Mesenchymal stromal cell interactions with pancreatic islets and liver cells modulate viability and functionality

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La Faculté des sciences, sur le préavis de Monsieur L. H.BÜHLER, professeur ordinaire et directeur de thèse (Faculté de médecine, Département de chirurgie), Madame B. GALLIOT, professeure associée et codirectrice de thèse (Département de génétique et évolution), Monsieur J. SEEBACH, professeur ordinaire (Faculté de médecine, Département de médecine interne spécialités), Madame C. GONELLE-GISPERT, docteure (Faculté de médecine, Département de chirurgie), Madame R. BOTTINO, professeure (Institute of Cellular Therapeutics, Allegheny Health Network, Pittsburgh, Pensylvania, U.S.A.), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

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1. ABBREVIATIONS

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CCL4	Carbon tetrachloride
CD	Cluster of differentiation
CK	Cytokeratin
DC	Dendritic cells
E	Epithelial
GVHD	Graft versus host disease
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HNF	Hepatocyte nuclear factor
HSC	Hematopoietic stem cells
ICAM	Intercellular adhesion molecule
IDO	Indoleamine 2,3dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin
ISCT	International Society of Cellular Therapy
MCP	Monocyte chemoattractant protein
MFGE8	Milk fat globule-EGF factor 8
MHC	Major Histocompatibility Complex
MMP	Matrix metalloproteinase

MSC	Multipotent mesenchymal stromal cell
N	Neural
NK	Natural killer
NOD	Non-obese diabetic
Pdl	Programmed death-ligand
PDX-1	Pancreatic and duodenal homeobox 1
PEG	Poly(ethylene glycol)
PGE2	Prostaglandin E2
ROCK	Rho associated kinase
STZ	Streptozotocin
T1D	Type 1 diabetes
TGF	Transforming growth factor
Th	T helper
TIMP	Tissue inhibitors of metalloproteinases
TNF	Tumor necrosis factor
Treg	T regulatory
VEGF	Vascular endothelial growth factor

2. SUMMARY

Mesenchymal stromal cells (MSCs) actively contribute to their environment by secretion of cytokines, growth factors and extracellular matrix molecules. Their contribution to tissue regeneration remains to be explored. Likewise, a better understanding of molecular mechanisms leading to the beneficial effects in tissue repair processes will ultimately help to define the functional role of MSCs and their use for regenerative applications.

In this study, we first explored the potential of human MSCs to sustain the function of isolated human islets of Langerhans. Co-culture studies revealed that MSCs, together with islets, promoted insulin secretion. This effect was dependent on cell-to-cell contact, and was absent when islets were cultured alone or in co-culture with MSCs without direct contact. Using qPCR analysis, we identified expression of the adhesion molecule N-cadherin in islets and MSCs. Specific blocking antibodies against N-cadherin significantly decreased the MSC-induced increase in insulin secretion, without affecting insulin secretion by islets alone. Furthermore, the transplantation of co-encapsulated islets and MSCs into immunocompetent and streptozotocin-induced diabetic mice revealed a significant prolongation of graft survival, compared to mice transplanted with encapsulated islets alone.

Human MSCs were investigated for the improvement of porcine hepatocyte survival and albumin secretion. To this aim, we optimized and standardized a high-yield isolation protocol for hepatocytes from porcine liver. We demonstrated *in vitro* that MSCs enhance both the metabolic function of hepatocytes and albumin secretion. Co-encapsulation of hepatocytes with MSCs, using a novel biocompatible PEG hydrogel, showed increased viability and albumin secretory functions.

In conclusion, our data suggests that MSCs provide beneficial structural and paracrine signals for both human islets and porcine hepatocytes. As a mechanism, we reveal that MSCs increase islet function via N-cadherin interactions, demonstrating that paracrine signals are insufficient on their own. Therefore, co-encapsulation of hepatocytes and islets with MSCs might represent a valuable strategy to increase the viability of cells in microcapsules for clinical approaches, for future treatments in T1D and acute liver failure.

3. RÉSUMÉ EN FRANÇAIS

Les cellules multipotentes mésenchymateuses stromales (MSC) contribuent activement à leur environnement en sécrétant des cytokines, des facteurs de croissance et des molécules de la matrice extracellulaire. Toutefois, leur contribution à la régénération tissulaire est encore à explorer. Une meilleure compréhension du mécanisme moléculaire menant à des effets bénéfiques dans le processus de réparation des tissus permettrait ainsi de définir le rôle fonctionnel des MSC et leur utilisation pour des applications de régénération.

Dans cette étude, nous avons d'abord exploré le potentiel des MSC humaines pour soutenir les fonctions des îlots de Langerhans humains. Des études ont révélé que les MSC, en co-culture avec les îlots, améliorent la sécrétion d'insuline. Cet effet est absent quand les îlots sont cultivés seuls ou en co-culture sans contact direct, il est donc dépendant du contact intercellulaire. Par analyse de qPCR, nous avons identifié l'expression de la molécule d'adhésion N-cadherine dans les îlots et les MSC. Des anticorps bloquants spécifiques anti-N-cadhérine diminuent significativement l'augmentation de sécrétion d'insuline induite par les MSC sans affecter la sécrétion d'insuline des îlots seuls. De plus, la transplantation d'îlots et de MSC co-encapsulés, dans des souris immunocompétentes et rendues diabétiques par injection de streptozotocine, a montré une prolongation de la survie du greffon en comparaison avec des souris transplantées avec des îlots encapsulés seuls.

De plus, nous avons optimisé et standardisé un protocole à haut rendement pour l'isolement d'hépatocytes de porc. Les MSC humaines ont été étudiées pour améliorer la survie et la sécrétion d'albumine des hépatocytes porcins. *In vitro*, les MSC soutiennent la sécrétion et la fonction métabolique des hépatocytes. La co-encapsulation des hépatocytes avec des MSC utilisant un nouvel hydrogel PEG biocompatible a montré une augmentation de la viabilité et de la fonction de sécrétion d'albumine.

En conclusion, nos résultats suggèrent que les MSC fournissent un bénéfice structurel ainsi que des signaux paracrines aux îlots humains et aux hépatocytes porcins. Concernant le mécanisme, nous avons révélé que les MSC augmentent la fonction des îlots *via* des interactions utilisant la N-cadhérine, démontrant ainsi que les seuls signaux paracrines ne sont pas suffisants. Par conséquent, la co-encapsulation des hépatocytes et des îlots avec des MSC peut représenter une stratégie valable dans le cadre clinique pour augmenter la viabilité des cellules dans les microcapsules pour de futurs traitements du diabète de type 1 et de l'insuffisance hépatique aigue.

4. INTRODUCTION

4.1 Multipotent mesenchymal stromal cells

Mesenchymal stromal cells (MSCs) were discovered by Friedenstein in 1970, when he isolated adherent fibroblast-like cells from the bone marrow of a guinea-pig (1). MSCs originate from the mesoderm and are now isolated from almost all tissues throughout the body (2). MSCs reside in the stromal adherent fraction of the bone marrow, where they sustain the homeostatic turnover of non-hematopoietic stromal cells, thus regulating hematopoietic stem cells (HSCs) maintenance (2-4). In fact, after bone marrow irradiation in mice and consequent death of all progenitor cells, the infusion of MSCs together with HSCs improves the recovery of hematopoiesis, compared to mice infused with HSCs alone (5). Similarly, *in vitro* cultured MSCs sustain hematopoiesis for up to six months (6). HSCs are sustained by several cells derived from MSCs, such as reticular cells, adipocytes and osteoblasts, as well as macrophages (7). These cells together constitute the hematopoietic stem cell "niche", which regulate HSC function through cellular contact and paracrine signaling (8). Indeed, MSCs secrete many cytokines that promote cell renewal, such as stem cell factor (SCF), leukemia inhibitory factor (LIF), stromal cell-derived factor-1 (SDF-1), transforming growth factor (TGF)-β and others that promote hematopoietic cell maturation, such as granulocyte-macrophage (GM) colony-stimulating factor (CSF) and G-CSF (9). Hence, MSCs sustain HSC function by regulating quiescence and self-renewal through cell contact and secreted molecules (10).

