Universidade Federal do Rio Grande do Sul

Aspectos Genéticos e Celulares do Diabetes Mellitus Tipo 1

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Apresentação

Esta tese de doutorado está organizada em duas partes para facilitar o entendimento. A primeira diz respeito aos aspectos celulares, que foi o objetivo inicial desse trabalho, com a investigação de duas abordagens para uso de células-tronco mesenquimais no tratamento do Diabetes mellitus tipo 1 (DM1). No capítulo 1 é apresentado um artigo de revisão, publicado na revista Science Progress, sobre a biologia e as aplicações de células-tronco mesenquimais, o qual introduz o assunto para os próximos dois capítulos. O Capítulo 2 refere-se à tentativa de diferenciação de células-tronco mesenquimais em células-produtoras de insulina *in vitro*, com o objetivo de criar uma nova fonte de células para reposição das células β perdidas, enquanto o Capítulo 3 consiste no co-transplante destas células com ilhotas pancreáticas em um modelo de camundongos diabéticos.

A segunda parte refere-se aos aspectos genéticos do Diabetes mellitus tipo 1. Esse trabalho começou no início do meu doutorado com uma parceria entre o Laboratório de Imunogenética da UFRGS e o Instituto da Criança com Diabetes do Rio Grande do Sul (ICDRS), intermediada pelo Prof. Israel Roisenberg. Essa parceria resultou na criação de um banco de DNA de pacientes com DM1. Além disto, dois trabalhos foram publicados investigando a associação entre variantes alélicas no gene da proteína tirosino-fosfatase 22 (Capítulo 4) e de sistemas polimórficos do HLA classe I, II e KIR (Capítulo 5) com a susceptibilidade a essa doença.

Sumário

Lista de abreviaturas

ALCAM - *activated leukocyte-cell adhesion molecule* ou CD166

AMP - *adenosine monophosphate* (monofosfato de adenosina)

ATP - *adenosine triphosphate* (trifosfato de adenosina)

CD133 – *cluster of differentiation 133* ou Prominina

CK19 – *cytokeratin 19* (citoqueratina 19)

CTLA-4 - *cytotoxic T-lymphocyte antigen 4* (antígeno de linfócito T citotóxico 4)

DAPT - *N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester*

db-cAMP - *N6,2'-O-dibutyryladenosine 3':5' cyclic monophosphate*

DM - Diabetes mellitus

DMSO - *dimethyl sulfoxide* (dimetilsulfóxido)

DM1 - Diabetes mellitus tipo 1

DM2 - Diabetes mellitus tipo 2

EDTA - *ethylenediamine tetraacetic acid* (ácido etilenodiamino tetra- acético)

ERAS - *embryonic stem cell expressed Ras*

ESC - *embryonic stem cell* (célula-tronco embrionária)

SFB - soro fetal bovino

FGF - *fibroblast growth factor* (fator de crescimento de fibroblasto)

FGF8 - *fibroblast growth factor 8* (fator de crescimento de fibroblasto 8)

GLP-1 – *glucagon like peptide-1* (peptídeo similar ao glucagon 1)

GLUT2 – *glucose transporter-2* (transportador de glicose-2)

HLA - *human leucocitary antigen* (antígeno leucocitário humano)

HGF - *hepatocyte growth factor* (fator de crescimento de hepatócito)

HNF3B - *hepatocyte nuclear factor 3, beta* (fator nucler hepático 3 beta) ou FOXA2.

HNF6 - *hepatocyte nuclear factors 6* (fator nucler hepático 6)

IBMX - *3-isobutyl-1-methylxanthine* (3-isobutil-1-metilxantina)

ID2 - *inhibitor of DNA binding 2*

IGF-1 – *insulin-like growth factor-1* (fator de crescimento similar à insulina)

LACZ - gene da β-galactosidase

MESP1 - *mesoderm posterior 1* (mesoderme posterior 1)

MET - *N-methyl-N'-nitro-N-nitroso-guanidine HOS tranforming gene*

MSC - *mesenchymal stem cell* (célula-tronco mesenquimal)

NEUROD1 - *neurogenic differentiation 1* (fator de diferenciação neurogênica 1)

NGN3 – *neurogenin3* (neurogenina3)

NKX2.2 – *NK2 homeobox 2*

NKX6.1 – *NK6 homeobox 2*

NOD - *non obese diabetic* (diabético não obeso)

OCT4 - *octamer-4* ou *POU5F1*

PAX4 - fator de transcrição envolvido na diferenciação de células β

PC1/3 - *prohormone convertase 1*

PC2 - *prohormone convertase 2*

PDX1 - *pancreatic and duodenal homeobox-1* ou IPF-1

PSC - *pancreatic stem cell* (Célula-tronco pancreática)

PTPN22 – *protein tyrosine phosphatase non-receptor 22*

P75NTR - *p75 neurotrophin receptor*

SOX2 - *sex determining region Y-box 2*

SOX17 - *sex determining region Y-box 17*

STZ - *streptozotocin* (estreptozotocina)

SUR1 - *sulfonylurea receptor 1* (receptor de sulfoniluréia 1)

TGF-β – *transforming growth factor beta*

VP7 – proteína do capsídeo do rotavírus

VNTR - *Variable number tandem repeat*

Wnt3a – membro da família Wnt

Resumo

O Diabetes mellitus tipo 1 (DM1), na maioria dos casos, é causado pela destruição de células β pancreáticas, levando à hiperglicemia. Atualmente a única fonte de novas células β e os únicos tratamentos capazes de restaurar o padrão fisiológico de secreção de insulina nesses pacientes são o transplante de pâncreas e de ilhotas pancreáticas. O transplante de ilhotas apresenta problemas relacionados a enxertia, devido principalmente a baixa vascularização, o que leva à morte de células β nos primeiros dias pós-transplante. Células-tronco mesenquimais apresentam características interessantes para o tratamento do DM1. A primeira aplicação explorada nesse trabalho foi a capacidade de diferenciação de MSCs humanas e murinas em células produtoras de insulina (CPIs). A identidade das células isoladas foi confirmada pela caracterização imunofenotípica e pela capacidade de diferenciação adipogênica e osteogênica *in vitro*. Quatro protocolos de diferenciação em CPIs foram testados em MSCs derivadas de ilhotas pancreáticas e um em MSCs derivadas de rim murino. A análise da expressão gênica de insulina em células diferenciadas em todos os protocolos testados mostrou níveis insignificantes ou nulos de expressão desse hormônio. A segunda aplicação explorou o co-transplante de ilhotas pancreáticas com MSCs derivadas de rim em camundongos diabéticos. Os resultados mostraram aumento da taxa de cura e melhora na glicemia pós-transplante, bem como uma tendência ao aumento do conteúdo total de insulina em animais co-transplantados em comparação com animais que receberam apenas ilhotas. Não houve diferenças no peso e teste de tolerância à glicose entre os grupos. Foi observado aumento na vascularização do enxerto nos animais que receberam MSCs. Paralelamente, foi estudada a associação de variantes alélicas dos genes PTPN22, KIR, HLA classe I e II e a susceptibilidade ao desenvolvimento de DM1 em uma população do Rio Grande do Sul. Foi observada associação entre o alelo 1858T e o risco aumentado de DM1. A genotipagem do KIR e HLA-C mostrou uma frequência maior de alelos do grupo 2 do HLA-C em controles não diabéticos, bem como o genótipo 2DL1/C2+, sugerindo um papel protetor desse genótipo. Além disso, indivíduos com haplótipo KIR2DL2/DR3+ e KIR2DL2/DR3/DR4+ tem risco aumentado de desenvolvimento de DM1. MSCs parecem possuir baixa capacidade de diferenciação em células β *in vitro*, entretanto, possuem efeitos benéficos importantes quando co-transplantadas com ilhotas pancreáticas. Essa aplicação tem grande potencial e deveria ser testada em estudos clínicos com o objetivo de melhorar a enxertia e diminuir o número de ilhotas necessárias para cada paciente.

Abstract

Type 1 Diabetes (DM1), in almost all the cases, is caused by the destruction of beta-cells by cells of the immune system, leading to hyperglycemia. The only source for new beta-cells available is through the pancreas and islet transplantation, two treatments able to restore insulin secretion pattern in this patients. Islet transplantation presents issues related to grafting, caused mainly by poor vascularisation post-transplant, leading to beta-cell death in the first days after transplantation. Mesenchymal stem cells have interesting characteristics to the treatment of DM1. The first application explored in this work was testing the capacity of differentiation of human and mouse MSCs into insulin-producing cells (CPIs). Identity of isolated cells was confirmed by immunophenotyping and potential of adipogenic and osteogenic differentiation *in vitro*. Four protocols were tested in human islet-derived MSCs and one in mouse kidney-derived MSCs to generate CPIs. Analysis of insulin expression in differentiated cells from all protocols showed no or very little expression levels of this hormone. The second application was to evaluate the role of MSCs in the co-transplantation with pancreatic islets in diabetes mice. Our results showed an increased number of cured mice and a decrease in glycemic levels post transplant in islet+MSCs group, as well as a tendency to an increase in total insulin content in islet+MSCs compared with islet-only group. No differences could be found in weight and intraperitoneal glucose tolerance test between groups. An increase in graft vascularisation was observed in MSCs-receiving animals. At the same time, we studied the association of allelic variants in PTPN22, KIR, HLA class I and II genes and its association with the developing of DM1. We reported and association of the 1858T allele and an increased risk of DM1. Genotyping shows an increased frequency of group 2 alleles (C2) of HLA-C in controls as well as the 2DL1/C2+ genotype, suggesting a protective role of this genotype. Moreover, individuals with KIR2DL2/DR3+ and KIR2DL2/DR3/DR4+ haplotypes have increased risk of developing DM1. MSCs seem to have low capacity of *in vitro* differentiation in a beta-cell phenotype, however, they exert important benefic effects when co-transplanted with pancreatic islets in diabetic mice. This application has great potential and should be tested in clinical trials aiming the improvement of islet grafting and decrease in the number of islets needed for transplantation.

INTRODUÇÃO GERAL

Diabetes mellitus

Definição

O diabetes mellitus (DM) compreende um conjunto de doenças caracterizadas por altos níveis de glicose no sangue, causados pela não produção, produção insuficiente ou falta de resposta à insulina fabricada pelas células β localizadas nas ilhotas de Langerhans do pâncreas (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). Quando a insulina deixa de desempenhar o seu papel, ocorre reduzida captação de glicose pelas células do tecido muscular e adiposo, assim como a maior produção desse açúcar no fígado, levando à hiperglicemia. O glucagon é um hormônio com ação oposta à da insulina, sendo liberado quando os níveis de glicose estão baixos, estimulando o uso de outras fontes energéticas como gordura e proteína. Sendo assim, esses dois hormônios possuem um papel fundamental na regulação dos níveis de glicose sanguíneos, e qualquer desequilíbrio entre eles pode levar ao desenvolvimento do diabetes (Bansal e Wang, 2008).

Classificação

Existem dois tipos mais comuns de DM. O diabetes mellitus tipo 1 (DM1), cuja hiperglicemia na maioria dos casos é causada pela destruição das células β do pâncreas pelo sistema imune. Alguns dos sintomas apresentados são: poliúria, perda de peso, sede excessiva e cansaço. A maioria dos pacientes depende de injeções diárias de insulina exógena para sobreviver, mantendo a glicemia próxima dos níveis normais, embora, em longo prazo, possam desenvolver complicações crônicas relacionadas à hiperglicemia.

O diabetes mellitus tipo 2 está intimamente ligado a obesidade e manifesta-se principalmente em pessoas acima de 40 anos, podendo ocorrer em qualquer idade. Usualmente caracteriza-se por resistência à insulina associada a graus variados de deficiência na secreção de insulina pelas células β. As causas do diabetes tipo 2 continuam desconhecidas, porém, tanto fatores genéticos quanto ambientais, como obesidade, falta de exercício e infecções, parecem desempenhar um papel importante no desenvolvimento da doença. Em baixa freqüência existem ainda o diabetes gestacional e outros tipos

específicos de diabetes associados a várias patologias (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003).

Complicações crônicas

A incapacidade de controlar adequadamente os níveis glicêmicos leva, à longo prazo, a danos nas células de diversos órgãos. Esse dano é sentido principalmente em células endoteliais da micro e macrovasculatura (Sweet et al., 2009). Por isso, o DM é um fator de risco para o aparecimento de doenças como cardiopatias (Nichols et al., 2001), nefropatias (Loon, 2003) e retinopatias (Conway et al., 2009).

Aronow et al. (1999) estudaram 2.737 pessoas idosas com e sem diabetes mellitus, mostrando que pacientes diabéticos têm um risco 1,3 vezes maior de desenvolver problemas cardíacos congestivos. Segundo dados do NIDDK (National Institute of diabetes & Digestive & Kidney Disease, 2005) o diabetes é a causa mais comum de problemas renais, correspondendo a mais de 40% dos novos casos, sendo os pacientes com diabetes tipo 1 mais propensos a esse tipo de doença. De 20 a 40% dos pacientes com DM1 apresentam problemas renais aos 50 anos, embora estas complicações possam aparecer antes dos 30 anos. Estima-se que 50% dos diabéticos possuam alguma forma de neuropatia, embora muitos casos sejam assintomáticos.

Epidemiologia

Dados da Organização Mundial de Saúde de 2000 mostram que aproximadamente 171 milhões de pessoas no mundo tinham diabetes, e que esse número poderá chegar a 366 milhões até 2030. Esse aumento deverá ocorrer principalmente nos países em desenvolvimento, devido ao crescimento e envelhecimento da população, obesidade, hábitos alimentares incorretos e sedentarismo. No Brasil, o número estimado de Diabéticos em 2000 era de pouco mais de 4,5 milhões, prevendo-se que esse número ultrapasse os 11 milhões em 2030 (World Health Organization, 2010).

A Federação Internacional de Diabetes (IDF) desenvolveu o Atlas do Diabetes, com dados de 172 associações em 132 países. Dados de 2010 mostram que aproximadamente 480 mil crianças (0-14 anos) têm DM1 nas regiões analisadas. Dados da América Central e Caribe mostram que 36,9 mil crianças de 0-14 anos têm DM1, com uma incidência anual de 5,8 mil novos cassos diagnosticados. A estimativa de gastos com DM

na região está em torno de 8,1 bilhões de dólares (Atlas do Diabetes, 4ª edição, Federação Internacional do Diabetes, 2009).

O pâncreas

O pâncreas é uma glândula mista com uma porção endócrina e outra exócrina. A porção exócrina é formada por uma glândula acinosa cujas células têm morfologia característica de células secretoras de proteínas, importantes no processo de digestão. O órgão é revestido por uma cápsula delicada de tecido conjuntivo que divide a glândula em lóbulos (Junqueira e Carneiro, 1999).

Em 1869, Langerhans descreveu no pâncreas pequenos aglomerados de células parecidos com pequenas "ilhas", as quais, posteriormente, passaram a ser chamadas de ilhotas de Langerhans. Em seguida, Kuhne e Lea identificaram nas ilhotas, redes de capilares que apontavam para uma possível função endócrina. A relação entre o diabetes e o pâncreas só foi provada em 1889, quando von Mering e Minkowski retiraram o pâncreas de animais de laboratório e viram que eles passavam a produzir altas quantidades de urina contendo glicose (Ham, 1963). Assim, as ilhotas de Langerhans constituem a porção endócrina do pâncreas, estando distribuídas por todo o órgão.

O número de ilhotas em humanos varia, ficando em torno de um milhão, sendo que cada ilhota possui em média 1500 células (Pisania et al., 2010). Estima-se que as ilhotas correspondam a 1-1,5% do volume do órgão em humanos (Junqueira e Carneiro, 1999).

As ilhotas são compostas por três diferentes tipos celulares principais; em humanos, células β, células α e células δ correspondem a aproximadamente 54% (28-75), 35% (10-65) e 11% (1.2-22) do total de células das ilhotas, já em camundongos as frequências ficam em 75% (61-88), 19% (9-31) e 6% (1-13), com uma heterogeneidade maior presente em ilhotas humanas (Brissova et al., 2005). Em menor número existem ainda as células PP que produzem o polipeptídeo pancreático e as células épsilon que produzem grelina. Envolvendo cada ilhota há uma fina camada de tecido conjuntivo, além de uma rede de capilares sanguíneos que penetra entre as células (Junqueira e Carneiro, 1999, Cabrera et al., 2006).

Células β pancreáticas

A captação de glicose pelas células pode ser ou não dependente de insulina, dependendo do tecido. No cérebro, a maior parte da captação se dá na ausência de insulina, já no músculo e tecido adiposo esse hormônio é usualmente indispensável. A insulina fica estocada em vesículas secretoras no citoplasma das células β e, em resposta a estímulos, é liberada por exocitose devido, principalmente, a flutuações internas de Ca^{2+} (Halban et al., 2001). A figura 1 traz um esquema da via envolvida na secreção de insulina em resposta à glicose.

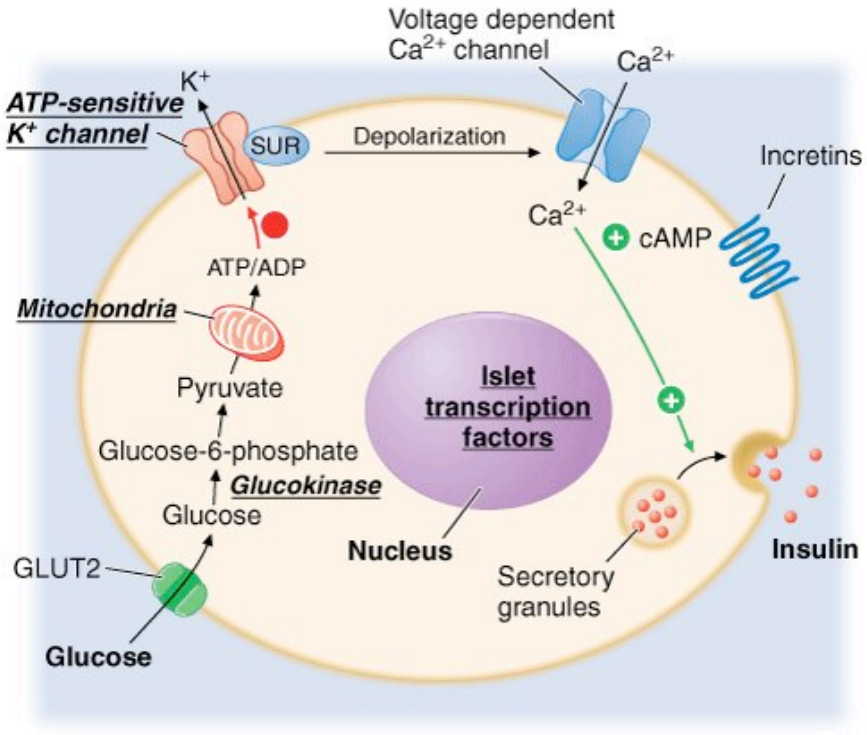

Figura 1. Esquema detalhando os eventos que envolvem a secreção de grânulos de insulina em resposta à glicose em células β pancreáticas. Retirado de Fauci et al., Harrison's Principles of Internal Medicine.

A glicose entra nas células β pancreáticas pelo transportador GLUT-2, sendo imediatamente fosforilada em glicose-6-fosfato pela enzima glucoquinase. Ao final da glicólise é formado piruvato, que no processo de geração de energia que ocorre nas mitocôndria produz ATP, levando ao fechamento dos canais de potássio (subunidade KIR6.2 e SUR1) sensíveis a ATP. Ocorre, então, a despolarizacão da membrana

plasmática e a entrada de Ca^{2+} na célula por canais de cálcio dependentes de voltagem. O Ca^{2+}, por fim, leva à liberação da insulina armazenada em vesículas próximas a membrana plasmática das células (Figura 1).

Diabetes e o sistema imune

Como dito anteriormente, a maioria dos pacientes com DM1 apresenta um quadro de autoimunidade. A análise do pâncreas de doadores cadavéricos com diagnóstico recente de DM1 mostrou que linfócitos T citotóxicos ($CD8^+$) e macrófagos ($CD68^+$) são os tipos celulares mais freqüentes nos infiltrados ao redor das ilhotas, comparado com uma baixa freqüência de linfócitos T auxiliares ($CD4^+$), linfócitos B ($CD20^+$) e plasmócitos ($CD138^+$). Células *natural killer* ($CD56^+$) e células T regulatórias ($FoxP3^+$) raramente foram encontradas (Willcox et al., 2008). A presença de anticorpos para insulina (IAA), descarboxilase do ácido glutâmico (GAA) ou proteína tirosino-fosfatase IA2 (IA-2AA) pode ser detectada na maioria dos pacientes com DM1 mesmo antes do diagnóstico. A presença de múltiplos autoanticorpos pode ser observada a medida que a doença progride (Barker et al., 2004, Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003).

O desencadeamento do DM1 pode ser atribuído tanto a fatores genéticos quanto a desencadeantes ambientais. Infecções virais ou bacterianas são associadas à predisposição ou proteção ao DM1 (Salminen et al., 2003, Dotta et al., 2007, Goldberg e Krause, 2009). Entretanto, existem vários resultados conflitantes na literatura e o mecanismo pelo qual isso ocorre ainda não foi comprovado (revisado por Goldberg e Krause, 2009). Uma das explicações pela qual esses patógenos iniciam o processo autoimune denomina-se mimetismo molecular, ocorrendo por uma semelhança de sequência entre proteínas presentes nas ilhotas e peptídeos encontrados nos patógenos (van der Werf et al., 2007). No caso descrito por Honeyman et al., (2010) a similaridade é entre peptídeos da proteína VP7 do rotavírus e as proteínas tirosino fosfatase associada ao insulinoma Ag 2 (IA2) e descarboxilase de ácido glutâmico 65 (GAD65).

Aspectos celulares do DM1

Tratamentos

A insulina foi isolada pela primeira vez por Frederic Banting e Charles Best em 1921 no Canadá. A partir daí foram desenvolvidos novos métodos para purificação de extrato pancreático para aplicação terapêutica. A nova técnica de DNA recombinante e os avanços na biotecnologia permitiram que, em 1978, fosse produzida a primeira proteína humana recombinante, a insulina. Estas descobertas trouxeram esperança aos pacientes, transformando o diabetes de uma doença fatal em uma doença com tratamento.

Pacientes com DM tipo 2, na maioria das vezes, podem ser tratados com dieta, exercícios, drogas anti-hiperglicêmicas via oral ou injeções de insulina, dependendo do quadro apresentado. Com relação ao DM tipo 1, o tratamento padrão consiste de injeções diárias de insulina exógena via subcutânea. São necessárias várias injeções diárias e um controle rigoroso dos níveis de glicose sanguínea nesses pacientes, podendo ocorrer eventos freqüentes de hipoglicemia grave. O tratamento com insulina possibilita que os pacientes sobrevivam por longos períodos de tempo. Entretanto, o acompanhamento clínico desses pacientes mostrou que, após anos de terapia com insulina, a grande maioria passa a apresentar uma série de complicações e doenças. Estava claro que, embora as injeções de insulina mantivessem os níveis de glicose próximos do normal, esse controle não era totalmente eficiente.

Embora a terapia com insulina seja eficaz, este é um tratamento paliativo, além de ser considerado incômodo pela maior parte dos pacientes, dificultando a adesão ao tratamento. Vias de administração alternativas são estudadas para amenizar esse problema, como por exemplo a insulina inalável (Exubera). Embora com algumas contra-indicações, é uma ótima alternativa para pacientes com DM1 (Skyler et al., 2008).

Dentre as estratégias de tratamento definitivo para o DM1, capazes de corrigir a dependência de insulina e potencialmente restaurar o padrão fisiológico de secreção de insulina, está o transplante de pâncreas e de ilhotas pancreáticas (American Diabetes Association). O transplante alogenéico de pâncreas é bastante utilizado atualmente, mas ainda apresenta desvantagens como mortalidade perioperatória e significante morbidade, além da necessidade de administração de drogas imunossupressoras para evitar a rejeição do órgão. Assim, esta abordagem é recomendada apenas quando associada ao transplante

renal, em pacientes com insuficiência renal decorrente do diabetes e em pacientes com controle metabólico difícil, com crises de hipoglicemia grave (Martins et al., 2010, American Diabetes Association).

O transplante de ilhotas oferece vantagens em comparação ao transplante de pâncreas por ser um procedimento mais simples, já que as ilhotas são isoladas do pâncreas de doadores cadavéricos e injetadas via veia porta, ficando depositadas no fígado. Esse procedimento já foi aprovado como terapêutico no Canadá, mas permanece em fase experimental nos demais países (Eliaschewitz et al., 2009).

Entre 1974 e 2003, foram realizados 705 transplantes de ilhotas pancreáticas em paciente com diabetes tipo 1 (Bretzel et al., 2004). Os resultados estão ainda longe do ideal: a taxa de sobrevivência um ano após o transplante é de 97%, mas apenas 20% dos pacientes se tornam independentes de insulina. Muitos grupos estão empenhados em aumentar a eficácia dos transplantes, para que um número maior de pacientes alcance a independência de insulina. O primeiro estudo com resultados satisfatórios foi realizado por um grupo canadense da Universidade de Alberta, em Edmonton no Canadá. Sete pacientes com diabetes tipo 1 que apresentavam hipoglicemia severa e instabilidade metabólica foram submetidos ao transplantes de ilhotas pancreáticas. Todos os pacientes alcançaram independência de insulina em um ano de acompanhamento, mostrando uma alta taxa de sucesso quando imunossupressores não glicocorticóides foram usados e um número adequado de ilhotas foi infundido (Shapiro et al., 2000). O acompanhamento a longo prazo desses pacientes, entretanto, mostrou que, após 5 anos, apenas 10% dos pacientes transplantados permaneciam independentes de insulina (Ryan et al., 2005). Um conjunto de fatores deve ser considerado para o sucesso do transplante, como minimização dos efeitos nocivos da preservação e do processamento das células durante o transplante, diminuição dos efeitos tóxicos sobre as ilhotas com o uso de imunossupressores não esteróides e padronização do número de células injetadas para suprir a produção de insulina e restaurar a glicemia em longo prazo.

Embora o transplante de ilhotas esteja mostrando resultados animadores, não se pode ignorar os riscos de uma terapia em longo prazo com imunossupressores, sendo importante determinar o risco/benefício para os pacientes que irão receber esse tipo de tratamento (Stevens et al., 2001, Eliaschewitz et al., 2009).

