UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

DANIELA APARECIDA MASCHIO

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Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do Título de Doutora em Biologia Celular e Estrutural na área de Biologia Tecidual.

Thesis presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of Doctorate in Cellular and Structural Biology, in the area of Tissue Biology.

ESTE ARQUIVO DIGITAL CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA DANIELA APARECIDA MASCHIO E ORIENTADA PELA PROFA. DRA. CARLA BEATRIZ COLLARES BUZATO.

Orientadora: PROFA. DRA. CARLA BEATRIZ COLLARES BUZATO

Campinas

2018

Agência(s) de fomento e nº(s) de processo(s): CNPq, 140815/2015-5; CAPES
ORCID: https://orcid.org/0000-0002-8236-2026

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca do Instituto de Biologia
Mara Janaina de Oliveira - CRB 8/6972

M375p

Maschio, Daniela Aparecida, 1983-
Papel da via de sinalização Wnt canônica na proliferação e função secretora da célula beta pancreática em condições *in vitro* e na pré-diabetes / Daniela Aparecida Maschio. – Campinas, SP : [s.n.], 2018.

Orientador: Carla Beatriz Collares Buzato.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Células beta pancreáticas. 2. Células MIN6. 3. Pré-diabetes. 4. Dieta hiperlipídica. 5. Via de sinalização Wnt. I. Collares-Buzato, Carla Beatriz, 1965-. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: The role of the canonical Wnt signaling in the proliferation and secretory function of the pancreatic beta cell *in vitro* conditions and during prediabetes
Palavras-chave em inglês:
Pancreatic beta cells
MIN6 cells
Prediabetes
High-fat diet
Wnt signaling pathway
Área de concentração: Biologia Tecidual
Titulação: Doutora em Biologia Celular e Estrutural
Banca examinadora:
Carla Beatriz Collares Buzato [Orientador]
Silvia Cristina Crepaldi Alves
José Roberto Bosqueiro
Leonardo dos Reis Silveira
Lúcia Elvira Alvares
Data de defesa: 05-11-2018
Programa de Pós-Graduação: Biologia Celular e Estrutural

Comissão examinadora

Profa. Dra. Carla Beatriz Collares Buzato

Profa. Dra. Silvia Cristina Crepaldi Alves

Prof. Dr. José Roberto Bosqueiro

Prof. Dr. Leonardo dos Reis Silveira

Profa. Dra. Lúcia Elvira Alvares

Os membros da Comissão Examinadora acima assinaram a Ata de Defesa, que se encontra no processo de vida acadêmica do aluno.

Dedicatória

Aos meus pais,
dedico esta tese e todas as minhas conquistas, sem toda dedicação deles recebida certamente eu não teria chegado até aqui.

Agradecimentos

Aos meus pais e às minhas irmãs pelo amor, carinho e pelo apoio deles recebido sempre.

À minha orientadora a Profa. Dra. Carla Beatriz Collares Buzato pela confiança, por sua prontidão em todos os momentos que necessitei e pelos ensinamentos que levarei para comigo.

Aos meus colegas do Laboratório de Comunicação de Adesão Celular, Ricardo Beltrame, Leandro Canuto, Valquíria Matheus, Joquebede Nascimento e Ariane De Sant'ana por toda ajuda na bancada, pelo aprendizado e pela amizade que constituímos durante esses anos.

Aos técnicos do Departamento de Bioquímica e Biologia Tecidual, Cintia Rizoli, Natália Santos, Thais Holtz e especialmente à Suseli Tada pela imensa ajuda técnica prestada a este trabalho.

A todos os professores, alunos e funcionários do Departamento de Bioquímica e Biologia Tecidual pela disponibilidade e ajuda e prestada, especialmente aos colegas do laboratório Large.

Aos professores Helena Barbosa Sampaio e Henrique Marques pela concessão do uso dos seus equipamentos e laboratórios.

Aos professores e funcionários do Programa de Biologia Estrutural e Funcional pela contribuição direta e/ou indireta para a execução deste trabalho.

À Liliam Alves Senne Panagio pela eficiência e auxílio na ajuda burocrática.

À Beatriz Silva de Toledo pela dedicação e auxílio na ajuda burocrática.

À Universidade Estadual de Campinas por sua excelência no acesso ao saber durante os anos de pós-graduação.

Ao Programa de Pós-Graduação em Biologia Celular e Estrutural e à sua coordenação pelo incentivo à pesquisa científica e ao aprimoramento acadêmico de seus alunos.

Aos professores membros da banca avaliadora agradeço por prontamente aceitaram fazer parte da mesma.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pelo auxílio financeiro.

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pelo auxílio financeiro.

RESUMO

Tem havido um grande interesse na determinação das vias de sinalização envolvidas na proliferação celular e função secretora das células beta pancreáticas para aplicação deste conhecimento em terapias moleculares e celulares na diabetes. Em especial, a via de sinalização Wnt canônica ou Wnt/β-catenina, que apresenta a proteína β-catenina como uma molécula sinalizadora juntamente com a Wnt, foi recentemente relacionada à diabetes tipo 2 quando foi descoberta em humanos uma relação entre a diabetes e mutações no gene que codifica o fator de transcrição TCF7L2 dessa via. Nós observamos previamente uma possível ativação dessa via, revelada pelo aumento significativo do conteúdo proteico de β-catenina ativada e Ciclina D1/2, em ilhotas hiperplásicas de animais pré-diabéticos, alimentados por 60 dias com uma dieta hiperlipídica (HFD, do inglês *High-Fat Diet*). Até então, não havia estudos que comprovassem diretamente o envolvimento desta via de sinalização no processo de hiperplasia compensatória da célula beta durante a pré-diabetes experimental. Sendo assim, esta Tese de Doutorado visou investigar a ativação da via Wnt canônica durante a hiperplasia compensatória da massa de célula beta observada na fase inicial da diabetes mellitus tipo 2 em modelo *in vivo* e expandir para um modelo *in vitro*. Para isso, foram realizadas as seguintes etapas experimentais: 1) a caracterização metabólica de camundongos C57BL/6/JUnib expostos à HFD por 30 ou 60 dias e seus controles (CTL 30d ou CTL 60d) alimentados com ração padrão pelo mesmo período de tempo; 2) a análise da ativação da via Wnt canônica em ilhotas hiperplásicas (obtidas de animais expostos à HFD 60d) e não hiperplásicas (obtidas de animais expostos à HFD 30d) e em ilhotas dos respectivos controles, por meio de imunofluorescência em criocortes do pâncreas, Western Blot, RT-PCR e qPCR de genes/proteínas associados à via Wnt canônica em homogenizados de ilhotas isoladas; 3) a análise da expressão gênica dos 19 subtipos de Wnts por RT-PCR e qPCR em homogenizados de ilhotas isoladas, do pâncreas total e do tecido adiposo peripancreático (controle positivo) dos camundongos expostos à HFD 60d e do seu grupo controle (CTL 60d), além de embriões de 12 dias (controle positivo); 4) a análise, por imunofluorescência em criocortes do pâncreas desses animais, da expressão proteica de determinados Wnts, selecionados na etapa anterior, para se determinar em quais tipos celulares do pâncreas/ilhotas são expressos, e por fim; 5) experimentos *in vitro* com a linhagem de células beta MIN6 para analisar se a co-cultura com ilhotas hiperplásicas (do grupo HFD) ou não hiperplásicas (do grupo CLT) tem efeito sobre a proliferação e/ou função secretora da célula beta desta linhagem. Nossos resultados confirmaram que os camundongos alimentados com HFD por 60 dias apresentaram alterações metabólicas características da pré-diabetes tipo 2. No grupo dos animais HFD 60d (com ilhotas hiperplásicas), além de um aumento no conteúdo proteico de β-catenina ativada e Ciclina D1/2, há uma translocação nuclear da β-catenina nas células beta e um aumento significativo na expressão de mRNA de Ctnnb1 (β-catenina), Ccnd1 (Ciclina D1), Ccnd2 (Ciclina D2), c-Myc, Ins2 (Insulina 2) e Coup-TFII, associado com diminuição do inibidor Axina 2, em homogenizados de ilhotas isoladas desses animais em relação aos grupos HFD 30d e controle. Esses resultados confirmam a ativação da via Wnt canônica durante a hiperplasia compensatória das células beta. Ainda, no grupo dos animais HFD 60d e no grupo CTL, a análise da expressão gênica dos 19 subtipos de Wnts revelou que a maioria deles são expressos nas células das ilhotas, como os Wnts 2, 2b, 3, 3a, 4, 5a, 5b, 6, 7a, 7b, 8a, 8b, 9a, 9b e 11, e que, no pâncreas, há expressão desses mesmos subtipos com exceção dos Wnts 3, 6, 7a e 7b. Entre todos os Wnts, os Wnts: 3a e 5b apresentaram um aumento significativo da expressão gênica em ilhotas hiperplásicas de camundongos HFD em comparação aos CTL, e o Wnt8b demonstrou ser expresso especificamente pelas células beta das ilhotas pancreáticas no grupo controle. Em adição, observamos que a co-cultura da linhagem MIN6 com ilhotas hiperplásicas ou não hiperplásicas não alterou significativamente a função secretora das células beta MIN6, mas induziu o aumento significativo da proliferação celular nesta linhagem *in vitro*. Essa indução da proliferação celular, no entanto, foi parcialmente bloqueada pelos inibidores Wnt, IWR-1 e IWP-2. Em conclusão, nossos dados indicam que a via de sinalização Wnt canônica está ativada durante o processo de hiperplasia compensatória da célula beta na pré-diabetes, e que Wnts liberados possivelmente por células das ilhotas podem estar envolvidos nesse processo.

ABSTRACT

There has been a great interest in determining the signaling pathways involved in cell proliferation and secretory function of the pancreatic beta cells for applying this knowledge in molecular and cellular therapies of diabetes. In particular, the canonical Wnt or Wnt/β-catenin signaling pathway, which has the β-catenin as a signaling molecule together with Wnts, has recently been associated to the onset of type 2 diabetes mellitus (T2DM). A link has been described between diabetes and mutations in the gene encoding the TCF7L2 transcription factor of the Wnt pathway. We have previously observed a possible activation of this pathway revealed by a significant increase in the protein content of active β-catenin and Cyclin D1/2 in hyperplastic islets of prediabetic animals fed a high-fat diet (HFD) for 60 days. Besides this observation, studies directly addressing the involvement of this signaling pathway in the process of compensatory beta-cell hyperplasia seen during experimental prediabetes are limited. Therefore, this Ph.D. Thesis aimed at investigating the activation of the canonical Wnt pathway during the compensatory hyperplasia of the beta-cell mass observed during the early phase of T2DM using *in vivo* and *in vitro* models. For this purpose, the following steps were performed: 1) the metabolic characterization of C57BL/6/JUnib mice exposed to HFD for 30 or 60 days and their respective control groups (CTL 30d or CTL 60d) fed a chow diet for the same period of the time; 2) the analysis of the activation of the canonical Wnt pathway in hyperplastic islets (obtained from animals exposed to HFD 60d) and non-hyperplastic islets (obtained from animals exposed to HFD 30d) and in islets of the respective control groups, by means of immunofluorescence, Western Blot, RT-PCR and qPCR of genes/proteins associated with the canonical Wnt pathway in isolated islet homogenates; 3) the analysis of the gene expression of all 19 Wnt subtypes (described in mice) by RT-PCR and qPCR in homogenates of isolated islets, whole pancreas and peripancreatic adipose tissue (positive control for the Wnts) obtained from the animals exposed to HFD 60d and their controls (CTL 60d), as well as whole 12-day mouse embryos (positive control for the Wnts) homogenates; 4) the immunofluorescence of some Wnts, screened in the previous step, to determine in which pancreas/islet cell types they are expressed, and finally; 5) *in vitro* experiments with MIN6 beta cell lineage were performed to analyze whether co-culture with hyperplastic or control islets has an effect on the proliferation and/or secretory function of this beta cell lineage. Our results confirmed that mice fed a HFD for 60 days display metabolic changes characteristic of type 2 prediabetes. In the HFD 60d group (with hyperplastic islets), a significant increase in the protein content of active β-catenin and Cyclin D1/2 was seen as well as a nuclear translocation of β-catenin in beta cells and a significant increase in the expression of mRNA of Ctnnb1 (β-catenin), Ccnd1 (Cyclin D1), Ccnd2 (Cyclin D2), c-Myc, Ins2 (Insulin 2) and Coup-TFII, as well as a decrease in Axin2, in isolated islet homogenates from these animals in relation to the CTL 60d group. These results confirm the activation of the canonical Wnt pathway during the compensatory beta-cell hyperplasia. In addition, the analysis of the gene expression of the 19 Wnt subtypes showed that the majority of Wnts are expressed in hyperplastic and non-hyperplastic islets, such as Wnt subtypes 2, 2b, 3, 3a, 4, 5a, 5b, 6, 7a, 7b, 8a, 8b, 9a, 9b and 11, and that, in the pancreas, there is expression of these same subtypes, except for Wnts 3, 6, 7a and 7b. Among all the Wnts, the Wnt3a and Wnt5b showed a significantly increased expression in hyperplastic islets of prediabetic mice when compared to CTL mice, and Wnt8b was shown to be expressed specifically by pancreatic beta cells. Furthermore, under *in vitro* conditions, we observed that co-culture with hyperplastic or non-hyperplastic islets did not significantly change the secretory function of the MIN6 beta cells but induced a significant increase in cell proliferation in this lineage, which was partially blocked by the Wnt inhibitors IWR-1 and IWP-2. In conclusion, we demonstrated that murine pancreas/islet cells can secrete Wnts and that Wnts released by islets may participate in the regulation of the beta-cell mass under type 2 prediabetes induced by HFD.

LISTA DE ABREVIATURAS

AGLs	Ácidos graxos livres
Axina1/2	Proteinas fosfatases de suporte do complexo de degradação da proteína β-catenina (do inglês, *Scaffolding protein*)
APC	Proteína do adenoma polipose do cólon (do inglês, *Adenomatous Polyposis Coli*)
Akt	Serina/treonina cinase (do inglês, *V-akt murine thymoma viral oncogene homolog 1,2 ou 3*)
Bcl2	Refere-se a uma família de genes/proteínas antiapoptóticos, descoberta em linfoma de células B 2; (do inglês, *B-cell lymphoma 2*)
CAMs	Moléculas de adesão celular (do inglês*, Cell Adhesion Molecules*)
CoupTF-II	Receptor nuclear órfão (do inglês, *Chicken ovalbumim upstream promoter transcription factor II*)
CTL	Controle (animais do grupo alimentado com dieta padrão)
CK1	Proteína caseína-cinase 1 (do inglês, *Casein Kinase 1*)
Cx36	Conexina 36, proteína funcional da junção comunicante (do inglês*, Connexin 36*)
Dact-1	Proteína Dapper Homologue-1 (do inglês, Dishevelled-binding antagonist of beta-catenin 1)
Dvl	Proteína Dishevelled (do inglês, *Dishevelled - cytoplasmic scaffolding protein*)
FOXO	Fator de transcrição relacionado à adipogênese (*do inglês, Forkhead boxO (FoxO) transcription factor)*
Fz	Proteína transmembrana 7 passos (do inglês, *seven-pass transmembrane receptors (Fz, Fzd or Frizzled)*
GDM	Diabetes Mellitus Gestacional (do inglês, *Gestational Diabetes Mellitus*)
GLUT2	Transportador de glicose tipo 2 (do inglês, *Glucose transporter type 2*)
GLUT4	Transportador de glicose tipo 4 (do inglês, *Glucose transporter type 4*)
Groucho	Família de proteínas co-repressoras transcricionais relacionadas ao gene Gro (Grg) nos vertebrados.
GSK-3β	Glicogênio sintase cinase-3 beta (do inglês*, Glycogen Synthase Kinase-3beta*)

HFD	Dieta hiperlipídica (do inglês, *High Fat Diet*)
HLAs	Antígenos leucocitários humanos (do inglês, *Human leukocyte antigens*)
IκB-β	Cinase IκB-β (do inglês, *IκB-β Kinase*), IKB participa do complexo enzimático que resulta na cascata de transdução do sinal de NF-κB.
IL-6	Interleucina-6 (do inglês, *Interleukin-6*)
JNK1/MAPK8	Proteína cinase c-Jun ou proteína cinase ativadora de mitose (do inglês *c-Jun N-Terminal Protein Kinase 1* ou *Mitogen-activated protein kinase 8*)
IR	Receptor de insulina (do inglês, *Insulin receptor*)
IRS1/2	Substrato do receptor de insulina 1 e 2 em camundongos (do inglês*, Insulin receptor substrate 1 and 2*)
LRP5/LRP6	Proteína 5 e 6 relacionada ao receptor de LDL (do inglês, *Low density lipoprotein receptor related protein 5 and 6*).
MIN6	Linhagem celular de insulinoma de camundongo (do inglês, *Mouse Insulinoma Clone 6*)
mTor	Proteína cinase alvo da rapamicina em mamíferos (do inglês, mammalian target of rapamycin)
NF-kβ	Fator de transcrição nuclear kappa β, (do inglês, *Nuclear factor kappa beta*), relacionado com a resposta celular à inflamação
PTEN	Fosfatase homóloga da tensina, é um supressor de tumor multifuncional (do inglês, *Phosphatase and tensin homolog*)
PDK1	Proteína cinase-1 dependente de fosfoinositídeo (do inglês, *Protein 3-phosphoinositide-dependent protein kinase-1*)
PI3K	Proteína fosfoinositídeo 3-cinase (do inglês, *Phosphatidylinositol-4,5-Bisphosphate 3-Kinase*)
PIP2	Fosfatidilinositol (4,5) bisfosfato, é um fosfolipídio da membrana citoplasmática que age como mensageiro secundário (do inglês, *Bisphosphate do Phosphatidylinositol 4,5*)
PIP3	Fosfatidilinositol (3,4,5)-trifosfato (do inglês, *Bisphosphate do Phosphatidylinositol 4,5*)
qPCR	PCR quantitativo
RE	Retículo endoplasmático
Ror	Receptor órfão ligado ao receptor tirosina cinase (do inglês, *Receptor tyrosine kinase-like orphan receptor (ROR) 1 and 2*)

ROS	Espécies reativas de oxigênio (do inglês, *Reactive Oxygen Species*)
RT-PCR	Transcriptase reversa (do inglês, *Reverse transcription polymerase chain reaction*)
RTKs	Proteínas cinases receptoras (do inglês, *Receptor yrosine kinases*)
Ryk	Receptor de tirosina cinase (do inglês, *Receptor-like tyrosine kinase*)
SOCS	Proteínas supressoras da sinalização de citocinas (do inglês, *Suppressor of cytokine signaling*)
SFRP1	Proteína 1 relacionada à *frizzled* secretado (do inglês, *Secreted Frizzled-Related Protein 1*)
SNP	Polimorfismo de nucleotídeo único (do inglês, *Single nucleotide polymorphism*)
T1DM	Diabetes Mellitus tipo 1 (do inglês, *Type 1 Diabetes Mellitus*)
T2DM	Diabetes Mellitus tipo 2 (do inglês, *Type 2 Diabetes Mellitus*)
Tlr4	Receptor do tipo Toll 4 (do inglês, *Toll-like-4 receptor*)
TCF/LEF	Fator de transcrição da célula T/Fator de ligação ao estimulador linfóide (do inglês, *T Cell specific factor/lymphoid enhancer-binding factor*)
TCF2L7	Fator de transcrição 2 semelhante ao 7 (do inglês, *Transcription Factor 2-Like 7).*
TNF-α	Fator de necrose tumoral-α (do inglês, *Tumor Necrosis Factor-α*)
Wnt	Refere-se a uma família de glicolipoproteínas. É um nome "hibrido", proveniente da junção de dois nomes dado ao mesmo gene descoberto independentemente, o gene *Wingless* (*Wg*) de *Drosophila* e o gene *Int1* de camundongos.
Wnt/Ca^{2+}	Via de sinalização Wnt/Ca^{2+} (do inglês, *Wnt signaling pathway* Ca^{2+})
Wnt/PCP	Via de sinalização Wnt de polaridade celular planar (do inglês, *Wnt signaling pathway polar cell polarity*)
β-TRCP	Proteínas contendo repetições beta-transducina (do inglês, Beta-transducin repeat-containing proteins)

SUMÁRIO

Capítulo 1

1. INTRODUÇÃO AO TEMA DA TESE

1.1 Diabetes Mellitus

A diabetes mellitus é uma doença endócrino-metabólica que atingiu proporções epidêmicas mundialmente e, segundo dados do primeiro relatório global sobre diabetes da Organização Mundial de Saúde (OMS), o número de adultos com diabetes atingiu 422 milhões em 2014. São duas as principais formas dessa doença: a Diabetes Mellitus Tipo 1 (T1DM - do inglês*, Type 1 Diabetes Mellitus*) – também conhecida como diabetes juvenil ou diabetes insulino-dependente; e a Diabetes Mellitus Tipo 2 (T2DM - do inglês*, Type 2 Diabetes Mellitus*) – também conhecida como diabetes do adulto ou diabetes não insulino-dependente. Uma terceira forma menos comum da doença é a Diabetes Mellitus Gestacional (GDM – do inglês, *Gestational Diabetes Mellitus)* que apresenta uma patogênese semelhante à T2DM, porém é uma condição temporária que se apresenta durante o período gestacional (World Health Organization, 2016).

A T1DM é classificada como uma doença autoimune crônica que resulta de uma combinação de predisposição genética/hereditariedade e exposição a causas ambientais como, por exemplo, a alimentação desbalanceada e certas infecções virais. Esses fatores desencadeiam uma desregulação imunológica que leva à morte das células beta e consequente deficiência na produção de insulina (Varela-Calvino *et al.*, 2000; Cooper *et al.*, 2012; Acharjee *et al.*, 2013; Pugliese *et al.*, 2016)

Tem sido demonstrado que a suscetibilidade genética à T1DM envolve mutações (polimorfismos) de genes tais como os que codificam o complexo genético do sistema antígeno leucocitário humano (HLAs - do inglês, *Human Leukocyte Antigens*), os genes envolvidos na função das células beta e os polimorfismos na região promotora do gene da insulina que, por sua vez, afetam os níveis de expressão de insulina (Singal & Blajchman 1973; Nerup *et al*., 1974; Noble & Erlich, 2012; Pugliese *et al*., 2016).

Apesar da predisposição genética ser a maior responsável pelo desenvolvimento da T1DM, infecções virais também tem sido relacionadas a essa doença, como a infecção causada pelo vírus Coxsackie tipo B4 (*Coxsackie virus B4*) (Varela-Calvino *et al*, 2000; Acharjee *et al*., 2013). Em adição, a exposição a substâncias antigênicas no início da vida, como certos ingredientes da dieta como a albumina sérica bovina, a beta-caseína e

o glúten, também parecem contribuir para o desenvolvimento da T1DM (Buschard, 2011; Acharjee *et al*., 2013).

A T2DM, por sua vez, acomete 90-95% das pessoas com diabetes em todo o mundo (IDF 2017). Está estabelecido que a obesidade e o sedentarismo são os principais fatores que contribuem para o desencadeamento da T2DM, embora possam haver outros fatores como a predisposição genética e ambientais que contribuem para isso (Prentki & Nolan 2006; Tripathy & Chavez, 2010; Kahn & Davidson, 2014; DeFronzo *et al*., 2015). A maioria dos pesquisadores reconhece que a T2DM é uma doença multifatorial que tem uma patogênese extremamente complexa e que é improvável que exista um único gatilho ligado a sua etiologia (Shoelson, Lee, & Goldfine 2006; Jonietz, 2012; Kahn & Davidson, 2014).

É consenso, contudo, que o quadro chamado de resistência periférica à insulina, onde a ação da insulina está prejudicada nos tecidos/órgãos periféricos, juntamente com a inadequação funcional das células beta, representem os principais sinais da T2DM e que ambos os acontecimentos são essenciais para a manifestação completa da doença (Butler *et al*., 2003; Nolan, Damm, & Prentki, 2011;Tripathy & Chaves, 2010; Mezza *et al*., 2014).

A GDM, por sua vez, reflete a secreção de insulina materna prejudicada em relação à demanda anterior à gravidez, em respostas a estressores metabólicos temporários impostos pela placenta (como hormônios e outros mediadores) e pelo feto, onde o resultado é a intolerância à glicose como ocorre na T2DM (Metzger *et al*., 2007; Kim, 2014; Chiefari *et al*., 2017). Assim, a GDM apresenta um quadro metabólico semelhante à T2DM, porém muitas vezes as características da doença não são claramente evidentes como na T2DM; essa foma da doença, no entanto, está se tornando cada vez mais comum e tem sido frequentemente associada à obesidade materna (Coustan, 2013).

1.2 Diabetes Mellitus tipo 2

1.2.1 Etiologia

Tem crescido o interesse nos estudos da T2DM para melhor entender a sua etiologia e evolução e, dessa forma, desenvolver novas terapias de tratamento para essa forma mais prevalente de diabetes.

Na T2DM, assim como na T1DM, há também uma associação com fatores hereditários; por exemplo, em famílias com indivíduos diabéticos tipo 2, os descendentes têm um risco aumentado de desenvolver a doença (Jonietz, 2012; DeFronzo *et al*., 2015). Além disso, estudos de associação genômica demonstraram que variantes genéticas comuns estão associadas com a T2DM (Tripathy & Chavez, 2010; Herder & Roden, 2011; Jonietz, 2012). Entre esses estudos, o polimorfismo de nucleotídeo único (SNP) no gene TCF7L2 mostrou uma forte associação com T2DM e foi relacionada com a disfunção das células beta (Tripathy & Chavez, 2010; Herder & Roden, 2011; Jonietz, 2012).

A maioria da hereditariedade (85%), contudo, não pode ser explicada pelos SNPs atualmente identificados. Acredita-se, atualmente, que a T2DM pode não ter uma causa genética única, mas envolver também interações gene-ambiente e mecanismos epigenéticos (como metilação do DNA e modificações na cromatina). Ainda, alguns pesquisadores sugerem que genes diferentes e fatores ambientais podem levar ao desenvolvimento da doença que difere de pessoa para pessoa (Lyssenko & Laakso, 2013; Herder & Roden 2014; DeFronzo *et al*., 2015).

Outros fatores que podem contribuir para o desenvolvimento da T2DM incluem obesidade, toxinas ambientais e a própria microbiota intestinal (Jonietz, 2012). O risco de desenvolver T2DM é substancilmente aumentado com a obesidade e com as alterações metabólicas que acompanham uma dieta altamente energética, que incluem o aumento de ácidos graxos livres circulantes e citocinas inflamatórias (Golson *et al*., 2010). Ainda, estudos recentes sugerem que a obesidade e a T2DM estão associadas a uma disbiose intestinal (DeFronzo *et al*., 2015). As investigações do metagenoma humano demonstraram correlações altamente significativas de bactérias intestinais, certos genes bacterianos e vias metabólicas associadas à T2DM. Por exemplo, as bactérias intestinais produtoras de butirato, como *Roseburia intestinalis* e *Faecalibacterium prausnitzii,* apresentam concentrações menores em indivíduos com T2DM (Tilg & Moschen, 2014). Corroborando esses estudos, tem sido demonstrado que o butirato e outros ácidos graxos de cadeia curta são capazes de exercer efeitos imunometabólicos, anti-obesidade e anti-diabético (Tilg & Moschen, 2014; Matheus *et al*., 2017).

1.2.2 Patogênese

Quanto à sua patogênese, a T2DM pode ser dividida em duas fases, a pré-diabetes e uma fase mais avançada da doença (ou fase evidente da doença). Na pré-diabetes, o primeiro sinal é o desenvolvimento do quadro de resistência periférica à insulina, principalmente dos tecidos adiposo, músculo esquelético, e fígado, que resulta inicialmente em hiperglicemia moderada (Tripathy & Chaves, 2010; Mezza *et al*., 2014). Na fase evidente da T2DM, em razão da não adequação funcional das células beta e diminuição da sua massa, há um substancial declínio na secreção de insulina e consequente aumento da glicemia no organismo, o que leva a um quadro de hiperglicemia mais elevado e descompensado (Liu *et al*., 2010).

Há controversa entre os pesquisadores sobre os fatores que poderiam desencadear a resistência periférica à insulina. Alguns pesquisadores acreditam que apenas o excesso de lipídios livres no organismo (não acumulados nos adipócitos) seja suficiente para causar a resistência à insulina (Kahn, Hull, & Utzschneider 2006; Ding *et al*., 2010). Outros acreditam que o acúmulo de tecido adiposo e a sua inflamação lançam na corrente sanguínea citocinas e outros fatores inflamatórios que promovem uma inflamação sistêmica no organismo, afetando também as ilhotas pancreáticas, sendo os principais responsáveis pelo desencadeamento da resistência tecidual à insulina (Shoelson *et al*., 2006; Lackey *et al*., 2014; Herder & Roden 2014). Mais recentemente, porém, tem se aceitado que ambas as possibilidades, separadamente ou associadas, podem resultar nesse quadro (De-Fronzo *et al*., 2015)

Em indivíduos com T2DM, as concentrações de ácidos graxos livres (AGLs) são significativamente mais altas do que em indivíduos saudáveis (Miles *et al*., 2003). O excesso de AGLs pode induzir a geração de espécies reativas de oxigênio (ROS), resultando em lipotoxicidade associada ao estresse do retículo endoplasmático (RE. (Ly *et al*., 2017). No RE, a liberação de Ca^{2+} devido ao estresse oxidativo induzido pelos AGLs (principalmente o palmitato) resulta em sobrecarga citosólica e mitocondrial de Ca^{2+}, que promove a geração de ROS nas mitocôndrias (Rieusset, 2015; Ly *et al*., 2017). Em conjunto, o estresse do RE e a disfunção mitocondrial podem causar uma sinalização de insulina prejudicada e aumento anormal na gliconeogênese nos hepatócitos e comprometimento da função secretora das células beta, que contribuem para o quadro de resistência à insulina durante a T2DM (Lowell & Shulman, 2005; Ma *et al*., 2011; Rieusset, 2015).

