UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA

MESENCHYMAL STEM CELLS FOR THE CELL-BASED THERAPY OF TYPE 1 DIABETES

AN EXPERIMENTAL APPROACH

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DOUTORAMENTO EM MEDICINA

Fisiopatologia

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DOUTORAMENTO EM MEDICINA (Fisiopatologia)

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SUMÁRIO[1]

As células estaminais adultas obtidas a partir do estroma de tecidos humanos, representam uma fonte celular potencialmente atractiva para a terapia celular da Diabetes Mellitus. Todavia, não existem ainda resultados definitivos que permitam a sua aplicação clínica.

Objectivos

1. Avaliação do potencial de diferenciação endócrina das células estaminais mesenquimatosas humanas, imortalizadas pela telomerase (hMSC-TERT);

2. Análise comparativa da plasticidade e assinatura molecular de células humanas derivadas do ilhéu pancreático (hIPC) e células estaminais mesenquimatosas derivadas da medula óssea (hBM-MSC).

Estratégias de investigação

1. Diferenciar *in vitro* as hMSC-TERT em células produtoras de insulina através de:

   a) Cultura em meio específico pró-endócrino;

   b) Expressão ectópica de dois genes reguladores do pâncreas endócrino humano, neurogenina3 (hNGN3) e pancreatic-duodenal homeobox 1 (hPDX-1) nas células hMSC-TERT.

2. Comparar a assinatura molecular funcional das hIPC e hBM-MSC por:

   a) Caracterização do fenotipo imunológico;

[1] Resumo até 300 palavras, de acordo com o artigo 40º do DR nº153, II série de 5 Julho de 2003 que regulamenta a elaboração das teses de doutoramento.

b) Diferenciação *in vitro* das hIPC e hBM-MSC em linhagens mesenquimatosas e endócrinas;

c) Análise transcripcional de marcadores do mesênquima e genes associados ao carácter estaminal. Identificação de genes-alvo relacionados com a origem e função das hIPC e hBM-MSC.

Resultados

As hMSC-TERT são diferenciáveis em fenótipos do pâncreas endócrino com capacidade de síntese de insulina. Tal como ocorre na pancreatogénese, a expressão de NGN3 contribui para a activação do PDX1 nas hMSC-TERT. A ausência de resposta insulínica à glicose indica que as hMSC-TERT modificadas não atingiram a maturidade funcional de células-beta nativas;

As hIPC e hBM-MSC apresentam características de MSC, tendo todavia, funções tecidulares distintas que reflectem diferenças moleculares relacionadas com a "especificidade mesodérmica" e "memória posicional" destas células. As hIPC, encontram-se num estado de transição epitelial-mesenquimatosa .

Conclusões

As populações MSC, são células estaminais adultas com níveis distintos de plasticidade e compromisso tecidular. Estudos futuros permitirão identificar as MSC com maior potencial para substituição ou regeneração de células beta na *Diabetes tipo 1.*

SUMMARY[2]

Human adult mesenchymal stem cells (hMSC) represent a potential source for cell-based therapies in Diabetes Mellitus. Yet, till now, no definitive data exist for clinical application.

Aims

1. Evaluation of the pancreatic endocrine differentiation potential of human-mesenchymal stem-cells, immortalized with telomerase (hMSC-TERT);

2. Comparative analysis of the plasticity and molecular signature of human-islet-derived pancreatic cells (hIPC) and bone-marrow-derived hMSC (hBM-MSC).

Research strategies:

1. In vitro differentiation of hMSC-TERT into insulin producing cells through:

a) Endocrine-promoting culture conditions;

b) Ectopic expression of two key regulatory genes of human endocrine pancreas, neurogenin3 (*hNGN3*) and pancreatic-duodenal homeobox 1 (*/hPDX-1*) genes in hMSC-TERT.

2. Comparison of the functional signature of hIPC and hBM-MSC by:

a) Immunophenotype characterization;

b) *In vitro* mesenchymal and endocrine differentiation of primary hBM-MSC and hIPC;

[2] Abstract up to 300 words, in accordance with the 40th article of DR nº153, II series of 5th of July 2003, which regulates PhD thesis elaboration.

c) Transcriptome analysis for detection of mesenchymal and adult stem-cell related gene markers. Identification of target genes, related to the origin and function of hBM-MSC and hIPC.

Results

We could demonstrate that hMSC-TERT can be differentiated *in vitro* into pancreatic endocrine phenotypes with the ability to synthesise and store insulin. Similar to pancreatic development, in hMSC-TERT, NGN3 contributes to the activation of PDX-1 promoter. Insulin secretion in a glucose-sensing manner was not detected in our cell system, which indicates that a mature state of beta-cell was not attained in these phenotypes;

hIPC and hBM-MSC display very similar MSC phenotypes. However, our data suggest distinct functions of these populations, which are related to a "mesodermal tissue specification" and "positional memory" of these cells. In hIPC, we confirmed a state of epithelial-mesenchymal transition.

Conclusions

MSC populations are adult stem cells with distinct levels of plasticity and commitment. Future studies will help to identify those MSC populations with greater potential to replace or regenerate beta cells in *type 1 Diabetes*.

PALAVRAS CHAVE

Diabetes Mellitus tipo 1, Terapia Celular da Diabetes, Células Progenitoras/Estaminais Adultas, Células Estaminais Mesenquimatosas

KEYWORDS

Type 1 Diabetes Mellitus, Cell-based therapy, Adult progenitor/stem cells, Mesenchymal stem cells

ÍNDICE

PREFÁCIO

A prática da Medicina sempre foi uma actividade em constante evolução e envolta de interrogações. Os doentes questionam acerca da doença, das suas causas, do tratamento e da existência ou não de cura. Os médicos, acerca do diagnóstico e das atitudes terapêuticas mais adequadas. Apesar das soluções que decorrem do conhecimento actualizado, da experiência e do senso clínico, a verdade é que para muitas perguntas não existem ainda respostas. Este facto justifica uma curiosidade constante na busca do conhecimento mais actual e consequentemente dos avanços da Ciência.

Interroguei-me muitas vezes, se os médicos têm um papel devidamente relevante nos caminhos da investigação clínica e básica. Em particular, se esta última deverá ser uma área estritamente reservada a biólogos, químicos e outros profissionais das ciências básicas.

Na verdade acredito que o treino diário do raciocínio clínico, baseado no relacionamento/associação de ideias, bem como a actualização do conhecimento através de bibliografia, palestras ou discussões com colegas, são ferramentas ideais para colocar as questões científicas mais adequadas e urgentes.

Em muitos países a prátca médica corre a par com a investigação científica básica, tendo os médicos um papel decisivo nas linhas de investigação emergentes.

Todas estas inquietações, associadas ao gosto pela pesquisa e uma curiosidade nunca satisfeita, conduziram-me, já numa fase sólida da carreira como Pediatra dedicada à Endocrinologia e Diabetologia Infantil, a investir na ciência directamente aplicada à Medicina.

A ideia motivadora foi a de que a *Diabetes tipo 1* (uma doença mortal até à descoberta da insulina em 1921) possa, de facto, vir a ter cura.

Tal como em muitas outras doenças degenerativas, as estratégias terapêuticas baseadas na substituição ou regeneração das células afectadas parecem ter um futuro prometedor na cura da *Diabetes Mellitus*.

De facto, a terapia celular da Diabetes é já uma realidade através dos transplantes de ilhéus pancreáticos humanos. Contudo, o método é ainda ineficaz, a fonte celular humana é limitada e até hoje, a obtenção da cura definitiva ainda não foi possível.

O objectivo último deste trabalho experimental foi investigar outras fontes celulares passíveis de substituir a função do pâncreas endócrino lesado que pudessem ser transplantadas em doentes com *Diabetes tipo1,* de modo a atingir a independência insulínica.

Este trajecto, árduo mas fascinante, tornou possível compreender melhor a complexidade molecular e funcional da *célula beta* do pâncreas. Permitiu aprofundar conceitos sobre biologia do desenvolvimento. Estimulou a aquisição de conhecimentos recentes no campo da biologia dos tecidos adultos, no que respeita a vias de sinalização de morte e proliferação celular. Resultou, por fim, na identificação de células estaminais / progenitoras com hierarquias de pluripotência, níveis de plasticidade e assinaturas moleculares distintas.

Ainda que os resultados obtidos fiquem sempre aquém dos objectivos idealizados reconheço, no entanto, o valor inestimável dos conhecimentos adquiridos, nomeadamente: conceitos báscos de biologia molecular, genética e química; métodos laboratoriais avançados; formulação de hipóteses científicas; concepção de projectos de investigação e elaboração de artigos para revistas.

Foi estimulante deparar, nesta área de investigação, com um nível deveras elevado de competitividade que obriga a ideias muitissimo inovadoras, eficiência no trabalho

experimental e rapidez na publicação dos resultados. Reunir estas condições não foi tarefa fácil para quem, como eu, estava no início desta actividade.

Os resultados e conclusões de três anos de trabalho, exclusivamente laboratorial e as publicações daí resultantes, são o objecto desta tese de doutoramento.

Como bolseira, devo um agradecimento à Sociedade Europeia de Endocrinologia Pediátrica, pela possiblidade de, durante dois anos, ter podido desenvolver as ideias e linhas de investigação necessárias para este projecto, num centro europeu conceituado. Devo o subsequente suporte para prosseguir os trabalhos, publicar os resultados e defender esta tese de doutoramento, à Fundação para a Ciência e Tecnologia, através de uma "Bolsa Individual de Doutoramento".

RESUMO

Introdução

A Diabetes Mellitus é uma doença de elevada e crescente incidência com um grande impacto socioeconómico. As terapêuticas actualmente disponíveis, destinadas à normalização da glicémia, se bem que razoavelmente eficazes e largamente utilizadas, são exclusivamente sintomáticas. Surge pois a necessidade de actuar no *primum movens* da doença, com uma finalidade curativa, ou seja, regenerando ou substituindo as células lesadas dos ilhéus de Langerhans.

As estratégias baseadas na terapêutica celular são abordagens promissoras que visam a cura da Diabetes tipo 1 (DT1) bem como de formas avançadas de Diabetes tipo 2 (DT2). Revelam, no entanto limitações; o transplante de ilhéus pancreáticos, por exemplo, tem tido um alcance limitado devido não só à escassez de orgãos de dadores mas também por implicar uma imunosupressão prolongada. A investigação na área das células estaminais poderá permitir ultrapassar estes problemas quer pela geração de células produtoras de insulina passíveis de ser transplantadas quer pela regeneração de células beta a partir de células progenitoras existentes no organismo.

Estudos de diferenciação de células estaminais embrionárias e células estaminais adultas em células fenotípicamente semelhantes às células beta do pâncreas endócrino mostraram-se bastante encorajadores. Assim, células estaminais mesenquimatosas (mesenchymal stem/stromal cells (MSC)) adultas obtidas a partir do estroma da medula óssea (hBM-MSC), representam uma possível fonte celular para a repovoação de células insulínicas uma vez que existem em abundância, são fáceis de obter e apresentam baixa imunogenecidade. Por outro lado, foi possível diferenciar determinadas células oriundas dos ductos, ácinos e ilhéus de Langerhans em células

produtoras de insulina. Destas células, têm particular interesse as isoladas a partir do ilhéu pancreático, comummente designadas de células progenitoras derivadas do ilhéu (Islet-derived Progenitor Cells (IPC)). As IPC têm sido alvo de intensa investigação, sobretudo no que respeita à sua origem germinativa (epitelial ou mesenquimatosa) e à sua função nestes tecidos, nomeadamente ao seu carácter progenitor e potencial para substituir ou regenerar as células beta lesadas.

Objectivos

Neste estudo pretendeu-se estabelecer um modelo celular que permitisse avaliar de forma segura e reproduzível a capacidade de diferenciação das hBM-MSC em células produtoras de insulina, ultrapassando os problemas da variabilidade de dadores e da heterogeneidade de populações de MSC primárias.

Um segundo objectivo consistiu em analisar a plasticidade e assinatura molecular das hIPC primárias de modo a determinar o seu potencial de substituição/ regeneração de células insulínicas e compará-las com as hBM-MSC.

Métodos

I. Diferenciação *in vitro* de hMSC-TERT em células produtoras de insulina

a) Análise da linha celular humana de células estromais mesenquimatosas isoladas a partir da medula óssea de um índivíduo adulto saudável e imortalizadas pela telomerase (hMSC-TERT) no que respeita à expressão de dois genes marcadores de células estaminais, *NESTINA* e *C-MET/HGFR*, utilizando técnicas de RT-PCR, citometria de fluxo e imunocitoquímica.

b) Cultura das hMSC-TERT em meio específico pró-endócrino durante 9 dias. Após este período de tempo, procedeu-se à análise da expressão de genes marcadores da diferenciação endócrina através de RT-PCR.

c) Sobreexpressão nas hMSC-TERT de dois genes fundamentais para a diferenciação e funcionamento do pâncreas endócrino *hNGN3* e/ou *hPDX1*, através de técnicas de lipotransfecção, de forma a obter três linhas celulares estáveis, nomeadamente hMSC-TERT-NGN3, hMSC-TERT-PDX1 e hMSC-TERT-NGN3/PDX1. Seguidamente, procedeu-se à análise da expressão de genes específicos do ilhéu incluindo os *PAX4, PAX6, NEUROD1, PDX1, GLUT2, GCK e INSULINA*) através de RT-PCR. A síntese proteica de insulina e péptido-C, foram analisadas nestas linhas celulares por western-blotting e imunocitoquímica.

d) Posteriormente, analisou-se a actividade funcional do *hNGN3 e do PDX1* nas novas linhas celulares utilizando métodos de quantificação da actividade dos genes promotores/activadores do PDX1 e da Insulina, nomeadamente os ensaios SEAP e Luciferase. O conteúdo e secreção de insulina foram avaliados por um método ELISA.

II. Comparação da assinatura molecular funcional das hIPC e hBM-MSC primárias

e) Detecção dos marcadores de superfície hematopoiéticos (CD14, CD34, CD45) e mesenquimatosos (CD29, CD44, CD54, CD73, CD90 e CD105) em três dadores de hIPC (n=3) e e cinco de hBM-MSC (n=5) através de citometria de fluxo.

f) Cultura das hIPC e hBM-MSC em condições indutoras de diferenciação osteogénica, condrogénica e adipogénica de acordo com protocolos standardizados. Paralelamente, procedeu-se à cultura das células hIPC e hBM-MSC em meios de diferenciação endócrina durante 5 dias.

g) Análise de marcadores genéticos do mesênquima (FNT, THY1, SNAI2, P4HA1, MMP2 e VIM) e do pâncreas endócrino (p. ex. PAX4, PAX6, NGN3, NEUROD1, PDX1, GLUT2, GCK e INSULINA) nas duas populações, por RT-

PCR. Detecção da síntese proteica de vimentina e insulina por imunocitoquímica de fluorescência.

h) Determinação do perfil de expressão genómica das hIPC de três dadores e das hBM-MSC de 5 dadores, a partir do RNA total isolado destas amostras e utilizando técnicas de hibridização por microarray, nomeadamente Affymetrix Gene Chips HG-U133 Plus 2.0.

i) Avaliação quantitativa do transcriptoma nos dois grupos de células utilizando o método de análise de significância dos microarrays (significance analysis of microarrays (SAM)). Parte dos resultados obtidos pelos microarrays foi confirmada por RT-PCR.

j) Identificação de genes alvo relacionados com a capacidade de diferenciação endócrina nas hIPC, utilizando a PCR em tempo real (qPCR).

Resultados

I. Diferenciação *in vitro* de hMSC-TERT em células produtoras de insulina

A cultura das hMSC-TERT em meio pró-endócrino, assim como a expressão ectópica dos genes *hNGN3* e *hPDX1* induziram nestas células a expressão de genes anteriormente não expressados e relacionados com o pâncreas endócrino nomeadamente os *PAX4, PAX6 , NEUROD1, INSULINA, GLUT2, GLUCAGON e SOMATOSTATINA*.

A sobreexpressão de *NGN3* nas hMSC-TERT promoveu a expressão de *NEUROD*, *PAX4* e *PDX1*, à semelhança da conhecida cascata de factores de transcrição observada na pancreatogénese. A expressão de *PDX1* foi suficiente para desencadear o processo de diferenciação endócrina nas hMSC-TERT, o que sugere a existência de um "compromisso" endodérmico nesta população de células de origem mesodérmica.

As três novas linhas celulares hMSC-TERT geradas, mostraram capacidade de produção e secreção de insulina. No entanto, em nenhuma delas se detectou uma resposta insulínica sensível à concentração de glucose.

II. Determinação da assinatura molecular funcional das hIPC e hBM-MSC primárias

As células hIPC isoladas a partir do ilhéu pancreático e as células hBM-MSC oriundas da medula óssea, apresentam um fenótipo imunológico sobreponível entre si.

A expressão de genes do *core mesenquimatoso* bem como a de genes associados a um carácter estaminal é significativa, tanto nas hIPC como nas hBM-MSC, sugerindo que ambos os tipos celulares apresentam um fenotipo característico de MSC. Contudo, as hIPC revelaram uma capacidade de diferenciação mesenquimatosa (osso, cartilagem e tecido adiposo) reduzida. As hBM-MSC por sua vez, mostraram uma capacidade de diferenciação no sentido endócrino algo rudimentar.

A partir dos dados obtidos por microarray, procedeu-se a uma análise genética *in silico* (utilizando programas informáticos) dos grupos ontológicos importantes que veio confirmar o envolvimento das duas populações hIPC e hBM-MSC em processos biológicos distintos. Detectaram-se também diferenças significativas entre os dois tipos de células no que respeita ao padrão de expressão genética das cadherinas e dos factores de transcrição relacionados com as vias de diferenciação mesodérmicas e endodérmicas nomeadamente a família de genes *HOX* , os genes *SOX17* e *ISL-1*.

Nas hIPC, em particular, verificou-se uma expressão significativa de genes relacionados com um estado de transição funcional celular (ex. genes *TCF21*, *TGFb*) que não se encontrou nas hBM-MSC.

Conclusões

Concluí-se que nas hMSC-TERT é possível induzir a activação de genes-chave do pâncreas endócrino, bem como a produção e secreção de insulina. Esta linha celular humana constitui assim um modelo adequado para a investigação de células mesenquimatosas derivadas da MO (hBM-MSC) como fonte de células-alternativas para substituição ou regeneração de células beta. No entanto, para que estas hMSC possam vir a ser utilizadas na terapêutica celular da diabetes será necessário induzir um estado mais avançado de maturação endócrina.

O segundo estudo revelou que as hIPC são células com carácter mesenquimatoso oriundas da mesoderme mas que apresentam um fenótipo específico relacionado com o tecido em que residem, o ilhéu pancreático. Além do mais, estas células encontram-se num estado evidente de transição seja epitelio-mesenquimatosa (EMT) ou mesenquimatosa- epitelial (MET) que corresponde a um estadio intermédio de diferenciação e as coloca numa posição priveligiada de "stand-by".

Será pois importante prosseguir este estudo de modo a compreender 1) se as hIPC são essenciais para a regeneração do ilhéu e 2) se é possível completar o seu estado de transição para um fenotipo endócrino maturo.

Os nossos estudos acrescentaram novos conhecimentos relativamente às características moleculares das populações MSC presentes em diferentes tecidos e à sua potencial aplicação em estratégias de terapêutica celular da DT1.

ABSTRACT

Background

Diabetes Mellitus is a disease of increasing incidence, with important socioeconomic impact. So far, all available therapies have been directed to normalize glycemic level. Although effective, they only minimize symptoms, as well as preventing or delaying long-term vascular complications .Therefore, new approaches acting at the *primum movens* of the disease urge to be developed.

Cell-based strategies such as pancreatic islet transplantation hold the promise to cure Type1 diabetes (T1D) and advanced type 2 diabetes (T2D). However, this approach is severely limited by shortage of donor organs and the need of life-long immunosuppression. Stem-cell research might overcome this problem by generation of transplantable insulin-producing cells or regeneration of mature beta-cells. Search for *in vitro* differentiation of embryonic and adult stem cells into insulin phenotypes led to promising results.

Adult mesenchymal stem cells (MSC) obtained from the bone marrow (hBM-MSC) represent an attractive potential source for beta-cell replacement since they are abundantly available and low immunogenic. Possible pancreatic progenitor/stem cells isolated from the ducts, acini and islet of Langerhans have been described to differentiate into insulin producing phenotypes. Particularly, islet derived cells, commonly designated islet derived progenitor cells (IPC) have been under intensive investigation regarding their epithelial or mesenchymal origin, stem cell character and capacity to replace beta cells in T1D.

Objectives

In this study we intended to establish a reliable and reproducible model of human mesenchymal stem cells in order to evaluate the potential of hBM-MSC to be directed into insulin-producing cells and to overcome the donor variability and heterogeneity of primary MSC.

In a second approach, it was our goal to determine the plasticity and molecular signature of primary hIPC, compare them to hBM-MSC and to evaluate their potential for replacement or regeneration of beta-cells.

Methods

I. *In vitro* differentiation of hMSC-TERTtowards insulin producing cells

k) Evaluation of the expression of two stem/progenitor markers NESTIN e C-MET/HGFR in the telomerase immortalized human mesenchymal stem cell line obtained from the bone marrow of a healthy young male adult (hMSC-TERT), by means of RT-PCR, flow-cytometry and immunocytochemistry.

l) Culture of hMSC-TERT in specific endocrine promoting conditions for 9 days. After this period, expression of pancreatic endocrine gene markers were analysed by means of RT-PCR.

m) Stable transfection of hMSC-TERT with two key regulatory genes of pancreatic endocrine maturation and function *hNGN3 and/or hPDX1* giving rise to the generation of three new cell lines (hMSC-TERT-NGN3, hMSC-TERT-PDX1 and hMSC-TERT-NGN3/PDX1). Islet gene markers expression including *PAX4, PAX6, NEUROD1, PDX1, GLUT2, GCK and INSULIN*) were analysed by RT-PCR. Insulin and C-peptide protein synthesis were detected by western blotting and immunocytochemistry.

n) Subsequently, functional analysis of new generated cell lines was performed using promoter gene activity assays, namely SEAP and Luciferase reporter gene assays. Insulin content and secretion were evaluated by an ELISA.

II. Comparison of the functional signatures of primary hIPC and hBM-MSC

o) Detection of hematopoietic (CD14, CD34, CD45) and mesenchymal (CD29, CD44, CD54, CD73, CD90 e CD105) surface antigen markers in primary hIPC (n=3) and hBM-MSC (n=5) was perfomed by flow-cytometry (FACS).

p) hIPC and hBM-MSC were expanded and subjected to osteogenic, chondrogenic and adipogenic differentiation media according to standardized protocols. On the other hand, hIPC and hBM-MSC were cultured under endocrine differentiation conditions for 5 days.

q) Mesenchymal gene markers (*FNT*, *THY1*, *SNAI2*, *P4HA1*, *MMP2* e *VIM*) and pancreatic endocrine differentiation markers (*PAX4, PAX6, NGN3, NEUROD1, PDX1, GLUT2, GCK e INSULINA*) were analysed by RT-PCR. Insulin and vimentin protein synthesis were demonstrated by fluorescence immunocytochemistry.

r) Genome-wide gene expression profiling from hIPC (n=3) and hBM-MSC (n=5) was performed by microarray analysis of total RNA expression using Affymetrix Gene Chips HG-U133 Plus 2.0 .

s) Transcriptional profiling within the two groups of samples was evaluated using the significance analysis of microarrays (SAM) approach. RT-PCR was used to partly confirm the microarray results.

t) Expression of those target genes which where significantly differently expressed in hIPC and hBM-MSC was confirmed by means of real-time PCR (qPCR).

Results

I. *In vitro* differentiation of hMSC-TERT towards insulin producing cells

Specific culture conditions alone as well as hNGN3 and hPDX1 ectopic expression could induce pancreatic endocrine gene expression in hMSC-TERT including *PAX4, PAX6, NEUROD1, INSULIN, GLUT2, GLUCAGON* and *SOMATOSTATIN*.

In hNGN3 overexpressing hMSC-TERT, endogenous *NEUROD*, *PAX4* and *PDX1* were activated similarly to the transcription factors cascade observed in pancreatogenesis.

PDX1 was apparently sufficient to trigger endocrine differentiation in hMSC-TERT, suggesting an endodermal commitment in this mesenchymal population.

Although overexpressing NGN3 and/or PDX1 hMSC-TERT were able to produce and secrete insulin, no glucose sensitivity was detected in either of the new-generated hMSC-TERT cell lines.

II. Comparison of the functional signature of primary hIPC and hBM-MSC

The results demonstrated that hIPC isolated from human pancreatic islets and hBM-MSC obtained from human bone marrow display an overlapping immunophenotype. The significant expression of genes of the mesenchymal core and the expression of stem cell-related genes in both cell types, strongly suggests the MSC character othese cells. However, hIPC showed reduced differentiation potential towards mesenchymal lineages (bone, cartilage and fat) compared to hBM-MSC, which on the other hand exhibited a rudimentary endocrine differentiation capacity.

In silico analysis (by means of informatics) of the obtained microarray data, demonstrated from a panel of important gene onthology groups that hIPC and hBM-MSC are involved in very distinct biological processes. Moreover, detailed analysis of differentially expressed genes revealed marked differences in the expression pattern of

cadherin family genes, of key transcription factors for mesodermal and endodermal pathways like the HOX genes family and the *SOX17* and *ISL-1* genes.

In particular, in hIPC, a significant expression of genes known to be related to a cellular transitional state such as *TCF21* and *TGFb* genes was detected. This was not found in hBM-MSC.

Conclusions

hMSC-TERT can be induced to activate pancreatic endocrine genes, therefore representing a reliable cell system to investigate the hMSC, as alternative source for β-cell replacement or regeneration. However, higher endocrine maturation must be achieved, in order to obtain functional hMSC that might be suitable for the cell-based therapy of T1D and advanced T2D.

Our results also suggest that hIPC are MSC, which maintain a specified phenotype related to the tissues where they reside. They display clear signs of epithelial-mesenchymal transition (EMT) or MET, but are determinedly arrested in an intermediate state of differentiation. Further investigation is necessary to reveal if they are essential for islet regeneration and if they may complete their transitional state towards a mature endocrine phenotype.

Our studies provided important knowledge to the molecular and functional signature of adult MSC populations derived from distinct tissues, giving new insight towards alternative cell sources to be used in cell-based therapies of T1D.

LISTA DE ABREVIATURAS / LIST OF ABBREVIATIONS[3]

ABCC8	human Sur1 gene
ABCG2	human ATP binding cassette group 2 gene
AGEs	advanced glycation endproducts
AGN	human aggrecan gene
ALP	human alkaline phosphatase gene
AMPK- AMP	protein kinase or 5' adenosine monophosphate-activated protein kinase
APC	antigen presenting cells
ATG	anti-thymocyte globulin
bHLH	basic helix loop helix
BMI	body mass index
BMI1	human polycomb group repressor gene 1
BM-MSC	bone marrow-derived mesenchymal stem cells
BSA	bovine serum albumin
BSP	human bone sialoprotein II gene
CDH1	human E-cadherin gene
CDH4	human retinal cadherin gene
CDH6	human fetal kidney cadherin gene
CDH11	human osteoblast cadherin gene
CFA	complete Freund's adjuvant

[3] Reúne as abreviaturas em lingua portuguesa e inglesa, utilizadas no documento.

c-met/MET	protooncogene that encodes the protein MET also known as HGF receptor
COL II	human collagene type II gene
COLX	human collagen type X gene
COS7 cells	African Green Monkey SV40-transf'd kidney fibroblast cell liine. They are useful in virus replication studies and transfection studies.
CSII	continuous subcutaneous insulin infusion
Cy2	reactive water-soluble fluorescent dye of the cyanine dye family. Cy2 is excited maximally at 510 nm in the green region of the visible spectrum
Cy3	reactive water-soluble fluorescent dye of the cyanine dye family. Cy3 is excited maximally at 649 nm in the red region of the visible spectrum
Dapi	4' - 6- Diamidino-2-phenylindole (DAPI) forms fluorescent complexes with natural double-stranded DNA (blue staining). Useful tool in various cytochemical investigations.
DCCT	diabetes control and complications trial
DPP-4	dipeptyl peptidase 4
DT1	Diabetes Mellitus tipo 1
DT2	Diabetes Mellitus tipo 2
EF1alpha	human housekeeping gene.
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transition
ES	embryonic stem cells

FACS	fluorescent activated cell sorting
FasL R	Fas ligand receptor
FBS	fetal bovine serum
FC	fold change
FCS	fetal calf serum
FGF	fibroblast growth factor
FGFR1	human fibroblast growth factor receptor 1 gene
FGFR2	human fibroblast growth factor receptor 2 gene
FITC	fluorescein isothiocyanate label
FNT	human fibronectin gene
FPG	fasting plasma glucose
G6P	glucose 6 phosphate
GAD65	glutamic-acid decarboxilase 65 antibody
GCG	human glucagon gene
GCK	human glucokinase gene
GFP	green fluorescence protein
GLP-1	human glucagon-like-peptide-1 gene
GLUT2	human glucose transporter member 2 gene
GO analysis	gene ontology analysis
GOstat analyses	statistical analysis of the gene ontology groups.
HbA1c	glycated haemoglobin A1c
hBM-MSC	human bone marrow-derived mesenchymal stem cells
HES	human hairy enhancer of split gene
HGF	hepatocyte growth factor
hHCG	human chorionic gonadotrophin

hIPC	human pancreatic islet- progenitor cells
HMGB1	human high mobility group box 1 gene
hMSC-TERT	telomerase immortalized human mesenchymal stem cells
HNF1β	human hepatic nuclear factor 1 homeobox B gene (Mody 5)
HNF1α	human hepatic nuclear factor 1 homeobox A gene (Mody 3)
HNF4α	human hepatic nuclear factor 4 alpha (Mody 1)
hNGN3-pcDNA 3.1^{+}	expression vector that includes the cytomegalovirus promoter-driven pcDNA 3.1+ sequence, the human NGN3 sequence and the neomycin-resistance gene.
HOXA	human homeobox gene family A
hPDX1-pcDNA6/V5-His-A	expression vector that includes the cytomegalovirus promoter-driven pcDNA6/V5-His-A sequence, the human PDX1 sequence and the blasticidinb resistance gene.
HPRT	human hypoxanthine phosphoribosyltransferase, used as housekeeping gene
HSC	hematopoietic stem cells
HSP60	heatshock protein 60
hUCB	human umbilical cord blood cells
IA	insulin antibody
IFNγ	interferon gamma
IGF-1	insulin-growth factor 1
IL-1b	interleukin -1 beta
IL-1Ra	interleukin 1 receptor antagonist
IL-8	interleukin -8

INS	human insulin gene
INS-1E	rat pancreatic beta cell line
ISL1	human islet 1 gene
IPC	islet derived progenitor cells
IPF1	human PDX-1 gene
iPS	induced pluripotent stem cells
K_{ATP}	ATP potassium channels
KCJJ	human Kir6.2 protein gene
Klf4	human Krueppel-like factor 4 gene
KRB	Krebs-Ringer buffer
LADA	latent autoimmune diabetes in the adult
LDL	low density lipoproteins
LPL	human lipoprotein lipase gene
LUC	luciferase
mAB	monoclonal antibody
MAF	human v-maf musculoaponeurotic fibrosarcoma oncogene homolog gene
MAF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B
MAFK	v-maf musculoaponeurotic fibrosarcoma oncogene homolog K
MDI	multiple daily injections
MEIS	human meis homeobox 1gene
MEIS2	human meis homeobox 2 gene
MET	mesenchymal epithelial transition
MHCI	major histocompatibility complex 1
MHCII	major histocompatibility complex II

MIDD	maternal inherited deafness and diabetes
MMP2	human alpha I subunitmatrix metalloproteinase 2 gene
MMP2	matrix metalloproteinase-2
MMP9	matrix metalloproteinase-9
MODY	maturity onset of diabetes of the young
MSC	mesenchymal stem/stromal cells
MtDNA	mitochondrial desoxiribonucleic acid
NANOG	human homebox transcription factor Nanog gene: Encodes a transcription regulator involved in inner cell mass and embryonic stem (ES) cells proliferation and self-renewal. Imposes pluripotency
NDM	neonatal diabetes mellitus
NEUROD1	human neurogenic differentiation 1 gene: encodes pancreatic endocrine differentiation transcription factor and regulates expression of the insulin gene
NGN3 or Neurog	human neurogenine 3 transcription factor gene. Member of the subfamily of basic- helix-loop-helix (bHLH) transcription factors, involved in the determination of neural precursor cells in the neuroectoderm and essential for development of endocrine pancreas.
Ngn3	mouse neurogenine 3 transcription factor gene
NOD mice	non obese diabetic mice
NODAL	human Nodal gene, member of the TGF-beta superfamily. Essential for mesoderm formation and axial patterning during embryonic development.