Além dos problemas de rejeição e a necessidade de administração de imunossupressores, o principal limitante dessas terapias é a fonte escassa de doadores e conseqüentemente a insuficiência de células β pancreáticas (Shapiro et al., 2000, Stevens et al., 2001). cada

Células-tronco

Nos últimos anos se têm buscado fontes alternativas para obtenção de células-produtoras de insulina (CPIs), utilizando células-tronco isoladas de diferentes locais (Scharfmann, 2003), como será exemplificado e discutido no capítulo 2 dessa tese. Essas células podem ser classificadas quanto à origem em células-tronco embrionárias e do adulto. Células tronco-embrionárias são obtidas da massa celular interna de blastocistos pré-implantação, podendo ser mantidas indefinidamente *in vitro* e originar qualquer tipo celular do organismo. Embora tenham características de grande interesse e diversas aplicações clínicas, existem restrições e problemas técnicos e éticos que limitam o estudo e uso de células-tronco embrionárias humanas (Donovan e Gearhart, 2001). Células-tronco do adulto possuem uma grande heterogeneidade, com morfologia, nichos e características bastante diversos (Walker et al., 2009). Células-tronco mesenquimais são um tipo de células-tronco do adulto presentes em todos os órgãos/tecidos do organismo (da Silva Meirelles et al., 2006), com grande potencial de diferenciação em células maduras de origem mesodérmica, embora sua diferenciação em células de origens endodérmica e ectodérmica ainda seja controversa (da Silva Meirelles et al., 2008). As características e aplicações de células-tronco mesenquimais serão discutidas mais profundamente nos capítulos 1 e 2 dessa tese.

Aspectos genéticos do DM1

A genética do DM1

Embora a etiologia do DM1 seja bastante estudada, ainda não se sabe exatamente os mecanismos que desencadeiam essa doença, embora se possa atribuir a fatores genéticos e ambientais (Fernandes et al., 2005). Estudos epidemiológicos apontam uma concordância de aproximadamente 50% em gêmeos monozigóticos (Kyvik et al., 1995). Dessa forma, a presença de fatores ambientais também é importante no desenvolvimento dessa doença. Várias regiões do genoma parecem estar relacionadas ao DM1. Podemos citar,

principalmente, os alelos de classe II do HLA, VNTR no gene da insulina e no gene correspondente ao CTLA-4, uma molécula importante na ativação de linfócitos T (Redondo et al., 2001, Kantárová et al., 2006, Concannon et al., 2009). Outros polimorfismos parecem estar associados ao DM1, como descrito abaixo.

Genes do HLA e susceptibilidade a DM1

A região do complexo principal de histocompatibilidade, em humanos denominado de antígeno leucocitário humano (HLA), é localizada no braço curto do cromossomo 6. Essa região contém três classes de genes, denominados de genes de classe I, classe II e classe III, conhecidos por serem altamente polimórficos. Moléculas de HLA classe II apresentam antígenos na superfície externa da membrana de células apresentadoras de antígenos e são importantes na determinação do repertório de antígenos que serão reconhecidos por linfócitos T auxiliares ($CD4^+$). A forte associação entre genes do HLA classe II, principalmente dos genes DQ e DR, e predisposição ao desenvolvimento de DM1 em diferentes populações já é conhecida a décadas (Marques et al., 1997, Saruhan-Direskeneli et al., 2000). Aproximadamente 40% do risco genético de desenvolvimento de diabetes tipo 1 pode ser atribuído a genes do HLA. Como a região está em forte desequilíbrio de ligação, normalmente a correlação é feita por haplótipos e não por alelos (Kantárová et al., 2006). Podemos citar, como haplótipos de predisposição já descritos, DQA1*0501-DQBl*0201, denominado DQ2, herdado em bloco com DRBl*0301 (DR3) e DQA1*0301-DQBl*0302 (DQ8), também herdado em bloco com DRBl*0402 (DR4) (Nepom et al., 2001, Cerna et al., 2003). No Brasil já existem estudos de associação entre variantes do HLA e desenvolvimento de DM1. Marques et al. (1997) estudaram a distribuição dos alelos do HLA-DRB1 numa população miscigenada do sudeste do Brasil, encontrando uma freqüência maior dos alelos DR3 e DR4 em indivíduos diabéticos quando comparado com a amostra controle. Nahas et al., 2000 também encontraram associação significante dos antígenos de HLA-DR3 e –DR4 em indivíduos diabéticos, além de alelos de proteção como HLA-DR2 e –DR7. A freqüência dos alelos e haplótipos de HLA variam bastante de acordo com a população estudada, o que pode explicar as diferenças encontradas na incidência de DM1 em regiões distintas do mundo, ou seja, a incidência de DM1 estaria associada a freqüência de alelos de susceptibilidade ou proteção (Dorman et al., 1990, Petrone et al., 2002). A população brasileira é formada por

indivíduos de origens étnicas diversas (Moraes et al., 1993). Louzada-Junior et al. (2000) caracterizaram os alelos de HLA DQB1 e DRB1 numa amostra de caucasianos, negros e mulatos da região nordeste de São Paulo. Esse estudo mostrou a heterogeneidade dessa população, com indivíduos apresentando uma mistura de alelos e haplótipos tipicamente de caucasianos, negros e índios.

Sistema KIR e susceptibilidade ao DM1

A família de genes que codifica para receptores KIR (Killer-cell Immunoglobulin-like Receptors) encontrados em células *natural killer* (NK), permitem com que essas células reconheçam antígenos apresentados no contexto do MHC classe I. As células NK atuam como primeira linha de defesa no combate a infecções virais e reconhecimento e destruição de células tumorais no organismo (Sivori et al., 2010).

Associações entre DM1 e infecções causadas por vírus sugere um papel importante de células NK no desencadeamente dessa doença. Células NK foram encontradas em ilhotas pancreáticas de camundongos NOD antes mesmo da presença de células T, o que os autores atribuem ser um papel de sentinelas dessas células (Brauner et al., 2010). Alba et al. (2008) mostraram que a superexpressão de interferon-β em células β acelera o processo de destruição dessas células, com papel importante de células NK. A depleção dessas in vivo desacelera esse processo.

Associações entre diferentes alelos de KIR com DM1 já foram descritas na literatura (van der Slik et al., 2003, Santin et al., 2006, Ramos-Lopez et al., 2009). Entretanto, ainda existem muitas lacunas sobre esse sistema polimórfico, que vão desde o desconhecimento de ligantes para alguns dos receptores, até os mecanismos envolvidos na ativação ou inibição da ação citotóxica dessas células. Além disso, existem outros grupos de receptores importantes na função e resposta de células NK que atuam em paralelo (Pegram et al., 2010), sendo difícil apontar uma relação direta entre variantes alélicas e a susceptibilidade ou proteção em pacientes com DM1.

PTPN22 e susceptibilidade ao DM1

PTPN22 é o terceiro lócus, depois do HLA-DR/DQ e da insulina, com maior associação com risco de desenvolver DM1 (Wellcome Trust Case Control Consortium,

2007). Vários polimorfismos já foram descritos para o gene *PTPN22*, embora a substituição C1858T pareça estar realmente associada ao desenvolvimento do DM1 (Zoledziewska et al., 2008).

Além de linfócitos T, essa proteína também está presente em células NK. Um estudo *in vitro* mostrou, recentemente, a associação entre o alelo 1858T do PTPN22 e redução no número de células NK em cultura (Douroudis et al., 2010), entretanto não se sabe se esse desbalanço também pode ocorrer in vivo. A associação do alelo 1858T e a susceptibilidade ao DM1 já foi descrita para diversas populações, sendo esses e outros aspectos dessa associação melhor abordados no capítulo 5 dessa tese.

OBJETIVOS

Este trabalho teve como objetivo geral o estudo de aspectos genéticos e celulares do Diabetes mellitus tipo I. Os objetivos foram divididos em duas categorias, por se tratarem de abordagens diferentes à investigação desta doença. Estes objetivos podem ser detalhados como segue:

Estudos celulares:

Objetivo 1 - Isolar e caracterizar células-tronco mesenquimais de ilhotas humanas e rim de camundongo, investigando sua capacidade de diferenciação em células-produtoras de insulina *in vitro* (Capítulo 2).

Objetivo 2 - Investigação do efeito do co-transplante de células-tronco mesenquimais murinas e ilhotas pancreáticas em um modelo de camundongos diabéticos (Capítulo 3).

Estudos genéticos:

Objetivo 3 - Avaliar a associação do alelo 1858T da proteína tirosino fosfatase tipo 22 (PTPN22) com o Diabetes mellitus tipo 1 em uma população brasileira (Capítulo 4).

Objetivo 4 - Avaliar a associação de genótipos dos receptores de células natural killer (KIR) e antígeno leucocitário humano-C (HLA–C) com o Diabetes mellitus tipo 1 em uma população brasileira (Capítulo 5).

CAPÍTULO 1. Artigo publicado na revista Science Progress.

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Biology and applications of mesenchymal stem cells

*PEDRO CESAR CHAGASTELLES, NANCE BEYER NARDI AND MELISSA CAMASSOLA**

ABSTRACT

Undifferentiated adult stem cells are responsible for cell replacement in adult organisms. Initially isolated from the bone marrow, they are now known to be distributed throughout the organism as a whole, with a perivascular location. They are defined by properties which include proliferation as adherent cells, a defined immunophenotype, and the capacity to differentiate in vitro *into osteoblasts, adipocytes and chondroblasts. Mesenchymal stem cells (MSCs) are considered as one of the most promising cell types for therapeutic applications. Mechanisms responsible for this therapeutic role are not well understood, and may involve differentiation or, as most evidences point out, paracrine activity. The ability to modulate the immune system opens a wide range of applications, mainly for autoimmune diseases and graft-versus-host disease. Preclinical and clinical studies show promising results, but controversial results are still reported, indicating the need for further basic and preclinical investigation on their therapeutic potential. This review will focus on recent advances in understanding MSC biology and applications in cell therapy.*

Keywords: *mesenchymal stem cells, adult stem cells, cell therapy, biology, applications*

Introduction

By definition, stem cells are able to replicate giving rise to other stem cells and to differentiate into at least one specialized cell type. Stem cells are currently classified according to their origin as embryonic and adult stem cells. Embryonic stem cells (ESCs) are isolated from the inner mass of blastocysts, can be expanded indefinitely *in vitro* and are pluripotent, which means that they have the capacity to originate all types of tissue-specific cells of the

organism. In the post-natal organism, undifferentiated adult stem cells (ASCs) are responsible for the replacement of cells that are lost naturally or by tissue injury. They may be isolated from virtually any organ or tissue, without ethical issues as with ESCs, and since they can be obtained from the patient, they are not immunologically rejected when used for therapeutic purposes.

For cell therapy applications, one of the most promising ASC types is the mesenchymal stem cell (MSC). This review will focus on recent advances in understanding MSC biology and its applications in cell therapy.

Organ-specific stem cells

During the last few years, intensive research has focused on ASCs, showing that each organ or tissue has its own compartment of stem cells. These organ-specific stem cells are slow cycling cells responsible for cell replacement in a process that involves proliferation and differentiation[1]. The rate of replenishment by stem cells

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will depend on the rate of cell death by apoptosis or tissue injury, which is variable for different organs or tissues. Some organs, such as the skin and intestine, have a fast renewing rate (high turnover), with all epithelial cells being replaced every 5 days in mice, for example. Some of the main characteristics of tissue-specific stem cells are presented in Table 1.

The *in vivo* behaviour of stem cells is intimately linked to their environment, or ''niche'', which gives the conditions for stem cells to be maintained in an undifferentiated state (self-renew) and to differentiate when required. This process is mediated by cell-to-cell contact, by components of extracellular matrix and soluble factors. The niche is well described for some types of ASCs, such as the haematopoietic stem cell (HSC). The post-natal niche of HSCs is the bone marrow, where MSCs play an important role[14].

Mesenchymal stem cells

MSCs are a special type of adult stem cells, initially isolated from the bone marrow[15]. These cells were first studied by Friedenstein[16] and the number of preclinical and clinical studies that employ these cells has increased exponentially in the last years[17]. Pittenger *et al.*[18] described a population of adherent cells that may be expanded in culture and could originate differentiated adipocytes, osteoblasts and chondrocytes. MSCs cells are also negative for CD14, CD34 and CD45 and positive for SH2 (CD105) and SH3 (CD73). According to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cell Therapy, the minimal criteria to define human MSCs are the capacity of plastic-adherence when in standard culture conditions. Cells must be positive for CD105, CD73 and CD90, and negative for CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. Additionally, they must have the functional capacity to differentiate *in vitro* into osteoblasts, adipocytes and chondroblasts[19]. The name mesenchymal stem cells should only be used for cells that match these criteria, plastic adherent non-characterized cells should be named as multipotent mesenchymal stromal cells[20].

No exclusive MSC marker has been described, which makes difficult the isolation of MSCs from fresh organs and tissues. The same technical problem happens to HSCs and other tissue-specific stem cells but some markers, such as CD34 for HSCs and Stro-1 for MSCs, allow the enrichment of specific stem cells populations.

In spite of intensive investigation on the *in vitro* characterization of these cells, the biology and role of MSCs *in vivo* is still poorly

Table 1 *Characteristics of adult stem cells that include mesenchymal or MSC-like stem cells and other types of tissue-specific stem cells*

	Stem cell	Organ/niche	Surface markers	Progeny	Ref.
MSC-like stem cells	Mesenchymal stem cells	All vascularizated organs and tissues/ Perivascular niche	CD45 −, CD14 −, CD34 −, HLA −, DR −, CD73 +, CD90 +, CD105 +	Adipocytes, osteoblasts, chondrocytes and myoblasts	2
	Dental pulp stem cells	Teeth	Similar to MSCs	Adipocytes, osteoblasts, chondrocytes and myoblasts	3
	Adipose-derived stem cells	Fat tissue	Similar to MSCs	Adipocytes, osteoblasts, chondrocytes and myoblasts	4
Tissue-specific stem cells	Haematopoietic stem cells	Bone marrow/ Endosteal and vascular niche	CD45 +, CD34 +, CD38 −	All hematopoietic stem cells (lymphocytes, macrophages, erythrocytes...)	5
	Liver stem cells	Liver/Canals of hearing	CK7 +, CK19 +, CD133 +, c-kit	Hepatocytes or cholangiocytes	6
	Pancreatic stem cells	Pancreatic ducts	CD133 +, CK19 +, c-met +	All islets subtypes, acinar and ductal cells	7

Table 1 *Characteristics of adult stem cells that include mesenchymal or MSC-like stem cells and other types of tissue-specific stem cells* (cont.)

	Stem cell	Organ/niche	Surface markers	Progeny	Ref
Tissue-specific stem cells	Intestinal stem cells	Intestinal crypt (+4 position)	Sox-4, sFRP5, Dcamkl1, phosph PTEN and phospho-AKT	Enterocytes, globet cells, enteroendrocrine cells and paneth cells	8
	Epidermal stem cells	Skin/basal layer	b1 integrin, a6 integrin, P63	Spinous cells, granular cells and stratum corneum	9
	Kidney stem cells	Controversial/ Bowman's capsule, glomeruli, papilla	CD133+, CD24+, Sca-1+, Lin-	Tubular phenotype, mesangial cells	10
	Cardiac stem cells	Heart	Lin-, c-kit+	Myocytes, endothelial cells and smooth muscle cells	11
	Satellite cells or muscle stem cells	Muscle/surface of muscle fibres	Pax7+	Myoblasts, myofibres	12
	Neural stem cells	Subventricular zone	Nestin, CD133+, NCAM	Neurons, oligodendrocytes, astrocytes	13

understood. Recent studies demonstrate that MSCs can be isolated from virtually all organs and tissues, suggesting that they reside in association with blood vessels in a perivascular niche[2,17]. Pericytes are cells localized on the abluminal side of blood vessels in close association with endothelial cells. These cells can receive special names depending on in which organ they are localized. A recent review of our group proposes that MSCs and pericytes can be the same cell type[17]. This affirmation is based on similarities between them, such as the presence of several surface proteins (stro-1, nestin, α-smooth muscle actin, CD44, CD90 and CD105). Speculations about the identity of MSCs suggest that they may be also closely related to conventional fibroblasts, based on shared characteristics that include phenotype, differentiation capacity, immunosuppressive properties, distribution in the organism, and growth potential[21]. Further studies need to be performed to determine if they are exactly the same cell type or are cells sharing generic properties but with a specialized function. We draw attention to the importance of characterizing the cells in basic, preclinical and clinical studies, mainly because it is possible to find more than one type of stem cells (tissue-specific and MSCs) in organs and tissues.

Transdifferentiation x paracrine effect

MSCs are currently considered as the adult stem cells with the greatest potential for therapeutic applications[22]. Mechanisms responsible for this therapeutic role are not well understood, as happens with other types of adult stem cells, and may involve differentiation or paracrine activity[23].

The embryonic origin of MSCs is uncertain but some evidence suggests that they derive from mesoangioblasts from the embryonic dorsal aorta (mesoderm germ layer), which explains their facility to differentiate *in vitro* into adipocytes, osteoblasts, chondrocytes and myocytes[17] (Figure 1). Transdifferentiation of mesenchymal stem cells (mesodermal origin) into neurons (ectodermal origin) or hepatocytes and beta cells (endodermal origin) has already been suggested, but remains highly controversial, due to the possibility of technical artefacts involved with *in vitro* culture systems[24]. Protocols for differentiating MSCs into insulin-producing cells, for example, show that differentiated cells can produce little insulin compared to a beta cell[25] or they do not differentiate completely *in vitro*[26]. Barnabé *et al*.[27] induced the differentiation of rat mesenchymal stem cells to a neuronal phenotype with a

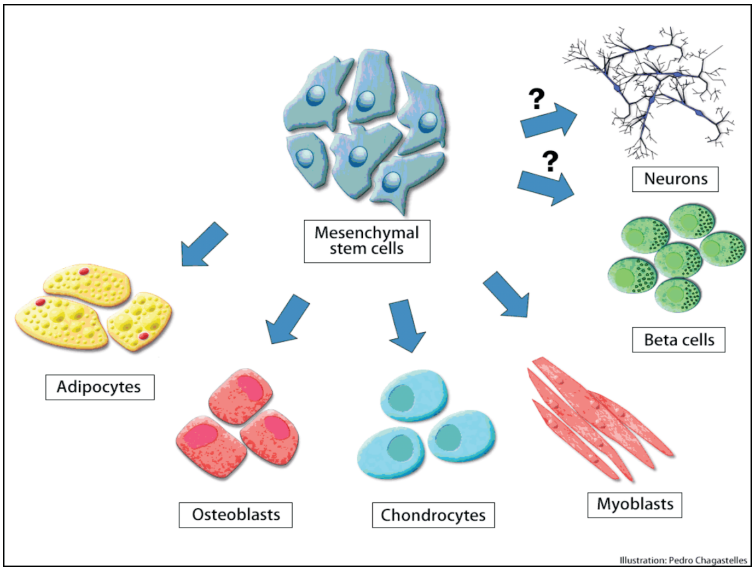

Figure 1 *Mesenchymal stem cells are currently considered the adult stem cell type of greatest plasticity, but differentiation into cells of different germinal layers is still under debate.*

combination of chemical compounds. Resulting cells showed a neuronal-like morphology as well as the expression of neuronal markers, but lack of basic functional neuronal properties. Nevertheless, several studies still aim to differentiate MSCs into several specialized cell types *in vitro*, through the combination of cytokines, growth factors and biomolecules[22]. In some cases, the differentiation process can be improved by *ex vivo* genetic manipulation with vectors expressing key transcription factors specific for each cell type[26].

MSC therapy in several preclinical disease models has shown very little *in vivo* differentiation of the transplanted cells, so that transdifferentiation is not recognized as the main mechanism that explains improvements after treatment[17,28]. On the other hand, MSCs produce and secrete a vast panel of cytokines, growth factors and chemokines, with angiogenic, immunosuppressive, anti-apoptotic and proliferative properties[29]. An example of that is hepatocyte growth factor (HGF) secreted by MSCs, that induces the proliferation of tissue-specific stem cells such as the neural stem cells and epithelial progenitor cells[7]. *In vivo*, dental pulp mesenchymal stem cells induce the proliferation and differentiation of endogenous neural stem cells when transplanted in the hippocampus

of mice[3]. Other MSC-produced factors, such as VEGF, bFGF, angiopoietin-2 and FGF4 among others that are produced by MSCs, mediate angiogenesis[29]. Most evidence, therefore, suggests the paracrine effect as the major mechanism responsible for tissue regeneration and for the success of MSCs in protocols of therapy[17].

MSCs and the culture dish

The frequency of mesenchymal stem cells in the bone marrow is very low, so that, for clinical applications, they must be expanded *in vitro* for weeks or months. This expansion process can result in undesirable effects such as the loss of ''stemness'', senescence and genetic instability[22]. Human bone marrow MSCs normally expand *in vitro* but progressively present telomere shortening and do not express hTERT transcripts or telomerase activity[30]. Cell culture, however, is generally not followed by chromosomal abnormalities, suggesting that MSCs are safe for cell therapy even after *in vitro* expansion. MSCs from other species, such as rhesus macaques, may present tetraploidization at late passages, as well as alterations in cell cycle, cell cycle checkpoint and apoptosis[31]. Murine MSCs acquire chromosomal abnormalities early in culture, can became malignant and originate tumours in mice[32].

The expansion process requires the addition of factors that induce MSC proliferation. Foetal calf serum is most generally used, but for human applications cells must be cultivated free of xenomaterials to avoid contamination with pathogens and a possible immune response against residual antigens. The alternative is to use autologous serum or a serum-free culture medium supplemented with factors that still must be well defined for expansion of MSCs for clinical application[28].

Mesenchymal stem cells and the immune system

One important property of MSCs that emerged when mechanisms responsible for their therapeutic potential were explored, is the ability to modulate the immune system. *In vitro* and *in vivo* studies have explored the mechanisms by which MSCs exert immunosuppressive effects[33]. MSCs are not immunogenic, so that they may be used in allotransplantation (same species) or even in xenotransplantations (different species) without rejection. They are able to inhibit the proliferation of subsets of T lymphocytes and B lymphocytes, as well as the differentiation, maturation and function of antigen-presenting cells. The proliferation and cytotoxicity of NK cells in

the presence of MSCs is inhibited and the generation of regulatory T cells is favoured[34]. These effects are mainly exerted by soluble factors, and MSCs are also known to express high levels of toll-like receptors 3 and 4, two important molecules that recognize virus-derived double-strand RNA and lipopolysaccharides from gram-negative bacteria, respectively. However, the presence of these antigens inhibit the capacity of MSCs to suppress $CD4^+$ T lymphocytes[35], indicating a mechanism of regulation in which MSCs starts to inhibit the immune response only after the infection is under control. MSCs have thus a central role on the modulation of immune responses, which opens a wide range of applications, mainly for autoimmune diseases and graft-versus-host disease (GVHD)[36].

In vivo function of MSCs

As proposed by our group[17], MSCs have a perivascular location and contribute to blood vessel stabilization and tissue homeostasis. This model proposes that, besides replenishing tissues with MSCs, MSCs have a more active role in the repair of focal tissue injury. In the case of tissue injury, MSCs secrete a panel of cytokines and factors which control the immune response to avoid an autoimmune process. They also act to stimulate the formation of new blood vessels, inhibiting local apoptosis and stimulating the proliferation of tissue-specific stem cells.

Therapeutic applications of MSCs

Characteristics presented by MSCs, such as their expansion potential, ease of collection, plasticity and immunosuppressive activity, make them attractive candidates for clinical cell therapy trials[37]. The cells can be administered locally or systemically, due to their ability to migrate to sites of lesion. A large number of preclinical studies have also been performed with MSCs, but results have been heterogeneous[22], possibly because of a lack of standardization of disease models, tissue culture conditions and characterization of the cells employed. Preclinical studies also differ in the number of cells employed, route and time of administration. This rapidly expanding field is represented below by considering the use of MSCs in cardiac and autoimmune diseases, as well as their potential applications in the treatment of cancer.

Cardiac diseases

Clinical stem cell therapy in cardiac diseases started with several phase I and II studies employing bone marrow mononuclear cells (BMMCs). BMMCs proved to be safe for therapy but with a modest effect in improving the cardiac function[38,39]. Some phase I and II studies are now recruiting patients for therapy of myocardial infarction, dilated cardiomyopathy and ischaemic heart disease with autologous MSCs, according to the data of the National Institutes of Health (ClinicalTrials.gov). In some cases, the studies do not specify the type of cell to be used. Only three studies have been published so far and it is too early to anticipate any results, except for the safety of the procedure[28].

Autoimmune diseases and GVHD

While the immunosuppressive effects of MSCs are clearly seen *in vitro*[40], results in animal models are more controversial. GVHD, which occurs after bone-marrow transplantation and is caused by the immune activity of the transplanted cells against host tissue, can be treated by MSCs in mice. Other studies, however, showed no effect of MSCs[41], nor that MSCs can prevent but not treat GVHD in mice[42]. Few studies have used MSCs in autoimmune models, but positive results were described for autoimmune encephalitis and autoimmune diabetes[43], while contradictory results were observed on experimental rheumatoid arthritis[44,45]. Several phase I and II studies are recruiting patients for GVHD treatment with MSCs, and results from these studies are expected to clarify the real therapeutic benefits of MSCs[46]. In September 2009, Osiris Therapeutics, Inc., Columbia, MD, announced preliminary results for two Phase III trials evaluating the use of a preparation of MSCs specially formulated for intravenous infusion (Prochymal) for the treatment of GVHD. While significant improvements in response rates in difficult-to-treat liver and gastrointestinal GVHD were observed, neither trial reached its primary endpoint. Based on these results, Osiris Therapeutics, Inc plans to apply for a broadening of the entry criteria to include patients with severe GVHD of the liver. Clearly, more preclinical studies are needed, so that the *in vivo* immunosuppressive effect of mesenchymal stem cells is known in greater detail, allowing the design of more efficient clinical trials.

Mesenchymal stem cells and cancer

Studies evaluating the therapeutic potential of MSCs for cancer are producing interesting results. Injected MSCs seem to have tropism for glioma sites, probably attracted by cytokines released by tumour cells[47]. This behaviour is of great interest, since MSCs can be modified genetically to produce anti-tumourigenic molecules that will be released in or in the vicinity of tumour sites, whereas healthy tissue remains preserved[48]. In some situations, however, MSCs can accelerate tumour growth and metastasis. The allogeneic cotransplantation of MSCs with a melanoma lineage, for instance, has been shown to favour tumour growth[49]. This effect may be explained by the immunosuppressive effect of MSCs, which prevents normal immune responses against malignant cells. Other unwanted properties are the capacity for inducing neoangiogenesis as well as the production of several other anti-apoptotic and mitogenic factors, that accelerate tumour growth. They may also have a role as tumour-associated stromal cells, contributing to tumour progression[50]. Further studies are necessary to determine the true potential of MSCs on different types of tumours.

Conclusions

MSCs have a perivascular niche in the organism, which explains their isolation from any vascular tissue. MSCs easily differentiate into osteoblasts, adipocytes, chondrocytes and myoblasts *in vitro*, but it is still unclear why this would be important for MSCs located in non-mesenchymal tissues such as the brain or liver. The immunomodulatory, anti-apoptotic and regenerative properties of MSCs suggest they have a major role in tissue homeostasis and regeneration. Preclinical studies show promising results in some disease models, but controversial results are still reported, indicating the need for further basic and preclinical investigation on their therapeutic potential. Clinical trials with MSCs are in the preliminary stages, but have already shown the safety and feasibility for different diseases.

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CAPÍTULO 2. Diferenciação de células-tronco mesenquimais isoladas de ilhotas humanas e rim murino em células-produtoras de insulina *in vitro*.