A prejudicada sinalização da insulina está associada a falhas nos mecanismos moleculares de fosforilação do receptor de insulina (IR) e, principalmente, do substrato do IR (IRS1/2), bem como à consequente falha na atividade de PIK3 (Tripathy & Chavez, 2010). A enzima PI3K pode atuar recrutando Akt que é uma enzima chave nos vários eventos de sinalização intracelulares que resultam da sinalização da insulina como os relacionados ao metabolismo celular da glicose (Huang & Czech, 2007; Watson & Pessin, 2006). Nos tecidos adiposo e muscular, por exemplo, Akt atua nos mecanismos envolvidos na reciclagem do transportador de glicose tipo 4 (GLUT4) desses tecidos (Ng *et al*., 2008). É sabido que, em indivíduos com T2DM, obesos e resistentes à insulina, a prejudicada sinalização da insulina leva ao comprometimento da reciclagem das vesículas contendo GLUT4 nesses tecidos que aumenta substancialmente a glicemia, principalmente devido ao tecido muscular que exige uma elevada captação de glicose. (Ng *et al*., 2008; Tripathy & Chavez, 2010; Li *et al*., 2012) (**Figura 1**).

Como mencionado anteriormente, a inflamação sistêmica é um contribuinte para a resistência à insulina. O aumento dos níveis de citocinas pró-inflamatórias, tais como interleucina-6 (IL-6) e fator de necrose tumoral-α (TNF-α), bem como o aumento do número de macrófagos e de outras células inflamatórias, observado no tecido adiposo, no soro de pacientes e animais obesos e em pacientes insulino-resistentes (Golson *et al*., 2010, Huang *et al*., 2018). As citocinas pró-inflamatórias induzem a resistência à insulina por ativação de cinases, incluindo a cinase IκB-β, JUN 1 (JNK1; também conhecida como MAPK8) e a p38 MAPK, que podem contribuir para a fosforilação dos resíduos de serina/treonina de IRS1/2 e estimular a produção de supressores de sinalização de citocinas (SOCS), que bloqueiam a ação de IRS1/2 em vários células/tecidos, como músculo, hepatócitos, e adipócitos (De-Fronzo *et al*., 2015).

As vias inflamatórias também podem ativar a via de sinalização do fator de transcrição nuclear kappa β (NF-kβ) (Sriwijitkamol *et al*., 2006; Jia *et al*., 2014) que promove a transcrição de genes envolvidos em cascatas inflamatórias e geração do estresse oxidativo, o que piora ainda mais o quadro inflamatório. Foi demonstrado, por exemplo, que em indivíduos normoglicêmicos, mas obesos e resistentes à insulina, e em indivíduos com T2DM, há um aumento da expressão gênica e do conteúdo proteico do receptor Toll-like-4 (Tlr4), um mediador chave de respostas pró-inflamatórias (Sriwijitkamol *et al*., 2006; Reyna *et al*., 2008). Corroborando esses dados, tem sido

demonstrado que camundongos deficientes em Tlr4 (Tlr4LKO) são protegidos da resistência à insulina e inflamação do tecido adiposo unilocular que são causadas com exposição à dieta hiperlipídica (Jia *et al*., 2014).

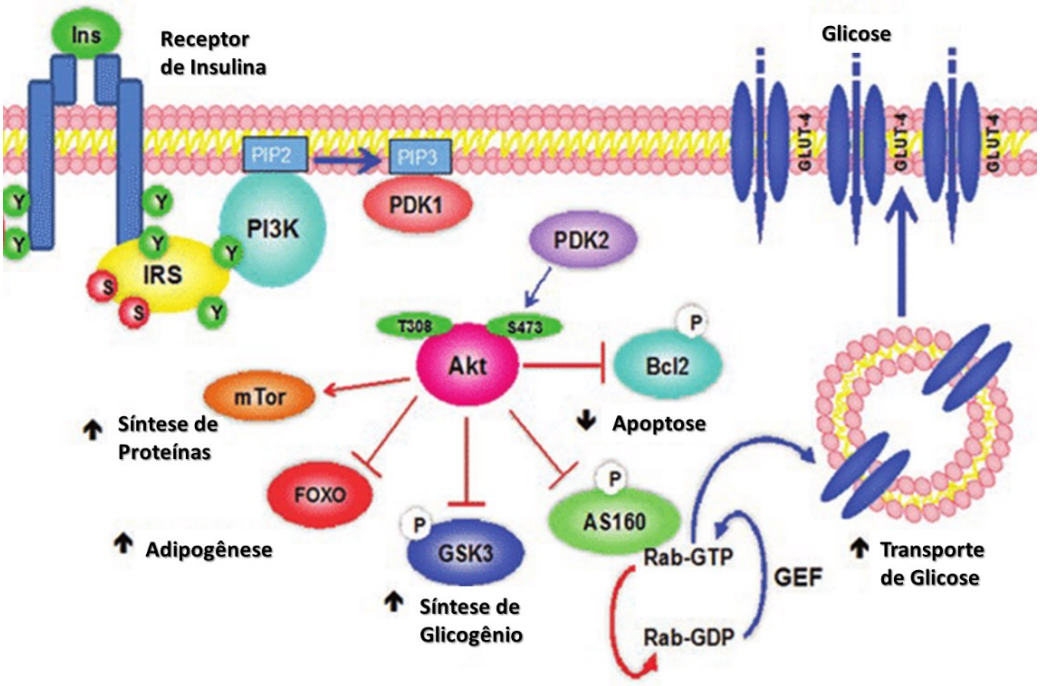

Figura 1. Via de sinalização da insulina nos tecidos periféricos: adiposo e muscular esquelético. A interação da insulina promove fosforilação do receptor de insulina (IR) e subsequente fosforilação da tirosina dos substratos receptores de insulina (IRS1/2) (Sun *et al*., 1991). IRS ativa a cinase fosfatidilinositol-3 (PI3K), que, então, fosforila um fosfolipídio da membrana celular, o fosfatidilinositol (4,5) bisfosfato (PIP2), convertendo-o em fosfatidilinositol (3,4,5)-trifosfato (PIP3). PIP3 e outros alvos do IRS, por sua vez, ativam uma variedade de alvos intracelulares que têm efeitos específicos para cada tipo de célula/tecido (Golson *et al*., 2010). Entre as vias mediadas por IRS, encontra-se a via PI3K/Akt, que desempenha um papel crucial na ativação e na regulação de diversos acontecimentos metabólicos, incluindo a captação de glucose e a síntese de glicogênio, proteínas e lípidos. Akt: proteína cinase B; AS160: Substrato de Akt; GLUT4: transportador de glicose tipo 4; GSK3: proteína cinase glicogênio sintase 3; Bcl2: refere-se a uma família de genes/proteínas anti-apoptóticos; mTor: proteína/sinalização relacioda à síntese proteica; FOXO: fator de transcrição relacionado à adipogênese; IRS: substrato do receptor de insulina; PDK1: proteína cinase 1 dependente de fosfoinositídeo; PDK2: proteína cinase 2 dependente de fosfoinositídeo; PI3K: fosfatidilinositol-4,5- bisfosfato 3-cinase. Adaptado de Vázquez-Jiménez *et al*., 2017. *Gac Med Mex 153*(7), 852-863.

1.3 Pâncreas e célula beta pancreática

O pâncreas é o órgão central nos estudos que buscam melhor entender como ocorre o desenvolvimento da T2DM e que procuram um potencial terapêutico de agentes que tem como alvo de ação este órgão para, dessa forma, melhorar a homeostase da glicose. O pâncreas é uma glândula acessória ao sistema digestório, cujo parênquima é formado por uma porção exócrina e uma endócrina (El-Gohary *et al*. 2012; Pandiri, 2014). A porção exócrina do pâncreas compreende 90% de toda a sua massa e é composta pelas células acinares, centroacinares e células do ducto (ou ductais). A porção endócrina compreende cerca de 1-2% da massa e corresponde às ilhotas pancreáticas. O restante da glândula é constituído pelo interstício com os vasos sanguíneos e linfáticos, nervos e o estroma de tecido conjuntivo (El-Gohary *et al*., 2012; Logsdon & Ji, 2013; Pandiri, 2014).

A porção exócina do pâncreas é constituida principalmente pelas células acinares que se organizam em ácinos serosos responsáveis pela produção, armazenamento e secreção de grandes quantidades de enzimas, como a amilase, lipase e várias proteases, necessárias para a digestão e absorção de nutrientes (Logsdon & Ji, 2013; Antonucci *et al*., 2015). Localizadas centralmente nos ácinos, estão as células centroacinares que formam uma interface entre o ácino e o ducto intercalar. O ducto intercalar é contínuo com os ductos intralobulares e ambos são revestidos pelas células ductais. Os ductos intralobulares se fundem para formar os ductos interlobulares que desembocam no ducto pancreato-hepático (biliar), que, por sua vez, abre-se diretamente no lúmen duodenal (**Figura 2**) (Pandiri, 2014).

No pâncreas endócrino, as ilhotas pancreáticas, também chamadas de ilhotas de Langerhans, são as unidades morfofuncionais que o compõem. Em ratos e camundongos, dependendo da região que ocupa, o pâncreas pode ser anatomicamente dividido em cabeça (duodenal), corpo (lobo gástrico) e cauda (esplênica). Curiosamente, as ilhotas pancreáticas não estão distribuídas uniformemente pelo parênquima pancreático em cada uma dessas regiões, apresentando-se em menor número na região da cabeça/corpo e em maior número na região da cauda (El-Gohary *et al*., 2012, Wang *et al*., 2013; Pandiri, 2014).

Alguns pesquisadores sugerem que as células que revestem os ductos e as células acinares do pâncreas podem servir como fonte de novas células beta (Juhl, Bonner-Weir, & Sharma, 2010; Yoneda *et al*., 2013). Mais recentemente, estudos demonstraram

que ocorre um maior número de agrupamentos de ilhotas próximos a ductos, provenientes de neogênese, e a presença de células ductais insulino-positivas, que foram relacionadas ao aumento da massa de células beta em ilhotas de humanos não diabéticos, mas com resistência à insulina, (Mezza *et al*., 2014).

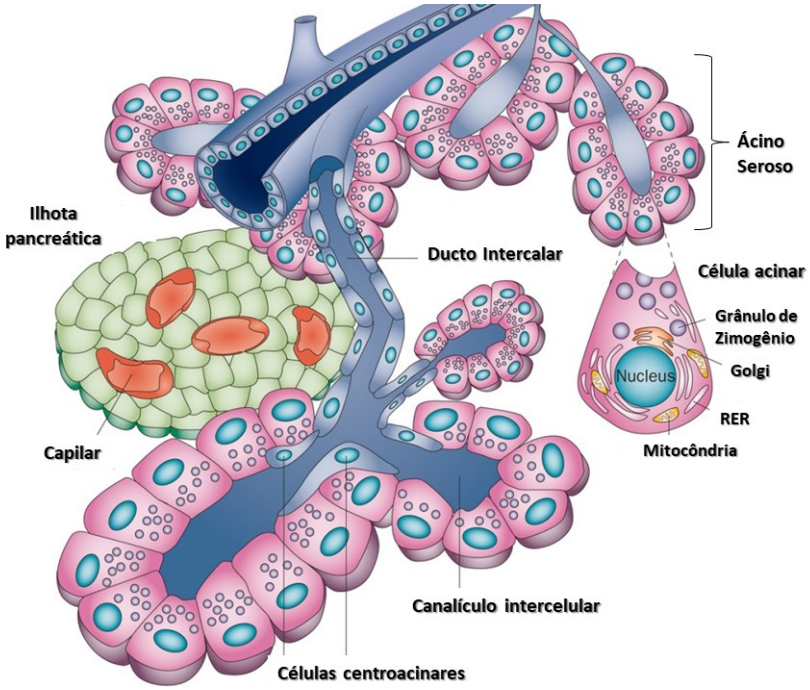

Figura 2. Esquema da organização histológica do pâncreas. O parênquima pancreático é constituido por uma porção exócrina e uma endócrina. A porção exócrina compreende 90% de toda a sua massa, sendo composta pelas células acinares, centroacinares e células do ducto (ou ductais). As células acinares são o principal tipo celular e constituem os ácinos serosos, responsáveis pela produção, armazenamento e secreção de enzimas digestórias. A porção endócrina compreende cerca de 1-2% da massa e corresponde às ilhotas pancreáticas que consistem em um aglomerado de diferentes tipos de células organizadas ao redor de pequenos vasos. O restante da glândula é constituído pelo interstício com os vasos sanguíneos e linfáticos, nervos e o estroma de tecido conjuntivo. Adaptado de Logsdon, C. D. & Ji, B. (2013) *Nat. Rev. Gastroenterol. Hepatol*. *10*:362–370.

As ilhotas são constituídas por cinco tipos celulares; as células beta, β, ou B, secretora de insulina com ação hipoglicemiante; as células alfa, α, ou A, responsáveis pela secreção do hormônio glucagon com função hiperglicemiante; as células delta, δ, ou D, responsáveis pela secreção de somatostatina, hormônio de ação parácrina, que regula a liberação de insulina e glucagon; as células épsilon, ε, ou E, produtoras de

grelina, hormônio cuja função no pâncreas ainda não está bem estabelecida, mas possivelmente esteja relacionada à inibição da secreção de insulina; e as células γ, F, ou PP, produtoras do polipeptídeo pancreático que parecem exercer uma função inibidora na secreção do pâncreas exócrino (Orci, 1975; Ekblad & Sundler, 2002; Kanno and Go, 2002; Andralojc, Mercalli, & Nowak, 2009; Pandiri, 2014).

Em cada ilhota, as diferentes células se conectam, de forma homotípica ou heterotípica, por meio das junções intercelulares do tipo oclusão, comunicante e aderente e desmossomos (Orci, 1976). As junções celulares e suas proteínas constitutivas participam de processos tais como a adesão, homeostase celular, comunicação intercelular, bem como da organização e citoarquitetura das ilhotas e são cruciais para o perfeito funcionamento da ilhota (Cirulli *et al.*, 1993; Jain & Lammert, 2009; Carvalho *et al.,* 2012; Defronzo *et al*., 2015; Collares-Buzato, 2013).

Em certas condições patológicas, podem ocorrer alterações das junções e de suas proteinas estruturais que causam uma desregulação da secreção endócrina pela ilhota, sobretudo na secreção de insulina pelas células beta. Por exemplo, a diminuição na expressão da proteína Cx36 e no número de canais da junção comunicante está relacionada ao comprometimento da secreção de insulina pela célula beta que ocorre na pré-diabetes experimental em camundongos (Carvalho *et al*., 2012). Além disso, foi observado também que moléculas de adesão celular (CAMs) como as N-, E- e VE-caderinas e a α-catenina apresentam distribuição e expressão alteradas nas ilhotas de camundongos obesos e diabéticos, o que foi associado também à disfunção secretora da célula beta nesses animais (Falcão *et al*., 2016).

Na ilhota pancreática, as células beta são o tipo celular mais abundantemente encontrado, compreendendo aproximadamente 60% ou 80% de todas as células em ilhotas humanas e de roedores, respectivamente (Cabrera *et al*., 2006; Bosco *et al*., 2010) (**Figura 3**). A célula beta, embora seja um tipo celular altamente diferenciado, possui uma capacidade adaptativa surpreendente em condições fisiopatológicas como ocorre na T2DM e na obesidade. Nessas condições, há um aumento dinâmico na massa de célula beta, por meio dos processos de hiperplasia e hipertrofia, como forma de aumentar a síntese de insulina para compensar o quadro de resistência à insulina inicial e assim manter a normoglicemia do organismo (Sone & Kagawa, 2005; Liew & Andrews, 2009; Collares-Buzato, 2015; Giaccari *et al*., 2009; Figeac et al., 2010; Li &

Lail, 2015; Saisho *et al.*, 2013; Mezza *et al*., 2014; Oliveira *et al*., 2015, Falcão *et al*., 2016)

A capacidade adaptativa da célula beta varia no decorrer da evolução da T2DM. Na pré-diabetes, frente ao quadro de resistência periférica à insulina, as células beta inicialmente aumentam a biossíntese de insulina e sua capacidade secretora. Posteriormente, este aumento na produção de insulina é sustentado ao longo prazo por um aumento relativo da massa de células beta. Esta resposta é tão eficiente que os níveis normais de glicose no sangue são mantidos, mesmo com a resistência periférica à insulina (Kahn *et al*., 2006; revisado por Liew & Andrews 2009; Saisho *et al.*, 2013; Collares-Buzato, 2015).

Na fase mais avançada da T2DM, devido ao aumento da produção de insulina e sua liberação continua por tempo prolongado, sob as condições de resistência periférica à insulina, ocorre um esgotamento funcional das células beta que leva à sua disfunção e, consequente, morte celular por apoptose (Collares-Buzato, 2015). Como consequência, há então um declínio contínuo na secreção de insulina que leva à hiperglicemia progressiva (Hollingdal *et al*., 2000; Conget *et al*., 2001). O quadro de hiperglicemia constante tem efeitos tóxicos (glicotoxicidade) ao organismo, também para a célula beta, e efeitos nocivos sobre a sensibilidade à insulina nos tecidos periféricos, dessa forma se estabelece a fase mais evidente da T2DM (Rahier *et al*., 2008; Mezza *et al*., 2014; Satin *et al*., 2015).

Assim, os mecanismos que regulam a massa e a função secretora da célula beta têm sido objeto de pesquisas por grupos interessados no desenvolvimento de novas terapias celulares/moleculares para diabetes, uma vez que a manutenção da massa e da adequada função secretora das células beta são fundamentais para prevenir a progressão da diabetes, conforme descrito anteriormente (Liew & Andrews, 2009; Figeac *et al*., 2010; Amisten *et al*., 2017; Kuljanin *et al*., 2017). Vários fatores de proliferação celular parecem regular o ciclo celular da célula beta tais como: as Ciclinas e as proteínas cinases dependentes de Ciclina; os fatores que agem através dos receptores de tirosina cinases (RTKs); os fatores que agem através da via JAK/STAT e os fatores que agem através de receptores acoplados à proteina G. (Yesil & Lammert, 2008; Lee & Nielsen, 2009).

A sinalização Akt/PI3K parece também ter um papel na proliferação de células beta. A deleção específica do gene que codifica a PDK1 (proteína cinase 1 dependente de

fosfolipídios), agonista dessa via Akt, causa a redução da massa celular beta levando à deficiência de insulina (Hashimoto *et al*., 2006). Em contraste, a deleção específica em células beta da fosfatase homóloga da tensina (PTEN), um regulador negativo da sinalização PI3K, leva a um aumento da massa celular das ilhotas e mantém a função das células beta (Nguyen *et al*., 2007).

Em adição, a via de sinalização Wnt canônica (ou Wnt/β-catenina), que é uma via muito estuda durante o desenvolvimento embrionário e está relacionada aos mecanismos de proliferação celular, tem sido considerada importante na biologia da célula beta (Logan & Nusse, 2004; Clevers, 2006; Rulifson *et al.,* 2007; Murtaugh, 2008). Uma estreita relação entre o grau de expressão de variantes de seu fator de transcrição TCF7L2 e a T2DM tem sido documentada em seres humanos (Lyssenko *et al.*, 2007). Polimorfismos em TCF7L2 estão associados a uma deficiência na secreção de insulina, sugerindo um papel importante da via Wnt canônica na função secretora das células beta (Krützfeldt & Stoffel 2010; Sorrenson *et al*., 2016).

Além disso, demonstrou-se que a inibição de TCF7L2 em ratos neonatos resulta em alteração do crescimento normal das células beta, principalmente por meio da inibição da proliferação celular durante o desenvolvimento pós-natal do pâncreas endócrino. Inversamente, em ratos neonatos com T1DM, a ativação da via Wnt canônica induzida por meio da inibição de GSK-3β (um inibidor da via) teve um efeito estimulatório significativo na regeneração da massa de célula beta nesses animais. Ainda, *in vitro*, a inativação de GSK-3β resultou na estimulação da proliferação das células beta, que foi mediada pela estabilização de β-catenina e indução de Ciclina D (Figeac *et al*., 2010). Mais recentemente, foi demonstrado que camundongos *knockdown* condicionais para TCF7L2, alimentados com uma dieta hiperlipídica, apresentaram uma redução significativa na massa de células beta em comparação com camundongos selvagens tratados com a mesma dieta (Mitchell *et al*., 2015).

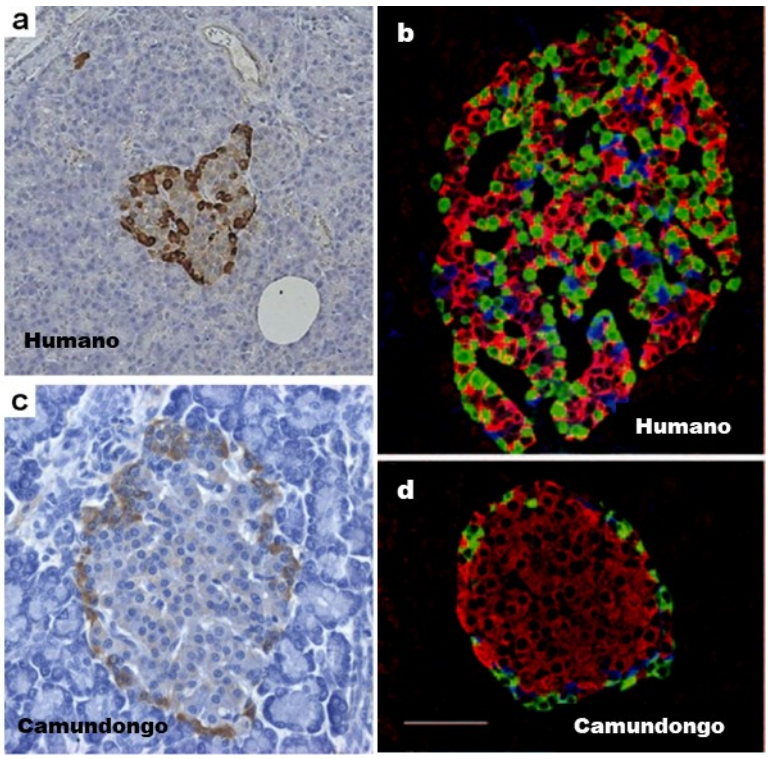

Figura 3. Fotomicrografias de ilhotas humanas (a,b) e de ilhotas de camundongo (c,d). Nas imagens (a) e (c), a marcação é por Hematoxilina de Ehrlich e imunoperoxidase para glucagon. Nas imagens (b) e (d), a marcação foi feita por imunofluorescência para insulina (vermelho), glucagon (verde) e somatostatina (azul). Figura adaptada de Cabrera *et al*., 2006, *PNAS* 103(7):2334-2339 e Collares-Buzato CB, *Molecular Nutrition and Diabetes, Elsevier/Academic Press, San Diego, 2015, 115-130.*

1.4 Vias de Sinalização Wnt

As proteínas Wnts fazem parte de uma conservada família de glicolipoproteínas, com 19 subtipos descritos até o momento nos mamíferos. Essa diversidade permite complexidade e especificidade nas sinalização por Wnt (revisado por MacDonald, Tamai, & He, 2009). São três as mais conhecidas vias de sinalização ativadas pelas proteínas Wnts. A mais estudada é a *via Wnt canônica* (ou Wnt/β-catenina), que é regulada pela quantidade de um co-ativador transcripcional, a β-catenina, que por sua vez controla os principais programas de expressão gênica do desenvolvimento embrionário e da vida adulta dos animais (revisado por revisado por MacDonald, Tamai, & H, 2009; revisado por revisado por Clevers & Nusse, 2012). Estudos têm relacionado a via Wnt canônica à T2DM e ao quadro de resistência periférica à insulina (Abiola *et al.,* 2009; Figeac *et al*., 2010, Schinner, 2010; Kuljanin *et al*., 2017).

Além da via Wnt canônica, outras duas vias de sinalização bem conhecidas que podem ser ativadas pelas proteínas Wnts são: a *via Wnt/PCP* (de polaridade celular planar), que coordena a polarização das células durante o desenvolvimento do epitélio através GTPases da família Rho; e a *via Wnt/*Ca^{2+}, que desempenha um papel importante na manutenção da homeostase do organismo através ativação de proteínas heterotriméricas G, as quais ativam a fosfolipase C, que estimula o aumento intracelular de Ca^{2+} (Langenbacher & Chen, 2008; revisado por Katoh & Katoh 2017). Essas vias de sinalização celular, no entanto, não têm sido relacionadas diretamente à T2DM.

As proteínas Wnt interagem com um receptor chamado *Frizzled* (Fz) nas três vias de sinalização mencionadas anteriormente, mas no caso da via Wnt canônica também há interação com co-receptores chamados LRP5/6 (*Low-Density Lipoprotein Receptor-Related Proteins 5 and 6*) (revisado por Lloyd-Lewis *et al*., 2013). Após a interação de Wnt com o receptor e co-receptores, uma reação em cascata é desencadeada resultando na acumulação de β-catenina no citoplasma (revisado por Murtaugh, 2008 e Welters & Kulkarni, 2008). Na ausência do estímulo Wnt, o nível de β-catenina citoplasmática é regulado de duas formas: através da sua interação com a molécula caderina nos sítios de adesão intercelular da membrana plasmática, onde tem a função de estabilizar as interações célula-célula, como ocorre em células epiteliais e nas células beta pancreáticas, por exemplo, e também por meio de sua fosforilação por um complexo proteico de degradação (revisado por Murtaugh, 2008; Maschio *et al*., 2016).

Denominado complexo Axina, esse complexo citoplasmático de degradação é constituído pelas proteínas Axina e APC (*Adenomatous Polyposis Coli*), ambas são codificadas por genes supressores de tumor, e pelas cinases CK1 (*Casein Kinase 1*) e GSK-3β (*Glycogen Synthase Kinase*-3β). Ambas cinases fosforilam sequencialmente a região amino terminal da β-catenina, o que resulta em seu reconhecimento pela β-TRCP (uma subunidade da ubiquitina ligase E3), subsequente ubiquitinação e degradação proteossômica dessa proteína chave da via (revisado por MacDonald, Tamai, & H, 2009) (**Figura 4**).

São dois os tipos de Axinas relacionadas à regulação da via Wnt canônica: a Axina 1, que é frequentemente associada apenas ao complexo Axina/APC/CK1/GSK-3β participando, portanto, no mecanismo inibitório clássico da via, e a Axina 2, que tem seu gene expresso como produto da ativação da via Wnt canônica, sendo um regulador de *feedback* negativo da via (Rulifson *et al*., 2007; Leung, *et al*., 2002). Em certos tipos

de câncer associados com defeitos na via Wnt canônica, embora uma expressão aumentada de Axina 2 tenha sido observada, isso não parece ser suficiente para regular negativamente os níveis de β-catenina e inibir o processo de proliferação celular desordenada (Leung, *et al*., 2002).

Quando há sinalização Wnt canônica, ocorre a desestabilização do complexo de fosforilação da β-catenina mediada pela proteína citoplasmática Dvl (*Disheveled*) e, dessa forma, leva ao acúmulo de β-catenina no citoplasma. Uma fração do *pool* citoplasmático de β-catenina é deslocada para o núcleo onde interage com fatores de transcrição, como o TCF/LEF (*T Cell Specific Factor/Lymphoid Enhancer-binding Factor*), que por sua vez, deslocam proteínas *Groucho* (Gro) repressoras de genes como c-Myc e Ciclina D associados ao processo de proliferação celular (revisado por Murtaugh, 2008; Verkaar, Cadigan, & Van-Amerongen, 2013; Stamos & Weis, 2013) (**Figura 4**). No núcleo, a Axina 2 pode também formar um complexo com β-catenina e TCF para reprimir diretamente a expressão de c-Myc, que é um gene alvo da Wnt canônica (Li *et al*., 2015; Rennoll *et al*., 2014).

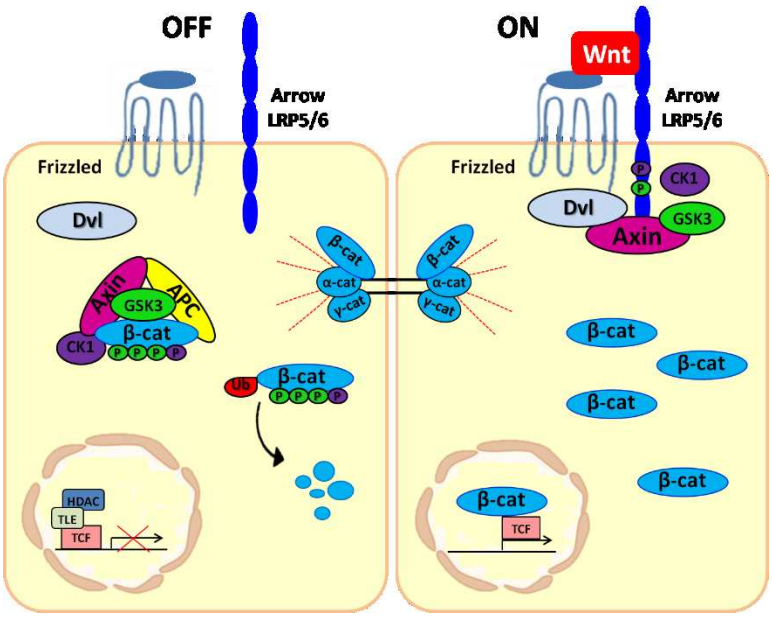

Figura 4. Via de sinalização Wnt canônica. Na ausência de sinalização por Wnt (esquerda), a proteína citoplasmática β-catenina está ligada por um complexo de degradação constituído pelas proteínas Axin/APC/CK1/GSK3. A fosforilação de β-catenina por GSK-3β tem como alvo a ubiquitinação e a degradação proteossômica. Quando ocorre sinalização Wnt (direita), a sua ligação com o receptor Frizzled e com o co-receptor LRP5/6 ativa a proteína Disheveled (Dvl), que perturba o complexo de degradação, recrutando Axina, e permitindo assim um aumento do nível citoplasmático

de β-catenina não fosforilada. Com isso, uma fração do *pool* citoplasmático transloca-se para o núcleo e associa-se às proteínas de ligação ao DNA como os da família TCF. Adaptado de Logan & Nusse, 2004, *Annu. Rev. Cell Dev. Biol. 20*:781–810 e revisado por Murtaugh, 2008 e MacDonald, Tamai, & He, 2009.

