progression, there is an upregulation of *Wnt11*, which could indicate that in the early stages of heart disease, when an adaptive hypertrophy response develops, Wnt11 signaling might possibly exert a role in directing the CPCs differentiation towards cardiac cells, increasing the global amount of cardiac tissue.

As a matter of fact, Nagy *et al.* [233] has recently demonstrated that Wnt11 seems to be required for the correct co-localization of the cell adhesion molecules N-cadherin and β-catenin. Wnt11 knockouts showed an irregular organization of the developing ventricular wall, and this might be due to the improper localization of these two adhesion effectors. Since during hypertrophy development, alterations in cardiomyocytes morphology and orientation can be observed, one can also hypothesize that an increase in Wnt11 will aid to the proper morphological changes observed.

INTRACELLULAR REGULATION: THE KINASES

A key step in canonical Wnt signaling activation is highlighted by LRPs phosphorylation/activation. GSK-3 accounts for most of LRPs phosphorylation revealing a positive role for GSK-3 in canonical Wnt signaling [42, 44]. In addition, we also found that GRKs, proteins that traditionally phosphorylate and desensitize GPCRs, are also kinases responsible for the phosphorylation/activation of LRPs [47].

Our reports are rather interesting, since we confirm upregulation of *GSK-3β* in diabetics, which could support its role as a regulator of LRP6 phosphorylation and subsequent canonical Wnt signaling activation. In accordance, as we observe both a decline after six-weeks of treatment, for *GSK-3β* and *LRP6*, we might correlate their decreased levels, supporting their cooperation.

Regarding *GRK6*, it is impossible to correlate a direct involvement with LRP6 due to its various roles in cells namely desensitization of GPCRs and thence, other signaling pathways activation.

REGULATION OF HYPERTROPHY

It has been demonstrated by Masuelli *et al.* [123] a decrease of *GSK-3β* expression with subsequent accumulation of β-catenin, in hypertrophic cardiomyopathic hearts. However, our results and others [121-122, 240-241] demonstrate the opposite observation, but it is important to emphasize that Masuelli *et al.* [123] samples were extracted from hamster and human models at different time points of disease progression.

Additionally and of interest, *GSK-3β* overexpression has been demonstrated to function as a negative regulator of cardiac hypertrophy by inhibiting the expression of hypertrophic genes. Haq *et al.* [122] observed that GSK-3β inhibits ET-1-induced hypertrophy in neonatal rat cardiomyoytes. In an opposite manner, ET-1, a cardiac hypertrophic neurohormone, is reported to inhibit GSK-3β activity through activation of a Wnt-independent mechanism involving the Akt pathway. In addition, both ET-1 and GSK-3β had been reported to stimulate the expression of BNP, an indirect inhibitor of cell hypertrophy, through regulation of GATA4. At the same time, Morisco *et al.* [240] demonstrated that GSK-3β is also able to negatively regulate BNP [242]. These contrasting reports apparently highlight the existence of a possible feedback mechanism in the

regulation of hypertrophy and thence, at least, we checked the expression levels of these genes, during disease progression (from an early stage to six-weeks of induced-diabetes).

Our results show a significant upregulation of *ET-1* and *GSK-3β*, at four-weeks of treatment, suggesting that in an early stage of diabetic cardiomyopathy, both of these genes might be synergistically acting to induce an adaptive heart model. Yet, after some time, we observe a sharp decrease in *ET-1* expression with subsequent upregulation of *GATA4* and *BNP*, suggesting that at six-weeks of diabetes, the cardioprotective abilities of the heart presumably try to counteract hypertrophy. Nonetheless, the slightly decline in *GSK-3β* expression might suggest a possible decrease of its levels and function overtime, correlating to the Masuelli *et al.* [123] findings. In fact, as both ET-1 protein and its overexpressed mRNA levels are found in long terms of STZ-induced diabetes [243-244], we are thus expected to observe upregulation of *ET-1*, once more, overtime, as we start to observe a decline of *GSK-3β* expression. Our results are this way of great interest, as we demonstrate a possible time window, where *ET-1* downregulation can be observed and possible used for pharmacological intervention of hypertrophy regulation.