OC	osteocalcin gene
OCT	human Pou domain family genes, classically related with stem cell character
OCT1	POU domain class 2, transcription factor 1gene (POU2F1 gene)
OCT3	human solute carrier family 22 member 3 (SLC22A3) gene
OCT4	human POU domain, class 5, transcription factor 1 pseudogene 1 (Pou5F1 gene)
OGTT	oral glucose tolerance test
P4HA1	human prolyl 4-hydroxylase, alpha polypeptide I gene
PAX4	human paired box 4 gene
PAX6	human paired box 6 gene
PBS	phosphate buffered saline
PDX1	human pancreatic and duodenal homeobox 1 gene: encodes a transcription factor involved in early pancreatic development, beta cell maturation and glucose-dependent regulation of insulin gene expression (Mody4)
Pdx1	mouse pancreatic and duodenal homeobox 1 gene:
PE	phycoerythrin label (stains red)
PERV	porcine endogenous retrovirus
PFA	paraformaldehyde
PNDM	permanent neonatal diabetes mellitus
POU	class 2 homeobox 1 gene
PP	human pancreatic polypeptide gene
PPARγ2	human peroxisome proliferator- activator receptor gama 2 gene
PPARγ	peroxisome proliferator-activated receptors gama

pSEAP2	basic secreted alkaline phosphatase gene's expression vector.
PTD	protein transducer domain
qPCR	real time protein chain reaction
ROS	reactive oxygen species
rPdx-1	recombinant pancreatic duodenal homeobox transcription factor of the mouse
RT-PCR	everse transcription polymerase chain reaction
RUNX2	human runt-related transcription factor 2 gene
RUNX3	human runt-related transcription factor 3
SAM	significance analysis of microarrays
SD	standard deviation
SEAP	secreted alkaline phosphatase
SNAI1	human snail1 gene
SNAI2	human snail2 gene
SOX17SRY	(sex determining region Y)- box 17 gene
SOX2	SRY (sex determining region Y)-box 2
SST	human somatostatin gene
SU	sulphonylureas
T1D	Type 1 diabetes
T2D	Type 2 diabetes
T3D (neonatal,	Type 3 diabetes or diabetes due to specific mechanisms monogenic, diabetes secondary to other diseases)
***TCF21* (*Pod-1*)**	human transcription factor 21 gene
TEDDY	the environmental determinants of diabetes in the youth
TF	transcription factor

TGFA	human transforming growth factor, alpha gene
TGFB1	human transforming growth factor beta-1 gene. Encodes a member of the transforming growth factor beta (*TGFb)* family of cytokines
TGFB2	human transforming growth factor, beta 2 gene
TGFBR1	human transforming growth factor, beta receptor 1 gene
THY1	human Thy-1 cell surface antigen gene (CD90 antigen)
TNFα	tumor necrosis factor alpha
Treg	Lymphocyte T regulatory cells
tRNA	transfer ribonucleic acid. Small RNA molecule that transfers a specific active aminoacid to a growing polypeptide chain at the ribosomal site of protein synthesis during translation.
UC-MSC	umbilical cord-derived mesenchymal stem cells
VDCC	voltage dependent calcium channel
VIM	human vimentin gene
Vit D	vitamin D
VSEL	very small embryonic- like stem cells

LISTA DE PUBLICAÇÕES RELACIONADAS COM O PROJECTO / LIST OF PUBLICATIONS RELATED TO THE RESEARCH PROJECT

1. Functional Signature of Human Islet-derived Precursor Cells compared to Bone Marrow-derived Mesenchymal Stem Cells.
Limbert C, Ebert R, Schilling T, Paeth G, Benisch P, Klein-Hitpass L, Seufert J, Jakob F.

Stem Cells Dev. 2009 Nov 6.

2. In vitro (re)programming of human bone marrow stromal cells toward insulin-producing phenotypes.
Limbert C, Seufert J.

Pediatr Diabetes. 2009 Sep;10(6):413-9. Epub 2009 Jul 13. Review.

3. Effects, of high glucose on mesenchymal stem cell proliferation and differentiation
Li YM, Schilling T, Benisch P, Zeck S, Meissner-Weigl j, Schneider D, **Limbert C**, Seufert

J, Kassem M, Schütze N, Jakob F, Ebert R.

Biochem Biophys Res Commun. 2007 Nov 9; 363(1):209-15. Epub 2007 Sep 5.

4. Biology of Mesenchymal Stem Cells
Jakob F, **Limbert C**, Schilling T, Benisch P, Seefried L, Ebert R.

Current Rheumatology Reviews, 2008, 4, 148-154

5. Beta-cell replacement and regeneration: Strategies of cell-based therapy for type 1 diabetes mellitus.

Limbert C, Päth G., Jakob F., Seufert J.

Diabetes Res Clin Pract. 2008 Mar; 79(3): 389-99. Epub 2007 Sep 12.

6. Human Krüppel-like factor 11 inhibits human proinsulin promoter activity in pancreatic beta cells.

Niu X, Perakakis N, Laubner K, **Limbert C**, Stahl T, Brendel MD, Bretzel RG, Seufert J, Päth G.

Diabetologia. 2007 Jul;50(7):1433-41. Epub 2007 May 4. Erratum in: Diabetologia. 2007 Sep;50 (9):2031.

7. Glucose-dependent expansion of pancreatic beta-cells by the protein p8 in vitro and in vivo.

Päth G, Opel A, Gehlen M, Rothhammer V, Niu X, **Limbert C**, Romfeld L, Hügl S, Knoll A, Brendel MD, Bretzel RG, Seufert J.

Am J Physiol Endocrinol Metab. 2006 Dec; 291(6):E1168-76.

A- INTRODUCTION

1. DIABETES

1.1. Definition

Diabetes Mellitus is a metabolic disease of multiple etiologies characterized by chronic hyperglycaemia, with disturbances of the carbohydrate, fat and protein metabolism, due either to defects in insulin secretion, in insulin action or in both [1]. Overt diabetes is defined by fasting plasma glucose (FPG) ≥ 126 mg/dl or 2 hours PG ≥ 200 mg/dl after oral glucose tolerance test (OGTT) [2]. The two major forms, type 1 diabetes (T1D) and advanced type 2 diabetes (T2D) are characterized by a progressive loss of functional beta cell mass within the pancreatic islets, whereby resulting from distinct pathogenic mechanisms. T1D is a chronic autoimmune disease characterized by absolute insulin deficiency resulting from the progressive immune-mediated destruction of pancreatic islet beta cells. T2D results from a combination of both, impaired insulin action and deficient pancreatic beta cell function. Both forms progress to long-term micro and macro vascular disease, which accounts for the high morbidity of diabetes.

1.2. The silent epidemics

The social and economical impact of diabetes is enormous. In all its forms, it affects nearly 200 million people worldwide [3]. According to recent forecasts the global diabetes prevalence of 2,4% will explode to 4,8% within the next 30 years with the highest absolute rise to be expected in India. Demographic changes such as aging of the world population and urbanization in developing countries, which are related to changes in diet, reduced physical activity and stress are strongly associated to the rising numbers in diabetes across the world (Fig 1).

T1D represents 10% of all cases of diabetes, affecting mainly children and young adults [3]. It is thought to be triggered by as yet unidentified environmental factors in genetically susceptible individuals, the major genetic contribution coming from loci within the HLA complex, in particular HLA class II. The incidence of T1D has been increasing worldwide at an annual rate of approximately 3%. [4]. According to the latest results from the EuroDiab (Epidemiology and Prevention of T1D) study group the greatest increase in incidence of T1D is observed among the youngest age groups (children <5 years old), which number is expected to double from 2005 to 2020 [5]. This means that with a younger age of onset, the prevalence of T1D < 15 years old in Europe will dramatically rise. The actual prediction is an increase of 94.000 cases in 2005 to 160.000 in 2020, with major health care implications. Interestingly, over the study period of 15 years, the rise in incidence was most significant in countries with typically low incidence rates [5]. Such rapid increasing incidence of T1D within distinct ethnic groups cannot be explained by a rising transmission of the susceptibility genes. Instead, the declining proportion of newly diagnosed children with high-risk genotypes suggests that environmental pressures are now able to trigger T1D in genotypes that previously would not have developed the disease during childhood [4, 6]. These epidemiologic observations stir up most important questions regarding the pathogenesis of diabetes. Which are the important environmental factors? Which mechanisms of action are involved: do they directly influence beta cell function or do they rouse epigenetic modifications that affect gene penetrance and genetic susceptibility? Currently, identification of possible environmental triggers of diabetes is a priority carried on by a study group of american and european diabetes centers, The Enviromental Determinants of Diabetes in the Youth (TEDDY) [7]. These include diet (vit D deficiency, early exposure to cow milk proteins and gluten, food composition), climate (exposure to ultra

violet radiations), low exposure to childhood infections, high birth weight, early weight gain and most important of all, childhood obesity [8, 9]

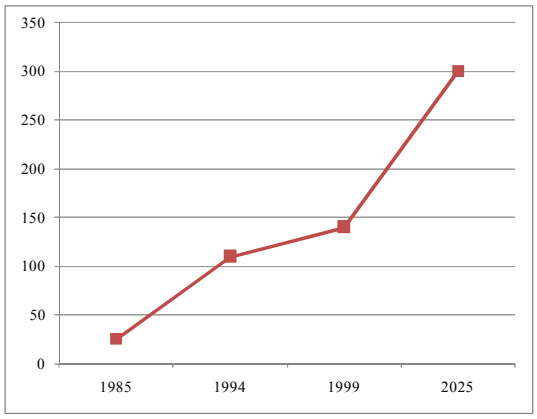


Fig 1: Prevalence of Diabetes Worldwide

By the year 2025 the number of subjects with diabetes will have increased approximately 10-fold compared to figures observed in 1985. Adapted from Metabolic disease Group, Sanger Institute, UK

Indeed, it is the increasing prevalence of obesity in all continents and ages, which is catching major attention [10, 11] (Fig 2). In the WHO European Region, about 30-80% of the adult population, is overweight - has a body mass index (BMI) over 25. Obesity (BMI over 30) affects up to a third of the adult population (Fig 3). Considered an important risk factor for diabetes, overweight people will steadily contribute for the growing public health burden of this disease [12].

Also childhood obesity constitutes an acute health issue. About 20% of children are overweight, and a third of these are obese [13]. In the last 10 years and due to the obesity epidemic, increasing number of children and adolescent are affected by abnormalities of glucose metabolism such as glucose intolerance, metabolic syndrome or T2D [14-16].

Obesity induces beta cell apoptosis in predisposed individuals, raising the incidence of both, T1D and T2D. The underlying pathophysiologic mechanisms may be explained by the “accelerator hypothesis” according to which, the body mass index influences the

three key factors that variably accelerate beta cell apoptosis such as *individual constitution*, *insulin resistance* and *autoimmunity*. Consequently, weight gain induces insulin resistance, which leads to blood glucose disturbances. This is followed by glucose toxicity that results in beta cell apoptosis, inflammation and autoimmunogenecity in a genetic predisposed subset [9].

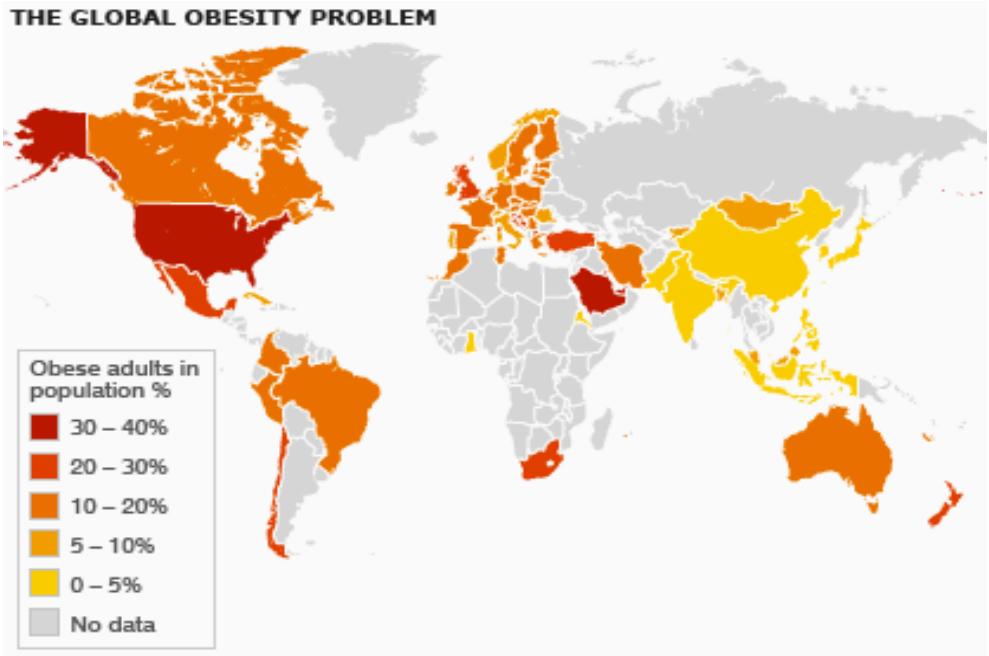


Fig 2: Prevalence of Obesity worldwide

An overweight individual is defined as having a Body Mass Index (BMI) ≥ 25. Obesity is defined as having a BMI ≥ 30 (weight ≥ 30/m2). Adapted (source) from World Health Organization.

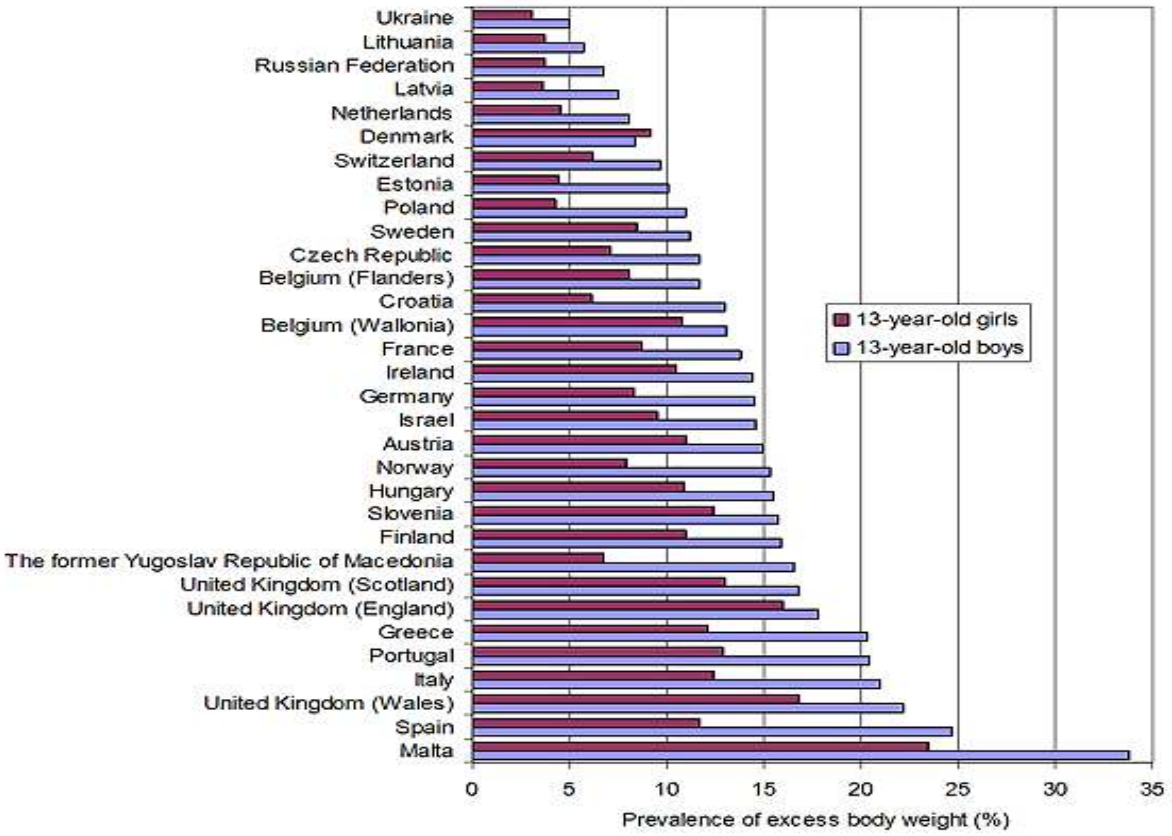


Fig 3: Prevalence of excess body weight (including obesity) among 13-year-olds in countries of the WHO European Region, 2001/2002

Shows the average prevalence of obesity and excess body weight in 13-year-olds in 35 countries and sub-regions in the Region that participated in the HBSC 2001/2002 survey. The prevalence of overweight and obese adolescents ranges from 3% to almost 35% in 13-year-olds. Source: Health Behaviour in School-aged Children (HBSC) 2001/2002 survey http://www.euro.who.int/Document/e82923.pdf

1.3. Inflammation: a pathogenic link between Diabetes Mellitus and Obesity

The concept that T1D is caused by a defect in insulin production and T2D is the result of defective insulin action does no longer hold true. Both these mechanisms that lead to impaired glucose control are pathogenic players in both types of Diabetes Mellitus.

In fact, increasing experimental data and epidemiological evidence show that insulin resistance is linked not only to T2D but also to T1D. Moreover, obesity, which is related to insulin resistance, is increasing worldwide hand in hand with T1D [17].

This observation led to the formulation of the "accelerator hypothesis" which suggests that insulin resistance, together with genetic predisposition and autoimmunogenecity contribute as "accelerators" to the onset of T1D [9, 18]. It is known that insulin resistance caused by obesity does not trigger or increases islets antibodies, but it influences beta cell dysfunction and the onset of T1D [19].

On the other side, it is now well accepted that beta cell apoptosis is a common feature of T1D and T2D, being responsible for impaired insulin secretion in both diseases. Years of basic research have shown that finding one pathogenic model that integrates all the mechanisms involved is rather impossible since there are numerous intervenients and they seem to differ in T2 and T1D.

While insulin resistance is a key player in the pathogenesis of T2D, it is not sufficient for T2D to occur since many obese patients with insulin resistance never develop diabetes. This means that, the genetic pattern related to beta cell function, survival and regeneration in each individual is crucial to determine the capacity of beta cells to survive in response to increased insulin demands [20, 21]. Notably, none of the T2D genes is affected in T1D except for PPARγ, which is an inflammation-related gene [22, Qu, #329].

Furthermore, recent data suggest that environmental factors acting early in life may modify HLA gene penetrance for T1D risk [23]. The rising incidence and decreasing age at diagnosis of T1D is accounted for by the impact of environment on children with lower-risk HLA class II genes, who previously would not have developed T1D in childhood [23]. Since T1D and T2D are genetically different diseases, it has been

suggested that enviromental factors involved in the genetic susceptibility of T1D might be different from those influencing T2D genetics [24].

Although distinct genetic and environmental stimulus are triggering T1D and T2D, a common intracellular pathway in the beta cell has been identified with consequences on the regulation of apoptosis, insulin secretion and beta cell regeneration [24] (Fig 4). Preclinical data and molecular studies strongly indicate that the innate immune system is activated not only in T1D but also in T2D. This corresponds to a systemic inflammatory state characterized by increased circulating levels of acute-phase proteins, cytokines and chemokines. Ehses et al have demonstrated that a specific T2D milieu (high glucose and fat concentrations) induces the pancreatic islets to produce cytoquines (IL-1b) and chemokines (IL-8 from the classical chemiotactic factor CXC family) [25] leading to a state of insulitis, similar to pre-T1D. These islet-derived cytokines (from beta, alpha and endothelial cells) and those produced by recruited immune cells can support adaptation and repair of islet cells, in a short time period. However continuous metabolic stress causes deleterious auto-inflammatory effects leading to beta cell apoptosis, impaired cell regeneration and insulin secretion (glucolipotoxicity)[26].

Similarly, obesity induces a state of lipotoxicity with increased circulating leptin, fat and glucose due to insulin resistance. Lipo and glucotoxicity cause increased levels of circulating cytokines and chemokines, activating the innate immune system and triggering a general systemic inflammatory state. In parallel, islet cells respond to this metabolic stress with an insulitis process [27, 28], which allows at first for beta cell adaptation in response to increased insulin demands. As in diabetes, continuos metabolic stress will activate intra-cellular signalling pathways, which interfere with the fine balance between apoptosis and regeneration of the beta cell. In T1D, the inherent

autoimmunogenic susceptibility leads to activation of the cellular (e.g.LT8 and LT4) and later on, the humoral immune system, which accelerates the loss of beta cell mass. As a result, rapid insulin deficiency and insulin dependency, arises.

Therefore, the recognition that inflammation constitutes a common pathogenic link between T1D, T2D and obesity along with evidences from the experimental and epidemiological side, makes clear that 1) epidemic evolution of these diseases are not independent from each other. 2) understanding the role of islet-derived cytoquines and chemokines such as IL-1b and IL-8, opens the door to targeted clinical interventions aimed at remodeling islet inflammatory response in order to promote beta cell regeneration or to inhibit beta cell apoptosis.

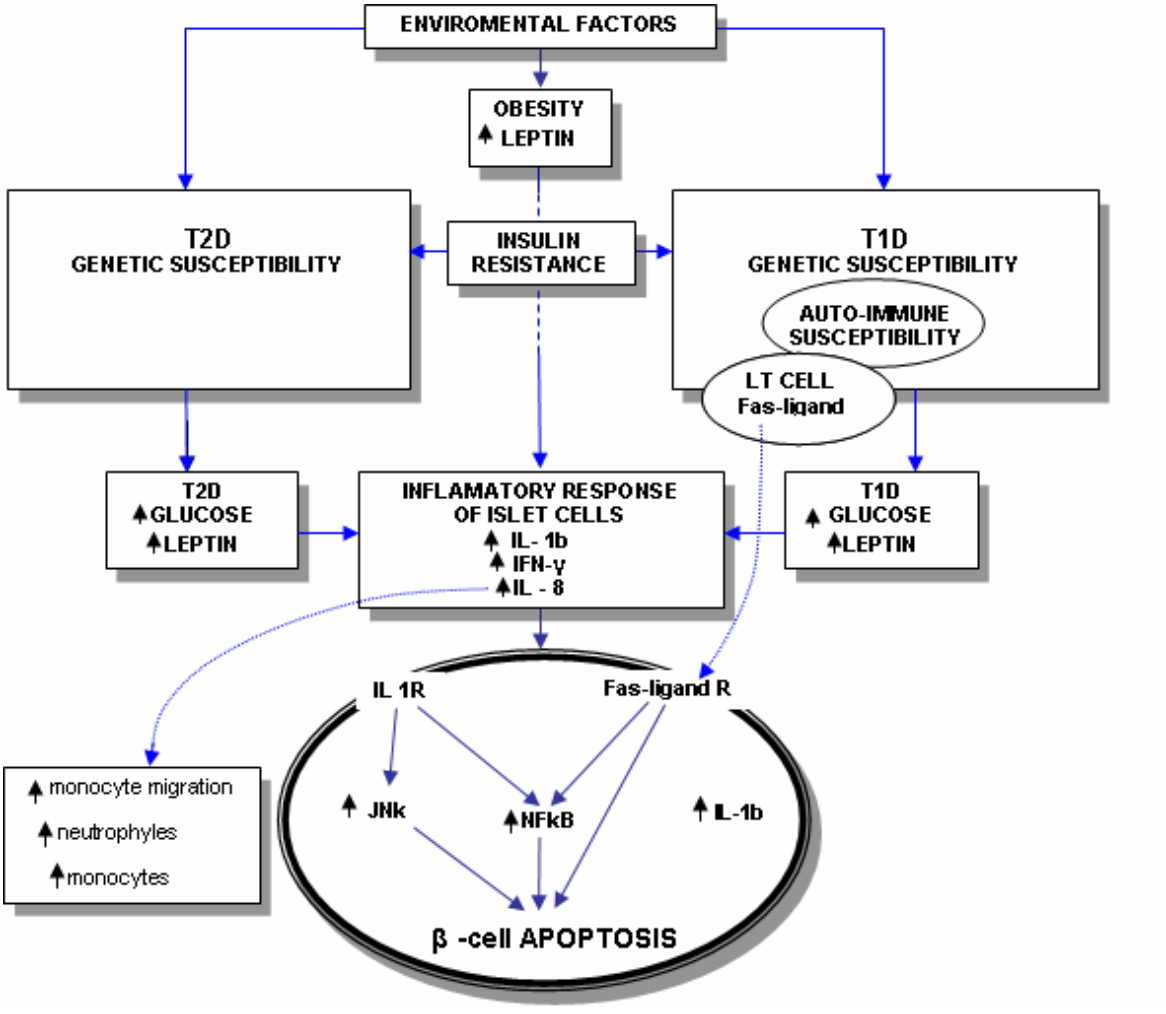


Fig. 4: Inflammation: a pathogenic link between Diabetes Mellitus and Obesity

Environmental factors are determinant for the onset of T2D, T1D and obesity by influencing the specific genetic susceptibility for these diseases. In obesity, circulatory levels of fat and leptin increase and insulin resistance frequently occurs with disturbances of insulin action resulting in higher glucose concentration. Gluco and lipotoxicity induces an inflammatory response via the production of cytokines and chemokines by islet cells (mainly alpha, beta and endothelial cells) and by inflammatory cells of the innate immune system, which is also activated.

In a first phase, inflammatory response of the islet exerts a protective and regenerative effect by stimulating beta cell function and replication, in order to re-establish metabolic control. However, sustained production of islet cytokines and chemokines, due to continuous metabolic stress, leads to deregulation of apoptosis pathways within the beta cell. Increased islet cytokines, in particular IL-1b and IFNγ induce apoptosis by activation of the MAPK/JNK pathways. In addition, IL-1b stimulates expression of Fas-ligand receptor (FasL R) in the beta cell, which in cases of T1D genetic susceptibility, will sensitize beta cell to the T cell mediated apoptosis via the FasL. In T2D, glucose and possibly leptin will activate the same inflammatory response with disruption of common intracellular apoptotic pathways as in T1D.

1.4. Other types of Diabetes

Historically, diabetes classification has been based on the therapeutic approach towards correction of hyperglycaemia resuming Diabetes Mellitus to insulin-dependent and non insulin-dependent forms.

In the last ten years, thanks to improved understanding of the disease, diabetes has been classified according to the pathogenesis of glucose intolerance in:

1) Type 1 diabetes (T1D) (autoimmune)

2) Type 2 diabetes (T2D) (non autoimmune)

3) Type 3 diabetes (T3D) Diabetes due to specific mechanisms such as neonatal diabetes (NDM), maturity onset diabetes of the young (MODY) and diabetes associated to other diseases (secondary diabetes and syndromes)

4) Gestational diabetes (insulin resistance during pregnancy) [1].

Nevertheless, based on clinical and laboratorial diagnostic and more recently on molecular genetic testing, other forms of diabetes have been identified.

Latent autoimmune diabetes in the adult (LADA) or Type 1,5 diabetes, accounts for 5-10% of diabetic older patients [29]. Characterized by insulin resistance, low BMI, low C-peptide and presence of anti-islet antibodies (especially anti glutamic acid descarboxilase 65 (GAD65), it is thought to be an autoimmune diabetes in adults with slowly progressive beta-cell failure [30].

Monogenic diabetes is considered a rare type of diabetes mellitus (1-2%) [31] and is caused by one or more mutations in a single gene. Generally, it affects children in the first 6 months of life and includes permanent or transient neonatal diabetes. However, it may also arise in young adults < 25 years of age (former MODY). This form of diabetic disease should be suspected whenever T1D or T2D occurs with unusual clinical features or family history of diabetes [32].

In monogenic diabetes, most mutations occur in beta-cell key regulatory genes, which result in beta-cell dysfunction, decreased pancreatic growth or even subtotal pancreatic agenesis. Well-characterized mutations have been identified at genes encoding the glucose-transporter-2 (*GLUT2*), Glucokinase (*GCK*) and mitochondrial DNA (*mtDNA*), leading to reduced glucose sensing capacity. Mutations at the Kir6.2 and Sur1 protein genes (*KCJJ* and *ABCC8* genes, respectively) of the membrane K_{ATP} channels typically affect insulin secretion mechanisms of the beta-cell. Insulin synthesis and packaging are disrupted in cases of pancreatic transcription factor's mutations such as *HNF1*α (MODY3), *HNF4*α (MODY1), *HNF1*β (MODY5), *NEUROD1* (MODY6), CEL (MODY7) and *IPF1* (MODY4) (Fig 5). Among these, the most common is *HNF1*α (MODY3). Depending on the number of mutations in one single gene, severity of the

clinical features varies from mild insulinopenia to pancreatic atrophy with permanent neonatal diabetes mellitus (PNDM/ MODY4).

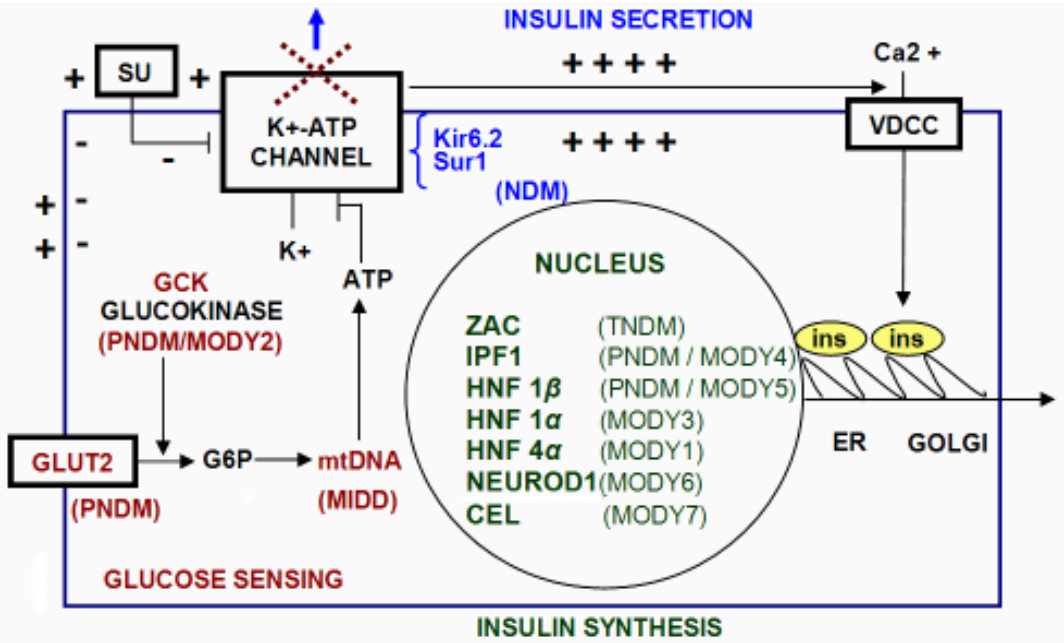


Fig 5: Pathogenesis of Monogenic Diabetes - pancreatic beta cell and the most frequent genes implicated in monogenic diabetes.