Introdução

Terapia celular para o Diabetes

Como dito anteriormente, a administração de insulina é o único tratamento disponível para a maioria dos pacientes com Diabetes mellitus do tipo 1 (DM1), entretanto, ele não é a cura para essa doença. Entre as terapias que podem oferecer a independência de insulina estão o transplante alogénico de pâncreas ou de ilhotas pancreáticas. Além de outros problemas relacionados ao transplante, a escassez de doadores torna essa terapia disponível para uma parcela muito pequena desses pacientes (Harlan et al., 2009).

A busca por fontes alternativas de células-produtoras de insulina a partir dos diferentes tipos de células-tronco disponíveis tem sido alvo de muitos estudos nos últimos anos (Scharfmann, 2003, Borowiak e Melton, 2009). A terapia celular para diabetes pode envolver duas abordagens principais: a primeira tem o objetivo de interromper a destruição das células β por células do sistema imune. Para isso, muitos trabalhos utilizam abordagens com terapias imunossupressoras ou indução de tolerância (Ludvigsson et al., 2010). O transplante de medula óssea associado a terapias imunossupressoras tem sido empregado com sucesso em pacientes com diagnóstico recente de DM1 (Couri et al., 2009). Entretanto, essa alternativa é válida apenas quando a doença está no começo. Estimativas apontam que 80-95% das células β já estão destruídas quando os pacientes são diagnosticados com diabetes tipo 1 (Gale, 2002). Assim, devem ser buscadas terapias associadas que interrompam a autoimunidade mas que também originem novas células.

Entre as células comumente utilizadas na diferenciação em CPIs, podemos citar as células-tronco embrionárias, células-tronco mesenquimais (isoladas de várias origens), células-troco pancreáticas e hepáticas (Nir e Dor, 2005, Bonner-Weir e Weir, 2005, Borowiak e Melton, 2009). Diferentes abordagens podem ser utilizadas para diferenciação *in vitro*. Podemos citar como principais, a utilização de moléculas/fatores solúveis e matrizes extracelulares, na tentativa de mimetizar *in vitro* o processo de diferenciação pancreático, e a manipulação genética das células, utilizando vetores que superexpressem

fatores de transcrição envolvidos no processo de diferenciação de células β pancreáticas (Courtney et al., 2010).

Para que a terapia com células-tronco seja vantajosa em relação à administração de insulina, além de induzir a produção desse hormônio pelas células, seu correto processamento, estocagem e secreção, regulados em resposta a sinais fisiológicos, devem ser garantidos (Efrat, 2004), sendo esses os principais desafios desse campo de pesquisa.

Desenvolvimento pancreático

O conhecimento dos eventos que ocorrem durante o desenvolvimento do pâncreas é essencial para a elaboração de protocolos de diferenciação, e para entender melhor o papel de cada tipo de célula e de suas interações. As etapas que levam a formação das células maduras do pâncreas a partir do zigoto são reguladas precisamente, e inúmeros fatores são responsáveis por esse processo (Slack, 1995, Borowiak e Melton, 2009).

A primeira evidência morfológica da formação do pâncreas se dá pela condensação do mesenquima que recobre o endoderme do tubo intestinal. Dessa forma, o endoderma se evagina em direção ao mesenquima e forma o broto dorsal. Pouco depois ocorre a formação do broto ventral a partir da região caudal do broto hepato/biliar. Essas estruturas começam a alongar e na região mais apical do broto aparecem ramificações. Posteriormente elas fusionam para formar o ducto pancreático principal. (revisado por Gittes GK, 2009). A partir daí começam os processos de diferenciação e formação das células maduras do pâncreas. As etapas estão ilustradas na figura 1, abaixo.

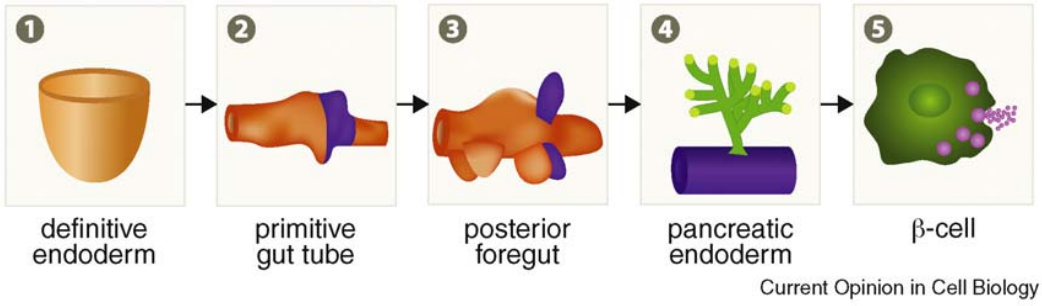


Figura 1. Ilustração das etapas de formação de células β durante o desenvolvimento pancreático (retirado de Borowiak e Melton, 2009).

Sabe-se que células do pâncreas (células ductais, ácinos e células de ilhotas) são formadas a partir da camada de células epiteliais do endoderma. Evidências sugerem que o

mesenquima não contribui para a formação dessas células, mas que outros tipos celulares encontrados no pâncreas, como fibroblastos, sistema linfático e músculo liso se originam do mesenquima que envolve os rudimentos pancreáticos (Slack, 1995, Borowiak e Melton, 2009).

Interação epitélio-mesenquima

Sabe-se que o mesenquima tem papel importante no controle do crescimento e diferenciação das células epiteliais durante o desenvolvimento (Slack, 1995, Wells e Melton, 1999, Gittes, 2009). O primeiros estudos que evidenciaram essa interação mostraram que rudimentos pancreáticos íntegros são capazes de se desenvolver normalmente *in vitro*, entretanto, separando-se as células epiteliais do mesenquima o crescimento e diferenciação em células maduras é interrompido. Essa capacidade foi restaurada pelo co-cultivo epitélio-mesenquima separados por membranas permeáveis que permitem a passagem de fatores, mostrando a importância dessa interação (Golosow e Grobstein, 1962). Além disso, Gittes et al. (1996) mostraram que células epiteliais do rudimento pancreático implantadas na cápsula renal de camundongos foram capazes de originar ilhotas pancreáticas, mas não células ductais ou acinares; quando cultivadas em Matrigel (matriz rica em laminina) as células formaram estruturas semelhantes a ductos pancreáticos e quando cultivadas com mesenquima ocorreu a diferenciação em células acinares. Dessa forma, o mesenquima parece ser indispensável à formação de células acinares, mas não de células endócrinas. Alguns fatores produzidos pelo mesenquima, importantes em fases específicas do processo de proliferação/diferenciação pancreática, já foram identificados. Podemos citar o FGF10, ácido retinóico, activina A e HGF, como descrito na tabela 1.

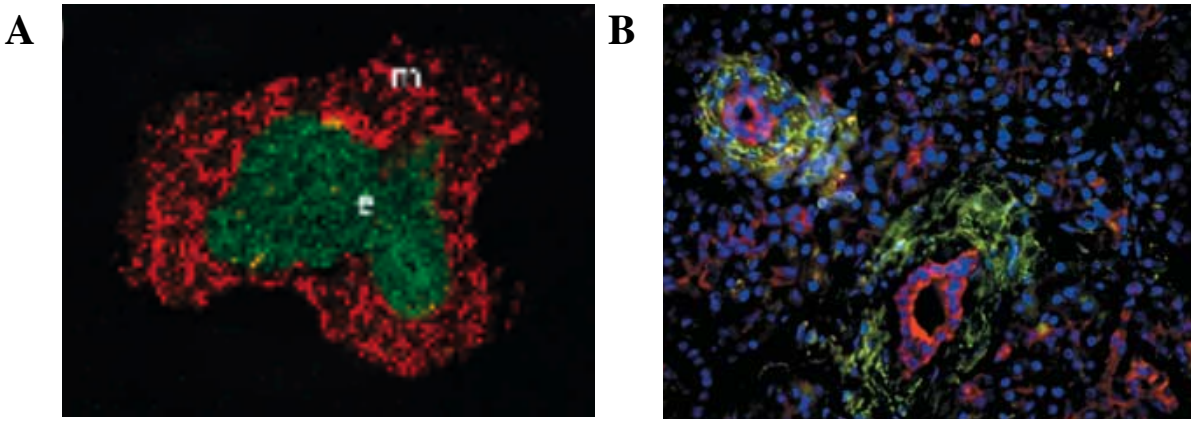


Figura 2 A. Rudimento pancreático (E12.5) de rato mostrando células epiteliais no centro, positivas para pan-citoqueratina (verde) e o mesenquima, ao redor do rudimento, marcado para vimentina (vermelho) (retirado de Miralles et al., 1998). B. Mostrando pâncreas de camundongo adulto com células expressando citoqueratina 19 (vermelho) e vimentina (verde) (retirado de Seeberger et al., 2009).

Tanto na fase embrionária (Miralles et al., 1998) quanto adulta (Seeberger et al., 2009) podemos encontrar células com fenótipo epitelial e mesenquimal, não havendo co-expressão desses dois marcadores em ambos os estágios (Figura 2).

Regeneração do pâncreas pós-natal

O processo de regeneração e reposição de novas células do pâncreas após o nascimento, especialmente de células β, ainda não está totalmente elucidado. Já foi observado que novas células β podem ser repostas por diferentes mecanismos (Bonner-Weir e Weir, 2005). Dor et al. (2004) demonstraram que a auto-duplicacão, ou seja, e replicação das próprias células β nas ilhotas contribui para a reposição de novas células endócrinas. Alguns trabalhos sugerem que novas ilhotas podem ser formadas a partir da diferenciação de células progenitoras presentes no ducto pancreático (Xu et al., 2008, Li et al., 2010). Ainda não existe um consenso sobre qual o principal mecanismo de regeneração do pâncreas, sendo provável que vários contribuam simultaneamente para tal.

Sabe-se que o processo de regeneração é dependente do tipo de injúria criado. Um dos modelos utilizados é a pancreactomia parcial. Em camundongos, por exemplo, com a remoção de 50-70% do pâncreas os animais permanecem normoglicêmicos e normoinsulinêmicos (Peshavaria et al., 2006, Bonal et al., 2008), sendo a regeneração atribuída principalmente à proliferação de células β previamente existentes (Dor et al.,

2004). Já a pancreactomia de 90% leva a um processo de regeneração que, apesar de não recuperar totalmente o tamanho do órgão estimula a neogênese a partir de células do ducto (Sharma et al., 1999).

A administração de aloxana ou estreptozotocina causa morte específica de células β. Na dose adequada, a presença de células β é residual, os animais apresentam baixa capacidade de recuperação e acabam morrendo após algumas semanas se não for introduzida insulina exógena. Por ser um dano pontual, acredita-se que esse não seja o estímulo adequado para que ocorra neogênese de células endócrinas (Bonal et al., 2008).

O modelo de injúria por ligação temporária do ducto principal do pâncreas leva a interrupção do fluxo de enzimas pancreáticas que passam a ser liberadas no órgão, acarretando na destruição de células acinares, que são repostas após algumas semanas pós injúria. Esse modelo parece ser mais fisiológico comparado com os citados anteriormente, visto que ocorre proliferação ductal e neogênese de células acinares e endócrinas. Esse processo é acompanhado da expressão de *NGN3* em células ductais, um gene fundamental para a geração de novas células β (Bonal et al., 2008, Xu et al., 2008).

Assim, os resultados de diferentes trabalhos podem variar dependendo do modelo de injúria utilizado. O contexto inflamatório, como ocorre no último exemplo, parece ser importante para um processo eficiente de regeneração (revisado por Bonal et al., 2008).

Células-tronco pancreáticas (PSCs) e diferenciação em CPIs

Conforme citado anteriormente, células-tronco têm sido encontradas em diferentes órgãos e locais no adulto. Um dos nichos propostos para as células-tronco pancreáticas é o ducto (Bonner-Weir et al., 2000, Bonner-Weir e Weir, 2005). O isolamento dessas células mostra que elas possuem um fenótipo epitelial e expressam *CK19* (Yatoh et al., 2007), *CD133*, *MET* (Hori et al., 2008) e anidrase carbônica II (Inada et al., 2008), marcadores já utilizados para sua purificação. Quando transplantadas in vivo, originam células que expressam insulina, glucagon, somatostatina e PP (Hori et al., 2008). Quando diferenciadas *in vitro*, produzem insulina e glucagon (Bonner-Weir et al., 2000). Apesar da biologia de PSCs ainda ser pouco conhecida, está seria a célula mais apropriada para reposição de CPIs, sendo o principal empecilho a dificuldade na obtenção. Com o estabelecimento de um protocolo eficiente para diferenciação de células-tronco pancreáticas, elas poderiam ser

co-purificadas em procedimentos de isola mento de ilhotas, já que esse material acaba sendo descartado durante procedimento.

Células-tronco embrionárias e CPIs

Por serem as células com maior plasticidade, as células-tronco embrionárias são a população mais estudada na tentativa de obtenção de células produtoras de insulina para o tratamento do diabetes. Apesar de serem tão promissoras, a maioria dos trabalhos até o momento falhou na tentativa de diferenciar de forma eficiente ESCs murinas ou humanas em CPIs. Os protocolos até o momento utilizam três diferentes abordagens: manipulação genética para superexpressar genes chave no desenvolvimento pancreático, seleção de células em cultura e diferenciação espontânea.

Entre os trabalhos que utilizaram manipulação genética, podemos citar os genes *PDX1* (Miyazaki et al., 2004), *PAX4* (Liew et al., 2008) e *NGN3* (Treff et al., 2006). Em todos os trabalhos houve indução da expressão de insulina e de genes presentes em células β. Entretanto, a maioria dos trabalhos apenas mostra um aumento relativo da expressão de insulina, não comparando com a quantidade produzida por ilhotas pancreáticas.

Em outro estudo, Assady et al. (2001), diferenciou espontaneamente ES humana *in vitro*, apresentando expressão de insulina e outros genes importantes para a função de células β. Apenas 60-70% dos corpos embrióides produziram insulina, embora a quantidade não tenha sido quantificada. Em 2001, Lumelsky et al. desenvolveram um protocolo para diferenciação de células-produtoras de insulina CPIs a partir de mESCs. Após seleção de células nestina^{+} os resultados mostraram expressão gênica de insulina e alguns marcadores de diferenciação, bem como secreção de insulina em resposta à glicose. Apesar disso, o conteúdo de insulina foi 50 vezes menor comparado com células β e as células não reverteram o diabetes em animais induzidos com estreptozotocina. Posteriormente, um estudo que reproduziu esse protocolo mostrou que o conteúdo de insulina foi resultado da captação desse hormônio do meio de cultura e não da sintetize pelas células (Hansson et al., 2004). Hori et al. (2002) utilizaram um inibidor da fosfoinositídeo 3-quinase para diferenciação de mESCs. Apesar de induzir expressão de insulina, quando transplantadas na cápsula renal as células diminuíram moderadamente os níveis glicêmicos mas não levaram à cura dos animais. Blyszczuk et al. (2004) também utilizaram diferenciação espontânea em uma linhagem de ESCs modificada para expressar

constitutivamente o fator de transcrição *PAX4*, seguido de indução com meio N_2 + nicotinamida. Além de expressar e secretar insulina em resposta à glicose, as células transplantadas diminuíram os níveis glicêmicos nos animais transplantados. Boyd et al. (2008) compararam os protocolos publicados por Lumelsky, Hori e Blyszczuk, mostrando um maior eficiência na indução de insulina 1 no protocolo publicado por Blyszczuk, além de ter sido o único a reverter a hiperglicemia em 33% dos animais transplantados. Entretanto, foi detectada a formação de teratomas na cápsula renal dos animais.

Até o momento o estudo mais promissor para a obtenção de CPIs a partir de ESCs empregou um protocolo que reproduz os eventos conhecidos que ocorrem durante o desenvolvimento pancreático, desde ESCs até células β maduras (D'Amour et al., 2006). Esses estudos foram desenvolvidos pela empresa ViaCyte (www.viacyte.com), e o protocolo denominado de NovoCell.

O protocolo é composto por 5 fases que vão desde células-tronco embrionárias indiferenciadas até células com fenótipo de células β, passando por fenótipos intermediários (mesoendoderme, endoderme definitiva, tubo intestinal primitivo, endoderme posterior do intestino anterior, endoderme pancreática/precursores pancreáticos). A diferenciação foi acompanhada pela expressão de genes específicos de cada etapa (vide figura Anexo 1).

Mas a estratégia de maior sucesso foi a diferenciação *in vitro* de ESCs até progenitores pancreáticos e posterior implantação *in vivo*, permitindo que as células completassem a sua diferenciação em ambiente apropriado (Kroon et al., 2008).

Ainda existem muitas limitações ao uso de ES em humanos, que dizem respeito à ineficiência da técnica de diferenciação *in vitro,* formação de teratomas e indução de resposta imunológica, questões pouco conhecidas ainda que impedem a terapia com essa fonte celular em humanos.

Células-tronco mesenquimais e diferenciação em CPIs

Células-tronco mesenquimais apresentam a vantagem de serem facilmente isoladas e expandidas *in vitro*, permitindo a obtenção de um número suficiente de células para utilização terapêutica. Entre os locais de isolamento de MSCs com maior potencial estão a medula óssea, tecido adiposo, polpa de dente e cordão umbilical, embora essas células

possam ser isoladas de todos os órgãos e tecidos vascularizados de um organismo (da Silva Meirelles et al., 2006).

Múltiplas abordagens para diferenciar MSCs em um fenótipo de CPIs já foram utilizados, que vão desde o cultivo com extrato pancreático, passando por combinações de fatores de crescimento/citocinas e diferentes tipos de matrizes por diferentes períodos de tempo em cultura e, por fim, a manipulação genética, assim como ocorre para ESCs, sendo os genes mais utilizados o *PDX1*, *NGN3* e *NEUROD1* (Tabela 1).

Ainda não foi descrito um protocolo eficiente para diferenciação de MSCs que induza a produção de insulina em níveis próximos aos de células β, bem como a secreção de insulina regulada por glicose. De maneira geral os trabalhos pecam ao não realizar análises essenciais para demonstrar essas características.

Indutores comumente utilizados em protocolo de diferenciação em ESCs e MSCs.

Muitos dos protocolos utilizados para diferenciação de MSCs em CPIs são baseados nos protocolos de ESCs. A seguir está a tabela com uma lista dos indutores mais utilizados na diferenciação em CPIs *in vitro* e o efeito sobre células-tronco embrionárias, células-tronco mesenquimais e ilhotas pancreáticas, quando descrito

Tabela 1. Função e efeito de indutores sobre células-tronco embrionárias, células-tronco mesenquimais e ilhotas pancreáticas.

Indutor	Características	Tipo de célula	Efeito
Indutores Protéicos			
Activina A	Membro da superfamília do TGF-β. Possui ação antagônica à folistatina.	Produzido em vários tipos de MSCs (Djouad et al., 2010)	Diferenciação de ESCs em endoderme definitiva (Sulzbacher et al., 2009) Papel na diferenciação osteogênica e condrogênica de MSCs (Djouad et al., 2010) Ativador autócrino de células estreladas pancreáticas, induzindo produção de colágeno (Ohnishi et al., 2003)
Betacelulina	Membro da família do fator de crescimento epidermal (EGF)	Expressão em células epiteliais mas não mesenquimais do pâncreas embrionário (Thowffequ et al., 2007)	Aumenta a produção de insulina e *PDX1* e diminui a produção de amilase e glucagon durante o desenvolvimento embrionário (Thowfeequ et al., 2007) Inibe a diferenciação osteogênica de hMSCs (Genetos et al., 2010) Junto com PDX1, induz expressão de insulina e peptídeo-C, além de um fenótipo epitelial em MSCs (Li et al., 2008)
Fator de crescimento de hepatócito (HGF)	Citocina com múltiplas funções no organismo. Papel no desenvolvimento embrionário de vários órgãos.	Secretado por células mesenquimais, se liga ao receptor c-MET em células epiteliais (Sonnenberg et al., 1993).	Induz a proliferação de células β fetais (Otonkoski et al., 1996) Envolvido na migração de MSC para sítios de lesão, não estimula a sua proliferação (Neuss et al., 2004) Efeito anti-apoptótico e mitogênico sobre ilhotas pancreáticas (Dai et al., 2003)
Fator de crescimento	Membro da família FGF.	Secretado pelo	Proliferação de progenitores pancreáticos ($PDX1^{+}$) (Norgaard et al., 2003)

de fibroblasto 10 (FGF10)	Mitógeno para vários tipos celulares. Indispensável ao desenvolvimento pancreático.	mesenquima durante o desenvolvimento pancreático.	Indução de diferenciação adipogênica em hADSCs (Zhang et al., 2009)
Fator de crescimento similar à insulina (IGF-I)	Estimula a proliferação e inibe apoptose em múltiplos tipos celulares do organismo.	Produzido e secretado primariamente pelo fígado.	Induz a proliferação e diferenciação de células β (Agudo et al., 2008, Tsaniras et al., 2010) Indução de diferenciação condrogênica em MSCs (Longobardi et al., 2009)
Exendina-4 (análogo do GLP-1)	É um peptídeo sintético de 39 aminoácidos. Mimetiza a ação do GLP-1 (incretina),.	Descoberto na saliva do lagarto "Monstro-de-Gila" no México. O GLP-1 é secretado por células L do intestino.	Induz diferenciação de ESCs em células produtoras de insulina *in vitro* (Hui et al., 2010) Proliferação de células β e neogênese a partir de células ductais (Xu et al., 1999) Estimula a liberação de insulina por células β (Nauck e Meier, 2005)
Indutores Não-protéicos			
Nicotinamida	Forma do ácido nicotínico (Vitamina B_3).	Induz proliferação e diferenciação de ESCs (Vaca et al., 2003) Diferenciação e maturação de células β fetais (Otonkonski et al., 1993)	
Glicose	Monossacarídeo	Induz a expressão de insulina em células β pancreáticas (Mosley et al., 2003)	
Ácido retinóico	Forma oxidada da Vitamina A. Envolvido na diferenciação de diversos tipos celulares.	Estocado em células estreladas do pâncreas (Senoo et al., 2009)	Diferenciação de células β a partir de progenitores pela indução da expressão de *NGN3* (Oström et al., 2008) Inibição da diferenciação adipogênica (Marchildon et al., 2010)

Ciclopamina	Composto alcalóide. Inibe a via de sinalização Hedgehog (Hh)	A inibição da sinalização de Hh é necessária nos eventos iniciais do desenvolvimento pancreático. Aumenta a expressão de *PDX1* e insulina (D'Amour et al., 2006, Tsaniras et al., 2010)	
		Inibe a proliferação de MSCs derivadas de ilhotas (Gallo et al., 2008)	
db-cAMP	Forma não degradável do AMP cíclico. Mantém a sinalização por segundo mensageiro.	Induz a atividade da proteína quinase A (PKA)	Induz diferenciação osteogênica em hMSCs (Siddappa et al., 2008)
DAPT	Inibidor da γ-secretase. Inibe a via de sinalização NOTCH	Inibição de NOTCH permite expressão de *NGN3* durante o desenvolvimento pancreático (D'Amour et al., 2006)	
		Inibição de NOTCH diminui a proliferação e diferenciação condrogênica e aumenta a diferenciação adipogênica em combinação com dexametasona em hMSCs (Vujovic et al., 2007)	
Butirato de sódio	Inibidor de histona desacetilase	Ativa genes chave no desenvolvimento pancreático	Diferenciação em mesoendoderme e endoderme definitiva. (Haumaitre et al., 2008, Tsaniras et al., 2010)
			Inibe a proliferação e diminui a capacidade de diferenciação adipogênica e condrogênica de hMSCs isoladas de tecido adiposo e cordão umbilical (Lee et al., 2009)
2-mercaptoetanol	Antioxidante (quelante de radicais livres)	Sem efeito aparente sobre a diferenciação em CPIs, aumenta a resistência de celular ao dano oxidativo causado pela cultura, além de permite a absorção de cistina pelas células (Pruett et al., 1989)	
Taurina	Derivado do aminoácido cisteína.	Produzida no pâncreas, é o principal constituinte da bile.	Indução de diferenciação de mADSCs em células-produtoras de insulina (Chandra, et al., 2009)
			Diminui a fibrose pancreática, inibe a expressão de colágeno tipo 1, *TGFβ* e *MMP2* em células estreladas pancreáticas (Shirahige et al., 2008)

Os protocolos utilizam combinações variadas desses indutores, não havendo um consenso. O conhecimento adquirido com os estudos sobre o desenvolvimento embrionário permite que se desenhe mais adequadamente protocolos para ESCs. No caso da MSCs, por ser uma célula relativamente nova, ainda existem lacunas sobre a biologia dessas células para permitir uma escolha mais consciente dos indutores, como ocorre para ESCs.

Materiais e métodos

Isolamento de MSCs

Células-tronco mesenquimais de rim de camundongo C57Bl/6 foram isoladas conforme da Silva Meirelles et al., 2006. Ilhotas pancreáticas foram isoladas de pacientes cadavéricos como descrito por Huang et al., 2004. Após o isolamento, ilhotas foram dissociadas pela incubação em solução de 0,2% de EDTA (Sigma-Aldrich, Poole, UK) e incubação a 37° C por 5 min com pipetagem a cada 2 min. As células foram contadas e 10^6 células/poço (placa de 6 poços) foram plaqueadas em Dulbecco's Modified Eagle's Medium (DMEM) suplementado com 10% de soro fetal bovino inativado (Gibco, Paisley, UK), 1% de glutamax e 1% de solução de penicilina/estreptomicina. Após as culturas atingirem confluência, as células foram tripsinizadas, após lavagem com solução de PBS livre de Ca^{2+} e Mg^{2+}, pela incubação em solução de tripsina-EDTA (Sigma), e plaqueadas em garrafas novas. A taxa de repique foi determinada empiricamente para que fossem feitos 2 repiques semanais.

Citometria de fluxo

A imunofenotipagem de MSCs derivadas de ilhotas foi realizada pela incubação das células com anticorpos anti-CD13, -CD44, -CD69, -CD73, -CD90, -CD105, -CD117, -KDR e -HLA-DR (Becton Dickinson, San Diego, CA) conjugados com isotiocianato de fluoresceína (FITC) ou ficoeritrina (PE). Após tripsinização, as células foram lavadas com PBS, incubadas com os anticorpos por 30 min a 4° C e lavadas novamente com PBS para remover o excesso de anticorpos não ligados. A análise foi feita em um citômetro fluxo (FACScalibur, Becton Dickinson), equipado com argon laser de 488 nm. Os gráficos foram gerados no programa WinMDI, versão 2.8.