ENERGY BALANCE: METABOLIC ROLES OF PPARS

Normal insulin-producing organisms are characterized through production of insulin, a hormone that is central to the regulation of energy and glucose metabolism in the body. In response to high blood sugar levels, β-cells, a specific type of cells in the pancreas, stimulate the production of insulin with its subsequent release to the blood. Then, circulating insulin is sensed by the liver, muscle and fat tissue cells, which in turn, through activation of the PI3K/Akt pathway results in the translocation of glucose transporter proteins, such as GLUT4, to the plasma membrane, leading to the transport of glucose from the blood to the intracellular milieu. In an opposite manner, non-insulin producing organisms, such as type 1 diabetic patients, are unable to use glucose as a source of energy. Hyperglycemia is thus defined as the result of an excessive amount of circulating glucose, due to an inability of glucose transporters translocation to the plasma membrane.

This way, as type 1 diabetic hearts are unable to use glucose as a source of energy they must solely depend on fatty acids oxidation that is under the regulation of PPARs. Our data is of great significance, because as we detected upregulation of *PPARγ* during

progression of the disease state, we suggest that PPARγ activity could be directly regulating heart's energetic balance, through increased fatty acids oxidation.

PROTECTIVE SYSTEM: THE ROLE OF FOXOS

Increased fatty acids oxidation, as well as hyperglycemia, leads to increased ROS production, resulting in oxygen toxicity. A family of transcription factors called FOXO has emerged in the last years, as key players in oxidative stress resistance, by regulating several intracellular cascades involved in the trans-activation of antioxidant enzymes, amongst other functions. It has been demonstrated that FOXOs-mediation of downstream target genes transcription, is exerted by FOXOs-β-catenin interactions, which directly compete with TCF transcription factors for a free β-catenin limited pool [147-148, 175]. In addition, Schmidt *et al.* [245] demonstrated that FOXO3 inhibits cell cycle progression through inhibition of CyclinD1, which might correlate with the low levels of *CyclinD1* by us detected, since we observe upregulation of *Foxo3*. In addition, we also demonstrate that along disease progression, *Foxo3* has a more pronounced expression, which might correlate to a more central role, when compared to *Foxo4*.

This result is of major importance, since it might support a negative regulation exerted by *Foxos* in *CyclinD1* expression, by directly shifting the β-catenin pool towards an antioxidant response, rather downstream activation of canonical Wnt target genes. Moreover, Ni *et al.* [246] recently demonstrated that FOXO1 and FOXO3 inhibit cardiac hypertrophy, yet, through a non-canonical Wnt pathway. Since our previous studies support progression of hypertrophy, we cannot exclude that our *Foxo* levels are a direct response to regulation of cardiac hypertrophy and in simultaneous, could be counteracting ROS production through antioxidant genes induction. In addition, it is even plausible to consider FOXOs-induced apoptosis after some time.

Our data is of great significance, since important canonical Wnt signaling genes are upregulated, as seen by increased *Wnt2b*, *LRP6* and *GSK-3β* expression. Because diabetes promotes increased levels of ROS, we can assume that canonical Wnt signaling activation, through β-catenin stabilization could have a determinant role in antioxidant gene expression, when associated to FOXOs, shifting β-catenin from the co-activators TCF/LEF, resulting in downstream downregulation of *CyclinD1*.

Although our results support a main role for GSK-3β in LRP6 activation, we still cannot exclude a simultaneous role in the regulation of hypertrophy, where GSK-3β could easily detain various pools within cells.

PPAR-FOXO INTERACTIONS: A POSSIBLE MECHANISM ON GLUT4 TRANSCRIPTION REGULATION

A convergence between PPARs and FOXOs has also been made, where FOXOs were demonstrated to negatively regulate PPARs. Dowell *et al.* [150] discovered that FOXO1 disrupts the DNA-binding activity of PPARγ/RXRα heterodimers, thence suppressing PPARγ activity. It is however interesting to observe that no correlation between *PPARγ* and *Foxo1* can be made, since *Foxo1* levels decrease, even if some, in the diabetic model. Nevertheless, because we are using diabetic animal models and only FOXO1 has been studied to suppress PPARγ activity in insulin-responsive genes, it is plausible to assume that other FOXO members could suppress it as well.