De acordo com as vias que ativam, as proteínas Wnts podem ser classificadas como canônicas ou não canônicas (revisado por MacDonald, Tamai, & He, 2009; Liu & Habener 2010). Assim, existem proteínas Wnts reconhecidas como canônicas, como as Wnts 1, 3, 3a, 8a e 8b, e outras conhecidas como não canônicas, como as Wnts 4, 5a, 7a, 7b e 11 (Bernard *et al.,* 2008; Buechling & Boutros, 2011). No entanto, existem controvérsias sobre o modo de ação e regulação dessas proteínas. Tem sido sugerido que as proteínas Wnts podem agir de maneiras diferentes dependendo do tecido ou da célula que atuam, não sendo, portanto, membros intrinsecamente canônicos ou não canônicos (revisado por Verkaar, Cadigan & Van-Amerongen, 2013; Gonzalez & Rodríguez, 2017). Por exemplo, algumas evidências sugerem que a Wnt5a pode ativar a via Wnt canônica ou inibí-la dependendo do receptor que interage (Mickels & Nusse 2006, Kuljanin *et al*., 2017). Também foi descrita a possibilidade de uma proteína Wnt poder inibir a ação da outra, como ocorre, *in vitro*, com a Wnt4 (conhecida como não canônica) que pode inibir a ação da Wnt3a (conhecida como canônica) de forma a redirecionar a β-catenina para a membrana plasmática, bloqueando, dessa forma, a sinalização Wnt canônica (Bernard *et al.,* 2008; Bowen *et al.*, 2016).

Além das três principais vias de sinalização Wnt mencionadas anteriormente, também foi relatado que os Wnts podem se ligar aos receptores tirosina cinase Ror e Ryk podendo promover uma outra forma de regulação da sinalização Wnt, que independe de Fz, mas que também pode regular a via Wnt canônica por meio de mecanismos intracelulares que modulam a atividade da β-catenina (Roarty & Rosen, 2010) (**Figura 5)**. Assim sendo, os estudos têm sugerido que, devido à grande variedade de membros Wnts, receptores Fz, co-receptores, co-mediadores, e proteínas efetoras intracelulares, as sinalização Wnt são provavelmente muito mais integradas do que se pensava inicialmente, podendo haver mecanismos de regulação entre elas ditadas pelas interações ligante-receptor e por meio de suas proteínas efetoras (Roarty & Rosen, 2010; Green, Nusse, & van Amerongen, 2014).

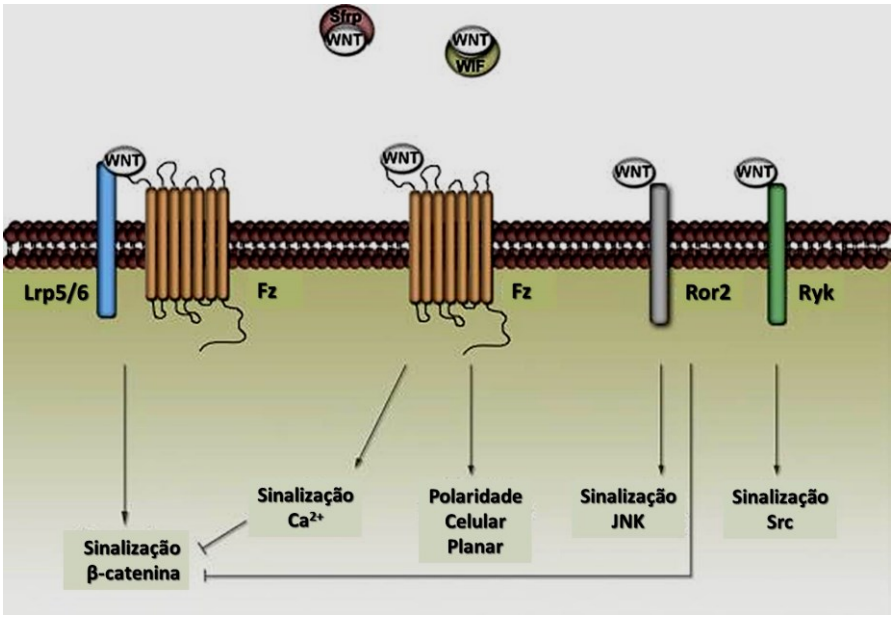

Figura 5. **As sinalização Wnt são diversificadas, apresentando uma regulação também diversificada e podendo haver *crosstalk* entre elas.** Fatores inibidores de Wnt (Wifs) e proteínas Frizzled-relacionadas (Sfrps) podem modular a sinalização Wnt, por meio do sequestro de Wnts dos seus receptores. Além disso, outras vias de sinalização Wnt também podem regular a via Wnt canônica. Adaptado da revisão por Roarty & Rosen, 2010, *Current Opinion in Pharmacology 10*:643–649.

2. JUSTIFICATIVA

Embora os estudos anteriores indiquem que a sinalização Wnt canônica esteja associada à proliferação das células beta em certas condições experimentais (Figeac *et al*., 2010; Mitchell *et al*., 2015), pouco é conhecido sobre as proteínas Wnts no pâncreas endócrino no contexto da pré-diabetes tipo 2. Por exemplo, não se sabe se as células da ilhota pancreática as secretam, qual seria a sua forma de secreção, ou se essas proteínas desempenhariam um papel autócrino/parácrino/endócrino na regulação da massa de células e/ou função secretora das ilhotas. Foi verificado que ilhotas humanas expressam mRNAs que codificam os 10 subtipos de receptores Fz para Wnt, tanto os relacionados à via Wnt canônica quanto os aqueles relacionados à sinalização Wnt não-canônica, mas a relevância funcional desses receptores ainda não está estabelecida (Amisten *et al*., 2013, 2017). Além disso, foi relatado que um antagonista de Fz, a proteína SFRP1 (*Secreted Frizzled-Related Protein 1*), inibe a secreção de insulina em ilhotas de humanos e de ratos e na linhagem de célula beta Ins-1, mas não se sabe se essa inibição ocorre através da sua interação com receptores Fz ou com as proteínas Wnts (Schinner

2010; Salehi *et al*., 2012; Amisten *et al.,* 2013).

Nosso grupo de pesquisa tem utilizado camundongos C57BL/6/JUnib alimentados com uma dieta hiperlipídica (HFD) por 60 dias para induzir alterações metabólicas características da pré-diabetes (Carvalho *et al*., 2012; Oliveira *et al*., 2014; 2015). Nesta fase da pré-diabetes, verificamos, em trabalho preliminar, que, em homogeneizados de ilhotas hiperplásticas isoladas de camundongos, há aumento significativo na expressão de mRNA de *Ctnnb1* (β-catenina), *Ccnd1* (Ciclina D1) e *Ccnd2* (Ciclina D2), que foi acompanhado por aumento significativo no conteúdo proteico de β-catenina e Ciclina D, o que pode ser indicativo de ativação da via Wnt canônica (Maschio, 2014).

3. HIPÓTESE

A hipótese inicial desta Tese de Doutorado é que a via Wnt canônica está envolvida na expansão compensatória da massa de célula beta na pré-diabetes tipo 2, e que células do pâncreas exócrino e endócrino (incluindo a própria célula beta) secretam Wnts que, por sua vez, poderiam regular de forma parácrina e/ou autócrina esta via.

4. OBJETIVOS

4.1 Objetivo geral

O objetivo geral dessa Tese de Doutorado foi investigar: 1) se a via Wnt canônica tem papel na hiperplasia compensatória das células beta durante a pré-diabetes experimental em camundongos machos C57BL/6/JUnib alimentados com dieta hiperlipídica (HFD) por 60 dias; 2) se as células do pâncreas exócrino e endócrino são capazes de expressar as proteinas Wnts e se a expressão gênica pode estar alterada nos grupos experimentais estudados; e 3) se, em condições *in vitro,* ilhotas isoladas de camundongos podem secretar fatores solúveis (Wnts) que, por sua vez, podem induzir a proliferação celular ou alteração na função secretora da linhagem de célula beta MIN6.

4.2 Objetivos específicos

Os objetivos específicos foram:

1) Investigar a ativação da via Wnt canônica em ilhotas hiperplásicas (obtidas de animais expostos à HFD por 60 dias) e não hiperplásicas (obtidas de animais expostos à HFD somente por 30 dias) e em ilhotas dos respectivos animais controles alimentados com ração padrão pelo mesmo período de tempo. Para isso foram executadas as seguintes etapas: imunofluorescência indireta, em criocortes do pâncreas, para análise da distribuição celular da proteína β-catenina ativada (forma funcional da proteína), e RT-PCR e qPCR, em homogenizados de ilhotas isoladas, para análise de expressão gênica de proteinas associadas à ativação dessa via, tais como *Ctnnb1* (β-catenina), *Ccnd1* (Ciclina D1), *Ccnd2* (Ciclina D2), *c-Myc* e *Tcf7l2*, além das duas isoformas de insulina presente em camundongo, *Ins 1* (Insulina 1) e *Ins 2* (Insulina 2), e de proteínas inibidoras/moduladoras da via como as Axinas 1 e 2, o GSK-3β, Dact-1 e CoupTF-II;

2) Analisar a expressão gênica dos 19 subtipos de Wnts (descritos em camundongos) por meio de RT-PCR e qPCR em homogenizados de ilhotas isoladas, de pâncreas e de tecido adiposo peripancreático (controle positivo) no grupo dos animais com ilhotas hiperplásicas, ou seja expostos à HFD por 60 dias, e no seu grupo controle (CTL), além de homogeneizados de embriões de camundongos com 12 dias de desenvolvimento (também usado como controle positivo);

3) Analisar, por imunofluorescência indireta, em criocortes do pâncreas dos animais HFD 60d (com ilhotas hiperplásicas) e controle (CTL) (com ilhotas não hiperplásicas), quais tipos celulares do pâncreas exócrino ou endócrino (ilhotas) expressam determinados Wnts selecionados na etapa anterior;

4) Por fim, analisar *in vitro* na linhagem celular de células beta MIN6, se a co-cultura com ilhotas isoladas hiperplásicas ou não hiperplásicas, tem alguma influência sobre a proliferação celular e/ou função secretora das células desta linhagem.

5. ESTRUTURA DA TESE

Os resultados dessa Tese estão apresentados nos **Capítulos 2** e **3** e foram organizados na forma de manuscritosartigos científicos, redigidos em Inglês. No **Capítulo 2**, encontra-se o artigo publicado em revista internacional indexada,

intitulado: *"Activation of the Wnt/β-catenin pathway in pancreatic beta cells during the compensatory islet hyperplasia in prediabetic mice*", onde demonstramos que ilhotas pancreáticas hiperplásicas de camundongos pré-diabéticos, alimentados com HFD por 60 dias, apresentaram translocação nuclear da proteína β-catenina ativa em células beta pancreáticas, que foi associada com aumento significativo do conteúdo protéico e expressão gênica de β-catenina, bem como de ciclinas D1/2, c-Myc e insulina 2 (genes alvos da via Wnt canônica) mas não de *Tcf7l2* (fator de transcrição). Em contraste, essas alterações não foram observadas em ilhotas pancreáticas de camundongos tratados com HFD por apenas 30 dias, os quais não apresentam hiperplasia significativa da célula beta pancreática. Além disso, são apresentados os resultados adicionais ao trabalho publicado, relacionados à expressão dos inibidores/moduladores da via (GSK-3β, Axina 1/2, Dact-1 e Coup-TFII), além de *Ins 1* (Insulina 1), nas ilhotas isoladas dos grupos experimentais.

No **Capítulo 3,** é apresentado o manuscrito a ser submetido para publicação em revista internacional indexada. No artigo intitulado: "*Wnts, expressed by pancreatic islet cells, may be involved in the compensatory beta-cell proliferation"* são apresentados os resultados que confirmam a expressão gênica de Wnts no pâncreas como um todo e em ilhotas hiperplásicas isoladas de camundongos pré-diabéticos e que mostram que o co-cultivo de células MIN6 com ilhotas de camundongos controle e de pré-diabéticos não afeta a secreção de insulina, mas resulta em aumento significativo da proliferação celular, o qual foi parcialmente inibido por antagonistas da via Wnt, o IWR-1 e o IWP-2. No Capítulo 4, encontram-se as considerações finais e a conclusão dessa Tese e, por fim, no **Capítulo 5**, estão as referências utilizadas no presente **Capítulo 1**.

Capítulo 2

ARTIGO CIENTÍFICO I

Activation of the Wnt/β-catenin pathway in pancreatic beta cells during the compensatory islet hyperplasia in prediabetic mice[1]

ABSTRACT

The Wnt/β-catenin signaling pathway, also known as the canonical Wnt pathway, plays a role in cell proliferation and differentiation in several tissues/organs. It has been recently described in humans a relationship between type 2 diabetes (T2DM) and mutation in the gene encoding the transcription factor *TCF7L2* associated to the Wnt/β-catenin pathway. In the present study, we demonstrated that hyperplastic pancreatic islets from prediabetic mice fed a high-fat diet (HFD) for 60d displayed nuclear translocation of active β-catenin associated with significant increases in protein content and gene expression of β-catenin as well as of cyclins D1, D2, c-Myc, Ins2 (target genes of the Wnt pathway) and Coup-TFII (Wnt pathway regulator), but a decrease in Axin2 expression (inhibitor of the pathway). Meanwhile, these alterations were not observed in pancreatic islets from 30d HFD-fed mice, that do not display significant beta cell hyperplasia. These data suggest that the Wnt/β-catenin pathway is activated in pancreatic islets during prediabetes and may play a role in the induction of the compensatory beta cell hyperplasia observed at early phase of T2DM.

[1] Parte dos dados desse capítulo foram publicados no periódico *Biochemical and Biophysical Research Communications 47*8(1) 534-1540 (2016). Anexo 3 - autorização para reproduzir o artigo.

1. INTRODUCTION

The pathogenesis of type 2 diabetes mellitus (T2DM) involves the development of an intolerance to glucose and a peripheral resistance to insulin that can be partially compensated by insulin hypersecretion and/or pancreatic beta cell mass expansion [1-3] The increase in beta cell mass occurs mainly during the prediabetes phase and as consequence of hypertrophy and hyperplasia of this cell type [1,4] Hyperplasia, in turn, is result of beta cell self-replication and/or neogenesis from other pancreatic cells [5,6]. The mechanisms linking obesity and insulin resistance to beta cell hyperplasia are not fully known.

The Wnt/β-catenin signaling pathway has been reported to be involved in cell growth and differentiation in several tissues/organs, including the endocrine pancreas [7-9]. Wnts, belonging to the Wnt family of secreted glycoproteins, interact with its receptor, known as Frizzled, and co-receptor, called LRP5/6 (low-density lipoprotein related receptor proteins 5 and 6), at the membrane surface of the target cell and trigger a cascade reaction resulting in β-catenin accumulation in the cytoplasm (reviewed by [8,10]. This cytoplasmic β-catenin pool is shifted to the nucleus where interacts with the transcription factor TCF/LEF (specific T Cell Factor/Lymphoid Enhancer-binding Factor) and activates the expression of several target genes related to cell proliferation and survival [8,10]. In the absence of Wnt stimulation, the cytoplasmic level of β-catenin is kept low by the interaction with the adhesion molecule cadherin at intercellular adhesion sites as well as by the proteossomal degradation triggered by the phosphorylation of β-catenin by the APC/Axin/CK1/GSK-3β complex as reviewed by [8,9].

Evidences for the implication of canonical Wnt pathway in beta cell proliferation and function come mainly from experiments *in vitro*, using beta cell lineages [11-13], or *in vivo* models, employing genetically modified rodents [14-16]. Nevertheless, *in vivo* studies, particularly those employing *Tcf7l2*-deficient mice, have gathered conflicting results concerning the role of this Wnt effector on beta cell function [14,16]. Yet, a putative role of the Wnt signaling pathway in the beta cell plasticity during type 2 prediabetes has not been directly addressed.

In this work, we investigated a possible activation of the Wnt/β-catenin signaling pathway in the compensatory beta cell hyperplasia in a model of type 2 prediabetes [4]. For that, C57BL/6 male mice were fed a high-fat diet (HFD) for different periods of

time (30 or 60 days), which differ regarding the stage of the prediabetes (i.e. before and after the beta cell mass expansion is established). We firstly characterized metabolically and morphometrically our model and, then investigated the pancreatic islet cell distribution, protein content and gene expression of some proteins associated to the activation of the Wnt/β-catenin pathway (i.e, β-catenin, *TCF7L4*, cyclins D1 and D2, c-Myc), as well as of inhibitors (i.e. GSK-3β, Axin1, Axin2) and regulators/modulators of this pathway (i.e. Coup-TFII (Chicken ovalbumim upstream promoter transcription factor II) and Dact-1 (Dishevelled-binding antagonist of beta-catenin 1) [40,41] in both control (fed a chow diet, 4.5% lipid content, w/w) and HFD mice.

2. MATERIAL AND METHODS

2.1. Animal treatment and metabolic evaluation

Male C57BL/6/JUnib mice were obtained from the breeding colonies maintained at the University of Campinas (UNICAMP, Brazil). The animals were housed at 25±1°C on a 12h light/12h dark cycle, and had free access to water and food. When aged 4-5 months (with a body weight mean of 30.1±0.16g), the animals were divided in two groups. The treated group was fed a high-fat and hypercaloric diet (HFD) for 30 days (HFD 30d) or for 60 days (HFD 60d) containing 21g% lipids (mainly lard) (w/w), 50g% carbohydrates and 20g% proteins (4.7 kcal/g). The control group (CTL) was fed a standard rodent diet containing 4.5g% lipids (w/w), 53g% carbohydrates and 23g% proteins (2.9 kcal/g) for the same periods of time. All animals were weighed before starting the diet period and at the end of it; values were expressed as percentage of body weight gain over the initial body weight. The measurement of fast and post-prandial glycemia, insulinemia and response to the insulin tolerance test (ITT; values expressed as area under curve (AUC)) were done as previously described [4,17]. Insulin concentration in plasma samples was measured using the Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem Inc.). All blood samples for all biochemical analysis were collected between 9:00 and 11:00 am. All experimental protocols used were approved by the Ethics Committee on Animal Use (CEUA) of UNICAMP under protocols #2815-1 and #3443-1).

2.2 Immunohistochemistry for insulin and pancreas morphometry

Pancreas were fixed for 18h in 4% paraformaldehyde (in 0.05M Tris-buffered saline, TBS, pH 7.4), sectioned in 3 fragments of similar sizes and separately embedded in paraffin. Two sections per block were randomly selected and processed for insulin immunoperoxidase reaction as previously described [4,17,18]. All islets and pancreas sections were photographed with a digital camera coupled to a Nikon Elipse E800 microscope. The *relative area of the beta cell* was determined by dividing the sum of the total areas of the insulin-positive cells (beta cells) by the total area of the pancreas section, that was measured using the free software ImageTool (http://ddsdx.uthscsa.edu/dig/itdesc.html).

2.3. Immunolocalization of active β-catenin in mouse pancreas cryosections

The cell localization of β-catenin was performed in -20°C acetone-fixed pancreas cryosections by triple labeling of active β-catenin, insulin (by indirect immunofluorescence) and DAPI (4',6-diamidino-2-phenylindole). For that, the pancreas sections were 2h-incubated with a primary antibody that detects only the active (unphosphorylated) form of the β-catenin (see Table S1 in supplementary material) at room temperature (RT). After washings with TBS, the pancreas sections were incubated with their respective secondary antibody conjugated with FITC for 2h at RT. For co-immunolabeling with insulin, the sections were incubated sequentially with an anti-insulin antibody followed by their specific secondary antibody conjugated with TRITC and DAPI (dilution 1:1000, Sigma) [19]. All sections were mounted in a commercial antifading agent (Vectashield, Vector Laboratories) and observed using an inverted fluorescence microscope coupled to an image capture system (Observer-Z1; Zeiss - AxioCam MRC). Pancreas sections from control and HFD-fed mice were analyzed and photographed during the same session using identical parameters of the fluorescence microscopy. The nuclear acumulation of active β-catenin in beta cells was determined by detecting the co-labeling of β-catenin and DAPI using the GIMP free software (http://www.gimp.org/). The values obtained were normalized against the area of the islet using the free software ImageJ (http://rsbweb.nih.gov/ij/), and expressed as pixels/islet area. Table S1 (Supplementary material) displays all the antibodies and the

respective dilutions employed in immunohistochemistry and Western Blot (as described below).

2.3 Pancreatic islet isolation

Each pancreas was inflated by injecting 3mL of 0.8mg/mL type V collagenase (Sigma, Cat#C9263) in Hank's balanced salt solution (supplemented with 3mg/mL bovine serum albumin plus 5.6 mM glucose, pH 7.4) and added to 2mL of this collagenase solution (totalizing 4mg of the collagenase/pancreas). Each pancreas was then incubated for 10min at 37°C for enzymatic digestion. Islets were then isolated in gradient of Histopaque®1077 (Sigma) and individually collected under a dissecting microscope. Pools of 300 isolated islets were homogenized in an anti-protease cocktail for immunoblotting or added to RNAlater® (Ambion) for Real Time-PCR as previously described [19,20].

2.5 Western Blot

Aliquots of islet homogenates containing 50µg total protein were applied to a 8 or 10% SDS-PAGE gel and then the samples were fractionated by electrophoresis as previously described [17,19]. Membranes were blocked with 5% dry skimmed milk in TBS containing 0.05% Tween20 (TTBS), overnight at 4°C, and then incubated for 2h at RT with one of the primary antibodies depicted in Table S1 (Supplementary material), followed by incubation with their respective secondary antibody conjugated with HRP. After washings, the membranes were revealed using an enhanced chemiluminescence kit (Chemiluminescent Substrate, Pierce), followed by autoradiography (Amersham) or detection using a chemilumininescence imaging system (GeneGnome XRQ, SynGene). Band intensities were quantified by optical densitometry using software ImageJ and normalized against β-actin band densitometry (internal control).

2.6. Quantitative real-time PCR

Total RNAs were extracted from pools of 300 isolated islets using the RNAqueous kit (Ambion) before being reverse-transcribed into cDNAs. Quantification of mRNAs encoding *Ctnnb1*, *Ccnd1*, *Ccnd2*, *c-Myc*, *Tcf7l2*, *Ins1, Ins2, Gsk3b, Axin1,*

Axin2, Dact-1, Coup-TFII, Gapdh, *Actb1* and *Rps29* were performed using the 7500 ABI system. The primers used in this study are shown in Table S2 in supplementary material. The specificities of amplifications were verified by size characterisation of the amplification products on 2% agarose gel and by melting-curve analyses. The absolute quantities of target transcripts were normalized against the endogenous control *Gapdh* [21], that was demonstrated to be the best internal control for the experimental conditions tested in comparison with *Actb1* and *Rps29* (data not shown).

2.7 Statistical analyses

All numerical results were expressed as means ± standard error of the mean (SEM). For comparison between two groups, statistical significance was assessed using Student's t test (two-tailed). The significance level was set at $p<0.05$. All statistical analyses were performed using the GraphPad Prism Version 5.00 for Windows (GraphPad Software, La Jolla, USA).

3. RESULTS

HFD exposure for 30d or 60d induced significant weight gain above the control levels (HFD 30d 17.7 ± 2.8% (25) *vs* CLT 30d 5.3 ± 0.8% (25); HFD 60d 43.3 ± 2.1% (63) *vs* CLT 60d 9.1± 1.3% (52), ***$p<0.0001$); however, only treatment for 60d with this diet resulted in metabolic alterations tipically of prediabetes such as a marked insulin peripheral resistance (as revealed by the increased ITT AUC; Fig.1j) associated with fast and fed hyperglycemia (Fig.1f,g) and fast and fed hyperinsulinemia (Fig.1h,i). Corroborating the hyperinsulinemia state, 60d HFD mice showed a significant increase in gene expression of insulin 1 in comparison with CLT mice, as revealed by qPCR in homogenates of isolated islets (60d HFD 6134 ± 676.8 (5) *vs* 60d CLT 3146 ± 690.4 (5) *insulin1/Gadph* mRNA ratio; **$p<0.002$). Meanwhile, 30d-exposure to HFD resulted only in an increased postprandial glycemia (Fig.1b) and postprandial insulinemia (Fig.1d) without significant changes in the other metabolic parameters analyzed (Fig.1a,c,e).

The metabolic alterations induced by 60d HFD exposure were accompanied by a significant increase in the relative volume of pancreatic beta cells (Fig.3a) which was

not observed in the pancreas from 30d HFD-fed mice (Fig.2a). This result is in accordance with previous works [4,17], which also showed that the beta cell expansion seen in 60d HFD-fed mice was mainly result of hyperplasia due to beta cell replication.

In the next step of this study, we verified the degree of activation of the canonical Wnt pathway in pancreatic islets at these two distinct stages of prediabetes in mice: when the presence of hyperplastic pancreatic islets (Fig.3a) associated with beta cell expansion (as seen after 60d HFD exposure) is established and when no significative morphometric changes in beta cell mass are detected (as seen in 30d HFD-fed mice) (Fig.2a).

As shown in Fig.2, no alterations of the immunolocalization of active β-catenin (which was immunodetected mainly at the intercellular region and cytoplasm of islet cells) (Fig.2b), neither in the protein cell content (c) or gene expression of β-catenin (d) were seen in islet homogenates of 30d HFD-fed mice. In contrast, hyperplastic islets from 60d HFD-fed mice showed nuclear translocation of active β-catenin (Fig.3c) accompanied by an increase in the islet content of this protein (Fig.3d) and β-catenin mRNA transcripts (Fig.3f). In addition, homogenates of islets isolated from these prediabetic mice displayed an increment in protein islet content of cyclin D1/2 (Fig.3e) as well as an increase in gene expression of cyclins D1 (Fig.3g) and D2 (Fig.3h), c-Myc (Fig.3i) and insulin 2 (Fig.3j), that have been shown to be target genes of the Wnt pathway [11,13, 15, 22], but not of TCF7L2, the transcription factor (Fig.3k).

As shown in Fig.4, only Axin2 and Coup-TFII showed alterations in gene expression between the experimental groups among all the Wnt regulators studied. Isolated islets from mice of the HFD 60d group showed a significant decrease in Axin2 gene expression in relation to the CTL group (Fig. 4c). In contrast, the Coup-TFII nuclear receptor showed a significant increase in its gene expression in the HFD 60d group relative to CTL (Fig. 4d). We also observed, by immunohistochemistry, that Dact-1 is relatively well expressed on both exocrine and endocrine pancreas (Fig. 4e), but there was no difference in the degree of gene expression of this protein (Fig 4f), as well as for GSK-3β (Fig. 4a) and Axin1 (Fig. 4b), in islet homogenates of HFD 60d mice relative to CTL.

4. DISCUSSION

Pancreatic beta cells play a critical role in the pathogenesis of T2DM being capable of an adaptive response that involves changes in their secretory function and mass to compensate for the peripheral resistance to insulin in rodents and humans [1-6, 23-25]. Initially, beta cells increase the biosynthesis and release of insulin and then an expansion of beta cell mass usually occur to promote a further increase in insulin plasma concentration required to maintain normoglycemia [1-6, 23,24]. Later, due to a continuously high insulin demand as result of the peripheral resistance to this hormone, beta cells gradually enter in functional failure and death, leading to a permanent hyperglycemic state which requires then exogenous insulin administration [1, 3, 23, 25,26].

There has been an increasing interest in studying intracellular pathways involved in pancreatic beta cell proliferation and secretory function in order to apply this knowledge in molecular and cellular therapies of diabetes [2,24]. The canonical Wnt signaling pathway, well known as the Wnt/β-catenin pathway, has been investigated as inductor of cell proliferation and differentiation in several tissues/organs during embryonic development and in adults. Mutations in members of the Wnt pathway are invariably linked to human congenital defects and some diseases such as cancer [27, 28].

The role of the canonical Wnt signaling pathway in the endocrine pancreas development and physiology was firstly reported by Rulifson and co-workers [12]. They showed that perinatal activation of β-catenin in beta cells of bitransgenic RIP-Cre, β-cat^{active}mice, and lead to an increased proliferation of this cell type while overexpression of Axin had an opposite effect in transgenic mice [12]. Several *in vitro* studies have reinforced this result [11-13]. Employing different beta cell lineages (i.e. MIN6, NIT-1beta, INS-1 cells) and isolated islets, they demonstrated that the activation of the Wnt/β-catenin pathway induced by exposure to synthetic Wnt3a or Wnt-enriched culture media resulted in significant beta cell proliferation as well as an enhancement of the glucose-stimulated insulin secretion [11-13]. However, loss-of-function studies in adult rodents have yielded some controversal data [14,16,29,30]. Conditional knocking-out of proteins associated to activation of the canonical Wnt pathway (i.e. LRP5, TCF4, β-catenin) was shown to have no repercussion in beta cell mass and insulin secretion [14,29] or to impact negatively on beta cell proliferation and secretory function [16,30].

Considering the problems underlying the use of gene knock-out and knock-in technology, whose results should be therefore interpreted with some caution [31-33]. We have taken a different approach to investigate whether the canonical Wnt pathway is activated during the compensatory beta cell mass expansion associated to type 2 prediabetes. By employing HFD-fed mice at two different stages of diabetes, i.e. before (30 days) and after (60 days) the establishment of a significant insulin resistant state, we decided to check whether an increased beta cell mass was associated with cell signs that are typically described in the literature as related to canonical Wnt pathway activation, such as nuclear translocation of β-catenin and increased expression of key proteins of this signaling pathway [34-37].