Diferenciação adipogênica e osteogênica

A diferenciação adipogênica de MSCs foi induzida pelo cultivo de culturas confluentes em meio DMEM contendo 20% de soro fetal bovino (Gibco), 2,5 μg/ml de insulina, 100 μM de indometacina, 5 μM de roziglitazona e 10^{-7} M de dexametazona. Para diferenciação osteogênica as células foram cultivadas em DMEM contendo 10% de soro fetal bovino, 10 mM de β-glicerofosfato, 5 μg/ml de ácido ascórbico e 10^{-7} M de dexametazona. As culturas foram mantidas nos meios de indução por 30 dias com trocas de meio a cada 3-4 dias. Culturas não diferenciadas (controle) foram mantidas pelo mesmo período de tempo no meio descrito para o isolamento e manutenção das MSCs. A revelação da diferenciação foi feita pela coloração com *Alizarin Red S* ou *Oil Red O*, que coram matriz de cálcio e lipídeos, respectivamente. Adicionalmente, culturas adipogênicas foram contra-coradas com hematoxilina. Todos os reagentes e corantes da marca Sigma.

Diferenciação em células-produtoras de insulina

Para a diferenciação das MSCs em CPIs foram utilizados quatro protocolos. A tabela 2 detalha o tipo celular utilizado, as condições e os indutores utilizados nos protocolos.

Tabela 2. Detalhes dos protocolos utilizados para diferenciação de MSCs em células produtoras de insulina.

	Tipo Celular	Condições de diferenciação	Referência
Protocolo 1 (NovoCell)	hMSCs derivadas de ilhotas	**Estágio 1: Endoderme definitiva (3 dias)** - RPMI + Glutamax + 100 ng/ml Activina A + 25 ng/ml Wnt3a (sem SFB) - Activina A por 3 dias - Wnt3a durante o primeiro dia - Dias 2 e 3, adiciona-se 0,2% de SFB **Estágio 2: Tubo intestinal primitivo (3 dias)** - RPMI + Glutamax + 2% SFB + 50 ng/ml FGF10 + 0,25 μM KAAD-ciclopamina **Estágio 3:** endoderme posterior do intestino anterior **(3 dias)** - DMEM + Glutamax + 1% B27 + 2μM ácido retinóico + 0,25 μM KAAD-ciclopamina + 50 ng/ml FGF10 (Duração: 3 dias) **Estágio 4: Endoderme pancreática** - DMEM + Glutamax + 1% B27 + 1 μM DAPT + 50 ng/ml Exendina-4 Ao final do estágio 4, as células foram tripsinizadas e cultivadas metade em plástico tratado para permitir a aderência das células e a outra metade em plástico de baixa aderência (Nunc). **Estágio 5: Células endócrinas maduras (3 dias)** - CMRL + Glutamax + 1% B27 + 50 ng/ml Exendina-4 + 50 ng/ml IGF-1 + 50 ng/ml HGF (Duração: 3 dias)	D'Amour et al., 2006
Protocolo 2	hMSCs derivadas de ilhotas	CMRL 1066 + 2% SFB (Hyclone) + 1 mM db-cAMP + 1 mM Ácido retinóico Duração: 7 dias/trocas de meio a cada 2 dias	Adaptado de Milne et al., 2005
Protocolo 3	hMSCs derivadas de ilhotas	CMRL 1066 + 2% SFB (Hyclone) + 100 ng/ml de Activina A + 10 nM de Betacelulina + 10 mM de Nicotinamida + 50 ng/ml de Exendina-4 + 50 ng/ml de HGF. Duração: 7 dias/trocas de meio a cada 2 dias	-
Protocolo 4	hMSCs derivadas de ilhotas e mMSCs derivadas de rim	**Estágio 1: (3 dias)** Meio: DMEM-F12 + 1% BSA + ITS (5 mg/l insulina, 5 mg/l transferrina, 5 mg/l selênio) + 17,5 mM glicose + 4 nM de Activina A + 1 mM de Butirato de sódio + 50 mM 2-mercaptoetanol. Concentração: 2 x 10^5 células/cm^2. **Estágio 2: (2 dias)** Meio: DMEM-F12 + 1% BSA + ITS + 17,5 mM glicose + 0,3 mM de taurina. **Estágio 3: (5 dias)** Meio: DMEM-F12 + 1,5% BSA + ITS + 17,5 mM glicose + 1X Aminoácido não-essenciais + 3 mM de taurina + 100 nM de Exendina-4 + 1 mM Nicotinamida Obs: Placas de baixa aderência (Nunc) na concentração	Adaptado de Chandra et al., 2009

Extração de RNA e síntese de cDNA

O RNA foi extraído das culturas utilizando o RNeasy Mini Kit (Qiagen, Crawley, UK). A quantidade e pureza do RNA foi determinada no aparelho Nanodrop (ND-1000). O cDNA foi sintetizado com o kit QuantiTec Reverse Transcription (Qiagen) conforme instruções do fabricante. Para cada reação, 1 μg de RNA foi utilizado na síntese de cDNA.

Curvas-padrão

A construção das curvas-padrão de cada gene foi realizada pela amplificação dos fragmentos com primers específicos (vide tabela Anexo 3) por PCR convencional. O programa utilizado para amplificação foi: 95º C por 10 min, seguido de 40 ciclos de 95º C por 10 s, 58º C por 20 s e 72 por 20 s, com passo final à 95º C por 5 min. Os fragmentos foram plotados em gel de agarose 2% e as bandas purificadas com o kit QIAquick (Qiagen). O DNA purificado foi ressuspendido em 20 μl de água e quantificado no Nanodrop. O cálculo utilizado na determinação do número de moléculas para montagem da curva-padrão foi:

(X ng/μl DNA / [tamanho do fragmento de PCR em pares de base x 660]) x 6.022 x 10^{23} = Y moléculas/μl

A concentração foi ajustada para 10^{9} cópias/2 μl a partir da qual foram feitas diluições seriadas de 10 X até a concentração de 10^{1} cópias/2μl em água contendo 10 μg/ml de tRNA.

PCR quantitativo em tempo real (qPCR)

A quantificação absoluta da expressão gênica foi realizada com o kit QuantiFast SYBR Green PCR (Qiagen). Para tal, o cDNA foi diluído 5x em água e foram utilizados 2 μl em cada reação, em triplicada. Exceto para as curvas-padrão que foram feitas em apenas um poço. Foram utilizados os aparelhos de PCR em tempo real RotorGene (Corbett Life Science) para o protocolo 1 e LightCycler 480 (Roche Applied Science) para os demais protocolos. Cada reação de 25 μl era composta por 12,5 μl do master mix (contendo tampão, dNTPs, polimerase ativada pelo calor), 0.5 μM de cada primer e 2 μl de cDNA. O programa de dois passos utilizado foi: desnaturação por 5 min a 95º C seguido de 40 ciclos de 95º C por 10 s, 58º C por 20 s. As imagens dos géis de agarose foram obtidas com o sistema de captura Gene Genius (Syngene, Cambridge, UK).

RT-PCR

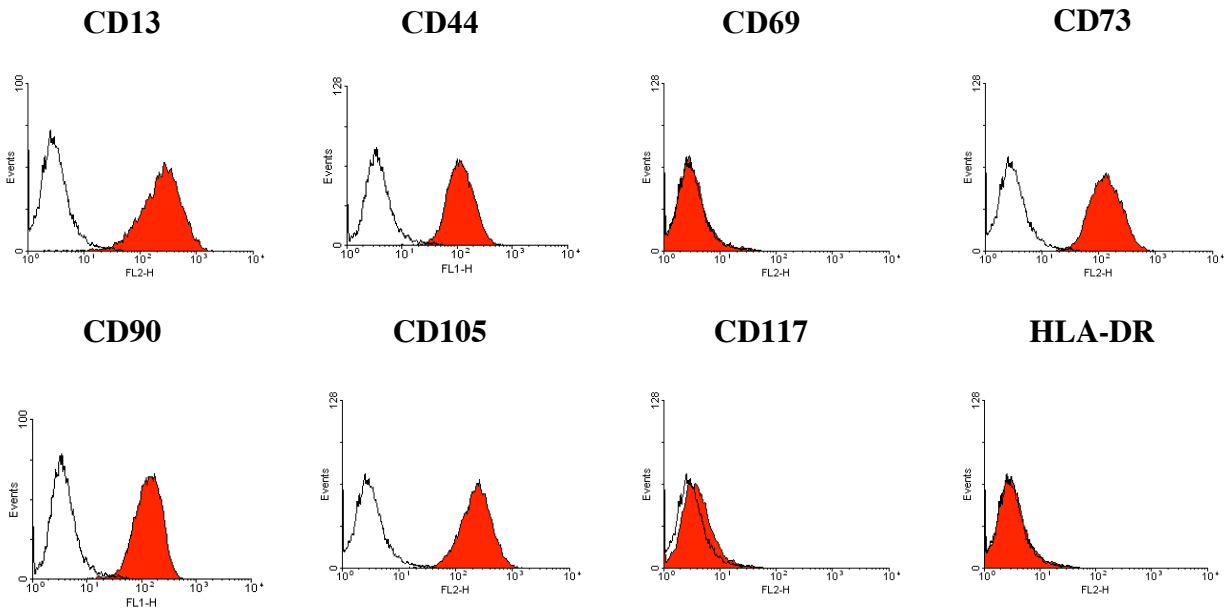
As reações de PCR foram preparadas com kit da Promega (Promega, Southampton, UK). Cada reação de 20 µl era composta por 10 µl do master mix (contendo tampão, dNTPs, polimerase), 0.5 µM de cada primer e 2 µl de cDNA não diluído. As reações foram amplificadas em termociclador (Eppendorf UK Limited, Cambridge, UK) utilizando o seguinte programa: desnaturação por 5 min a 95º C seguido de 40 ciclos de 95º C por 10 s, 58º C por 20 s e 72º C por 20s e um ciclo final a 72º C por 5 min. As imagens dos géis de agarose foram obtidas com o sistema de captura Gene Genius (Syngene, Cambridge, UK).

Resultados

Caracterização de células-tronco mesenquimais

Células-tronco mesenquimais derivadas de ilhotas pancreáticas foram isoladas de 4 doadores diferentes e denominadas de Panc1, 2, 3 e 4. As culturas apresentaram capacidade de proliferação *in vitro*, sendo expandidas por pelo menos 10 passagens (dados não mostrados). Culturas de células foram analisadas por citometria de fluxo quanto a presença de diversas proteínas de superfície. Células isoladas de três pacientes diferentes mostraram um mesmo padrão, sendo negativas para CD69, CD117 e HLA-DR e positivas para CD13, CD44, CD73, CD90 e CD105 (Figura 3).

A



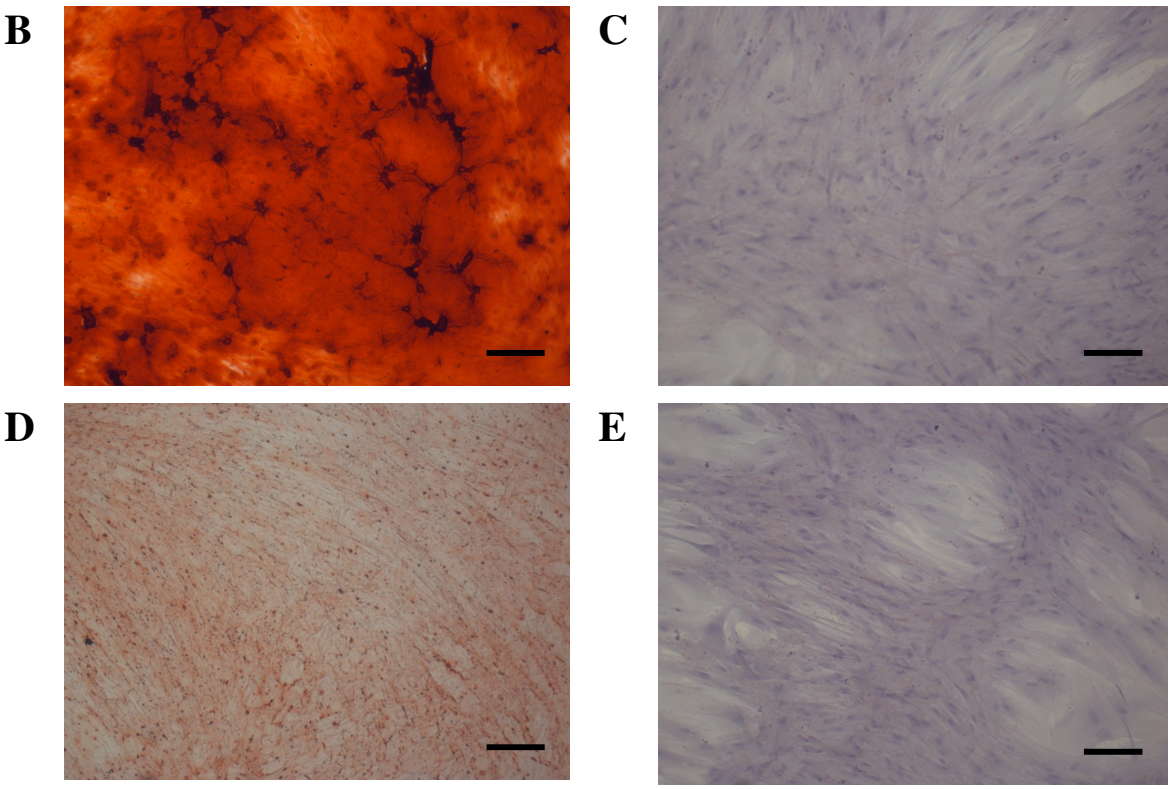


Figura 3. Caracterização de células-tronco mesenquimais derivadas de ilhotas pancreáticas humanas. (A) Padrão de marcadores de superfície de culturas celulares na 5ª passagem, para os marcadores CD13, CD44, CD69, CD73, CD90, CD105, CD117 e HLA-DR, por citometria de fluxo. Diferenciação osteogênica (B) e adipogênica (C) e respectivos controles (D e E) de culturas de MSCs derivadas de ilhotas pancreáticas humanas. Aumento de 100X, barra de escala 100 μm. Pelo menos 5.000 eventos por amostra foram coletados. Dados representativos de experimentos com linhagens de três doadores diferentes.

Após indução com meios de diferenciação específicos, as células foram capazes de se diferenciar em osteoblastos e secretar matriz mineralizada (Figura 3A) mas não em adipócitos (Figura 3B). As imagens 3C e 3D mostram as culturas não diferenciadas cultivadas em meio de manutenção.

Diferenciação em CPIs in vitro

Protocolo 1 (NovoCell)

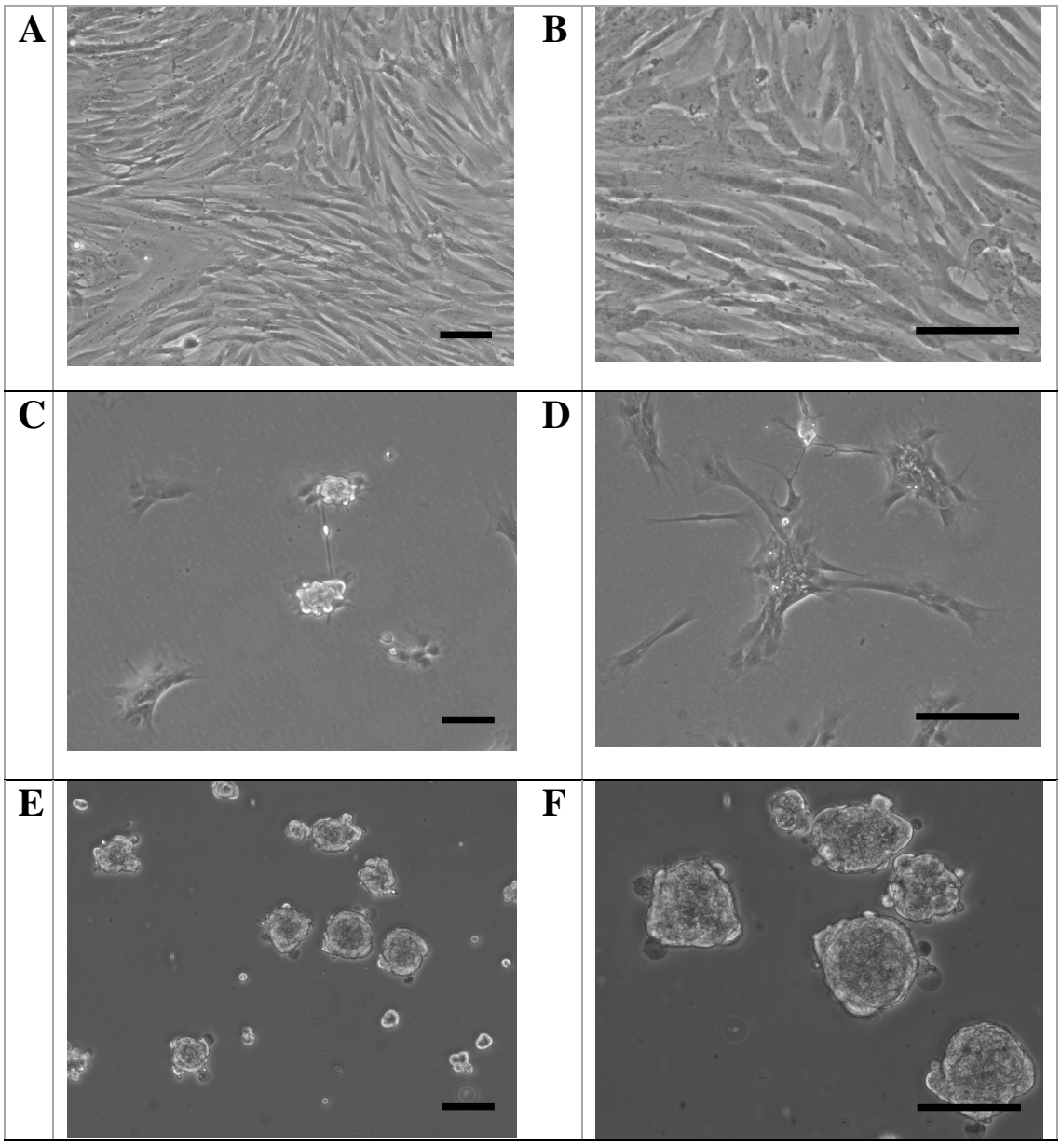


Figura 4. Morfologia de uma cultura de células-tronco mesenquimais isoladas de ilhotas humanas (hMSC Panc3) diferenciadas com o Protocolo NovoCell. Controle (A, D), diferenciadas em placas não-tratadas (B, E) e placas tratadas (C, F) ao final do estágio 5. Aumento de 100x (A, B e C) e 200x (D, E e F). Barra de escala, 100 μm.

A figura 4 mostra imagens de culturas de hMSCs não diferenciadas (4A e 4B) crescendo como monocamada, e culturas diferenciadas com o Protocolo 1 (NovoCell) nos quais as células foram cultivadas a partir do estágio 4 em placas que permitem a aderência

das células (4C e 4D) e placas de baixa aderência celular (4E e 4F). Em placas de baixa aderência, as células se organizaram em estruturas tridimensionais muito similares à ilhotas pancreáticas (4E e 4F) enquanto em plástico normal a maioria das células se aderiu, formando alguns focos de células aglomeradas (4C e 4D).

Real-time PCR (qPCR)

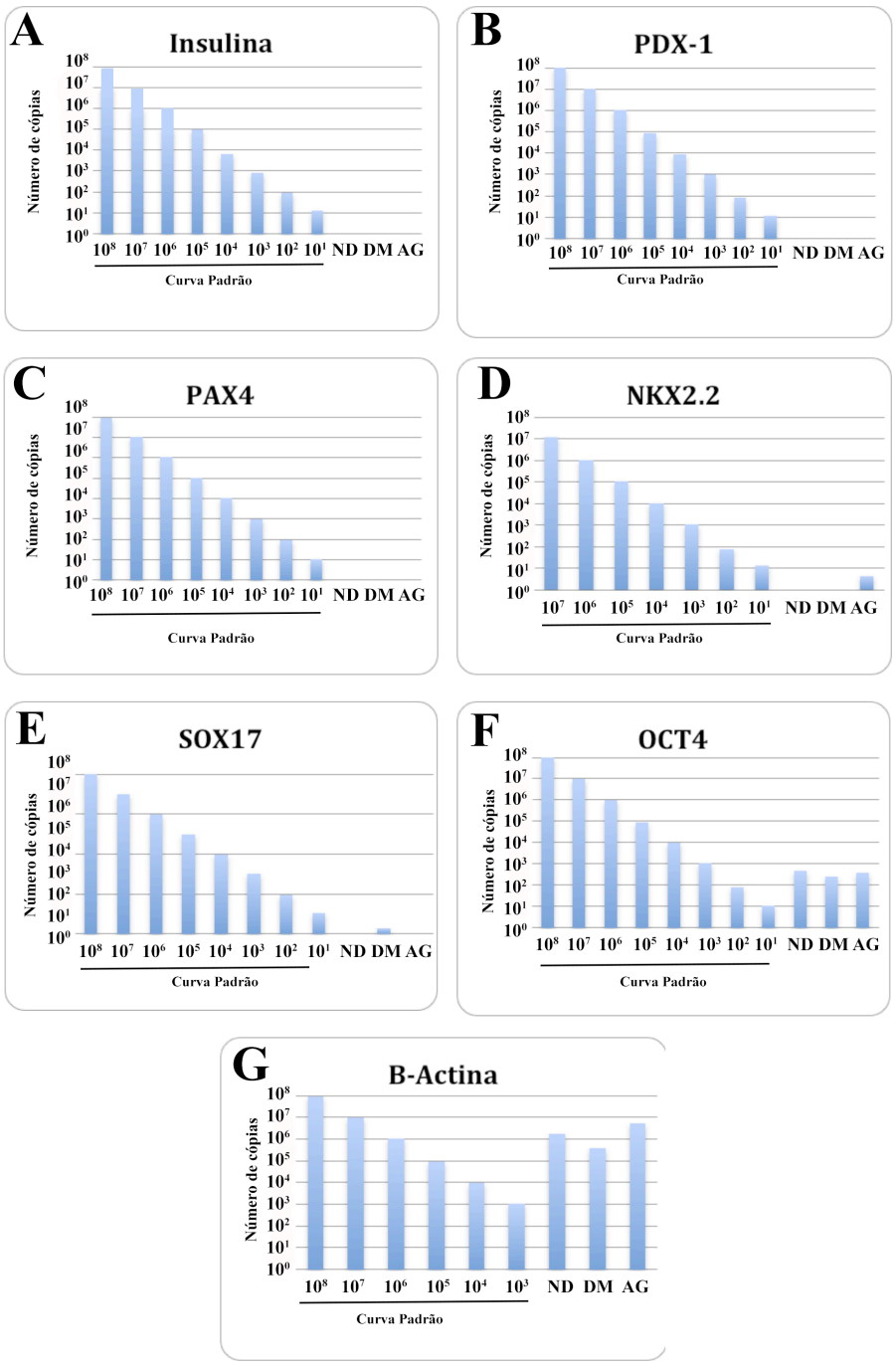
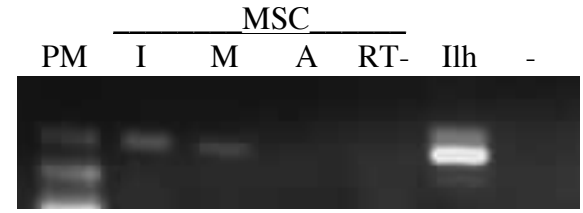


Figura 5. Quantificação absoluta da expressão dos genes *INS* (A), *PDX1* (B), *PAX4* (C), *NKX2.2* (D), *SOX17* (E) *OCT4* (F) e *ACTB* (G) em culturas de MSCs isoladas de ilhotas humanas não diferenciadas (ND) e diferenciadas em plástico tratado (DM) e plástico de baixa aderência (AG) no último estágio (Estágio 5) do Protocolo Novocell. Para cada gene foi preparada uma curva padrão com concentrações conhecidas de cada fragmento. Os resultados estão expresos em número absoluto de cópias. Eixo Y em escala logarítmica. Dados relativos a um único experimento. Eficiências das reações: *INS* (0.95), *PDX1* (0.98), *PAX4* (0.96), *NKX2.2* (0.99), *SOX17* (0.97), *OCT4* (0.98) e *ACTB* (0.97).

Os resultados da quantificação absoluta de genes envolvidos na diferenciação em culturas diferenciadas com o protocolo Novocell (Figura 5) mostram que apesar de observadas diferenças morfológicas, não houve indução da expressão de *INS* (5A), *PDX1* (5B), *PAX4* (5C), *NKX2.2* (5D) e *SOX17* (5E). MSCs isoladas de ilhotas expressam níveis baixos de *OCT4* (5F). Como controle interno foi utilizado o gene da B-actina (5G). As amostras plotadas em gel de agarose confirmam a ausência de expressão de insulina (Figura 6B). Quando foi feito PCR convencional para insulina com 5 vezes a quantidade de cDNA, foi possível observar bandas muito fracas na cultura indiferenciada (I) e diferenciada em monocamada (M), mas não em aglomerados (A) (6B).

A



B

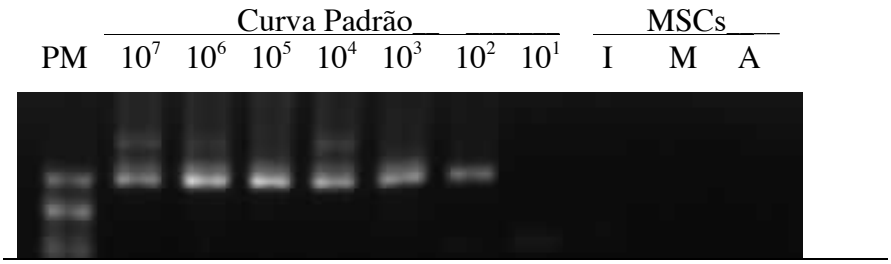


Figura 6. Produto de PCR convencional (A) e qPCR (B) em gel de agarose 2% de culturas de MSCs isoladas de ilhotas pancreáticas humanas diferenciadas e não-diferenciadas em CPIs utilizando o protocolo NovoCell. Curva padrão entre 10^7 e 10^1. M=MSCs diferenciadas em monocamada, A= MSCs diferenciadas em aglomerados (placas de baixa aderência), I= MSCs indiferenciadas (controle), Ilh= ilhotas.