MSCs are preferentially isolated from the bone marrow, where they represent 0.01%-0.0001% of the nucleated cells (11). Given that MSCs are scarce in the bone marrow, MSCs are often isolated from several other abundant tissues, most commonly the adipose tissue (12-16), the umbilical cord (17, 18) and Wharton's jelly (19), but also from the amniotic membrane (20),

placenta (21), dental pulp (22), tonsils (15, 23), lung (24), pancreas (25), liver (26), dermis (27, 28) and skeletal muscle (29).

To have a consensus about how to identify MSCs, the International Society of Cellular Therapy (ISCT) defined cells as Multipotent Mesenchymal Stromal Cells when they express or lack combination of surface markers, listed in Table 1 (11, 30-34).

Positive (ISCT)	CD73, CD90, CD105
Other positive markers	CD13, CD29, CD44, CD46, CD51, CD54, CD55, CD59, CD106, CD146 (MCAM), CD166, CD271, ICAM-1, ITGA11, STRO-1, VCAM-1, CXCR4, Sca-1, Nestin, PDGF Rα, PDGF Rβ, Integrin α1
Negative (ISCT)	$CD14^-$, $CD34^-$, $CD45^-$, $CD79a^-$, human leukocyte antigen (HLA) class 2
Other negative markers	$CD\text{-}11b^-$, $CD19^-$, $CD31^-$, $CD36^-$, $CD40^-$, $CD80^-$, $CD86^-$, von Willebrand $factor^-$

Table 1. Surface markers of MSC.

MSCs adhere to plastic support under *in vitro* culture conditions. Indeed, MSCs are adherent cells, with proliferative and self-renewal capacities and a spindle shaped morphology similar to fibroblast, although they maintain limited cell sprouts compared with fibroblasts (31, 35).

Furthermore, the ISCT defined MSCs by their *ex vivo* capacity to differentiate into osteocytes, chondrocytes and adipocytes. MSCs have limited differentiation capacities compared to embryonic stem cells or with induced pluripotent stem cells (IPS) that have differentiation potential similar to embryonic stem cells. However, these extended differentiation capacities implicate a high risk of teratoma development which remains minimal in MSCs.

The tissue source of MSCs may direct their ability to differentiate. In fact, after isolation from the pancreas, one study showed that MSCs expressed embryonic markers, such as Oct-4

(Pou5f1-POU domain, class 5, transcription factor 1), Sox2 (sex determining region Y-box 2) and Rex-1 (zinc finger protein 42), and presented high proliferative capacities and telomerase activity, demonstrating their undifferentiated state (25). Independently, another group retrieved pancreatic MSCs, which were positive for classical markers, such as cluster of differentiation (CD)13, CD29, CD44, CD73, CD90, CD105, nestin and vimentin and negative for von Willebrand factor, CD31, CD34, CD45, cytokeratin (CK) 19 and CA19.9, suggesting that the origin of these cells was not ductal, endothelial or hematopoietic (14, 36). Nevertheless, pancreatic MSCs expressed constitutively specific markers of differentiation (25) and had the potential to differentiate into adipocytes and osteocytes (14, 36). Moreover, after culture with specific induction factors, MSCs derived from the pancreas, expressed Pdx-1 (pancreatic and duodenal homeobox 1), insulin, connecting peptide or C peptide and Glut-2 (glucose transporter-2); whereas MSCs derived from the bone marrow under the same conditions only expressed Glut-2 and insulin (37). These data suggest that the differentiation potential of MSCs is dependent on their origin. Nonetheless, MSCs derived from the pancreas or the bone marrow manifest the same immunomodulatory capacity to suppress T cells after anti-CD3 and anti-CD28 stimulation (38).

4.1.1 Secreted molecules and paracrine effects

The precise molecular mechanisms leading to the beneficial effects by MSCs remain largely unknown. However, previous studies have identified some relevant molecules synthetized and released by MSCs. Indeed, MSCs secrete trophic molecules that could be classified into cytokines, growth factors, receptors and binding proteins (39). The principal trophic factors that are secreted by MSCs are: interleukin (IL)-6, TGF-β, hepatocyte growth factor (HGF), prostaglandin E2 (PGE2) and vascular endothelial growth factor (VEGF). Further, MSC-

conditioned medium or MSC-derived vesicles are responsible for proliferative, angiogenetic, anti-fibrotic and immunomodulatory effects (40).

4.1.1.1 Effect on cell viability

MSCs secrete trophic molecules that have the capacity to rescue injured cells, accelerate tissue repair and decrease apoptosis (40). A protein screen on a secretome derived from human MSCs, revealed a correlation between MSCs-derived VEGF and cell proliferation and development. Indeed, systemic injection of MSC-conditioned medium, led to increased survival in mice with acute liver failure (41). Moreover, VEGF has an anti-apoptotic effect (42). Additionally, HGF protects cells from apoptosis, while its neutralization suppresses the protective effects (43). HGF, together with VEGF and insulin-like growth factor (IGF)-1, protect renal cells from acute injury after ischemia reperfusion (44).

Furthermore, MSC-conditioned medium has the capacity to improve hepatocyte proliferation, stimulate angiogenesis, trigger anti-inflammatory cytokines and increase expression of hepatic genes relevant for proliferation, such as tumor necrosis factor (TNF)-α and IL-6 (45, 46). Moreover, MSC-conditioned medium increases in turn, VEGF and matrix metalloproteinase (MMP)-9 expression in hepatocytes, suggesting multiple mechanisms behind the increased cell survival and proliferation (45, 46).

4.1.1.2 Angiogenic effect

MSCs facilitate wound healing and are also implicated in promoting angiogenesis (39). Several factors released by MSCs are known to promote angiogenesis. For example, VEGF, which increases endothelial cell migration and proliferation, thus promoting angiogenesis (39) as demonstrated in several animal models, such as dogs, rats and mice (42). Further, MMP-1 is involved in angiogenesis, in particular MMPs mediate the destruction of the basement

membrane, allowing endothelial cells to migrate and restore the capillary structure. Moreover, MMP-2 and MMP-9 derived from MSCs enhance elastin production during wound healing (47). Likewise, HFG is involved in the acceleration of the wound healing and angiogenesis (42).

4.1.1.3 Anti-fibrotic effect

Several studies report that MSC-conditioned medium exerts protective effects towards liver fibrosis in mouse animal models. Indeed, MSC-conditioned medium is efficient in decreasing α-smooth muscle actin, type I collagen and MMP-2 expression, molecules involved in fibroblast activation (31). Moreover, the secretome of umbilical cord-derived MSCs contains several proteins that are protective towards liver fibrosis in mice. In particular, milk fat globule-EGF factor 8 (MFGE8), injected in mice with liver injury, elicited anti-fibrotic effects that are reversed by the neutralization of the protein with a specific antibody. The hepatic level of MFGE8 in patients with cirrhosis was also reduced, correlating with the anti-fibrotic properties of MFGE8 (48). Further, IL-1Ra and stanniocalcin-1, derived from MSCs, are, in part, responsible for the reduction of fibrosis in bleomycin-induced lung fibrosis in mice (49, 50).

4.1.2 Differentiation capacity

MSCs are characterized by the capacity to differentiate into several cell types (figure 1). MSCs originate from the mesodermal embryonic lineage, and have the capacity to differentiate principally into cells derived from the mesodermal lineage, such as osteocytes, chondrocytes and adipocytes, as well as muscle cells, myofibroblasts, cardiomyocytes and endothelial cells (31, 51, 52).

MSCs also have the capacity to trans-differentiate into cells derived from other germline lineages, as endoderm. MSCs can differentiate into islets (37) and hepatocytes (53, 54). Furthermore, they have the capacity to trans-differentiate into cells derived from the ectoderm cell lineage, such as neurons and epithelial cells (11, 55). However, the crossing of the lineage barrier into cell types others than the mesodermal lineage remains controversial.

After *in vitro* differentiation of MSCs into osteocytes, calcium deposits are visible in the culture (31). The activation of the pathway leading to bone formation is well known. Runx2 (runt-related transcription factor 2), Osterix and β-catenin are the principal transcription factors involved in the osteogenic differentiation (56). Runx2 promotes MSCs to a pre-osteoblastic commitment, thus inhibiting chondrogenic and adipogenic differentiation (57). Wnt, Notch and BMP activate Runx2, which also takes place through the translocation of Smad into the nucleus (58). Runx2 is essential for osteoblast differentiation since the suppression of Runx2 blocks Osterix, and its absence, leads in turn to arrest cortical bone and trabeculae formation in bones (59). β-catenin is regulated by Wnt and its inhibition prone MSCs to differentiate into adipocytes or chondrocytes (60). Additionally, CBF-1α (core binding factor-1 α) is involved in osteogenic differentiation (61).