As already stated, PPARγ agonists have a preponderant role in the regulation of glucose homeostasis. Glucose is uptaken by cells through GLUT4, which in turn is negatively regulated by PPARγ activity. In an opposite manner, it has been demonstrated that FOXO1 upregulates *GLUT4* gene expression by directly binding to the *GLUT4* promoter and indirectly, via repressing PPARγ activity. Interestingly, FOXOs are negatively regulated by Akt upon survival signals, such as insulin. In response to insulin, Akt is activated, phosphorylates FOXO1, promoting its nuclear exclusion, which in turn cannot suppress PPARγ activity, leading to *GLUT4* transcription repression. These reports are of major interest, because as we are using type 1 diabetic models that are unable to stimulate insulin, Akt activity is suppressed. This way, we should observe FOXOs' nuclear import/activation and further FOXOs-PPARγ-mediated activation of *GLUT4* transcription. Yet, type 1 diabetic patients have attenuated or repressed *GLUT4* transcription and it is still not clear how this precise negative regulation occurs.

From our results we could suggest that, as we observe upregulation of canonical Wnt signaling genes, β-catenin could be directly engaging to FOXO members, in order to activate antioxidant genes transcription, and thus releasing PPARγ-mediated repression. This way, activation of canonical Wnt signaling could aid to answer such incongruence's and confusions, where β-catenin might indirectly route FOXOs' away from PPARγ, thence

inhibiting FOXOs-suppression activities, resulting in an attenuated or repressed *GLUT4* transcription.

CONCLUSIONS

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In the presence of a stress situation the heart triggers cardioprotective responses, which enable the heart to pump enough blood to meet the metabolic demands of the organism. This cardioprotective balance is the result of several adaptive hypertrophic responses-activation that through extracellular matrix alterations lead to thickening and increased myocardium mass, with subsequent cell hypertrophy. In addition, these stress situations can also cause disequilibrium in the metabolic needs of the cardiac tissue, activating specific pathways to again achieve its internal balance. Our data sheds a new light over the role of Wnt signaling in the heart, through identification and association of various distinct signaling pathways and in simultaneous, the characterization of some of its effectors. We demonstrated that, at least, two Wnts are involved in the early stages of diabetic cardiomyopathy. Since both Wnts are also important during heart specification in embryonic development, one can conclude that during the early stages of heart disease, embryonic signaling pathways are activated, presumably having a similar role (plus others) to those observed during embryogenesis. Activation of Wnt pathways might have an important role not only in the adaptation of the cardiac tissue to stress conditions (namely in the hypertrofic response), but also in the establishment of a metabolic equilibrium through an interaction with pathways known to have a preponderant role in these processes.

Our observations may also support the notion that in the adult organism exists groups of multipotent cells capable of undergoing differentiation into a certain cell type, in the presence of the correct stimulus. It is of crucial importance the understanding of such stimulus and how their action can be modulated. Since Wnt signaling is regarded as an important target for pharmaceutical intervention in other diseases, such as cancer, Alzheimer and osteoporosis, it is important to understand the risks and/or benefits that such interventions represent to cardiac function. Taking our results, one example that can be given of such risk, is a compound that could be developed to downregulate canonical Wnt signaling in cancer (since, in most cases, this pathway is highly activated). One can hypothesize that dowregulation of canonical Wnt signaling at the receptors level would impair the ability of cardiac cells to fight against oxidative stress, because no stabilization

of β-catenin would be observed. This would cause a premature aging of the cardiac tissue, with possible rapid progression to heart failure.

Near future experiments will try to uncover which cell population is the preferential target for each of the Wnt pathways, as well as try to determine the degree of activation of each branch by assessing the phosphorylated levels of important effectors.