Protocolo 2, 3 e 4

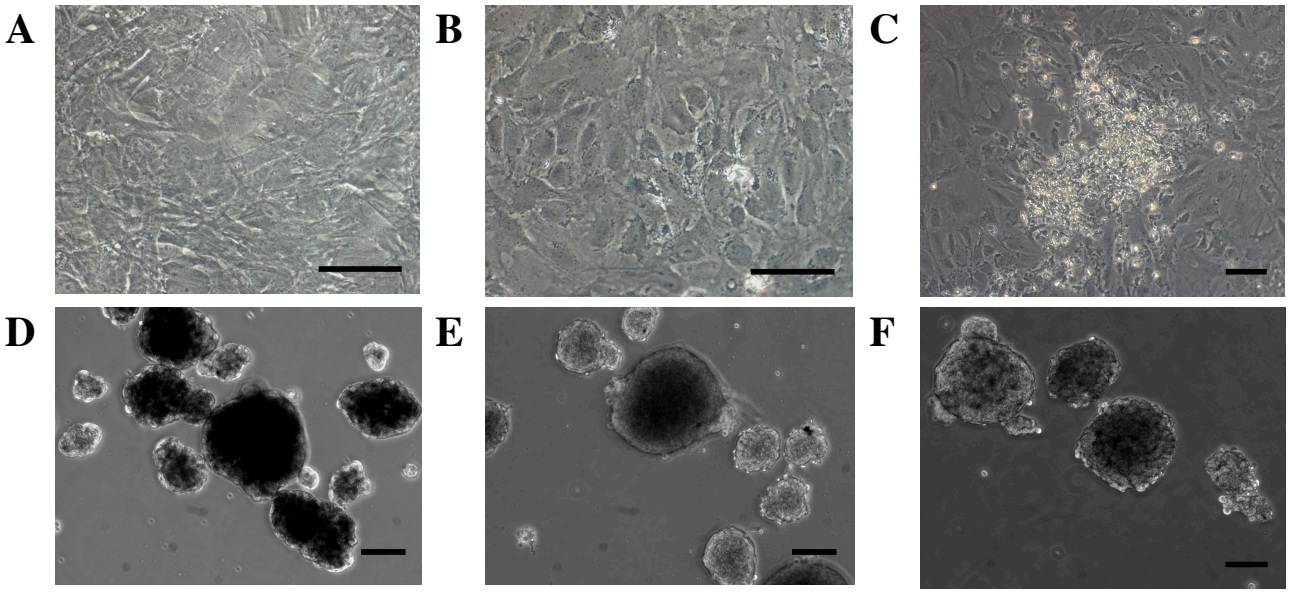


Figura 7. Morfologia de culturas de MSCs (A-E) e ilhotas pancreáticas de camundongos (F). (A) Células hMSCs (Panc4) não diferenciadas, (B) diferenciadas com o protocolo 2 após 7 dias, mostrando a presença de grânulos no citoplasma ao redor do núcleo e (C) foco de células que se desprenderam e que possivelmente estão morrendo. (D) MSCs isoladas de rim de camundongo e (E) hMSCs isoladas de ilhotas pancreáticas humanas, ambas diferenciadas com o protocolo 4. Aumento de 100x (C-F) e 200x (A e B). Barra de escala, 100 μm.

Após 7 dias de diferenciação de hMSCs de ilhotas pancreáticas, as mudanças morfológicas observadas foram o aparecimento de grânulos dentro das células (Figura 7B) que não estavam presentes no grupo controle (Figura 7A) e a ocorrência de focos de células aparentemente em processo de morte celular (Figura 7C). Não foram observadas mudanças morfológica entre culturas controle e diferenciadas com o protocolo 3 (dados não mostrados).

Quando cultivamos MSCs em plástico que impedem a sua aderência, as células tendem a formar aglomerados de diferentes tamanhos (Figuras 7D e 7E) que se assemelham bastante a ilhotas pancreáticas (Figura 7F). Apesar da semelhança morfológica, as culturas de MSCs de pâncreas humano e rim murino não produziram insulina *in vitro*. Os resultados mostram que pouco mais de dez cópias foram detectadas em culturas de rim murino diferenciadas com o protocolo 4 (Figura 8A), e quando plotadas em gel de agarose mostram uma banda fraca tanto em culturas diferenciadas quanto em não diferenciadas (controle) (Figura 8C). Com relação a MSCs de ilhotas humanas, menos de dez cópias detectadas em culturas diferenciadas com os protocolos 2, 3 e 4 (Figura 8B).

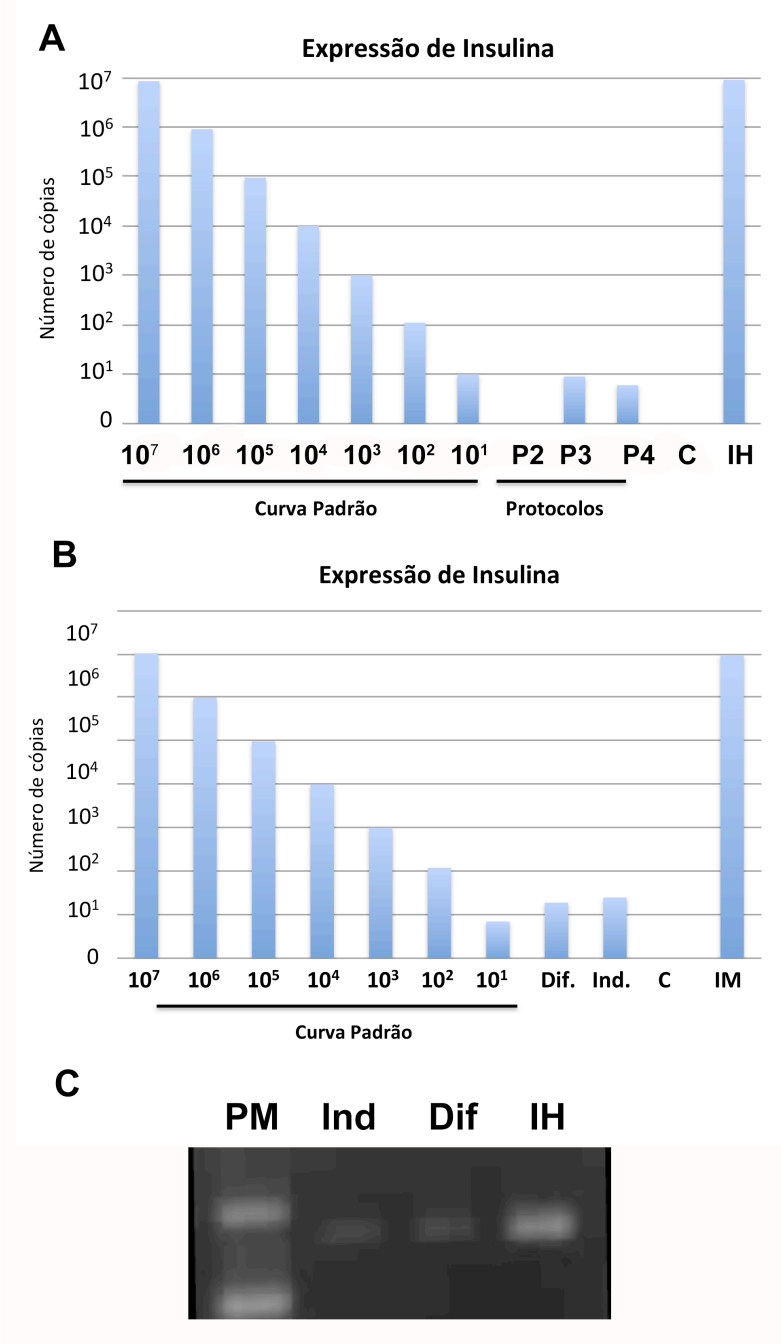


Figura 8. Quantificação absoluta da expressão do gene *INS* em culturas em MSCs isoladas de ilhotas humanas diferenciadas com o Protocolos 2 (P2), 3(P3) e 4(P4) (A) e *Ins1* em MSCs isoladas de rim murino diferenciadas com o Protocolo 4 (B). Os resultados demonstram o número total de cópias. **(C)** Produto de qPCR em gel de agarose 2% de culturas de MSCs isoladas de rim de camundongo indiferenciadas (Ind) e diferenciadas (Dif) e em CPIs utilizando o protocolo 4. PM=marcador de peso molecular, Dif=diferenciada, Ind=indiferenciada, IH=Ilhota Humana, IM=Ilhota Murina. Dados relativos a um único experimento.

Discussão e conclusões

No início dos estudos sobre células-tronco da medula óssea, foi descrito um tipo celular capazes de originar células maduras dos três folhetos embrionários (Jiang et al., 2002) que foi encontrado também em outros órgãos além da medula óssea (Jiang et al., 2002b), conferindo a essas células uma plasticidade comparável a de ESCs. Entretanto, esses resultados não puderam ser reproduzidos da mesma maneira pela comunidade científica. A capacidade de diferenciação de MSCs em células derivadas do endoderme e ectoderme tem sido investigada, mas apesar de haver expressão de alguns genes específicos, a eficiência de diferenciação é baixa e as células não tem a mesma funcionalidade que células β (Tabela 3).

Devido à localização perivascular das MSCs *in vivo*, espera-se que se possa isolar essas células de qualquer tecido ou órgão vascularizado. Ilhotas pancreáticas são ricamente vascularizadas, possuindo uma rede de capilares sanguíneos onde a insulina é liberada. MSCs já haviam sido isoladas de ilhotas pancreáticas humanas (Eberhardt et al., 2006, Gallo et al., 2007, Davani et al., 2007) e do tecido exócrino (Baertschiger, 2008), ambos mostrando características morfológicas e fenotípicas similares às observadas nesse trabalho. Outros trabalhos já haviam observado que MSCs isoladas de ilhotas pancreáticas tem capacidade limitada de diferenciação adipogênica, apresentando vacúolos lipídicos pequenos (Eberhardt et al., 2006, Gallo et al., 2007, Davani et al., 2007). Essas diferenças quanto a capacidade de diferenciação adipogênica e ostogência dependendo do local de isolamento já foram observadas anteriormente em camundongos (da Silva Meirelles et al., 2006).

O protocolo 1 (NovoCell) foi desenvolvido para mimetizar as etapas do desenvolvimento embrionário do pâncreas. No trabalho de D'Amour et al. (2006), foram diferenciadas 5 linhagens diferentes de hESCs, mas apenas uma delas foi capaz produzir insulina em níveis muito próximos aos de células β. Mostrando que existem diferenças intrínsecas na capacidade de diferenciação de linhagens de hESCs. Além disso, apenas de 7-12% das células ao final do protocolo eram positivas para insulina, e a capacidade de processamento (clivagem do precursor em insulina e peptídeo C) se assemelha a de células β fetais imaturas. Isso mostra que a diferenciação não é totalmente eficiente e que o protocolo ainda pode ser otimizado.

A idéia de diferenciar MSCs com esse protocolo foi a de que se MSCs estivessem em alguma das etapas intermediárias, elas seriam capazes de originar CPIs. Não foi possível evidenciar expressão de insulina, nem de genes presentes em células β como *PDX1*, *PAX4* e *NKX2.2*, o que nos sugere que MSCs não se encontram em nenhuma das etapas intermediárias do desenvolvimento de células β. Além disso, a expressão de *OCT4* analisada por qPCR parece ser discreta. Quando comparado com hESC a expressão de genes de pluripotência em MSCs é muito baixa (Sanaz Ajami, comunicação pessoal). Analisando a Tabela 1, vemos que os diferentes indutores parecem ter efeitos diferentes sobre ESCs e MSCs, provavelmente por estarem em estágios de desenvolvimento diferente, o que explicaria os resultados negativos com esse protocolo. Resultados similares foram encontrados com a aplicação do protocolo NovoCell em células de polpa de dente humano (Sanaz Ajami, comunicação pessoal), sugerindo que o local de onde MSCs são isoladas não parece influenciar a sua capacidade de diferenciação em CPIs.

Burns et al. (2005) diferenciaram células-tronco neurais (NSCs) em CPIs *in vitro*. O tratamento com db-cAMP e ácido retinóico elevou de 50-100 vezes o número de cópias do gene da insulina. Entretanto, os níveis de peptídeo C estavam abaixo do limite de detecção do ensaio, indicando baixa expressão/produção de insulina. NSCs diferenciadas expressaram *PC2*, *GLUT2*, *KIR6.2* e *GCK*, além de serem positivas para PDX-1, NKX2.2, ISLET-1 e HNF3β. Os autores argumentam que uma baixa freqüência de células (aproximadamente 7%) de toda a população diferenciada apresentaram grânulos secretórios.

A única modificação feita em nosso protocolo (Protocolo 2) foi o aumento na sua duração, passando de 2 para 7 dias, baseado no fato de que os processos ocorrem mais rapidamente em ratos e camundongos do que em humanos. Em nosso trabalho, aplicando o mesmo protocolo em hMSCs derivadas de ilhotas, não foi possível detectar a expressão de insulina por qPCR (Figura 7). Entre as hipóteses para essa diferença podemos citar o tipo celular, visto que células neurais apresentam várias características em comum com células β, incluindo alguns elementos responsáveis pela secreção de insulina (Yang et al., 1999, Burns et al., 2005).

O Protocolo 3 utilizou os indutores mais frequentemente empregados em artigos que diferenciam MSCs em CPIs. Nosso protocolo se assemelha aos indutores utilizados por Timper et al., 2006, por exemplo, com a diferença que eles utilizaram pentagastrina e

nós utilizamos betacelulina (Tabelas 2 e 3). Os resultados mostram que MSCs isoladas de tecido adiposo humano tiveram aumento na expressão de insulina, glucagon e somatostatina após diferenciação, e presença de marcação com peptídeo C (Timper et al., 2006). Um protocolo semelhante ao utilizado por Timper foi empregado por Wu, 2009 (Tabela 3). A imagem de imunocitoquímica para peptídeo C mostra uma superexposição nas culturas diferenciadas o que pode ter dado a impressão de fluorescência aumentada. Além disso, o trabalho não contempla dados de expressão gênica, importantes para comprovação de síntese de novo de insulina. Os dados também demonstram que a diferenciação é acompanhada por diminuição na viabilidade celular, com aumento no número de células apoptóticas, provavelmente pela não adição de soro ao meio de cultura. Como descrito nos resultados, não foi possível observar expressão de insulina (Protocolo 3) aplicando-se protocolos semelhantes aos da literatura.

O protocolo comprovadamente mais eficiente publicado para produção de insulina a partir MSCs utilizou células de tecido adiposo de camundongos. Após 10 dias de diferenciação em placas de baixa aderência, foi observado aumento de genes *FOXA2* e *GATA4* (marcadores de endoderme definitiva) e *CK19* (marcador de células epiteliais). Houve aumento da expressão, comparado com células não diferenciadas, de genes como *PDX1*, *NGN3*, *NEUROD1* entre outros, avaliados qPCR relativo. Comparado com a expressão de ilhotas de camundongos, células no décimo dia de diferenciação tinham expressão de insulina mil vezes menor, embora o conteúdo de insulina tenha sido aproximadamente 1/10 do total apresentado em ilhotas (Chandra et al., 2009). O transplante das células reverteu a hiperglicemia em camundongos diabéticos, confirmado por nefrectomia ao final do experimento. O protocolo 4 é baseado no protocolo de Chandra et al., 2009, com a única diferença que em vez de GLP-1 nós utilizamos Exendina-4, além da origem das células. Nossos resultados mostram a expressão de níveis muito baixos de insulina nas culturas controle e diferenciadas, não sendo possível reproduzir os dados publicados por eles.

Dos 25 trabalhos analisados na Tabela 3, vemos que 12 deles utilizaram PCR convencional (RT-PCR) não quantitativo para detectar a presença de insulina, 11 utilizaram PCR quantitativo (qPCR) relativo, e desses 11 apenas 3 comparam os níveis de expressão das culturas diferenciadas com ilhotas pancreáticas para analisar a eficiência do protocolo. Ainda, 2 trabalhos não analisaram a expressão de insulina por PCR.

Uma análise dos três trabalhos mostrou que os níveis de insulina foram: 1% (Davani et al., 2007), 0.1% (Chandra et al., 2009) e 0.6% (Chao et al., 2008) dos níveis encontrados em ilhotas pancreáticas. No trabalho de Karnieli et al., 2007 foi feita uma estimativa do conteúdo de insulina, que ficou em 1% comparado com ilhotas. Ainda, do total de trabalhos, apenas 10 realizaram estudos *in vivo*, e desses, 8 reverteram a hiperglicemia levando a cura, mas apenas 3 realizaram nefrectomia ao final do experimento como forma de confirmar que a melhora se devia às células transplantadas. Podemos argumentar que a expressão de insulina encontrada nos trabalhos pode ser conseqüência das condições artificiais de cultivo *in vitro*, e que isso não ocorreria *in vivo* com as células.

Estudos que investigaram a capacidade de diferenciação de células indiferenciadas da medula óssea transplantadas *in vivo* em modelos de diabetes mostraram que essas células não são capazes de se diferenciar e produzir insulina (Choi et al., 2003, Lechner et al., 2004, Lavazais et al., 2007).

Um artigo que comparou a eficiência de ESCs diferenciadas espontaneamente ou induzidas com o protocolo NovoCell, chama atenção para a importância da quantificação da expressão em protocolos de diferenciação. Apenas dessa maneira é possível comparar a eficiência do protocolo tanto em relação às células não diferenciadas quanto comparando com ilhotas (Courtney et al., 2010). Outros parâmetros importantes que devem ser analisados caso haja expressão de insulina são a expressão de genes envolvidos na regulação da secreção de insulina em resposta à glicose, como *GLUT2*, *PC1/3*, *GCK*, *KIR6.2* e *SUR1*, além da capacidade de secreção de insulina (preferencialmente peptídeo C) em resposta à glicose, também utilizando como parâmetro ilhotas pancreáticas isoladas.

Nos experimentos *in vivo*, para comprovar a funcionalidade das células diferenciadas transplantadas, é importante a realização de nefrectomia ao final do experimento, o que comprova que a melhora dos níveis glicêmicos não ocorreu em virtude da regeneração do pâncreas.

Baseado nas características apresentadas pelas MSCs, da Silva Meirelles et al. (2008) discute sobre a função *in vivo* dessas células. Entre as funções propostas estão a estabilização de vasos sanguíneos (com sobreposição de identidade com pericitos) e homeostase tecidual, ajudando no reparo de injúrias (secreção de fatores antiapoptóticos e mitogênicos) e sobre células do sistema imune (controle da inflamação).

Podemos tentar explicar a ineficiência ou baixa eficiência na diferenciação de MSCs em células-produtoras de insulina olhando para a origem dessas células durante o desenvolvimento, embora a origem embrionária de MSCs ainda não seja clara (da Silva Meirelles et al., 2008 Geerts et al., 2004). Devido à facilidade de diferenciação de MSCs em células encontradas em tecidos de origem mesodérmica tais como ossos, cartilagem, músculo esquelético e cardíaco, seria bastante provável que elas se originassem desse folheto embrionário. Durante o desenvolvimento embrionário, o mesoderma se origina da camada de células do ectoderma pela migração de um grupo de células através da linha primitiva. Essas células vão se localizar entre o ectoderma e o endoderma na região posterior do embrião (Wells e Melton, 1999).

A expressão transitória do fator de transcrição *MESP1* foi identificada como um dos eventos iniciais para o começo da especificação da mesoderme durante a gastrulação, ocorrendo durante o processo de migração das células para formação da linha primitiva (Saga et al., 1996). Asahina et al. (2009) investigaram a origem embrionária de células estreladas do fígado. Para tal, utilizaram camundongos *MESP1*-Cre, o que permitiu traçar (pela expressão de gene repórter *LACZ*) as células que se originaram de células que em algum momento do desenvolvimento expressaram *MESP1*. Os resultados mostraram que três populações de células mesenquimais no fígado se originaram do mesoderma (*LACZ*$^{+}$): células estreladas hepáticas (DESMINA^{+}, P75NTR^{+} e α-SMA$^{+/-}$), células submesoteliais (DESMINA^{+} P75NTR^{+} ALCAM^{+} -PDGFRα^{+}), e células mesenquimais perivasculares (DESMINA^{+} P75NTR^{+} α-SMA^{+}), além de células endoteliais. Não foi observada expressão de *LACZ* em hepatoblastos (origem endodérmica). Entre os eventos decorrentes da expressão de *MESP1* em células mesodérmicas estão a diminuição da expressão de genes envolvidos na pluripotência (*lD2*, *ERAS*, *OCT4*, *NANOG* e *SOX2*) e na especificação de células da endoderme (*SOX17*, *FOXA2*, *FGF8* e do gene *T*) (Bondue et al., 2008). Além do fígado, sabe-se que o pâncreas, pulmão, rins e intestino também possuem células estreladas com funções similares (Senoo et al., 2009, Zhao e Burt, 2007). Células estreladas (hepáticas ou pancreáticas) e células-tronco mesenquimais apresentam várias características em comum. Entre elas podemos citar a morfologia fibroblastóide em cultura, a localização perivascular e marcadores de superfície semelhantes (Zhao e Burt, 2007, Meirelles et al., 2008), bem como capacidade imunossupressora (Chen et al., 2006) e papel na vascularização e fibrose (Masamune et al., 2008), capacidade de diferenciação

adipogênica (Mato et al., 2009). Desta forma, parece haver uma sobreposição de identidade entre células estreladas e MSCs, como sugerido por da Silva Meirelles et al., 2008. Sendo esses dois tipos celulares o mesmo, fica clara a origem mesodérmica das MSCs. Outros fatores também suportam à origem mesodérmica, como a expressão de vimentina (tanto no mesenquima embrionário quanto em MSCs), o seu papel parácrino tanto no desenvolvimento embrionário quanto na fase adulta. Especula-se que MSCs também podem ser originadas de mesoangioblastos, um precursor comum para células mesenquimais e endoteliais (Minasi et al., 2002) também com origem mesodérmica.

Dessa forma, apesar do pâncreas ou fígado serem classificados como órgãos de origem endodérmica, nem todas as células desses órgãos parecem ser originárias desse folheto. Células do mesenquima que também compõem esses órgãos teriam uma origem mesodérmica. Essa seria uma das explicações da dificuldade de células-tronco mesenquimais de se diferenciarem em CPIs e explicaria porque células-tronco pancreáticas (ou progenitores pancreáticos) presentes nos ductos teriam essa habilidade.

Sordi et al. (2010) demonstraram recentemente o isolamento de duas populações de células do pâncreas. Uma delas possui fenótipo epitelial ($CD133^{+}CD73^{-}$), e a outra mesenquimal ($CD133^{-}CD73^{+}$), lembrando que CD133 é uma marcador de células ductais e que CD73 é uma das proteínas expressas por MSCs. A análise da expressão de genes como *PDX1*, *HNF6*, *NEUROD1*, *NKX6.1*, *PAX4*, mostrou uma maior expressão em células epiteliais comparado com mesenquimais.

Baseado no exposto acima, acreditamos que células-tronco mesenquimais não são boas candidatas para originar células-produtoras de insulina devido a:

- Baixa eficiência de diferenciação em protocolos publicados até o momento (quando quantificada).
- Provável origem mesodérmica dessa células.
- Incapacidade de diferenciação *in vivo* quando transplantadas em animais.
- Existência de uma suposta célula-tronco pancreática ou progenitor pancreático localizado nos ductos.
- Evidências de um papel de "coadjuvante", tanto durante o desenvolvimento embrionário (controle da diferenciação, pela interação epitélio:mesenquima) quanto na fase pós-natal (regeneração tecidual; indução de proliferação de células diferenciadas; diferenciação de

progenitores pancreáticos; promoção da vascularização e controle da inflamação) mediada principalmente por fatores secretados por elas.

Assim, células com características de células-tronco mesenquimais podem ser isoladas de ilhotas humanas após procedimento de isolamento de ilhotas pancreáticas. MSCs aderem e proliferam por várias passagens *in vitro* e expressam marcadores esperados para MSCs. São capazes de diferenciação osteogênica, mas tem capacidade de diferenciação adipogênica limitada. Após diferenciação utilizando quatro protocolos diferentes, essas células não foram capazes de expressar insulina *in vitro* avaliado por qPCR. MSCs isoladas de rim de camundongo diferenciadas com um protocolo publicado previamente expressaram quantidades insignificantes de insulina, também avaliado por qPCR.

Tabela 3. Síntese dos protocolos e principais resultados obtidos em artigos que tentaram diferenciar células-tronco mesenquimais (MSCs) em células-produtoras de insulina (CPIs).
qPCR=PCR quantitativo em tempo real, RT-PCR= PCR normal
Em branco=não analisado, ICQ=imunocitoquímica, IHQ=imunohistoquímica, NC= não compara, C=compara, NR=não responde, R=responde
As letras m (camundongo), r (rato) e h (humano) antes de MSCs indicam.