In a physiological state, glucose enters the beta cell through passive diffusion which is facilitated by the glucose transporter 2 (GLUT2). It is phosphorylated into Glucose 6P (G6P) by glucokinase enzyme (GCK) and transformed into ATP via glycolysis or via Krebs cycle inside the mithocondria which involves mithocondrial DNA (mtDNA). ATP induces closure of the K^+-ATP channels at the cellular membrane, preventing K^+ efflux, subsequent membrane depolarization and opening of voltage dependent calcium channels (VDCC) with cellular Ca^{2+} influx. Enhanced intracellular calcium levels, induces fusion of insulin vesicles with the membrane and stimulates insulin secretion. Sulphonylureas (SU), induce insulin secretion by closing the K^+-ATP channels.

Mutations in different genes lead to disruption of different functions in beta cell, including **glucose sensing** (red), **insulin synthesis** (green) and **insulin secretion** (blue) mechanisms. Parenthesis refers to the corresponding type of monogenic diabetes. Different mutations in the same gene may lead to several phenotypes of the same disease. NDM - neonatal diabetes mellitus; TNDM - transitory neonatal diabetes mellitus; PNDM - permanent neonatal diabetes mellitus; MIDD - maternal inherited diabetes and deafness. Adapted from Slingerland A.S. Rev.Endocr Metab Disord (2006) 7:171-185

Monogenic diabetes associated with defects in the peripheral cell is very rare. It results from mutations in the insulin receptor gene, which leads to receptor degradation, no glucose-uptake and severe insulin resistance (Rabson-Mendenhall syndrome or Leprechaunism). In addition, insulin resistance may also be associated with familial lipodystrophies conditions.

Molecular genetic diagnosis is essential in monogenic diabetes as it determines the treatment that a patient should receive [33]. For instance, patients with HNF1alpha mutation are very sensitive to low dose sulphonylureas; glucokinase MODY patients need no treatment at all and patients with neonatal diabetes due to Kir6.2 mutation can discontinue insulin and be well controlled on high dose sulphonylurea tablets [34].

Mitochondrial diabetes usually refers to the specific A-G mutation at the 3243 position from the tRNA (Leu (UUR)) gene in the mitochondrial DNA (mtDNA) [35, 36]. It results in maternal-transmitted diabetes associated with sensorial deafness and short stature (MIDD), which manifests in young adult ages. Depending on the proportion of mtDNA affected, several phenotypes have been recognized. Meanwhile other mutations in mitochondrial or nuclear genes have been identified [37].

Interestingly, recent studies strongly suggest that aging, age-related metabolic and degenerative disorders such like Alzheimer's disease, cancer or T2D present mitochondrial dysfunction as a common underlying phenomenon [38].

Mitochondrias are power factories with an independent genome (mtDNA) that generate ATP by oxidizing reduced equivalents from the citric acid cycle, via the respiratory chain. Several studies have shown that aging, oxidative stress and chronic hyperglycaemia promote mutated mitochondrial DNA (mtDNA) and damage of transfer RNA (tRNA) and mitochondrial proteins. In other words, mitochondria is considered one of the key regulators of longevity. Injured mitochondria promotes respiratory chain

deficiencies in several tissues like heart, skeletal muscle and brain, which in turn favours aging-related disorders [39] (Fig 6). Metabolic active tissues with high- energy demand, like the pancreas, are especially vulnerable to mitochondrial aging. Thus, in older individuals, aged/injured pancreatic mitochondria induce deficits of insulin secretion, leading to hyperglycaemia and direct acceleration of diabetic nephropathy in overt T2D [40 41].

At present, an intense ongoing debate about the role of reactive oxygen species (ROS) and oxidative stress in the aging process exists. While there is a tendency to automatically link mitochondrial dysfunction to increased generation of ROS, the experimental support for this concept is weak. In fact, respiratory-chain-deficient mice with tissue-specific mtDNA depletion or massive increase of point mutations in mtDNA typically have aging-related phenotypes without evidence of increased oxidative stress [42]. Thus, mtDNA mutations causing respiratory chain dysfunction may accelerate aging by provoking bioenergy deficit in physiologically crucial cells, by decreasing the signal threshold for cell death, by inducing senescence in stem cells, or by some other mechanism.[43].

An increasing number of specific mutations associated to genetic susceptibility for diabetes have been identified due to recent advances on molecular genetic diagnosis [22].These findings make us to consider that in the near future the etiological classification may give place to a genetic classification of Diabetes Mellitus. This would represent a milestone as far as the progress of treatment towards cure is concerned.

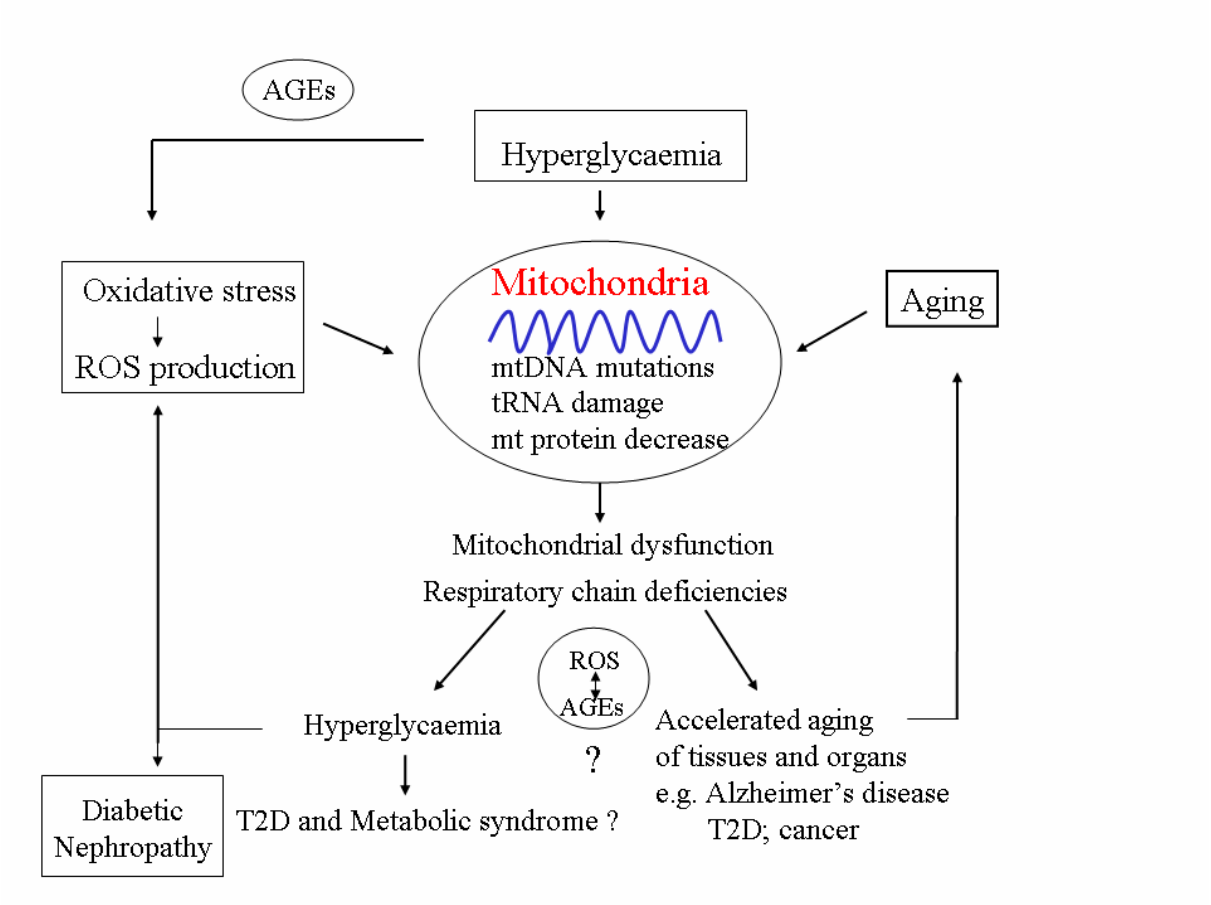


Fig 6: The role of mitochondrial dysfunction in diabetes and other age-related disorders

Well-characterized mitochondrial DNA (mtDNA) mutations (e.g. A3243G) leads to inherited mitochondrial diabetes like the MIDD syndrome. Hyperglycaemia per si, aging and localized tissue oxidative stress have been related to injury of mitochondrial DNA, RNA and protein, which provoke respiratory chain deficiencies in physiological crucial cells. This favours chronic hyperglycaemia and acceleration of aging. Advanced glycation end products (AGEs) result from non-enzymatic protein glycosylation and contribute to reactive oxygen species (ROS) formation (superoxide, peroxide and free radicals) in the mitochondria. Both seem to play a key role in mitochondrial dysfunction, diabetic microvascular disease and other age-related disorders. Wether mitochondrial dysfunction leads to increased ROS production and therefore induces a self-perpetuating cycle of ROS/AGEs formation, is still not proven.

2. CURRENT MAIN THERAPEUTICAL STRATEGIES IN DIABETES

2.1. Oral Antidiabetic Agents

Treatment of diabetes mellitus has been focused on strategies to revert hyperglycemia upon low-carbohydrate diet, physical exercise and tight glycemic control, based on the knowledge of the deleterious effects of hyperglycemia in several tissues and organs. Thus, standard pharmaceutical agents for T2D include **insulin secretagogues** (sulphonylureas and meglitinides), **insulin sensitizing drugs** (biguanides and glitazones), **incretin hormones** (Glucagon-like-peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP)) **analogues** (exendine-4) and **dipeptidyl peptidase 4** (**DPP-4**) **inhibitors**. Sulphonylureas and meglitinides are both K^+ATP channel closers, which enhance insulin secretion from the beta cell. Oral biguanides (metformin) and glitazones (thiazolidinediones) are insulin sensitizers that stimulate the utilization of glucose mainly by the liver (metformin) and skeletal muscle and fat (glitazones). Incretins are intestinal hormones that exert multiple glucose-lowering effects, such as to stimulate glucose-dependent insulin secretion, inhibit glucagon secretion, delay gastric emptying, reduce food intake and also promote glucose utilization in peripheral tissues.

Since circulatory incretin hormones, namely GLP-1 and GIP, are rapidly inactivated by the enzyme DPP-4, several long lasting analogues have been developed with similar glucose lowering effects. GLP-1 analogues have the disadvantage that they must be administered subcutaneously. DPP-4 inhibitors, by inhibiting the activity of DPP-4 enzyme, indirectly increase endogenous GLP-1 concentration.

2.2. Insulin preparations

Insulin replacement is essential for T1D. Also in later stages of T2D, beta cell mass is reduced by about 50%, and 20-30% of these patients require exogenous insulin to control hyperglycemia, when oral hypoglycemic agents fail [44].

Most advanced **insulin preparations** are now available in the market. Well-tolerated synthetic human insulin compounds have long replaced former porcine and bovine derived insulin, which could cause allergic reactions (it was back in 1978 that Genentech started producing human synthetic insulin using recombinant DNA technology). Progress in genetic engineering techniques made available both, rapid-acting insulin analogues (e.g. lispro and aspart insulin) and long acting insulins (e.g. glargine and detemir). These allow better glycemic control, flexible dietary regimens and most important, lead to a remarkable improvement on quality of life of T1D patients [45].

In 1993, the Diabetes Control and Complications Trial (DCCT) demonstrated that 10 years of intensive therapy of T1D significantly reduced the risk of development and progression of microvascular and neurological complications [46]. Since then, efforts have been made to achieve near normal glycemia in T1D. The combination of continuous glucose monitoring with intensified insulin regimens using **multiple daily injections** (MDI) therapy could effectively improve metabolic control and reduce the incidence of long term micro and macrovascular complications in T1D [47 48, 49].

Continuous subcutaneous insulin infusion (CSII) constitutes an alternative to MDI for those T1D and T2D patients under intensive insulin regimens. The secretor function of beta cells in response to physiological stimulus is complex and difficult to duplicate. Yet, CSII is the most physiological insulin delivery system currently available. With this method, it is possible to simulate the pattern of insulin secretion with a continuous

basal delivery and a superimposed mealtime "boluses". The CSII depends on the utilization of insulin pumps, which allows more flexibility in life style, very small and precise insulin delivery and thus reduces the risk of severe hypoglycaemias and ketoacidosis, especially in childhood [50, 51]. Moreover, by improving long-term metabolic control (lower glycated haemoglobin A1c (HbA1c)) in adults and adolescents, CSII may lower the incidence of late complications in T1D [52]. Recent meta-analysis of randomized controlled trials, comparing CSII and MDI in T1D and T2D patients show 1) lower HbA1c in adult T1D patients treated with CSII, but no differences in HbA1c of T2D patients treated with CSII compared to those on MDI.2) the effect of CSII on hypoglyceamias in T1D resulted in a sligth reduction, whereas in T2D, CSII and MDI had similar outcomes. The impact in patients with hypoglycemia unawareness or recurrent severe hypoglycemia is not known because of lack of data [53].

Inhaled insulin represents a therapeutic alternative to the subcutaneous insulin injections. Two inhaled human insulin have been developed for the treatment of T1D and T2D. One is delivered as a dry power and the other in a liquid form, which provides more precise and smaller dosis of insulin. Both are used as pre-meals insulin bolus. Although, the lung tissues absorb them as fast as subcutaneous short acting insulin, the effect does not last long enough to maintain the basal needs [54]. A meta analysis of 16 open-label trials (4023 patients; age range, 18 to 80 years) have shown that inhaled insulins constitute an effective noninvasive option for premeal insulin administration, with slight less efficacy in glycemic control than subcutaneous regular insulin but increased patient acceptability [55]. In children (6 to 11 years) with T1D, a three month randomised trial suggested that inhaled insulin compounds might be an effective and safe alternative to short acting analogues [56], particularly in those with resistance to subcutaneous insulin. Another open-label randomized controlled trial was recently

performed in oder to analyse two-year efficacy and safety of inhaled insulin compound in 385 T1D patients. Here, no differences between the SC and inhaled insulin group were found concerning insulin doses or hypoglycemic events. As in previous studies, HbA1c was significantly higher in the inhaled group (0.44%). Patients treated with inhaled insulin, developped larger (reversible) decline in diffusing capacity of the lung for carbon monoxide (CO). Greater weight gain was seen with SC insulin treatment [57]. In summary, the key benefit of inhaled insulin compounds seems to be patient satisfaction and improved quality of life, which are significantly improved, due to reduce number of daily injections. However none of the inhaled insulin compounds could be implemented in the clinical use till now. Besides the respiratory side effects, the well-recognized lower bioavailabilities and hence higher doses of inhaled insulin required make it less cost-effective than injected insulin [58].

2.3. Limitations of current therapies

2.3.1. Secondary effects of insulin therapy

Current therapeutic agents for the treatment of DM are considered to be safe.

With the new generation of highly purified molecules and rDNA human insulins, severe **immune reactions** due to the use of exogenous insulin such as anaphilatic reactions are rarely seen and only few patients have mild hypersensitivity complications. **Lipodistrophy** (lipoatrophy and lipohypertrophy) at the injection sites became also rare due to the increasing use of rapidly absorbed analog insulins (e.g. insulin lispro, insulin aspart), which are also more anergic [59]. Conversely, exceptional cases of lipoatrophy have been described in patients receiving rapid acting insulin analogs via CSII [60] or detemir [61]. These findings suggest that local complications (abcess formation,

lipodystrophies and mild immune reactions) remain a potential problem even with new insulin therapeutic technologies.

Insulin resistance and **unexplained hypoglycaemia** episodes have been both attributed to anti-insulin antibody (IA) formation that occurs in almost every diabetic patient receiving exogenous insulin. Numerous studies suggested that insulin resistance, which manifests by increased insulin dose requirements, results from neutralization of the biological effect of circulating insulin by IA. Conversely, a correlation between IA levels and a prolonged effect of insulin causing hypoglycaemia was described in early reports. In fact, most studies published since 1980 have shown no evidence for a significant relationship between IA and average glycemia [62]. No proof exists that humoral response to exogenous insulin affects insulin dose requirements, glycemic control or contributes to beta cell failure or long-term complications [63]. Besides, the prevalence and titers of insulin antibodies in insulin-treated patients have markedly decreased, mainly as a consequence of the improvements in the purity of insulin preparations and because of the changes of species of insulin (human insulin).

In recent years, **intensive insulin treatment** using MDI and specially CSII has been shown to improve life quality, metabolic control and reduce the risk of long term diabetes complications in both, T1D and advanced T2D [52]. Nevertheless, in a recent prospective randomised study it was observed that in adult T2D patients with independent cardiovascular disease or additional cardiovascular risk factors, tight metabolic control was associated with unexpected deleterious effects. Indeed, intensive therapy to target normal HbA1c levels increased episodes of severe hypoglycaemia, induced significant weight gain and did not reduce cardiovascular complications.

Moreover, higher mortality rate was observed in these patients [64]. These conflicting results reinforce the idea that no insulin regimen or delivery system can ensure physiologic glycaemic control in order to provide satisfactory prevention of diabetes complications.

Although **inhaled insulins** may substitute subcutaneous short-acting insulin delivery in T2D patients, significant disadvantages dissuades physicians to advice this method. Based on observational studies, severe hypoglycemia is more frequent with inhaled insulin than with antidiabetic oral agents. Also, inhaled insulin causes mild to moderate nonprogressive dry cough and mild decreases in respiratory function in some individuals with T1 and T2D [55, 57]. The latter is due the activation of IGF1 receptor in the lung, which leads to cell proliferation, enhanced connective tissue deposition and consequent reduction in gas exchange and lung compliance. In addition, inhaled insulin is unsuitable during intercurrent respiratory illness; it excludes children, smokers and individuals with significant pulmonary disease.

2.3.2. Secondary effects of antidiabetic oral agents

Current antidiabetic oral agents are widely used in T2D and some cases of monogenic diabetes, with high efficacy in a significant percentage of patients.

The metabolic actions of **metformin**, occur via activation of AMP- activated protein kinase (AMPK) mainly in the liver, but also in the heart and peripheric tissues [65]. AMPK enhances ATP-producing catabolic pathways, which reduces gluconeogenesis and increases glucose uptake in metabolic active cells including skeletal muscle [66, 67]. Other beneficial effects such as lowering of serum low-density lipoproteins (LDL) and tryglycerides, stimulation of fibrinolysis and inhibition of oxidative stress have been

reported indicating thus, important additional cardiovascular benefits of this agent [68]. The most common side effect of metformine is gastrointestinal intolerance resulting from the drug high concentration in upper intestinal tract leading to bowel irritation. More distally, metformin may alter bile salt absorption, which increases fluid retention and triggers loose stools and diarrhoea in some patients [69]. These symptoms can be avoided by initial low doses and slow titration of the drug. Lactic acidosis is the most severe complication of metformin therapy. Its incidence, however, is very low and most cases occur with concomitant kidney or liver impaired functions [70].

Glitazones, influence glucose and fat metabolism via binding to the nuclear peroxisome proliferator-activated receptors gama (PPARγ) at the heart, skeletal muscle and mainly at fat tissue. They facilitate glucose and lipid uptake, stimulate glucose oxidation, decrease free fatty acid level and ameliorate insulin resistance. PPARα and γ agonists have controversial pleiotropic effects namely on inflammation, vascular function, and vascular remodelling. While some studies have shown an increased risk for coronary heart disease with rosiglitazone, pioglitazone treatment appears to have a protective role against cardiovascular complications. At present, safety and clinical indication of PPAR agonists remain undetermined due to the incidence of heart disease in metabolic syndrome and T2D.

Hypoglycaemia and weight gain are common side-effects of insulin secretagogues such as **sulphonylureas and meglitinides**. Moreover, recent studies have suggested that closure of the K_{ATP} channels by the sulphonylureas tolbutamide and glibenclamide may induce Ca2+ dependent beta cell failure and apoptosis in T2D [71, 72]. In vivo clinical studies comparing insulin and glibenclamide in T2D patients corroborate these findings [73]. Given the possible deleterious effect of some sulphonylureas, alternative insulin secretagoges are under investigation. Repaglinide and nateglinide sulphonylureas, of the

meglitinides group, are short acting closers of the K_{ATP} channels, which do not appear to induce beta cell apoptosis in their circulating concentrations [72]. Curiously, and in contrast to sulphonylureas, K_{ATP} channel openers may protect the remaining beta cells from exhaustion by inhibiting their secretory function. Improvement of insulin secretion has been observed after short treatment with **diazoxide** in recent diagnosed T1D and T2D patients [74].

Treatment with incretine mimetics like GLP-1 analogues and DPP-4 inhibitors provoke mild and transient gastrointestinal side effects.

3. STRATEGIES FOR THE FUTURE: "FROM TREATMENT TO CURE"

Exogenous insulin therapy has been the single approach to treat T1D so far. Major improvements in insulin peptide activity, insulin delivery devices and glucose monitoring have been observed over the past years. Nevertheless, advances on the pathogenesis of diabetes, in its different forms, have raised much interest of investigators towards therapies targeting the cause of the disease.

It is now well accepted that the cure for T1D and for many cases of T2D requires either regeneration or replacement of insulin producing cells. Thus, finding a functional substitute for the "missing beta-cell" or restoring regeneration capacities is a major goal in the field of diabetes research [75]. On the other hand, modulation of the immunesystem and anti-inflammatory approaches in both T1D and T2D are crucial therapeutic research lines that may contribute to solve key pathogenic factors of the disease.

3.1. Beta cell regeneration

Adult endocrine pancreas is not a quiescent cell population. Like most tissues, islet cells are dynamically regulated by expansion and reduction mechanisms. There is enough evidence for beta-cell regeneration in animal models [76]. In humans, increases in metabolic demand such as obesity, pregnancy and a first stage of insulin resistance, increases beta-cell mass [77]. Conversely, subjects with diabetes (either T1D or T2D) show a decrease in beta-cell mass compared to weight-matched individuals [78].

Although it is certain that beta-cell mass regeneration does occur, it has been largely discussed whether this happens through self-replication of mature beta-cells [79], through neogenesis of progenitor cells residing in the pancreas [80-83] or recruited from other tissues (bonafide differentiation) [84]. There have been studies suggesting that endogenous regeneration of beta-cell mass in rodents differs from that in humans. In

diabetic mice models, self- replication is the main process [79], whereas in humans, neogenesis from progenitor cells seems to be the predominant mechanism of compensation [80, 83, 85, 86]. On the other hand, collected evidence over the past decade indicates that, *in vivo*, regeneration mechanisms of the adult endocrine pancreas vary according to the metabolic need signals and pancreatic injury levels (e.g. mild to severe destruction of the endocrine compartment). [87].

Anyhow, loss of beta cell mass is the hallmark of most forms of diabetes. Thus, development of therapeutical strategies based on endogenous islet regeneration or ex vivo proliferation is an attractive option.

Ex vivo expansion of human islets has been very difficult. After a period of time, insulin production and secretion capacity decline in these cells, probably caused by aging mechanisms and *in vitro* dedifferentiation of beta-cells. In recent years, however, *in vivo* and *ex vivo* experiments with human tissue using biomolecules have shown promising results. Beta-cellulin, which has been long recognised as a beta-cell mitogen, [88] promotes beta cell regeneration in diabetic mice [89]. Activin A, another regulatory peptide of cell growth and differentiation, has been shown to induce endocrine differentiation in human foetal pancreatic cells [90]. When administered in combination, beta-cellulin and activin A significantly increase islet regeneration and ameliorates glycemic control [91].

Observations in the 90% pancreatectomized mice model provided first evidences for the major role of insulin growth factor -1 (IGF-1) in the regeneration or replication of beta-cells in the injured pancreas [92]. Other factors such as hepatocyte growth factor (HGF) and fibroblast growth factor (FGF), particularly when combined with serum free medium, have also been successful in expansion of cultivated pancreatic islets [93, 94].

Insulinotropic agents, such as nicotinamid have been shown to prevent insulitis and to induce beta cell proliferation *ex vivo* as well as *in vivo* [95, 96]. Also gastrin and epidermal growth factor (EGF) have been reported to enhance beta-cell mass expansion, both *in vitro* and *in vivo* [97]. In culture, however, islet-treated cells show a short life-time, display progressive loss of insulin secretion and dedifferentiate. *In vivo,* combined therapy with gastrin and EGF partially restored beta cell mass and reversed hyperglycaemia, in non-obese diabetic (NOD) mice [97, 98]. More recently, it was shown that combination therapy with gastrin and EGF in NOD diabetic mice transplanted with syngeneic islets inhibits autoimmune recurrence that normally occurs after transplantation and delays rejection of syngeneic grafts [99].

Another group of intestinal peptides, the incretines, are already successful therapeutic agents in T2D. Indeed, glucagon -like peptide1 (GLP-1) and its analogue exendin-4 (exenatide) are able to promote beta-cell mass expansion and inhibit apoptosis *in vitro and in vivo* [100, 101].These peptides are also associated to a number of important physiological effects such as inhibition of glucagon release, delay of gastric emptying and reduction of appetite [102]. Molecular studies have shown that Exendin-4 enhances beta-cell proliferation by stimulation of cell cycle regulators such as cycline-A2 via the cAMP dependent pathway. PDX1 transcription factor is essential for cAMP activation in the beta-cell.[103, 104]. Whether incretins exert protective and proliferative effect on beta-cells in a T1D context remains unclear. In animal models of T1D, there is much less evidence for a beta-cell preserving effect [105].

Nevertheless, combination therapy with GLP-1 and gastrin restores normoglycemia in diabetic NOD mice by increasing the pancreatic beta-cell mass and reducing the autoimmune response.[106]

3.2. Immunomodulation in Type 1 Diabetes Mellitus

In vivo restoration of beta-cell mass might prove beneficial for the reversal of hyperglycaemia. Yet, for this to be effective, the expanded beta-cells must be protected from ongoing autoimmune destruction by immunesuppression or even better, by immunomodulation approaches.

First approaches towards suppression of T lymphocytes were performed using cyclosporine [107, 108]. However, withdrawal of cyclosporine led to recurrence of the symptoms implying lifelong administration of the agent with the known risks of chronic immunosuppression.

First studies, using monoclonal antibody against CD3 in NOD mice, have shown that short-term treatment could induce remission of established diabetes [109]. Anti-CD3 exerts an immunomodulatory effect that involves the transforming growth factor beta (TGFβ)-producing regulatory T cells (Treg). By stimulation of the Treg cells (CD4+, CD25+, foxp3+), antiCD3 IgG induces a state of immunotolerance and blocks the autoimmune destruction allowing islets regeneration and prevention of the disease[110]. In this context, a two-phase clinical trial was performed in recent-onset T1D patients using two humanized recombinant antiCD3 antibodies- OKT3 or ChAglyCD3, to evaluate the long-term metabolic effects of this immunoregulatory strategy in a randomised placebo-control trial [111, 112]. Results showed a higher C-peptide level and lower insulin dosis requirements in treated patients after 18 months of trial, suggesting that antiCD3 therapy has the capacity to preserve existing beta-cells. While it confers a metabolic benefit no insulin independence was observed. Moreover, side effects such as flue-like symptoms as well as signs of clinical and laboratorial Epstein - Barr virus (EBV) infection were detected in all patients. Consistent with transient cytokine release following the first infusions, all symptoms disappeared 5-10 weeks after treatment.

Other immunomodulatory regimens to prevent destruction of beta-cell mass are currently on final clinical trial phases. Diapep277 is a 24 aminoacid peptide with similariries to the heat shock protein 60 (HSP60), which is a beta cell membrane auto-antigen that contributes to the autoimmune reaction in T1D and LADA. Diapep277 has been suggested to exert immunomodulatory effects on diabetogenic T lymphocytes, therefore protecting beta cell from ongoing immune destruction and sustain insulin secretion without causing immunosupression [113]. Results from several clinical trials revealed that treatment of adult T1D patients with Diapep277 significantly inhibits a decrease in stimulated C-peptide secretion (thus preserving beta cell function) without adverse effects. Also, it ameliorates glycemic control compared to placebo treated patients [114]. However, in children at early onset of T1D, Diapep277 appears to have no beneficial effect in preserving beta-cell function or improving metabolic control [115].

A recent approach, is the vaccination against the main beta-cell autoantigen, the glutamic acid decarboxylase 65 (GAD65), using adenoviral recombinant GAD65 peptide. This therapy has been effective in reducing pancreatic insulitis and insulin requirements in newly diagnosed T1D mice [116]. Results of a phase II clinical trial on 70 recent diagnosed T1D patients (10-18 years old) treated with recombinant GAD peptide indicate, that two dosis of GAD autoantigen leads to preservation of residual beta cell mass by stimulation of an antigen-specific T cell population and induces long- lasting modulation of the general B cell memory [117].

The beneficial effects on preservation of insulin secretion, common to all this immunomodulatory agents, are more notorious in patients that still have a high beta-cell reserve at start of the treatment (in the first 3 months after diabetes onset).

Other most interesting agents with recognized immunomodulatory effects in T1D such as human chorionic gonadotrophic hormone (hHCG) [118], TGFβ1 [119] and humanized

anti-thymocyte globulin (ATG) [120] are currently under investigation. Complete Freund's adjuvant (CFA), an antigen presenting cells (APC) activator, has shown to upregulate the immunosuppressive T regulatory (Treg) cells (CD4+, CD25+ and foxp3+), thus preventing the onset of diabetes in NOD mice [121]

3.3. Anti-inflammatory intervention in T1D and T2D

Inflammation plays a major role in the pathogenesis of T1D. Interleukine-1 beta (IL-1b), tumor necrosis factor alpha (TNFα) and interferon gamma(IFNγ) are pro-inflammatory cytokines involved in the course of insulitis that occurs during the autoimmune process of T1D. IL-1, in particular, modulates the interaction between the innate and adaptive immune systems. The apoptotic effects of IL-1 pathway on beta-cell and its role on T-lymphocyte regulation point to this molecule as beeing a potential interventional target in autoimmune diabetes mellitus [122]. Interestingly, Ehses and al. have shown that islets produce cytokines and chemokines in response to a T2D milieu of circulating nutrients [25]. These findings suggest that opposite to previous knowledge, the pathophysiology of beta-cell apoptosis and islet failure observed in advanced T2D also entails an inflammatory process in response to the metabolic stress (in particular glucose toxicity) [24]. In fact, beta-cells of T2D, when compared to those of non- diabetic patients have shown increased IL-1b concentration[123]. Moreover, gene array analysis of islet- RNA from T2D patients revealed enhanced IL-1b expression, induced by glucose and IL-1b auto-stimulation in beta-cells [124]. Hence, modulation of intra-islet cytokines such as IL-1b may represent a promising therapeutic approach in T2D and T1D. Indeed, in a randomised double-blind placebo clinical trial with T2D patients, the administration of IL-1 receptor antagonist (IL-1Ra) significantly improved glycemic control, ameliorated insulin secretion and reduced inflammatory markers [125]. Even though, only

44% of the treated patients responded to this therapy, which seems to be associated with lower levels of IL-1 Ra- predisposition in these individuals. In animal models of T1D, genetic or pharmacological blockage of IL-1 action also reduces disease incidence. Presently, clinical trials have been started to study the feasibility, safety and efficacy of IL-1 therapy in patients with T1D (AIDA study group)[122].