SUPPLEMENTS

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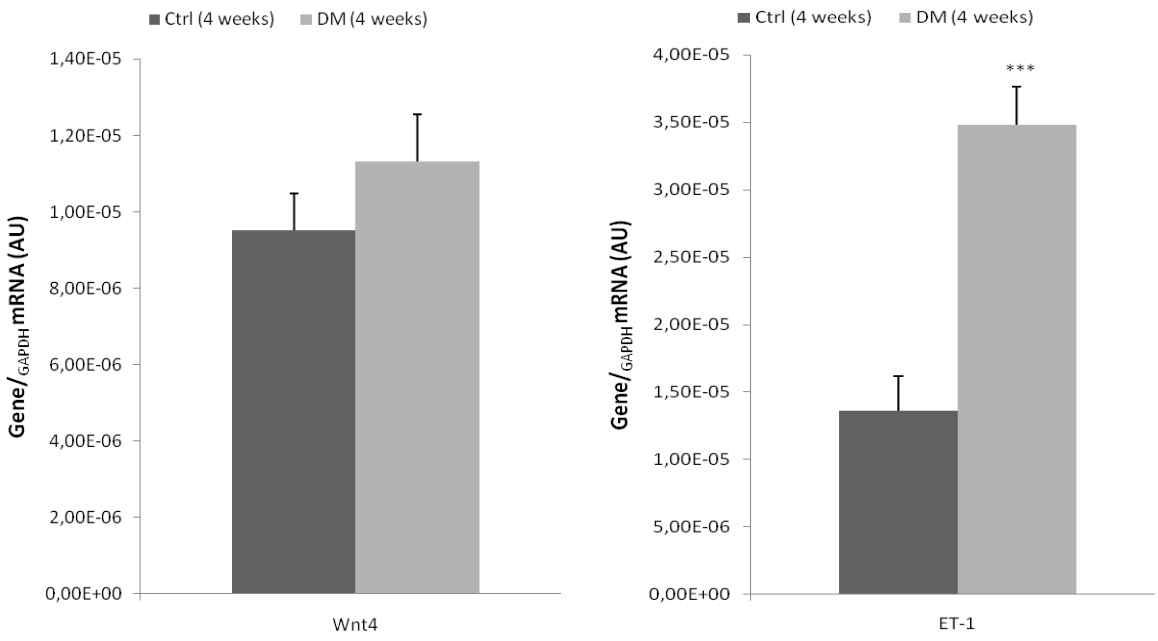
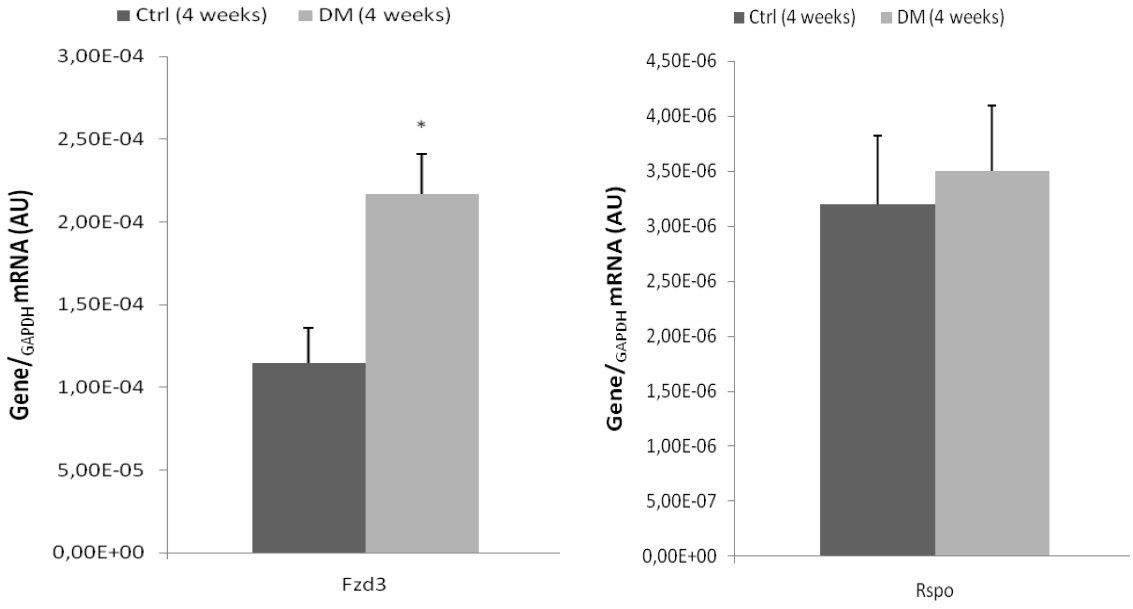
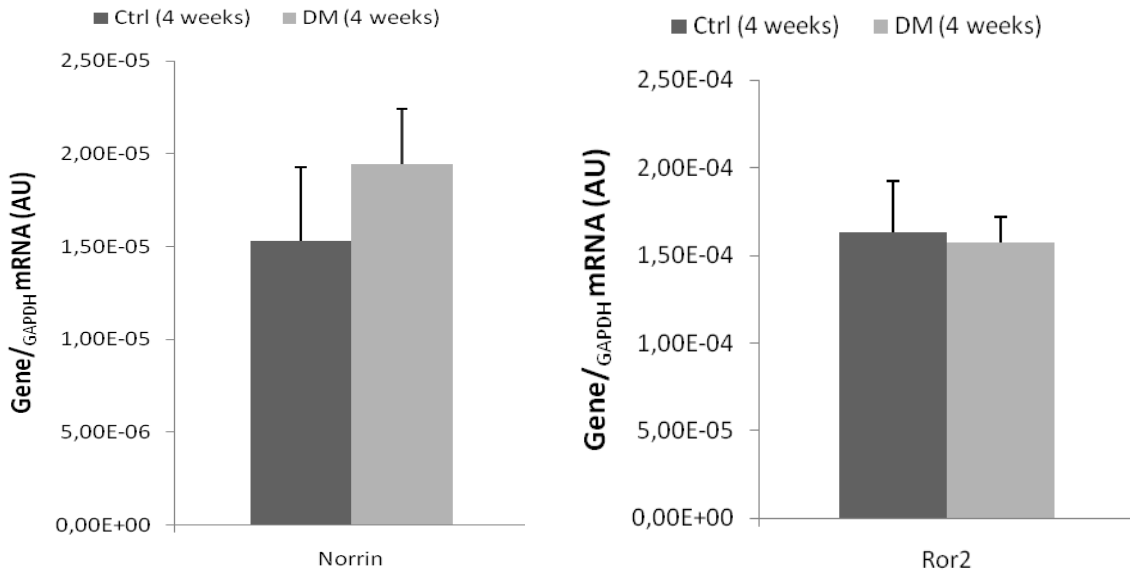