Sox9 and ZNF145 (zinc-finger protein 145) are the principal transcription factors involved in chondrogenic differentiation (56, 62). Inhibition of ZNF145 diminishes chondrogenic differentiation, while its activation overexpresses Sox9 and chondrocyte formation (63). Additionally, upregulation of FOXO3A (forkhead box O3) correlates with MSC differentiation into chondrocytes (64).

Accumulation of lipid droplets reveals MSC differentiation into adipocytes (31, 35). PPARγ-1 (peroxisome proliferator-activated receptor-γ), PPARγ-2 and EBF-1 (early B cell factor) are the principal transcription factors involved in the differentiation of MSCs in adipocytes (65-

68). Also, Twist-1, Sox2 and Oct4 expression is involved in adipogenic differentiation (51); however, GATA2 and Foxa1 expression inhibits differentiation of MSC into adipocytes (69).

Several other transcription factors have been shown to be implicated in the differentiation of MSCs into cell types such as cardiomyocytes and skeletal muscle cells. Cardiomyocytes differentiate from MSCs via GATA-4 (70) and Nkx2.5 (Nk2 homeobox 5) (71, 72). GATA-4 is regulated by Wnt11 (73), and by histone acetylation, methylation and DNA methylation (74).

For differentiation into skeletal muscle cells, Pax3 (paired box protein 3), Pax7 (75), MyoD (myogenic differentiation 1) and Myf-5 (myogenic factor 5) are implicated (76).

Smooth muscle cells are differentiated via GATA6 and SRF (serum response factor) transcription factors (77).

Further, it has been shown that MSCs differentiate into cells normally derived from endoderm such as pancreatic islet cells and hepatocytes. MSCs derived from adipose tissue, human bone marrow, and tonsils can differentiate into pancreatic islet-like cells (12-15). It has been suggested that, rat bone marrow-derived MSCs can differentiate into insulin-producing β cells (78). Pdx1 positive MSCs are inclined to differentiate into functional insulin-producing cells, and Pax4 promotes Pdx1, the expression of which has been suggested to be important for differentiation into beta cells (79). Moreover, human adipose tissue derived MSCs can differentiate into insulin-, glucagon- and somatostatin-expressing cells (14).

Early studies showed that bone marrow derived MSCs also differentiate into hepatocyte-like cells after culture in Matrigel and culture medium supplemented with fibroblast growth factor-4 and HGF. Hepatocyte nuclear factor (HNF)-3β, GATA4, α-fetoprotein, transthyretin and, weakly, CK19 were expressed after 1 one week, whereas bi-nucleated cells expressing

CK18, HNF-1α and HNF-4 appeared after a few weeks. Cells secreted urea and presented cytochrome P450, suggesting a functional maturation of hepatocytes. In accordance to this, hepatocyte-like cells derived from rat and mouse MSCs secreted albumin, while human derived hepatocyte-like cells did not (54, 80).

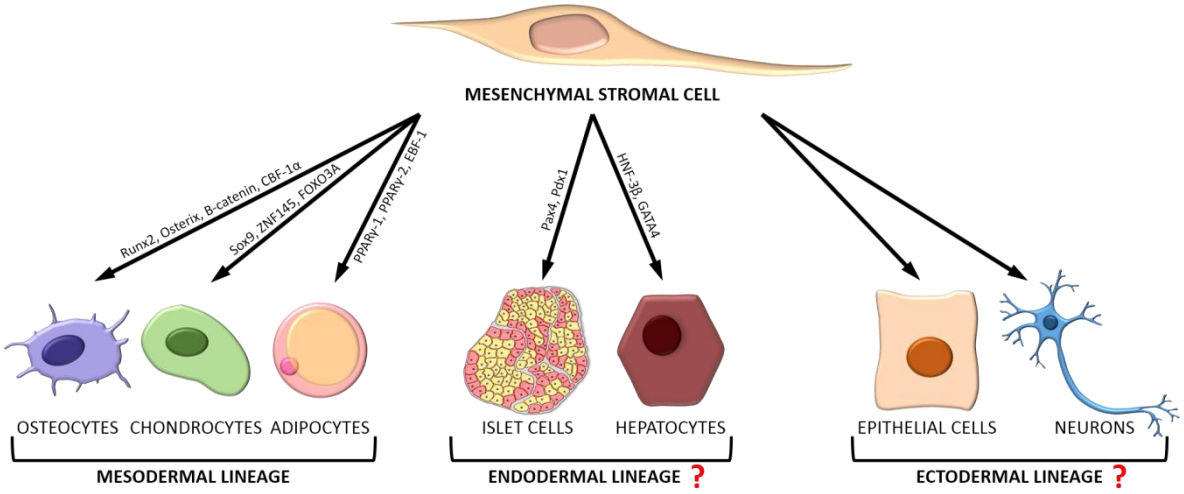

Figure 1. Differentiation capacity of MSCs

Whereas the differentiation capacity of MSCs into osteocytes, chondrocytes and adipocytes has clearly been demonstrated, differentiation of MSCs into pancreatic beta cells, hepatocytes, cardiomyocytes, neurons and smooth muscle cells remains controversial and needs confirmation. Actually, most studies analyze gene expression after cell differentiation; however studies demonstrating mature and fully differentiated cells are still missing.

4.1.3 Immunomodulatory effects

MSCs carry low immunogenicity, due to their low levels of human leukocyte antigen (HLA) class 1 and class 2 molecules expression. Moreover, they do not express co-stimulatory molecules that activate the immune system, such as CD40, CD80 and CD86 (81). Furthermore, MSCs possess immunomodulatory functions towards the adaptive and the innate

immune systems, which occur via paracrine signaling or through cell-to-cell contact directly with immune cells (81-83).

MSCs have the capacity to suppress T cell activation and proliferation, for both CD4 and CD8 T lymphocytes (84) and this suppression is principally HLA independent (85). TGF-β, IL-10, indoleamine 2,3-dioxygenase (IDO) and HGF are involved in T cell inhibition. Indeed, their blocking in the MSC-conditioned medium, reversed suppression of T cell activation (81, 86). T cell inhibition is also caused by monocyte chemoattractant protein (MCP)-1, secreted by MSCs (87), as demonstrated in encephalomyelitis mouse model (81). MSCs convert T helper (Th) 1 cells to a Th2 phenotype, a cell involved in an anti-inflammatory response, and further induce IL-4 secretion, a typical cytokine produced by Th2 cells (88). PGE2 and MSC-derived vesicles are involved in the switch to a Th2 response (81, 89). MSCs also inhibit Th17 functionality (90), but sustain function and proliferation of T regulatory (Treg) cells that immunomodulate the immune system (91, 92). The expression of T reg *in vitro* depended on cell-to-cell contact with MSCs and blocking of programmed death-ligand (Pdl)-1 impaired Treg proliferation, proving that Pdl-1 is involved in the supportive effect of MSCs on Treg cells (93, 94). MSCs exert the same effect *in vivo*, as shown in mouse transplant recipients treated with MSCs, where the proliferation of effector T cells was inhibited and Treg expansion is induced in the spleen, mesenteric lymph nodes and peritoneal lavage (91). Treg proliferation and function *in vivo* is mediated by IDO (95). T cells express CD25, which is the receptor of IL-2 that is a strong T cell activator (96). CD25 is blocked by MMP-2 and MMP-9, secreted by MSC. Blockade of CD25 decreased T cell responsiveness and function (91, 97). Inhibition of MMP *in vitro* reactivated T cells (97, 98). The injection of an inhibitor of MMP-2 and MMP-9 was associated to earlier graft rejection *in vivo*, compared to non-treated mice (97). Hence, the neutralization of MMP-2 and MMP-9 inhibited the immunosuppressive effects of MSCs. Furthermore, MSCs can suppress the activation of CD8 cytotoxic T cell

(99); however, when CD8 T cells are activated prior to the incubation with MSCs, MSC-mediated-inhibitory effect is absent (100). Also, MSCs promote the differentiation of CD8 regulatory T cell that are known to inhibit lymphocyte proliferation (101).