3.4. Cell-based therapy

Cell therapies are based on the utilization of autologous or allogenic uncommitted multipotent stem/progenitor cells to replace or regenerate injured cells and tissues that account to the pathogenesis of multiple chronic and degenerative diseases. Basic and clinical research accomplished in the last years strongly suggest that stem/progenitor cells can be 1) induced *ex vivo* to proliferate and/or differentiate into specific cell lineages or 2) they can be *in vivo s*timulated to proliferate or to regenerate injured tissues. 3) Genetically modified stem/progenitor cells could also be used as a delivery system for therapeutic molecules into specific damaged tissues. On the other hand, development of therapies targeting cancer related stem- cells and its microenviroment represents a most promising approach in cancer research [126]. Therefore, recent knowledge in stem cell biology has been providing enough evidence to the potential of "specific cells" to be used as tools or targets in regenerative medicine and cancer therapies. Parkinson disease, multiple sclerosis, acute myocardial infarction and bone fractures are some of the conditions where cell therapy approaches have been applied with positive outcome. Experimental settings for the cell-based therapy of neonatal hypoxia in animal models have also shown promising results. Moreover, efforts to identify, isolate and characterize tissue-resident progenitor cells in organs such as retina,

lung, intestinal tract, liver and pancreas, which are often affected by irreversible conditions, also represent major goals in stem cell biology research.

3.5. Cell-based therapy in diabetes

Cell-based therapy for T1D and advanced T2D, as in many other disorders has become a main research issue in diabetes field. There is not much doubt that functional insulin phenotypes are required in order to replace damaged beta-cells, so that insulin independence can be achieved in these patients.

Endogenous insulin secretion could be restored for the first time, in 1966, after pancreas organ transplantation in a renal end-stage diabetic patient. But this recipient died 2 months later from surgical complications. Since then important lines of investigation have been developed towards finding an alternative source of functional beta- like cells [75] (Table 1).

Islet Transplantation
Human pancreatic islet transplantation
Xenotransplantation (porcine origin)
In vitro generation of human insulin producing cells
Generation of beta-cell lines (rodent and human)
Stem cells:
Embryonic stem cells
Adult stem / progenitor cells
Pancreatic (ductal, acinar, islet derived cells)
Extrapancreatic (liver, CNS, bone-marrow, fat)
Induced pluripotent stem cells (iPS)
In vivo reprogramming of beta cells

Table 1: Potential sources for cell-based therapy in type 1 diabetes mellitus

Adapted from Limbert C. et al. Diabetes Res Clin Pract. 2008 Mar; 79(3): 389-99. Epub 2007 Sep 12.

3.5.1. Islet Transplantation

Pancreatic islet transplantation represents an almost ideal approach to cure Diabetes Mellitus type 1. Data from the Edmonton protocol have convincingly demonstrated that in selected T1D patients (recurrent episodes of hypoglicemia, difficult metabolic control), human islet-transplantation (through the portal vein into the liver) leads to insulin independence and normalization of glycemic control [127]. Compared to pancreas organ transplantation, human pancreatic islets engraftment is technically easier, has lower morbidity and permits storage of the islet graft in tissue culture or cryopreservation for banking [128]. The low morbidity of the procedure and the potential for inducing tolerance to the grafted tissue define islet transplantation as a promising strategy for correcting diabetes in young patients, including children [129, 130]. However, due to shortage of organs and life long immunosuppression this therapy can only be offered to a very limited number of patients. Besides, insulin independence lasts no longer than 2 years in 90% of transplanted subjects [131] (Fig 7). This fact might be due to ongoing autoimmune destruction or even to the aggressive immunosuppressive regimens, which inhibit beta-cell regeneration and prevent the normalization of glucose homeostasis [132]. Typically, about 850.000 islets (11.000 islet equivalents/Kg or 2 human pancreas) are required for a single transplantation, which must be repeated to achieve insulin independence.

As a result of these difficulties, intensive search for alternative sources of transplantable insulin producing cells has been undertaken in the last few years.

Grafts of porcine islets represent a valuable source for obtaining beta-cells. However, the natural immunological inter-species barrier is of great disadvantage and several techniques have been developed to avoid xenotransplant immune rejection. Alginate-

based microcapsules to involve the grafted islets have proven to confer immunoisolation and to allow for metabolic and nutrient changes that are essential for islet survival [133]. Recently, better results have been achieved by inducing donor-specific tolerance in islet graft recipients. Both, the administration of T-cell suppressive adjuvants, such as CTLA4 (CD152) Ig+ combined with anti-CD154 monoclonal antibody (mAB) or treatment with anti-adhesion molecule LFA-1 (CD11a) together with anti-CD154 mABs, prolonged viability of microencapsulated porcine islets and restored normoglycemia for more than 1 year in transplanted diabetic mice [134, 135].

Another approach is the utilization of islet grafts obtained from transgenic pigs, which do not express xenogenic surface antigens [136].

Yet, pig islet xenotransplantation in humans is far from being clinically applicable due to risk of transmission of porcine pathogens to the human species. Particularly, the porcine endogenous retrovirus (PERV) zoonose, constitutes a major obstacle, since it is integrated in the pig genome and might be transmitted to the human cells *in vitro*, even if the islet's source is a pathogen-free pig herd [137].

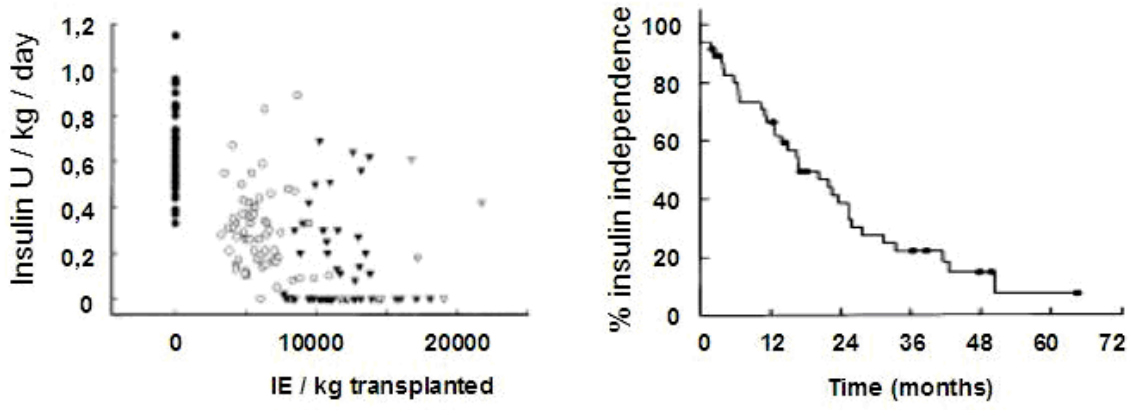


Fig 7: Five years follow-up of transplanted patients of the Edmonton's protocol.

Insulin independence requires repeated islet transplantation. Insulin independence significantly decreases after 24 months. Adapted from Ryan et al., 2005, Diabetes.

3.5.2. Beta cell lines

Rodent cell lines are extremely useful for studies on islet cell biology and on the transcriptional cascade of endocrine differentiation. Insulin secretory physiology has been characterised in tumor cell lines derived from primary culture of insulinomas developed in transgenic mice expressing the large T-antigen of SV40 in pancreatic beta-cells. Nevertheless, due to their tumorigenic and allogenic characteristics, these animal cell lines cannot be used for clinical purposes.

Therefore, great effort has been made in order to immortalize human beta cells [138, 139]. This has proved to be a very difficult task because human beta-cells do not proliferate *in vitro* and dedifferentiate very rapidly in culture. Moreover, most of the human beta-cell lines generated tend to display an unstable phenotype, including loss of regulated insulin secretion in culture conditions.

A reversibly immortalised human beta-cell line was established through introduction and subsequent removal of two immortalising genes, SV40T and hTERT [140]. Transplantation of these cells into streptozotocin-induced diabetic mice resulted in apparent control of blood glucose levels for longer than 7 months. Although this human pancreatic beta-cell line seems to be functionally equivalent to primary cells, the use of highly genetically manipulated cells for clinical applications should be avoided, since their phenotypic features are not fully known and their potential for tumours formation is not excluded.

3.5.3. Stem cells

Stem cells are uncommitted cells with self-renewal capacity (symmetric division), which under specific conditions are able to differentiate into mature somatic cells (asymmetric division) (Fig 8).

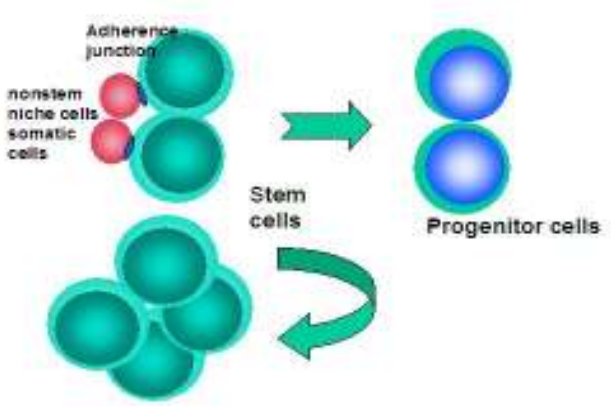


Fig 8: The stem cell niche.

> Somatic cells residing in stem cell niches are main regulators of stem cell differentiation and proliferation. Published in Jakob F, Limbert C, et al. Current Rheumatology Reviews, 2008, 4, 148-154

Embryonic stem cells (ES) are pluripotent diploid cells, derived from the inner mass of the mammalian blastocyst. They indefinitely proliferate in an undifferentiated state and can be induced to differentiate into cells of all three germ layers, ectoderme, mesoderme and endoderme, both *in vivo* and *in vitro* (Fig 9).

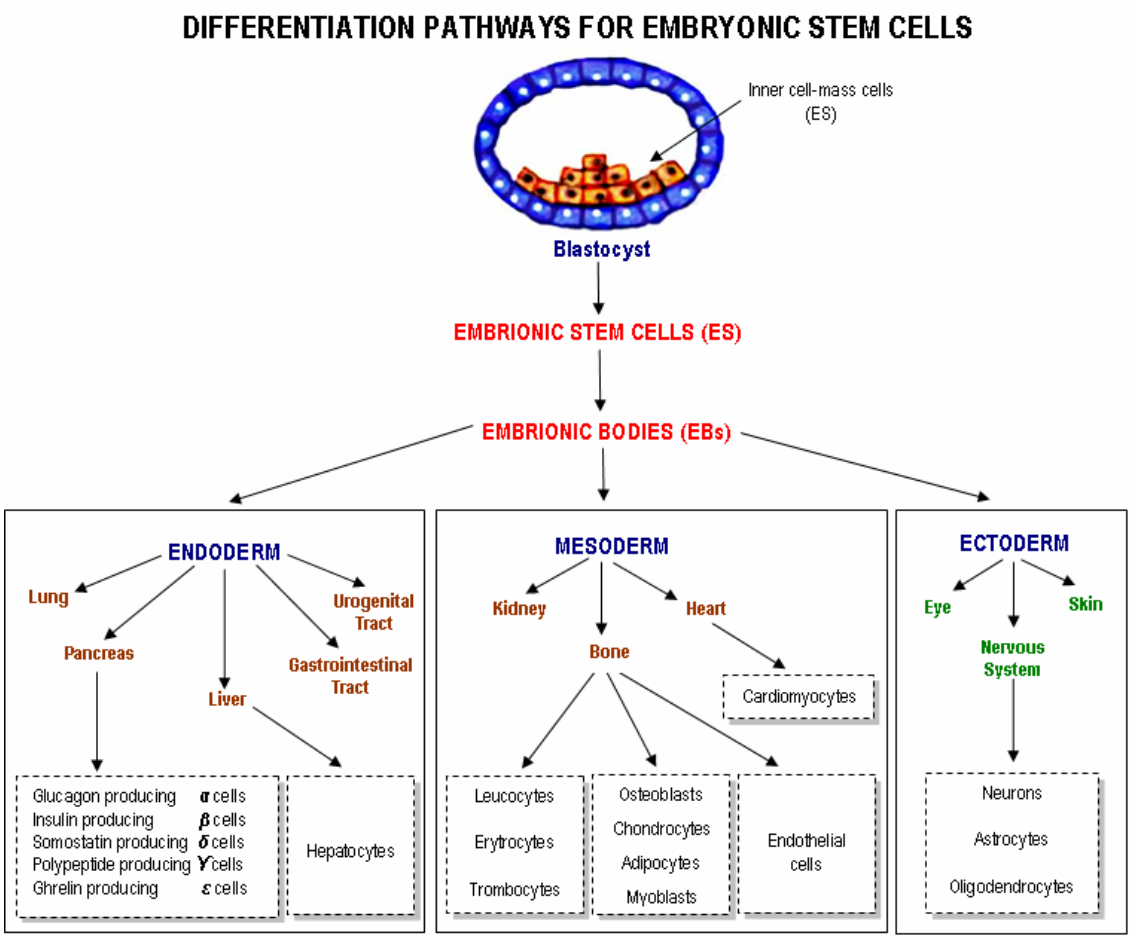


Fig 9: Schematic diagram showing the possible differentiation pathways of ES

Adapted from Mimeault, M. et al. Stem Cells 2006; 24:2319-2345

Adult stem cells are present in most adult tissues. They reside in well-identified stem cell niches, which have been already recognized in several organs including intestinal, gonadal, hematopoietic tissues and hair follicle [141, 142] (Fig 10). Adult stem cells remain in its undifferentiated state until injury or unbalanced cell turnover occurs (proliferation versus apoptosis). Specific somatic cells residing within the niches play a key repressive or inductive regulatory role on stem cell fate [143] (Fig 10).

Identification of different stem cell populations for clinical diagnosis, basic research and therapeutical strategies has been one of the most challenging tasks in stem cell research. Expression of classical stem-cell related markers such as CD45, CD34, CD133, nestin

and/or ABCG2 in adult stem cells as well as OCT-4, SSEA-4 and Nanog in embryonic stem cells (ES) and very small embryonic-like stem cells (VSEL) [144] have been of great help to isolate and characterize these cell populations. However, recent advances in molecular biology and flow-cytometry techniques, (with multi-parameter analysis of over 6-8 markers at the same time), strongly suggest that the same stem cell type may display different phenotypes and that different stem cell populations show an overlapping immunophenotype [145]. One hypothesis to explain these findings is that organ-specific environment induces the different expression patterns, which points out to a commitment of recognized stem cells to the tissue where they reside. Thus, concepts such as "functional signature" and "positional memory" from the developmental biology may be applied to recognized stem cell populations [146]. Another explanation might be that phenotypes differ because of different isolation and enrichment procedures.

Several in vitro and in vivo experiments have suggested that adult stem cells from different origins could be induced to replace or regenerate injured tissues by overwhelming germ layer boundaries [147, 148]. Yet, understanding the key molecular mechanisms that control stem cell fate in order to enable effective manipulation of cell populations is still required. In this context, DNA-encoded small molecule libraries, which have been useful in proofing biological systems in cancer research are now being adopted for stem cell-based therapies. Recent literature has described several natural and synthetic small molecules with the capacity to control stem cell self-renewal by targeting signal transduction pathways that affect DNA replication, cell differentiation and apoptosis. Examples of these compounds are retinoids, Activin-A, TGFbeta1, epidermal and hepatocyte growth factors [149]. Undoubtedly, chemical approach to stem

cell biology and regenerative medicine seems to hold great promise as a tool for cell replacement therapies.

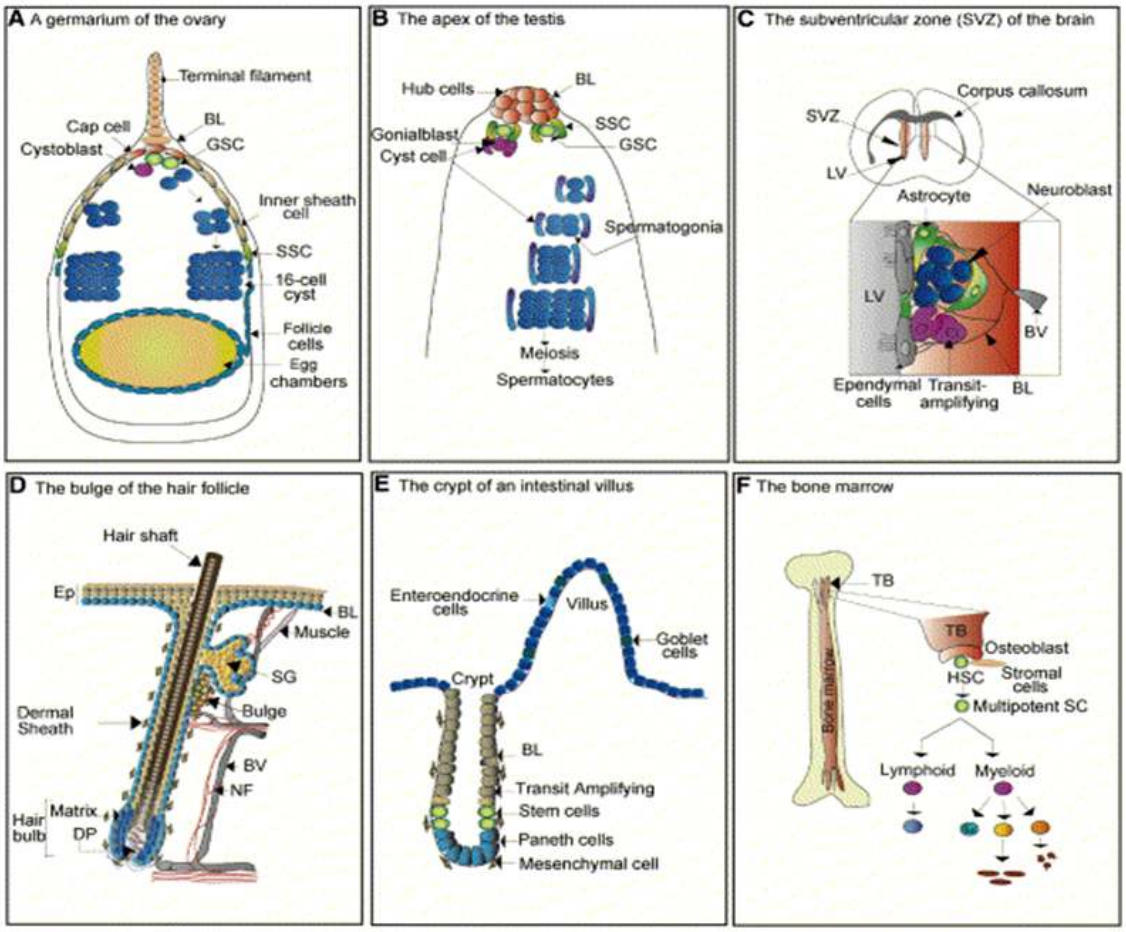


Fig 10: The stem cell niches and their neibourghs

> Stem/progenitor cells reside in several adult tissues. Specific somatic cells take part of the stem cell niche environment. Source: Fuchs E. et al, Cell 2004, Vol. 116, 769–7

Embryonic stem cells (ES) and diabetes

A remarkable step in diabetes cell-therapy research was achieved with the first experiments on mouse ES, demonstrating that such cells might constitute an unlimited supply of beta-cells [150]. Subsequent studies have shown that, rodents [151, 152], monkey [153] and humans ES [154, 155] could be induced to differentiate into insulin-producing cells.

Nevertheless, either with techniques of cellular genetic manipulation [150, 156] or with utilisation of specific culture conditions [157, 158] beta cell-like phenotypes did not exhibit significant insulin content or a physiological regulation of insulin secretion. Actually, insulin secretion in differentiated ES never exceeded 1,6% of the amount that a native beta-cell typically secretes.

In other remarkable studies, mouse and human ES could be successfully differentiated into definitive endoderm (supposed precursors for pancreatic cells) [159, 160]. Further induction towards beta-like cells showed a considerable quantity of insulin production but, so far, no glucose-responsive insulin secretion was observed in these phenotypes [161-163].

Importantly, ES investigation has been crucial to uncover developmental steps of the human pancreatic endocrine cell *in vitro* since up to this point almost all available data could only be obtained from animal models. One example is the recent knowledge that specific small molecules, such as FGF2, FGF4, retinoic acid and activin-A play a major role in the activation of major signalling pathways required for the differentiation of ES into pancreatic endoderme. Such discoveries have been of great help to further understand embryonic pancreas development and to improve the methodology for high-efficiency beta-cell differentiation *in vitro* [164-166].

Along with major ethical concerns regarding the harvesting of human ES, a crucial limitation of ES transplantation is the associated risk of teratomes and teratocarcinomes in humans. This risk has been delaying the breakthrough of ES for the development of new forms of regenerative medicine [167].

Adult stem cells within the pancreas

Intensive efforts have been made to identify pancreatic stem/progenitor cells within the endocrine pancreas since its isolation and *in vitro* proliferation may represent an ideal solution for a cell-based replacement therapy in T1D.

The first evidence for the existence of a pancreatic stem/progenitor cell came from studies in developing rodent embryos which demonstrated that exocrine and endocrine cells share a common origin of pancreatic endoderm [168]. Data showing the potential of very early murine embryonic pancreatic Pdx1+, Cpa-1+ cells to differentiate into all cell lineages strongly indicates the presence of a multipotent pancreatic stem cell [169]. Using a single cell cloning approach, Seaberg et al could identify rare multipotent precursor cells isolated from islets and ducts of adult mouse pancreas capable of producing neuronal populations of cells and exocrine, endocrine and stellate pancreatic cells [170]. Interestingly, embryonic and even adult pancreatic cells expressing the basic helix loop helix neurogenin 3 gene (NGN3/Neurog), can only give rise to cells of the endocrine pancreas [170, 171]. Thus, **Ngn3 is described as a key control factor for endocrine cell fate specification in uncommitted pancreatic progenitor cells, being proposed as a marker of islet precursor cells** [172]. Yet, the existence of a "true" pancreatic endocrine stem cell has been strongly questioned. Genetic lineage tracing studies have demonstrated that in steady states, pregnancy and animal models with 50-70% pancreatic injury, physiological beta-cell regenerative process corresponds to the replication of existing mature beta-cells, and not to differentiation of pancreatic progenitor cells [79, 132, 173]. These data conflicts with several other studies that corroborate the existence of pancreatic precursor cells in pancreatic islets [76, 81], ducts [80] and acini [82], within the adult and fetal pancreas.[170, 174, 175]. More recently, using a unique pancreatic

injury model of duct ligation, Xu and co-authors have produced evidence for *de novo* generation of beta-cells from Ngn3+ pancreatic progenitor cells. Again, these cells could be found in islets, ducts, acini and epithelial enriched populations [86].

Based on the previously described confirmed data, we can hypothezise that adult pancreatic regeneration occurs upon several rescue mechanisms depending on the physiological needs and injury degree. Apparently, a spectrum of pancreatic progenitors with several lineage potentials resides in the adult pancreas. Then, perhaps under steady-states, pregnancy, post natal growth and milder injuries, recruitment of existing mature beta cells may be sufficient to restore glucose homeostasis, whereas severe beta cell destruction (90% pancreactomy or in duct ligation models) implicates differentiation of stem/progenitor cells residing in the pancreas[87].

The isolation of a distinct stem/progenitor cell within the endocrine pancreas depends on the identification of specific progenitor markers. Nestin, an intracytoplasmic filament protein present in neuro-precursor cells was suggested as candidate marker by several groups [176, 177]. Nestin^{+} islet-derived cells display endocrine differentiating capacity. However, further investigation revealed its transient expression profile in very early phases of pancreatic development, which explains the recent observations that nestin+ cells located in the primitive pancreas generate both, the cells of endocrine and exocrine lineages [178, 179]. In addition, nestin seems to be implicated in assimetrical division of stem cells [180] and so far it is considered a characteristic marker of multi-lineage progenitor cells.

Suzuki et al identified a cluster of surface antigen markers to obtain enriched populations of stem/ precursor cells from the adult and fetal mouse pancreas.These cells were positive for the hepatocyte growth factor (HGF) receptor gene (c-met/MET) and negative for the hematopoietic markers CD45, c-kit , FLk-1 and TER119 [181]. In humans,

however, the receptor c-Kit, which is known as an important hematopoietic precursor cell marker has been found to be dynamically expressed during foetal pancreas neogenesis. Its phosphorilation may be involved in early beta cell differentiation and survival, proposing c-kit as marker for human pancreatic progenitor cells [182]. The conserved surface markers CD133 and CD49low were detected in mouse foetal pancreatic epithelium (at E13,5 weeks) and might also be relevant for the detection of stem/progenitor cells within the pancreas (Ngn3+ cells) [183].

Extrapancreatic stem cells and diabetes

Increasing knowledge concerning adult stem cells properties, namely self-renewal and high plasticity, has raised the interest for alternative cell sources, outside the pancreas, to be used in cellular-based strategies for Diabetes Mellitus. Indeed, extrapancreatic stem cells from liver [184, 185], central nervous system [186] and bone marrow [84, 187] have been described to differentiate into insulin-producing phenotypes. Also umbilical cord-derived mesenchymal stem cells (UC-MSC) have been found to differentiate in vivo into insulin-producing cells and to ameliorate glycemic control and glomerular hypertrophy when transplanted in T2D mice model [188] as well as in partial pancreactomized mice [189]. MSC isolated from human umbilical cord Wharton's jelly, which are easily available, present no risk of discomfort for the donor, and low risk of rejection also possess insulin producing ability *in vitro* and *in vivo*[190]. Moreover, the observation of spontaneous expression of pancreatic endocrine transcription factors in human adipose tissue- derived MSCunder specific culture conditions, also suggests the endodermal switching capacity of these cells [191]. In spite of this, none of these studies could generate true beta-cells with the capacity to secrete significant amounts of insulin in a glucose-sensing manner.

Bone marrow derived–mesenchymal stem cells (BM-MSC)

The multilineage differentiation capacity of mesenchymal stromal cells (MSC) has been firstly described in the bone marrow (BM) [192]. In BM, there are at least two distinct populations of cells, both with high plasticity; hematopoietic stem cells (HSC) - the precursors of all mature blood cells [193], and mesenchymal stem cells (MSC). BM-MSC is a specific population within marrow stromal cells that has been shown to differentiate into mesodermal lineages, including fat [194], bone [195], cartilage [196], muscle [197] and also towards epithelial [198], neuroectodermal [199] and endodermal phenotypes [200]. Although no specific markers have been identified until now, isolation of BM-MSC is based on the selection of CD45-, CD14-, CD29+, CD90+, CD105+ and CD79+ bone marrow derived cells.

Due to their migration and homing capacity, mesenchymal cells play a fundamental role in embryonic morphogenesis, tissue repair and regeneration [201]. In BM-MSC, expression of several chemokine receptors, such as CXCR4 receptor for stem-cell factor 1 strongly suggests these properties [202]. Furthermore, extensive *in vitro* studies have suggested that BM-MSC exert immunosuppressive effects via modulation of both cellular and humoral immune pathways [203-206]. In diabetes, one of the mechanisms by which MSC can prevent rejection of allogenic islet grafts is through the immunosuppressive activity of matrix metalloproteinase-2 and-9 (MMP2 and MMP9). Both metalloproteinases, seem to reduce surface expression of CD25 on responding T-cells [207].

Due to their immunophenotype, BM-MSC are recognized as non-immunogenic cells (MHCI+, MHCII -, CD80-, CD86-, CD40-). This feature would allow for their allogenic transplantation with no need for immunosupression. On the other hand, the stromal supporting nature of MSC offers an additional advantage for maintenance of the

grafts or regeneration of injured tissues (Fig 11). Thus, MSC immunomodulatory and tissue supporting properties have raised the possibility of establishing allogeneic MSC banks for tissue regeneration. These facts are strongly reflected in the current exponential growth in stem cell research in the pharmaceutical and biotechnology communities

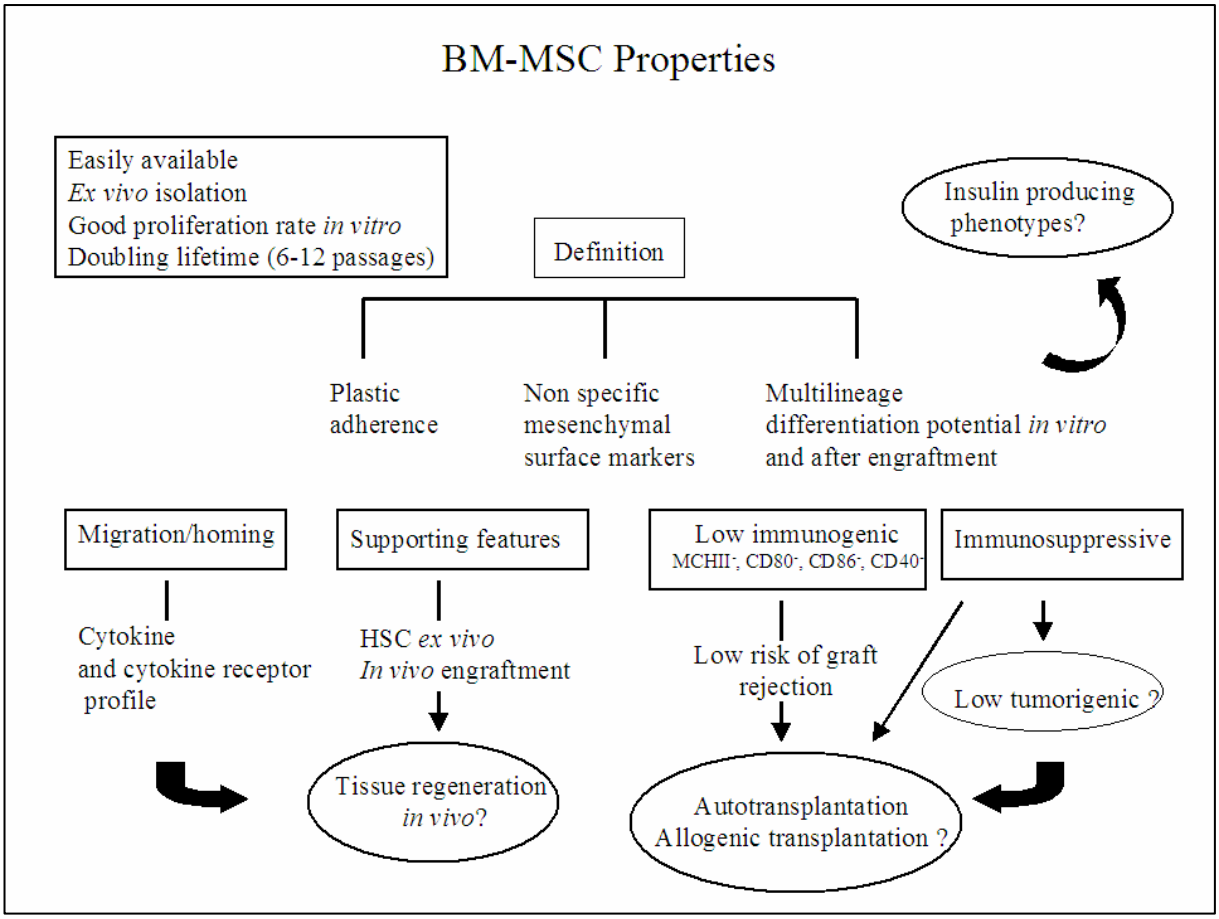


Fig 11: The bone marrow derived mesenchymal stem cell (BM-MSC) properties

Bone marrow derived mesenchymal stem cells (BM-MSC) are defined by plastic adherence, expression of unspecific surface antigen markers such as CD90, CD105, CD79, CD29 and differentiation capacity into mesenchymal lineages including bone, cartilage, fat and muscle. Functional characterization of BM-MSC, have shown most important features, which renders these cells very attractive for cell-based therapies and in particular for beta-cell replacement in T1D. Published in Limbert C et al. Pediatr Diabetes. 2009 Sep; 10(6):413-9. Epub 2009 Jul 13. Review.