Origem	Protocolo	Expressão gênica	Análises *in vitro*				Análises *in vivo*				Referência
			ICQ Insulina	**ICQ Peptídeo C**	**Resposta à Glicose**	**Comparação Com ilhotas**	**IHQ Insulina**	**IHQ Peptídeo C**	**Glicose / Cura**	**Nefrectomia**	
hMSCs e rMSCs (tecido adiposo)	Lentivírus contendo *PDX1*, *PDX1-VP16* DMEM (23 mM glicose)	qPCR (expressão relativa de insulina)	+		R	NC	+		+/-		Lin et al., 2009
mMSCs (tecido adiposo do epidídimo)	DMEM-F12 (17.5 mM glicose) + 1% BSA + 1X ITS + 4 nM de Activina A + 1 mM de Butirato de sódio + 50 mM 2-mercaptoetanol (3 dias) DMEM-F12 (17.5 mM glicose) + 1% BSA + ITS + 0.3 mM de taurina (2 dias) DMEM-F12 (17.5 mM glicose) + 1.5% BSA + 1X ITS + 1X Aminoácido não-essenciais + 3 mM de taurina + 100 nM de Exendina-4 + 1 mM Nicotinamida (5 dias) Em placas de baixa aderência.	qPCR (expressão relativa de insulina). Compara com ilhotas murinas.	+	+	R	C	+	+	+/+	+	Chandra et al., 2009

hMSCs (Wharton's Jelly)	- Meio condicionado neuronal (7 dias) - DMEM-F12 (25 mM glicose) + 2% SFB + NAD + B27 (7 dias) - DMEM-F12 (25 mM glicose) + 2% SFB + NAD + B27 + Meio condicionado (7 dias)	qPCR (expressão relativa de insulina)	+		R	C*	+		+/+		Chao et al., 2008
hMSCs (ilhotas)	CMRL-1066 + 1X ITS + 1% BSA (fração V livre de ácidos graxos) (5 dias)	qPCR (Aproximadamente 1% dos níveis de expressão de insulina de ilhotas)			R	C		+			Davani et al., 2007
hMSCs (ilhotas)	Linhagem imortalizada DMEM-F12 (17.5 mM glicose) + B27 + N2 + 2 nM Activina A + 10 mM Nicotinamida + 10 nM Exendina-4 + 100 pM HGF + 10 nM Pentagastrina (4 dias) Em placas de baixa aderência.	RT-PCR para insulina.		+	NR	NC					Eberhardt et al., 2006
hMSCs (medula óssea)	Lentivírus contendo *PDX1*	qPCR (expressão relativa)/#Estimativa de 1% do conteúdo de ilhotas	+		R	#NC	+	+	+/+	+	Karnieli et al., 2007
mMSCs (medula óssea)	DMEM + 55 nM Tricostatina A (3 dias) 1:1 DMEM/DMEM:F-12 (25 mM glicose) + 10% SFB + 10 nM GLP-1 (7 dias)	RT-PCR para insulina	+	+	R	NC					Tayaramma et al., 2006
hMSCs (medula óssea)	Plasmídeos contendo *PDX1*, *NGN3* e *NEUROD1* CMRL1066 (16.5 mM glicose) + 10% SFB + 25 pM Activin A + 200 pM Betacelulina + 10 mM Nicotinamida (6 dias)	RT-PCR para insulina			NR	NC	+		+/+		Zhao et al., 2008
hMSCs (medula óssea)	Estágio 1 – DMEM (5.56 mM glicose) + 10% SFB + 1% Penicilina/estreptomicina, com 5	qPCR (expressão relativa de insulina)	+		NR	NC					Lin et al., 2010

	μg/mL de Fibronectina ou laminina. Estágio 2 - DMEM/F-12 (25 mmol/l glicose) + ITSA + 0.45 mM IBMX + 5 μg/mL de Fibronectina ou laminina. Estágio 3 – DMEM-F12 (5.56 mM glicose) + 10 mM Nicotinamida + B27 e N2 com ou sem 5 μg/mL de Fibronectina ou laminina Estágio 4 – Mesmo que estágio 3, mas com 25 mM glicose.							
hMSCs (sangue de cordão umbilical)	Transdução com gene *NOTCH1* ou adição de 50 μM de DAPT ao meio de diferenciação. Meio de diferenciação: DMEM (25 mM glicose) + 10% SFB + 10^{-6} M Ácido retinóico (1 dia) DMEM (25 mM glicose) + 10% SFB (2 dias) DMEM (LG) + 10% SFB + 10 mM Nicotinamida + 20 ng/ml EGF plaqueadas em gel matriz extracelular (6 dias) DMEM (LG) + 10% SFB + Exendina 4 (6 dias)	qPCR (expressão relativa de insulina)	-	NR	NC	D	-/-	Hu et al., 2010
rMSCs (medula óssea)	Transfecção com plasmídeo contendo *PDX-1* DMEM (5.5 mM glicose) + 1% DMSO (3 dias) DMEM (25 mM glicose) + 1% DMSO + 10% SFB (9 dias)	qPCR (expressão relativa de insulina).		NR	NC			Yuan et al., 2010
rMSCs (medula óssea)	DMEM-LG + 10 ng/mL bFGF + 10 ng/mL EGF + 2% B27 (6 dias) DMEM-LG + 10 ng/mL HGF + 10 ng/mL Betacelulina + 10 ng/ml Activina A + 10 mM Nicotinamida + 2% B27 (6 dias)	RT-PCR para insulina.	+			+	+/+	Zhang et al., 2009

hMSCs (medula óssea e Wharton's Jelly)	DMEM-F12 (17.5 mM glicose) + 10 mM Nicotinamida + 2 nM Activina A + 10 nM Exendina 4 + 100 pM HGF + 10 nM Pentagastrina (plástico de baixa aderência)	Não analisa expressão de insulina. RIA para insulina	+		NC			Wu et al., 2009
hMSCs (sangue de cordão umbilical)	DMEM (25 mM glicose) + 10% SFB + 10^6 M Ácido retinóico (1 dia) DMEM (25 mM glicose) + 10% SFB (2 dias) DMEM (LG) + 10% SFB + 10 mmol/L nicotinamida + 20 ng/ml EGF plaqueadas em gel matriz extracelular (6 dias) DMEM (LG) + 10% SFB + Exendina 4 (6 dias)	RT-PCR para insulina.	+	+	NR	NC		Gao et al., 2008
hMSCs (pâncreas exócrino)	CMRL 1066 + 10% SFB (1 dia) em plástico baixa aderência DMEM (25 mM) + 1X ITS + 10 μM Vitamina C + 10 mM Nicotinamida + 50 μM 2-mercaptoetanol + 100 ng/ml Activina A (2 dias) DMEM (25 mmol/L) + 1X ITS + 10 μM Vitamina C + 10 mM Nicotinamida + 50 μM 2-mercaptoetanol + 100 ng/ml Activina A (2 dias) DMEM (25 mM) + 1X ITS + 10 μM Vitamina C + 10 mM Nicotinamida + 50 μM 2-mercaptoetanol + 100 ng/ml Activina A + 10 ng/ml HGF (15 dias)	RT-PCR para insulina. Negativo para insulina	-			NC		Baertschiger et al., 2008
mMSCs (medula óssea)	DMEM (25 mM glicose) + 10% SFB + 2 nM Activina A + 1 nM Betacelulina. OU DMEM (25 mM glicose) + 10% SFB + 100 ng/ml de CnP + 1 nM Betacelulina/deta4.	RT-PCR para insulina	+		NR	NC	+/+	Hisanaga et al., 2008
hMSCs	Estágio 1 – DMEM (5.56 mM glicose) + 10%	NA/FISH para mRNA de				NC		Chang et al.,

(medula óssea)	SFB + 1% Penicilina/estreptomicina, com 5 μg/mL de fibronectina. Estágio 2 - DMEM/F-12 (25mM glicose) + ITSA + 0.45 mM IBMX + 5 μg/mL de Fibronectina. Estágio 3 – DMEM-F12 (5.56 mM glicose) + 10 mM Nicotinamida + B27 e N2 com ou sem 5 μg/mL de Fibronectina Estágio 4 – Mesmo que estágio 3, mas com 25 mM glicose.	insulina e peptídeo C					2007
rbMSCs (medula óssea)	Estágio 1 – DMEM (5.5 mM glicose) + 10% SFB + 1% Penicilina/estreptomicina, com 5 μg/mL de Fibronectina. Estágio 2 - DMEM/F-12 (25 mM glicose) + ITSA + 0.45 mM IBMX + 5 μg/mL de Fibronectina. Estágio 3 – DMEM-F12 (5.5 mM glicose) + 10 mM Nicotinamida + B27 e N2 com ou sem 5 μg/mL de Fibronectina Estágio 4 – Mesmo que estágio 3, mas com 25 mM glicose.	RT-PCR para insulina	+	+	R	NC	Chang et al., 2008
hMSCs (ilhotas)	RPMI 1640 + insulina (10 mg/ml), transferrina (5.5 mg/ml), Selenito de sódio (6.7 ng/ml) + 1% BSA	qPCR (expressão relativa de insulina)		+	NR	NC	Gallo et al., 2007
hMSCs (medula óssea)	Estágio 1 - DMEM (25 mM glicose) + 0.5 mmol/L 2-mercaptoetanol (2 dias) Estágio 2 – DMEM + 1% Aminoácidos não-essenciais + 20 ng/ml bFGF + 20	RT-PCR para insulina	+		R	NC	Sun et al., 2007

	ng/ml EGF + 2% B27 + 2 mM L-glutamina (8 dias) Estágio 3 - 10 ng/ml Betacelulina + 10 ng/ml Activina A + 2% B27 + 10 mM Nicotinamida (8 dias)									
hMSCs (medula óssea)	Transdução com adenovírus contendo *PDX1* + DMEM-LG + 2% B27 + 10 nM GLP-1 (7 dias)	RT-PCR para insulina	+	+	R	NC	+	+/+	+	Li et al., 2007
hMSCs (tecido adiposo)	DMEM/F12 (17.5 mM glicose) + 10 mM Nicotinamida + 2 nM Activina A + 10nM Exendin-4 + 100 pM HGF + 10 nM pentagastrina + B-27 + N-2 e 1% Penicilina/estreptomicina	qPCR (expressão relativa de insulina)		+		NC				Timper et al., 2006
rMSCs (medula óssea)	DMEM (LG) + 10% SFB + extrato pancreático de ratos (homogeneizado de pâncreas após 48h de pancreactomia parcial) (14 dias)	RT-PCR para insulina	+		R	NC				Choi et al., 2005
hMSCs (tecido adiposo	DMEM (LG) + 10% SFB + extrato pancreático de ratos (homogeneizado de pâncreas após 48h de pancreactomia parcial) (14 dias)	qPCR (expressão relativa de insulina)		+		NC				Lee et al., 2008
mMSCs (medula óssea)	RPMI (23 mM glicose) + 10% SFB (2-4 meses) RPMI (5.5 mM glicose) + %5 SFB + 10 mM Nicotinamida (7 dias) RPMI (5.5 mM glicose) + %5 SFB + 10 mM Nicotinamida + 10 nM Exendina-4 (5-7 dias)	RT-PCR para insulina	+	+	NR	NC		+/+		Tang et al., 2004

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ARTICLE

Co-transplantation of mesenchymal stem cells maintains islet organisation and morphology in mice

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Abstract
Aims/hypothesis Recent studies have shown that mesenchymal stem cells (MSCs) secrete several factors that improve survival and function of transplanted islets. Implantation of islets beneath the kidney capsule results in morphological changes, due to interactions of the graft with the host, thus impairing islet function. We co-transplanted MSCs with islets to determine their effects on the remodelling process and studied graft function in a mouse model of minimal islet mass.
Methods Islets were syngeneically transplanted, either alone or with kidney-derived MSCs, underneath the kidney capsule of streptozotocin-induced diabetic C57Bl/6 mice. Blood glucose levels were monitored and intraperitoneal glucose tolerance tests carried out. Hormone contents of grafts and pancreas were assessed by radioimmunoassay. Graft morphology and vascularisation were evaluated by immunohistochemistry.
Results MSCs improved the capacity of islet grafts to reverse hyperglycaemia, with 92% of mice co-transplanted with MSCs reverting to normoglycaemia, compared with 42% of those transplanted with islets alone. Average blood glucose concentrations were lower throughout the 1 month monitoring period in MSC co-transplanted mice. MSCs did not alter graft hormone content. Islets co-transplanted with MSCs maintained a morphology that more closely resembled that of islets in the endogenous pancreas, both in terms of size, and of endocrine and endothelial cell distribution. Vascular engraftment was superior in MSC co-transplanted mice, as shown by increased endothelial cell numbers within the endocrine tissue.
Conclusions/interpretation Co-transplantation of islets with MSCs had a profound impact on the remodelling process, maintaining islet organisation and improving islet revascularisation. MSCs also improved the capacity of islets to reverse hyperglycaemia.

Keywords Diabetes · Graft morphology · Islet architecture · Mesenchymal stem cells · Transplantation

Abbreviations
IPGTT Intraperitoneal glucose tolerance test
MSCs Mesenchymal stem cells

Introduction

The development of the Edmonton protocol led to marked improvements in the success rate of clinical islet transplantation [1]. Despite this, graft function progressively declines, with only 10% to 15% of patients remaining independent of insulin after 5 years [2–4]. One important confounding factor in islet transplantation is the loss of functional islets during the early post transplantation period [5–7], which has detrimental effects on the outcome of individual grafts, further exacerbating the scarcity of donor tissue. Important factors contributing to the loss of trans-

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planted islets include ischaemia due to inadequate vascularisation and deleterious responses of islet cells to an inflammatory, immunogenic environment [8]. Transplanted islets are avascular during the immediate post transplantation period [9] and must function in a hypoxic microenvironment [7], both of which contribute to ischaemic cell death and inflammatory events. An improved rate or extent of revascularisation of transplanted islets, or enhancement of islet survival should improve the outcome of islet transplantation.

Mesenchymal stem cells (MSCs) are adult progenitor cells, which can proliferate in vitro and give rise to differentiated mesenchymal cell types. MSCs have been isolated from many tissues and may be localised to a perivascular niche that is present in most, if not all, vascularised organs [10]. MSCs play a major role in tissue repair through localised immunosuppressive effects and through the release of soluble trophic factors to affect neighbouring cells [11]. These properties make MSCs excellent candidates for improving the survival of transplanted islets. Several recent studies in experimental animals have reported that co-transplantation of islets and MSCs produces superior outcomes to islet-alone grafts [12–19]. To date, these beneficial effects have been largely attributed to direct or indirect actions of MSCs in promoting islet survival and function by enhancing graft revascularisation [13, 17, 19] or by suppressing immune or inflammatory responses [12, 14–16, 18].

The kidney subcapsular site is most commonly used for islet transplantation in rodent studies [20], although this is known to result in remodelling of the islets within the graft tissue in terms of morphology and composition of endocrine cells [5, 21–23], which is associated with cell loss and disruption of normal islet function [5]. In this study we used the renal subcapsular site for implantation of a minimal islet mass. Our aims were to assess in a syngeneic mouse model of diabetes: (1) the effect of co-transplanting kidney-derived MSCs on the morphological and vascular engraftment; and (2) the function of the transplanted islets.

Methods

Experimental animals Male C567Bl/6 mice (Charles River, Margate, UK) aged 8 to 12 weeks were used as donors and recipients of grafts. Mice were made diabetic by i.p. streptozotocin injection (180 mg/kg; Sigma-Aldrich, Poole, UK) and those with a non-fasting blood glucose concentration of ≥20 mmol/l were used as recipients. Blood glucose concentrations were determined using a blood glucose meter and strips (Accu-Chek; Roche, Burgess Hill, UK) with blood obtained from a pin prick to the tail. All animal procedures were approved by our institution's Ethics Committee and carried out under licence, in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986.

Islet isolation Islets were isolated by collagenase digestion (1 mg/ml; type XI; Sigma-Aldrich) followed by density gradient separation (Histopaque-1077; Sigma-Aldrich). After washing with RPMI-1640 medium, islets were picked into groups of 150 for transplantation.

Mesenchymal stem cell isolation Kidney-derived MSCs were isolated from C57Bl/6 mice as previously described [10]. Kidneys were rinsed in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution containing 10 mmol/l sodium HEPES (Sigma-Aldrich) and cut into small pieces. The fragments were digested with collagenase type I (1 mg/ml; Sigma-Aldrich) for 30 to 45 min at 37°C and then triturated with a glass Pasteur pipette. Cells were pelleted by centrifugation for 10 min at 400×*g* at room temperature. After this, cells were resuspended in DMEM supplemented with 1% (vol./vol.) penicillin/streptomycin solution (Gibco BRL, Gaithersburg, MD, USA) and 10% (vol./vol.) FCS, seeded in six-well dishes (3 ml/well) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The medium was changed after 24 h, with removal of non-adherent cells. When cultures reached confluence, cells were trypsinised and subcultured in new flasks, at passage ratios empirically determined for two subcultures a week.

Immunophenotyping Kidney-derived MSCs were analysed for the presence of surface markers by flow cytometry. MSCs at the fifth passage were trypsinised, resuspended in PBS and incubated with the following FITC- or phycoerythrin-conjugated antibodies: CD11b, CD31, CD44, CD45, CD73, CD90.2 and stem cell antigen-1 (BD Pharmingen, San Diego, CA, USA). After 30 min incubation at 4°C, the cells were washed and resuspended in 0.5 ml PBS. Cells were analysed in a FACS calibur cytometer equipped with 488 nm argon laser (BD Pharmingen).

Adipogenic and osteogenic differentiation Adipogenic differentiation was induced by cultivation of confluent cultures in DMEM containing 20% (vol./vol.) FCS, 2.5 μg/ml insulin, 100 μmol/l indomethacin, 5 μmol/l rosiglitazone and 10 nmol/l dexamethasone. For osteogenic differentiation, confluent cultures were cultivated in DMEM containing 10% (vol./vol.) FCS, 10 mmol/l B-glycerophosphate, 5 μg/ml ascorbic acid and 10 nmol/l dexamethasone. Cultures were maintained in differentiation media for 1 month with medium changes twice a week. Cell differentiation was analysed by staining with Oil Red O or Alizarin Red S for adipogenic and osteogenic differentiation, respectively, as described previously [10].

Transplantation Mice were transplanted with 150 freshly isolated islets either alone or together with 25×10^4 kidney-derived MSCs. The number of transplanted islets was chosen to act as a minimal islet mass, intended to reverse hyperglycaemia in only a proportion of diabetic recipients. A lumbar incision was made, the kidney exposed and an incision made in the capsule. Islets alone or islets+MSCs that had been centrifuged into pellets in PE50 polyethylene tubing (Becton Dickinson, Sparks, MD, USA) were placed underneath the kidney capsule using a Hamilton syringe (Fisher, Pittsburg, PA, USA).

Graft function The body weight and blood glucose concentrations of recipient mice were monitored every 3 to 4 days. Reversal of hyperglycaemia was defined as non-fasting blood glucose concentrations ≤11.1 mmol/l for at least two consecutive readings, without reverting to hyperglycaemia on any subsequent day. All mice with blood glucose <11.1 mmol/l were given an intraperitoneal glucose tolerance test (IPGTT) 1 month after transplantation. Weight-matched, non-diabetic, non-transplanted male C567Bl/6 mice were used as controls. Fasting blood glucose concentrations were measured prior to i.p. injection of 2 g/kg glucose and then after 15, 30, 60, 90 and 120 min. In some animals, the islet graft-bearing kidneys were removed 2 days later to assess whether graft removal would result in a reversion to hyperglycaemia. Nephrectomised mice were killed 3 to 4 days later and the pancreas was also removed for histological examination. Other mice were killed so that the graft-bearing kidney and pancreas could be removed at the same time for analysis of hormone content or histological analysis.

Immunohistochemistry Graft bearing kidneys were fixed in 4% (vol./vol.) formalin and paraffin-embedded. Sections were stained for islet hormones and microvascular endothelial cells. For CD34 staining (detection of endothelial cells), antigen retrieval was required (2 min in 10 mmol/l citric acid solution, pH 6.0 in a pressurised cooker). Sections were incubated for 1 h at room temperature in the appropriate primary antibody as follows: polyclonal guinea pig anti-insulin antibody (1:1,000; Dako, Ely, UK), monoclonal mouse anti-glucagon antibody (1:1,000; Sigma-Aldrich), monoclonal rat anti-somatostatin antibody (1:50; AbCam, Cambridge, UK) or with a monoclonal rat anti-CD34 antibody (1:500; AbD Serotec, Kidlington, UK). Slides were then incubated for 1 h at room temperature with either a goat biotin anti-guinea pig antibody (1:200; Jackson Immunolaboratories, West Grove, PA, USA), a biotinylated link universal (Dako) or a rabbit biotinylated anti-rat antibody (1:200; Vector Laboratories, Peterborough, UK). Sections were incubated with streptavidin–horseradish peroxidase (Dako) and diaminobenzidine. For immunofluorescence labelling of insulin, a polyclonal guinea pig anti-insulin antibody (1:100; Jackson) was used (1 h at room temperature) with a Texas Red anti-guinea pig secondary antibody (1:40; Jackson; 1 h at room temperature).

Evaluation of graft morphology and vascular density For each animal five to nine tissue sections from different graft areas were evaluated for total endocrine area and vascular density. To evaluate the extent of islet fusion we measured the number and area of individual endocrine aggregates. An individual endocrine aggregate was defined as an area of insulin-positive tissue separated from any other adjacent insulin-positive tissue by ≥50 μm of non-endocrine tissue (insulin-negative). Total endocrine area refers to the sum of the area of all endocrine aggregates within an individual graft section. The demarcation of the islet graft was taken as the area of endocrine and non-endocrine tissue between the renal parenchyma and the kidney capsule. The number of endothelial cells was counted by an individual blinded to the treatments. Areas of endocrine and non-endocrine tissue were counted separately. Area and diameter were determined using Image J software (http://rsbweb.nih.gov/ij/) and the vascular density (number of endothelial cells per square millimetre) was determined.

Hormone measurement of islet grafts and serum The islet graft-bearing kidneys or pancreas were homogenised in acid-ethanol (0.18 mol/l in 70% [vol./vol.] ethanol) then sonicated. Tissue insulin, glucagon and somatostatin contents were measured using in-house RIA, as previously described [24, 25] and a commercially available somatostatin RIA kit (Euro-Diagnostica, Malmö, Sweden). Serum samples were obtained from non-fasted mice and insulin was measured using an ELISA (Mercodia, Uppsala, Sweden).

Statistical analysis Statistical analysis used Student's *t* test or ANOVA, as appropriate. Two-way repeated-measurement ANOVA was used with Bonferroni's post hoc test to analyse repeated measurements in the same animal at different time points. When data sets were not normally distributed or did not have equal variance, ANOVA on ranks was performed. A Kaplan–Meier survival curve was used to identify differences in the time to cure between groups. A p value of $p < 0.05$ was considered significant. All data are expressed as means ± SEM.

Results

MSC characterisation The characteristics of the adherent cells isolated from the whole kidney of male C57Bl/6 mice and expanded in vitro were similar to those previously

described in detail by our group [10]. The cells presented morphological characteristics of MSCs and their identity was confirmed by flow cytometry immunophenotyping. Thus, the cells were negative for CD11b, CD31 and CD45, which are markers of macrophages, endothelial cells and haemopoietic cells, respectively, but positive for CD44, stem cell antigen-1, CD73 and CD90.2, which are characteristic of MSCs. Cells were also able to differentiate in vitro into adipocyte- and osteoblast-like cells, as assessed by histological staining with Oil Red O or Alizarin Red S.

Graft function Co-transplantation of islets with kidney-derived MSCs produced superior transplantation outcomes to islet-alone grafts, as shown in Fig. 1. The average blood glucose concentrations of mice transplanted with islets+MSCs were significantly lower than in mice transplanted with islets alone at 3, 7, 14, 21 and 28 days after transplantation (Fig. 1a). After 1 month, only 8% of mice transplanted with islets+MSCs had not reverted to hyperglycaemia, compared with 58% of mice transplanted with islets alone (Fig. 1b). The average time to reverse hyperglycaemia for islet+MSC grafts was 7±2 days, with islet-alone recipients taking significantly longer (17±2 days, $p<0.001$, $n=13$). The median time to attain normoglycaemia was 4 and 17 days for islet+MSC and islet-alone grafts, respectively. There were no significant differences in the weights of mice in either transplant group on day 0 (24.2±0.7 and 23.2±0.3 g for islet-alone and islet+MSC recipients, respectively, $n=13$, $p>0.2$) or at 1 month after transplantation (25.9±0.7 and 26.1±0.4 g, $n=13$, $p>0.2$). MSC-alone grafts did not lower blood glucose concentrations in streptozotocin-induced diabetic mice (day 0, 23.7±1.6 mmol/l; day 3, 23.4±4.1 mmol/l, $n=3$), which had to be killed after a 3 day monitoring period due to excessive weight loss. Thus, MSCs alone had no capacity to reverse streptozotocin-induced diabetes. At 1 month after transplantation, non-fasted serum insulin concentrations were 432±114 pmol/l in islet+MSC mice vs 300±47 pmol/l in islet-alone mice ($n=11$, $p=0.25$). This was associated with lower blood glucose in the islet+MSC mice than in the islet-alone mice (8.2±0.8 mmol/l vs 17.1±3.2 mmol/l, $n=11$, $p=0.01$). At this time-point, IPGTTs were carried out in all transplanted mice with blood glucose <11.1 mmol/l and in weight-matched non-diabetic, non-transplanted controls. Glucose tolerance was similar in both transplant groups, but impaired in comparison to non-transplanted controls (Fig. 1c). At 2 days after the IPGTT, some of the mice with blood glucose <11.1 mmol/l in each transplant group were nephrectomised, with all nephrectomised mice reverting to hyperglycaemia within 2 days.

Graft morphology Figure 2 shows the typical morphology of graft material retrieved 1 month after transplantation, demonstrating that islets co-transplanted with MSCs maintained a morphology that more closely resembles that of endogenous islets in the pancreas, in contrast to the amorphous mass of endocrine tissue that forms in islet-alone grafts. Insulin immunostaining of islet-alone grafts revealed a single amorphous endocrine mass in the majority of sections analysed (Fig. 2a, c), where the transplanted islets have fused to form aggregated islet tissue. In contrast, there were rarely signs of any fusion between individual islets in the grafts of islet+MSC recipients. In these grafts, insulin immunostaining revealed endocrine aggregates with the appearance of normal islets separated by extensive areas of non-endocrine tissue (Fig. 2b, d). Although the total area of endocrine tissue per section (immunostained for insulin) was similar in islet-alone and islet+MSC grafts (Fig. 2e), it was clear that the graft morphology was different. We quantified the extent of islet fusion within the grafts of both

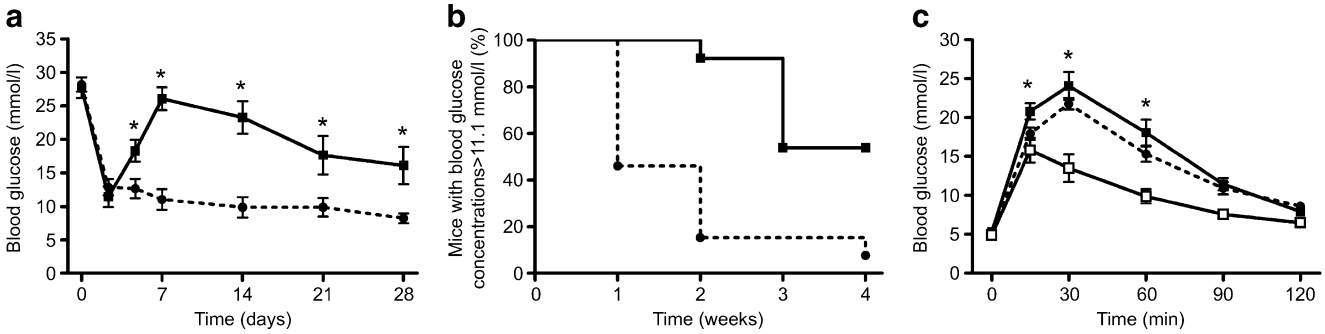

Fig. 1 Graft function. **a** Blood glucose concentrations of mice transplanted with 150 islets alone (continuous line) or those co-transplanted with 150 islets+25×10^4 kidney-derived MSCs (dashed line); *$p<0.05$ vs mice transplanted with islets+MSCs (repeated-measurement ANOVA with Bonferroni post hoc test, $n=13$). **b** Percentage of mice remaining diabetic (blood glucose concentration >11.1 mmol/l) after transplantation as above (**a**); $p=0.0005$ Kaplan–Meier, $n=13$ for both transplant groups. **c** IPGTTs in all mice with reversed hyperglycaemia after transplantion as above; IPGTT was conducted 4 weeks after transplantation. *$p<0.05$ vs non-diabetic non-transplanted controls (repeated-measurement ANOVA with Bonferroni post hoc test, $n=6$–12). Black squares, islets alone; black circles, islets+MSCs; white squares, control weight-matched, non-diabetic, non-transplanted mice

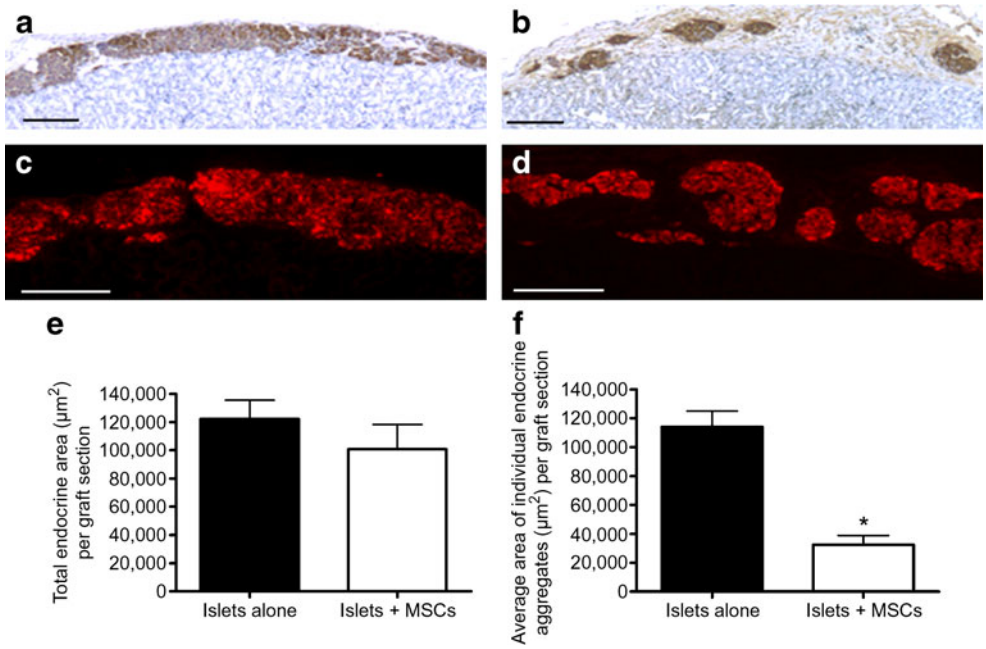

Fig. 2 Graft morphology. **a**, **c** Representative sections of islet-alone grafts and (**b, d**) islet+MSC grafts at 1 month post transplantation. **a** Islet-alone graft, where islets have aggregated to form a single amorphous endocrine mass. **b** Islet+MSC graft, where individual endocrine aggregates are separated by extensive areas of non-endocrine tissue. Original magnification (**a**, **b**)×40, scale bar 100 μm. **c** At a higher magnification (×100), islet-alone graft shows that the rounded morphology of individual islets can no longer be discerned, in comparison with (**d**) islet+MSC graft, where even when islets have aggregated, they still maintain a morphology comparable to that of endogenous islets in the pancreas, with individual islets still clearly distinguishable from each other. Scale bars, 100 μm (**c**, **d**). **e** Total endocrine area in graft sections; n=4–6 animals per group, $p>0.2$ Student's t test. **f** Average individual endocrine aggregate area in graft sections; n=4–6 animals per group, *$p<0.05$ vs islet-alone grafts, Student's t test

transplant groups by measuring the average area of each individual endocrine aggregate, defined as an individual mass of islet tissue that was separated from any adjacent endocrine aggregate by ≥50 μm in each graft section. The average area of each single endocrine aggregate in islet+MSC recipients was approximately fourfold smaller than that of the aggregates in islet-alone recipients (Fig. 2f). The total graft area (endocrine+non-endocrine tissue) was higher in islet+MSC grafts than in islet-alone grafts (322,596±38,919 vs 134,546±14,941, $p<0.05$ t test, n=4–6). Considering that the area of endocrine tissue was similar in both grafts, it was clear that the islet+MSC grafts contained large areas of non-endocrine tissue. Analysis of this tissue showed very few α-smooth muscle actin-positive cells (a marker we have previously shown to stain murine MSCs [10]); rather, this tissue was predominately extracellular tissue, consisting of loosely packed fibres, staining positively with van Geison, which strongly indicates the presence of collagen.