MSCs suppress also B cell lymphocyte function, as demonstrated by the co-culture of MSCs and splenic B cells. After stimulation with lipopolysaccharide *in vitro,* the presence of MSCs suppressed B cell proliferation, as demonstrated by decreased presence of CD138 positive plasma cells, and immunoglobulin (Ig)M release. Furthermore, MSC-conditioned medium also inhibited differentiation and IgM and IgG secretion in B cells activated by T cell-dependent or T cell-independent antigen stimulation. Similar effects were described in mice, where the injection of MSCs decreased the accumulation of alloreactive antibodies (82). These studies demonstrate that MSCs suppress B cells by trophic molecules *in vivo*; however MCP-1, IL-10, TGF-β, IDO seemed to play no role (102). Conversely, other studies report that IL-6, programmed death 1 pathway (103), IDO (104) and MSC-derived vesicles (81) are involved in B cell suppression. Nevertheless, there are some contrasting data reporting that MSCs support B cell proliferation and differentiation (105).

Natural killer (NK) T cells have a robust cytotoxic potential. MSCs modulate NK cytotoxicity in freshly cultured cells, decreasing proliferation and cytokine secretion, whereas in mature NK cells, MSCs exhibit no effect (104, 106). MSCs also modulate cytokine production and proliferation of NK cells (83).

In co-culture MSCs inhibit the capacity of dendritic cells (DC) to differentiate into mature and active DC. MSCs inhibit major histocompatibility complex (MHC) class II and CD1-α, which are molecules implicated in the antigen presentation, as well as co-stimulatory molecules CD40, CD80, and CD86 (107). MSCs can direct DC toward a tolerogenic phenotype, expressing decreased levels of MHC class II, with a diminished capacity to present antigens.

This inhibited CD80 expression and induced the PGE2, involved in the increased secretion of IL-10 in the conversion of DC into tolerogenic DC, and this has the potential to inhibit T and B cell activation in *in vitro* culture, and allogeneic islet transplantation *in vivo* (33). Moreover, MSCs diminish migration, endocytosis and maturation of DC (108, 109). MSC-derived vesicles co-cultured with DC, address the latter to an immature phenotype, with the reduction of activation markers and increased IL-10 and IL-6 production. This switch in DC marker expression and cytokine production drives DC to a tolerogenic phenotype, and these tolerogenic cells have the capacity to decrease Th17 and increase Treg cell numbers in *in vitro* co-cultures (110).

MSCs promote the differentiation of macrophages into M2 phenotype, thus promoting migratory capacities of monocytes and macrophages, and improving the phagocytic activity by cell-to-cell contact or by paracrine factors (83, 111). PGE2 is involved in macrophage regulation (112). MSCs decrease the secretion of inflammatory cytokines by activated macrophages (109); however MSCs boost macrophage activity, facilitating healing of wounds and injuries (113).

MSCs sustain neutrophil recruitment, by secreting cytokines that attract neutrophils and improve their functionality. STAT3 pathway is involved in the protection of neutrophils from apoptosis (114). Moreover, MSCs secrete IL-8 that modulate CD11b expression in neutrophils, regulating extravasation (115). MSCs improve neutrophil function in pathogen recognition; hence facilitating pathology resolution (83).

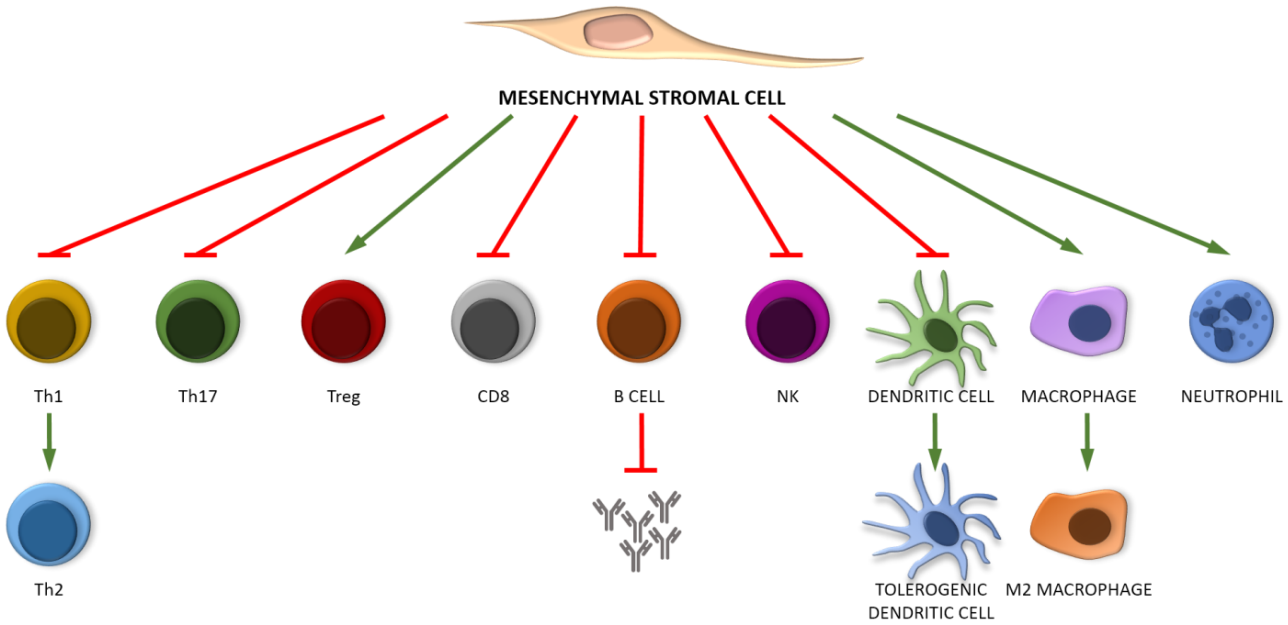

Figure 2. Immunomodulatory effects of MSCs on the different immune cells

4.1.4 Therapeutic potential

Autologous MSC transplantation could represent a solution for several pathologies, since MSCs exhibit limited immunogenicity, as they do not express HLA class 2, nor costimulatory molecules. For this reason, transplantation of allogeneic MSCs could represent an attractive therapeutic solution (4). Furthermore, MSCs have the potential to migrate towards injured sites and to alleviate the damage by proliferative, renewal, anti-apoptotic, anti-fibrotic and angiogenic capacities. These migratory capacities direct MSCs not only to the principal injured sites, but also to secondary injures, and allow the treatment of diseases that involve many organs (116).

MSCs have been successfully used for the treatment of osteogenesis imperfecta in children, where allogeneic MSC transplantation alleviated the symptoms of the pathology (117). Further, MSCs have been efficient in several pathologies implying cellular regeneration, such as ischemic heart disease, cerebral and myocardial infarction (118, 119), stroke (120), spinal and cord injury (121), brain injury (122), corneal and retinal diseases, Crohn's disease,

cartilage regeneration, acute and chronic liver failure (31) and ischemia reperfusion injury in the kidney and in the liver. MSCs were also effective in neurological and endocrinological pathologies, such as diabetes type 1 and 2 (17, 123-125). Moreover, since MSCs have immunomodulatory capacities, they have been applied for the treatment of autoimmune disorders such as lupus erythematosus systemicum (126-128) or of graft versus host disease (GVHD) (129).

GVHD is the immune reaction occurring after the transplantation of hematopoietic stem cells. T cells from the graft react towards the antigen presenting cell of the host, leading to damage in several organs (130). Since MSCs elicit clear immunomodulatory capacities *in vitro*, several clinical studies are ongoing to treat GVHD; however, the beneficial effects remain controversial, despite the absence of adverse symptoms (129, 131 , 132-134). Mainly, the immunosuppressive effect seems to be principally realized by paracrine factors, such as TGF-β, IL-10, PGE2, IDO and HGF (135, 136).

4.1.4.1 Type 1 and 2 diabetes

Type 1 (T1D) and type 2 (T2D) diabetes are endocrine diseases, characterized by glycemia dysregulation, caused by autoimmune beta cell destruction (T1D) or insulin resistance associated to impaired insulin and glucagon levels, which are the hormones responsible for the maintenance of blood glucose homeostasis (137, 138).

Several studies observed beneficial effects of MSCs in humans. Intravenous infusion of umbilical-cord derived MSCs, administered twice at two-week intervals for 24 months, improved C-peptide and HbA1c1 levels in type 2 diabetes patients. Also, MSC transplantation reduced exogenous insulin supply without exerting any side effects (17, 139).