Our data clearly showed that the expansion of beta cell mass associated with the establishment of a prediabetic state, as seen in 60d high fat diet-fed mice, was parallelled by β-catenin translocation to beta cell nuclei, as well as by a significant increase in β-catenin expression at gene and protein (in its active form) levels, which are indicative of canonical Wnt pathway activation. In contrast, mice treated for only 30 days with this high-fat diet did not show either significant change in beta cell mass or in β-catenin location and expression in pancreatic islets. In addition, isolated islets from 60d HFD-fed mice displayed increased expression of target genes of Wnt pathway related to beta cell proliferation and differentiation, such as *Ccnd1* [13,36,38], *Ccnd2* [11,12,15], *c-Myc* [35,38], and *Ins2* [22], but showed no significant changes in *Tcf7l2* expression, the major effector of the pathway. Although *Tcf7l2* seems to be a target gene of Wnt signaling *in vitro* [11], *in vivo* studies have reported a decrease in *Tcf7l2* islet expression at hyperinsulinemic state or after insulin exposure [39], indicating that the increase in *Tcf7l2* levels, usually used as a marker of Wnt pathway activation, should be revised.

Regarding the Wnt pathway inhibitors analyzed, that form the β-catenin inactivation complex (i.e. the LRP5/6/Axin/GSK-3β complex), only Axin2 showed alteration to its gene expression level in islets of the 60d HDF group in relation to CTL. It has been suggested that the Axin2 gene is targeted by the Wnt/β-catenin pathway being a negative feedback regulator of the pathway when it is activated [12,42]. In addition, Axin2 was identified in the cell nucleus of different human colorectal cancer cell lines (i.e. HCT116, SW480, and SW620), forming a complex with β-catenin and TCF with a possible function of directly repressing c-Myc expression [43,44].

Therefore, the decrease in Axin2 gene expression observed here in hyperplastic islets of prediabetic mice is in agreement with the maintenance of an activated state and increased expression of target genes of the Wnt/β-catenin pathway.

As part of the specific objectives of the present work, we also investigated in our model the expression of two regulatory/modulatory proteins of the canonical Wnt pathway, namely Coup-TFII (Chicken ovalbumin upstream promoter transcription factor II) and Dact-1 (Dapper Homologue-1). It is known that DACT family proteins modulate the Wnt and TGF-β signaling pathways and consequently can regulate several cellular processes such as cell proliferation, differentiation and migration, stem cell behavior, homeostasis, and tissue regeneration during embryonic and adult development [45,41]. The Dact-1 subtype appears to act as an antagonist of the Wnt/β-catenin pathway in various tissues/organs, and the decrease in its expression seems to be related to the tumorigenesis process [46,47]. As shown in the present work, Dact-1 is expressed on both the exocrine and endocrine pancreas, as demonstrated by immunohistochemistry and qPCR. However, the degree of Dact-1 gene expression did not show significant alteration in islets isolated from prediabetic mice compared to controls. The presence of Dact-1 in the pancreas is a novelty; nevertheless, the function of this protein, particularly in beta cells, needs further investigation.

Coup-TFII, also known as NR2F2, is an orphan nuclear receptor, which is involved in several vital processes, such as organogenesis, angiogenesis and metabolic homeostasis [48]. In the pancreas, Coup-TFII is expressed both during embryonic development and in adult tissues, being detected in pancreatic islets, more precisely in beta cells [49]. Bardoux et al (2005) demonstrated that genetically deficient mice for Coup-TFII in pancreatic beta cells display an impaired glucose sensitivity and abnormal insulin secretion. In addition, Coup-TFII seems to be a critical transcription factor for beta cell proliferation and survival in neonatal life [40]. *In vitro* experiments of Coup-TFII overexpression and silencing in beta cells, Boutant et al. [40] found that Coup-TFII gene silencing directly affected the expression of β-catenin and its target genes, such as Cyclin D1/D2 and c-Myc, inhibiting the process of beta cell proliferation and consequently affecting pancreatic development. Therefore, our data showing an increased expression of Coup-TFII in hyperplastic islets corroborates these works and also suggests a role of this transcription factor in the activation of the canonical Wnt pathway during compensatory beta-cell hyperplasia of prediabetic mice after HFD 60d.

Taken all together, our results indicate that the Wnt/β-catenin pathway is activated in pancreatic islets during prediabetes induced by high-fat diet in mice. In accordance with our data, Mitchell and co-workers [16] have interestingly shown that conditional *Tcf7l2*-null mice fed a high-fat diet showed a significant reduction in beta cell mass in comparison with wild-type mice treated with the same diet. Nevertheless, the interpretation of their results allow the following considerations: 1) the authors have not compared these animals fed a high-fat diet with their respective control mice (fed a regular diet) to verify whether they were actually prediabetics and whether the beta cells were actively proliferating as consequence; and 2) they described that the *Tcf7l2*-null mice displayed a significant decrease in the gene expression of GLP1r and insulin 2, that can interfere with beta cell mass independently of Wnt signaling [1-3]. To the best of our knowledge, our study for the first time addresses a possible role of the Wnt signaling in the induction of the compensatory beta-cell hyperplasia observed at the early phase of T2DM.

Future investigation will be necessary to determine the Wnt ligand(s) and its (their) source (autocrine, paracrine or endocrine) responsible for this beta cell expansion associated with the prediabetic condition.

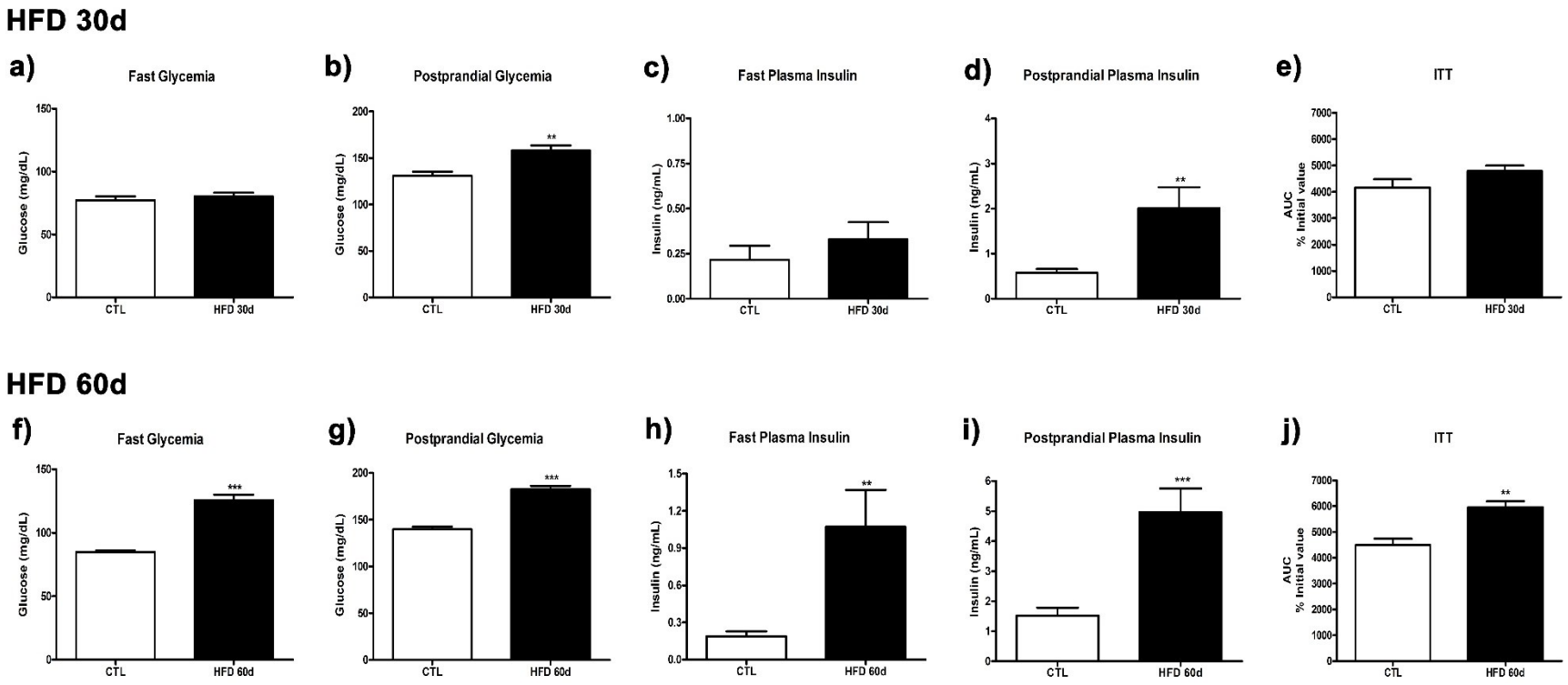

Figure 1. Exposure to high-fat diet (HFD) for 60 days, but not for shorter period (30 days), induces metabolic alterations indicative of prediabetes. As metabolic parameters, we have measured the fast (**a** and **f**) and postprandial glycemia (**b** and **g**), the fast (**c** and **h**) and postprandial insulinemia (**d** and **i**), and the response to the insulin tolerance test (ITT) (shown as AUC of ITT curve, **e** and **j**). All values represent the means + SEM (n= 7-35 animals/group). **p<0.001 or **p<0.005 and ***p<0.0001 as compared to its respective control (CTL) fed a regular diet (Student'st-test).

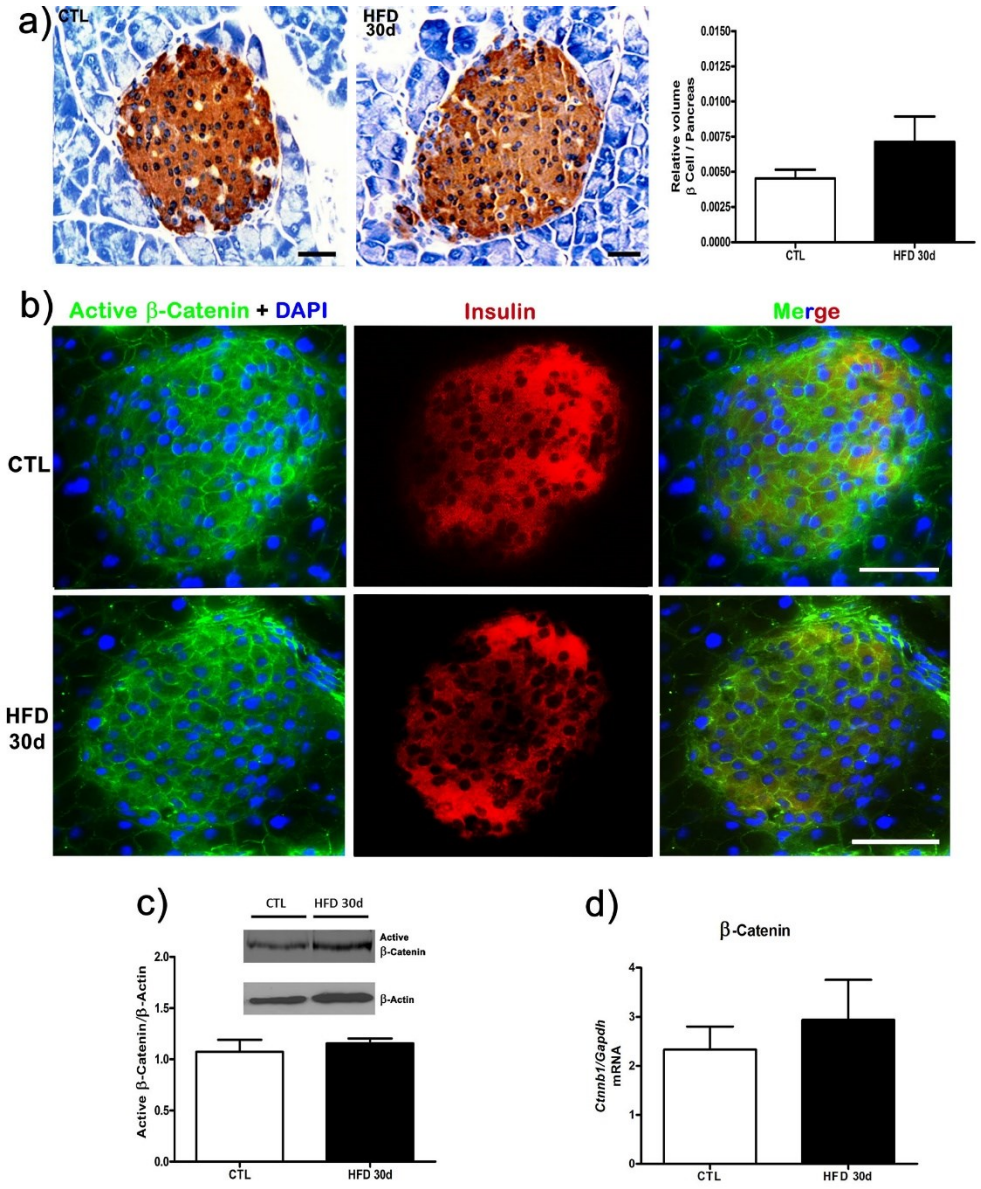

Figure 2. Exposure to HFD for 30 days induces no changes in relative beta cell volume neither in β-catenin location and expression in pancreatic islets. Panel (a) depicts islet sections processed for insulin immunoperoxidase. Morphometric analysis showed no significant difference in relative beta cell volume between the control (CTL) and 30d HFD groups. All values represent the means + SEM. Data were obtained from six pancreas sections from each animal (n = 4 animals/group) and a total of 240–259 islets were analyzed per group. Panel (**b**) shows photomicrographs of dual immunofluorescence for active β-catenin (green) and insulin (red) plus DAPI (blue) in CTL and HFD 30d mice. Active β-catenin was found at intercellular region and in the cytoplasm of islet cells similarly in all groups. No differences in protein (**c**) and gene expression (**d**) of β-catenin were seen in islet homogenates from HFD 30d group and the control. Data in **c** represent means + SEM of 5 membranes from 5 independent experiments. Data in **d** represent means + SEM of 5 islet pools/groups. Scale bars in **a** and **b**, 50 μm.

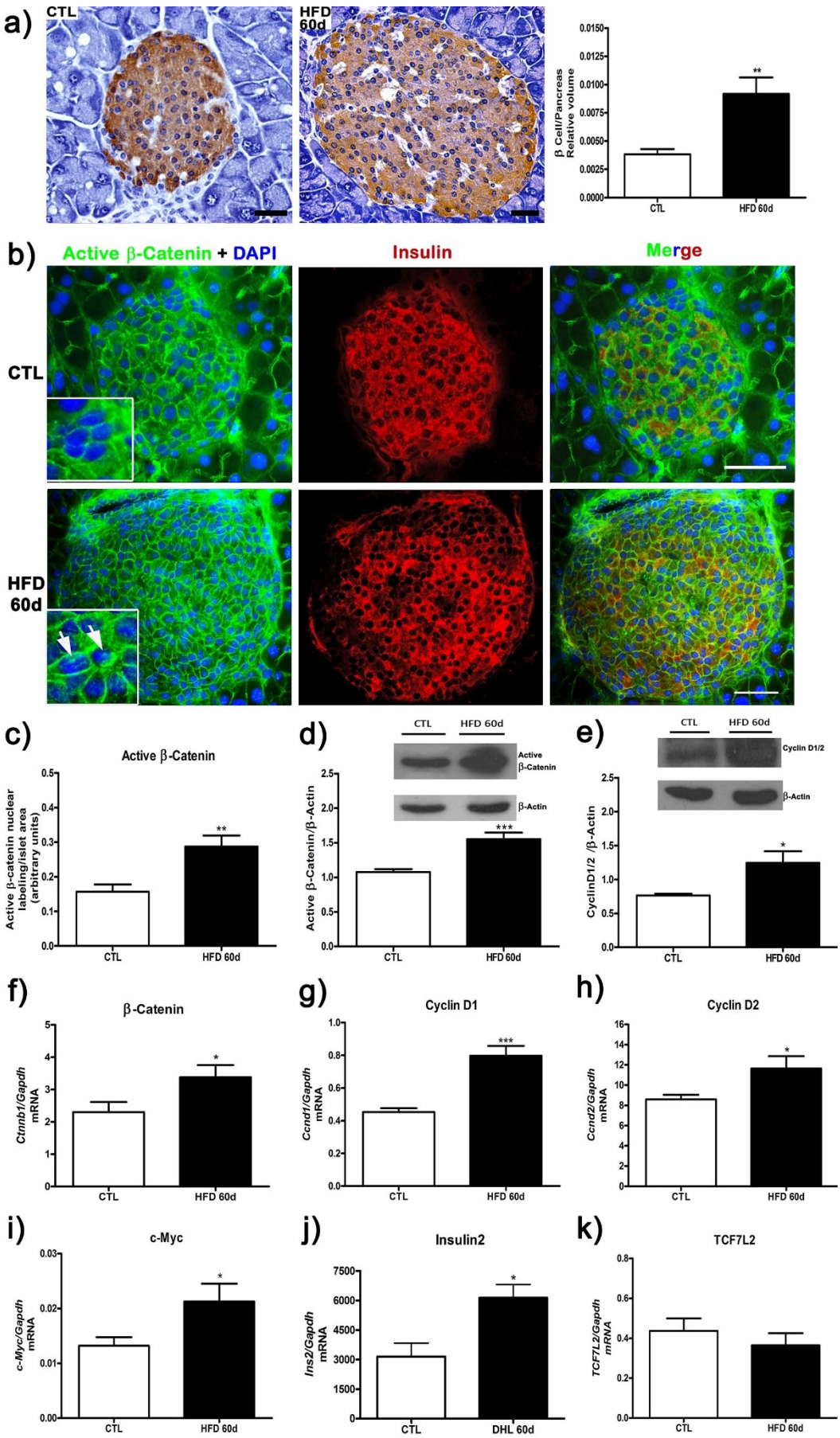
a)
CTL
HFD 60d
β Cell/Pancreas Relative volume
CTL
HFD 60d
b)
Active β-Catenin + DAPI
Insulin
Merge
CTL
HFD 60d
c)
Active β-Catenin
Active β-catenin nuclear labeling/islet area (arbitrary units)
d)
Active β-Catenin/β-Actin
Active β-Catenin
β-Actin
e)
CyclinD1/2 /β-Actin
Cyclin D1/2
β-Actin
f)
β-Catenin
Ctnnb1/Gapdh mRNA
g)
Cyclin D1
Ccnd1/Gapdh mRNA
h)
Cyclin D2
Ccnd2/Gapdh mRNA
i)
c-Myc
c-Myc/Gapdh mRNA
j)
Insulin2
Ins2/Gapdh mRNA
DHL 60d
k)
TCF7L2
TCF7L2/Gapdh mRNA

Figure 3. Prediabetic mice, exposed to HFD for 60 days, display beta cell expansion associated with activation of the Wnt/β-catenin signaling pathway in pancreatic islets. Panel (**a**) depicts islet sections processed for insulin immunoperoxidase. Morphometric analysis showed significant increase in relative beta cell volume in HFD 60d group, in comparison with the control group (CTL) (**p<0.008), which is indicative of beta cell mass expansion. All values represent the means + SEM. Data were obtained from six pancreas sections from each animal (n =5 animals/group) and a total of 296–399 islets were analyzed per group. Panel (**b**) shows photomicrographs of dual immunofluorescence for active β-catenin (green) and insulin (red) plus DAPI (blue) in CTL and HFD 60d mice. An increase in the co-localization of active β-catenin and the nuclear marker DAPI (inset), indicative of translocation of this protein to the nucleus (arrow), was detected in beta cells of prediabetic mice (HFD 60d group) (**b**) as compared to the control (CLT), and confirmed quantitatively (**c**) (50-51 islets/group) (**$p<0.008$). This was associated with a significant increase in active β-catenin islet content (**d**) (***$p<0.001$) and in *Cnntb1* gene expression (**f**) (*$p<0.05$) in HFD 60d mice as compared to the control. In addition, HFD 60d islets display significant increase in protein content of Cyclin D1/2 (**e**) (*$p<0.02$) as well as in gene expression of *Ccnd1* (**g**) (***$p=0.0002$), *Ccnd2* (**h**) (*$p<0.05$), *c-Myc* (**i**) (*$p<0.05$) and *Ins2* (j) (*$p<0.05$), but not of *Tcf7l2* (**k**), relative to CTL. Data in **d** and **e** represent means + SEM of 5-6 membranes from 5 independent experiments. Data in **f** to **j** represent means + SEM of 5-7 islet pools/group. Scale bars in **a** and **b**, 50 μm.

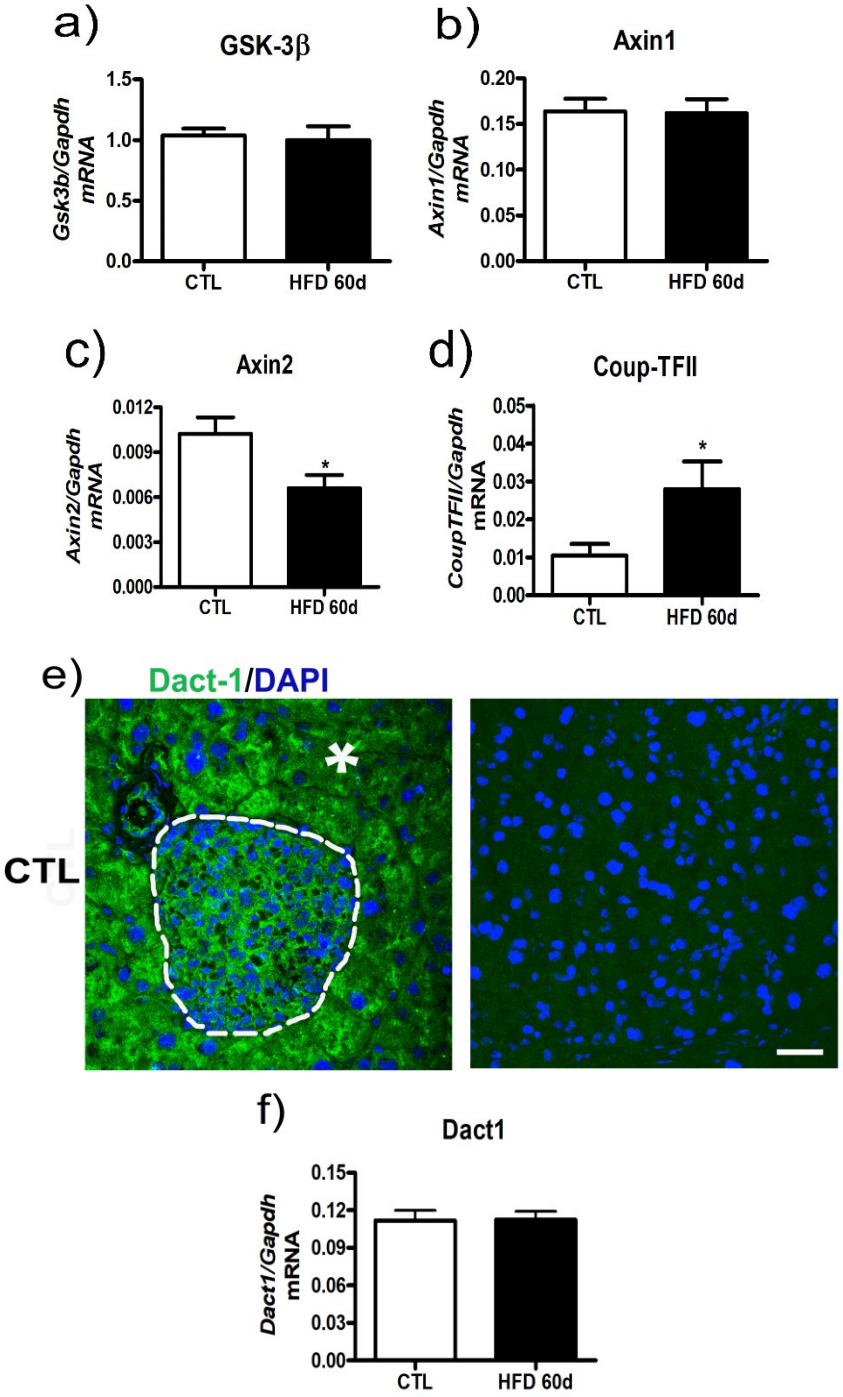

Figure 4. Expression of regulatory proteins are associated with the activation of the Wnt/β-catenin pathway in prediabetic mice exposed to HFD for 60 days. A significant decrease of *Axin2* expression (**c**) *(*p<0.02)* and a significant increase of *CoupTFII* expression *(*p<0.05*) (**e, left**) were observed in HFD 60d islets relative to CTL, and this can be associated with β-catenin translocation. Panel shows photomicrograph of immunofluorescence for Dact-1 (green) plus TO-PRO (nuclei in blue) in CTL mice; panel **e**, at right, shows the negative control of the immunoreaction. Dact-1 was immunodetected both in islet cells and in the exocrine pancreas (asterisk) of CTL mice. No difference in gene expression of *Dact1* was observed between the experimental groups, the CTL and HFD 60d mice (**f**) Data in (a) to (d) and (f) represent means + SEM of 5-7 islet pools/group. Scale bars in **e**, 50 μm.

REFERENCES

[1] C.B. Collares-Buzato, High-fat diets and β-cell dysfunction: molecular aspects, in: D. Mauricio (Eds.), Molecular Nutrition and Diabetes, Elsevier/Academic Press, San Diego, 2015, pp. 115-130.

[2] Y.C. Lee, J.H. Nielsen, Regulation of beta cell replication. Mol. Cell. Endocrinol. 297 (2009) 18-27.

[3] M. Prentki, C.J. Nolan, Islet beta cell failure in type 2 diabetes. J. Clin. Invest. 116 (2006) 1802-12.

[4] R.B. Oliveira, D.A. Maschio, C.P.F. Carvalho, C.B. Collares-Buzato, Influence of gender and time diet exposure on endocrine pancreas remodeling in response to high fat diet-induced metabolic disturbances in mice. Ann. Anat. 200 (2015) 88-97.

[5] Q. Li, Z-Q. Lail, Recent progress in studies of factors that elicit pancreatic β-cell expansion. Protein Cell. 6 (2015) 81–87.

[6] E. Montanya, Insulin resistance compensation: not just a matter of beta-cells? Diabetes 63 (2014) 832-834.

[7] T. Jin, The WNT signallig pathway and diabetes mellitus. Diabetologia 51 (2008) 1771-1780.

[8] L.C. Murtaugh, The what, where, when and how of Wnt/beta-catenin signaling in pancreas development. Organogenesis 4 (2008) 81-86.

[9] B.T. MacDonald, Tamai, & He, K. Tamai, X. He, Wnt/β-catenin signaling: components, mechanisms, and diseases. Dev. Cell. 17 (2009) 9-26.

[10] H.J. Welters, R.N. Kulkarni, Wnt signalling: relevance to β-cell biology and diabetes. Trends Endocrinol. Metab. 19 (2008) 349-355.

[11] S. Gui, G. Yuan, L. Wang, L., et al., Wnt3a regulates proliferation, apoptosis and function of pancreatic NIT-1 beta cells via activation of IRS2/PI3K signaling. J. Cell. Biochem. 114 (2013) 1488-1497.

[12] I.C. Rulifson, S.K Karnik, P.W Heiser, et al., Wnt signaling regulated pancreatic beta cell proliferation. Proc. Natl. Acad. Sci. USA. 104 (2007) 6247-6252.

[13] S. Schinner, F. Ülgen, C. Papewalis, et al., Regulation of insulin secretion, glucokinase gene transcription and beta cell proliferation by adipocyte-derived Wnt signalling molecules. Diabetologia 51 (2008) 147-154.

[14] S.F. Boj, J. H. van El, M. Huch, et al., Diabetes risk gene and Wnt effector Tcf7l2/TCF4 controls hepatic response to perinatal and adult metabolic demand. Cell 151(2012) 1595-1607.

[15] F.B.U. Figeac, M. Faro, N. Chelali, et al., Neonatal growth and regeneration of β-cells are regulated by the Wnt/β-catenin signaling in normal and diabetic rats. Am. J. Physiol. Endocrinol. Metabol. 298 (2009) 245-256.

[16] R.K. Mitchell, A. Mondragon, L. Chen, et al., Selective disruption of Tcf7l2 in the pancreatic β cell impairs secretory function and lowers β cell mass. Hum. Mol. Genetics 5 (2015) 1390–1399.

[17] R.B. Oliveira, C.P.F. Carvalho, C.C. Polo, et al., Impaired compensatory beta-cell function and growth in response to high-fat diet in LDL receptor knockout mice. Int. J. Exp. Path. 95 (2014) 296–308.

[18] C.P.F. Carvalho, J.C., Martins, D.A. da Cunha, et al., Histomorphology and ultrastructure of pancreatic islet tissue during in vivo maturation of rat pancreas. Ann. Anat. 188 (2006) 221-234.

[19] C.P.F. Carvalho, R.B. Oliveira, A. Britan, et al., Impaired β-cell-β-cell coupling mediated by Cx36 gap junctions in prediabetic mice. Am. J. Physiol. Endocrinol. Metab. 303 (2012) 144-151.

[20] V.T.F.L. Falcão, D.A. Maschio, C.C. de Fontes, et al., Reduced insulin secretion function is associated with pancreatic islet redistribution of cell adhesion molecules (cams) in diabetic mice after prolonged high fat diet. Histochem. Cell. Biol. 146 (2016) 13-31.

[21] S.A. Bustin,V. Benes, J. A. Garson, et al., The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55 (2009) 611-622.

[22] J. Lee, K. Kim, S.W. Yu, E-K. Kim, Wnt3a upregulates brain-derived insulin by increasing NeuroD1 via Wnt/β-catenin signaling in the hypothalamus. Mol. Brain 9 (2016) 1-24.

[23] H. Sone, Y. Kagawa, Pancreatic beta cell senescence contributes to the pathogenesis of type 2 diabetes in high-fat diet-induced diabetic mice. Diabetol. 48 (2005) 58-67.

[24] C.G. Liew, P.W. Andrews, Stem cell therapy to treat Diabetes Mellitus. Rev Diabet. Stud. 5 (2008) 203-219.

[25] S.E. Kahn, R.L. Hull, K.M. Utzschneider, Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 14 (2006) 840-846.

[26] T. Tomita, Apoptosis in pancreatic β-islet cells in type 2 diabetes. Bosn. J. Basic Med. 3 (2016) 162-79.

[27] H. Clevers, Wnt/beta-catenin signaling in development and disease. Cell 127 (2006) 469-480.