Graft composition Immunostaining for glucagon-positive alpha cells indicated that the normal core–mantle segregation of islet endocrine cells was altered in the grafts of islet-alone recipients, as shown in Fig. 3a. In contrast, the majority of alpha cells in islet+MSC grafts were located at the periphery of individual islets (Fig. 3b). In both transplant groups, somatostatin-positive delta cells were dispersed among the alpha cells. As for the alpha cells, delta cells were distributed throughout the islet-alone grafts (Fig. 3c), whereas they were primarily located in the islet mantle in the islet+MSC grafts (Fig. 3d).

Hormone content At 1 month after transplantation there was considerable variation in the graft insulin content between animals, ranging from 3.0 to 15.2 and 7.7 to 21.1 μg/graft for islet-alone and islet+MSC recipients respectively. The mean insulin content of islet+MSC grafts was approximately 60% higher than in islet-alone grafts, but this was not statistically significant ($p>0.05$; Fig. 4a). The graft glucagon and somatostatin contents were similar between transplant groups (Fig. 4b, c). The insulin content of the pancreas in all streptozotocin-treated mice was around ten times lower than that of the grafts. There was no difference in the pancreas insulin content for mice transplanted with islets alone or islets+MSCs (1.1±0.3 and 1.9±0.4 μg/pancreas, $p>0.2$, n=4). Immunohistochemical analysis of the pancreas for each transplant group at 1 month after transplantation revealed very few insulin-positive cells (data not shown), consistent with the greatly reduced insulin content, providing further confirmation that

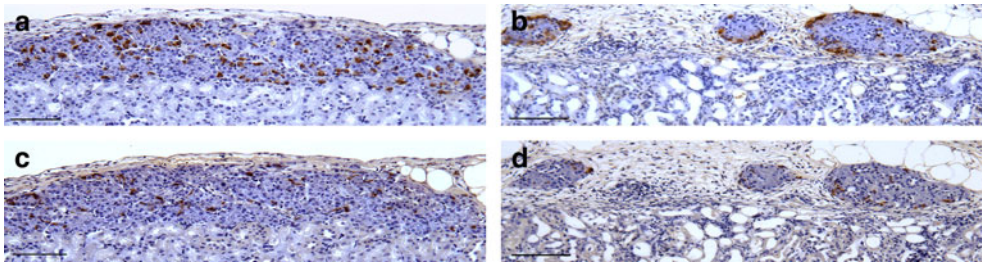

Fig. 3 Graft composition. Distribution of glucagon-positive alpha cells and somatostatin-positive delta cells. At 1 month after transplantation, consecutive sections were stained with glucagon (**a**, **b**) or somatostatin (**c**, **d**) antibodies, in grafts consisting of islets alone (**a**, **c**) or islets+MSCs (**b**, **d**). Images are representative of sections from four to six different animals. Original magnification ×100, scale bars 100 μm

endogenous beta cell regeneration in the pancreas did not contribute significantly to maintenance of normoglycaemia.

Vascular density CD34 antibodies were used to immunostain microvascular endothelium in endogenous pancreatic islets (Fig. 5a) and in islet grafts beneath the kidney capsule (Fig. 5b–d). The distribution of CD34-positive endothelial cells in islet+MSC grafts was similar to that of islets in the pancreas, with endothelial cells located throughout the islet mass (Fig. 5b). In contrast, islet-alone grafts contained areas of endocrine tissue that were devoid of any endothelial cells, with no detectable differences being observed between hyperglycaemic and normoglycaemic recipients (Fig. 5c, d). We quantified the number of endothelial cells in the endocrine and non-endocrine tissue in the field of view by counting CD34-positive cells. The vascular density of the endocrine tissue in grafts consisting of islets+MSCs was significantly higher than that of islet-alone grafts, as shown in Fig. 5e. However, the non-endocrine tissue in islet-alone grafts had a markedly higher vascular density than the non-endocrine tissue of islet+MSC grafts (Fig. 5e).

Discussion

We used a minimal islet mass transplantation model in streptozotocin-treated hyperglycaemic mice to demonstrate that co-grafting kidney-derived MSCs with islets increased the rate and number of recipients attaining normoglycaemia by 1 month after transplantation. Several reports have indicated that MSCs have beneficial effects in different transplantation models, including islet grafts [12–19], and several different mechanisms have been thought to account for these effects, some of which are unlikely to account for our observations. Our results do not support the suggestion that transplantation of MSCs alone is sufficient to reverse hyperglycaemia in streptozotocin-treated rodents, either by enhancing regeneration of pancreatic beta cells [26] or through other paracrine effects [11]. Thus, transplanting MSCs alone did not reduce blood glucose in our streptozotocin-treated mice and MSC co-transplantation had no significant effect on the very low levels of pancreatic insulin and beta cell numbers up to 1 month after induction of hyperglycaemia. Other studies have suggested that the immunosuppressive properties of MSCs enhance islet survival after transplantation by secreting cytokines [15, 16, 18] or metalloproteinases [14]. This mechanism may be important in allogeneic grafts [12, 14–16, 18], but immunomodulation is unlikely to be the sole mechanism accounting for the beneficial effects of MSCs in our syngeneic transplantation model.

MSCs secrete several trophic factors, many of which could have positive effects on islet cell viability and function in a co-transplant model [13, 27]. For example, in vitro co-culture experiments have shown that MSCs increase islet viability by upregulating anti-apoptotic genes

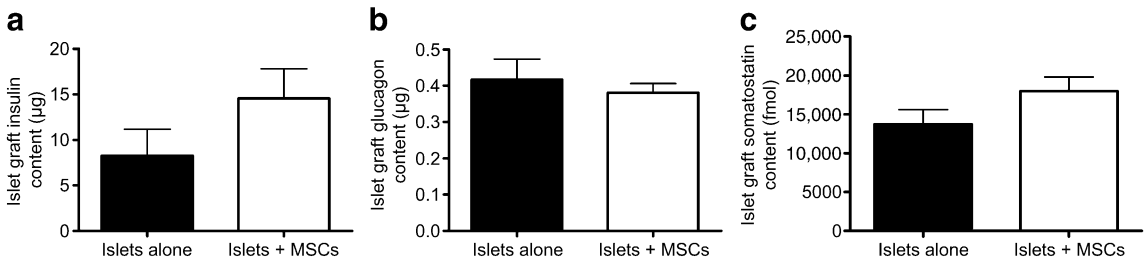

Fig. 4 Hormone content. **a** Insulin, (**b**) glucagon and (**c**) somatostatin content of grafts at 1 month after transplantation. Mice were transplanted with 150 islets alone (black bars) or 150 islets+25×10^4 kidney-derived MSCs (white bars). $n=4$, $p>0.05$ by Student's t test

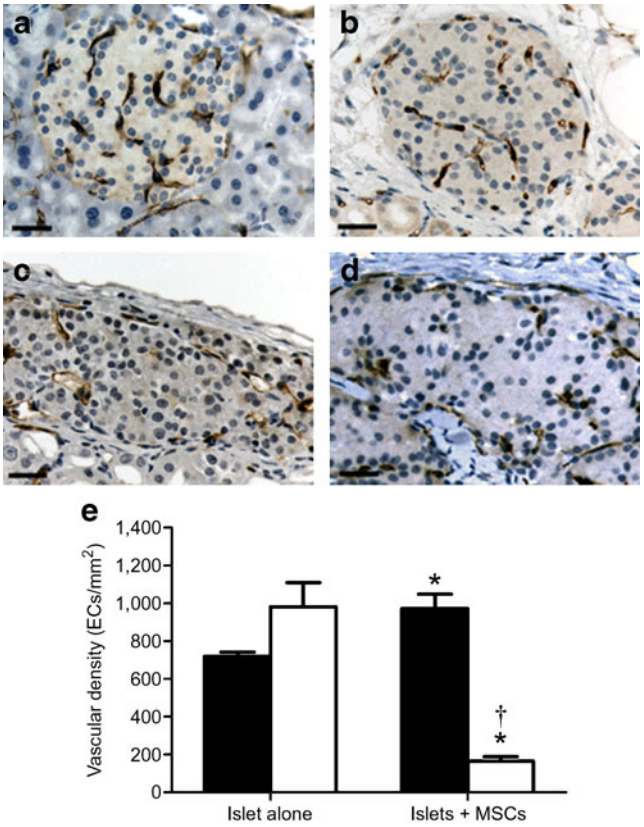

Fig. 5 Vascular density. Staining of endothelial cells (ECs) with CD34 antibodies in endogenous pancreatic islets (**a**), and in grafts of islet+MSC recipients with blood glucose <11.1 mmol/l at 1 month after transplantation (**b**), of islet-alone hyperglycaemic recipients (**c**) and of islet-alone recipients with blood glucose <11.1 mmol/l (**d**). Original magnification ×400, scale bars 25 μm. **e** Vascular density of endocrine components (black bars) and non-endocrine components (white bars) in 1 month grafts consisting of 150 islets alone or 150 islets+25×10^4 kidney-derived MSCs. *$p<0.05$ vs islet-alone and $^{\dagger}p<0.05$ vs endocrine tissue within the same transplant group, by two-way ANOVA with Bonferroni's post hoc test

and improve insulin secretory function by modulating islet ATP content [27]. These effects are likely to be important in the immediate post-transplantation period when improved glycaemic control will favour effective islet engraftment by maintaining function [6, 28] and enhancing revascularisation [29]. These factors alone are unlikely to account for the beneficial effects of MSCs in our experiments. Thus, the presence of co-transplanted MSCs did not cause significant increases in graft insulin content and serum insulin at 1 month after transplantation, suggesting that the enhancing effects of MSCs on graft function may involve other factors. Although in contrast to a recent report [17], MSC co-transplantation enhanced the ability of islet grafts to reverse hyperglycaemia, it did not improve glucose tolerance in mice after reversal of hyperglycaemia. Islet-alone and islet+MSC grafts both showed similar degrees of impaired glucose tolerance when compared with non-transplanted control animals. This is presumably a consequence of the deliberately low insulin content in the minimal mass islet grafts. It should be noted that glucose tolerance tests were not carried out in hyperglycaemic mice. These data suggest that the presence of MSCs helps in the reversal of hyperglycaemia, but does not increase functionality after reversal of hyperglycaemia compared with islet-alone grafts.

The rate of revascularisation of the islet graft is thought to have a major influence on graft survival and function, and on the reversal of hyperglycaemia [8]. Our results are consistent with previous observations that co-transplantation of MSCs improves islet revascularisation, at least in terms of endothelial cell numbers. MSCs derived from bone marrow [13] or pancreas [19] have been reported to increase the endothelial cell density of transplanted islets, consistent with a more effective revascularisation. MSCs may influence angiogenesis through several mechanisms. They secrete a range of angiogenic factors, including vascular endothelial growth factor, IL-6, IL-8, haemopoeitic growth factor and platelet-derived growth factor [19, 27, 30], which are known to enhance islet revascularisation [31–33]. In addition, MSCs secrete matrix metalloproteases [14], which facilitate migration of host-derived endothelial cells into the islets [34] degrading the extracellular matrix [35, 36].

Our morphological studies suggest that MSCs may also influence revascularisation and graft function by modulating the morphology of the graft. MSC co-transplantation had a profound impact on the remodelling process that occurs after transplantation, inducing a graft morphology that more closely represents that of islets in the endogenous pancreas, rather than the amorphous endocrine mass that formed at the graft site in islet-alone recipients as reported in previous islet transplantation studies [5, 6]. The MSC-induced changes in graft morphology were associated with an altered distribution of endothelial cells. Islet+MSC grafts showed a distribution of endothelial cells throughout the endocrine tissue, similar to that of islets in the pancreas, whereas the islet-alone grafts showed a largely peripheral distribution of endothelial cells, with large areas of endocrine tissue lacking endothelial cells. This is in accordance with previous studies demonstrating that the revascularisation of smaller islets after transplantation is more efficient than that of larger islets [37], an effect that is likely to be amplified by the large aggregates formed in our islet-alone grafts. The re-establishment of a blood supply is of obvious importance for the survival and function of the islets, but it is also clear that paracrine interactions between beta cells and endothelial cells are important in maintaining beta cell function [38]. The improved recovery of these interactions in the islet+MSC grafts may account for some beneficial effects of the MSC co-transplant in our model. Another striking observation from our analysis of endothelial cell distribution in the grafts was the low vascular

density in the non-endocrine tissue surrounding the islets in the islet+MSC grafts, when compared with islet-alone grafts, in which the surrounding non-endocrine parenchyma contained large numbers of endothelial cells, as reported previously [39–41]. These observations are consistent with earlier reports that transplanted islets induce increased vascularisation of the surrounding tissue to compensate for their low vascular density [42]. The MSC-dependent increase in islet endothelial cell density observed by us may negate the requirement for a compensatory increase in revascularisation of the adjacent non-endocrine tissue.

The MSC-induced alterations in graft morphology are also likely to influence graft survival and function independently of the rate or extent of revascularisation. A recent study using encapsulated islets, which are unable to revascularise, demonstrated improved transplantation outcomes from grafting of aggregates of smaller islets rather than of larger intact islets, consistent with an anatomical effect on function that is independent of revascularisation or endothelial cell density [43]. It is well established that islet organisation influences function by facilitating the numerous interactions between the islet cells [44] that are required for normal insulin secretion [45, 46]. The loss of organised islet anatomy in the islet-alone grafts, with alpha and delta cells distributed throughout the large endocrine aggregates is therefore likely to result in impaired function. Conversely, the MSC-dependent maintenance of normal islet size and organisation may contribute to the improved outcomes in the islet+MSC co-transplants. We did not see any evidence of MSCs remaining in the islet+MSC graft at 1 month, although they were detectable in grafts removed at 3 days. The non-endocrine component of the graft appeared to be largely composed of collagen fibres. Interestingly, in this context, Jalili et al. recently reported that a collagen matrix is beneficial to islet graft function [47].

In summary, we have demonstrated that co-transplantation of MSCs has a beneficial effect on the outcome of islet grafts for treatment of diabetic hyperglycaemia, confirming recent reports [12–18]. These effects may be due in part to enhanced revascularisation, as has been previously suggested [13, 17, 19]. However, our results also suggest that MSC-dependent effects on the anatomical remodelling of the graft may have a major effect on graft function by maintaining islet organisation and morphology.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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BRIEF COMMUNICATION

Association between the 1858T allele of the protein tyrosine phosphatase nonreceptor type 22 and type 1 diabetes in a Brazilian population

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Key words
1858T allele; Brazilian; lymphoid-specific tyrosine phosphatase; polymorphism; protein tyrosine phosphatase nonreceptor 22; type 1 diabetes mellitus

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Abstract

The 1858T allele of the protein tyrosine phosphatase nonreceptor 22 (PTPN22) gene has been associated to diabetes in different populations. We investigated a possible relationship between this polymorphism and type 1 diabetes in a cohort of Brazilian patients. A significantly higher frequency of the 1858T allele was observed in diabetic patients ($n = 211$) than in control individuals ($n = 241$). Additionally, the heterozygote genotype was also increased in the diabetic group. No association was observed between the PTPN22 T allele and gender, or between T carriers and age of onset of T1D. This work describes for the first time a strong association of the 1858T allele with type 1 diabetes in a Brazilian population, reinforcing the role of this variant as an important susceptibility factor for this disease.

Polymorphisms on the protein tyrosine phosphatase nonreceptor 22 (PTPN22) gene are associated with several autoimmune diseases. PTPN22 encodes a lymphoid-specific tyrosine phosphatase (Lyp) involved in the inhibition of immune response through dephosphorylation of proteins involved in the T cell receptor (TCR) signaling cascade (1). The substitution of C for T nucleotide at position 1858 causes the change of arginine to tryptophan (R620W) in the prolin-rich region, disrupting the binding of Lyp to a domain of Csk (2). Substitution gives rise to a more active phosphatase activity enzyme, indicating this variant as a gain of function mutation (3). The consequences of this mutation on regulation of the immune system are under investigation, and it has been described that $CD4^+$ T cells from diabetic patients carrying the PTPN22/Lyp 620W variant have decreased activation and proliferation as well as interleukin 2 production when stimulated with anti-CD3 and anti-CD28 (4). The 1858T allele was reported to be associated with type 1 diabetes in several populations including Italians (5), Germans (6), Danish (7), North Americans (8), English (9), Spanish (10), Ukrainian (11), and Estonian (12). No association was observed for an Asian Indian population (13). This allele has been also associated with other autoimmune diseases such as lupus erythematosus (14), rheumatoid arthritis (15), juvenile idiopathic arthritis (16), but not with some others such as inflammatory bowel disease (17), psoriasis and multiple sclerosis (16). The association is controversial for celiac disease (18, 19). The first study of this polymorphism in Latin America was performed in a Colombian population. The results describe association of 1858T allele with type 1 diabetes as well as Sjogren's syndrome and systemic lupus erythematosus (20). In the present study, we report for the first time an association of the 1858T polymorphism on the PTPN22 gene and type 1 diabetes in a Brazilian population.

Type 1 diabetic patients were recruited from Instituto da Criança com Diabetes do Rio Grande do Sul (Porto Alegre, RS, Brazil), and a previously collected DNA bank from healthy blood donors at Hospital de Clínicas de Porto Alegre

Table 1 Allelic and genotypic frequencies of the 1858T polymorphism in the protein tyrosine phosphatase nonreceptor type 22 in healthy controls ($n = 241$) and type 1 diabetic patients ($n = 211$)[a]

			Allelic		Genotypic		
Group	Age ± SD	Gender (% male)	C	T	CC	CT	TT
Control	43.50 ± 7.95	66.8	457 (94.81)	25 (5.19)	216 (89.63)	25 (10.37)	0 (0)
Patients	15.13 ± 5.72	52.6	360 (85.31)	62 (14.69)	152 (72.04)	56 (26.54)	3 (1.42)

[a]Data are expressed as absolute number (%). Patients *vs* control: T *vs* C; odds ratio(OR)(95%) = 3.14 (1.94–5.11); $P = 0.000$. TT + TC *vs* CC; OR(95%) = 3.35 (2.01–5.59); $P = 0.000$.

was used as control group. The study was approved by a Research Ethics Committee, and patients or responsible adults signed informed consent forms. Diagnosis of type 1 diabetes was based on World Health Organization criteria. Because the 1858T allele seems to be originated in Caucasian populations (8, 21), we studied only Caucasoid individuals. We considered those individuals who, according to their physical traits (specially skin color), do not seem to present admixture with non-European populations as Caucasoid. DNA was extracted from blood samples by a salting-out method. Genotyping for the PTPN22 C1858T SNP (rs2476601) was performed as described (8), with modifications. A total of 25 µl reaction was prepared by addition of 50–100 ng DNA, 2.5 µl of 10× Taq DNA polymerase buffer, 10 mM dNTP, 2 mM $MgCl_2$, 10 pmol of each primer, and 1 U of Taq DNA polymerase (Invitrogen Co, Carlsbad, CA). Polymerase chain reaction products were digested with 4 U of *Rsa*I (New England Biolabs Inc., Ipswich, MA) at 37°C overnight and identified by electrophoresis on 2.5% agarose gel stained with ethidium bromide. Homozygotes for T allele have no site for *Rsa*I, presenting only the 218 bp band; homozygotes for the C allele present two fragments of 176 and 46 bp and hetorozygotes present the three fragment lengths. The chi-squared test was used to determine if populations were in Hardy–Weinberg equilibrium. Statistically significant differences in allelic and genotypic frequencies between groups were assessed by the Fisher's exact test (significance level of 0.05) using WinPepi. Associations were estimated by the odds ratio (OR) with 95% confidence interval (CI).

A total of 211 type 1 diabetic patients and 241 healthy control individuals were enrolled in this study. Demographic data are shown in Table 1. Diabetic and control populations were in Hardy–Weinberg equilibrium. Significant differences in allelic and genotypic frequencies were found when comparing the control and diabetic groups (Table 1). The T allele is associated with an increased risk of developing type 1 diabetes (OR 3.14, 95% CI 1.94–5.11). We also found that T carriers have an increased risk of developing T1D compared with CC homozygotes (OR 3.35, 95% CI 2.01–5.59). The frequency of C/T heterozygotes among diabetic patients (26.54%) was significantly higher than in the control group (10.37%). No TT homozygotes were found in controls, whereas 1.42% of the diabetic patients had this genotype. A sex-specific association has been reported between T allele and female gender (6, 7). Although the higher frequency of males in the control group might have introduced a bias in the results, we did not observe any association between the T allele and gender in this study, in agreement with the most of the literature reports. Analysis of diagnostic data, available for 80.5% of the total T1D patients, did not show statistically significant difference when comparing the mean age of onset for CC homozygotes (9.19 ± 4.43 years) *vs* T carriers (8.89 ± 5.15 years). The genotypic frequencies observed in the present study are very similar to those described for diabetic patients but slightly lower than control groups in the United States (8) and Denmark (7). Our results also show a higher frequency of the T allele and heterozygotes in the diabetic group when compared with a Spanish population (10), and lower frequencies compared with an Ukrainian population (11).

Allelic and genotypic frequencies of C1858T polymorphism in different T1D case–control studied show enormous variability (Table 2). In Asian populations, such as Chinese (8), Japanese, and Korean (22), only the C allele could be found so far. There is no study involving a black African population and T1D patients; however, other studies show that, as for Asians, it is not polymorphic in Africans (21). In some populations such as Asian Indians (13), Italians from Sardinia (2), Colombians (20), and Azeri (23), the T allele as well as the frequencies of T carriers are low, varying from 2.8%–8% for the T allele and 5.03%–14.9% for T carriers. Populations from Italy (24), Germany (25), Spain (10), North America (2, 26), UK (9, 27), Netherlands (28), Denmark (29), and Brazil show intermediate frequencies for the T allele (10.4%–19%) and T carriers (19.7%–34.3%). The highest frequencies observed were in populations from North and East of Europe, including Ukraine (11), Finland (30), Germany (6), Estonia (12), and Czech Republic (23), with T allele and T carriers frequency varying from 19.3% to 25% and 35.5% to 42.1%, respectively. In the French study (31), T1D patients present T allele frequency of 29.6% and T carriers of 29.61%. The same tendency is observed in control populations. This data suggest a geographic gradient, with higher frequencies in Northeastern Europe, decreasing toward the Southwest. This suggests that the T variant originated in this region, from where it was spread and/or positively selected (32).