MSCs are also effective in the treatment of T1D and its complications. Indeed, using non-obese diabetic (NOD) mice, a mouse model of T1D, intravenous injection of undifferentiated MSCs, decreased glycemia and their survival was improved (140). Further, MSCs were detected in the pancreas after 23 days, where they differentiated into insulin-producing cells, as demonstrated by colocalization of GFP (fluorescent label marker of donor cells) and C peptide in the pancreas. MSCs reduced levels of reactive T cells, in particular Th1 and Th17, and increased levels of Treg in the spleen and locally in the pancreatic lymph nodes (19, 141). These results suggest that MSCs have the potential to migrate to the damaged site, to differentiate into insulin-producing cells, to repair damaged cells and to also decrease the autoimmune reaction towards islets in T1D mouse model. Similar beneficial effects had also been reported in streptozotocin (STZ)-induced and high-fat-diet diabetic rats (142). Interestingly, a study reported that the addition of liraglutide, an analogue of glucagon-like peptide-1, prolongs the ability of MSCs to preserve β cells in NOD mice (141).

It is also known that 20-40% of diabetic patients suffer of renal failure, as a consequence of diabetes (143). A study reports that MSC infusion in STZ induced-diabetic mice decreased levels of albuminuria and presented slight tubular dilatation in the kidney, suggesting that MSCs, after systemic injection, could migrate in the damaged sites and improve different injuries caused by the same pathology (144). A clinical phase 2 study revealed that infusion of MSCs in five patients with T1D and ketoacidosis significantly improved exogenous insulin uptake, with one patient becoming normoglycemic. Importantly, no patients developed side effects derived from MSCs (145).

4.1.4.2 Liver injury

Liver injuries comprise several pathologies that provoke damage to hepatic cell function. Despite the regenerative capacity of the liver, some injuries result in an extensive hepatocyte

loss, which does not allow a sufficient regeneration of functional hepatocytes for the survival of the patient (146). MSCs have been investigated for their use as cellular therapy to favor regeneration and also to decrease liver fibrosis (147).

Hepatitis C virus could be responsible for liver damage, through the progressive transformation of liver parenchyma into fibrotic tissue (148). Autologous transplantation of MSCs in the parenchymal liver tissue of patients with cirrhotic liver, decreased hepatic enzymes, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), bilirubin levels and extracellular matrix protein, leading to an improvement of liver function (149). MSCs can also reduce the hepatic fibrosis induced by the parasite Schistosoma mansoni (150). Furthermore, MSCs promote hepatocyte regeneration in a murine model of hepatic steatosis induced by a high-fat diet (151), however, a study report that MSCs exert no effects after infusion in the ischemia/reperfusion injury model (152). In cases of alcoholic cirrhosis, infusion of autologous MSCs improved the histological features, decreasing collagen deposition, α smooth muscle actin and TGF levels in 6 of 11 patients (153).

Acute liver failure consists in the fulminant destruction of hepatic cell and cause high mortality (154). MSCs have been infused in several animal models of acute liver failure, including mouse (155), rat (156) and pig (157) and MSCs exert anti-fibrotic and anti-apoptotic beneficial effects in all animal models used (158, 159). Recently, MSCs and MSC-conditioned medium were tested in a mouse model of fulminant hepatic failure and chronic liver fibrosis. Both were efficient in the recovery of hepatic failure; however MSCs were more efficient than MSC-conditioned medium in fulminant hepatic failure due to their immunosuppressive effects. On the other hand, MSC-conditioned medium was more efficient in chronic liver fibrosis since it decreased inflammatory responses (160). Contrarily to this, one study reported that MSC transplantation did not improve liver failure; however, systemic injection of extracellular vesicles from bone marrow-derived MSCs, reduced hepatic injury

and improved mice survival (161), demonstrating that microvesicles efficiently replace the paracrine effects of entire cells and suggesting that factors are transported and released via microvesicles. In light of these experiments, showing that paracrine effect seems to be more effective in rescuing liver failure, MSCs were encapsulated, prior to transplantation in mice with acute liver failure caused by bile duct ligation or carbon tetrachloride (CCL4) injection. After intraperitoneal transplantation of encapsulated MSCs, collagen deposition levels in the liver were decreased, probably through an action on MMP-9 (31). These data strengthened the idea that the beneficial effects of MSCs on liver fibrosis occurs primarily through paracrine effects and do not necessarily need cell-to-cell interactions.

4.1.4.3 Side effects

Despite the beneficial effects that MSCs exert in several pathologies in experimental animal models, the risks of a cell therapy with MSCs needs to be evaluated. MSCs may present some risks related to malignant transformation, donor morbidity after MSC withdrawal, in cases of autologous transplantation and viral exposure (39).

MSCs are mainly isolated from tissues and need an extensive *in vitro* expansion, prior to therapeutic applications. High proliferation might cause cellular modifications. In the mouse model, MSC *in vitro* expansion led to malignant modifications (162, 163); however, human MSCs did not differentiate into malignant cells (164, 165). Moreover, recent research showed that now no malignant transformations had been described in clinical applications.

In case of auto-transplantation, MSCs are isolated from bone marrow aspiration via the iliac crest. These surgical procedures may cause complications or undesirable effects in patients, especially in immunosuppressed patients.

Contrary to auto-transplantation, allo-transplantation of MSCs involves the risk of the transmission of pathogens such as viruses. Studies report that after *in vitro* culture, MSCs were carrying cytomegalovirus, herpes simplex virus, parvovirus B19 and varicella zoster virus infections (166-168).

4.2 Mesenchymal stromal cells and pancreatic islets

Islets of Langerhans fulfill the endocrine function of the pancreas and are principally composed of insulin-secreting β cells and glucagon-secreting α cells. Insulin and glucagon are the key players for the maintenance of glucose homeostasis in the blood, the dysregulation of which causes diabetes (169). Morphologically, human islets are organized in substructures, where α cells line the endothelial cells and are surrounded by adjacent β cells (170). Islets are located in the exocrine tissue of the pancreas and are surrounded by a thin stromal layer and are highly vascularized. Cellular interactions between the different cell types include cadherins and integrins and are essential for the regulated insulin and glucagon release (171, 172).

4.2.1 MSCs improve islet viability and functionality

It is mainly established that the beneficial effects of MSC are mostly derived from the secretion of immunomodulatory and cyto-protective factors. However, in relation to islets, MSCs may also play a role in the conditioning of the micro-environment, especially in isolated islets which suffer from isolation with enzymatic digestion and from the absence of their natural micro-environment. Therefore, MSCs may contribute to an increased function by mimicking the natural environment of islets. Indeed, co-culture of islets and MSC, showed that cell dispersion and the sprout formation of islets were both improved (173). Further, insulin secretion by islets was strongly enhanced by MSCs, as demonstrated by co-culture models of MSCs and islets of mouse (174), rat (175, 176) and human (177). Cell-to-cell contact between islets and MSCs seems to be compulsory to improve insulin secretion and that culture of MSCs and islets without contact maintains insulin secretion similar to levels secreted by the islet alone (174). Co-culture of rat islets and MSCs showed decreased levels

of soluble MCP-1, TNF-α, and increased levels of tissue inhibitors of metalloproteinases (TIMP)-1 and VEGF after one month, however these results were not observed in transwell culture conditions, where there is only a paracrine exchange (175, 176). Further, the co-culture of rat islets and MSCs improved islet viability and increased the expression of insulin 1, Pdx1, platelet endothelial-cell adhesion molecule 1 and VEGFa, suggesting that the presence of MSCs facilitates insulin secretion and that the expression of growth factors decreases inflammation (178). However, there are some reports showing that mouse islets present enhanced survival only after exposure to trophic molecules secreted by human MSCs and not in cell-to-cell contact culture conditions. IL-6, IL-8, VEGF, HGF and TGF-β were detected in the MSCs conditioned medium and were suggested to be involved in the beneficial effects observed (179).

Moreover, MSCs preserved the function and viability of isolated islets injured by cytokines, such as interferon (IFN)-γ, TNF-α and IL-1β (177) and of islets cultured under hypoxia/reoxygenation conditions (180). Supernatants obtained from co-culture of MSC with STZ damaged rat islets showed increased levels of IL-6 and TGF-β. Furthermore, the expression of anti-apoptotic genes in the damaged islets positively correlated with the presence of MSCs, suggesting a role of MSCs in regulating the expression of anti-apoptotic genes (181).