[28] B.R. Hu, A.S. Fairey, A. Madhav, at al., AXIN2 expression predicts prostate cancer recurrence and regulates invasion and tumor growth. Prostate 76 (2016) 597-608.

[29] T. Fujino, H. Asabab, M.J. Kang, et al., Low-density lipoprotein receptor-related protein 5 (LRP5) is essential for normal cholesterol metabolism and glucose-induced insulin secretion. Proc. Natl. Acad. Sci. USA. 100 (2003) 229-234.

[30] L. Elghazi, A.P. Gould, A.J. Weiss, et al., Importance of β-Catenin in glucose and energy homeostasis. Sci. Rep. 2 (2012) 693.

[31] M.I. McCarthy, P. Rorsman, A.L. Gloyn, TCF7L2 and diabetes: a tale of two tissues, and of two species. Cell Metabol. 17 (2013) 157-159.

[32] Z. Liu, J.F. Habener, Wnt signaling in pancreatic islets. in: M.S. Islam (Eds), The Islets of Langerhans. Springer, New York, 654 (2010) pp. 391-419.

[33] G. Teitelman, M. Kedees, Mouse insulin cells expressing an inducible RIPCre transgene are functionally impaired. J. Biol. Chem. 290 (2015) 3647-3653.

[34] H-Z. Chen, Q-F. Liu, L. Li, et al., The orphan receptor TR3 suppresses intestinal tumorigenesis in mice by downregulating Wnt signaling. Gut 61 (2012) 714-724.

[35] N. Hou, B. Ye, X. Li et al., Transcription Factor 7-like 2 mediates canonical Wnt/β-catenin signaling and c-Myc upregulation in heart failure. Circ. Heart Fail. 9 (2016) pii: e003010. doi: 10.1161/CIRCHEARTFAILURE.116.003010.

[36] Q. Shi, S. Luo, H. Jia, Wnt/β-catenin signaling may be involved with the maturation, but not the differentiation, of insulin-producing cells. Biomed. & Pharmacother. 67 (2013) 745–750.

[37] J. Zhang, J.R. Shemezis, E.R. McQuinn et al., AKT activation by N-cadherin regulates beta-catenin signaling and neuronal differentiation during cortical development. Neural Development 8 (2013) 7-23. http://www.neuraldevelopment.com/content/8/1/7.

[38] Z. Liu, J.F. Habener, Glucagon-like peptide-1 activation of TCF7L2-dependent Wnt signaling enhances pancreatic beta cell proliferation. J. Biol. Chem. 283 (2008) 8723-8735.

[39] J. Columbus, Y. Chiang, W. Shao, et al., Insulin treatment and high-fat diet feeding reduces the expression of three Tcf genes in rodent pancreas. J. Endocrinol. 207 (2010) 77-86.

[40] M. Boutant, O.H.P Ramos, C. Tourrel-Cuzin, et. al., COUP-TFII controls mouse pancreatic β-cell mass through GLP-1-β-catenin signaling pathways. PloS one. 7(1), year??? e30847.

[41] F.R. Schubert, D.R. Sobreira, R.G. Janousek, et al., Dact genes are chordate specific regulators at the intersection of Wnt and Tgf-β signaling pathways.
BMC Evol Biol. 14 (2014) 157.

[42] J.Y. Leung, F.T. Kolligs, R. Wu, Zhai, et al., Activation of AXIN2 expression by beta-catenin-T cell factor. A feedback repressor pathway regulating Wnt signaling. J Biol Chem. 277 (2002) 21657-21665.

[43] S.A. Rennoll, W.M. Jr. Konsavage, G.S. Yochum. Nuclear AXIN2 represses MYC gene expression. Biochem Biophys Res Commun. 443 (2014) 217–222.

[44] S. Li, W. Chunpeng, L. Xiaodong, et al., The roles of AXIN2 in tumorigenesis and epigenetic regulation. Familial Cancer. 14 (2015) 325–231

[45] S. Kivimäe, X.Y.Yang, B.N. Cheyette. All Dact (Dapper/Frodo) scaffold proteins dimerize and exhibit conserved interactions with Vangl, Dvl, and serine/threonine kinases. BMC Biochem. 12 (2011) 1471-1483.

[46] Yin X, Xiang T, Li L, et al., (2013). DACT1, an antagonist to Wnt/b-catenin signaling, suppresses tumor cell growth and is frequently silenced in breast cancer. Breast Cancer Res. 15(2):R23. doi: 10.1186/bcr3399.

[47] Y. Huang, P.Wang, H. Chen, et al., Myc-interacting zinc-finger protein 1 positively regulates Wnt signalling by protecting Dishevelled from Dapper1-mediated degradation. Biochem J. 15 (2015) 499-509.

[48] F.-J. LIN, J. QIN, K.TANG, et al., Coup d'Etat: an orphan takes control. Endocrine reviews. 32 (2011) 404–21.

[49] P. Zhang, M. Bennoun, C. Godard, et al., Expression of COUP-TFII in metabolic tissues during development. Mech Dev. 119 (2002) 109–111.

[50] P. Bardoux, P. Zhang, D. Perilhou, et al. Essential role of chicken ovalbumin upstream promoter-transcription factor II in insulin secretion and insulin sensitivity revealed by conditional gene knockout. Diabetes. 54 (2005) 1357–1363.

Supplementary Material

Table S1. Monoclonal and polyclonal primary and polyclonal secondary antibodies used in immunohistochemistry and Western Blot.

Monoclonal primary Ab (manufacturer)	Dilution	Polyclonal secondary Ab (manufacturer)	Dilution
Anti-active β-catenin (Millipore) Cat#05-665	1:300 (IF) 1:500 (WB)	Anti-Mouse IgG FITC Conjugate (Sigma) Cat#F9137 Anti-mouse IgG HRP Conjugate (Sigma) Cat#A9309	1:350 1:1500
Anti-Cyclin D1/2 (Millipore) Cat#06-137	1:200 (WB)	Anti-rabbit IgG HRP Conjugate (Sigma) Cat#F7512	1:1000
Anti-Insulin (Dako) Cat#A5164	1:75 (IF)	Anti-Guinea Pig TRITC Conjugate (Sigma) Cat#F7153	1:100
Anti-β-actin (Sigma) Cat#A2228	1:2000 (WB)	Anti-mouse IgG HRP Conjugate (Sigma)	1:2500

Table S2. Specific Primers used in qPCR experiments designed using the Primer Blast (NBCI).

Genes	Proteins	Forward (5'– 3')	Reverse (5'– 3')
Ctnnb1	β-CATENIN	CTGGGACTCTGCACAACCTT	CAGTGTCGTGATGGCGTAGA
Ccnd1	CYCLIN D1	TCAAGTGTGACCCGGACTGCCT	GTGGCCTTGGGGTCGACGTT
Ccnd2	CYCLIN D2	TAGGAGGCCCAAGTTGAATG	ACACCCGAGACCACAGAAAC
c-Myc	C-MYC	TCTCTGCCTCTGCCCGCGAT	TCGTGGCTGTCTGCGGGGTT
Tcf7l2	TCF7L2	GCTCCCAGACTACTCCGTTC	TTTTCAGTTCAAAGGCGGCG
Ins2	INSULIN2	GCAGAAGCGTGGCATTGTAG	TTCATTGCAGAGGGGTAGGC
Ins1	INSULIN1	ATGGGCCAAACAGCAAAGTC	TCACTAAGGGCTGGGGGTTA
Axin1	AXIN1	CCCGGAGCTATTCCGAGAAC	GGGTACCTCAGCATTGGCAT
Axin2	AXIN2	GAGGAGATCGAGGCAGAAGC	CACCTCTGCTGCCACAAAAC
Gsk3b	GSK-3β	TTGGACAAAGGTCTTCCGGC	AGCTTCCAGTGGTGTTAGCC
Coup-tfII	COUP-TFII	TGTGCTTTGGAAGAGTACGTTAG	CAATTGCTCTATGACTGAGGAGGA
Dact-1	DACT-*1*	ATCTCATAGGATGGTTGGAA	GCAATGACATCAAGGGAATT
Gapdh	GAPDH	TCAAATGGGGTGAGGCCGGTG	CACCCTTCAAGTGGGCCCCG
Actb1	β-ACTIN	GTGGATCAGCAAGCAGGAGT	AGGGTGTAAAACGCAGCTCA
Rps29	RPS29	GGGCGTCTGAAGGCAAGATGGG	TTGGAGCAGACGCGGCAAGAG

Capítulo 3

ARTIGO CIENTÍFICO II

Wnts are expressed by pancreatic islet cells and may be involved in the compensatory beta-cell proliferation

ABSTRACT

Wnt proteins act mainly as paracrine signals regulating cell proliferation and differentiation. The canonical Wnt pathway has recently been associated with the onset of type 2 diabetes in rodent and human but the underlying mechanisms are still unclear. Here, we aimed to screen for Wnt expressed by murine pancreas/islet cells; to investigate whether the Wnt gene expression profile can be changed in hyperplastic islets from type 2 prediabetic mice (fed either high-fat diet - HFD), and to verify whether soluble factors (namely Wnts) released by hyperplastic islets can induce secretion and proliferation of a beta-cell line *in vitro* condition. The majority of the Wnt subtypes are expressed by islet cells, such as Wnts 2, 2b, 3, 3a, 4, 5a, 5b, 6, 7a, 7b, 8a, 8b, 9a, 9b and 11, while in the whole pancreas homogenates were found the same subtypes, except Wnts 3, 6, 7a, and 7b. Among all the Wnts, the Wnts 3a and 5b showed a significantly increased gene expression in hyperplastic islets from prediabetic mice compared to those from control mice, and Wnt8b was expressed specifically by the pancreatic beta cells in both animal groups. Furthermore, *in vitro*, we observed that co-culture with hyperplastic or non-hyperplastic islets did not change the secretory function of the MIN6 beta cells but induced a significant increase in cell proliferation in this lineage, which was partially blocked by the IWR-1 and IWP-2 Wnt inhibitors. In conclusion, we demonstrated that murine pancreas/islet cells can secrete Wnts and that Wnts released by islets may participate in the regulation of the beta-cell mass under prediabetes or normal conditions.

1 INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a multifactorial disease with a complex pathogenesis and an onset that is associated with genetic predisposition and environmental factors, such as obesity and sedentarism (Kahn et al., 2006; Tripathy & Chaves, 2010; WHO, 2016). During the initial phase of T2DM, tissues and, organs - particularly the skeletal muscle, adipose tissue, and liver - show reduced sensitivity to insulin, a condition known as peripheral insulin resistance (Tripathy & Chaves, 2010; Collares-Buzato, 2016; Wu et al., 2014). In order to compensate this metabolic state, beta cells enhance the insulin output, sustained also by a marked increase in their mass within the islet. An overt T2DM develops under conditions of prolonged insulin resistance, where a functional exhaustion of beta cells followed by apoptosis occur, resulting in a continuous decline in insulin secretion and a consequent rise in blood glucose levels. (Collares-Buzato, 2016; Prentzi & Nolan, 2006; Wu et al., 2014).

Thus, beta cells play a central role in the evolution of T2DM, and although they are highly differentiated, they can compensate for the peripheral insulin resistance that occurs in prediabetes phase with a significant increase in beta-cell mass (Liew & Andrews, 2008; Sone & Kagawa, 2005; Collares-Buzato, 2016). This compensatory hyperplasia of beta cells is a result of self-replication and/or neogenesis from stem cells (Collares-Buzato, 2016; Prentzi & Nolan, 2006). Thus, the mechanisms regulating beta-cell mass have been the subject of research by groups interested in the development of novel therapies for diabetes (Liew & Andrews, 2008; Figeac et al., 2010; Kuljanin et al., 2017).

In this context, the Wnt signaling pathways and Wnt proteins are of particular interest (Jin et al., 2008; Kahn 2014). The Wnt proteins are part of a conserved family of glycolipoproteins, of which 19 subtypes have been described so far in human and mice (Mickels & Nusse, 2006). It is believed that this diversity allows complexity and specificity of the Wnt signaling. The canonical Wnt signaling pathway or Wnt/β-catenin is regulated by β-catenin, an adherens junction-associated protein that also acts as a transcriptional co-activator within the nucleus in conjunction with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors (Murtaugh, 2008; Welters & Kulkarni, 2008). This pathway is activated when Wnt proteins interact with a membrane receptor called Frizzled (Fz) and its co-receptor called LRP5/6 (Low-Density

Lipoprotein Receptor-Related Proteins 5 and 6) (Murtaugh, 2008; Welters & Kulkarni, 2008). Besides the canonical pathway, there are two others main non-canonical Wnt signaling, i.e. the planar cell polarity (PCP) pathway, which can regulate GTPases from the Rho family (reviewed by MacDonald, Tamai, & He, 2009; Clevers & Nusse, 2012), and the Wnt/Ca^{2+} pathway, that displays homeostatic regulation through heterotrimeric G protein-mediated phospholipase C activation and mobilization of intracellular Ca^{2+} (Kühl, 2004; Langenbacher & Chen, 2008).

Therefore, Wnt proteins are classified as canonical or non-canonical, according to the pathways they activate (reviewed by MacDonald, Tamai, & He, 2009; reviewed by Liu & Habener, 2010; Clevers & Nusse, 2012). However, it is also known that Wnt signaling is complexly integrated, where different Wnt subtypes are able to regulate each other (Roarty & Rosen, 2010; Green, Nusse, & van Amerongen, 2014). For example, the 'non-canonical' Wnt4 can inhibit the action of the 'canonical' Wnt3a and thus blocking canonical Wnt signaling *in vitro* (Bernard et al., 2008; Bowen et al., 2016). In addition, the same Wnt subtypes, as Wnt5a, can act through different signaling pathways depending on the experimental context, as occurs in glial cells (Mickels & Nusse 2006; Van-Amerongen et al., 2008; Gonzalez & Rodríguez 2017; Kuljanin et al., 2017), and are also able to activate or block the canonical Wnt pathway depending on the receptors they bind to (Roarty & Rosen, 2010; Kuljanin et al., 2017).

Studies have indicated that canonical Wnts act mainly as paracrine signals regulating cell proliferation and fate in several organs, including the endocrine pancreas (Figeac et al., 2010; Mitchell et al., 2015). Interestingly, a close link has been established between the degree of expression of variants of the TCF7L2 transcription factor and T2DM in humans (Lyssenko et al., 2007; Welters & Kulkarni, 2008; Figeac et al., 2010; Krützfeldt, 2010; Mitchell et al., 2015).

In addition, we have recently reported that the compensatory beta-cell expansion during high fat diet-induced prediabetes was accompanied by a significant increase in islet content and gene expression of several proteins associated to the canonical Wnt pathway, as β-catenin itself, which also is shifted to the beta-cell nucleus (Maschio et al., 2016). However, it is still unclear which Wnt subtypes are responsible for this beta-cell hyperplasia associated with the prediabetic and obese conditions, as well as the source of Wnt secretion (within or external to the pancreas). Recently, it has been suggested that a cross-talk between insulin-resistant adipose/skeletal muscular tissues

and the pancreas, mediated by the release of Wnt3a and Wnt4, may be involved in this phenomena (Kozinski et al., 2016).

Therefore, the aims of this study were as follows: 1) to screen for Wnt genes expressed by pancreas/islet cells; 2) to investigate whether the Wnt gene expression profile can be changed in the context of type 2 prediabetes; and 3) to verify whether soluble factors (namely Wnts) released by hyperplastic islets can induce secretion and proliferation of MIN6 beta-cell line *in vitro* condition. For that, we analyze gene expression by RT-PCR and absolute qPCR of 19 Wnt subtypes in homogenates of the whole pancreas and isolated islets from control and high-fat diet (HFD)-fed prediabetic mice, as well as in mouse peripancreatic adipose tissue (PAT) and 12-day embryos (used as positive controls). The tissue/cell distribution of selected Wnts was analyzed by indirect immunofluorescence in cryosections of pancreas of both experimental groups. Finally, we investigated the effect of co-culturing of MIN6 cells and islets, isolated from control and HFD mice, on cellular proliferation and insulin secretion in this beta cell line.

2 MATERIAL AND METHODS

2.1 Ethics statement

All experimental protocols used were approved by the Institutional Ethics Committee on Animal Use (CEUA) of the University of Campinas (UNICAMP, Brazil) under protocol #3443-1(Anexo 4).

2.2 Animals and diet

Adult male C57BL/6/JUnib mice were obtained from the breeding colonies maintained at the UNICAMP. All mice, aged 4 to 5 months, were maintained at standard housing conditions, on a 12-h light–dark cycle at a temperature of 25 ± 1 °C. Mice were divided in two experimental groups: the treated group that was fed a high-fat and hypercaloric diet (HFD) containing 21g% lipids (w/w) (4.7 kcal/g), and the control group (CTL) was fed a standard rodent diet containing 4.5g% lipids (w/w) (2.9 kcal/g) for 60 days. After the 60 days, the body weight gain, and the post-prandial glycemia were evaluated in these animals, before the euthanasia, as previously described (Maschio et al., 2016, Oliveira et al., 2015, Carvalho et al., 2012).

2.3 RT-PCR

Pancreatic islets were enzymatically isolated from mice of both experimental groups, according to the protocol described elsewhere (Maschio et al., 2016). Pools of 300 isolated islets were prepared for RNA extraction using the RNAqueous kit (Ambion, cat. AM1931), following the manufacturer's instructions. The extraction of RNAs from whole pancreas, peripancreatic adipose tissue (PAT) and mouse embryo (12 days) was done by homogenizing the tissue samples in Trizol Reagent (Invitrogen, cat. 15596018) (1 mL of Trizol per 50-100 mg of tissue) using a Polytron, and to each 1 mL of Trizol was added 0.2 mL volume of chloroform precipitation. The RNA pellets were suspended in nuclease-free water and the integrity of the RNAs was checked in 1% (w/v) agarose gel and by NanoDrop ND-1000 spectrophotometry (UV-Vis NanoDrop Technologies, USA). cDNA synthesis was performed from 0.5 µg RNA samples of isolated islets, whole pancreas, PAT and embryos using a High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). The primers for amplification of all 19 Wnt subtypes described in mouse, as well as of the receptor Frizzled (Fz) and co-receptors LRP5 and LRP6 were designed and tested against the *Mus musculus* genome (GenBank), Supplementary data, Table S1 shows the product length of the amplicons and Tm for each Wnts genes analyzed. RT-PCR reactions were performed in using the Mastercycler Nexus Gradient (Eppendorf) and the specificity of the amplificons was verified in 2% agarose gel.

2.4 Absolute quantitative PCR (qPCR)

Absolute qPCR was done to analyze the expression degree of Wnt subtypes, and Fz (receptor), LRP5 and LRP6 (co-receptors) in isolated islet samples. Absolute quantification of mRNAs was performed using a standard curve of 5 points with a decreasing dilution series, where the highest concentration (10^8) corresponds to 100,000,000 and the lowest concentration (10^3) to 1,000 copies of cDNA molecules (Bustin *et al.* 2009). The reactions were done using the 7500 ABI system and were standardized to a total volume of 20 µL/well, containing 10 µL SYBR (Applied Biosystems, cat. 4385612), 0.34 uL of each primer forward and reverse (10µM), 7.32 µL nuclease-free water and 2 µL cDNA (500 ng/uL). The primers used were identical to those used for RT-PCR analysis (Supplementary data, Table S1) and their specificity

for this technique was verified by the Melting-Curve, and the data were normalized against the endogenous control *Gapdh*.

2.5 Immunofluorescence for Wnt3a, Wnt5b and, Wnt8b in pancreas sections

The localization of specific Wnts in the endocrine pancreas was assessed by indirect immunofluorescence in - 20°C acetone-fixed cryosections of pancreas. For that, pancreas sections were 2% PFA-refixed at room temperature (RT), permeabilized with Triton X-100 for 5 min, blocked with 5% dry skimmed milk in TBS, and overnight-incubated with primary antibodies at 4°C. The primary antibodies: anti-Wnt3a (Abcam, cat. ab28472), anti-Wnt5b (Abcam, cat ab94914) and anti-Wnt8b (Invitrogen, cat. PA557723) were diluted in 3% dry skimmed milk containing 0.1% Tween20 (TTBS). After washings with TBS, the pancreas sections were incubated with the specific secondary antibody conjugated with FITC (diluted in 1% dry skimmed milk) for 2 h at RT. For co-immunolabeling with insulin, the sections were further incubated with an anti-insulin antibody followed by their specific secondary antibody conjugated with TRITC. All sections were mounted in ProLong® Gold Antifading media (cat. P36930, Invitrogen) and observed using a Zeiss LSM 510 Confocal Microscope (Hamburg, Germany). Supplementary data, Table S2 shows all the specific antibodies used and respective dilutions.

2.6 Co-culturing of MIN6 cells and isolated pancreatic islets: cell proliferation assay and immunofluorescence for active β-catenin

MIN6 cells (*Mouse insulinoma clone 6*) (passage from 17 to 21) were cultured in plastic flasks (Corning, USA) with Dulbecco's Modified Eagle Medium (DMEM) with high glucose (25mM) supplemented with L-glutamine (Cultilab – Campinas, Brazil), 15% fetal bovine serum, 100 IU of penicillin/mL and 100 μg of streptomycin/mL, and 71 μM β-mercaptoethanol in a 5% CO_2 humidified atmosphere at 37 °C (Incusafe Sanyo MCO-17A, Sanyo Electric Ltd., Japan).

For the cell proliferation assay, MIN6 cells were seeded at the density of 0.4×10^5 cells/well in 96-well plates (Nest Biotech Co. Ltd., China) containing supplemented DMEM. After 2 days of seeding, the culture medium was changed to DMEM/Ham's F-12 (Corning, cat. 10-090-CVR), without fetal bovine serum, but supplemented with L-glutamine, 15 mM HEPES and 100 IU of penicillin/mL and 100 μg of

streptomycin/mL. Then, MIN6 cells were co-cultured with isolated islets (30 size-matched islets/well) from control (CTL) or prediabetic mice (HFD), containing or not the canonical Wnt inhibitors, 10 μM IWR-1 (Sigma, cat. I0161) or 1 μM IWP-2 (Sigma, cat. I0536) for 24 h. The inhibitor concentrations were chosen based on the literature showing effectiveness *in vitro* conditions (Abraham, 2016; Chen et al, 2009; Wang et al., 2017). The BrdU solution, provided by the BrdU Cell Proliferation ELISA Kit (colorimetric) (Abcam, cat. ab126556), was added to MIN6-islet co-culture 5 h before the end of the 24 h incubation time. Then, the cells were fixed with the kit fixing solution and the colorimetric reaction was done as recommended by the manufacturer. The plates were read at 450 nm wavelength by a microplate reader (PowerWave XS2).

For immunofluorescence analysis, MIN6 cells were seeded at the density of 0.5 $\times 10^5$ cells/cm^2 on 24 mm (diameter) permeable inorganic membrane inserts (Millicell, Ireland). After co-culturing with islets, the cells were fixed in -20^{o}C methanol and kept at -20^{o}C until the immunoreaction for active β-catenin as previously described (Maschio et al. 2016) (Table S2).

In a set of experiments, MIN6 cells grown on 96-well plates were also exposed to adipose tissue-conditioned media for 24h to determine the cell proliferation using the BrdU Cell Proliferation ELISA Kit as described above. For that, adipose tissue samples (from the abdominal, pelvic and peripancreatic regions) were obtained from control and HFD-fed prediabetic mice at sterile conditions. Immediately after, these samples were weighted and added to 2mL of DMEM/Ham's F-12 (Corning, cat. 10-090-CVR), without fetal bovine serum, but supplemented with L-glutamine, 15 mM HEPES and 100 IU of penicillin/mL and 100 μg of streptomycin/mL. After removal of fibrous material and blood vessels, the adipose tissue was minced and washed three times in the tissue culture media followed by centrifugations. After the last wash, the volume of the tissue culture media was calculated to give a proportion of 100mg tissue/mL media. For culturing, 10 mL of adipose tissue suspension was transferred into 50mL culture flasks, kept at 37°C in a humidified atmosphere of 5% CO_2/95% air and cultured for 24 h. The conditioned medium was subsequently collected, carefully avoiding the lipid floating on the top, and kept frozen at −20°C until used. In some cases, the conditioned media were filtered with Acrodisc syringe filters (0.45 μm pore size) to ensure the removal of tissue fragments and complete sterilization.

2.7 Static insulin secretion in MIN6 cells

To test the secretory response to glucose, MIN6 cells were seeded at the density of 1.8×10^5 cells/well in 24-well plates and maintained as described in the previous section. After 48 h of seeding, the cells were pre-incubated for 30 min at 37 °C in 0.5 mL bicarbonate-buffered Krebs solution (BBKS; NaCl 112 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 25 mM, $NaHCO_3$ 1mM, HEPES 15mM; pH 7.4) containing 5.6 mM glucose and 3 mg/mL bovine serum albumin. Then, MIN6 cells were incubated for 24 h at 37°C in 1 mL BBKS supplemented with either 2.8 or 16.7 mM glucose, and aliquots of the supernatant were obtained and maintained at -20°C until insulin quantification. For the assays of MIN6 cells co-cultured with isolated islets, the cells were seeded at the same conditions as above described, and after 24 h they were co-cultured with islets (70 size-matched islets/well) isolated from CTL or HFD groups within DMEM/Ham's F-12. After this, the MIN6 monolayers were washed to remove the islets and further exposed, for 24 h, to 11 mM glucose-containing DMEM without fetal bovine serum. At the end of incubation period, supernatant aliquots were taken and maintained at -20°C until insulin quantification. Insulin concentration within the supernatant samples (secreted insulin) was measured using the Rat/Mouse Insulin ELISA kit (Millipore, USA).

2.8 Statistical analysis

All data are expressed as means ± SEM. Statistical difference between two experimental groups was determined using the Student's t-test. For multiple comparisons, One-way analysis of variance (ANOVA) was used, followed by the Bonferroni post-test to compare pairs of groups. The significance limit was set at $P < 0.05$. The statistical analyses were performed using the GraphPad Prism5 (GraphPad Software, La Jolla, USA).

3 RESULTS

3.1 Gain of weight and metabolic parameters

After 60 days of exposure to HFD, mice showed a significant increase, in relation to control mice, in body weight (CTL, 8.369 ± 2.036 n=24 *vs* HFD, 43.83 ± 3.493 (g) n=26, *p<0.0001*), in PAT mass (CTL, 0.0111 ± 0.0013 n=30 *vs* HFD, 0.0718

± 0.0179 (mg) n=23, *p=0.0003*) and postprandial glycemia (CTL, 132.1 ± 2.565 n=29 *vs* HFD, 172.2 ± 4.740 mg/dL n=28, p<*0.0001*). These results are in accordance with previous works demonstrating that the exposure to this HFD for the same period of time induces obesity and prediabetes in mice (Maschio et al. 2016, Chapter 2 of this thesis; Oliveira et al., 2015; Carvalho et al., 2012).

3.2 Wnt mRNA expression profile in mouse pancreatic islets, whole pancreas, PAT and embryos

Figure 1 shows the mRNA expression profile coding Wnt subtypes, revealed by RT-PCR, in isolated islets and whole pancreas from control and prediabetic animals (fed a HFD for 60d). Mouse embryo samples (12 days) and peripancreatic adipose tissue from animals of the control and 60d HFD groups were considered as positive controls of PCR reaction, since both embryonic tissues and adipose tissue are known to be important sources of Wnt secretion (Etheridge et al., 2004; Wada et al., 2013). Table 1 summarizes the results obtained. Our data demonstrate that pancreatic islet cells express the following Wnt subtypes: Wnt 2, 2b, 3, 3a, 4, 5a, 5b, 6, 7a, 7b, 8a, 8b, 9a, 9b and 11. Whole pancreas homogenates showed, in general, a more limited expression in terms of diversity and quantity of Wnt subtypes in relation to isolated islets, expressing Wnts 2, 2b, 3a, 4, 5a, 5b, 8a, 8b, 9a, 9b and 11. We believe that part of the Wnt mRNAs detected in the pancreas is probably due to the fact that the endocrine parenchyma (islets) is also represented in these samples, except Wnts 2, 2b, 3a and 9a that were relatively well expressed in the pancreas. As expected, the 12-day mouse embryo proved to be an adequate positive control, expressing all the Wnt subtypes. The peripancreatic adipose tissue expressed a considerable number of Wnts, 15 subtypes out of a total of 19, except Wnts 1, 7b, 10a and 16.

3.3 Differential gene expression of Wnt subtypes in isolated islets from control and HFD-fed prediabetic mice

Figure 2 (A) depicts the degree of gene expression of all Wnts identified in islets isolated from control and prediabetic mice, as revealed by absolute qPCR. The Wnt subtypes displayed distinct expression degree in control islets. The Wnt subtypes displayed distinct expression degree in control islets, being Wnt4 the most well

expressed one (Figure 2 B), while the Wnts 2, 7a and 7b were the least Wnt subtype expressed on islets (data not shown because the expression degree of these Wnt subtypes was below 10^3 copies of cDNA molecules).

In addition, among all the Wnt subtypes studied, we observed a significant increase in the gene expression of Wnt3a and Wnt5b in islets isolated from the HFD-fed prediabetic mice in relation to the CTL group (Figure 2 B, c and f). These two Wnt subtypes, as well as the Wnt8b, were immunodetected in pancreas cryosections, as shown in Figures 3 and 4. Wnt3a and Wnt5b co-localized with insulin-containing beta cells, although both Wnts were also found in non-insulin-secreting cells (probably alpha cells located at the periphery of islets) and in the exocrine pancreas (Figures 3 and 4). Meanwhile, Wnt8b seems to be expressed exclusively on beta cells, which are located within the islet core (Figure 5).

3.4 Islet cells and MIN6 cell line do express Wnt pathway receptors

We observed the expression of the Fz receptor, as well as the LRP5 and LRP6 co-receptors in isolated islets of the HFD and CTL groups, in embryo samples, and in MIN6 cells (Figure 6), demonstrating that islet cells and glucose-responsive beta cells, such as MIN6 cells, can potentially respond to Wnt signaling. However, the absolute quantitative analysis showed no difference in the expression of these genes in islets isolated from the control and HFD groups (Figure 6).