Reprogramming BM-MSC into insulin producing phenotypes

In recent years, increasing body of evidence suggests that BM-MSC have the potential to differentiate *in vitro* into insulin producing cells in mouse [187, 208, 209] and human [210-212] as well as to revert hyperglycemia in diabetic mice models [187, 209-211]. First results have been achieved by *ex vivo* expansion of plastic adherent BM-MSC and prolonged endocrine culture conditions [187, 208, 209]. More recently, an endocrine beta-cell like phenotype with higher differentiation efficiency was obtained by forced expression of a pancreatic endocrine key factor, the pancreatic duodenal homeobox 1 gene (PDX1) in primary BM-MSC. [210, 211]. , Also, epigenetic modification could induce differentiation of mice BM-MSC into insulin-producing cells, even in a glucose-sensing manner [213]. However, efficacy of BM-MSC differentiation has been low and highly variable. This might be related to donor-specific variability, heterogeneity of MSC populations, and non-optimized differentiation protocols. Still, there might be unknown BM-MSC populations with suitable endocrine commitment to be found.

iPS (induced pluripotent stem cells)

Reprogramming adult somatic cells implicates induction or repression of key regulatory genes, which are able to switch cells from one lineage into another or to dedifferentiate a mature phenotype back to a more primitive state in order to obtain pluripotency.

In pioneering experiments mouse and human fibroblasts have been successfully reprogrammed into ES-like stem cell lines. These induced pluripotent stem cells (iPS) were generated by retroviral or adenoviral transduction of 4 transcription factors (Oct3/4, Sox2, c-Myc and Klf4) that completely modified the epigenetic signature and phenotype of these cells [214, 215]. Using the same techniques, murine liver and stomach cells have also been reprogrammed into iPS cells [216]. iPS cells show characteristic

features of reprogrammed cells such as DNA demethylation, expression of endogenous pluripotency genes, give rise to teratomas, and contribute to multiple tissues, including the germ line, in chimeric mice.

More recent reports show that Oct4 alone is sufficient to reprogram directly adult and fetal mouse neural stem cells to iPS cells [217]. The therapeutic potential of iPS has been recently demonstrated by using reprogrammed fibroblasts for neural cell replacement in Parkinson's disease animal model. In this study, it has been demonstrated that iPS cells can be efficiently differentiated into neural precursor cells, giving rise to neuronal and glial cell types in culture. Upon transplantation into the fetal mouse brain, the cells functionally integrate into the brain regions and ameliorate the symptoms [218]. However, current methods for reprogramming require infection of somatic cells with multiple viral vectors, thereby excluding their use in transplantation medicine at this time. Anyhow, these are important achievements that raise hope in every field of regenerative medicine, including the ambitious target of producing patient-specific pluripotent stem cells. Noteworthy and extremely valuable in medical research, is the derivation of human iPS cell lines from patients with human genetic diseases to be used as a complement to animal models of disease [219].

In the context of T1D, generation of functional beta-cell like phenotypes from skin fibroblast- derived iPS has already been achieved [220]. In addition, Maher et al. were able to dedifferentiate adult somatic cells of two T1D patients, into iPS, which have the hallmarks of pluripotency and can be induced *in vitro* into insulin secreting cells [221].

3.6. In vivo regeneration of the endocrine pancreas

In a most challenging study, the combined injection of adenoviral transduced key pancreatic endocrine transcription factors, Pdx1, Maf-A and Ngn3 in diabetic mice was

capable of reprogramming mature exocrine cells into beta-cell like phenotypes[222]. In other recent report, recombinant Pdx1 protein (rPdx1) was directly injected via intra-peritoneal to mice with streptozotocin induced diabetes. Pdx1 transcription factor possesses a protein transducer domain (PTD) which facilitates its entry into the cells. By a mechanism of cellular transduction, rPdx1 treatment promoted beta cell regeneration, differentiation of liver cells into insulin producing cells and restoration of normoglyceamia in diabetic mice. Avoiding potential side effects associated with the use of viral vectors, this innovative PTD-based protein therapy may offer a promising way to treat patients with diabetes [223].

4. FUTURE PERSPECTIVES

Current therapeutic strategies of T1D are based on glyceamic control in order to minimize acute and late complications of diabetes.

Decades of investigation provided fundamental knowledge regarding genetics, pathogenesis and pathophysiology of diabetes, guiding the development of new targeted therapies.

Indeed, increasing amount of data lead us to expect that modern therapeutic approach of diabetes might soon be “curative” instead of symptomatic.

Considering the previously described lines of investigation and respective results, it is quite clear that the cure for T1D and advanced T2D will require a combination of distinct strategies. Cell-based therapies may recover endogenous insulin production but modulation of the autoimmune disease behind T1D is essential to achieve an effective and long-lasting therapeutic intervention.

Recovery of beta-cell function may be attained by stimulation of beta cell regeneration or by replacement of insulin-producing cells. It has been already demonstrated that endogenous beta cell-regeneration occurs, but the cellular and molecular pathways implicated in the proliferation of beta cell mass are still uncertain. Moreover, cells that can be differentiated into glucose-responsive insulin cell-types, without any risks after transplantation, have not yet been identified.

Most encouraging approaches, based on the utilization of natural or synthetic small molecules to selectively regulate stem cell fate *in vitro* and *in vivo* are under investigation. Also, development of therapeutic compounds to stimulate endogenous cell regeneration or to induce mechanisms of homing and migration are important research strategies in the regenerative medicine field.

In Diabetes, although progress is encouraging, major gaps in our understanding of developmental biology of the pancreas and adult beta-cell dynamics remain to be bridged before a therapeutic application is made possible.

B- RESEARCH PROJECT

1. OBJECTIVES

The main goal of the investigation presented in this dissertation was to evaluate the potential of adult MSC to be used as alternative sources for the cell-based therapy of T1D and advanced T2D.

In this context our objectives were:

I. *In vitro* differentiation of hMSC-TERT towards insulin producing cells

To determine the capacity of the human cell line hMSC-TERT, to be induced into the endocrine pathways and thereby beeing adopted as a suitable MSC cell model for further molecular endocrine diferentiation studies.

II. Comparison of the functional signature of primary hIPC and hBM-MSC

To characterize the primary hIPC population and to compare its multilineage differentiation capacity with primary hBM-MSC, the gold standard of MSC. To determine the molecular signature and functions of hIPC compared to hBM-MSC at the gene expression level. To identify key target genes, useful for the isolation of MSC, holding an endocrine reprogramming potential.

2. LINES OF INVESTIGATION/WORKSHEET

I. In vitro differentiation of hMSC-TERT towards insulin producing cells

This part of our project was entirely developed at the Laboratory for Metabolism, Endocrinology and Molecular Medicine of the Medizinische Klinik und Poliklinik II at the Universitätsklinikum Würzburg under the leadership and supervision of Professor Jochen Seufert and later on by Professor Franz Jakob.

This work has been submitted to Cytotherapy.

Evaluation of hBM-MSC as alternative source for cell-based therapy of T1D

To investigate the differentiation potential of adult MSC towards insulin producing cells, preliminary experiments were performed using primary human bone marrow-derived cells. Isolation, proliferation and immuno-characterization using hematopoietic, endohelial and mesenchymal surface antigen markers were undertaken and results were compared to other cell populations such as human umbilical cord cells (hUCB), pancreatic islet- progenitor cells (hIPC) and hMSC-TERT cell line.

In vitro differentiation of hMSC-TERT using specific culture conditions

Difficulties to acquire human cells due to ethical concern, the limited proliferation rate related to cell senescence in primary cells and donors-related variability, led us to investigate the potential of the hMSC-TERT cell line, as a suitable cell system for endocrine differentiation studies in human MSC phenotypes. This cell line was gently offered by Professor Kassem from the University of Odense, South Danemark. Simonsen et al., [224] previously demonstrated the differentiation potential of hMSC-TERT towards distinct mesodermal lineages, thus indicating its multipotent stem cell character.

To test the pancreatic endocrine differentiation capacity of hMSC-TERT, *in vitro* differentiation was induced by means of specific culture conditions. The expression of islet endocrine genes was analysed.

In vitro reprogramming of hMSC-TERT by ectopic expression of pancreatic endocrine key regulatory genes

Neurogenin3 (NGN3) and pancreatic duodenal homeobox 1 (PDX1) represent key developmental transcription factors in endocrine pancreatic organogenesis [225]. In mice adult pancreas, Ngn3 + cells display progenitor capacity within the injured islet [86] and Pdx1 is also required for maturation and function of adult beta cells [225]. To evaluate the effect of these genes on hMSC-TERT, we generated three new stable cell lines by transfection of hNGN3, hPDX1 or both genes in combination. In newly generated cell lines, transfected genes and their transcription factors (TFs) were assessed by RT-PCR and Western blotting, respectively.

Disclosure of molecular mechanisms involved in pancreatic endocrine differentiation in reprogrammed hMSC-TERT

Both, NGN3 and PDX1 play a major role in the pancreatic developmental transcription factor cascade [225]. To assess the role of these transcription factors in the molecular mechanism of endocrine differentiation in our adult MSC system, we used two reporter genes assays. SEAP reporter gene assay was performed to disclose whether hNGN3 and/or hPDX1 were able to activate human insulin promoter in hMSC-TERT. Luciferase assay was undertaken to determine whether hNGN3 directly activates the insulin promoter, or if its effect is mediated via the secondary induction of PDX1.

For detection of endocrine genes and proteins in reprogrammed hMSC-TERT, we used RT-PCR and Immunofluorescence, respectively.

Functional evaluation of insulin secretion in reprogrammed hMSC-TERT

A major goal in cell-based therapy of T1D is the generation of functional insulin-producing cells that are able to replace injured beta-cells, by releasing insulin in physiological concentrations and in a glucose-sensing manner.

We aimed to evaluate insulin expression and synthesis in newly generated hMSC-TERT - NGN3 /PDX1 using RT-PCR and Immunofluorescence. Insulin release and cellular content in response to glucose stimulation were assessed by an ELISA assay.

II. Functional signature of hIPC compared to hBM-MSC

The second part of our work was performed at the Laboratory for Musculoskeletal Research and Stem Cell Biology of the Orthopedic Department at the Universitätsklinikum Würzburg under the leadership and supervision of Professor Franz Jakob. The colaboration of this group, especially from Dr. Regina Ebert was extremely valuable concerning the large experience with the usage of primary human bone marrow derived MSC and well-established differentiation protocols in this lab.

Microarrays for determination of genome-wide gene expression profiles were performed in colaboration with the BioChip-Laboratory, Universitätsklinikum at the University of Essen in Germany. This study was recently published in Stem Cell and Development [146]

The results obtained in the first part of the project put in evidence limitations regarding the insulin secretion and glucose sensing mechanism of differentiated hMSC-TERT. Although hMSC-TERT behave as adult stem cells towards mesenchymal lineages, they could not achieve complete endocrine reprogramming. These observations suggest that different MSC populations have different multipotent character and not all of them can go through the same differentiation pathways

Therefore, to achieve objective II, we seek the following lines of investigation:

Characterization of hBM-MSC and hIPC

In part II of this project, our first aim was to characterize and compare two primary MSC populations isolated from distinct organs: the mesodermal derived hBM-MSC and the multipotent cell population called human pancreatic Islet-derived Progenitor Cells (hIPC) that can be obtained from explanted human islets. For this purpose, we evaluated in both populations, the surface antigen pattern using flow cytometry, stem cell and mesenchymal gene expression markers using RT-PCR/ qPCR and gene expression profiling by significant analyses of microarrays.

Multilineage differentiation potential of hIPC and hBM-MSC

Both, IPC and BM-MSC have been under extensive investigation as alternative sources for beta-cell replacement in T1D. Initially recognized as islet progenitor cells, IPC have been described to develop into insulin secreting cells *in vitro* and to improve glucose homeostasis *in vivo;* very similar reports were obtained on human and rodent BM-MSC [187, 210, 226-229].Besides, IPC express mesenchymal marker genes [230] and have been described to undergo mesenchymal differentiation just like BM-MSC [226-229]

In the present study, to further analyse and compare the multipotent capacity of these two MSC populations, we induced both, hBM-MSC and hIPC to differentiate, *in vitro,* towards mesenchymal and pancreatic endocrine cell lineages. Differentiated phenotypes were then evaluated for mesenchymal and endocrine gene markers using RT-PCR. Specific mesenchymal and beta cell proteins were analysed in the obtained phenotypes by means of immunohistochemical approach.

Molecular signature and function of hBM-MSC and hIPC

Although hIPC and hBM-MSC show common mesenchymal features, we found remarkable differences in their multipotent differentiation capacity. To identify the genes that are related to the distinct signatures and functional assignment of these two MSC populations, gene ontology clusters analysis and a candidate gene approach were performed in hIPC and hBM-MSC.

Previous studies have shown contrasting results concerning the origin of hIPC: some have traced hIPC to be of mesenchymal origin [230, 231] and others suggest that hIPC correspond to endocrine cells under epithelial mesenchymal transition (EMT) due to *in vitro* cultivation [232]. Therefore, to disclose the provenience of this cell population we performed a candidate gene approach in order to identify specific genes and pathways that might be determinant for the molecular signature of hIPC.

3. PERSONAL CONTRIBUTION INCLUDING ORIGINAL RESULTS

I. In vitro differentiation of hMSC-TERT towards insulin producing cells

I-1. Materials and Methods

Unless otherwise specified, all chemicals were purchased from Invitrogen, Karlsruhe,, Germany.

Cell Culture

hMSC-TERT as obtained from Moustapha Kassem were generated by retroviral transduction of bone marrow-derived MSC from a 33 year old healthy male donor and were routinely cultured as previously described [224]. Human pancreatic islets were isolated according to a modified semi-automated digestion-filtration method [233, 234] at the Giessen Islet Isolation and Transplantation Centre (Germany) from human "research" pancreases obtained from brain-dead multi-organ donors after legal consent and approval from the local ethics committee,. Prior to experiments, islets were cultured for 1-5 days in RPMI 1640 (w/o glucose) supplemented with 5.6 mM glucose, 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine,10 mM HEPES buffer, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 µg/ml streptomycin. Endocrine differentiation of hMSC-TERT was induced by two approaches; (i) specific medium and (ii) ectopic overexpression of human NGN3 and/or human PDX1 proteins. For approach (i), 2.5 x 10^5 cells/ well were seeded in 6 well plates and cultured for 8-9 days in a permissive endocrine differentiation medium. This consisted of serum-free DMEM/F12 containing 17.5 mM glucose, 10 mM nicotinamide, 2 nM activin-A, 10 nM exendin-4, 100 pM hepatocyte growth factor (Sigma-Aldrich GmbH, Schnelldorf, Germany), 1% bovine serum albumin (BSA), 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin [95, 185] further on named as cocktail medium. For approach (ii) we generated transgenic hMSC-TERT cell lines with stable overexpression of *NGN3* and/or *PDX1* genes. After transfection, cells were kept in standard medium

complemented with selection antibiotics as described below. COS-7 cells were routinely cultured in DMEM with 10% FBS, 100 units/ ml penicillin and 100 µg/ml streptomycin. INS-1E cells were routinely cultured in RPMI 1640 containing 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine,100 units/ml penicillin, 100 µg/ml streptomycin and 50 µM beta-mercapthoethanol. All cells were cultivated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Flow cytometry

Cells were labelled by a human c-met-specific mouse antibody (1 µl/5 x 10^5 cells) (Biomol GmbH, Hamburg, Germany) and an FITC-conjugated goat antibody against mouse IgG1 (Becton Dickinson GmbH, Heidelberg, Germany). Cells labelled by a FITC-conjugated isotype (1:100 dilution) (Becton Dickinson GmbH) served as negative control. Flow cytometry analysis (10,000 events) was performed using a FACSort apparatus and CellQuest software (Becton Dickinson GmbH)

Transgenic cell lines

The human *NGN3* coding sequence [235]was kindly provided by M. German and subcloned into a CMV promoter driven pcDNA3.1+ which includes a neomycin resistance gene (hNGN3-pcDNA 3.1+). The human *PDX1* coding sequence was subcloned into CMV promoter driven pcDNA6/V5-His-A comprising a blasticidin resistance gene (hPDX1-pcDNA6/V5-His-A). For transfection, hMSC-TERT were seeded in 6-well plates (10^5 cells/well). At 50-70% confluence, cells were stably transfected with human *NGN3*, *PDX1* or both genes, using the liposomal transfection reagent Metafectene (Biontex Laboratories GmbH, Martinsried/Planegg, Germany). 3 µl Metafectene and 1 µg DNA of each linearized vector were added to the cells,

according to the manufacturer's instructions. Transfection efficiency as determined by GFP-expression from transfected plasmids in independent experiments was 60% (data not shown). After 5 days, selection of transfected cells has been initiated with standard medium containing 600 µg/ml neomycin (Calbiochem/Merck KGaA, Darmstadt, Germany) or 7.5 µg/ml blasticidin (selection medium). Appropriate antibiotic concentrations used for selection were determined (kill curves) prior to experiments. After 6 weeks in selection medium, surviving successfully transfected cells were cultured in standard medium with 10 x lower concentrations of antibiotics (maintenance medium). All transgenic hMSC-TERT clones from each generated line were set together and were designated hMSC-TERT-NGN3, hMSC-TERT-PDX1 and hMSC-TERT-NGN3/PDX1, respectively. Experiments have been performed during the next 10 passages.

RT-PCR

Total RNA was isolated by NucleoSpin RNA II Purification Kit (Macherey-Nagel GmbH & Co KG, Düren, Germany) which include DNAse digestion. Prior to PCR amplification, RNA samples were tested by house keeping gene PCR for presence of genomic DNA. Only RNA free of genomic DNA was reversely transcribed into cDNA by using Superscript III. Additionally, for further detection of residual genomic DNA co-amplification, specific “intron-spanning” primers were used. PCR was performed using with the following conditions: 94 °C for 2 min; 94 ºC for 30 s, primer-specific annealing temperature for 30 s, 72 ºC for 30 s; 72 °C for 4 min (Primer sequences and conditions are listed in supplemental Tab 1). PCR products were separated by 1.5-2% agarose gel electrophoresis and visualised by ethidium-bromide staining.

Western Blotting

Proteins were extracted by RIPA lysis buffer (Upstate/Biomol GmbH) containing Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Mannheim, Germany). 15 µg protein/ lane were separated by 10% SDS gel electrophoresis and transferred to PVDF membranes by semi-dry blotting using Towbin transfer buffer. Ectopic and endogenous protein expression of NGN3 and PDX1 in hMSC-TERT was detected using rabbit anti-human NGN3 (1:500) or goat anti-human PDX1 (1:500) antibodies (both Santa Cruz Biotechnology Inc., Heidelberg, Germany) and the horseradish peroxidase-conjugated secondary antibodies donkey anti-rabbit IgG and rabbit anti-goat IgG (Dako Deutschland GmbH, Hamburg, Germany). Bands were visualised by ECL Plus Western Blotting Detection Reagent (GE Healthcare Europe GmbH, Freiburg, Germany). COS-7 cells transiently transfected with hNGN3-pcDNA3.1+ and/or hPDX1-pcDNA6/V5 His A served as positive control. Wild type hMSC-TERT and COS-7 cells were used as negative controls.

Reporter Gene Analyses

For secreted alkaline phosphatase (SEAP) reporter gene assay we used a -881 to +54 bp fragment of human insulin promoter subcloned into pSEAP2-Basic (-881hInsP-SEAP) 236. SV40 promoter driven pSEAP-Control and promotorless pSEAP2-Basic (Clontech/Becton Dickinson GmbH) were used as positive and negative control, respectively. For luciferase reporter gene assays, a -912 to +55 bp fragment of the mouse (m)Pdx1 promoter was subcloned into pGL3basic (Promega GmbH, Mannheim, Germany) to obtain the -912mPdx1P-Luc plasmid. SV40 promoter driven pGL3-Control and promoterless pGL3-Basic were used as positive and negative controls, respectively. All three generated cell lines and wild type hMSC-TERT were transiently transfected by the described vectors using Metafectene (Biontex) according to the

manusfacturer's instructions. Supernatants for assessment of secreted SEAP were obtained 36 h post transfection and measured using Great EscAPe SEAP Chemiluminescence Detection Kit (Clontech/Becton Dickinson GmbH). Values of alkaline phosphatase secretion were normalized to background activity of the promoterless SEAP2-Basic plasmid. Cell lysates for determination of luciferase activity were collected 48 h pos- transfection and measured using Luciferase Assay System (Promega GmbH). Values of luciferase activity were normalized to background activity of the promoterless pGL3-Basic plasmid.

Fluorescent immunocytochemistry

Mouse anti-human nestin (Chemicon, Schwalbach, Germany), guinea-pig anti-human insulin (1:500) (Dako Deutschland GmbH) and rabbit anti-human C-peptide (1:500) (Linco Research, Inc.) were used as primary antibodies. Anti-mouse IgG Cy3-conjugated (1:1000), anti-guinea-pig IgG Cy2-conjugated (1:1000) and anti-rabbit IgG Cy3-conjugated (1:1000) (all Dianova GmbH, Hamburg, Germany) were used as secondary antibodies. Cells were plated in 4 well culture slides (Becton Dickinson GmbH), cultured for 24 h, fixed in 4% paraformaldehyde for 30 min and air-dried. For immunostaining, cells were permeabilized with 0.1%Triton X 100 for 5 min, blocked by 5% species-specific normal serum for 30 min and incubated at room temperature with primary and appropriate secondary antibodies for 2 and 1 h, respectively. Cell nuclei were stained with DAPI. Slides were analysed by fluorescence microscopy (Carl Zeiss Jena GmbH, Jena, Germany) using Axiovision 4.4.1.0 software (Carl Zeiss Vision GmbH, Aarlen, Germany). Wild type hMSC-TERT served as negative and human islets as positive control. The number of insulin+ and C-peptide+ cells among the hMSC-TERT-NGN3, hMSC-TERT-PDX1 and hMSC-TERT-NGN3/PDX1 was quantitatively

determined in 3 independent immunostaining experiments by counting stained cells in 4 randomly taken microphotographs per experiment of each cell population using Adobe Photoshop CS8.0.1 (Adobe systems Inc.).

Insulin release and content

50.000 cells / well from each transgene cell line were seeded in 24 well plates and cultured for two days in maintenance medium. Prior to experiments cells were washed twice and pre-incubated for 1 h in Krebs-Ringer buffer (KRB) (Sigma-Aldrich GmbH) containing 0.1% BSA, followed by 1 h incubation in fresh KRB/0.1% BSA additionally supplemented with 0.5 mM 1-isobutyl-3-methylxanthine and 5 mM or 15 mM glucose. After collection of supernatants, cells were extracted in acetic acid containing 1% BSA and sonicated. Buffer and cell extracts were analysed using the human Insulin Ultrasensitive ELISA Kit (Mercodia, Upsala, Sweden) for specific detection of mature insulin. Insulin secretion into KRB and the remaining cellular insulin content were normalized to total cellular protein content of related samples. Wild type hMSC-TERT cells incubated in identical conditions were used as negative and rat INS-1E pancreatic beta cells as positive control.

Statistical analysis

All results are presented as mean values ± standard deviation (SD). Statistical analysis was performed using build in *t* test of GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com).

I-2. Results

hMSC-TERT express stem/progenitor cell markers

Nestin and c-met (also known as HGF-R, hepatocyte growth factor receptor) gene expression was analysed to characterise the stem-cell predisposition of hMSC-TERT. MSC of the developing pancreas express the neural stem cell marker nestin [237, 238], which has also been described in both adult human and rat islets of Langerhans [177] as well as in bone marrow derived MSC populations [239]. C-met is a surface marker of endodermal progenitor cells, such as hepatic [184, 240, 241] and pancreatic cells [181], and is also found in MSC populations [242]. In the present study, **both markers are detected in hMSC-TERT at mRNA levels by RT-PCR** (Fig. 12A). Nestin (Fig. 12B) and c-met (Fig. 12C) protein expression was demonstrated by immunofluorescence and FACS analysis, respectively. C-met was observed in about 80% of hMSC-TERT.

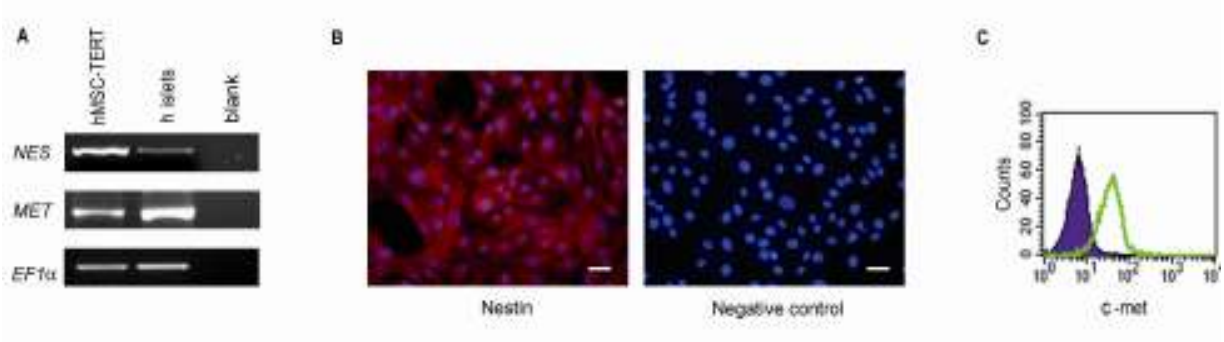


Fig. 12: Characterization of hMSC-TERT.

(A): RT-PCR analysis of human nestin (*NES*) and c-met (*MET*) gene expression in hMSC-TERT and human islets. (B): Immunofluorescence costaining for intracytoplasmatic nestin protein (cy3 red) and nuclei (dapi blue) on hMSC-TERT (left). For negative control hMSC-TERT were stained with secondary antibody (cy3) and nuclei (dapi) (right). Scale bar, 20 µm. (C): Detection of human c-met surface antigen in hMSC-TERT using flow cytometry.

hMSC-TERT can be induced towards a pancreatic endocrine fate

In a first approach, wild type hMSC-TERT were cultivated in serum-free cocktail medium to induce pancreatic endocrine differentiation by specific medium conditions. After 4-5 days in differentiation medium, cells become round-shaped, detach from the culture plate and form floating cell clusters (Fig. 13A right), whereas non-induced hMSC-TERT grow adherent and display a normal fibroblast-like shape (Fig. 13A left). While not specifically quantified, we observed that such cocktail-derived cell clusters exhibited reduced proliferation after 1 week and limited cell survival after 2 weeks in culture. These changes were not related to serum-free conditions since growth characteristics did not change after substitution of serum to BSA (serum-free) supplementation without addition of cocktail factors (data not shown). After 7-9 days, cocktail-treated hMSC-TERT clusters were analysed for specific endocrine pancreatic gene expression by RT-PCR and compared to untreated hMSC-TERT (Fig. 2B). **Cocktail-induced cell clusters newly express the pancreatic transcription factors *PAX4, ISL1*, *PDX1* and *NEUROD1* as well as the pancreatic hormones somatostatin (*SST*), pancreatic polypeptide (*PP*), and insulin (*INS*). Furthermore, induction of glucose transporter 2 (*GLUT2*) expression was also observed, whereas NGN3 or glucagon (*GCG*) could not be newly detected upon cocktail culture conditions.** Interestingly, in untreated hMSC-TERT, expression of the pancreatic alpha cell related transcription factor *PAX6* was also detected.

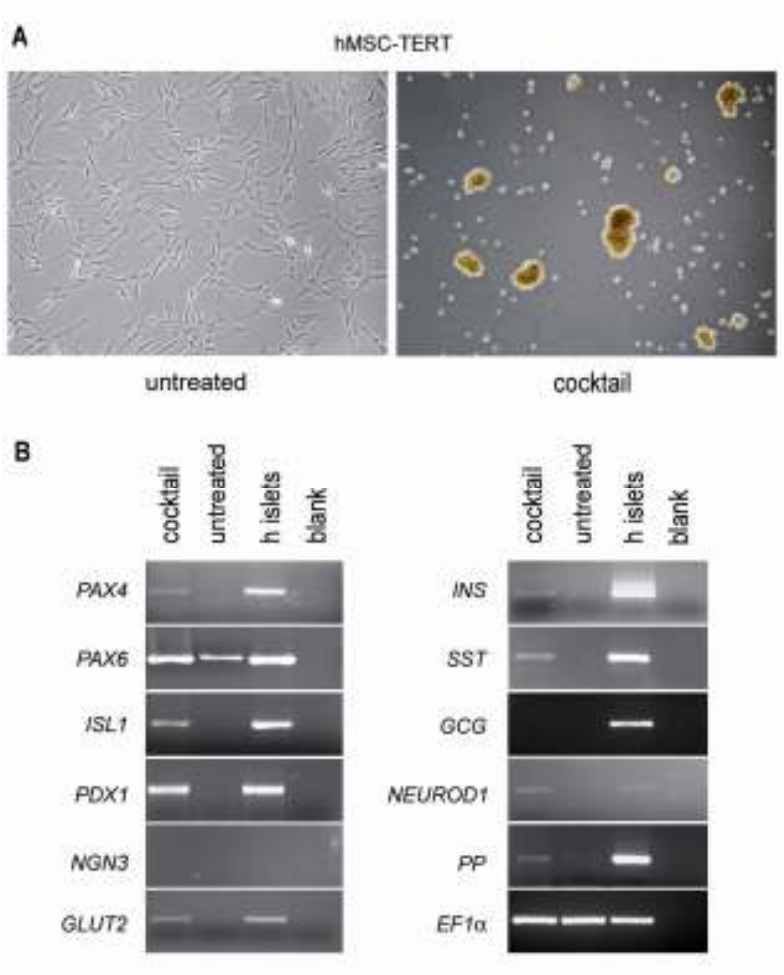


Fig. 13: Effects of endocrine promoting culture conditions (cocktail) on hMSC-TERT.

(**A**) Phase contrast images (10x magnification) of hMSC-TERT grown in standard medium (left), and hMSC-TERT cultivated in serum-free cocktail medium supplemented with 17 mM glucose and endocrine promoting factors (right). (**B**) Expression of pancreatic islet cell-related genes in induced hMSC-TERT-derived cell cluster compared to untreated MSC-TERT (negative control) and human primary islets (positive control). *PAX4,* paired box 4; *PAX6,* paired box 6; *ISL1*, ISL LIM homeobox 1; *PDX1*, pancreatic and duodenal homeobox 1; *NGN3*, neurogenin 3; *GLUT2*, glucose transporter 2; *INS*, insulin; *SST*, somatostatin; *GCG*, glucagon; *NEUROD1,* neurogenic differentiation 1; *PP*, pancreatic polypeptide; quality and amount of amplified cDNA was verified by EF1α house-keeping gene PCR. All data from Fig. 2 are representative (each n = 3).

Overexpression of human ectopic PDX1 and/or NGN3 in hMSC-TERT

In a second approach, we aimed to investigate the potential of ectopic gene expression on differentiation of adult human MSC into endocrine pancreatic lineages. Therefore, two pancreatic endocrine key genes, human NGN3 and PDX1 alone or in combination, were stably transfected in hMSC-TERT. These newly generated transgenic cell lines were cultured in maintenance medium, without any influence by other endocrine-permissive growth factors. Ectopic overexpression was stable within the investigated period of 10 population doublings and newly generated cell lines exhibited a proliferation rate comparable to wild type hMSC-TERT (data not shown). Stable overexpression of transgenes in hMSC-TERT cell lines was confirmed at the mRNA level by RT-PCR (Fig. 14A) and at the protein level by Western blotting (Fig. 14B). **As a major notion, we observed PDX1 expression not only in transgenic hMSC-TERT-PDX1 clones, but also in hMSC-TERT-NGN3. These results suggest that stable NGN3 overexpression in hMSC-TERT is sufficient to induce expression of endogenous PDX1 in these cells. On the other hand, NGN3 was not detected in hMSC-TERT-PDX1.** As expected, double transgenic hMSC-TERT-NGN3/PDX1 cells express both respective genes.