MSCs co-transplanted i*n vivo* with islets increase graft function and survival in syngeneic and allogeneic mouse and rat models (182-189). Also, islets pre-cultured with MSCs improved their outcome as a graft, demonstrating that exposure to paracrine factors exert potentiating effects on islets (174, 190). MSCs promote insulin secretion even in minimal mass islet transplantation studies. In such models, islets alone are incapable of reversing diabetes; however, the presence of MSCs along with islets allowed the transplanted diabetic rodent recipients to reach normoglycemia (191, 192).

Some studies show that MSCs promote islet graft function even in large animal models, such as non-human primates, where the presence of MSC enhanced islet graft survival and decreased the number of islets necessary to reverse diabetes in allo-transplantation models (193). Similarly, in a model of human bone marrow MSCs co-transplanted with neonatal porcine islets in mice, graft functionality is improved and diabetes is reversed faster compared to mice transplanted with islets alone (194). MSCs co-transplanted with islets exert immunosuppressive effects, and the presence of MSCs decrease T cell recruitment around the graft, suppress Th1 cell activation and increase the percentage of Treg in the blood of transplanted animals (182, 193, 195, 196). Further, MSCs decreased the levels of follicular B helper T cells and auto-antibodies in the spleen and in the lymph nodes of recipients (196).

4.2.2 Effects on islet vascularization

Several studies *in vivo* suggest that MSCs increase vascularization in islets (182, 197, 198). Also, MSCs differentiated into vascular endothelial cells, demonstrating an increased number of capillaries were present around the islet graft in the presence of MSCs compared to islet grafts without MSCs (191). VEGF and von Willebrand factor positive cells were localized after one week around the graft (192). A complete vascularization has been documented after subcutaneous transplantation in immunodeficient STZ-induced diabetic SCID mice after 84 days (186). MSCs migrated toward islet grafts to promote vascularization. After islet transplantation under the kidney capsule, MSCs infused by intraperitoneal injection migrated to the graft site and differentiated into vascular cells, producing VEGF (187).

4.2.3 Mechanism of islet-MSC cell-to-cell contact

It is known that cell-to-cell interactions within the islet cells, involving neural (N)- and epithelial (E)-cadherin, protect cells from apoptosis (199) and are also important to warrant sufficient insulin secretion after glucose stimulation (200). However, the mechanism of cell interaction between islets and MSCs is not clear.

Cellular adhesions and junctions are essential in the maintenance of the islet. Rho-associated kinase (ROCK) is responsible for cell polarity, morphology and motility (201) and its inhibition facilitate cellular stability, promoting adhesion through cadherins (202). In effect, the inhibition of ROCK in a co-culture of islets and MSCs compacted the structure of the heterogeneous co-culture (203). Moreover, islets cultured with MSC-conditioned medium expressed higher levels of pAKT and pERK and their neutralization totally inhibited islet proliferation capacities that were increased in the presence of MSCs (204). Elastin microfibril interface 1 (EMILIN-1), integrin-linked protein kinase (ILK) and hepatoma-derived growth factor (HDGF) positively correlate with the regenerative effects of MSCs (205). Furthermore, annexin A1 is involved in cell-to-cell contact between islets and MSCs (206). The addition of annexin A1 to islet culture *in vitro* mimics the effect of MSCs and increases insulin secretion; moreover, silencing of annexin A1 by si-RNA or genetic mouse knock-out models decreased insulin secretion capacities (206).

These studies explain in part the interactions occurring between MSCs and islets; however the molecules involved in the cell-to-cell contact or the secreted molecules that improve islet viability have not been fully identified.

4.3 Mesenchymal stromal cells and liver cells

Hepatocytes are the principal components of the liver parenchyma and they are arranged in linear cords to form a liver lobule, which is defined at a histological scale as a small division of the liver. These lobules are surrounded by the portal triad, composed of the bile duct, and the interlobular vein and artery where blood enters and flows into sinusoids of the lobules. Hepatocytes are responsible for the secretion of albumin, urea and drug metabolization, thus allowing medicaments to be eliminated (207).

4.3.1 MSCs sustain hepatocytes

MSCs furnish a stroma structure for hepatocytes and help to restore the lobes' morphology present in the liver (208). Hence, MSCs co-cultured with isolated hepatocytes promoted cell stability (209). MSCs and hepatocytes cultured in a three-dimensional culture fashion showed increased hepatocyte stability, and thus, functionality as well (210). Hepatocyte viability was also improved in a co-culture model with bio-artificial materials, such as poly(lactic-co-glycolic) acid (PLGA) scaffold or engineered liver scaffolds. It has been suggested that these structures sustain hepatocytes and foster cellular adhesion. The presence of MSCs further improves hepatocyte function and survival, probably facilitating the establishment of cell-to-cell contact and cell-to-matrix interactions (211-213). In fact, MSCs most probably condition the microenvironment of hepatocytes through the secretion of collagen, laminin and fibronectin, dermatan and chondroitin sulfate-proteoglycans, which are the principal components of the extra cellular matrix (208). Moreover, the presence of MSCs improves hepatocyte polarization; in fact, MSCs increased hepatocyte functions during the first week of co-culture and restored their polarity as shown by transmission electron microscopy (214).

4.3.1 MSCs improve hepatocyte viability and functionality

Several studies suggest that MSCs promote hepatocyte viability and proliferation as demonstrated by co-culture of human adipose-derived MSCs with primary human hepatocytes (209). As shown by others, the expression of hepatotrophic and anti-apoptotic genes might be involved in the beneficial effects of MSCs (215). Indeed, in the co-culture models with MSCs, hepatic cells in the G2/S phase of the cell cycle increased in number, demonstrating that hepatocytes are driven to proliferate (16).

Furthermore, MSCs improve hepatocyte functionality (210, 216). Indeed, previously cryopreserved hepatocytes co-cultured with MSCs secreted increased levels of albumin and urea (216). A similar effect was observed after co-encapsulation in alginate-poly-L-lysine polymer or in spheroids, where hepatocytes presented a significantly improved secretion of albumin and urea (16, 210). Contrarily to this, even though other studies reported that albumin and urea secretion were not affected by the presence of MSCs; however, hepatocytes co-cultured with MSCs presented an improved morphology, phenotypic stability, as shown by the expression of CK18 and HNF4α, and increased gene expression of cytochrome P450 (209).

The mechanism involved in these effects remains obscure; but, there is a study showing that he production of IL-6 in MSC-hepatocyte co-cultures is significantly increased in contrast to hepatocytes that are cultured alone. Interestingly, IL-6 neutralization *in vitro* prevented the increased levels of albumin and urea synthesis, present in the hepatocyte and MSC co-culture, demonstrating the involvement of IL-6 in the observed functional effects. Further, no differences in TGF-α or TNF-α levels were detected in supernatants of hepatocytes cultured alone or those cultured with MSCs (217). To understand the mechanism leading to the increased function of hepatocytes co-cultured with MSCs, Huang et al cultured hepatocytes

with MSCs and induced cell damage through the addition of acetaminophen in the culture. Effectively, acetaminophen induces cell death in hepatocytes via mitochondrial damage (218). The presence of MSCs improved cell viability, which correlated with decreased levels of lactate dehydrogenase, an indicator of reduced cytotoxicity. Furthermore, the presence of phosphorylated JNK and ERK was assessed, and they were overexpressed after injury induction. This overexpression of JNK and ERK was suppressed in hepatocytes co-cultured with MSC. This suggests that hepatocyte damage caused by acetaminophen activates JNK and ERK pathways, and that the presence of MSCs can inhibit the activation of this signaling (219).

Hepatocytes co-transplantated with MSCs or treated with MSC-conditioned medium before transplantation, exert benefic effect on liver injury *in vivo*. In fact, mouse primary hepatocytes culture, pretreated with MSC-conditioned medium and then transplanted into CCL4-treated mice exhibited an attenuation of early apoptosis through the activation of fibroblast-like protein-1 (220). Also the co-transplantation of MSCs together with hepatocytes improved liver functions and survival rate in rats (16), as shown by decreased levels of the hepatic enzymes, ALT and AST, in mice (211). The beneficial effects demonstrated i*n vivo* are suggested to be caused by paracrine molecules and by cell-to-cell contacts; however, the clear mechanisms involved in MSC and hepatocyte interactions leading to the beneficial effects are not well known.