3.5 Co-culturing of MIN-6 cells and mouse isolated islets does not alter insulin secretion but increases cell proliferation partially inhibited by Wnt pathway antagonists

We observed that 24h exposure of the MIN6 beta cells to the hyperplastic islets (isolated from HFD-fed prediabetic mice) and control ones did not significantly alter the secretory function (Figure 7c) but induced a significant increase in cell proliferation of this lineage that was partially inhibited by 10μM IWR-1 and 1μM IWP-2 canonical pathway inhibitors (Figure 7b). In accordance with the idea of a possible activation of the canonical Wnt pathway, MIN6 cells displayed an increase in the cytoplasmic amount of active β-catenin associated with a decrease in the intercellular content of this protein when co-cultured with islets isolated from both the CTL and HFD groups.

In order to investigate whether the adipose tissue from prediabetic mice could also affect the MIN6 proliferation index as suggested by others (Kozinski et al, 2016; Schinner et al., 2008), this beta cell line was exposed to adipose tissue-conditioned media (filtered or not) for 24h. As shown in Figure 8, the exposure to these conditioned media, in fact, inhibited significantly the proliferation of MIN6 cells as revealed by the BrdU proliferation assay.

4 DISCUSSION

The canonical Wnt pathway has been considered a signaling pathway with a possible repercussion in beta-cell biology based on the evidence of a close link between the degree of expression of TCF7L2 factor variants and type 2 diabetes in humans (Lyssenko et al. 2007). Polymorphisms in TCF7L2 are associated with a deficiency in insulin secretion, suggesting an important role of the canonical Wnt pathway in the secretory dysfunction of the pancreatic beta cell during diabetes (Krützfeldt, 2010). In addition, Figeac et al. (2010) have demonstrated in neonatal rats that inhibition of TCF7L2 results in alteration of normal compensatory growth of beta cells, primarily through the inhibition of cell proliferation. Conversely, in neonatal mice with T1DM induced by the administration of streptozotocin, the activation of the canonical Wnt pathway induced by inhibition of GSK-3β had a significant stimulatory effect on beta cell regeneration in these animals. Also *in vitro*, inactivation of GSK-3β resulted in stimulation of beta cell proliferation, which was mediated by β-catenin stabilization and Cyclin D induction (Figeac et al., 2010). Recently, we have suggested that the canonical Wnt pathway is activated in pancreatic islets during the prediabetic state in HFD-fed mice and may be involved in the compensatory hyperplasia of the beta cell (Maschio et al., 2016).

Although there is experimental evidence, such as those described above and others employing *in vitro* models (Lenz et al., 2014), which suggest an important role of the canonical Wnt pathway in beta-cell proliferation and function, it is yet unclear which subtypes of Wnt would participate in this process and what would be the source of these Wnts (i.e. which cells would be responsible to release them). Recently, Kozinski et al. (2016) suggested that alterations in the secretion profile of a canonical Wnt activator (Wnt3a) and inhibitor (Wnt4) from insulin-resistant tissues (adipose and

skeletal muscle tissues) during the development of T2DM may be responsible for triggering the increase followed by the decrease in beta-cell mass seen during the prediabetic and diabetic states, respectively. Nevertheless, given the fact that most of the Wnts act as paracrine and/or juxtacrine factors (MacDonald, Tamai, & He et al., 2009), we investigated in the present work whether Wnts released by pancreatic islets could potentially correlate with the beta-cell adaptation to systemic insulin resistance.

We demonstrated for the first time that islet cells are the source of several Wnt subtypes, expressing 15 subtypes out of 19 Wnts described in mice. In contrast, the whole pancreas showed a more limited expression in terms of diversity and quantity of Wnts in relation to islets, where only Wnt 2, 2b, 3a and, 9a showed a relevant expression in the (exocrine) pancreas. Among all the Wnt subtypes studied, we observed a significant increase in the gene expression of Wnt3a and Wnt5b in hyperplastic islets isolated from the HFD-fed prediabetic mice in relation to the CTL group.

The protein expression of these Wnts was confirmed by immunofluorescence that also showed Wnts 3a and 5b are expressed by beta cells, as well as by non-beta cells. These data may suggest that these Wnt subtypes released by islet cells could be involved in the compensatory beta-cell proliferation seen during prediabetes. In agreement with this idea, there is experimental evidence showing that Wnt3a, the most studied canonical Wnt subtype, can induce cell proliferation and improve insulin secretion in different beta cell lineages *in vitro* (Bowen et al., 2016; Gui et al., 2012; Schinner et al., 2008; Rulifson et al., 2007). In addition, an interesting study identified the expression of Wnt5b gene in human tissues such as adipose tissue, pancreas, and liver. This same study also reported that a single-nucleotide polymorphism (SNP) in the Wnt5b gene is strongly associated with type 2 diabetes in the Japanese population (Kanazawa et al., 2004).

Regarding the Wnt8b, this Wnt subtype has been shown to activate the canonical Wnt pathway in neuroepithelial cells and is involved in neurogenesis during the development of the central nervous system of different animal species (Hofmeister & Key, 2013; Fotaki et al. 2010). Interestingly, it is well known that, despite distinct embryological origin, pancreatic beta cells and neurons share several similarities, expressing common proteins/markers such as dopamine beta-hydroxylase, sodium-dependent voltage channels type II, neurofilament proteins, SNARE proteins, Cx36, the

transcription factors Islet-1, Pax-6 and Beta2, etc. (Van Arensbergen et al., 2010; Carvalho et al., 2010 Atouf et al., 1997). Therefore, our result demonstrating the islet expression of Wnt8b is in line with the putative neural origin of beta cells.

In order to investigate whether a soluble factor (i.e. Wnts) released by hyperplastic islet cells could induce cell proliferation *in vitro*, we have co-cultured the beta-cell line MIN6 with islets isolated from normal and prediabetic mice. Interestingly, we observed that 24h co-culturing of MIN6 cells with islets isolated from both HFD-fed prediabetic and control mice induced a significant increase in cell proliferation of this lineage, although it did not affect the glucose-induced insulin secretion. This effect on cell proliferation was associated with the translocation of active β-catenin to the cytoplasm of MIN6 cells and it was significantly inhibited by two canonical pathway inhibitors, the IWR-1 (an inhibitor of Wnt response that promotes β-catenin destruction likely by stabilizing the Axin-scaffolded destruction complexes) and the IWP-2 (an inhibitor of Wnt production/secretion) (Wang et al., 2017; Abraham, 2016; Chen et al., 2009). Nevertheless, this inhibition of MIN-6 proliferation co-cultured with mouse islet was partial, which could be explained by a limited effectiveness of the inhibitors to completely block the Wnt signaling pathway in our *in vitro* system or, alternatively, by the involvement of other signaling pathways in the phenomenon (activated by other factors released by islet cells, such as insulin/IGF-I, NGF, etc. (Alismail & Jin, 2014; Rosenbaum et al., 1998)). In addition, no difference in cell proliferation was observed between co-culturing with control and with HFD islets, despite the fact that we have seen an increased expression of determined Wnts (namely Wnts 3a, and 5b) in the latter. A possible explanation for this phenomenon is that, since the islet cells constitutively express several Wnts as revealed by qPCR, these factors could obscure the additional effect of those Wnts, overexpressed in HFD islets, on the *in vitro* proliferation of MIN6 cells.

In addition, the exposure to the adipose tissue-conditioned media had an opposite effect on MIN6 cell proliferation, inducing inhibition of this process. This observation is in contrast with previous works reporting that Wnts released by adipose tissue could induce cell proliferation (Kozinski et al, 2016; Wada et al., 2013; Schinner et al., 2008). This discrepancy between the results may be explained by the higher proportion of adipose tissue per volume of tissue culture media used in our study (2-fold) in comparison with the other works, which could result in relatively high

concentrations of cholesterol and free fatty acids within the conditioned media, that are known to have pro-apoptotic and/or inhibitory effects on beta-cell proliferation (Collares-Buzato, 2015).

In conclusion, we demonstrated for the first time that islet cells constitutively express several Wnt subtypes that may be involved in the regulation of beta-cell mass during normal and/or high insulin demanding conditions.

Table 1. Summary of mRNA expression of the 19 Wnt gene subtypes, from canonical (c) or noncanonical (nc) pathways, described in mice, in homogenates of isolated islets, whole pancreas, peripancreatic adipose tissue (PAT) and 12day-old mouse embryo. Legends: check=gene expression detected by RT-PCR; dash=gene expression undetected by RT-PCR.

Genes	Islets	Pancreas	PAT	Embryo12d
Wnt1 (nc)	-	-	-	✓
Wnt2 (nc)	✓	✓	✓	✓
Wnt2b (c)	✓	✓	✓	✓
Wnt3 (nc)	✓	-	✓	✓
Wnt3a (c)	✓	✓	✓	✓
Wnt4 (nc)	✓	✓	✓	✓
Wnt5a (c/nc)	✓	✓	✓	✓
Wnt5b (c)	✓	✓	✓	✓
Wnt6 (c)	✓	-	✓	✓
Wnt7a (c)	✓	-	✓	✓
Wnt7b (c)	✓	-	-	✓
Wnt8a (c)	✓	✓	✓	✓
Wnt8b (c)	✓	✓	✓	✓
Wnt9a (c)	✓	✓	✓	✓
Wnt9b (c)	✓	✓	✓	✓
Wnt10a (c)	-	-	-	✓
Wnt10b (c)	-	-	✓	✓
Wnt11 (nc)	✓	✓	✓	✓
Wnt16 (nc)	-	-	-	✓

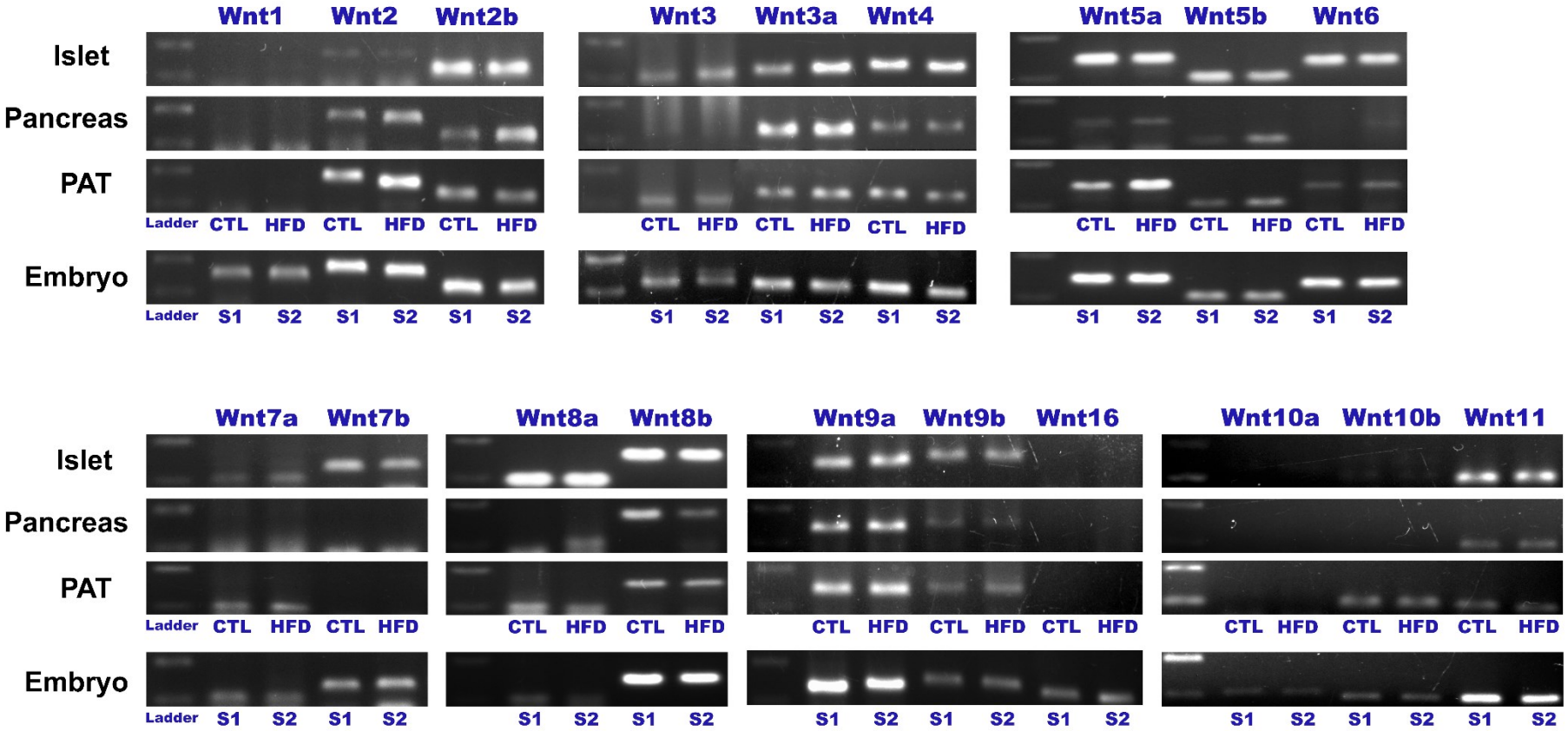

Figure 1. Gene expression profile of Wnt subtypes in mouse pancreatic islets, whole pancreas, peripancreatic adipose tissue (PAT) and 12 day-embryos. mRNA transcripts of all 19 Wnts described in mice were analyzed by RT-PCR in homogenates of isolated pancreatic islets, whole pancreas and PAT of control (CTL) and high-fat diet (HFD)-fed prediabetic mice. This analysis was also done in homogenates of 12 day-old mouse embryos (samples 1 and 2) (S1 and S2), taken as the positive control of the PCR reaction. Ladder: first band (100 pb); second band (200 pb). Table S1 (Supplementary Material) depicts the primer constructs, the amplicon sizes and the Tm used in the PCR analysis of the different Wnt subtypes. The PCR reactions were made in triplicates with at least 3 different biological samples. In the case of pancreatic islets, each biological sample contained 200-300 islets isolated from 2-3 mice.

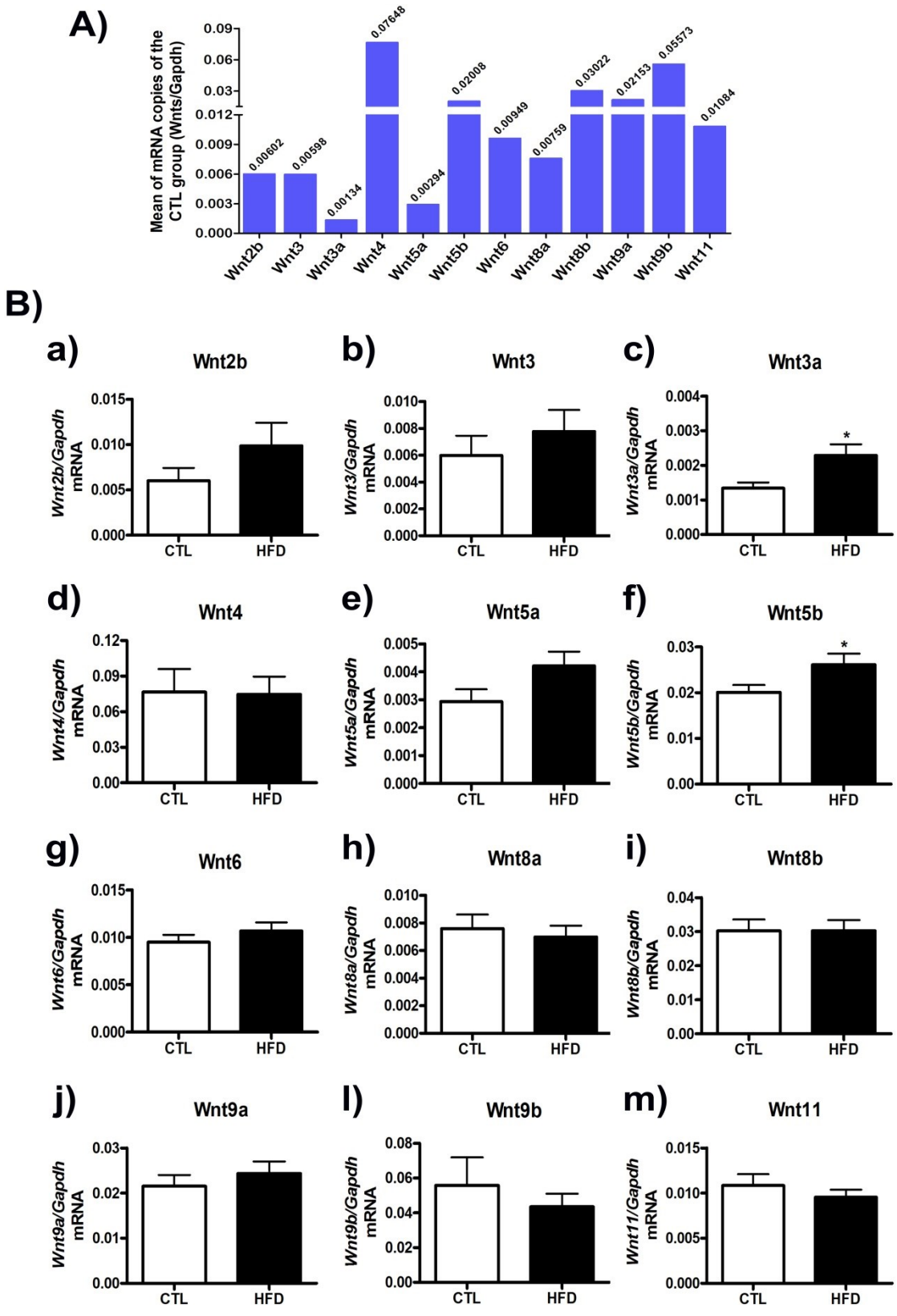
A)
Mean of mRNA copies of the CTL group (Wnts/Gapdh)
0.09
0.06
0.03
0.012
0.009
0.006
0.003
0.000
0.00602
0.00598
0.00134
0.07648
0.00294
0.02008
0.00949
0.00759
0.03022
0.02153
0.05573
0.01084
Wnt2b
Wnt3
Wnt3a
Wnt4
Wnt5a
Wnt5b
Wnt6
Wnt8a
Wnt8b
Wnt9a
Wnt9b
Wnt11
B)
a)
Wnt2b
Wnt2b/Gapdh mRNA
b)
Wnt3
Wnt3/Gapdh mRNA
c)
Wnt3a
Wnt3a/Gapdh mRNA
d)
Wnt4
Wnt4/Gapdh mRNA
e)
Wnt5a
Wnt5a/Gapdh mRNA
f)
Wnt5b
Wnt5b/Gapdh mRNA
g)
Wnt6
Wnt6/Gapdh mRNA
h)
Wnt8a
Wnt8a/Gapdh mRNA
i)
Wnt8b
Wnt8b/Gapdh mRNA
j)
Wnt9a
Wnt9a/Gapdh mRNA
l)
Wnt9b
Wnt9b/Gapdh mRNA
m)
Wnt11
Wnt11/Gapdh mRNA
CTL
HFD
*

Figure 2. The degree of expression of Wnt subtypes in pancreatic islets isolated from control mice (A) and quantitative expression of Wnts in islet homogenates from control (CTL) and high-fat diet (HFD)-fed prediabetic mice (B). Panel A) shows differences in the expression degree of Wnts in mouse pancreatic islets, as revealed by the absolute quantitative PCR analysis, in relation to Gapdh (the internal control). Panel B) shows that among all the Wnt subtypes studied, we observed a significant increase in the gene expression of Wnt3a and Wnt5b in islets isolated from the HFD-fed prediabetic mice in relation to the CTL group. The Wnts 2, 7a and 7b also were detectable by qPCR, but displayed a degree of expression below than 1,000 copies of cDNA molecules (data not shown). Data represent means + SEM of 8-11 islet pools/experimental group, where each islet pool contains 300-400 islets isolated from 2-3 mice. *$*p<0.05$* as compared to its respective control (CTL) fed a regular diet (Student's t-test).

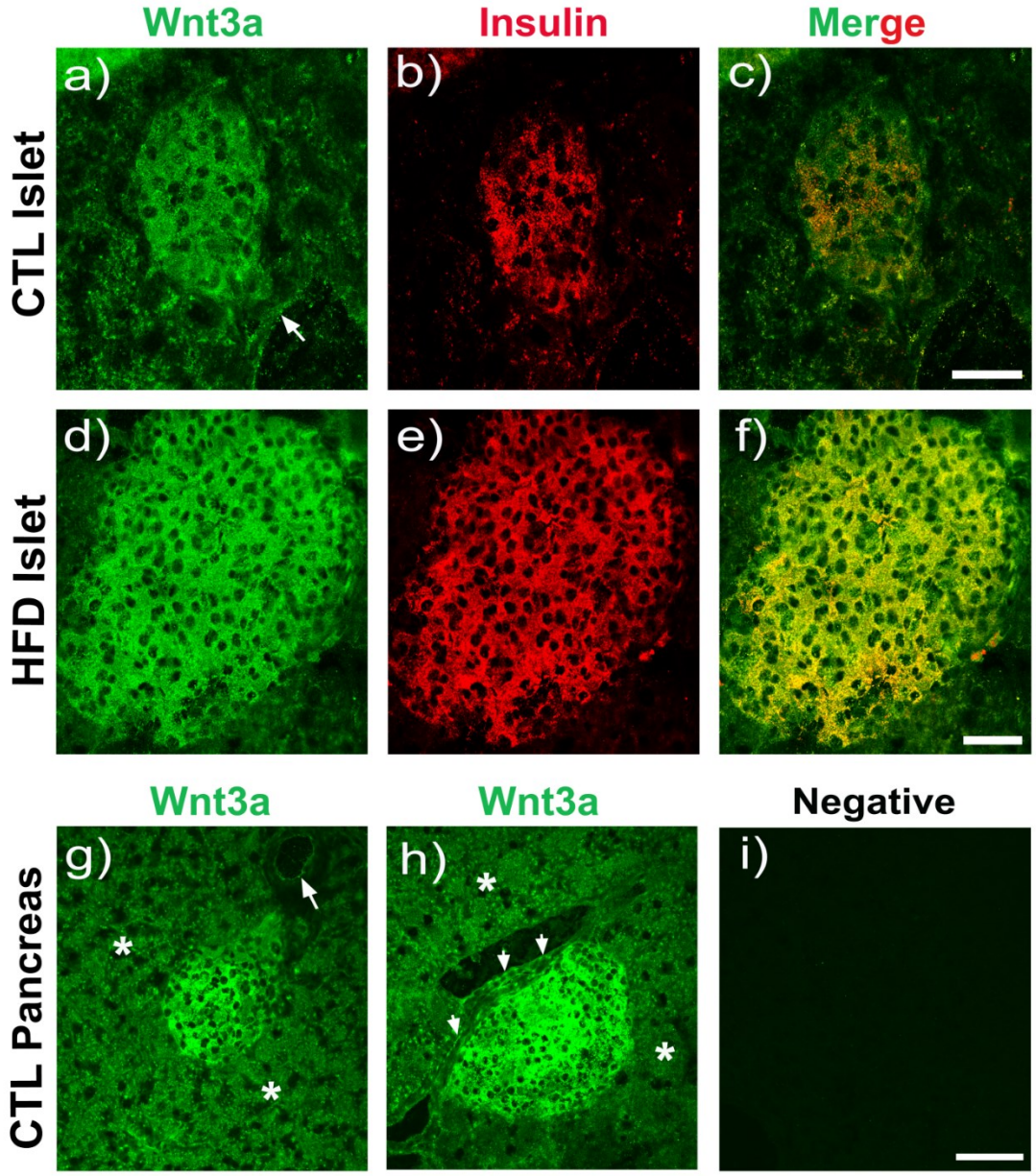

Figure 3. Immunolocalization of Wnt3a in the pancreas of control (CTL) and high-fat diet (HFD)-fed prediabetic mice. Confocal images of Wnt3a (in green) co-labeled with insulin (in red) in pancreas cryosections of CTL (a-c) and HFD (d-f) mice. Note that Wnt3a co-localized with insulin-containing beta cells, although this Wnt was also found in non-insulin secreting cells (probably alpha cells located at the periphery of islets) and in the exocrine pancreas (asterisks in g and h). Arrows in (a) and (g) indicate positive reaction for Wnt3a in endothelium. Arrowheads in (h) show Wnt3a immunoreaction in the epithelium lining the duct. Image (i) depicts the negative control of the reaction where the primary antibody (anti-Wnt3a) was omitted. All images are representative of islets of at least 3 animals per group. Scale bar in (c and f), 50 µm. Scale bar in (i), 100 µm.

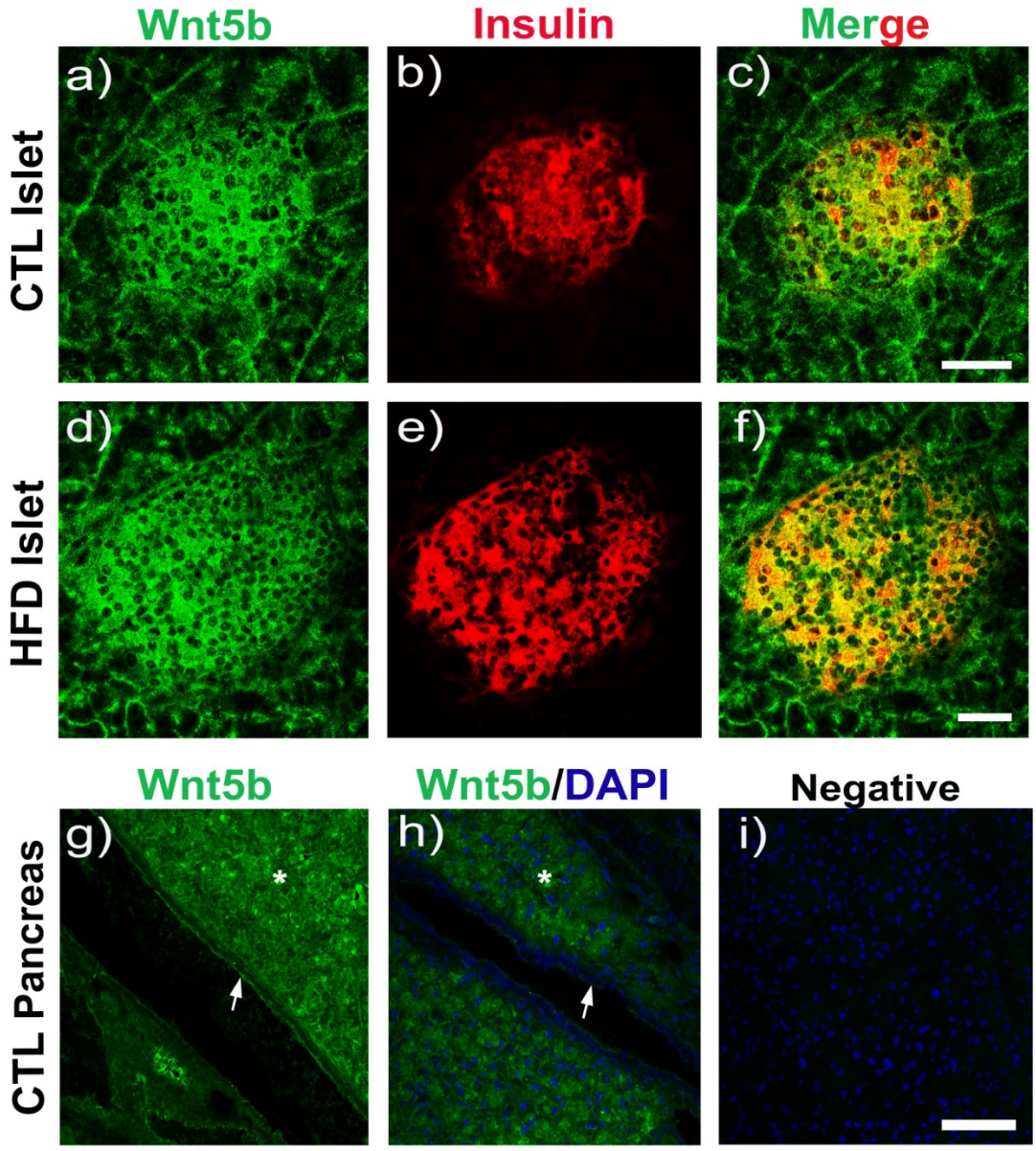

Figure 4. Immunolocalization of Wnt5b in the pancreas of controle (CTL) and high-fat diet (HFD)-fed prediabetic mice. Confocal images of Wnt5b (in green) (a,d) co-labeled with insulin (in red) (b,e) and DAPI (indicating the nuclei in blue) (h,i) in pancreas cryosections of mice. Note that Wnt5b co-localized with insulin-containing beta cells (c,f), although this Wnt was also found in non-insulin secreting cells (probably alpha cells located at the periphery of islets) and in the exocrine pancreas (g and h, asterisks). The arrow in (g) indicates a positive reaction for Wnt5b in endothelium. The arrow in (h) shows relatively low Wnt5b immunoreaction in the epithelium lining the duct. Image (i) depicts the negative control of the reaction where the primary antibody (anti-Wnt5b) was omitted. All images are representative of islets of at least 3 animals per group. Scale bar in (c and f), 50 µm. Scale bar in (i), 100 µm.