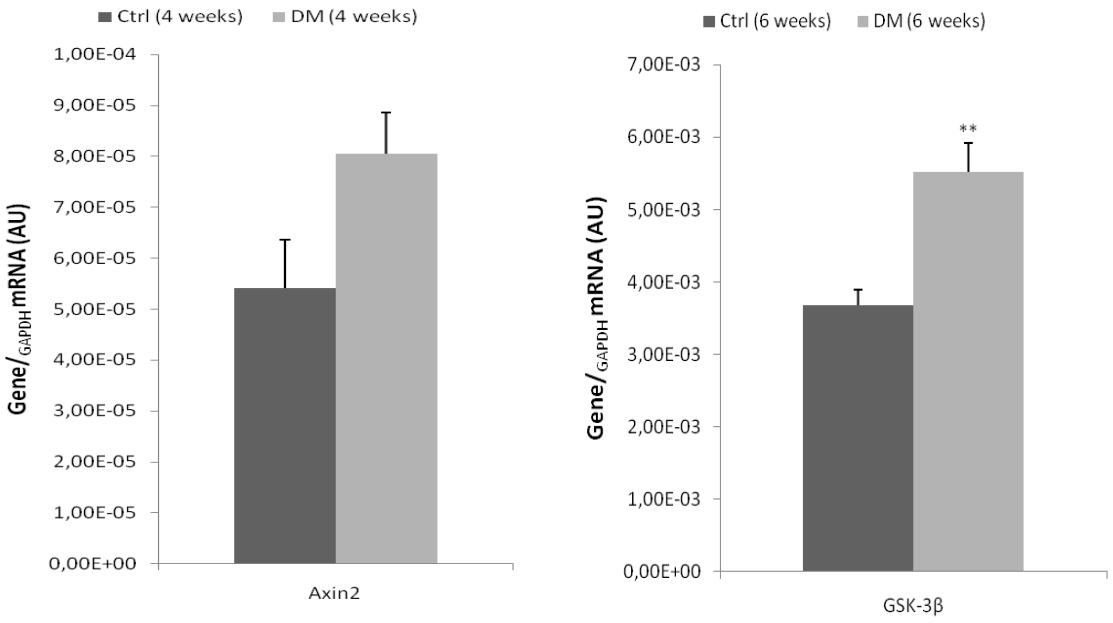
Expression of *Wnt4* and *ET-1* genes, respectively, in left ventricle heart samples of four-week animals, collected after treatment. Results are normalized for *GAPDH* and expressed in arbitrary units (AU). (***p < 0.001 versus Ctrl; Data are mean ± SEM)