Table 2 Allelic and genotypic frequencies of the PTPN22 C1858T polymorphism in T1D case–control studies[a]

Study (reference)	Country (ethnicity)	Group	*n*	Allelic		Genotypic		
				C	T	CC	CT	TT
Bottini et al. (2)	North America (C)	T1D	294	*81.0*	*19.0*	65.6	30.6	3.7
		Control	395	*88.4*	*11.6*	77.7	21.3	1
	Italy (C)	T1D	174	*95.1*	*4.9*	90.8	8.6	0.6
		Control	214	*97.9*	*2.1*	95.8	4.2	0
Smyth et al. (9)	UK (C)	T1D	1573	83	17	68.5	29	2.5
		Control	1718	89.6	10.4	80.2	18.8	1
Zheng and She (8)	United States (C)	T1D	396	*85.5*	*14.5*	73.2	24.5	2.3
		Control	1178	*91.4*	*8.6*	83.5	15.8	0.7
	China (AC)	T1D	103	0	0	0	0	0
		Control	302	0	0	0	0	0
Zhernakova et al. (28)	Netherlands (C)	T1D	334	82	18	67.7	28.7	3.6
		Control	528	91.3	8.7	83.3	15.9	0.8
Kahles et al. (6)	Germany (C)	T1D	220	80.7	19.3	64.5	32.3	3.2
		Control	239	88.7	11.3	78.2	20.9	0.8
Gomez et al. (20)	Colombia (U)	T1D	110	92	8	85.1	14	0.9
		Control	308	96	4	91.2	8.8	0
Hermann et al. (30)	Finland (C)	T1D	546	*76.2*	*23.8*	57.9	36.6	5.5
		Control	538	*86.1*	*13.9*	74.7	22.7	2.6
Fedetz et al. (11)	Ukraine (C)	T1D	296	78.9	21.1	63.2	31.4	5.4
		Control	242	85.9	14.1	72.8	26.4	0.8
Steck et al. (26)	United States (C)	T1D	690	83.8	16.2	69.9	28	2.2
		Control	515	91	9	82.5	16.9	0.6
Chelala et al. (31)	France (C)	T1D	885	70.4	29.6	*70.39*	*27.46*	*2.15*
		Control	442	82.1	17.9	*82.13*	*16.51*	*1.36*
Santiago et al. (10)	Spain (C)	T1D	316	89.1	10.9	79.7	18.7	1.6
		Control	554	93.3	6.7	87.2	12.3	0.5
Nielsen et al. (29)	Denmark (C)	T1D	253	84	16	71.9	24.1	4.0
		Control	354	90.8	9.2	81.6	18.4	0
Kawasaki et al. (22)	Japan (A)/Korea (A)	T1D	688/69	0	0	0	0	0
		Control	861/111	0	0	0	0	0
Cinek et al. (23)	Czech Republic (C)	T1D	372	*79.2*	*20.8*	*62*	*34*	*3.8*
		Control	400	*89.7*	*10.3*	*80.8*	*18*	*1.2*
	Azerbaijan (Az)	T1D	160	*97.2*	*2.8*	*95*	*4.4*	*0.63*
		Control	271	*99.6*	*0.4*	*99.26*	*0.74*	*0*
Baniasadi and Das (13)	India (AI)	T1D	129	96.5	3.5	93.8	5.4	0.8
		Control	109	97.25	2.75	94.5	5.5	0
Petrone et al. (24)	Italy (C)	T1D	558	89.6	10.4	80.3	18.8	0.9
		Control	545	95.4	4.6	91.1	8.6	0.4
Smyth et al. (27)	UK (C)	T1D	7434	82.2	17.8	67.67	29.12	3.21
		Control	7053	90.4	9.6	81.7	17.44	0.86
Douroudis et al. (12)	Estonia (C)	T1D	170	75	25	58.2	33.5	8.3
		Control	230	86.1	13.9	74.8	22.6	2.6
Dultz et al. (25)	Germany (C)	T1D	70	89.3	10.7	78.6	21.4	0
		Control	100	92	8	86	12	2
Present study	Brazil (C)	T1D	211	85.31	14.69	72.04	26.54	1.42
		Control	241	94.81	5.19	89.63	10.37	0

A, Asian; AI, Asian Indians; Az, Azeri; C, Caucasoid; PTPN22, protein tyrosine phosphatase nonreceptor 22; T1D, type 1 diabetes; U, unknown.
[a]Numbers in italic were calculated based on informations from the studies.

The association of the 1858T allele with several other autoimmune diseases suggests a disruption in the immunological tolerance mechanism. As the 620W variant seems to suppress the TCR signaling cascade, it can affect the selection of T cells during the thymic development resulting on the escape of autoreactive T cells to the periphery (32).

We report here for the first time an association of the 1585T allele and type 1 diabetes in a Brazilian population, stressing

the possibility that this polymorphism may confer increased risk for developing autoimmune diabetes.

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Association of killer cell immunoglobulin-like receptors and human leukocyte antigen–C genotypes in South Brazilian with type 1 diabetes

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ABSTRACT

Type 1 diabetes mellitus (T1D) is a multifactorial and chronic autoimmune disease caused by the deficiency of insulin synthesis and or by its secretion or action defects. Genetic and environmental factors are known to be involved in its pathogenesis. The human leukocyte antigen complex (human leukocyte antigen (HLA)) constitutes the most relevant region contributing with 50% of the inherited risk for T1D. Natural killer cells (NK) are part of the innate immune system recognizing class I HLA molecules on target cells through their membrane receptors, called killer immunoglobulin-like receptors (KIR). The aim of our study is to evaluate the association between the KIR genes and HLA alleles in patients with T1D and healthy controls. Two hundred forty-eight T1D patients and 250 healthy controls were typed for HLA and KIR genes by PCR-SSP. Our results showed an increase of C2 in controls ($p = 0.002$). The genotype 2DL1/C2+ was also more common in controls ($p = 0.001$), as well as haplotype association KIR2DL2/DR3/DR4+ and the combination with only DR3+ ($p < 0.001$; $p < 0.001$). The maximum protection was seen when KIR2DL2/DR3-were absent when the combination of KIR2DL1/C2+ were present ($p < 0.001$) and the maximum risk was observed when KIR2DL2/DR3/DR4+ were present in the absence of KIR2DL1/C2- ($p = 0.005$). Our results confirmed the association of the KIR2DL2/DR3 increasing risk for T1D and suggest a protective role of KIR2DL1/C2.

1. Introduction

Type 1 diabetes mellitus (T1D) is an autoimmune disease in which insulin-producing pancreatic beta cells are destroyed by an aberrant T-cell mediated immune response [1]. The presence of antibodies to insulin, glutamic acid decarboxylase (GADA), tyrosine phosphatase IA-2 and, more recently, the zinc transporter (ZnT8) have been found in individuals at risk or who have recently developed T1D [2–7].

T1D is a multifactorial and polygenic disease, in which the concordance rate in twins is approximately 30–50%, demonstrating that genetic susceptibility is relevant to its etiology. One of the most important genetic factors known to be involved in the autoimmune pathogenesis of this disease is the human leukocyte antigen (HLA) [8,9]. HLA class II DR4 and DR3 were shown to be associated with the development of T1D, whereas the combination of the two susceptible alleles, DR3 and DR4 together, conferred a higher risk [10].

HLA class I molecules are recognized by natural killer (NK) cells through killer immunoglobulin-like receptors (KIR). Inhibitory KIR molecules bind to target cell HLA class I molecules and prevent the attack of NK cells on normal cells [11]. When an activating KIR binds to its ligand, activating signals are generated leading to the destruction of target cells [12].

To date, 17 KIR genes and pseudogenes have been described on human chromosome 19q13.4 [13]. Eight KIR receptors are inhibitory (2DL1, 2DL2, 2DL3, 2DL5A, 2DL5B 3DL1, 3DL2 and 3DL3), seven are activating (2DL4, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 2DS5 and 3DS1), and two are pseudogenes (2DP1 and 3DP1). Of these, four KIR genes (3DL3, 3DP1, 2DL4, 3DL2) are always present and are considered framework genes [14,15].

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Based on the dimorphism in position 80 (epitope for KIR binding), all HLA-C alleles can be divided into two groups: the C1 group carrying asparagine, and the C2 group carrying lysine at this position. The C1 group consists of HLA-Cw1, -Cw3, -Cw7, -Cw8, -Cw13, -Cw14. The C2 group consists of HLA-Cw2, -Cw4, -Cw5, -Cw6, -Cw17, -Cw 18. KIR2DL2, 2DL3 and 2DS2 bind HLA-C1 ligands, whereas KIR2DL1 and 2DS1 bind HLA-C2 ligands. The inhibitory KIR3DL1 recognizes HLA-B Bw4 allotypes and KIR3DL2 binds HLA-A3 and HLA-A11 [16]. However, the HLA ligands for several KIR genes are not yet identified.

This difference in the HLA-C generates different interactions with the KIR receptors. The KIR-HLA interaction is differentiated by the intensity of the connection and similarity between the receptors and their ligands. The receptors are connected weakly to antigens of group C1 and strongly in the group C2 and the inhibitory receptor has more affinity to the HLA receptors [17].

Because of KIR specificity for HLA class I allotypes, and their extensive polymorphisms, it is reasonable to imagine that KIR gene variation affects resistance and susceptibility to several diseases. KIR genotypes and HLA ligand patterns have been recognized for diseases and disease conditions such as hepatitis C [18], psoriasis vulgaris [19,20], psoriatic arthritis [21,22], rheumatoid arthritis [23], celiac disease [24], ulcerative colitis [25,26], Crohn's disease [27], human immunodeficiency virus [28], recurrent miscarriage [29], and leprosy [30], as well as in T1D [31–39].

In the present study, we examined 15 KIR genes and HLA ligands in a group of 248 T1D patients and compared them with findings in 250 healthy controls, aiming at the identification of patterns of KIR genotypes and HLA ligands that could be more clearly associated with susceptibility to this disease. To the best of our knowledge, this is the first study of KIR genes in a Brazilian Caucasian population with T1D.

2. Subjects and methods

2.1. Patients

To analyze the combination of KIR genotypes and HLA-C ligands, we studied 248 T1D Causasian children and adolescents from Hospital Nossa Senhora da CONCEIÇÃO, 0–18 years of age, and 250 unrelated, healthy, gender- and geography-matched controls, from Hospital de Clínicas de Porto Alegre, Brazil. The diagnosis was based on the consensus on T1D published by the Expert Committee on the Diagnosis for the Classification of Diabetes Mellitus [40].

This study was approved by the Research Ethics Board of Hospital de Clínicas de Porto Alegre (IRB0000921) and all parents signed an informed consent for participating in this study.

2.2. Methods

Blood samples were collected into tubes containing ethylenediaminetetraacetic acid (EDTA). DNA was extracted using a salting-out procedure [41]. DNA samples were genotyped using polymer chain reaction–single-strand polymorphism (PCR-SSP) for 15 KIR genes (2DS1, 2DS2, 2DS3, 2DS5, 3DS1, 2DS4, 2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1, 3DL2, 3DL3, 2DP1). The PCR primers and conditions were based on previous reports [42]. Internal control was included in each PCR reaction. The combination used to achieve a 10-μl volume reaction was 10 ng of genomic DNA, 500 nM of specific primers, 2.5 U of Taq polymerase, 0.08 μl of PCR buffer, 0.3 μl of MgCl, and 10 μl of distilled water, which was amplified by the Gene Amp PCR system 9700 (Perkin Elmer, Norwalk, CT).

Temperature cycling conditions for PCR reaction were as follows: denaturation for 3 minutes at 94°C, followed by four cycles of 15 seconds at 94°C, 15 seconds at 65°C, 15 seconds at 72°C; 21 cycles of 15 seconds at 94°C, 15 seconds at 60°C, and 30 seconds at 72°C; five cycles of 15 seconds at 94°C, 1 minute at 55°C, 2 minutes at 72°C, and a final elongation step at 72°C for 7 minutes. Resulting products were visualized under ultraviolet light after electrophoresis in 1% agarose gels containing ethidium bromide.

HLA typing Cw epitope C1 (Cw 01, 03, 07 {01-06}, 08, 12 {02, 03, 06}, 14, 16 {01, 03, 04}, and C2 (Cw 02, 04, 05, 06, 0707, 12 {04, 05}, 15, 1602, 17, 18) was done using PCR-SSP, as described by Jones et al., 2006 [25]. HLA-Bw4 was also done using PCR-SSP described by Bunce et al. [43]. HLA-DR was typed by polymerase chain reaction with sequence-specific oligonucleotides (PCR-SSO) (LABType SSO; One Lambda, Canoga Park, CA).

2.3. Statistical analysis

Comparison of the KIR gene frequency with the control group was executed by Pearson χ^2 with continuity correction and in a few, where the expected difference between the two groups was small, Fisher's exact test was employed. Odds ratios (OR), confidence intervals, (95% CI), and significance values ($p < 0.05$) were calculated using SPSS for Windows version 16.0 (SPSS Inc., Chicago, IL). The number of genes used was adjusted for with the Bonferroni correction.

3. Results

Individual gene frequencies for the 15 tested KIR loci are shown in Table 1. The frequencies of the KIR genes in our control group were similar to those in other studies of Brazilian populations [44–46]. The framework genes KIR2DL4, KIR3DL2, and KIR3DL3 were present in all individuals, as expected, and every individual also carried either one or both of KIR3DL1/KIR3DS1 and KIR2DL2/KIR2DL3, which segregate as alleles at the same locus.

Overall, there were no significant differences in the frequency of any of the 15 KIR genes in the patient cohort compared with the control group. HLA-C group 2 were increased in controls when compared with T1D patients ($p = 0.002$). No significant differences in HLA-C group 1 and Bw4 frequencies were detected between T1D patients and controls.

The status of activating genes KIR2DS1 and KIR2DS2 were analyzed in conjunction with the presence of their HLA-C ligand (Table 2). However, no significant differences were observed. When we analyzed KIR2DL1/C2 ligand, we found a protective factor for T1D when compared with controls ($p = 0.001$). There was no associa-

Table 1
KIR gene frequencies (%) in healthy unrelated individuals ($n = 250$) and T1D patients ($n = 248$)

KIR gene	Controls		T1D patients		p Value[a]
	N	%	*N*	%	
2DL1	244	97.6	237	95.6	NS
2DL2	136	54.4	122	49.2	NS
2DL3	216	86.4	218	87.9	NS
2DL4	250	100.0	246	99.2	NS
2DL5	124	49.6	139	56.0	NS
3DL1	244	97.6	236	95.2	NS
3DL2	250	100.0	248	100.0	NS
3DL3	250	100.0	248	100.0	NS
2DS1	91	36.4	115	46.4	NS
2DS2	134	53.6	131	52.8	NS
2DS3	83	33.2	84	33.9	NS
2DS4	238	95.2	236	85.2	NS
3DS1	106	42.4	118	47.6	NS
2DP1	250	100.0	248	100.0	NS
2DS5	85	34.0	92	37.1	NS
Bw4	171	68.4	188	75.8	NS
C1	180	72.0	186	76.2	NS
C2	179	71.6	144	58.4	0.002

T1D, type 1 diabetes mellitus.
C1 group: HLA-Ow 01, 03, 07 (01–06), 08, 12 (02, 03, 06), 14, 16 (01, 03, 04). C2 group: HLA-Ow 02, 04, 05, 06, 0707, 12 (04, 05), 15, 1602, 17, 18. Bw4: HLA-B08, 13, 27, 44, 51, 52, 53, 57, 58.
[a]Chi-Square Test or Ficher's exact test with Bonferroni correction.

Table 2
KIR combinations and HLA ligands frequencies in healthy controls ($n = 250$) and T1D patients ($n = 248$)

	Controls		T1D patients		p Value[a]
	n	(%)	n	(%)	
2DL2/C1	102	(40.8)	94	(37.9)	NS
2DS2/C1	100	(40.0)	101	(40.9)	NS
2DL3/C1	153	(61.2)	165	(67.3)	NS
3DL1/Bw4	166	(66.4)	179	(72.2)	NS
3DS1/Bw4	67	(26.8)	91	(36.7)	0.018
2DL1/C2	176	(70.4)	137	(55.6)	0.001
2DS1/C2	63	(25.2)	67	(27.1)	NS

T1D, type 1 diabetes mellitus.
C1 group: HLA-Cw 01, 03, 07 (01–06), 08, 12 (02, 03, 06), 14, 16 (01, 03, 04). C2 group: HLA-Cw 02, 04, 05, 06, 0707, 12 (04, 05), 15, 1602, 17, 18. Bw 4: HLA-B 08, 13, 27, 44, 51, 52, 53, 57, 58.
[a]Chi-square test.

tion of T1D and other combinations of inhibitory KIR genes and corresponding ligands (Table 2).

With the intention of observing how KIR genes affect the disease, we stratified our patients and controls for the presence of KIR2DL2 with HLA class II alleles HLA-DR3, -DR4, and -DR3/DR4 in both groups (Table 3). We found that individuals carrying KIR2DL2 gene together with HLA class II alleles were more likely to be patients than controls, with the exception of HLA DR4, which showed no significant association. The gene HLA DR3 associated with KIR2DL2 was increased in patients when compared with controls, these difference reaching statistical significance ($p < 0.001$). When we analyzed the patients by HLA haplotype DR3/DR4, the association was even greater, showing a higher risk for T1D ($p < 0.001$).

A further analysis was made between the different combinations of inhibitory KIR2DL1 with their corresponding HLA-C ligand and KIR2DL2 with high-risk for T1D HLA class II alleles (Table 3). The reason for making this association was to explain the effect of genetic variation at the KIR locus and its ligand in combination with other genes which show disease susceptibility. The presence of activated KIR2DL1/C2+ in the absence of KIR2DL2/DR3, as well as KIR2DL2/DR3/DR4, was increased in controls, conferring protection for T1D ($p < 0.001$; $p < 0.001$). By contrast, when the combination KIR2DL1/C2-with the 2DL2/DR3/DR4+ and 2DL2/DR3+ was present, we found a higher risk for T1D ($p = 0.005$; $p = 0.005$).

4. Discussion

A report from Van der Slik et al. [31] showed that the combination of the activating KIR2DS2 gene, together with its putative HLA ligand, was present more frequently in T1D patients than in controls ($p = 0.030$). Shastry et al. [38] found susceptibility for T1D in the presence of KIR2DL2-C1 and the absence of 2DS1, 2DS2 ($p < 0.001$), whereas Middleton et al. [33] found that KIR2DS5 was significantly decreased in patients versus controls ($p = 0.043$). A study by Santin et al. [35] observed no association between the KIR gene content and susceptibility to T1D. Park et al. [34]. found an association in group A KIR haplotypes.

In a report published on Japanese T1D patients, the authors did not detect any difference in KIR gene frequencies between patients and controls [37]. Still, their results suggest that certain combination of KIR genes might be associated with age at onset of the disease.

Our study failed to identify any association of susceptibility to disease, although we found a protective factor for inhibitory KIR2DL1 and its C2 ligand ($p = 0.001$). When considering only the KIR2DL1 receptor without its respective ligand, statistical significance was not found. HLA and KIR interaction occurs through the innate immune response. This system is the first line of defense against pathogens, working to recognize common components of pathogens so that further immune responses can be signaled in the presence of foreign pathogens. The innate system uses multiple cell types, including macrophages, dendritic cells, NK cells, neutrophils, and epithelial cells, each of which has its own specific function in an innate response. NK cells are involved in destroying target cells, as well as interacting with antigen presenting cells and T-cells [47]. Although a reduced activation of NK cells has been reported in long standing type 1 diabetes [48], it is unclear whether this alteration is a consequence rather than a cause of disease, as prolonged hyperglycemia could also explain this phenomenon.

Other studies suggest that the balance between innate and acquired immunity is important, so that an imbalance could lead to T1D. Nikitina-Zake et al. [32] found combined association of MICA4 and KIR2DL2. The same author noted maximum risk when KIR2DL2 and DR3/DR4 were together. When analyzing the combination KIR2DL2 and HLA DR3, we also found a risk associated with the disease, but we did not find association between KIR2DL2 and HLA DR4. Furthermore, the combination of inhibitory receptor KIR2DL2 in the presence of haplotype DR3/DR4 was highly significant, leading to greater susceptibility to T1D.

Our study found a strong protective factor for gene 2DL1 with its C2 ligand and inhibitory 2DL2 conferred a high risk when associated with HLA-DR3/DR4. We observed that this combination has a greater protective factor in the absence of 2DL2 concurrently with HLA DR3 ($p < 0.001$) and the haplotype DR3/DR4 ($p < 0.001$). Furthermore, when the 2DL2 with HLA DR3 and DR4 are present, the combination 2DL1/C2 failed to protect, and individuals have certain susceptibility for disease. However, when the patients have

Table 3
KIR and HLA ligands in controls ($n = 250$) and T1D patients ($n = 248$)

	Controls		T1D patients		p Value[a]	OR	95% CI
	(n/x)	%	(n/x)	%			
KIR2DL2+							
DR3	(14/53)	26.4	(39/53)	73.6	<0.001	7.76	3.02–20.21
DR4	(31/68)	45.6	(37/68)	54.4	NS	–	–
DR3/DR4	(5/30)	16.7	(25/30)	83.3	<0.001	25.0	5.52–122.42
KIR2DL1/C2−							
KIR2DL2/DR3+	(3/17)	17.6	(14/17)	82.4	0.005	21.78	2.98–183.40
KIR2DL2/DR3/DR4+	(1/11)	9.1	(10/11)	90.9	0.005	100.0	4.1–4743.9
KIR2DL1/C2+							
KIR2DL2/DR3+	(11/36)	30.6	(25/36)	69.4	0.008	5.17	1.7–15.94
KIR2DL2/DR3/DR4+	(4/19)	21.1	(15/19)	78.9	0.008	14.06	2.42–89.89
KIR2DL2/DR3−	(148/221)	67.0	(73/221)	33.0	<0.001	0.24	0.16–0.36
KIR2DL2/DR3/DR4−	(155/238)	65.1	(83/238)	34.9	<0.001	0.29	0.19–0.42

T1D, type 1 diabetes; OR, odds ratio; 95% CI, 95% confidence interval; n, positive; x, total.
[a]Chi-square test or Fisher exact test.

an absence of 2DL1/C2 but showed KIR2DL2 with HLA haplotype DR3/DR4, the susceptibility for disease were higher. Individuals who did not have this haplotype, but showed only the DR3 also had a high risk factor.

Recently, Ramos-Lopez et al. [39] investigated the rs2756923 polymorphism (G/A), which according to the location within the KIR2DL2 gene conferred susceptibility to T1D in a series of individuals from Germany and Belgium. The investigators observed that genotype GG was more frequent in T1D than in healthy controls. This mutation in the receptor KIR2DL2 might explain the difference found between our study and others.

Collectively, the above-mentioned results, as well as those obtained by other groups, suggest that various genetic factors could be of importance for the development of T1D. Furthermore, several polymorphisms in a given individual may contribute to the individual risk of developing the disease. Our data, combined with other reports, points to a significant association of the KIR gene system with T1D, suggesting that KIR genes may have a pathogenic role in this disease.

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CONCLUSÕES GERAIS

- MSCs podem ser isoladas *in vitro* a partir de ilhotas pancreáticas. Essas células possuem marcadores característicos de MSCs, sendo capazes de proliferar e diferenciar *in vitro* em osteoblastos. As células não apresentaram capacidade de diferenciação adipogênica. Aplicando quatro protocolos de diferenciação em células-produtoras de insulina *in vitro* em hMSCs-derivadas de ilhotas e um protocolo em mMSCs-derivadas de rim, os níveis de expressão de insulina foram nulos ou insignificantes, mostrando uma baixa capacidade de difenciação dessas células em um fenótipo de células β pancreáticas.
- O co-transplante singenéico de MSCs derivadas de rim murino com ilhotas pancreáticas em camundongos diabéticos demonstram melhora dos níveis glicêmicos, aumentando a taxa de reversão/cura nos animais. Não foram observadas diferenças significativas nos níveis de glucagon, somatostatina e insulina, embora pareça haver uma tendência ao aumento de insulina em camundongos que receberam ilhotas + MSCs. Camundongos co-transplantados com MSCs apresentaram aumento na vascularização do transplante, sendo um dos possíveis motivos para as diferenças observadas entre os grupos. Não foram observadas diferenças no peso e capacidade de resposta à glicose entre os dois grupos. Esses resultados demontram o potencial de MSCs no co-transplante de ilhotas pancreáticas.
- A análise do polimorfismo C1858T no gene PTPPN22 mostrou associacão entre o alelo 1858T e o risco aumentado do desenvolvimento do DM1 numa população de pacientes do Rio Grande do Sul, corroborando resultados obtidos em outras populações ao redor do mundo.
- A genotipagem do sistema polimórfico de receptores de superfície KIR de células *natural killer*, não mostrou diferença nas frequências quando comparado com o grupo controle. A análise conjunta com alelos de HLA classe I (ligantes do KIR) mostrou aumento na frequência de alelos de HLA-C do grupo 2 (C2) em pacientes controles, bem como uma proteção ao DM1 conferida pelo KIR2DL1/C2. A análise conjunta com alelos de HLA classe II também mostrou risco aumentado do desenvolvimento de DM1 em pacientes KIR2DL2+DR3 e KIR2DL2+DR3/DR4.

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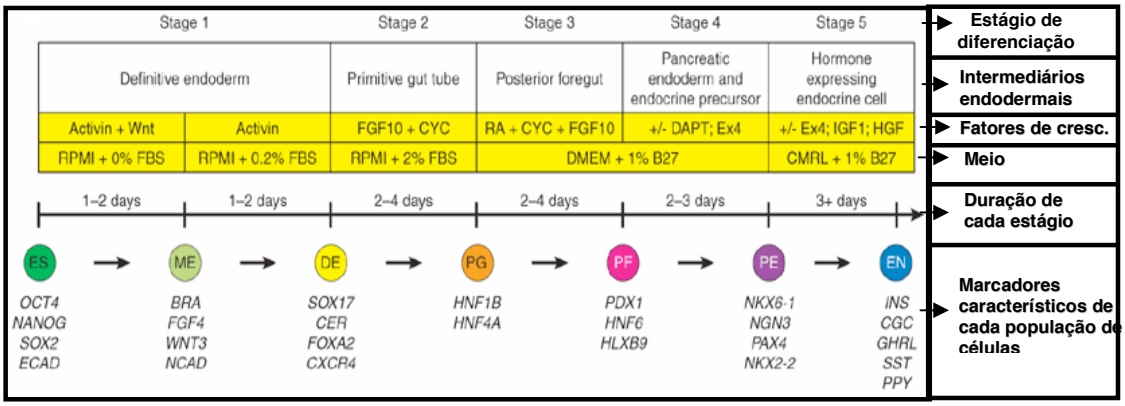
Anexo 1. Detalhamento do Protocolo Novocell testado para diferenciação de células-tronco embrionárias em células produtoras de insulina. (retirado de D'Amour et al., 2006)



Anexo 2. Lista de reagentes utilizados para diferenciação em células produtoras de insulina.

Fatores

- Activina A humana recombinante - *R&D Systems*
- Soro Fetal Bovino - *Hyclone*
- B27 suplemento - *Invitrogen*
- Wnt-3a murino recombinante - *R&D Systems*
- FGF10 humano recombinante - *R&D Systems*
- KAAD-ciclopamina - *Toronto research chemicals*
- Ácido all-trans retinóico - *Sigma*
- DAPT - *Sigma*
- Exendina-4 (50ng/ml): *Sigma*
- IGF-1 (50ng/ml): *Sigma*
- HGF humano recombinante (50ng/ml): *Sigma*
- Betacelulina - *R&D Systems*
- Nicotinamida - *Sigma*
- ITS 100x – *Sigma*
- Taurina – *Sigma*
- Aminoácidos não-essenciais - *Invitrogen*
- dibutyryl-cyclic AMP – *Sigma*

Meios de cultura

- RPMI 1640 - *Sigma*
- DMEM - *Hyclone*
- CMRL 1066 - *Invitrogen*

Anexo 3. Lista de primers utilizados no trabalho de diferenciação de células-tronco mesenquimais em células-produtoras de insulina.

Gene	Primer Direto 5'-3'	Primer Reverso 5'-3'	Tamanho Produto (bp)
Humanos			
INS	AGGCTTCTTCTACACACCCAAG	CACAATGCCACGCTTCTG	139
SOX17	GGCGCAGCAGAATCCAGA	CCACGACTTGCCCAGCAT	61
PDX1	AAGTCTACCAAAGCTCACGCG	GTAGGCGCCGCCTGC	51
PAX4	GGGTCTGGTTTTCCAACAGAAG	TCAGCCCCTGGGAAGCA	90
NKX2.2	GGCCTTCAGTACTCCCTGCA	GGGACTTGGAGCTTGAGTCCT	67
OCT4	TGGGCTCGAGAAGGATGTG	GCATAGTCGCTGCTTGATCG	78
ACTB	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAACGCA	140
Murino			
Ins1	AATCAGAGACCATCAGCAAGC	GGGACCACAAAGATGCTGTT	136