4.4 Cell encapsulation

Cellular transplantation could represent an attractive and easier therapeutic solution compared to full organ transplantation. Clinical islet cell transplantation is a therapeutic option in T1D, with difficult management and hypoglycemia unawareness. In acute liver failure, cell transplantation could be a solution when full liver transplantation cannot be offered. The use of animals as cell donors represents a possible new source for tissues to overcome the issue of limited availability. Cell encapsulation has the advantage to protect transplanted cells from the immune system, while also permitting oxygen and nutrients to reach these encapsulated cells by diffusing in between the polymer. Successful encapsulation would obviate the problem of systemic immunosuppression in cell transplant recipients. Permeability of the polymer is various but normally does not exceed pore sizes that allow the passage of molecules of >/= 100 kDa, preventing antibodies or immune cells to penetrate into the capsule (Figure 3), however allowing oxygen, hormons and nutrients to pass through (221).

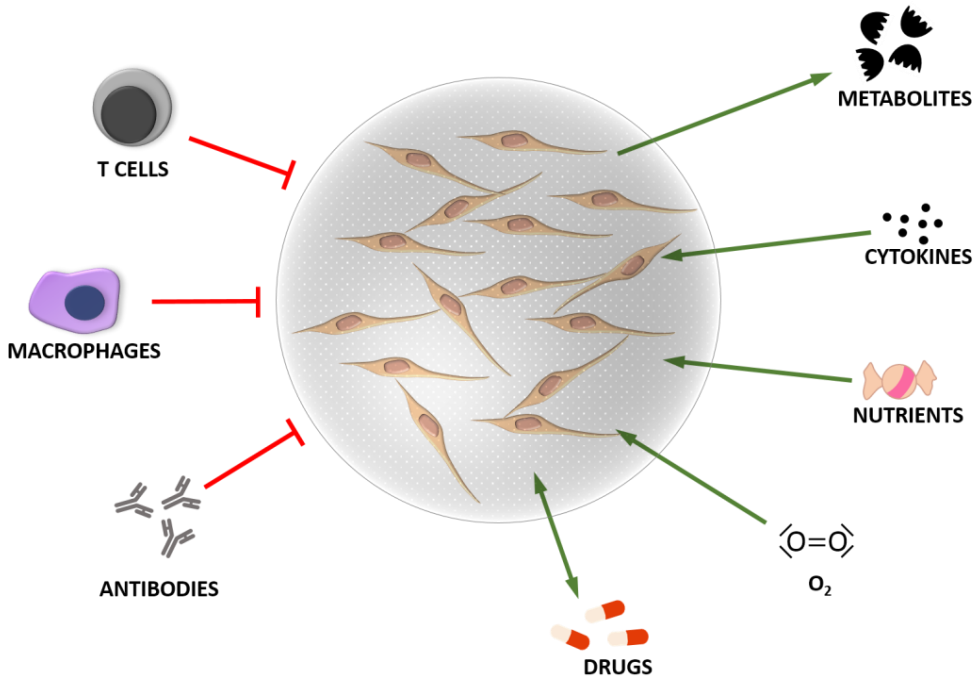

Figure 3. Bead properties.

Several polymers, such as agarose, chitosan and collagen, have been tested during recent decades and the polymer that remain widely used is alginate (222). Alginate is a hydrogel, being composed of a high quantity of water, and this renders the polymer biocompatible. Nevertheless, alginate is not ideal since microcapsules are mechanically unstable, leading to desegregation of capsules and to the release of immunogenic material over time (223). For these reasons, studies are ongoing to develop more suitable polymers or linking alginate with other polymers, in particular with poly(ethylene glycol) (PEG) (224, 225). These more advanced polymers are more stable and suitable for cell encapsulation (226).

4.4.1 Islet encapsulation and transplantation outcome

Islet cell transplantation is used worldwide to treat unstable cases of T1D and the number of patients waiting for transplantation increases constantly. Life-long harmful immunosuppressive treatments are necessary. With the aim to avoid immunosuppression treatments and to use new unlimited cell sources, encapsulation studies have been performed with islets (227).

In a pig-to-primate xenotransplantation model encapsulated islets were incorporated into a monolayer device and transplanted subcutaneously. Control animals transplanted with free islets immediately rejected the graft; however, diabetic primates transplanted with encapsulated islets achieved and maintained normoglycemia up to six months without immunosuppressive treatments, until breakage of the microcapsules (228). Similarly, in another study, islets were encapsulated using the TheraCyte macro capsules and implanted subcutaneously in non-human primates. Diabetes in the recipient was induced by partial pancreatectomy hyperglycemia was reduced after transplantation of macro-encapsulated islets. After retrieval at 3, 6 and 12 months, the device presented viable islets, onset of

vascularization and minimal fibrosis (229). Clinical allo-transplantation using encapsulated islets has been performed in T1D patients. Encapsulated islets were injected in the peritoneal cavity; under such conditions patients continued to require exogenous insulin supply (230-232). However, no infections or immune reactions were reported. Occasionally capsules were attached to the peritoneum, or formed fibrotic lumps (230, 231). In 2007, one patient underwent an intraperitoneal xenotransplantation of encapsulated porcine islets. Exogenous insulin supply was reduced by 30% during the first 12 weeks and returned to pre-transplant levels 49 weeks after transplantation (233).

Furthermore, several studies using various types of polymers demonstrated that co-encapsulation of rodent islets, together with MSCs, improved insulin secretion and increased graft survival in rodents after transplantation (234-237). Until today, only few studies investigated the effect of human MSCs on human islets, and their co-encapsulation and transplantation has, to our knowledge, not been described so far.

4.4.2 Hepatocyte encapsulation and transplantation outcome

Several types of microcapsules have been tested for hepatocyte encapsulation (238), using biomaterials such as alginate, polyurethane, chitosan and polycaprolactone. Hepatocytes adhered to all polymers, suggesting that the tested polymers conferred structural support to hepatocytes (239, 240).

Encapsulation of primary hepatocytes with alginate, PEG, chitosan, silk sericin-alginate-chitosan and three-layered alginate-chitosan-alginate polymers maintained typical morphology, albumin and urea secretion, and maintained their metabolic capacities for up to four months (241-244). Nevertheless, encapsulation with PEG resulted in major cell death one

day after encapsulation (245), demonstrating the technical challenge of primary hepatocytes encapsulation.

Baboons, transplanted with encapsulated pig hepatocytes, recovered from acute liver injury induced by 75% hepatectomy and warm ischemia. The intraperitoneal transplantation allowed 75% of baboons to recover from liver injury, while 25% developed liver failure after 21 days. Control animals developed liver failure after six to ten days (246).

Co-encapsulation of rat hepatocytes together with MSCs showed enhanced albumin secretion (247, 248), improved ammonia metabolism and urea synthesis (249), decreased bilirubin levels (248) and improved graft survival and function for up to four months in allo-transplantation models (250, 251).

Previously, in our laboratory, an efficient technique of isolation and encapsulation of primary pig (252) and human (253) hepatocytes had been established. Until today, no standardized protocols for clinical applications are available. Further experimental studies transplanting encapsulated hepatocytes needs to be performed, to evaluate the potential therapeutic usefulness for the treatment of acute liver failure.

5. RESULTS

5.1 Aims

The aim of this thesis was to assess whether bone-marrow derived human MSCs have a supportive effect on the function of human islets of Langerhans and porcine hepatocytes. These studies aimed to contribute to the elucidation of molecular mechanisms involved in the beneficial effects observed by using culture-expanded MSCs. Furthermore, one global objective was to analyze the usefulness of MSCs in survival and function of encapsulated islets and hepatocytes intended to cell therapies for the treatment of T1D and acute liver failure.

Specific Aim I:

The first aim was to analyze whether MSCs and human islet co-culture improves human β cell function, *i.e.* insulin secretion, using *in vitro* approaches. Further we aimed to analyze the molecular mechanisms such as cell-to-cell contact involved in the beneficial effects observed. Additionally, we analyzed the effects of MSCs co-encapsulated with human islets on graft function and survival after transplantation in diabetic mice.

Specific Aim II:

The second aim was, firstly, to optimize a protocol to obtain high yield preparations of viable porcine hepatocytes and, secondly, to encapsulate hepatocytes in newly developed polymers for transplantation purposes. We further analyzed the effect of MSCs on free and encapsulated porcine hepatocyte viability, albumin secretion and metabolism in order to evaluate the usefulness of co-encapsulation of hepatocytes and MSCs for future clinical applications.