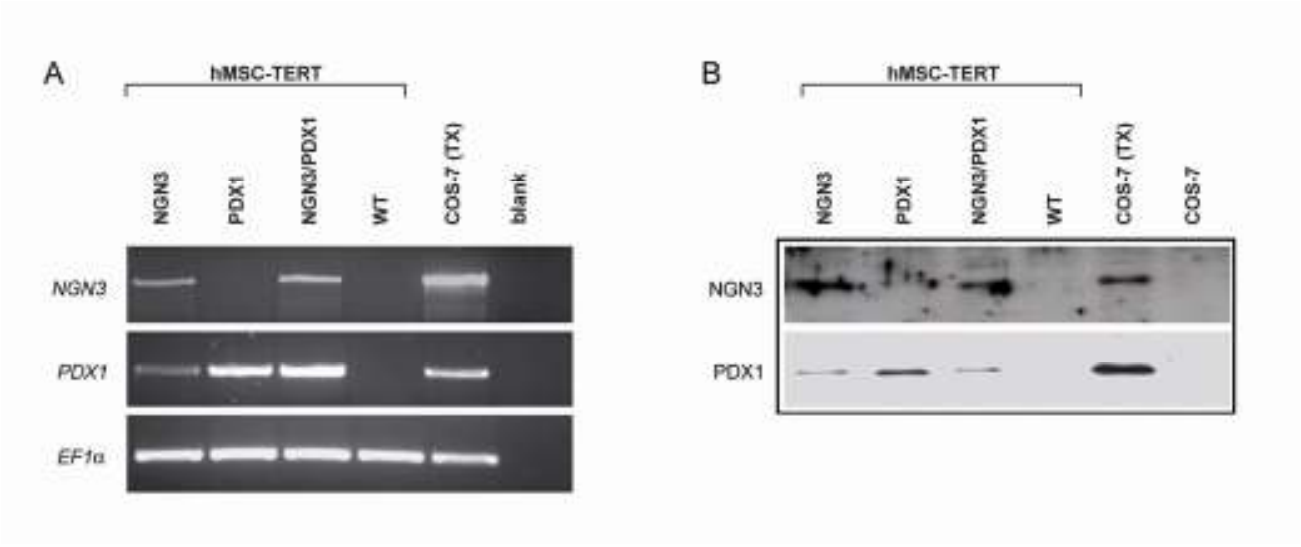


Fig. 14: Expression of ectopic genes in transgenic hMSC-TERT

(**A**) RT-PCR evaluation of *NGN3* and *PDX1* transcripts in hMSC-TERT overexpressing NGN3, PDX1 or both NGN3 and PDX1. (**B**) Detection of NGN3 and PDX1 protein synthesis by Western blot in transgenic cell lines. COS-7 transiently transfected with NGN3 or PDX1 served as positive and wild type hMSC-TERT and COS-7 as negative controls. All data from Fig. 3 are representative (each n = 3).

Insulin and PDX1 promoter activity in hMSC-TERT transgenic cell lines

To establish whether the human insulin gene was transcriptionally activated upon overexpression of NGN3 and/or PDX1 in hMSC-TERT cells, a human insulin promoter fragment (-881 to +54) driving expression of the SEAP reporter gene was transiently transfected into the new generated transgenic cells and hMSC-TERT wild type. Transfection efficiency as defined by control vector activity showed similar values of SEAP in the four transfected cell lines (data not shown). At 36 h after transfection, **human insulin promoter activity, normalised to pSEAP2-Basic, was significantly induced in transgenic hMSC-TERTcell lines as compared to wild type controls** (Fig. 15A). **Besides, combined overexpression of NGN3/PDX1 provides no additional promoter activation over single stimulation of NGN3 or PDX1.**

We further investigated transcriptional *PDX1* gene activity in transgenic hMSC-TERT cell lines by Luciferase reporter gene assay (Fig. 15B). Transfection efficiency was

similar in all cell lines as revealed by luciferase activity of the control vector (data not shown). Similar to insulin promoter activation, ***mPdx1*** **promoter activity normalised to pGL3-Basic was significantly induced in transgenic as compared to wild type hMSC-TERT thereby supporting the observation that ectopic NGN3 substantially induces endogenous PDX1 expression (Fig.** 14). Activation of the *mPdx1* promoter in NGN3 and PDX1 single transgenic hMSC-TERT was statistically similar. NGN3/PDX1 double transgenic cells demonstrated a statistically significant increase versus NGN3 but not versus PDX1 single transgenic cells.

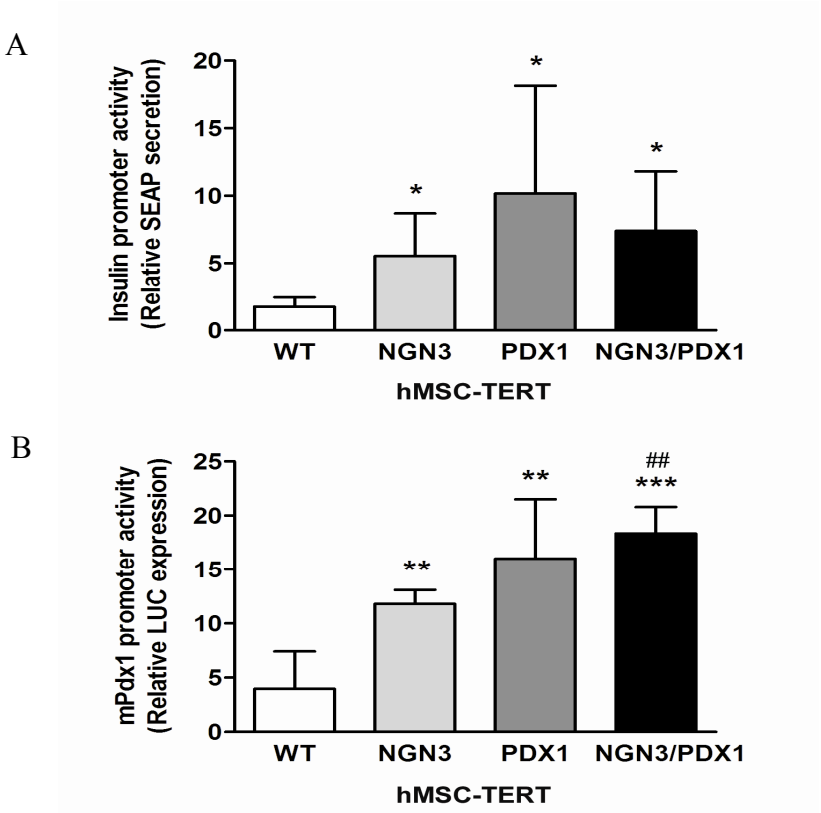


Fig. 15: Insulin and Pdx1 promoter activity in transgenic cell lines.

(**A**) Using a -881hInsP-SEAP reporter plasmid, insulin promoter activity was evaluated by SEAP reporter gene assay in hMSC-TERT-NGN3, hMSC-TERT-PDX1, hMSC-TERT-NGN3/PDX1 and wild type hMSC-TERT (n = 6). Values of secreted alkaline phosphatase were normalized to the activity of the promoterless SEAP2-Basic plasmid (**B**) Evaluation of *PDX1* promoter activity by Luciferase (LUC) gene reporter assay in the same cell lines using a -912mPdx1P-LUC reporter plasmid (n=4). Values of luciferase activity were normalized to background activity of the promoterless pGL3-

Basic plasmid. Columns in Fig. 4 represent means ± SD (*, versus wild type; #, versus NGN3; *, $p < 0.05$; **, $p<0.01$; *** / ###, $p < 0.001$).

Expression of pancreatic endocrine genes in transgenic hMSC-TERT

Expression of a panel of pancreatic islet-related and beta cell-specific genes was analysed by RT-PCR to evaluate the differentiation stage of hMSC-TERT following NGN3, PDX1 and NGN3/PDX1 overexpression (Fig. 16A). **Induction of endocrine pancreatic transcription factor genes such as NEUROD1, PAX6, PAX4 and ISL1 was observed.** Notably, low levels of PAX6 expression observed in wild type hMSC-TERT, were enhanced by overexpression of NGN3 and/or PDX1. **Other beta cell-specific genes such as GCK, GLUT2 and INS were also newly detected in all transgenic cell lines.** Additionally, endocrine pancreatic gene expression such as **GCG, PP and SST genes was induced** in parallel. **This may demonstrate the potential of NGN3 and PDX1 to trigger an overlap of alpha, PP and delta cell-specific reprogramming in hMSC-TERT**.

Synthesis and storage of insulin and c-peptide in transgenic hMSC-TERT

To evaluate the maturation level of NGN3 and/or PDX1-induced endocrine phenotypes, synthesis of insulin protein was analysed by means of insulin and C-peptide fluorescent immunostaining in transgenic hMSC-TERT cell lines. (Fig. 16B and 16C) demonstrate insulin (green) and C-peptide (red) immunofluorescence in representative ocular fields of all 3 transgenic cell lines as compared to negative immunostaining in wild type hMSC-TERT. Human islets served as positive control. Quantification of cell numbers displaying **C-peptide positive immunofluorescence demonstrated that about 35 to 40% of transgenic hMSC-TERT were reprogrammed by overexpression of either NGN3, PDX1 or both (Fig. 5B).**

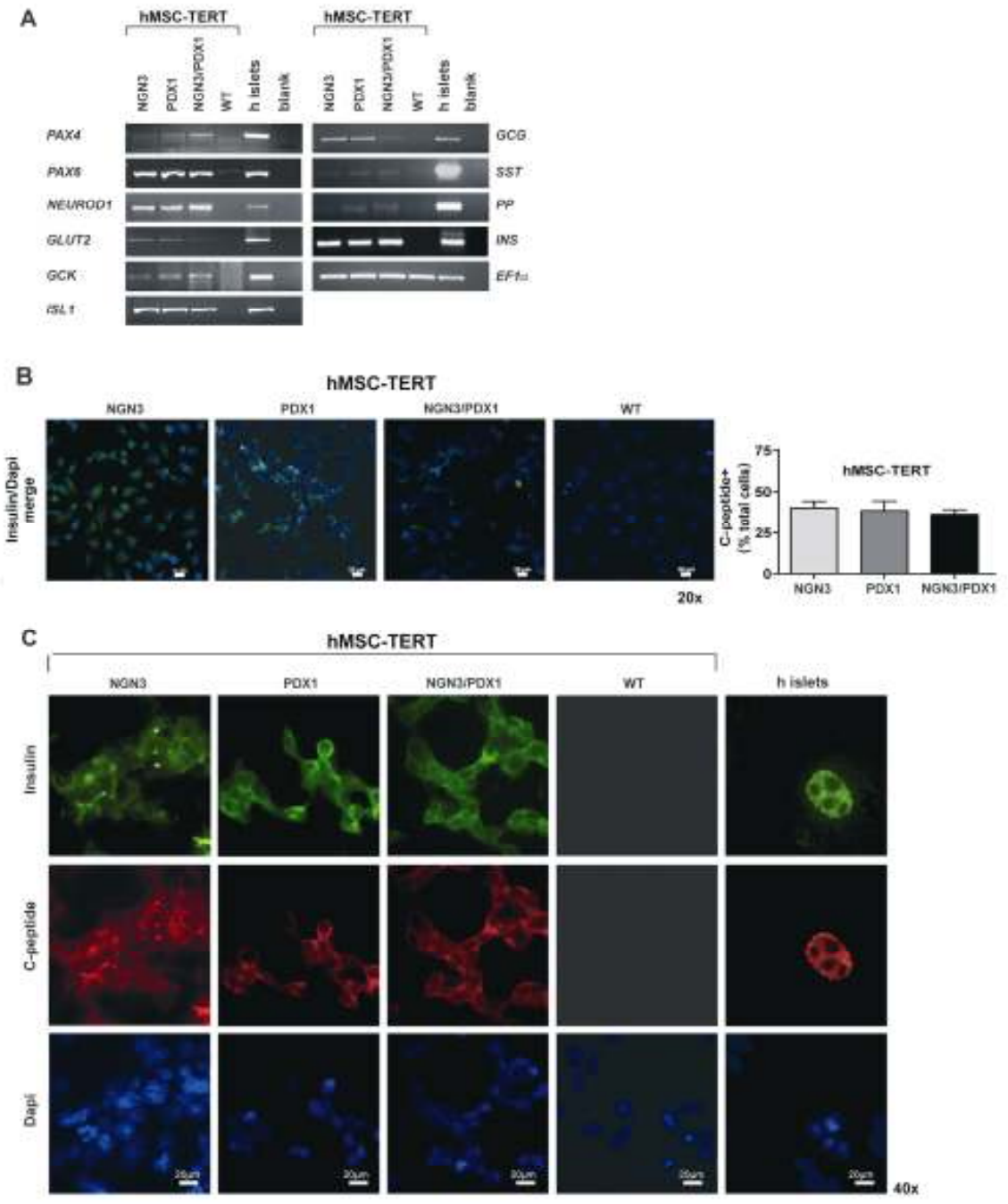


Fig. 16: Endocrine differentiation capacity of transgenic cell lines.

(**A**) Activation of pancreatic islet-related gene markers in hMSC-TERT-NGN3, hMSC-TERT-PDX1, hMSC-TERT-NGN3/PDX1, wild type hMSC-TERT (negative control) and human islets cells (positive control). *GCK*, glukokinase; for other abbreviations see legend of (Fig. 13). Representative gels (n = 3). **(B)** Immunostaining of Insulin (cy2, green) and nuclei (DAPI, blue) in the 3 generated hMSC-TERT cell lines compared to hMSC-TERT wild-type. Merge image shows that not all cells stain for insulin. Scale

bar, 20 µm. Representative pictures (n = 3). Number of C-peptide+ cells in wild type hMSC-TERT and differentiated transgenic cell lines overexpressing NGN3, PDX1or both NGN3 and PDX1. Columns in Fig. 5B represent mean ± SD (n = 3). **(C)** Immune fluorescence of Insulin (cy2, green), C-peptide (cy3, red) and nuclei (DAPI, blue) in differentiated cells detected in hMSC-TERT-NGN3, hMSC-TERT-PDX1, and hMSC-TERT-NGN3/PDX1 populations, and compared to hMSC-TERT wild-type. Scale bar, 20 µm. Representative pictures (n = 3).

Insulin release and content in transgenic hMSC-TERT

We finally tested whether NGN3 and/or PDX1 transgenic hMSC-TERT cell lines were able to store (content) and secrete insulin. In wild type hMSC-TERT, no insulin was detected within the cells, nor was there any insulin secretion observed. In contrast, **detectable insulin content and secretion was measured to a variable extend in all three transgenic cell lines** (Fig. 17). While these results parallel the findings observed in the gene and protein expression studies (Figs. 13 and 16), **insulin content and secretion was not enhanced by increasing glucose concentrations from 5 to 15 mM in all of the transgenic hMSC-TERT cell lines** (Fig. 17). Moreover, INS-1E insulin secretion under identical conditions shows that insulin production in transgenic cell lines is below biological significance levels.

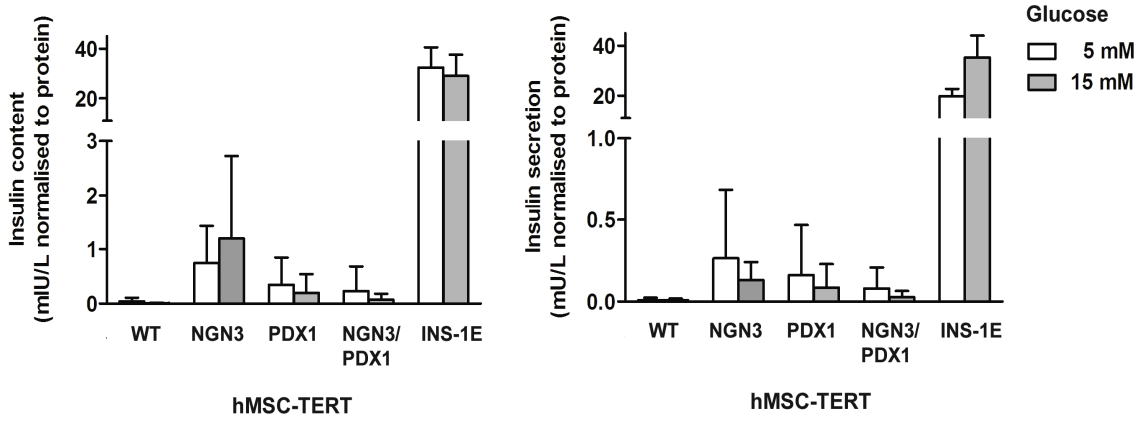


Fig. 17: Insulin response to glucose stimulation in transgenic cell lines

hMSC-TERT-NGN3, hMSC-TERT-PDX1, and hMSC-TERT-NGN3/PDX1 were stimulated with 5 (grey bars) or 15 mM (black bars) glucose. Treated in identical

conditions, wild-type hMSC-TERT and INS-1E cells served as negative and positive controls respectively. (**A**) Secreted insulin was measured in supernatants and (**B**) insulin content was determined after protein extraction from investigated cells by ELISA. All columns in (Fig. 17) represent values normalised to related cellular protein content and were plotted as mean ± SD (n = 3).

II. Functional Signature of hIPC compared to hBM-MSC

II-1. Materials and Methods

Cell culture

All media, fetal calf serum (FCS) and supplements were obtained from PAA (Linz, Austria).

Human pancreatic islets were isolated at the Giessen Islet Isolation and Transplantation Centre (Germany) from human pancreases (n=3) obtained from brain-dead multi-organ donors after obtaining legal consent and approval from the local ethics committee, according to a modified semi-automated digestion-filtration method [233, 234]. hIPC were obtained from freshly isolated human islets of 3 different donors as previously described [177] and expanded in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 1 U/ml penicillin (P), 100 µg/ml streptomycin (S), 50 µM β-mercaptoethanol (Sigma, Schnelldorf, Germany), 1 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine and 20 ng/ml recombinant human fibroblast growth factor 2 (rHu bFGF), and 20 ng/ml epidermal growth factor (rHu EGF) (both Promocell, Heidelberg, Germany). Cells were plated onto 75 cm^2 flasks (TPP, Trasadingen, Switzerland) and kept at 37ºC in a humidified atmosphere consisting of 5% CO_2 and 95% air. After 4-5 days the non-adherent cells were removed and the phenotypically similar adherent fibroblast-like cells were cultivated 4 weeks on monolayer prior to experiments. All experiments were performed at passage 3 or 4.

hBM-MSC were harvested from bone marrow of femoral heads from 5 otherwise healthy patients (n=5) undergoing total hip arthroplasty. Informed consent was obtained from each patient and experiments were performed upon approval of the Local Ethics Committee. Whole bone marrow cells were initially seeded at a density of 6.6 x $10^5/cm^2$

to obtain hBM-MSC by plastic adherence. After 2 to 3 days of cultivation, non-adherent cells were removed and adherent cells were washed twice with phosphate buffered saline (PBS). Cells were cultivated and expanded in DMEM/Ham's F-12 (1:1) medium supplemented with 10% fetal calf serum (FCS), 1 U/ml P, 100 µg/ml S, and 50 µg/ml L-ascorbic acid 2-phosphate. The culture medium (hBM-MSC medium) was changed every 3 to 4 days [243]. All experiments were performed after 1 to 2 passages.

Flow Cytometry

hIPC (at passages 3, 4, 6, 12) and hBM-MSC (at passage 1-2) were detached with PBS-0,5% EDTA and re-suspended in PBS-1% bovine serum albumin (BSA) in a round bottom polystyrene tube and labeled with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies according to the manufacturer's instructions. The antibodies used were: anti-human CD45-PE, CD14-PE, CD34-PE, CD-HLA-DR-FITC, CD13-PE, CD29-FITC, CD44-FITC, CD54-FITC, (ImmunoTools, Friesoythe, Germany), CD73-PE, CD90-PE, (BD Biosciences, Heidelberg, Germany) and CD105-FITC (Biotrend, Köln, Germany). Cells labeled with IgG1-FITC/IgG2a PE-conjugated antibody (BD Biosciences) served as negative control. Prior to FACS analysis, cells were washed twice and resuspended in 100 µl of PBS-1% BSA solution. Flow cytometry analysis (10,000 events) was performed using a BD FACScan and CellQuest-software (both BD Biosciences, Heidelberg, Germany).

Mesenchymal differentiation of hIPC and hBM-MSC

For adipogenic and osteogenic differentiation, hIPC and hBM-MSC were seeded at a density of 3 x 10^4 cells per cm^2 and incubated in differentiation medium for 14 days (adipogenic) and 28 days (osteogenic) as previously described [243].

Cells maintained in expansion medium served as negative controls.

For chondrogenic differentiation, a cell culture system of high-density pellets was applied. To obtain chondrospheres, 2.5 x 10^5 cells were resuspended in chondrogenic differentiation medium, which contained 10 ng/ml TGFb1 (R&D Systems, Wiesbaden, Germany), centrifuged at 250 x g and cultured in tubes for up to 21 days as previously described [243]. Pellets maintained in chondrogenic medium without addition of TGFb1 served as negative controls.

Pancreatic endocrine differentiation of hIPC and hBM-MSC

For endocrine differentiation, hIPC and hBM-MSC were seeded in ultra low-attachment 6-well plates (Corning, Schiphol-Rijk, Netherlands) and cultured for 4 days under specific endocrine conditions (cocktail medium), consisting of serum-free DMEM/F12 containing 17.5 mM glucose, 10 mM nicotinamide, 2 nM activin-A, 10 nM exendin-4, 100 pM hepatocyte growth factor (Sigma-Aldrich GmbH, Schnelldorf, Germany), 1% bovine serum albumin (BSA), 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin [185, 191]. Floating cluster-like formations were handpicked from cocktail medium and further analysis was performed. hIPC and hBM-MSC maintained in expansion medium served as negative controls.

RNA isolation and RT-PCR

Total RNA was obtained from differentiated and undifferentiated hIPC and hBM-MSC from 3 and 5 donors, respectively using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany, www.macherey-nagel.de) according to the manufacturer's instructions. Reverse transcription was performed using BioScript reverse transcriptase and cDNA was amplified with BIOTAQ DNA Polymerase (both Bioline, Luckenwalde,

Germany) using the following parameters: 94°C, 2 min; primer-specific number of cycles: 94ºC, 30 s; primer-specific annealing temperature, 30 s; 72ºC, 30 s. The human specific primer pairs were designed to be located in different exons based on GeneBank sequences to distinguish the PCR products from possible genomic DNA contamination. PCR products were separated by 1.5-2% agarose gel electrophoresis, visualized by ethidium bromide staining and photographed by BioCapt gel documentation system (LTF, Wasserburg, Germany). Primer sequences, number of cycles and annealing temperatures are listed in Suppl. Tab. 2.

Real-Time PCR (qPCR)

cDNA samples were investigated for mRNA levels by qPCR using Light Cycler 2.0 (Roche) and Q-PCR SYBR Geen Capillary Mastermix (Abgene/Thermo Fisher Scientific). Concentrations of mRNA levels in samples were calculated from external standard curves generated for each specific primer pair (dilution series: 1:1; 1:10; 1:100; 1:1,000 and 1:10,000) by Light Cycler Software 3.5 (Roche). Every run with samples included an internal standard (1:1,000) from the same cDNA sample as used for generation of standard curves to normalise reaction efficiencies. Standard curves were generated from triplicates, while samples and internal standards were analysed in duplicates. Bench-tested qPCR primer pairs for human /HPRT/ (house-keeping gene) (Quantitect Hs_HPRT1), human /MAF/ (Quantitect Hs_MAF), human/ISL1/ (Quantitect Hs_ISL1), human/SOX17/ (Quantitect Hs_SOX17), human /HOXA2/ (Quantitect Hs_HOXA2), human /HOXA10/ (Quantitect Hs_HOXA10), human /TCF21/ (Quantitect Hs_TCF21), were obtained from commercial supplier (Qiagen). qPCR conditions (according to general Light Cycler instructions): 95°C for 10 min; 40 cycles: 95°C for 15 s; 55°C (manufacturer's recommendation for all Quantitect primer)

for 10 s; 72°C for 20 s; followed by melting curve analyzation for specificity of qPCR products .

Cytochemical analysis

Adipogenic differentiation was examined by staining of intracytoplasmic lipid vesicles with Oil Red O. Cells were fixed in 4% paraformaldehyde (PFA) and incubated in 0.3% (wt/v) Oil Red O in 60% isopropanol (Merck, Darmstadt, Germany). Cell nuclei were counterstained with hematoxylin.

Chondrogenic differentiation was visualized by staining of the sulphated proteoglycans with Alcian Blue. Chondrospheres were fixed in 4% PFA, dehydrated with graded ethanol series and embedded in paraffin and sectioned at a thickness of 4 µm. Sections were incubated in 1% (w/v) Alcian Blue pH 1.0 and counterstained with Nuclear Fast Red.

Osteogenic differentiation was evaluated by staining for cytoplasmic alkaline phosphatase (ALP) (ALP Leucocyte Kit 86-C, Sigma Aldrich GmbH). For detection of calcium mineralization in the extracellular matrix, monolayers were fixed with methanol and stained with Alizarin Red S (1% w/v) (Chroma-Schmidt GmbH, Stuttgart, Germany).

Immunofluorescence analysis

Rabbit anti-human C-peptide (Linco Research, St. Charles, USA), guinea-pig anti-human insulin and mouse anti-human vimentin (clone v9) (both Dako, Hamburg, Germany) were used as primary antibodies, anti-rabbit IgG-Cy3 at 1:500, 1:500 and 1:2000 dilutions, respectively, in blocking buffer. Anti-rabbit IgG Cy3-conjugated and anti-guinea-pig IgG-Cy2 at 1:1000 dilution (both Dianova, Hamburg, Germany) and

anti-mouse IgG Alexa 555 (Invitrogen, Karlsruhe, Germany) at 1:2000 were used as secondary antibodies.

hBM-MSC and hIPC , human pancreatic islet cryosections (8 µm) and free-floating cell clusters generated in cocktail medium were fixed in 4% PFA prior to staining, permeabilised with 0.1% Triton X 100, blocked in 5% serum and incubated at room temperature with primary and appropriate secondary antibodies for 60 min and 30 min, respectively. Cell nuclei were counterstained with DAPI. Slides were analyzed by fluorescence microscopy (Carl Zeiss, Jena, Germany) using Axiovision 4.6.1.0 software (Carl Zeiss Vision, Aalen, Germany) and laser scanning confocal microscopy (Zeiss LSM 510) with Zeiss LSM 510 software (Esslingen, Germany).

Genome-wide gene expression profiling of hIPC and hBM-MSC populations

Hybridization experiments were performed using Affymetrix Gene Chips HG-U133 Plus 2.0 (54,000 probesets for 47,400 transcripts and 38,500 genes, High Wycombe, United Kingdom) and the corresponding kits according to the Affymetrix GeneChip Expression Analysis Technical Manual version 2 (www.affymetrix.com). Total RNA expression of 3 individual hIPC and 5 individual hBM-MSC preparations (at passage 3 and 1, respectively) was detected with an Affymetrix Gene Chip Scanner 3000 and analyzed by Affymetrix GeneChip Operating Software 1.4. Transcriptional profiling within the two groups was evaluated using the significance analysis of microarrays (SAM) approach (http://www-stat.stanford.edu/~tibs/SAM/) [244].

To assess differentially expressed genes between hIPC and hBM-MSC groups, pre-defined conditions were established according to Affimetrix Gene Chip methodology. The number of “Present” calls for a given gene had to be greater than 50% in at least one of the groups and only those genes that displayed a signal $\log_2$ ratio of < -1 or > 1,

i.e. with a fold change (FC) lower than 0.5 and greater than 2, were taken into account. All other probesets were stated as "not differentially expressed". In order to get reliable data, the q-value, i.e. false discovery rate had to be less than 10%. For heatmap creation, the program Spotfire DecisionSite for Functional Genomics 9.1.1 (Spotfire AB) (TIBCO Software Inc., Göteborg, Sweden) was used.

To evaluate gene ontology clusters, further analysis was attained using GOstat (http://gostat.wehi.edu.au) [245] together with the Benjamini and Hochberg correction.

II-2. Results

Overlapping immunophenotypes of hIPC and hBM-MSC

To evaluate the immunophenotype of adult hBM-MSC and hIPC, both cell populations were compared by means of surface antigen characterization using flow cytometry analysis. Both hBM-MSC and hIPC were negative for the hematopoietic markers CD14, CD34 and CD45, while both expressed the mesenchymal markers CD29, CD44, CD54, CD73, CD90 and CD105. **According to recently established minimal criteria, these results reveal a MSC immunophenotype in both, hBM-MSC and hIPC** [246] (Fig. 18a).

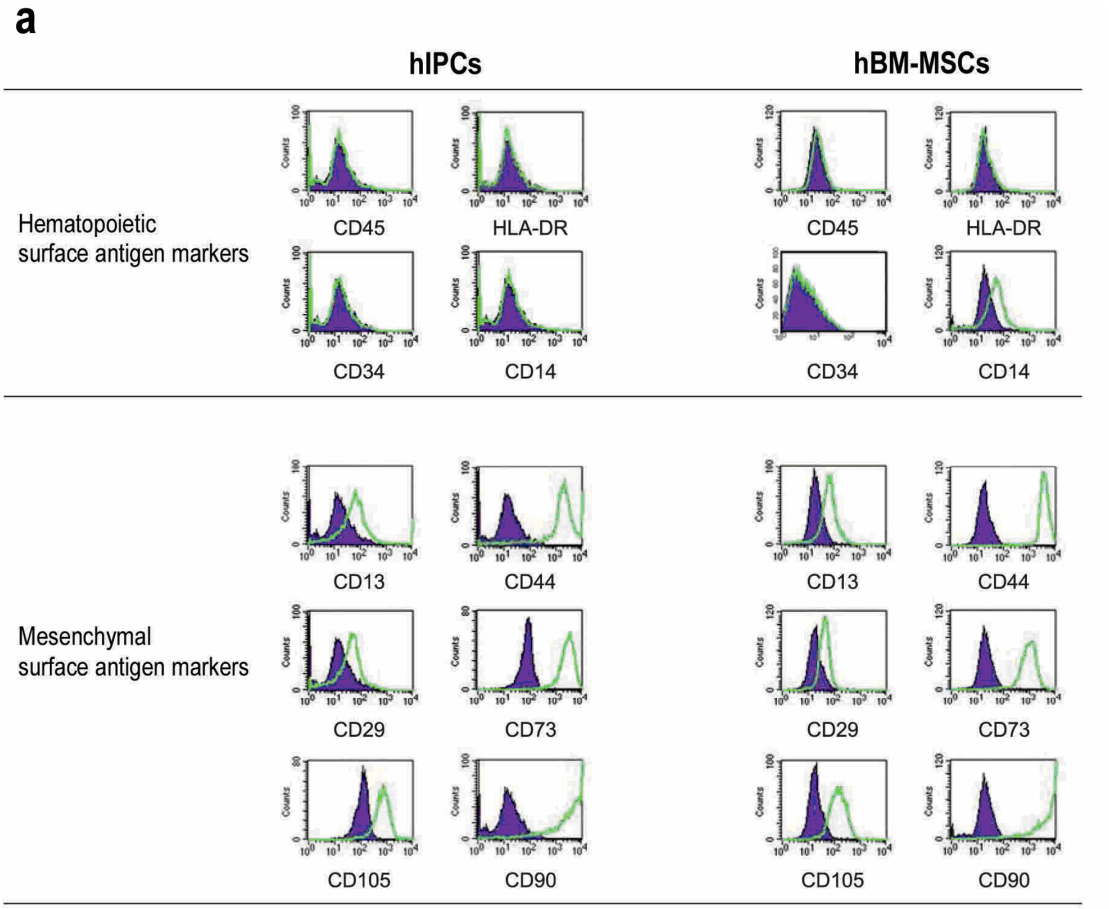
Similar mesenchymal gene marker expression in hIPC and hBM-MSC

The **expression of mesenchymal marker genes** [247] was analyzed in both cell populations. Transcripts for fibronectin (*FNT*), Thy1 (*THY1*), snail2 (*SNAI2*), prolyl 4-hydroxylase, alpha I subunit (*P4HA1*), matrix metalloproteinase 2 (*MMP2*) and vimentin (*VIM*) **were detected in both hIPC and hBM-MSC.** Although to a lesser extent, **mRNA for the majority of investigated mesenchymal genes was also found in extracts of intact human pancreatic islets (hislets)** (Fig. 18b). At the protein level,

the classical mesenchymal marker vimentin was present in the investigated cell populations as well as in cells within the human islets (Fig. 18d).