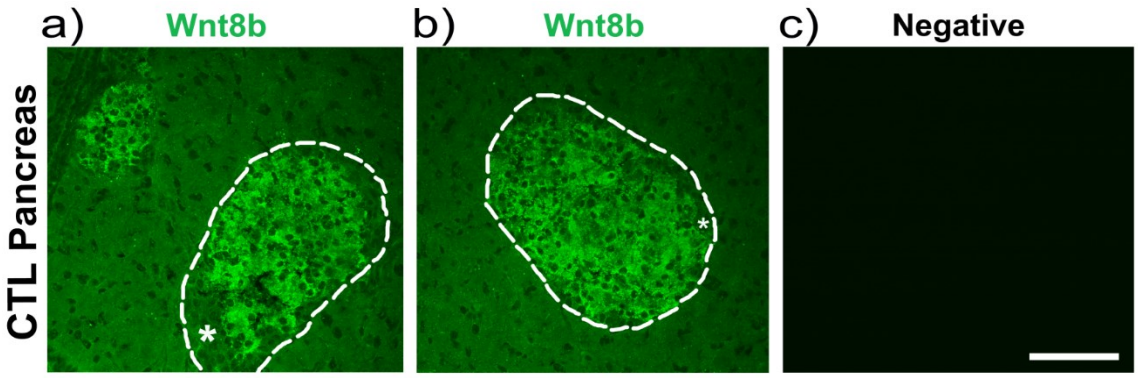

Figure 5. Immunolocalization of Wnt8b in the pancreas of control (CTL) mice. Confocal images of Wnt8b (in green) in pancreas cryosections of CTL (a, b) mice. Due to the low signal obtained from the immunofluorescence for Wnt8b, it was not possible to perform dual labeling with insulin. Note that Wnt8b seems to be expressed exclusively on beta cells, which are located within the islet core. No signal was observed in non-beta cells (asterisks in a and b), cells and structures of the exocrine pancreas. Image (c) depicts the negative control of the reaction where the primary antibody was omitted. Scale bar, 100 μm.

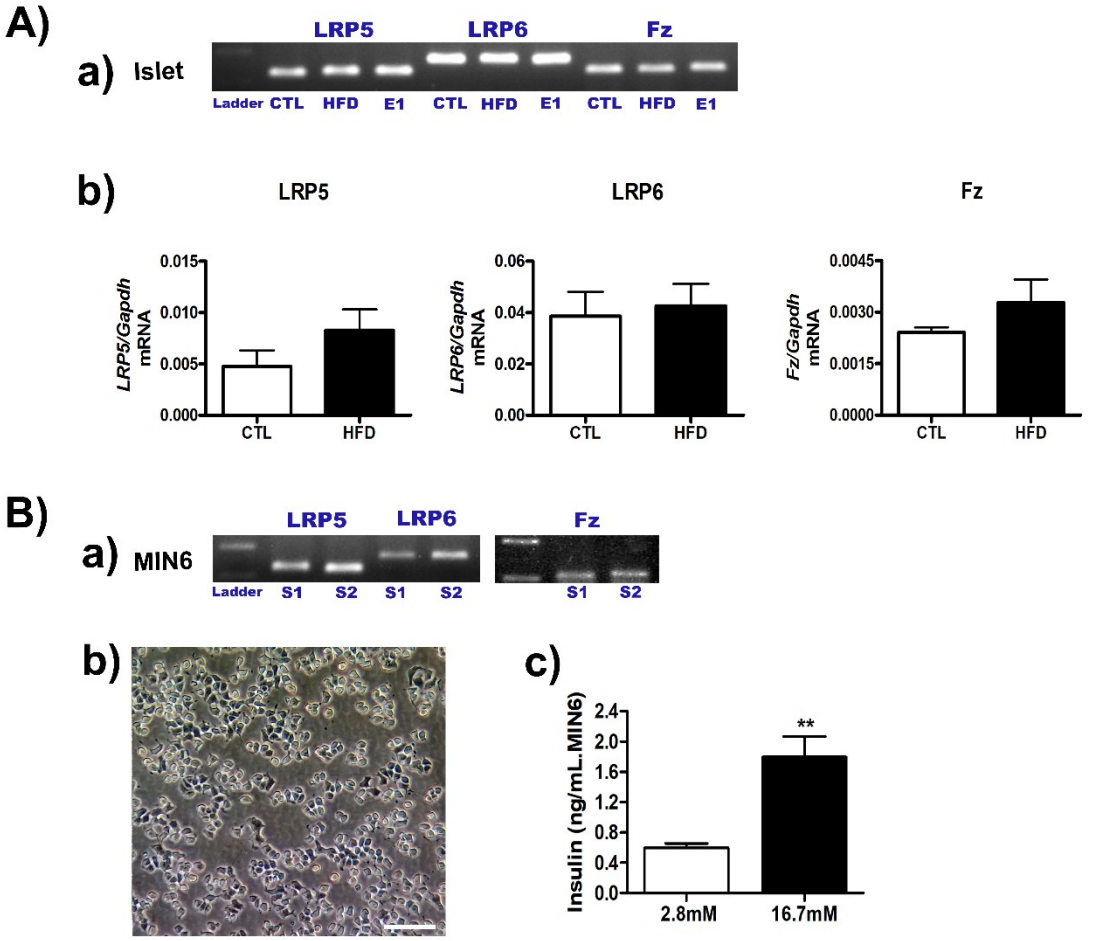

Figure 6. Mouse pancreatic islets and MIN6 beta cell line express receptor and co-receptors of the canonical Wnt pathway. Panels (A, a) and (B, a) show the expression of the *Frizzle* (Fz) receptor and the LRP5/6 co-receptors of the canonical Wnt pathway as revealed by RT-PCR. Panel (A, b) shows no significant difference in gene expression of Fz, LRP5, and LRP6 in islets isolated from high-fat diet (HFD)-fed prediabetic mice and control (CTL) ones. Panel (B, b) depicts a contrast-phase photomicrograph of MIN6 cells cultured in flasks, and in panel (B,c), it is possible to observe that MIN6 cells used in this work display a proper glucose-stimulated insulin secretion when exposed to basal (2.8mM) and supraliminal (16.7mM) glucose concentrations. The values in (A,b) represent the means + SEM (n=6-8 islet pools/group; where each islet pool contains 300-400 islets isolated from 2-3 mice). The values in graph (B,c) represent the means + SEM (n=3 MIN6 monolayers/group). ***p<0.003* as compared 2.8mM relative to 16.7mM of glucose (Student's t test).

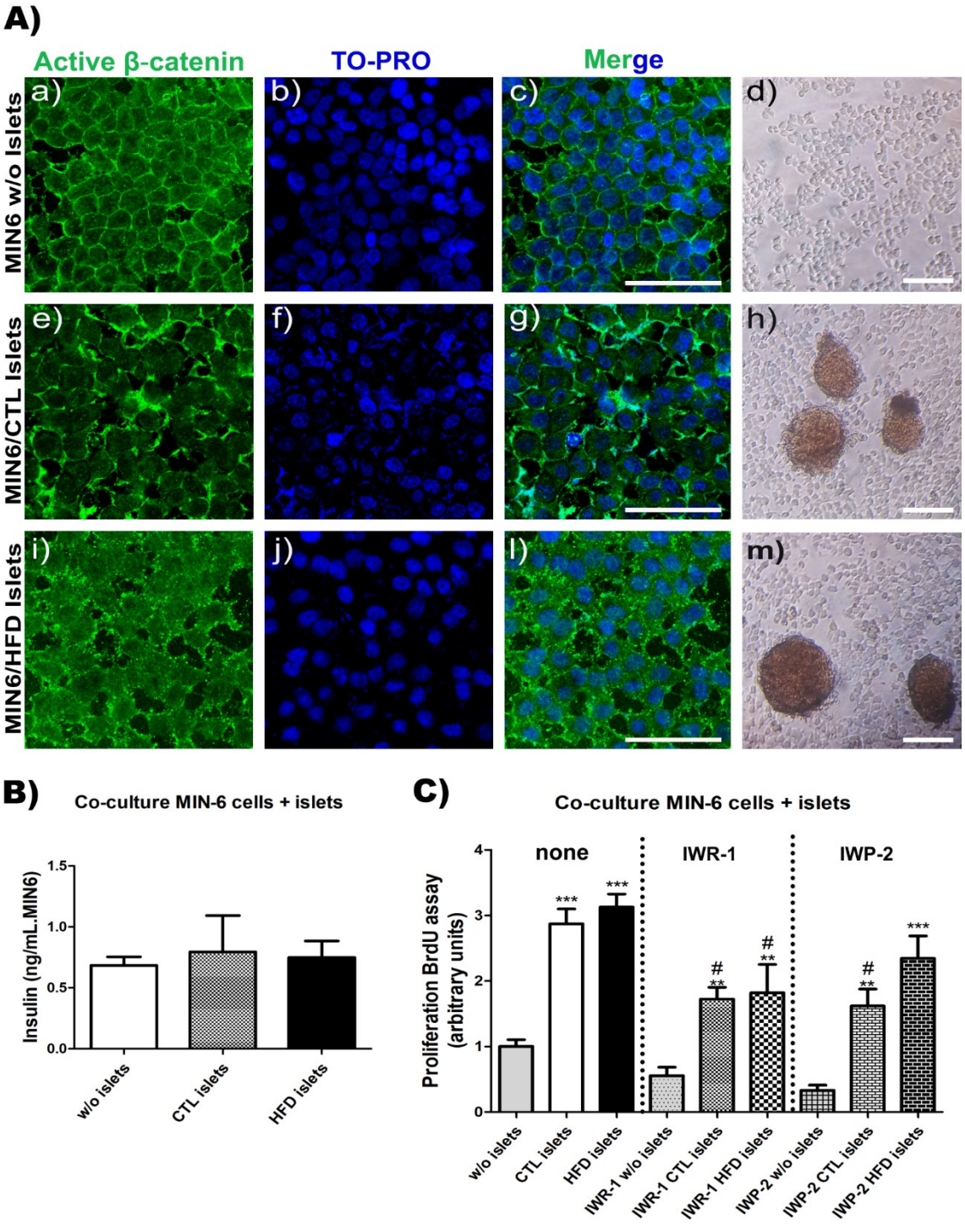

Figure 7. Co-culturing of MIN6 cells and pancreatic islets, isolated from control (CTL) and high-fat diet (HFD)-fed prediabetic mice, does not affect insulin secretion but induces an increase in cell proliferation. Panel (A) shows photomicrographs of immunofluorescence for active β-catenin (green) plus TO-PRO (blue) in MIN6 cells that display an increase in the cytoplasmic amount associated with a decrease in intercellular content of this protein when co-cultured with islets isolated from both the CTL and HFD groups. The immunolabeling reaction was done in triplicate. In panel (C), the cell proliferation analysis by BrdU incorporation demonstrates that co-culturing with islets isolated from both the CTL and HFD groups

promotes an increased cell proliferation in MIN6 cells, which is partially inhibited by the treatment with Wnt pathway antagonists, IWR-1 and IWP-2. Meanwhile, in panel (B), co-culturing with CTL and HFD islets does not alter the glucose-stimulated insulin secretion in MIN6 cells. The values in graph B represent the means + SEM (n=4-5 monolayers/group). ***p<0.01*; ****p<0.001* in comparison with MIN6 monolayers that were not co-cultured with islets (w/o islets); *#p<0.05* in comparison with the respective group without the Wnt inhibitor.

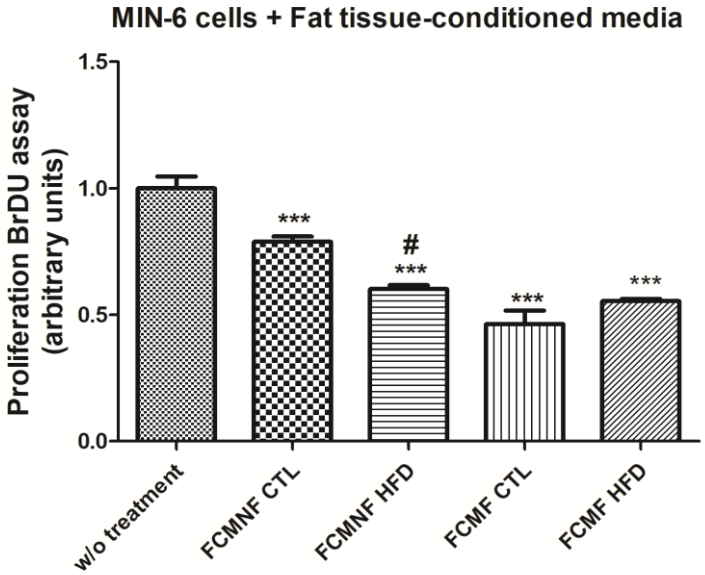

Figure 8. Exposure of MIN6 cells to adipose tissue-conditioned media inhibits cell proliferation. Conditioned media were prepared by culturing fragments of adipose tissue (collected from abdominal, pelvic and peripancreatic regions of control (CTL) and high-fat diet (HFD)-fed prediabetic mice) for 24h in DMEM without serum (100 mg adipose tissue per mL of tissue culture medium). The conditioned media were filtered or not before exposed to MIN6 monolayers for 24h. The cell proliferation analysis by BrdU incorporation demonstrates that exposure to these conditioned media significantly decreased the proliferation index in MIN6 monolayers in comparison to those exposed to DMEM medium. ****p<0.001* in comparison with MIN6 monolayers cultured with DMEM media (no treatment); *#p<0.05* in comparison with the respective CTL islets group. Legends: FCM-CTL=fat-conditioned media from CTL mice; FCM-HFD=fat-conditioned media from HFD-fed prediabetic mice; FFCM=filtered fat-conditioned media.

REFERENCES

Abraham, S. T. (2016). A role for the Wnt3a/β-catenin signaling pathway in the myogenic program of C2C12 cells. *In Vitro Cellular & Developmental Biology - Animal, 52*(9), 935-941.

Alismail, H., Jin, S., Kloppel, G., Lohr, M., Habich, K., Oberholzer, M., … & Bonner-Weir, S. (2014). Microenvironmental stimuli for proliferation of functional islet β-cells. *Cell & Bioscience*, *4*(1), 12.

Atouf, F., Czernichow, P., & Scharfmann, R. (1997). Expression of neuronal traits in pancreatic beta cells. *Journal of Biological Chemistry*, *272*(3): 1929.

Bernard, P., Fleming, A., Lacombe, A., Harley, V. R., & Vilain, E. (2008). Wnt4 inhibits β-catenin/TCF signalling by redirecting β-catenin to the cell membrane. *Biology of the Cell*, *100*(3), 167-177.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., ... & Wittwer, C. T. (2009). Why the need for qPCR publication guideline: The case for MIQE. *Clinical Chemistry 55*(4), 611-622.

Bowen, A., Kos, K., Whatmore, J., Richardson, S., & Welters, H. J. (2016). Wnt4 antagonises Wnt3a mediated increases in growth and glucose stimulated insulin secretion in the pancreatic beta-cell line, INS-1. *Biochemical and Biophysical Research Communication*s, *479*(4),793-799.

Carvalho, C. P. F., Barbosa, H. C. L., Britan, A., Boschero, A. C., & Meda, P. (2010). Beta cell coupling and connexin expression change during the functional maturation of rat pancreatic islets. *Diabetologia, 53*(7):1428-1437.

Carvalho, C. P. F., Oliveira, R. B., Britan, A., Santos-Silva, J. C., Boschero, A. C., Meda, P., & Collares-Buzato, C. B. (2012). Impaired β-cell-β-cell coupling mediated by Cx36 gap junctions in prediabetic mice. *American Journal Physiology Endocrinology and Metabolism*, *303*(1), E144-E151.

Collares-Buzato, C. B. High-Fat Diets and β-Cell Dysfunction: Molecular Aspects. in *Molecular Nutrition and Diabetes* (ed. Mauricio, D.) 115-130 (Elsevier, 2015).

Chen, B., Dodge, M. E., Tang, W., Lu, J., Ma, Z., Fan, C. W., … & Lum, L. (2009). Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nature Chemical Biology*, *5*(2), 100-107.

Clevers, H., & Nusse, R. (2012). Wnt/β-Catenin Signaling and Disease. *Cell*, *149*(6), 1192-1205.

Etheridge, S. L., Spencer, G. J., Heath, D. J., & Genever, G. (2004). Expression profiling and functional analysis of wnt signaling mechanisms in mesenchymal stem cells. *Stem Cells*, *22*(5), 849-860.

Figeac, F., Uzan, B., Faro, M., Chelali, N., Portha, B., & Movassat, J. (2010). Neonatal growth and regeneration of β-cells are regulated by the Wnt/β-catenin signaling in normal and diabetic rats. *American Journal Physiology Endocrinology and Metabolism 298*(2), E245-E256.

Fotaki, V., Larralde, O., Zeng, S., McLaughlin, D., Nichols, J., Price, D. J., … Mason, J. O. (2010). Loss of Wnt8b has no overt effect on hippocampus development but leads to altered wnt gene expression levels in dorsomedial telencephalon. *Developmental Dynamics*, *239*(1):284-296.

Green, J., Nusse, R., & van Amerongen, R. (2014). The Role of Ryk and Ror Receptor Tyrosine Kinases in Wnt Signal Transduction. *Cold Spring Harbor Perspectives in Biology, 6*(2), a009175.

Gui, S., Yuan, G., Wang, L., Zhou, L., Xue, Y.,Yu, Y.,...& Wang, D. W. (2013). Wnt3a regulates proliferation, apoptosis and function of pancreatic NIT-1 beta cells via activation of IRS2/PI3K signaling. *Journal Cellular Biochemistry, 114*(7), 1488-1497.

Gonzalez, P., & Rodríguez, F. J. (2017). Analysis of the expression of the Wnt family of proteins and its modulatory role on cytokine expression in non activated and activated astroglial cells. *Neuroscience Research*, *114*, 16-29.

Hofmeister, W., & Key, B. (2013). Frizzled-3a and Wnt-8b genetically interact during forebrain commissural formation in embryonic zebrafish. *Brain Research*, *1506*, 25-34.

Jin, T. (2008).The WNT signalling pathway and diabetes mellitus. *Diabetologia, 51*(10), 1771-1780.

Kahn, M. (2014). Can we safely target the WNT pathway? *Nature Reviews Drug Discovery*, *13*(7),513-532.

Kahn, S. E., Hull, R. L. & Utzschneider, K. M. (2006). Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature*, *444*(7121), 840-846.

Kanazawa, A., Tsukada, S., Sekine, A., Tsunoda, T., Takahashi, A., Kashiwagi, A., …& Maeda, S. (2004). Association of the Gene Encoding Wingless-Type Mammary Tumor Virus Integration-Site Family Member 5B (WNT5B) with Type 2 Diabetes. *The American Journal of Human Genetics*, *75*(5), 832-843.

Kozinski, K., Jazurek, M., Dobrzyn, P., Janikiewicz, J., Kolczynska, K., Gajda, A., & Dobrzyn, A. (2016). Adipose- and muscle-derived Wnts trigger pancreatic β-cell adaptation to systemic insulin resistance. *Scientific Reports*, *6*, 31553.

Krützfeldt, J., & Stoffel, M. (2010). Regulation of wingless-type MMTV integration site family (WNT) signalling in pancreatic islets from wild-type and obese mice. *Diabetologia*, *53*(1), 123-127.

Kühl, M. (2004). The WNT/calcium pathway: biochemical mediators, tools and future requirements. *Frontiers in Bioscience, 9*, 967-974.

Kuljanin, M., Bell, G. I., Sherman, S. E., Lajoie, G. A., Hess, D. A. (2017). Proteomic characterisation reveals active Wnt-signalling by human multipotent stromal cells as a key regulator of beta cell survival and proliferation. *Diabetolo*gia, *60*(10), 1987-1998.

Langenbacher, A., & Chen, J. N. (2008). Calcium signaling: A common thread in vertebrate left-right axis development. *Developmental Dynamics*, *237*(12), 3491-3496.

Lenz, A., Toren-Haritan, G., Efrat, S. (2014). Redifferentiation of Adult Human β Cells Expanded In Vitro by Inhibition of the WNT Pathway. *PLOS ONE, 9*(11), e112914.

Liew, C. G., & Andrews, P. W. (2009). Stem Cell Therapy to Treat Diabetes Mellitus, The *Review of Diabetic Studies, 5(*4), 203-219.

Liu, Z., & Habener, J. F. (2010). Wnt signaling in pancreatic islets. in: M.S. Islam (Eds), The Islets of Langerhans. Springer, New York, 654, pp. 391-419.

Lyssenko, V., Lupi, R., Marchetti, P., Del Guerra, S., Orho-Melander, M., Almgren, P., … Groop, L. (2007). Mechanisms by which common variants in the TCF7L2 gene increase risk of type 2 diabetes. *The Journal of Clinical Investigation*, *117*(8), 2155-2163.

MacDonald, B. T, Tamai, K., & He, X. (2009). Wnt/β-catenin signaling: components, mechanisms, and diseases. *Developmental Cell*, *17*(1), 9-26.

Maschio, D. A., Oliveira, R. B., Santos, M. R., Carvalho, C. P. F., Barbosa-Sampaio, H. C. L., & Collares-Buzato, C. B. (2016). Activation of the Wnt/β-catenin pathway in pancreatic beta cells during the compensatory islet hyperplasia in prediabetic mice. *Biochemical and Biophysical Research Communications*, *478*(4), 1534-1540.

Mikels, A. J., & Nusse, R. (2006). Wnts as ligands: Processing, secretion and reception. *Oncogene*, *25*(57), 7461-7468.

Mitchell, R. K., Mondragon, A., Chen, L., Mcginty, J. A., French, P. M., Ferrer, J., … & Da Silva Xavier, G. (2015). Selective disruption of Tcf7l2 in the pancreatic β cell impairs secretory function and lowers β cell mass. *Human Molecular Genetics*, *24*(5), 1390-1399.

Murtaugh, L. C. (2008). The what, where, when and how of Wnt/β-catenin signaling in pancreas development. *Organogenesis*, *4*(2), 81-86.

Oliveira, R. B., Maschio, D. A., Carvalho, C. P. F., Collares-Buzato, C. B. (2015). Influence of gender and time diet exposure on endocrine pancreas remodeling in response to high fat diet-induced metabolic disturbances in mice. *Annals of Anatomy*, *200*, 88-97.

Prentki, M., & Nolan, C. J. (2006). Islet β cell failure in type 2 diabetes. *Journal of Clinical Investigation*, *116*(7), 1802-1812.

Roarty, K., & Rosen, J. M. (2010). Wnt and mammary stem cells: Hormones cannot fly wingless. *Current Opinion in Pharmacology*, *10*(6), 643-649.

Rosenbaum, T., Vidaltamayo, R., Sánchez-Soto, M. C., Zentella, A., & Hiriart, M. (1998). Pancreatic beta cells synthesize and secrete nerve growth factor. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(13), 7784-7788.

Rulifson, I. C., Karnik, S. K., Heiser, P. W., Berge, D., Chen, H., Gu, X., … & Kim, S. K. (2007). Wnt signaling regulates pancreatic β cell proliferation, *104*(15), 6247-6252.

Schinner, S., Ülgen, F., Papewalis, C., Schott, M., Woelk, A., Vidal-Puig, A., Scherbaum, W. A. (2008). Regulation of insulin secretion, glucokinase gene transcription and beta cell proliferation by adipocyte-derived Wnt signalling molecules. *Diabetologia*, *51*(1), 147-154.

Sone, H., & Kagawa, Y. (2005). Pancreatic beta cell senescence contributes to the pathogenesis of type 2 diabetes in high-fat diet-induced diabetic mice. *Diabetologia*, *48*(1), 58-67.

Tripathy, D., & Chavez, A. O. (2010). Defects in insulin secretion and action in the pathogenesis of type 2 diabetes mellitus. *Current Diabetes Reports*, *10*(3),184-191.

Van Amerongen, R., Mikels, A., & Nusse, R. (2008). Alternative Wnt signaling is initiated by distinct receptors. *Science Signaling*, *1*(35), re9.

Wada, N., Hashinaga, T., Otabe, S., Yuan, X., Kurita, Y., Kakino, S., … Yamada, K. (2013). Selective Modulation of Wnt Ligands and Their Receptors in Adipose Tissue by Chronic Hyperadiponectinemia. *PLoS ONE*, *8*(7), e67712.

Wang, W., Yu, X., Wu, C., Jin, H. (2017). Il-36γ inhibits differentiation and induces inflammation of keratinocyte via Wnt signaling pathway in psoriasis. *International Journal of Medical Sciences*, *14*(10), 1002-1007.

Welters, H. J., & Kulkarni, R. N. (2008). Wnt Signaling : Relevance to B-Cell Biology and Diabetes. Review: *Trends in Endocrinology and Metabolism 19*(10), 349-355.

WHO Diabetes Country Profiles 2016 WHO Diabetes Country Profiles 2016 Explanatory Notes Background. (h.d.). (http://www.who.int/diabetes/country-profiles/diabetes_profiles_explanatory_notes.pdf)

Wu, Y., Ding, Y., Tanaka, Y., & Zhang, W. (2014). Risk factors contributing to type 2 diabetes and recent advances in the treatment and prevention. *International Journal of Medical Sciences*, *11*(11), 1185-1200.

Supplementary material

Table S1. Primers used in PCR analysis of Wnts and the respective Amplicon size and Tm.

Genes	Forward (5'– 3')	Reverse (5'– 3')	Amplicon	Tm
Wnt1	CTGTGCGAGAGTGCAAATGG	GATGAACGCTGTTTCTCGGC	116 pb	59°C
Wnt2	TTGCCCGTGCCTTTGTAGAT	GAGCCACTCACACCATGACA	142 pb	59°C
Wnt2b	TGACTACCTGAGGAGGCGAT	GACAAGATCAGTCCGGGTGG	115 pb	59°C
Wnt3	CTACCGCAACAGAACAGGCT	GTCACGAGTAGAGGCAGCTT	100 pb	59°C
Wnt3a	TCCATTGGTGCTGCTACGTC	GAACCCTGCTCCCGTGTTAG	96 pb	59°C
Wnt4	TCGGACAACATCGCCTATGG	ACCCGCATGTGTGTCAAGAT	146 pb	59°C
Wnt5a	AACCCTGTTCAGATGTCAGAAG	CTGCATGTGGTCCTGATACAAG	117 pb	58°C
Wnt5b	AAATGCACCGAGGTTGTGGA	ACTTTTGTGAGGCGGAGAGG	86 pb	59°C
Wnt6	CAGCAGGACATCCGAGAGACA	CTGACAACCACACTGTAGGAGC	111 pb	59°C
Wnt7a	CTCTTTCTCAGCCTGGGCAT	TACAGATGATGCTCGCACCC	82 pb	59°C
Wnt7b	TTCCACTGGTGTTGCTTCGT	CAAAATAATGGCGAGGGCCG	106 pb	59°C
Wnt8a	CCTTCATTCATGCCATCCGC	CGCAGTTTTCCAAGTCACCC	81 pb	59°C
Wnt8b	GCTAACCGGGAGACAGCATT	CCCAGTTGTCCATTTCGGGA	128 pb	59°C
Wnt9a	CGGGGACAACCTCAAGTACA	ATCACCTTCACACCCACGAG	117 pb	59°C
Wnt9b	GATTGAGTCGCAGAGCCCAT	TAGGGGTCTCGTTGAGCAGA	124 pb	59°C
Wnt10a	TCAGCCGAGGTTTTCGAGAG	CCGCAAGCCTTCAGTTTACC	106 pb	59°C
Wnt10b	CGGATGGAAGGGTAGTGGTG	GCTGGGAGATGGGGAAAGTC	95 pb	59°C
Wnt11	GGCAGTGCAACAAGACTTCC	CCACCACTCTGTCCGTGTAG	90 pb	59°C
Wnt16	CCCATCAGAAACACCACAGGA	GCGGCAGTCCACAGACATTA	111 pb	59°C
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	123 pb	60°C
Frizzled	CACGGCAGCTACTTCCACAT	GCAGAGCCCAGTCAGTTCAT	105 pb	59°C
LRP5	CCA TTGTGTTGCACCCTGTG	CAGGACATGCCGATCTCTCC	104 pb	59°C
LRP6	CAGACGGGACTTGAGATTGGT	TCTTCGCTGACATCACTCCAG	138 pb	59°C

Table S2. Primary and secondary antibodies used in immunohistochemistry.

Primary (manufacturer)	Dilution	Secondary (manufacturer)	Dilution
Anti-Wnt3a (Abcam) cat. ab28472	1:30	Anti-Rabbit IgG FITC Conjugate (Sigma) cat. F7512	1:50
Anti-Wnt5b (Abcam) cat ab94914	1:50	Anti-Rabbit IgG FITC Conjugate (Sigma) cat. F7512	1:50
Anti-Wnt8b (Invitrogen) cat. PA557723	1:30	Anti-Rabbit IgG FITC Conjugate (Sigma) cat. F7512	1:50
Anti-active β-catenin (Millipore) cat. 05-665	1:300	Anti-Mouse IgG FITC Conjugate (Sigma) cat. F9137	1:350
Guinea Pig anti-insulin (Dako) cat. A0564	1:75	Anti-Guinea Pig TRITC Conjugate (Sigma) cat. T7153	1:100

Capítulo 4

1. CONSIDERAÇÕES FINAIS

- A exposição à uma dieta hiperlipídica e hipercalórica (HFD), por 30 ou 60 dias, causa alterações metabólicas características da pré-diabetes tipo 2 em camundongos machos C57BL/6. Acompanhando as alterações metabólicas, após 60 dias de tratamento, ocorre uma hiperplasia compensatória significativa da massa de células beta que não é observada após 30 dias de tratamento.

- A expressão de proteínas envolvidas na ativação da via Wnt canônica está significativamente aumentada durante a expansão compensatória da massa de célula beta verificada em camundongos pré-diabéticos (HFD 60d). Além disso, ocorre a translocação nuclear da proteina β-catenina nas células beta pancreática desses animais. Ainda, em homogeneizados de ilhotas hiperplásicas isoladas, em comparação aos do grupo controle, há aumento significativo na expressão de mRNA de Ctnnb1 (β-catenina), Ccnd1 (Ciclina D1), Ccnd2 (Ciclina D2), Ins2 (insulina 2) e Coup-TFII e diminuição do inibidor Axina 2, o que indica uma ativação e possível participação da via Wnt no processo de hiperplasia compensatória da massa de células beta observada nesta fase da pré-diabetes experimental.