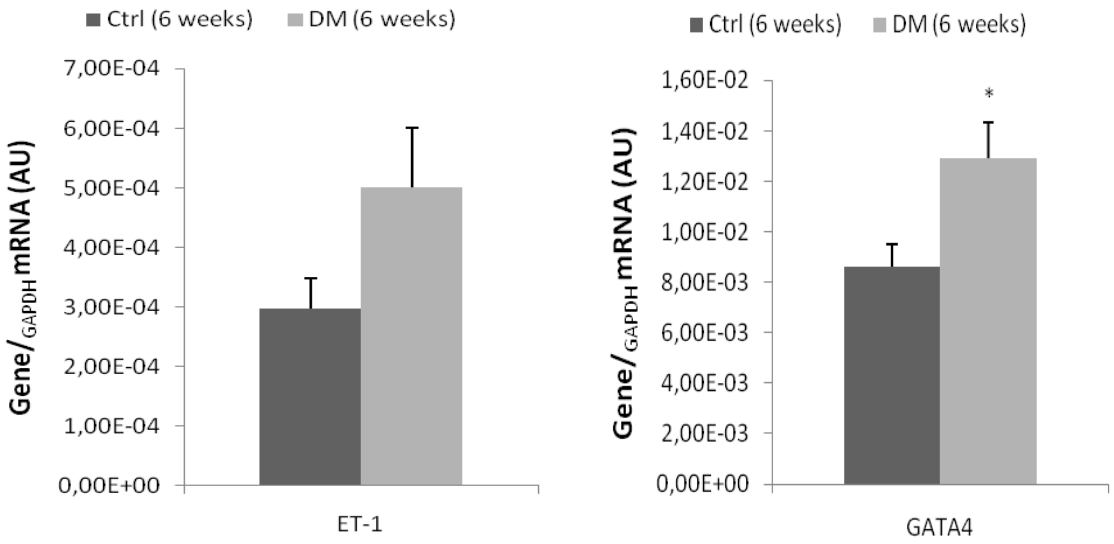
Expression of *Fzd3* and *Rspo* genes, respectively, in left ventricle heart samples of four-week animals, collected after treatment. Results are normalized for *GAPDH* and expressed in arbitrary units (AU). (*p < 0.05 versus Ctrl; Data are mean ± SEM)



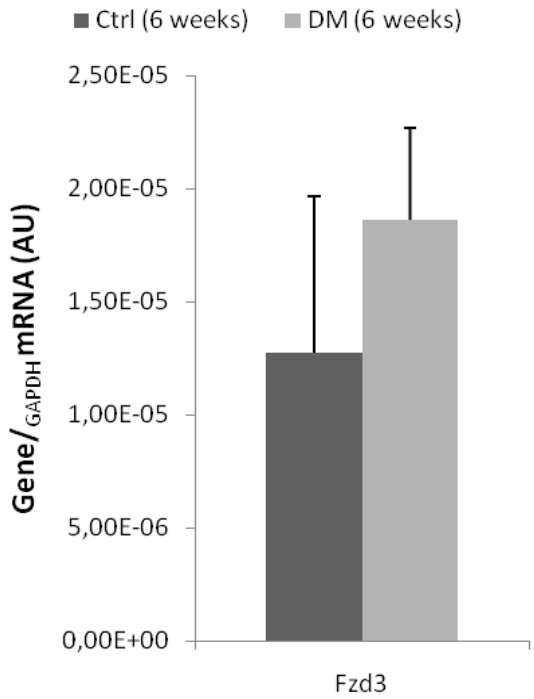
Expression of *Norrin* and *Ror2* genes, respectively, in left ventricle heart samples of four-week animals, collected after treatment. Results are normalized for *GAPDH* and expressed in arbitrary units (AU). (Data are mean ± SEM)



Expression of *Axin2* and *GSK-3β* genes, in left ventricle heart samples of four- and six-week animals, respectively, collected after treatment. Results are normalized for *GAPDH* and expressed in arbitrary units (AU). (**p < 0.01 versus Ctrl; Data are mean ± SEM)



Expression of *ET-1* and *GATA4* genes, respectively, in left ventricle heart samples of six-week animals, collected after treatment. Results are normalized for *GAPDH* and expressed in arbitrary units (AU). (*p < 0.05 versus Ctrl; Data are mean ± SEM)



Expression of *Fzd3* gene, in left ventricle heart samples of six-week animals, collected after treatment. Results are normalized for *GAPDH* and expressed in arbitrary units (AU). (Data are mean ± SEM)

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