Expression of genes related to stemness and development

Development and stemness-associated gene transcripts were detected in both hIPC and hBM-MSC. There was similar expression in both populations of e.g. high mobility group box 1 *(HMGB1)*, hairy enhancer of split *(HES)* and Pou domain *(OCT)* genes, polycomb group repressor gene *1 (BMI1)* as well as ATP binding cassette group 2 (ABCG2) transcripts. In contrast, Meis homeobox and some homeobox b *(HOXb)* family genes showed higher expression levels in hIPC (Fig 18c; Suppl. Tab. 1). As expected, embryonic stem cell markers or pluripotency markers like *POU5F1*, *NANOG* and *Nodal* were not expressed in either cell population (data not shown).



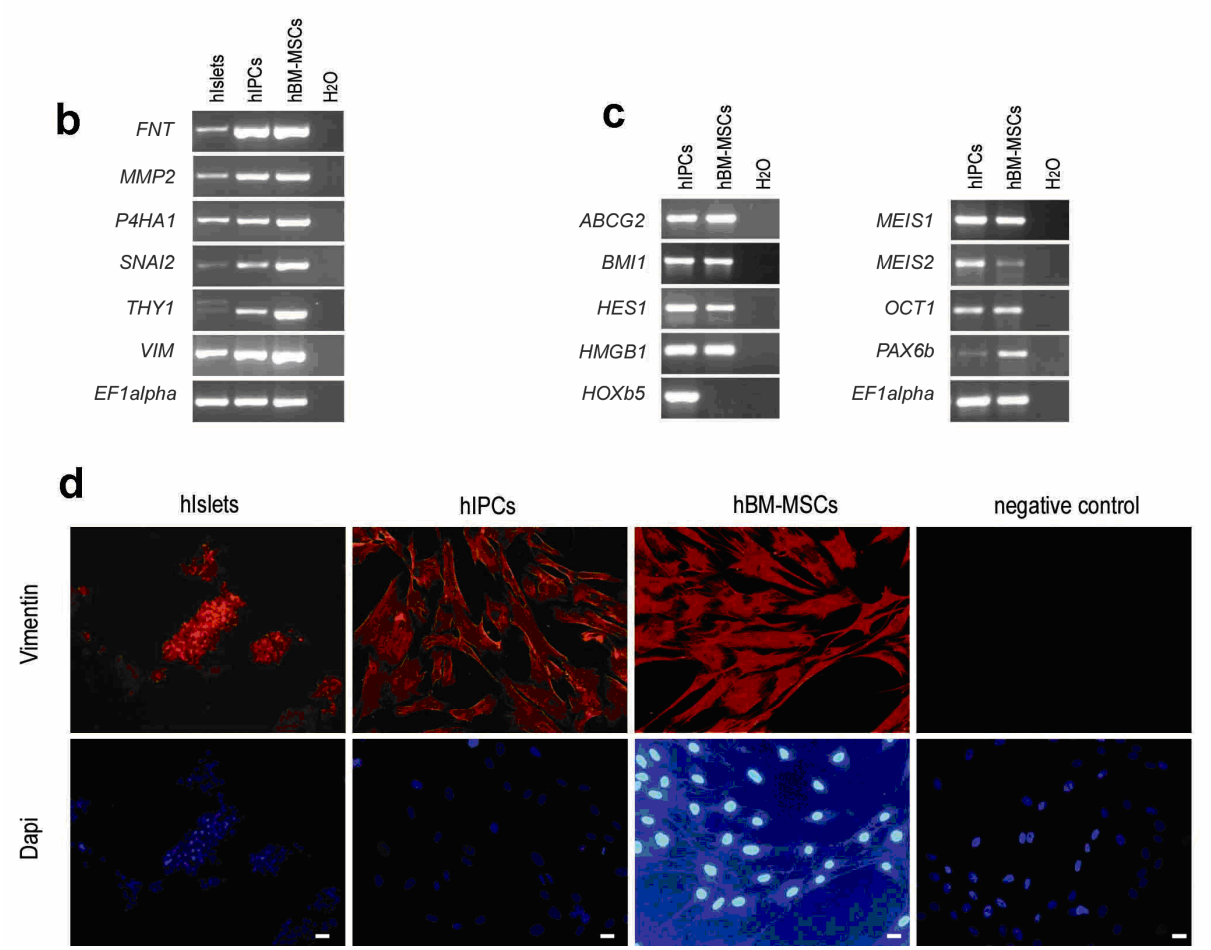


Fig. 18: Characterisation of hIPC and hBM-MSC.

18a: Surface antigen characterisation of hIPC (*left panel*) and hBM-MSC (*right panel*) by FACS analysis of hematopoietic surface markers (*upper panel*) and mesenchymal surface markers (*lower panel*). Representative histograms, from at least 4 independent experiments on distinct passages (hIPC ≥ passage 3, hBM-MSC at passage 1 or 2). **18b**:RT-PCR analysis of mesenchymal marker expression in hislets, hIPC and BM-MSC (n=3). *EF1alpha* was used as a housekeeping gene. Abbreviations: Fibronectin (*FNT*), matrix metalloproteinase 2 (*MMP2*), prolyl 4-hydroxylase, alpha I subunit (*P4HA1*), snail 2 (*SNAI2*), Thy1 (*THY1*), and vimentin (*VIM*) were amplified. **18c**: Representative expression of stemness-related genes in hIPC and hBM-MSC from 3 independent experiments. Abbreviations: ATP binding cassette group 2 (*ABCG2*), polycomb group repressor gene *1 (BMI1),* hairy enhancer of split 1 *(HES1),* high mobility group box 1 *(HMGB1)*, homeobox b 5 (*HOXb5*), meis homeobox 1 and 2 (*MEIS1, MEIS2*), POU class 2 homeobox 1 (*OCT1*), and paired box 6 (*PAX6b*). **18d:** Detection of vimentin (red) by fluorescence immunostaining in hislets, hIPC and hBM-

MSC (n=3). Cell nuclei were stained with DAPI (lower panel). Cells stained with secondary antibody were used as negative control. Bar represents 20 μm.

Mesenchymal differentiation potential of hIPC is reduced as compared to hBM-MSC

To compare mesenchymal differentiation capacities, adipogenic, chondrogenic and osteogenic differentiation were induced in hIPC and hBM-MSC at the same time. After adipogenic induction, transcripts for the adipogenic markers, lipoprotein lipase *(LPL)* and peroxisome proliferator-activated receptor *(PPARγ2)* were detected in both differentiated populations (Fig. 19a). However, **oil droplet formation, a later marker of adipogenesis, was found to a lesser extent in hIPC than in hBM-MSC** (Fig. 19b). After 3 weeks under chondrogenic conditions, **transcripts for specific chondrogenic markers s**uch as collagen type II *(COL II)* and Aggrecan *(AGN)* **were detected in hBM-MSC whereas in hIPC only the hypertrophic cartilage marker collagen type X (*COL X*) was expressed** (Fig. 19a). Alcian Blue staining indicated the presence of sulphated proteoglycans in both hIPC- and hBM-MSC-derived cell pellets (Fig. 19b). After 4 weeks in osteogenic conditions, both hIPC and hBM-MSC strongly stained for alkaline phosphatase *(ALP)*. Similar expression patterns for the osteogenic markers osteocalcin *(OC), ALP* and bone sialoprotein II (*BSP)* was detected, the latter however, showed a much higher expression in hBM-MSC (Fig. 19a). **The degree of *in vitro* mineralization of the extracellular matrix, which is an indicator of osteoblast maturation was clearly less pronounced in hIPC compared to hBM-MSC as shown by Alizarin Red staining** (Fig. 19b).

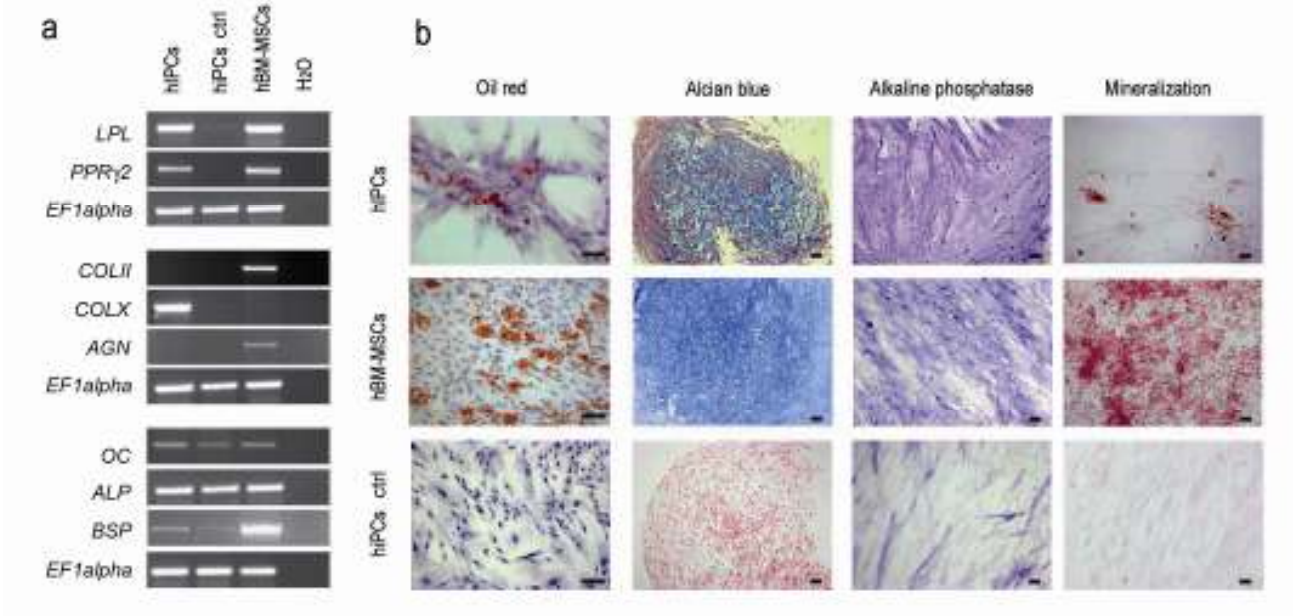


Fig. 19: Mesenchymal differentiation potential of hIPC and hBM-MSC.

19 a: Mesenchymal differentiation potential of hBM-MSC and hIPC monitored by RT-PCR detection of adipogenic markers (*upper panel*), chondrogenic markers (*middle panel*) and osteogenic markers (*lower panel*). Undifferentiated hIPC were used as negative control (hIPC ctrl). EF1alpha was used as a housekeeping gene. Abbreviations: Adipogenic gene markers: lipoprotein lipase *(LPL),* peroxisome proliferator-activated receptor γ2 *(PPARγ2);* chondrogenic markers: collagen type II *(COL II),* collagen type X (*COL X*), aggrecan *(AGN);* osteogenic markers: osteocalcin *(OC),* alkaline phosphatase *(ALP),* bone sialoprotein II (*BSP).* **19 b:** Mesenchymal differentiation potential of hIPC (*upper panel*) and hBM-MSC (*middle panel*) monitored by staining of oil droplets after adipogenic differentiation (*first column*), Alcian Blue staining after chondrogenic differentiation (*second column*), staining for alkaline phosphatase (*third column*) and mineralisation (*forth column*) after osteogenic differentiation. Undifferentiated hIPC (hIPC ctrl, *lower panel*) served as negative control. Bar represents 50 µm. Figure is representative of 3 independent experiments (hIPC at passage 3, hBM-MSC at passage 1 or 2).

Endocrine differentiation in hIPC is more effective than in hBM-MSC

Both cell types were kept in specific culture conditions to evaluate their endocrine differentiation potential. Within 4 days both cell types developed spherical cell clusters (Fig. 20a). Transcripts for the key transcription factor pancreatic-duodenal homeobox 1

(*PDX1/Ipf1*), as well as the early endocrine promoters of beta- and delta-cells *PAX6* and *PAX4* were detected in both hIPC and hBM-MSC after induction of endocrine differentiation (Fig. 20b). Neurogenin 3 *(NGN3),* a pancreatic endocrine progenitor cell marker, which is transiently expressed during differentiation into islet-like cells, was not detected in any of the treated populations (data not shown). On the other hand, **expression of specific genes involved in beta-cell function** such as the V-maf musculoaponeurotic fibrosarcoma oncogene homolog A *(MAFA)*, insulin *(INS)* and glucose transporter type 2 (*GLUT2)* w**ere observed in differentiated hIPC but not in hBM-MSC. Detection of mRNA for glucagon (*GCG*), somatostatin *(SST)* and P polypeptide *(PP)* in hIPC suggests that some of these cells underwent differentiation steps towards various islet-cell phenotypes. Synthesis of insulin protein was detected by immunostaining of hIPC with anti-insulin and anti-c-peptide antibodies (Fig. 20c), suggesting a high level of maturation in differentiated hIPC, which could not be observed in hBM-MSC (**data not shown).

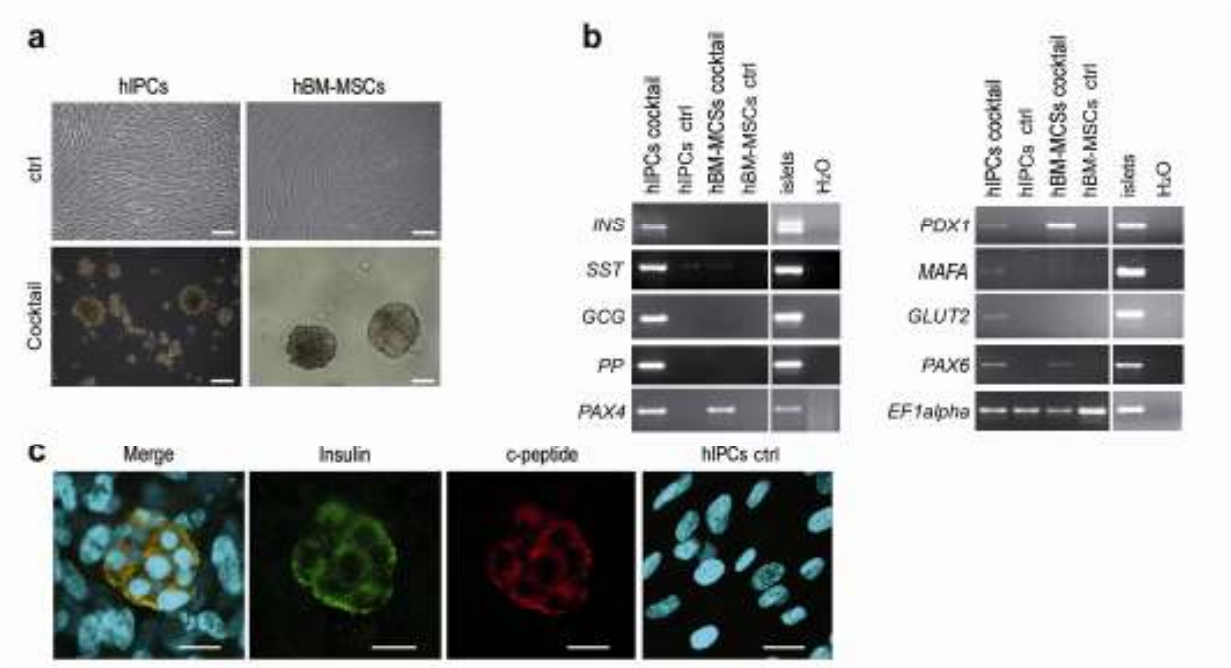


Fig. 20: Endocrine differentiation potential of hIPC and hBM-MSC

20 a: Cluster formation in hIPC (*left*) and hBM-MSC (*right*) after cultivation of cells in endocrine differentiation cocktail for 4 days. Bar represents 50 µm. **20 b:** Endocrine differentiation of hIPC and hBM-MSC monitored by detection of endocrine markers. RNA from human islets served as positive control (islets), undifferentiated hIPC and hBM-MSC as negative controls. EF1alpha was used as a housekeeping gene. Abbreviations: insulin (*INS*), somatostatin (*SST*), glucagon (*GCG*), pancreatic polypeptide (*PP*), V-maf musculoaponeurotic fibrosarcoma oncogene homolog A *(MAFA)*, pancreatic and duodenal homeobox 1 (*PDX1*), glucose transporter member 2 (*GLUT2*), paired box 6 (*PAX6*). **20 c:** Confocal imaging of endocrine differentiated hIPC immunostained for insulin (green) and c-peptide (red). Cell nuclei were stained with DAPI. Undifferentiated hIPC subjected to the same staining procedures served as negative control. Bar represents 20 µm. Figure is representative of 3 independent experiments (hIPC at passage ≥ 3, hBM-MSC at passage 1 or 2).

Detection of epithelial and mesenchymal cells in human pancreatic islet

To further evaluate the epithelial and mesenchymal cell populations residing in the islets of Langerhans, sections of isolated human pancreatic islets were analysed for the synthesis of insulin and vimentin by immunofluorescence. **Vimentin-positive cells (red) were found among the insulin-stained cells (green), which shows the existence of a mesenchymal cell-population, within the pancreatic islet** (Fig.21, left panel). The detection of **vimentin and insulin co-labeled cells (yellowish) in adult human islets *ex vivo* strongly suggest that** along with mature endocrine cells and mesenchymal cells, some others are found in an **epithelial-mesenchymal or mesenchymal-epithelial state of transition** (Fig. 21, right anel).

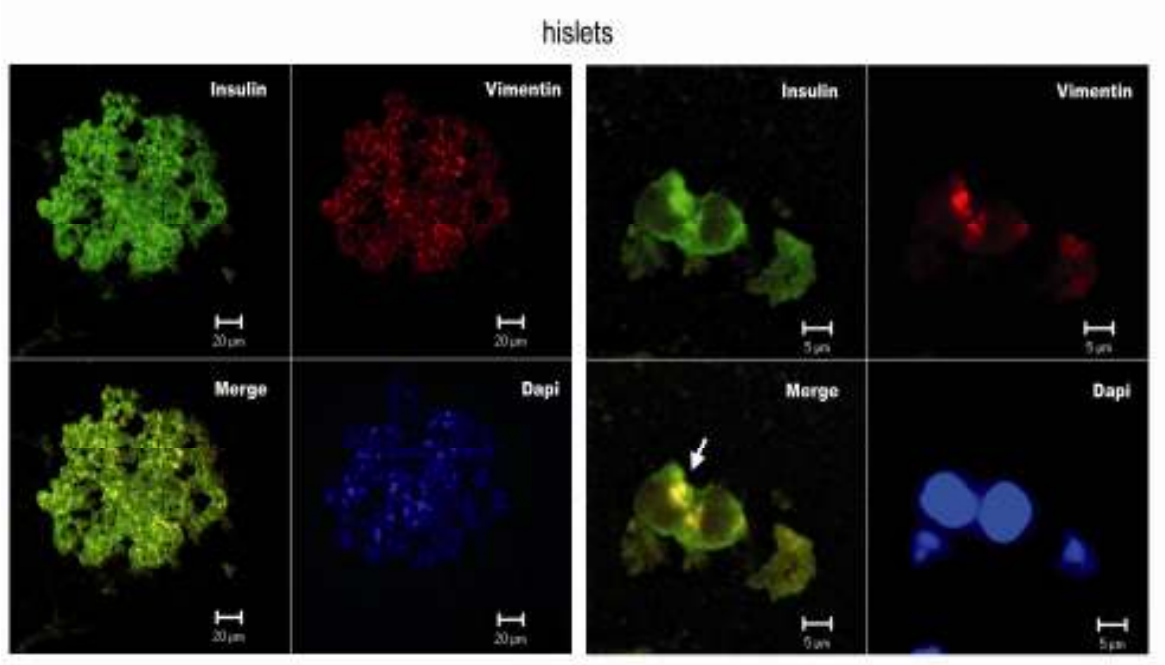


Fig 21: Detection of epithelial and mesenchymal cell markers in human pancreatic islets

Confocal imaging of a human pancreatic islet, co-stained with insulin (green) and vimentin (red). Cell nuclei were stained with DAPI. The left panel shows insulin/vimentin positive cells (yellow, *arrow*) on a section of whole pancreatic islet (bar represents 20 µm). The right panel shows single cells from the same islets labelled for insulin and vimentin (a*rrow*) (bar represents 5 µm) (n=3).

SAM-based genome-wide expression profiling in hIPC and hBM-MSC

The comparative analysis of gene hybridization signals from microarrays derived from hBM-MSC and hIPC was carried out using Significance Analysis of Microarrays (SAM). 22484 probesets were equally expressed in hIPC and hBM-MSC, 1606 were significantly higher expressed in hIPC (FC>2), 1995, were significantly lower expressed in hIPC (FC<0.5) (Fig. 22a).

Functional assignment of gene ontology clusters in hIPC and hBM-MSC

To assign functional categories to the identified gene clusters, microarray gene expression profiles of hIPC and hBM-MSC were examined using gene ontology (GO) analysis. **GOstat analysis in hIPC revealed a significant enrichment of functional**

gene clusters related to "gland development", "signal transduction" and "cell differentiation". In contrast, in hBM-MSC, ontology groups like most remarkably "skeletal development", but also "myogenesis" and "sensory organ development" were significantly overrepresented (Fig. 22b).

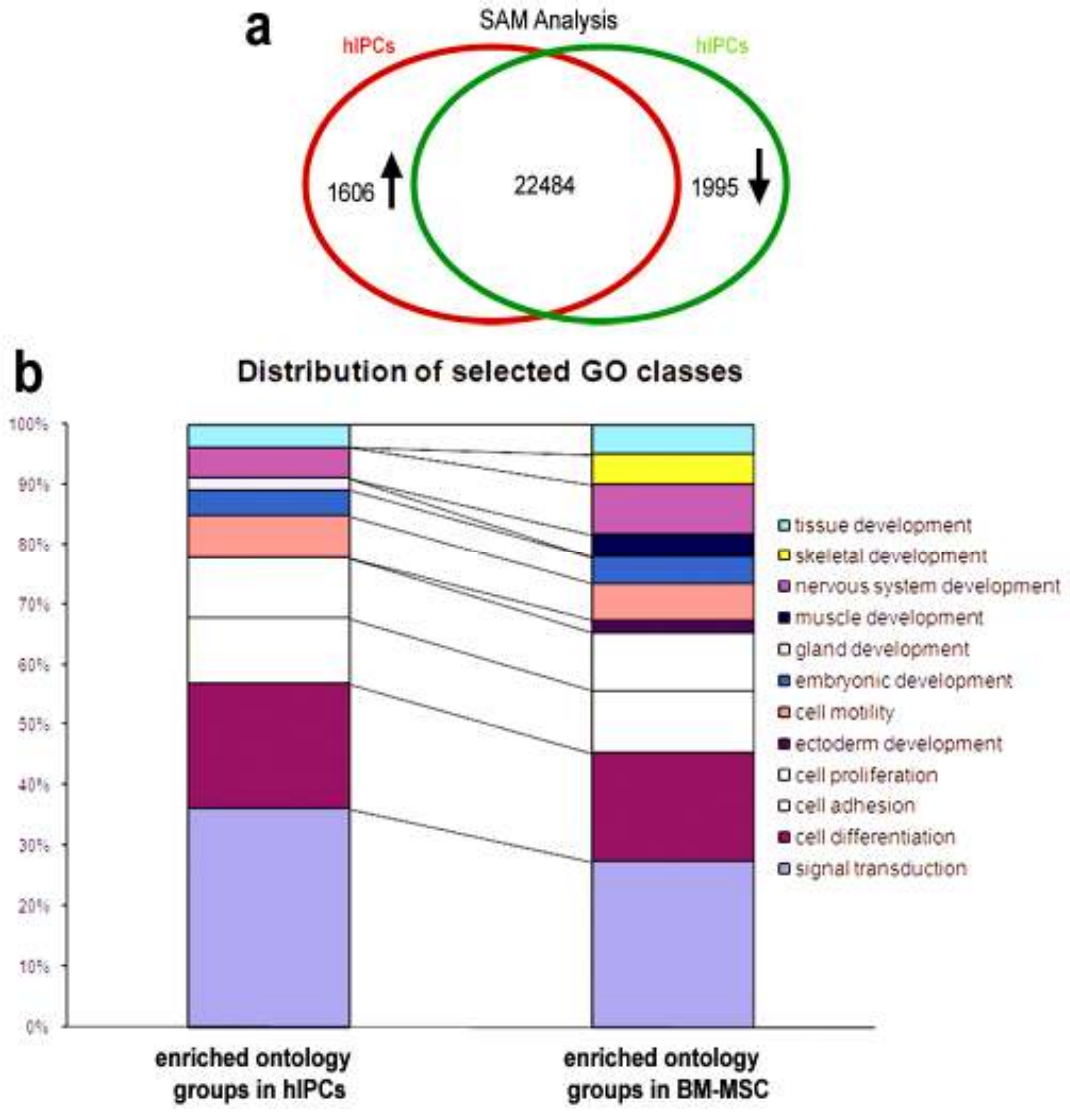


Fig. 22: Genome-wide expression profiling in hIPC and hBM-MSC.

22 a: Venn diagram of the number of probesets significantly upregulated (red) and downregulated (green) in hIPC (n=3) compared to hBM-MSC (n=5). Data were obtained by SAM (significance analysis of microarrays). **22 b:** GOstat analyses of gene ontology clusters in hIPC (n=3) and hBM-MSC (n=5). Enriched onthology groups in hIPC (left bar) and in hBM-MSC (right bar) are displayed.

Candidate gene clusters in hIPC and hBM-MSC

To identify relevant genes associated with mesenchymal or epithelial determination, cell-transition state and key organ morphogenesis, a candidate gene approach was used, based on the significantly differentially expressed genes in hIPC versus hBM-MSC which have been obtained by SAM. The expression pattern of key genes in both populations was compared on the basis of SAM data and verified by RT-PCR. Some of these genes were quantified by qPCR (Tab.1, Fig. 23a, b and c).

E-cadherin (*CDH1*), the classical epithelial marker was negative in hIPC albeit slightly expressed in hBM-MSC, while vimentin was strongly present in both populations (see also Fig. 18). Retinal cadherin (*CDH4*) was equally present in both populations according to RT-PCR analysis, in this case discongruent to SAM data. Also fetal kidney cadherin (*CDH6*) and osteoblast cadherin (*CDH11*) were equally expressed in hIPC and hBM-MSC. All other cadherins were either not differentially expressed or absent in either population (Tab. 1).

The expression pattern of E-cadherin repressor genes was very similar in hIPC and in hBM-MSC (Tab. 1) as shown by the expression of *SNAI1* and *SNAI2*, *TCF3* as well as *LOXL2*. In contrast, while *TWIST1* (FC 0.31) and *SIP1,* the E-cadherin promoter silencer (FC 0.34) were lower in hIPC, higher expression was found for *TWIST2* (mean FC 2.5), the high mobility group protein *HMGA2* (mean FC 2.8) and hepatocyte growth factor (*HGF*) (mean FC 4.0).

Finally, we looked for mesenchymal marker genes relevant in mesoderm specification and MSC differentiation versus those that are relevant for endocrine differentiation (Tab. 1 and Fig. 23a and c). hIPC displayed comparably low levels of fibroblast growth factor receptor 2 (*FGFR2)* (mean FC 0.06), which is active in MSC differentiation while mesenchyme-specific *MAFK* expression (FC 2.94) was relatively high. V-maf

musculoaponeurotic fibrosarcoma oncogene homolog (*MAF*/*c-MAF*), which is active in chondrocyte differentiation, was low (FC 0.04) in hIPC, as was its homolog *MAFB* (mean FC 0.17), which is largely expressed in developing endocrine pancreas and induces the glucagon promoter in adult alpha-cells. This indicates that hIPC on one hand display many mesenchymal features but their differentiation towards mature mesenchymal cells is hampered due to important signaling defects. *TCF21* (*Pod-1*), a basic helix-loop-helix transcription factor, is highly expressed in hIPC only (Fig. 23c). It represses terminal lineage maturation in myoblasts and is critically important for e.g. kidney organogenesis, suggesting that hIPC may be prohibited from mesenchymal maturation [248]. The expression pattern of members of the highly conserved homeobox gene family (*HOX*) is different in hIPC compared to hBM-MSC (Fig. 23b and c). *HoxA2*, *HoxA3*, *HoxA4* and *HoxA5* are significantly higher expressed in hIPC, while *HoxA7*, *HoxA9*, *HoxA10* and *HoxA13* are higher expressed in hBM-MSC. SRY sex determining box 17 (SOX17), a marker for definitive endoderm, was detected in hIPC but not in hBM-MSC (Fig. 23c)

Components of the TGFb signaling pathway were in part differentially expressed in hIPC (Tab. 1). While the expression of *TGFBR2* and *3* were similar, *TGFBR1* expression was lower (mean FC 0.37) in hIPC. TGFBR ligands were also differentially expressed. *TGFA* (FC 0.13) and *TGFB2* (FC 0.26) expression was reduced in hIPC, whereas *TGFB1* expression was unchanged. *SMAD1-5* expression levels were also similar, while the inhibitory *SMADs 6* and *7* were reduced (FC 0.23 and 0.48, respectively). *RUNX2* and *RUNX3*, genes associated with osteogenesis and differentiation into mesenchymal derived tissues, were significantly lower expressed in hIPC.