- A análise da expressão gênica dos 19 subtipos de Wnts demonstrou que a maioria deles são expressos em ilhotas isoladas e que no pâncreas como um todo são expressos em menor número. Os Wnts 3a e 5b mostraram um aumento significativo da expressão gênica em ilhotas hiperplásicas de camundongos pré-diabéticos em comparação com os do grupo controle. Foi demonstrada a expressão protéica desses Wnts, assim como o Wnt8b, pelas células beta pancreáticas por imunofluorescência indireta. Ainda, em condições *in vitro,* observamos que a co-cultura das células beta da linhagem MIN6 com ilhotas hiperplásicas ou com ilhotas não hiperplásicas não alterou significativamente a função secretora, mas induziu um aumento significativo da proliferação celular

desta linhagem que foi parcialmente inibido pelos inibidores farmacológicos IWR-1 e IWP-2 da via canônica, o que sugere que as ilhotas pancreáticas podem liberar fatores solúveis (Wnts) que, por sua vez, induziriam a proliferação da células beta.

2. CONCLUSÃO

Em conjunto, os nossos resultados demonstraram que a via Wnt canônica e as proteínas Wnts têm papel na biologia das células beta pancreática, pois em uma situação patológica como na pré-diabetes experimental foi possível observar que há uma regulação diferencial tanto da via como de alguns Wnts. O grande número de subtipos de Wnts expressos pelas ilhotas pancreáticas é uma observação interessante, mas que abriu novas questões e perspectivas de pesquisa, como: qual é a importância dessas proteínas para o pâncreas endócrino; se elas atuam por mecanismos de secreção autócrinos, parácrinos ou endócrinos; ou se têm funções distintas das suas funções clássicas já conhecidas; ou ainda se podem estar envolvidas em mecanismos que resultam na progressão da diabetes tipo 2.

Acreditamos que os resultados obtidos com o desenvolvimeno desta Tese de Doutorado tem capacidade para auxiliar em pesquisas futuras visando a melhor compreensão da biologia da célula beta pancreática e nos estudos de novas abordagens terapêuticas da diabetes mellitus tipo 2.

Capítulo 5

1. REFERÊNCIAS BIBLIOGRÁFICAS

Abiola, M., Favier, M., Christodoulou-vafeiadou, E., & Pichard, A. (2009). Activation of Wnt/b-Catenin Signaling Increases Insulin Sensitivity through a Reciprocal Regulation of Wnt10b and SREBP-1c in Skeletal Muscle Cells. *PLoS ONE 4*(12), 1–14.

Acharjee, S., Ghosh, B., Al-Dhubiab, B. E., & Nair, A. B. (2013). Understanding type 1 diabetes: Etiology and models. *Canadian Journal of Diabetes 37(4),* 269-276.

Amisten S., Salehi, A., Rorsman, P., and Jones P.M. (2013). An Atlas and Functional Analysis of G-Protein Coupled Receptors in Human Islets of Langerhans." *Pharmacology and Therapeutics 139*(3), 359–91.

Amisten, S., Mohammad Al-Amily, I., Soni, A., Hawkes, R., Atanes, P., Persaud, S. J., … Salehi, A. (2017). Anti-diabetic action of all-trans retinoic acid and the orphan G protein coupled receptor GPRC5C in pancreatic beta-cells. *Endocrine Journal, 64*(3), 325–338.

Andralojc, K. M., Mercalli, A., & Nowak, K. W. (2009). Ghrelin-producing epsilon cells in the developing and adult human pancreas. *Diabetologia 52*(3), 486-493.

Antonucci, L., Fagman, J. B., Kim, J. Y., Todoric, J., Gukovsky, I., Mackey, M., … Karin, M. (2015). Basal autophagy maintains pancreatic acinar cell homeostasis and protein synthesis and prevents ER stress. *Proceedings of the National Academy of Sciences, 112(*45), E6166–E6174.

Coustan, D.R. Gestational diabetes mellitus. (2013) *Clin Chem, 59*(9), 1310-1321.

Bernard, P., Fleming, A., Lacombe, A., Harley, V. R., & Vilain, E. (2008). Wnt4 inhibits β-catenin/TCF signalling by redirecting β-catenin to the cell membrane. *Biology of the Cell, 100*(3), 167-177.

Bosco, D., Armanet, M., Morel, P., Niclauss, N., Sgroi, A., Muller, Y. D., … Berney, T. (2010). Unique Arrangement of Alpha- and Beta-Cells in Human Islets of Langerhans. *Diabetes, 59*(5), 1202-1210.

Bowen, A., Kos, K., Whatmore, J., Richardson, S., & Welters, H. J. (2016). Wnt4 antagonises Wnt3a mediated increases in growth and glucose stimulated insulin secretion in the pancreatic beta-cell line, INS-1. *Biochemical and Biophysical Research Communications, 479*(4), 793-799.

Buechling, T., & Boutros, M. (2011). Wnt Signaling. Signaling at and Above the Receptor Level. *Current Topics in Developmental Biology 97*, 21-53.

Buschard, K. (2011). What causes type 1 diabetes? Lessons from animal models. *Acta pathologica, microbiologica, et immunologica Scandinavica,119*(132), 1-19.

Butler, A. E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R. A., & Butler, P. C. (2003). Beta-cell deficit and increased Beta-Cell apoptosis in humans with type 2 diabetes. *Diabetes, 52*(1), 102-110.

Cabrera, O., Berman, D. M., Kenyon, N. S., Ricordi, C., Berggren, P.-O., & Caicedo, A. (2006). The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. *Proceedings of the National Academy of Sciences, 103*(7), 2334-2339.

Carvalho, C. P. F., Oliveira, R. B., Britan, A., Boschero, A. C., & Meda, P. (2012). Impaired β-cell-β-cell coupling mediated by Cx36 gap junctions in prediabetic mice. *Am J Physiol Endocrinol Metab, 303*(1), E144-E151.

Cirulli, V., Halban, P. A., Rouiller, D. G. (1993). Tumor Necrosis Factor-a Modifies Adhesion Properties of Rat Islet B Cells. *J Clin Invest, 91,*1868-1876.

Clevers, H. (2006). Wnt/β-Catenin Signaling in Development and Disease. *Cell, 127*(3),469-480.

Clevers, H., & Nusse, R. (2012). Wnt/β-catenin signaling and disease. *Cell, 149*(6), 1192-1205.

Collares-Buzato, C.B. (2013). Junções Celulares. In H. F. Carvalho and S. M. Recco Pimentel (Eds.), A Célula, pp. 141-165. S. P., *Brasil: Manole* Ltda.

Collares-Buzato CB, High-Fat Diets and β-Cell Dysfunction: Molecular Aspects, in: D. Mauricio (Eds.), Molecular Nutrition and Diabetes, *Elsevier/Academic Press*, San Diego, 2015, pp. 115-130.

Chiefari, E., Arcidiacono, B., Foti, D., & Brunetti, A. (2017). Gestational diabetes mellitus: an updated overview. *Journal of Endocrinological Investigation, 40*(9), 899-909.

Conget, I., Manuel, J., Real, F., & Costa, À. (2001). Relación de la sensibilidad a la insulina y de la secreción de insulina con el grado de tolerancia a la glucosa en un grupo de personas de riesgo para desarrollar diabetes mellitus tipo 2. *Medicina Clínica, 7*(13), 491-492.

Cooper, J. D., Howson, J. M. M., Smyth, D., Walker, N. M., Stevens, H., Yang, J. H. M., … Rich, S. S. (2012). Confirmation of novel type 1 diabetes risk loci in families. *Diabetologia, 55*(4), 996-1000

DeFronzo, R. A., Ferrannini, E., Groop, L., Henry, R. R., Herman, W. H., Holst, J. J., … Weiss, R. (2015). Type 2 diabetes mellitus. *Nature Reviews Disease Primers, 1*, 15019.

Ding, Y. L., Wang, Y. H., Huang, W., Liu, G., Ross, C., Hayden, M. R., & Yang, J. K. (2010). Glucose intolerance and decreased early insulin response in mice with severe hypertriglyceridemia. *Experimental Biology and Medicine, 235*(1), 40-46.

Ekblad, E., & Sundler, F. (2002). Distribution of pancreatic polypeptide and peptide YY. *Peptides, 23*(2), 251-261.

El-Gohary, Y., Sims-Lucas, S., Lath, N., Tulachan, S., Guo, P., Xiao, X., … Gittes, G. K. (2012). Three-Dimensional Analysis of the Islet Vasculature. *Anatomical Record, 295*(9):1473-1481.

Falcão, V. T. F. L., Maschio, D. A., de Fontes, C. C., Oliveira, R. B., Santos-Silva, J. C., Almeida, A. C. S., … Collares-Buzato, C. B. (2016). Reduced insulin secretion function is associated with pancreatic islet redistribution of cell adhesion molecules (CAMs) in diabetic mice after prolonged high-fat diet. *Histochemistry and Cell Biology, 146*(1), 13-31.

Figeac, F., Uzan, B., Faro, M., Chelali, N., Portha, B., Figeac, F., … Movassat, J. (2010). Neonatal growth and regeneration of β-cells are regulated by the Wnt/β-catenin signaling in normal and diabetic rats. *Am J Physiol Endocrinol Metab 298*(2), 245-256.

Giaccari, A., Sorice, G., & Muscogiuri, G. (2009). Glucose toxicity: The leading actor in the pathogenesis and clinical history of type 2 diabetes - mechanisms and potentials for treatment. *Nutrition, Metabolism and Cardiovascular Diseases, 19*(5), 365-377.

Golson, M. L., Misfeldt A. A., Kopsombut, U. G, Petersen, C. P & Gannon M. (2010). High Fat Diet Regulation of β-Cell Proliferation and β-Cell Mass. *The open of Endocrinology* journal, 4: 10.2174/1874216501004010066.

Gonzalez, P., & Rodríguez, F. J. (2017). Analysis of the expression of the Wnt family of proteins and its modulatory role on cytokine expression in non activated and activated astroglial cells. *Neuroscience Research, 114*, 16–29.

Green, J., Nusse, R., & van Amerongen, R. (2014). The Role of Ryk and Ror Receptor Tyrosine Kinases in Wnt Signal Transduction. *Cold Spring Harbor Perspectives in Biology, 6*(2), a009175.

Guillén, M. A, Herranz, L., Barquiel, B., Hillman, N., Burgos, M. A., Pallardo, L. F. (2014). Influence of gestational diabetes mellitus on neonatal weight outcome in twin pregnancies. *Diabetic medicine, 31*(12), 1651-1656.

Hashimoto, N., Kido, Y., Uchida, T., Asahara, S. I., Shigeyama, Y., Matsuda, T., … Kasuga, M. (2006). Ablation of PDK1 in pancreatic β cells induces diabetes as a result of loss of β cell mass. *Nature Genetics, 38*(5), 589-593.

Herder, C., & Roden, M. (2011). Genetics of type 2 diabetes: Pathophysiologic and clinical relevance. *European Journal of Clinical Investigation, 41*(6), 679-692.

Hollingdal, M., Juhl, C. B., Pincus, S. M., Sturis, J., Veldhuis, J. D., Polonsky, K. S., … Schmitz, O. (2000). Failure of Physiological Plasma Glucose Excursions to Entrain High-Frequency Pulsatile Insulin Secretion in Type 2 Diabetes. *Diabetes 49*(8), 1334-1340.

Huang, S., & Czech, M. P. (2007). The GLUT4 Glucose Transporter. *Cell Metabolism, 5*(4), 237-252.

Huang, X., Liu, G., Guo, J. (2018). Corresponding address, Zhengquan Su1. The PI3K/AKT pathway in obesity and type 2 diabetes. (Review) *International Journal of Medical Sciences, 14*(11), 1483-1496.

Jain, R., & Lammert, E. (2009). Cell-cell interactions in the endocrine pancreas. *Diabetes, Obesity and Metabolism, 4*, 159-167.

Jia, L., Vianna, C. R., Fukuda, M., Berglund, E. D., Liu, C., Tao, C., … Elmquist, J. K. (2014). Hepatocyte toll-like receptor 4 regulates obesity-induced inflammation and insulin resistance. *Nature Communications, 5*, 3878.

Juhl, K., Bonner-Weir, S., & Sharma, A. (2010). Regenerating pancreatic B-cells: Plasticity of adult pancreatic cells and the feasibility of in vivo neogenesis. *Current Opinion in Organ Transplantation, 15*(1), 79–85.

Kahn, R., & Davidson, M. B. (2014). The reality of type 2 diabetes prevention. *Diabetes* Care, 37(4), 943-949

Kahn, S. E., Hull, R. L., & Utzschneider, K. M. (2006). Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature, 444*(7121), 840–846.

Kanno, T., & Go, S. O. (2002). Cellular Function in Multicellular System for Hormone-Secretion : Electrophysiological Aspect of Studies on A - , B - and D-Cells of the Pancreatic. *Islet, 42*, 79-90.

Katoh, M., & Katoh, M. (2017). Molecular genetics and targeted therapy of WNT-related human diseases (Review). *International Journal of Molecular Medicine, 40*(3), 587-606.

Kim C. (2014). Maternal outcomes and follow-up after gestational diabetes mellitus. (Review). *Diabetic medicine, 31*(3), 292-301.

Kramer, J., Moeller, E. L., Hachey, A., Mansfield, K. G., & Wachtman, L. M. (2009). Differential expression of GLUT2 in pancreatic islets and kidneys of New and Old World nonhuman primates. American Journal of Physiology. *Regulatory, Integrative and Comparative Physiology*, *296*(3), R786-R793.

Krützfeldt, J., & Stoffel, M. (2010). Regulation of wingless-type MMTV integration site family (WNT) signalling in pancreatic islets from wild-type and obese mice. *Diabetologia, 53*(1), 123-127.

Kuljanin, M., Bell, G. I., Sherman, S. E., Lajoie, G. A., & Hess, D. A. (2017). Proteomic characterisation reveals active Wnt-signalling by human multipotent stromal cells as a key regulator of beta cell survival and proliferation. *Diabetologia, 60*(10), 1987-1998.

Lackey, D. E., Burk, D. H., Ali, M. R., Mostaedi, R., Smith, W. H., Park, J., … Adams, S. H. (2014). Contributions of adipose tissue architectural and tensile properties toward defining healthy and unhealthy obesity. *AJP: Endocrinology and Metabolism*, *306*(3), E233-E246.

Langenbacher, A., & Chen, J. N. (2008). Calcium signaling: A common thread in vertebrate left-right axis development. *Developmental* Dynamics, 237(12), 3491-3496.

Lee, Y. C., & Nielsen, J. H. (2009). Regulation of beta cell replication. *Molecular and Cellular Endocrinology, 297*(1–2), 18-27.

Li, J., Malaby, A. W., Famulok, M., Sabe, H., Lambright, D. G., & Hsu, V. W. (2012). Grp1 Plays a Key Role in Linking Insulin Signaling to Glut4 Recycling. *Developmental Cell, 22*(6), 1286-1298.

Li, S., Wang, C., Liu, X., Hua, S., Liu, X. (2015). The roles of AXIN2 in tumorigenesis and epigenetic regulation. *Familial Cancer, 14*, 325-331.

Li, Q., & Lai, Z.-C. (2015). Recent progress in studies of factors that elicit pancreatic β-cell expansion. *Protein & Cell, 6*(2), 81-87.

Liew, C. G., & Andrews, P. W. (2009). Stem Cell Therapy to Treat Diabetes Mellitus. *The Review of Diabetic Studies, 5(*4), 203-219.

Liu, Z., & Habener, J. F. Wnt signaling in pancreatic islets. in: M.S. Islam (Eds), The Islets of Langerhans. *Springer*, New York, 654 (2010) pp. 391-419.

Lloyd-lewis, B., Fletcher, A. G., Dale, T. C., & Byrne, H. M. (2013). Toward a quantitative understanding of the Wnt/β-catenin pathway through simulation and experiment. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine, 5*(4), 391-407.

Logan, C. Y., & Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annual review of cell and developmental biology, 20*, 781-810

Logsdon, C. D., & Ji, B. (2013). The role of protein synthesis and digestive enzymes in acinar cell injury. *Nature Reviews Gastroenterology and Hepatology, 10*(6), 362-370.

Lowell, B. B. & Shulman, G. I. 2005. Mitochondrial Dysfunction and Type 2 Diabetes. *Science, 307*(5708), 384-387

Ly, L. D., Xu, S., Choi, S-K., Ha, C-M.,Thoudam, T., Cha, S-K. ... & Park, K-S. (2017). Oxidative stress and calcium dysregulation by palmitate in type 2 diabetes. (Review). *Experimental & Molecular Medicine, 49*, e291.

Lyssenko, V., & Laakso, M. (2013). Genetic Screening for the Risk of Type 2 Diabetes: Worthless or valuable? *Diabetes Care, 36*, S120-S126.

Lyssenko, V., Lupi, R., Marchetti, P., Del Guerra, S., Orho-Melander, M., Almgren, P., … Groop, L. (2007). Mechanisms by which common variants in the TCF7L2 gene increase risk of type 2 diabetes. *The Journal of Clinical Investigation*, *117*(8), 2155-2163.

Ma, Z. A., Zhao, Z., & Turk, J. (2011). Mitochondrial Dysfunction and β-Cell Failure in Type 2 Diabetes Mellitus. *Experimental Diabetes Research, 4*, 703538.

MacDonald, B. T, Tamai, K., and He, X. (2010). Wnt/β-catenin signaling: components, mechanisms, and diseases. *Developmental Biology, 17*(1), 9-26.

Maschio, D. A. Possível ativação da via de sinalização da WNT/β-catenina no processo de hiperplasia compensatória da célula beta pancreática em modelo animal de resistência periférica à insulina. Dissertação de Mestrado, Campinas, SP: [s.n.], 2014.

Maschio, D. A., Oliveira, R. B., Santos, M. R., Carvalho, C. P. F., Barbosa-Sampaio, H. C. L., & Collares-Buzato, C. B. (2016). Activation of the Wnt/β-catenin pathway in pancreatic beta cells during the compensatory islet hyperplasia in prediabetic mice. *Biochemical and Biophysical Research Communications, 478*(4), 1534-1540.

Matheus, V. A., Monteiro, L. C. S., Oliveira, R. B., Maschio, D. A., & Collares-Buzato, C. B. (2017). Butyrate reduces high-fat diet-induced metabolic alterations, hepatic steatosis and pancreatic beta cell and intestinal barrier dysfunctions in prediabetic mice. *Experimental Biology and Medicine, 242*(12), 1214-1226.

Mezza, T., Muscogiuri, G., Sorice, G. P., Clemente, G., Hu, J., Pontecorvi, A., … Kulkarni, R. N. (2014). Insulin resistance alters islet morphology in nondiabetic humans. *Diabetes, 63*(3), 994-1007.

Metzger, B. E. (2007) Long-term outcomes in mothers diagnosed with gestational diabetes mellitus and their offspring. *Clinical Obsterict and Gynecoogyl, 50*(4), 972-979.

Miles, J.M., Wooldridge, D., Grellner, W. J., Windsor, S., Isley, W. L., ... Klein, S. (2003). Nocturnal and postprandial free fatty acid kinetics in normal and type 2 diabetic subjects: effects of insulin sensitization therapy. *Diabetes, 52,* 675-681

Mikels, A. J., & Nusse, R. (2006). Wnts as ligands: Processing, secretion and reception. *Oncogene, 25*(57), 7461-7468.

Mitchell, R. K., Mondragon, A., Chen, L., Mcginty, J. A., French, P. M., Ferrer, J., ... Da Silva Xavier, G. (2015). Selective disruption of Tcf7l2 in the pancreatic β cell impairs secretory function and lowers β cell mass. *Human Molecular Genetics*, *24*(5), 1390-1399.

Murtaugh, L. C. (2008). The what, where, when and how of Wnt/β-catenin signaling in pancreas development. Organogenesis, 4(2), 81-86.

Nerup, J., Platz, P., Andersen, O.O., Christy, M., Lyngsoe, J., Poulsen, J.E., Ryder, L.P, ... (1974). HL-A antigens and diabetes mellitus. *Lancet, 2*(7885), 864-866

Ng, Y., Ramm, G., Lopez, J. A., & James, D. E. (2008). Rapid Activation of Akt2 Is Sufficient to Stimulate GLUT4 Translocation in 3T3-L1 Adipocytes. *Cell Metabolism, 7*(4), 348-356.

Nguyen, K.-T. T., Tajmir, P., Lin, C. H., Liadis, N., Zhu, X.-D., Eweida, M., … Woo, M. (2006). Essential role of Pten in body size determination and pancreatic beta-cell homeostasis in vivo. *Molecular and Cellular Biology, 26*(12), 4511-4518.

Noble, J. A., & Erlich, H. A. (2012). Genetics of type 1 diabetes. *Cold Spring Harbor Perspectives in Medicine, 2*(1), a007732.

Nolan, C. J., Damm, P., & Prentki, M. (2011). Type 2 diabetes across generations: From pathophysiology to prevention and management. *The Lancet, 378*(9786), 169-181.

Oliveira, R. B.; Carvalho, C. P.; Polo, C. C.; Dorighello, G. D.; Boschero, A. C.; Oliveira, H. C.; Collares-Buzato, C. B. (2014). Impaired compensatory beta-cell function and growth in response to high-fat diet in LDL receptor knockout mice.

International Journal of Experimental Pathology, 95(4), 296-308.

Oliveira, R. B., Maschio, D. A., Carvalho, C. P. F., & Collares-Buzato, C. B. (2015). Influence of gender and time diet exposure on endocrine pancreas remodeling in response to high fat diet-induced metabolic disturbances in mice. *Annals of Anatomy*, 200, 88-97.

Orci, L., Malaisse-Lagae, F., Amherdt, M., Ravazzola, M., Weisswange, A., Dobbs, R., … Unger, R. (1975). Cell contacts in human islets of langerhans. *Journal of Clinical Endocrinology and Metabolism, 41*(5), 841-844.

Orci, L. (1976). The microanatomy of the islets of langerhans. *Metabolism, 25*(11), 1303-1313.

Pandiri, A. R. (2014). Overview of exocrine pancreatic pathobiology. *Toxicologic Pathology, 42*(1), 207-216.

Prentki, M., & Nolan, C. J. (2006). Islet β cell failure in type 2 diabetes. *Journal of Clinical Investigation, 116*(7), 1802-1812.

Pugliese, A., Boulware, D., Yu, L., Babu, S., Steck, A. K., Becker, D., … Sosenko, J. M. (2016). HLA-DRB1∗15:01-DQA1∗01:02-DQB1∗06:02 haplotype protects autoantibody-positive relatives from type 1 diabetes throughout the stages of disease progression. *Diabetes, 65*(4), 1109-1119.

Rahier, J., Guiot, Y., Goebbels, R. M., Sempoux, C., & Henquin, J. C. (2008). Pancreatic β-cell mass in European subjects with type 2 diabetes. *Diabetes, Obesity and Metabolism, 10*(4), 32-42.

Rennoll, S. A., Konsavage, W. M. Jr, Yochum, G. S. (2014) Nuclear AXIN2 represses MYC gene expression. *Biochemical and Biophysical Research Communications, Biochemical and Biophysical Research Communications,* 443(1), 217-222.

Reyna, S. M., Ghosh, S., Tantiwong, P., Meka, C. S. R. M., Eagan, P., Jenkinson, C. P., … Musi, N. (2008). Elevated toll-like receptor 4 expression and signaling in muscle from insulin-resistant subjects. *Diabetes, 57*(10), 2595-2602.

Rieusset, J. (2015). Contribution of mitochondria and endoplasmic reticulum dysfunction in insulin resistance: Distinct or interrelated roles? *Diabetes & Metabolism, 41*(5), 358-368.

Roarty, K., & Rosen, J. M. (2010). Wnt and mammary stem cells: Hormones cannot fly wingless. *Current Opinion in Pharmacology, 10*(6), 643-649.

Rulifson, I. C., Karnik, S. K., Heiser, P. W., Berge, D., Chen, H., Gu, X., … Kim, S. K. (2007). Wnt signaling regulates pancreatic beta-cell proliferation. *Proceedings of the National Academy of Sciences, 104*(15), 6247-6252.

Saisho, Y., Butler, A. E., Manesso, E., Elashoff, D., Rizza, R. A., & Butler, P. C. (2013). β-Cell mass and turnover in humans: Effects of obesity and aging. *Diabetes Care, 36*(1), 111-117.

Salehi, A., Muhammed, S. J., Reinbothe, T. M., Tang, Y., Mahdi, T., Ha, S., …

Renstro, E. (2012). Secreted Frizzled-Related Protein 4 Reduces Insulin Secretion and Is Overexpressed in Type 2 Diabetes. *Cell Metabolism, 16*(5), 625-633.

Satin, L. S., Butler, P. C., Ha, J., & Sherman, A. S. (2015). Molecular Aspects of Medicine Pulsatile insulin secretion, impaired glucose tolerance and type 2 diabetes. *Molecular Aspects of medicine 42*, 61-77.

Schinner, S. (2010). Wingless-type MMTV integration site family (WNT) signalling in pancreatic beta cells-more complex than expected. *Diabetologia, 53*(9), 2073-2075.

Shoelson, S. E., Lee, J., & Goldfine, A. B. (2006). Inflammation and insulin resistance. *Journal of Clinical Investigation*, 116(7), 1793-801.

Singal, D. P., & Blajchman, M. A. (1973). Histocompatibility (HL-A) Antigens, Lymphocytotoxic Antibodies and Tissue Antibodies in Patients with Diabetes Mellitus. *Diabetes, 22*(6), 429-432.

Sone, H., & Kagawa, Y. (2005). Pancreatic beta cell senescence contributes to the pathogenesis of type 2 diabetes in high-fat diet-induced diabetic mice. *Diabetologia, 48*(1), 58-67.

Sorrenson, B., Cognard, E., Lee, K. L., Dissanayake, W. C., Fu, Y., Han, W., … Shepherd, P. R. (2016). A critical role for β-catenin in modulating levels of insulin secretion from β-cells by regulating actin cytoskeleton and insulin vesicle localization. *Journal of Biological Chemistry, 291*(50), 25888-25900.

Sriwijitkamol, A., Christ-Roberts, C., Berria, R., Eagan, P., Pratipanawatr, T., DeFronzo, R. A, Musi, N. (2006). Reduced skeletal muscle inhibitor of kappaB beta content is associated with insulin resistance in subjects with type 2 diabetes: reversal by exercise training. *Diabetes, 55*(3), 760-767.

Sriwijitkamol, A., Christ-Roberts, C., Berria, R., Eagan, P., Pratipanawatr, T., DeFronzo, R. A., ... & Weis, W. I. (2013). The b-Catenin Destruction Complex. *Cold Spring Harb Perspect Biol 5*, a007898.

Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A, Cahill, D. A., ... (1991) White MF.Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature, 352*(6330), 73-77.

Thorens, B. (2014). GLUT2, glucose sensing and glucose homeostasis. *Diabetologia, 58*(2), 221-232.

Tilg, H., & Moschen, A. R. (2014). Microbiota and diabetes: an evolving relationship. *Gut, 63(*9), 1513-1521.

Tripathy, D., & Chavez, A. O. (2010). Defects in insulin secretion and action in the pathogenesis of type 2 diabetes mellitus. *Current Diabetes Reports, 10*(3), 184-191

Varela-Calvino, R., Sgarbi, G., Arif, S., & Peakman, M. (2000). T-cell reactivity to the P2C nonstructural protein of a diabetogenic strain of coxsackievirus B4. *Virology, 274*(1), 56-64.

Vázquez-Jiménez, J. G., Roura-Guiberna, A., Jiménez-Mena, L. R., & Olivares-Reyes,

J. A. (2017). El papel de los ácidos grasos libres en la resistencia a la insulina. *Gaceta de México, 153*(7), 852-863.

Verkaar, F., Cadigan, K. M., & Amerongen, R. Van. (2013). Celebrating 30 Years of Wnt Signaling. *Science Signaling, 5* (254), mr2.

Vohr, B.R., Boney, C. M. (2008). Gestational diabetes: the forerunner forthe development of maternal and childhood obesity and metabolic syndrome? The *Jornal of maternal-fetal and neonatal medicine, 21,* 149-157.

Wang, X., Zielinski, M. C., Misawa, R., Wen, P., Wang, T. Y., Wang, C. Z., … Hara, M. (2013). Quantitative Analysis of Pancreatic Polypeptide Cell Distribution in the Human Pancreas. *PLoS ONE, 8*(1), e55501.

Watson, R. T., & Pessin, J. E. (2006). Bridging the GAP between insulin signaling and GLUT4 translocation. *Trends in Biochemical Sciences, 31*(4),215-222.

Welters, H. J., & Kulkarni, R. N. (2008). Wnt Signaling : Relevance to B-Cell Biology and Diabetes. (Review) *Trends in Endocrinology and Metabolism, 19*(10), 349-355.

WHO Diabetes Country Profiles 2016 WHO Diabetes Country Profiles 2016 Explanatory Notes Background. (n.d.).

Yesil, P., & Lammert, E. (2008). Islet dynamics: A glimpse at beta cell proliferation. *Histology and Histopathology, 23*(7), 883–895.

Yoneda, S., Uno, S., Iwahashi, H., Fujita, Y., Yoshikawa, A., Kozawa, J., … Shimomura, I. (2013). Predominance of β-cell neogenesis rather than replication in humans with an impaired glucose tolerance and newly diagnosed diabetes. *Journal of Clinical Endocrinology and Metabolism, 98*(5), 2053-2061.

ANEXOS

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CERTIFICADO

Certificamos que o projeto **"PAPEL DA VIA DE SINALIZAÇÃO WNT CANÔNICA NA PROLIFERAÇÃO E FUNÇÃO SECRETORA DA CÉLULA BETA PANCREÁTICA EM CONDIÇÕES IN VITRO E NA PRÉ-DIABETES"** (protocolo nº **3443-1**), sob a responsabilidade de **Profa. Dra. Carla Beatriz Collares Buzato / Daniela Aparecida Maschio**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

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O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **26 de junho de 2014**.

Campinas, 26 de junho de 2014.

Prof. Dr. Alexandre Leite Rodrigues de Oliveira
Presidente

Fátima Alonso
Secretária Executiva

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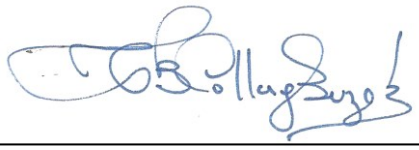

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