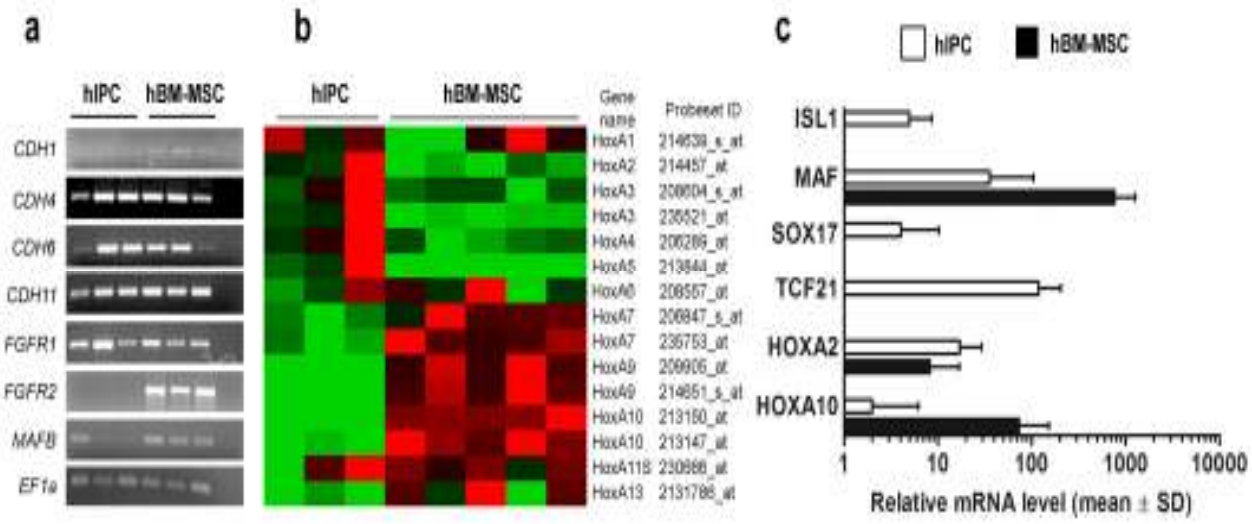


Fig. 23: Candidate gene clusters in hIPC and hBM-MSC

23 a: Detection of candidate gene expression in hIPC and hBM-MSC by RT-PCR. Abbreviations: E-cadherin (*CDH1*), retinal cadherin (*CDH4*), fetal kidney cadherin (*CDH6*), osteoblast cadherin (*CDH11*), fibroblast growth factor receptor 1 and 2 (*FGFR1, FGFR2*), v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (*MAFB*), **23 b:** Heatmap of the expression pattern of the highly conserved homeobox gene family A (HoxA) in different individual preparations of hIPC (n=3) and hBM-MSC (n=5). The gene names and the Affymetrix probeset IDs are shown. Red colour represents high expression (FC > 2), green colour represents low expression (FC < 0.5). **23 c:** Quantitative analysis of differentially expressed key genes in additional individual preparations of hIPC (n=5) and hBM-MSC (n=4) using qPCR. These donors are different from those used for the array analysis procedure thus mounting to eight and nine different donors for the respective primary cell populations. Abbreviations: insulin gene enhancer protein 1 (*ISL1*), v-maf musculoaponeurotic fibrosarcoma oncogene homolog (*MAF*), SRY (sex determining region Y)-box 17 *(SOX17)*, transcription factor 21 (*TCF21*), homeo box A2 *(HOXA2)* and homeo box A10 *(HOXA10)*. Data are expressed as means with standard deviations.

Tab. 1: Significance analysis of microarrays (SAM) of hIPC compared to hBM-MSC

Gene names, detection in hBM-MSC and hIPC (absent: A; present: P), analysed probeset IDs, fold changes (FC) of the expression values between hIPC and hBM-MSC, and the gene titles are shown. Fold changes marked with ** have highly significant q-values below 0.1. Where no difference between expression levels could be detected (ns: not significantly different), only the number of analysed probesets is given.

Gene Name	Detection in hBM-MSC	Detection in hIPC	Probesets analysed	Fold Change hIPC vs BM-MSC	Gene Title
Expression pattern of cadherins					
CDH4	A	P	220227_at	5.93**	cadherin 4, type 1, R-cadherin (retinal)
			206866_at	9.13**	
CDH6	P	P	3	ns	cadherin 6, type 2, K-cadherin (fetal kidney)
CDH11	P	P	3	ns	cadherin 11, type 2, OB-cadherin
Genes relevant in mesenchymal versus endocrine differentiation					
FGFR1	P	P	7	ns	fibroblast growth factor receptor 1
FGFR2	P	P	203638_s_at	0.02**	fibroblast growth factor receptor 2
			208228_s_at	0.1**	
MAF	P	P	206363_at	0.03**	v-maf musculoaponeurotic fibrosarcoma oncogene homolog
			209348_s_at	0.05**	
MAFB	P	P	218559_s_at	0.04**	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B
			222670_s_at	0.3**	
MAFK	P	P	226206_at	2.94**	v-maf musculoaponeurotic fibrosarcoma oncogene homolog K
TCF21	A	P	204931_at	475.32**	transcription factor 21 (POD-1)
Genes associated with EMT					
SNAI1	P	P	1	ns	snail homolog 1
SNAI2	P	P	1	ns	snail homolog 2
TCF3	P	P	3	ns	transcription factor 3
TWIST 1	P	P	213943_at	0.31**	twist homolog 1
TWIST 2	P	P	229404_at	2.92**	twist homolog 2 (Drosophila)
			1554163_at	2.07**	
LOXL2	P	P	2	ns	lysyl oxidase-like 2
SIP1	P	P	205063_at	0.34**	survival of motor neuron protein interacting protein 1
			3	ns	
HMGA	P	P	206074_s_at	3.36**	high mobility group AT-hook 1

Gene Name	Detection in hBM-MSC	Detection in hIPC	Probesets analysed	Fold Change hIPC vs BM-MSC	Gene Title
			210457_x_at	3.14**	
HMGA2	P	P	1558683_a_at	2.35**	high mobility group AT-hook 2
			1558682_at	3.53**	
			1561633_at	2.61**	
HGF	P	P	209960_at	6.48**	hepatocyte growth factor (hepapoietin A; scatter factor)
			210755_at	4.72**	
			209961_s_at	2.98**	
			210997_at	3.61**	
			210998_s_at	3.66**	
TGF beta signal transduction					
TGFBR1	P	P	224793_s_at	0.35**	transforming growth factor, beta receptor I
			206943_at	0.37**	
			236561_at	0.39**	
TGFBR2	P	P	2	ns	transforming growth factor, beta receptor II (70/80kDa)
TGFBR3	P	P	204731_at	ns	transforming growth factor, beta receptor III
TGFA	P	P	205016_at	0.13*	transforming growth factor, alpha
TGFB1	P	P	203085_s_at	ns	transforming growth factor, beta 1
TGFB2	P	P	220407_s_at	0.29**	transforming growth factor, beta 2
			209908_s_at	0.24**	
SMAD6	P	P	207069_s_at	0.23**	SMAD family member 6
SMAD7	P	P	204790_at	0.48**	SMAD family member 7
Genes relevant in mesenchymal differentiation					
RUNX2	P	A	1	ns	runt-related transcription factor 2
			236859_at	0.02**	
		P	232231_at	0.34**	
RUNX3	P	A	204197_s_at	0.17**	runt-related transcription factor 3
			204198_s_at	0.04**	
Genes relevant in endocrine differentiation					
ISL1	A	P	206104_at	21.89**	ISL1 transcription factor, LIM/homeodomain, (islet-1)
REN	A	P	206367_at	302.40**	renin
AGT	P	P	1	ns	angiotensinogen
AGTR1	P	P	205357_s_at	2.35**	angiotensin II receptor, type 1
			1	ns	

C-DISCUSSION, CONCLUSIONS AND FINAL CONSIDERATIONS

1. DISCUSSION

Research on cell-based therapies is developing very rapidly in many fields of Medicine as it holds the promise to cure chronic diseases in which tissue regeneration and cell replacement are required.

MSC are multipotent cells with paracrine effects and immunomodulatory properties, which present them as an ideal stem cell candidate for tissue engineering and regenerative medicine. In recent years it has come to light that MSC encompass plasticity that extends beyond the conventional bone, adipose, cartilage, and other skeletal structures, and has expanded to the differentiation of liver, kidney, muscle, skin, neural, and cardiac cell lineages.

With the present studies, we intended to evaluate the potential of human MSC to be utilized as a cell source for cell-based therapeutic strategies of T1D and advanced T2D.

In our first study, we investigated the potential of a well-characterized human MSC model (hMSC-TERT) [224], to be directed into the endocrine pathways of differentiation.

Our results provide evidence for the potential of telomerase immortalized hMSC-TERT to be reprogrammed towards pancreatic endocrine phenotypes by two different experimental approaches. **We demonstrate that hMSC-TERT display an endodermal pre-patterning phenotype, which may favour the endocrine pancreatic differentiation process. These multipotent MSC [224] respond to defined culture conditions with expression of several pancreatic endocrine lineage-related transcription factors, and also insulin and somatostatin genes. Furthermore, by stable overexpression of the two key pancreatic regulatory factors NGN3 and/or PDX1 in hMSC-TERT, we demonstrate the potential of these cells to be**

reprogrammed by defined genetic engineering procedures similar to other reprogramming approaches that were reported [210, 222].

Characteristically, hMSC-TERT cells retain a normal karyotype, can be differentiated into multiple mesodermal lineages [224] including an endothelial-like phenotype [249] and do not give rise to tumours when maintained under appropriate plating densities [250].

The detection of the neuronal protein nestin and the cell surface antigen c-met reinforces the multi-lineage differentiation potential of hMSC-TERT. Nestin represents a characteristic marker of multi-lineage progenitor cells [251] and c-met has been proposed to identify adult pancreatic precursor cells [181]. Previously, we found that c-met was expressed in 5% of freshly isolated bone marrow-derived MSC, while 80% of hMSC-TERT express this marker, suggesting that these cells originate from a c-met+ subpopulation of MSC thereby displaying an endodermal pre-patterning.

In our working hypothesis we postulated that telomerase-transduction may have sustained the multipotency of this clonal cell line. Validating this premise, we observed that treatment of hMSC-TERT with cocktail medium induces the expression of otherwise absent endocrine pancreatic genes including *ISL1*, *NEUROD1*, *PAX4*, *PDX1* and insulin. This response indicates an endodermal switching capacity, which is comparable to that described for human adipose-MSC treated with a similar cocktail medium [191]. The role of soluble factors such as nicotinamide, activin-A, beta-cellulin and exendin-4 combined to serum-free / high glucose medium as promoters of beta cell differentiation has been extensively investigated in previous studies, supporting the strong differentiation effect of the "cocktail medium" [95, 185, 252]. **Importantly, using hMSC-TERT the achieved reprogramming output was consistently reproducible. This contrasts with results obtained with primary MSC, ranging from absence of insulin expression [212] to amelioration of diabetes after transplantation of *in vitro***

generated insulin-producing MSC [187]. By this means, exploiting hMSC-TERT we could obviate the utilisation of heterogeneous MSC from different donors and different subpopulations, which unable reproducible data to be obtained [253].

Further investigation on cocktail-induced hMSC-TERT was not viable, in our study, due to phenomena of cellular apoptosis that occurred after 10 days of culture in serum free (SF) medium.

In a second approach, based on the ectopic expression of the two master regulatory endocrine genes, PDX1 and NGN3, we analysed both, the susceptibility of hMSC-TERT for endocrine reprogramming and specific molecular mechanisms of endocrine differentiation in human MSC. **NGN3 protein overexpression in hMSC-TERT results in endogenous PDX1 gene activation, which is essential for insulin promoter regulation in adult beta cells. In addition, we observed that NGN3 is involved in the activation of PDX1 promoter, which is a major regulator of insulin secretion in adult beta cells. This corroborates the idea that endocrine differentiation of MSC in vitro follows the hierarchy model of transcription factors involved in maturation and maintenance of beta cells 254**. Like others, we did not find *NGN3* gene activation due to ectopic *PDX1* expression in hMSC-TERT-PDX1 cells [210]. This may reflect the fact that *NGN3*, which is an important endocrine differentiation factor during development, is normally not expressed by mature beta cells [172]. Probably, as suggested by most recent work, in adult pancreas, endogenous NGN3 expression is activated whenever pancreatic injury occurs in order to stimulate beta cell mass regeneration [86, 255]. One reason why NGN3 is essential *in vivo* [172 86] but seems dispensable *in vitro* [210] may be the activation of alternative pathways to induce the endocrine differentiation cascade. Also, in our *in vitro* model, a transient expression of NGN3 cannot be excluded.

Other most important observation is that ectopic expression of NGN3 and/or PDX1 could induce hMSC-TERT to express, produce and store insulin protein. However, the amount of insulin produced by transgenic hMSC-TERT cells was low when compared to biological relevant levels synthesised by mature INS-1E beta cells. There may be two explanations for these results. First, activation of *SST*, *GCG* and *PP* genes in hMSC-TERT-derived phenotypes indicates that not every cell follows the specific reprogramming path towards beta cell differentiation, indicating variable differentiation responses to the same stimuli. Effectively, only 40% of hMSC-TERT-NGN3, 33% of hMSC-TERT-PDX1 and 30% of hMSC-TERT-NGN3/PDX1 were positive for insulin and C-peptide. Secondly, this observation may lie in the unavoidable polyclonal nature of generated stable cell populations, as suggested by 60% transfection efficiency in NGN3, PDX1 or NGN3/PDX1 new generated cell lines

Also, the insulin secretion capacity in response to glucose stimulation is reduced, suggesting that the molecular mechanism of insulin secretion may not be available in these cells. In contrast, retroviral PDX1 overexpression induces glucose-stimulated insulin release at high glucose dosages (> 15 mM) in freshly isolated human bone marrow-derived MSC [210], but noteworthy, such an approach failed to establish insulin gene expression in MSC samples in 5 out of 14 donors. This observation by others underscores the variability of primary MSC material and the need for a consistent cell culture model.

From our findings, we presume that to establish a fully functional beta cell phenotype, inclusion of yet unknown experimental parameters or other (transcription) factors is necessary. This consideration is supported by a recent *in vivo* study, in which from nine genes important for beta cell development that were subcloned into GFP-adenoviruses, higher GFP+/insulin+ ratio was only achieved when PDX1 and NGN3 were

administered together with MAFA adenoviruses [222]. Likewise, the addition of *MAFA* gene may also foster hMSC-TERT final endocrine maturation.

From our results in this study we can conclude that hMSC-TERT constitutes a suitable cell model to investigate the endocrine differentiation potential of hBM-MSC. The usage of hMSC-TERT made it possible to analyze the molecular mechanisms of the *in vitro* differentiation pathways in a controlled and reproducible way. Nevertheless, the here presented data and most recent knowledge clearly indicates, that further investigation is required to achieve terminal endocrine maturation of adult MSC, suitable to be used as a promising tool for cell based-therapy of diabetes.

The observations made in the previous study led us to hypothezise that, stromal derived/ mesenchymal stem cells (MSC) residing in distinct tissues might present different signatures (patterning and specification) and distinct multipotent functions.

Therefore, in a second project, it was our objective to compare two distinct candidate cell populations as tools or targets for the cell-based therapy of T1D: hBM-MSC and hIPC, obtained from mesodermal and endodermal derived tissues, respectively.

Here, we could show distinct functional signatures of these two MSC populations and identify specific key lineage regulatory genes in hBM-MSC and hIPC. Our data also indicate a clear transitional state of hIPC.

Likewise recent studies in rodents [230, 231] and in humans [226], our experiments strongly suggest that hIPC derive from mesenchymal cells within the pancreatic islet. This was shown by overlapping immunophenotypes of both cell types, similar expression levels of several mesenchymal markers in hIPC and hBM-MSC and a large vimentin-positive cell population in intact human islets.

In both populations, we detected genes that according to the literature have been related to functional stem cell characteristics like *BMI1* [215], *HES1* [256], *HMGB1* [257], and ABCG2 [258], which may indicate a role of both MSC populations as precursor cells.

Nevertheless and opposite to other recent studies [228, 230], the multilineage differentiating capacities of these two populations are very distinct: our direct comparison of hIPC and their differentiation potential with the "gold standard" of MSC reveals complementary deficits in cell maturation of hIPC and hBM-MSC into mesenchymal and endocrine pathways, respectively.

With genome-wide gene expression profiling, we identified differently expressed gene clusters in hIPC and hBM-MSC and unveiled a distinct functional assignment of the gene ontology groups in these cell types, providing evidence for a specified tissue signature and function of these two MSC populations.

Moreover, a candidate gene cluster analysis revealed important aspects related to the mesodermal pattern, endocrine versus mesenchymal commitment and cell transition state of hIPC. The vimentin^{+}/E-cadherin^{-} pattern and the comparable expression levels of other cadherin family genes showed the evident mesenchymal phenotype in both cell types.

HOX genes are known regulators of patterning and migration during gastrulation with respect to skeleton and endoderm [259]. **We observed that the *HOX* expression pattern in hIPC (*HoxA3-6*) is strongly related to the region of foregut and pancreas development, while *HOX* genes expressed in hBM-MSC (*HoxA7-13*) are related to skeletal development** [259, 260].

F**urthermore, *SOX17*, a marker for definitive endoderm, has only been detected in hIPC**. The variable expression level of *SOX17* in different hIPC preparations (Fig 6c) suggests different stages of endodermal commitment in these cells. It has been shown

that *SOX17* is transiently expressed during differentiation of endocrine precursors into islet-like cells, which makes it difficult to detect a consistent expression during the differentiation process [261, 262].

The concept of sustained signatures of MSC may provide important information for site-specific regeneration, based on the positional memory of MSC at the site of injury [263]. On the other hand, key genes for osteogenic differentiation such as *FGFR2* and *RUNX* genes [264] are repressed in hIPC, suggesting the defective mesenchymal differentiation potential of these cells.

Finally, our data present strong evidence for an intermediate phenotype of hIPC and for the maintenance of a positional mesenchymal signature. We found that *TCF21*, which is a key regulatory gene in MET processes, is highly induced in hIPC [248]. TGFb signalling is also important in EMT/MET and pancreas development [265]. Some components of TGFb signalling are repressed in hIPC, suggesting a permissive role of hIPC for islet cell replenishment. Recent contrasting data have traced hIPC to be either of mesenchymal origin [226, 230, 231] or to be a result of EMT from endocrine cells in culture [266 232, 267]. Our results emphasize the transitional state of this population. As the transitions are reciprocal processes, genes involved in EMT versus MET are very similar [268]. Thus, both mechanisms are possible, whereby their plasticity to either direction is more evident towards the endocrine lineage.

This work has brought new insights to the site-specific signatures and functions of both MSC types suggesting distinct potential of hBM-MSC and hIPC, and raises questions as to the usage of various MSC populations for cell-based manipulation of islets or any other tissue regeneration.

2. CONCLUSIONS

Due to their multipotent properties, MSC may have an almost unlimited differentiation potential once they have been reprogrammed. This might be achieved by specific culture conditions, genetic induction, silencing of key transcriptions factors or more recently through epigenetic modifications.

Our projects provided new insight to the biology and function of mesenchymal stem cells within and outside the pancreas. Using a well-characterized human mesenchymal stem cell model, we investigated the molecular mechanisms of endocrine differentiation in BM-MSC and the potential of these cells to be directed into the endocrine pathways of differentiation.

Furthermore, we compared the multilineage differentiation capacity of two MSC candidate cells, the hBM-MSC and the hIPC. In spite of the mesenchymal overllaping immunophenotype of both types of cells, differences in their stem cell character and cell commitment were observed. Genome-wide gene profiling analysis revealed very different molecular signatures, suggesting distinct mesodermal specification of the two MSC types and consequently distinct potential as tools and targets for beta cell replacement or regeneration.

While hMSC-TERT constitute a suitable cell model for the study of endocrine differentiation mechanisms of multipotent hBM-MSC, hIPC population, due to its functional signature and transitional state of differentiation may also contribute to beta-cell maintenance and/or replenishment by sustaining a complete mesenchymal-endocrine transition.

3. CONSIDERAÇÕES FINAIS E PERSPECTIVAS FUTURAS

O trabalho desenvolvido permite-me fundamentar algumas conclusões e discernir futuras linhas de actuação.

Os resultados obtidos indicam-nos que o potencial das populações MSC, nomeadamente das hIPC e hBM-MSC estão ainda por explorar. Outros estudos mostraram já que tanto as hIPC como as hBM-MSC quando transplantadas em ratinhos diabéticos levam à melhoria da glicémia nestes animais. Contudo, a resposta insulínica às variações da glicémia não é satisfatória o que indica uma diferenciação endócrina incompleta destas células *in vivo*.

A reprogramação genética das hIPC e hBM-MSC poderá optimizar a sua diferenciação endócrina. Uma linha de investigação poderá ser a indução da transição completa das hIPCs para células epiteliais endócrinas, identificando os mecanismos moleculares e os genes responsáveis pelo processo de MET. Outra linha de investigação, conhecendo agora o padrão de expressão de possíveis genes determinantes da assinatura molecular nestas células, poderá ser o desbloquear de certos genes (*HOX6-7*) ou a expressão de outros (*TCF21*). Estas abordagens poderão ser determinantes para a reprogramação *in vitro* de hBM-MSC, permitindo a sua utilização como fonte celular para o tratamento da *diabetes tipo 1*.

É também urgente reflectir sobre estratégias de regeneração pancreática, nomeadamente, na identificação de células ou biomoléculas que influenciem a regeneração endógena de células beta, estimulando mecanismos de proliferação, diferenciação ou migração celular.

Por outro lado, uma imunomodulação eficaz do processo autoimnune destruidor da massa de células beta, em combinação com a terapia celular, será sem dúvida imprescindível à cura da *diabetes tipo 1*.

Este é pois um campo de investigação que liga a medicina à investigação básica e se constitui um exemplo de Medicina translaccional por excelência. Devido aos avanços conseguidos pelos inúmeros grupos de investigadores, à pressão económica actual e ao impacto dos *media* na sociedade, existe um risco de oferta de terapias celulares curativas sem base científica rigorosa. São prova disso os centros médicos, de transplante de células estaminais já existentes na Europa, para o tratamento das mais variadas patologias, cuja idoneidade é questionável.

É minha firme intenção prosseguir na investigação das células estaminais adultas, candidatas à utilização em estratégias de terapia celular da *diabetes tipo 1*.

Procurando encontrar respostas a inúmeras questões surgidas ao longo deste trabalho e com a experiência e conhecimentos que ele me permitiu acumular, o passo natural seguinte é elaborar novos projectos de investigação visando esta e outras vertentes da terapia celular da diabetes.

Cito como exemplo de estudos actualmente propostos a entidades financiadoras:

1) Novos métodos de imagem para a detecção de células transplantadas em humanos (colaboração com o instituto de física e departamento de biologia de células estaminais da Universidade de Würzburg, proposto para uma bolsa de projecto Europeu Marie Curie);

2) O estudo das células progenitoras expressoras do gene *ISL1,* (colaboração com o Instituto Gulbenkian Ciencia, proposto à FCT;

3) O potencial dos análogos do glucagon-like-peptide 1 (GLP1) como regeneradores das células endócrinas na *diabetes tipo 1* (proposto a 2 empresas farmacêuticas);

4) Estudos para a avaliação do papel da *Vit D como imunomodulador na diabetes tipo 1* (colaboração com o departamento biologia de células estaminais da Universidade de Würzburg proposto para uma bolsa de projecto Europeu Marie Curie).

É minha expectativa que o desenvolvimento destas linhas de investigação contribua para:

a) Compreender as potencialidades dos tecidos e orgãos humanos, como possíveis fontes de células utilizáveis na regeneração e/ou substituição celular , para o tratamento definitivo da *diabetes tipo 1* e situções avançadas da *diabetes tipo 2*;

b) Desenvolver técnicas de detecção de células transplantadas sem risco de toxicidade humana;

c) Compreender o papel de determinadas biomoléculas envolvidas em mecanismos de imunomodulação essencial à prevenção e regeneração da massa de células beta do pâncreas endócrino.

É minha esperança contribuir para o desenvolvimento, em Portugal, da medicina translacional criando um Grupo de Trabalho neste campo da investigação e motivando jovens médicos a investir na sua formação nestas áreas tão fascinantes quanto inovadoras.

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F- DADOS COMPLEMENTARES / SUPPLEMMENTS

Suppl. Table 1: Primer Sequences and Conditions of PCR used in Project 1 and 2

Genes	sense primer antisense primer 5'-3'	Annealing Temperature (C°)	Cycles	Product size
ABCG2	GAAACCTGGTCTCAACGC TGGAGTCTGCCACTTTAT	50	35	317 bp
BMI1	TCGGAAAGTAAACAAAGACAAAGAG TAGGCAAACAAGAAGAGGTGGA	52	35	389 bp
CDH1	ACTGGACCATTCAGTACAAC GAACCGCTTCCTTCATAG	54	30	610 bp
CDH11	GGGGAGGAAGTAGGAAGAGTG ATACCTTATCGGGCTGTTGG	60	38	408 bp
CDH4	CAAAGACAACACGGCGGGAATC CGGGCACAATGTCCCTTCGTAA	66	38	458 bp
CDH6	ATTATCAGTATGTGGGCAAGTT GACATCAGACATTTCAGGGAC	60	38	314 bp
C-MET	CAATGTGAGATGTCTCCAGC CCTTGTAGATTGCAGGCAGA	57	35	560 bp
EF1alpha	AGGTGATTATCCTGAACCATCC AAAGGTGGATAGTCTGAGAAGC	56	35	235 bp
FGFR1	CGGGACATTCACCACATC CCACTGGCACCACCTATCT	56	27	1037 bp
FGFR2	CTCCTCCATGAACTCCAAC CTGGCTTATCCATTCTGTG	53	38	872 bp
FNT	ACAGTGGCAGAAGGAATA AGAACTCTAAGCTGGGTC	50	35-38	334bp
GLUC	ACTTTGTGGCTGGATTATTTGT GTTTGGCAATGTTATTCCTGTT	52	38	258 bp
GCK	CTGGTGAAGGTGGGAGAAG GCAGGAGATCATCGTGGC	55	38	312 bp
GLUT2	TCCAGCTACCGACAGCCTATTC AGATGGCACAAACAAACATCCC	55	38	257 bp
HES1	GTCAACACGACACCGGATAA AGCGGGTCACCTCGTTCA	57	35	310 bp
HMGB1	TCCATTGGTGATGTTGCGAAGA TCAGCCTTGACAACTCCCTTTT	53	35	176 bp
HOXB5	TTCACCGAAATAGACGAGG AAGACCTAAGACCAAACGAAA	53	40	893 bp
INS	GCAGCCTTTGTGAACCAACACC	58	38	209 bp

	TGTTCCACAATGCCACGCTTC			
ISL1	CAGGTTGTACGGGATCAAATGC GATGCTTCGCTTCTTGTCCTTG	60	35	508 bp
MAF	ATAGTGGGAGTAGCAAACAAAT AAGATCAAACGCAGCGTAAA	56	38	156 bp
MAFB	AGCCCGACCGAACAGAAGAC TGATGGTGGTGGTGGTGAGC	62	38	188 bp
MEIS1	ACCAGCCCTCTTGGAACA AGTCCCGTGTCTTGTGCC	57	35	315 bp
MEIS2	CAACCTCAACCCACTCAG TGCCCATTCCACTCATAG	53	35	400 bp
MMP2	CCTGATGTCCAGCGAGTG TCAGCAGCCTAGCCAGTCG	54	35-38	297 bp
NESTIN	AGAGGGGAATTCCTGGAG CTGAGGACCAGGACTCTCTA	58	35	495 bp
NEUROD1	CAGGCGCATAGACCTGCTA TTCTTGTCTGCCTCGTGCT	54	38	312 bp
NGN3	CTCAACTCGGCACTGGACG CAGGGAGAAGCAGAAGGAACAA	60	35	473 bp
OCT1	AATCCGTCAGAAACCAGTA GCAGGCGTCAAAGTAAGC	52	35	344 bp
P4HA1	GTTGGAGCTAGTGTTTGG CTGCCTTGTCTTCTGTGA	49	35-38	437 bp
PAX4	ACCGAGTCCTGCGGGCATTAC TGCCCACGCTGGAACTCTTTCT	60	38	210 bp
PAX6b	AATTCTGGGCAGGTATTACGA CAGCCATCTTGCGTAGGTT	56	35	366 bp
PDX1	CCCATGGATGAAGTCTACC GTCCTCCTCCTTTTTCCAC	55	35	262 bp
PP	GTGTACCCAGGGGACAATG CGTAGGAGACAGAAGGTGGC	53	35	207 bp
SNAI2	TTCCAGACCCTGGTTGCT CATTTGGCTTCGGAGTGA	53	35-38	283 bp
STT	TTTAGGAGCGAGGTTCGG TCAGGTTCCAGGGCATCA	54	38	306 bp
TCF21	TTGGAATGTGACGGGTTGAA TGGGCTCTATCCCTCTGGTA	58	38	553 bp
THY1	CCCGAACCAACTTCACCA GGCTTCCTGTCTCCTCCAT	54	35-38	291 bp
VIM	AGCTTCCTGTAGGTGGCA CAGTAGCGAGAAGAATGGT	52	35-38	356bp

Legend suppl Tab. 1:

Names of the amplified genes, sequences of the primer pairs, annealing temperatures, number of cycles and the size of the corresponding PCR product are shown (ATP binding cassette group 2 (ABCG2), polycomb group repressor gene 1 (Bmi1), E-cadherin (CDH1), retinal cadherin (CDH4), fetal kidney cadherin (CDH6), osteoblast cadherin (CDH11), hepatocyte growth factor receptor (C-MET), elongation factor 1 alpha (EF1alpha), fibroblast growth factor receptor 1 and 2 (FGFR1, FGFR2), Fibronectin (FNT), glucagon (GLUC), glucokinase (GCK), glucose transporter member 2 (GLUT2), hairy enhancer of split 1 (Hes1), high mobility group box 1 (Hmgb1), homeobox b 5 (HOXb5), insulin (INS), insulin gene enhancer protein 1 (ISL1), v-maf musculoaponeurotic fibrosarcoma oncogene homolog (MAF), v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB), meis homeobox 1 and 2 (MEIS1, MEIS2), matrix metalloproteinase 2 (MMP2), POU class 2 homeobox 1 (OCT1), neurogenic differentiation 1 (NEUROD1), neurogenin 3 (NGN3), prolyl 4-hydroxylase, alpha I subunit (P4HA1), paired box 4 (PAX4), paired box 6 (PAX6b), pancreatic and duodenal homeobox 1 (PDX1), pancreatic polypeptide (PP), snail 2 (SNAI2), somatostatin (STT), transcription factor 21 (TCF21), Thy1 (THY1), vimentin (VIM).

Suppl. Tab. 2: Expression of stemness-related genes in hIPC compared to hBM-MSC.

Gene Name	Probesets on U133Plus2 Array	Detection in hBM-MSC	Detection in hIPC	Affymetrix Probeset ID	Fold Change hIPC vs BM-MSC	Gene Title
Stemness-related Genes						
ABCG2	1	P	P	209735_at	ns	ATP-binding cassette, sub-family G (WHITE), member 2
BMI1	1	P	P	202265_at	ns	B lymphoma Mo-MLV insertion region (mouse)
HES1	2	P	P	203394_s_at	4.39	hairy and enhancer of split 1, (Drosophila)
				203395_s_at	3.5	
HMGB1	6	P	P	200679_x_at	ns	high-mobility group box 1
				200680_x_at		
				214938_x_at		
				224731_at		
				224734_at		
				216508_x_at		
HOXB2	1	P	P	205453_at	2.5**	homeobox B2
HOXB5	2	P	P	205601_s_at	8.75**	homeobox B5
				205600_x_at	7.07**	
POU2F1	3	P	P	206789_s_at	ns	POU domain, class 2, transcription factor 1
PAX8	8	P	P	121_at	ns	paired box gene 8
MEIS1	3	P	P	242172_at	3.89**	Meis1, myeloid ecotropic viral integration site 1 homolog (mouse)
				1559477_s_at	3.18**	
				204069_at	3.44**	
MEIS2	1	P	P	207480_s_at	ns	Meis1, myeloid ecotropic viral integration site 1 homolog 2 (mouse)

Legend suppl. Tab. 2: Gene names, detection in hBM-MSC and hIPC (absent: A; present: P), analysed probeset IDs, fold changes (FC) of the expression values between hIPC and hBM-MSC, and the gene titles are shown. Fold changes marked with ** have highly significant q-values below 0.1. Where no difference between expression levels could be detected, the number of analysed probesets is given (ns: not significantly different).