5.2 MSCs and human islets of Langerhans

"Multipotent mesenchymal stromal cells enhance insulin secretion from human islets via N-Cadherin interaction and prolong function of transplanted encapsulated islets in mice"

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MSCs co-cultured with human islets of Langerhans improved insulin secretion by cell-to-cell contact *in vitro*. The screening of adhesion molecules by qPCR in islets and in MSCs, revealed the expression of intercellular adhesion molecule (ICAM)-1 and N-cadherin in both cell types. Blocking of N-cadherin inhibited the increased insulin secretion in MSC-islets co-culture conditions, without affecting insulin secretion by islets cultured alone, demonstrating the involvement of N-cadherin interactions in the observed effect. Histology studies evidenced the contact of MSCs with islets, thus providing a cellular basement that surrounds islets and serves as a stromal structure. Co-encapsulation and co-transplantation of MSCs *in vivo* along with islets in diabetic mice significantly increased graft functionality and prolonged graft survival.

Personal contribution:

In this work, I designed and performed experiments, analyzed the data and wrote the manuscript.

Multipotent mesenchymal stromal cells enhance insulin secretion from human islets via N-Cadherin interaction and prolong function of transplanted encapsulated islets in mice

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Abstract

Background: Multipotent mesenchymal stromal cells (MSC) enhance viability and function of islets of Langerhans. We aimed to examine the interactions between human MSC and human islets of Langerhans that influence the function of islets.

Methods: Human MSC and human islets (or pseudo-islets, obtained after digestion and reaggregation of islet cells) were co-cultured and cell function was tested *in vitro* by static incubation for islets or pseudo-islets cultured with or without MSC. The expression of several adhesion molecules, notably ICAM and N-cadherin on islets and MSC was investigated by qPCR. The role of N-cadherin was analysed adding an anti-N-cadherin antibody in islets cultured with or without MSC for 24 hrs followed by insulin measurements in static incubation assays. Islets and MSC were co-encapsulated in new hydrogel microspheres composed of calcium alginate and covalently cross-linked polyethylene glycol. Encapsulated cells were transplanted intra-peritoneally in streptozotocin-induced diabetic mice and glycemia was monitored. Islet function was evaluated by intraperitoneal glucose tolerance test.

Results: *In vitro*, free islets and pseudoislets co-cultured in contact with MSC showed a significantly increased insulin secretion when compared to islets or pseudoislets cultured alone or co-cultured without cell-to-cell contact with MSC ($p<0.05$). The expression of ICAM-1 and N-cadherin was present on islets and MSC. Blocking N-cadherin prevented the enhanced insulin secretion by islets cultured in contact with MSC whereas it did not affect insulin secretion by islets cultured alone. Upon transplantation in diabetic mice, islets microencapsulated together with MSC showed significantly prolonged normoglycemia when compared with islets alone (median 69 and 39 days, respectively, $p<0.01$). Intraperitoneal glucose tolerance test revealed an

improved glycemic response in mice treated with islets microencapsulated together with MSC compared to mice transplanted with islets alone ($p<0.001$).

Conclusions: MSC improve survival and function of islets of Langerhans by cell-to-cell contact mediated by the adhesion molecule N-cadherin.

Keywords: mesenchymal stromal cells, human islets, N-cadherin, cell interaction, encapsulation

Background

Multipotent mesenchymal stromal cells (MSC) present immunomodulatory properties [1-3], thereby reducing the cell-mediated immune response [4]. MSC secrete bioactive molecules improving tissue regeneration by increasing vascularization [5-9], angiogenesis [10] and reducing apoptosis [5]. Co-transplantation of islets and MSC results in improved islet graft survival and function as demonstrated by syngeneic and allogeneic transplantation models in rodents and nonhuman primates [4-6, 9, 11], in a rat-to-mouse xenotransplantation model [3, 7] and in human to diabetic humanized NOD *scid* gamma mouse model [12]. However, the functionality of human islets co-transplanted with human MSC into immunocompetent diabetic mice has, to our knowledge, not been investigated so far. In such a setting, host immunity needs to be overcome. As a strategy to avoid immunosuppression-treatments, semipermeable microcapsules can be used to entrap cells [13, 14]. Hence, for the human to mouse islet transplantation, we used newly developed biomaterials enabling ionotropic interaction between alginate (Alg) molecules and covalent crosslinking between poly(ethylene glycol) (PEG) derivatives, which have high mechanical resistance [15].

Further, the molecular mechanism leading to improved islet graft survival and function is unclear. Several studies attributed the beneficial effect to factors released by MSC [16] and also to regulatory effects on the host immune system [4]. It is not known whether direct cell contact between islets and MSC plays a role in the beneficial effects of MSC on islet function. However, adhesion molecules such as cadherins and integrins are expressed in human islets [17, 18] and play a role in regulating insulin secretion. Notably, cadherin interactions on beta-cells play a role

in increased insulin secretion after glucose stimulation [19] and are implicated in protecting islets from apoptosis [20]. Morphological analysis of human islets demonstrated dispersed alpha- and beta-cells [21], and only recently, structures of sub-islets have been identified, where alpha-cells are organized around centrally located beta-cells [22]. This cell arrangement, based on cell interactions between alpha- and beta-cells, and also with stromal cells around islets, are also important for an optimal insulin response by beta-cells [23].

This prompted us to study the cellular interactions involved in the beneficial effects of human MSC on islets *in vitro*. Therefore, we investigated a possible involvement of intercellular adhesion molecules, such as epithelial (E)-cadherin, neural cell adhesion molecule (NCAM), epithelial (Ep)CAM-1, vascular (V)CAM-1, N-cadherin and intercellular (I)CAM-1. Furthermore, we aimed to assess the function of human islets co-encapsulated with MSC upon transplantation in diabetic mice.

Methods

Isolation and culture of human pancreatic islets and human MSC

Human islets were isolated following the Ricordi protocol [24], and their purity was assessed after dithizone staining and calculated using Metamorph (Metamorph, Universal Imaging, West Chester, PA, USA). Islets used for these experiments were 80-100% pure, and no handpicking was performed. Human islets were provided to research only when considered not suitable for clinical transplantation, through the JDRF award 1-RSC-2014-100-1-X, ECIT Islet for Basic Research program. The amount of islets was expressed in islet-cell equivalents (IEQ),

normalizing each islet to an average diameter of 150 μm. We considered that 1 IEQ contains 10^3 cells. Islets were cultured in HEPES-buffered CMRL1066-medium supplemented with 5.6 mmol/L glucose (Gibco-Invitrogen, Basel, Switzerland), 100 IU/ml penicillin, 100 mg/ml streptomycin (P-S, Gibco-Invitrogen) and 10% FCS (Gibco-Invitrogen) (complete CMRL), at 37°C in humidified air containing 5% CO_2.

MSC were obtained from the femoral head of patients undergoing total hip replacement as previously described and characterized by FACS analysis and their ability to differentiate into osteoblasts, chondrocytes and adipocytes [25]. Informed consent was given by all patients and the experimental procedure was approved by the local ethical committee of the University Hospitals of Geneva (NAC 01-015). Briefly, MSC were purified from crushed bone marrow by gradient centrifugation, and then cultured in Iscove's modified Dulbecco's medium (Cambrex, Verviers, Belgium) supplemented with 10% FCS, P-S and 10 ng/ml platelet-derived growth factor BB (PDGF-BB, PeproTech EC Ltd, London, UK) [26]. MSC from 3 different donors between passages 2 to 4 were used, and cultured at 37°C in humidified air containing 5% CO_2.

Preparation and culture of pseudoislets

Single islet cell suspensions and pseudoislets were prepared as previously described [22, 27]. Briefly, islets were rinsed in PBS (Gibco-Invitrogen) without calcium and incubated for 7 minutes in Accutase cell detachment solution (Sigma, St Louis, MO, USA) with occasional mixing by pipetting. The resultant single cell suspension was diluted with CMRL supplemented with 10% FCS, to stop the enzyme activity. Dissociated islet cells were counted and were cultured overnight at a density of $5x10^5$ cells in non-adherent 10cm-diameter petri dishes in

complete CMRL. To obtain pseudoislets, single islet cells (10^4), with or without MSC at a ratio of 3:1, were taken in 40 µl of complete CMRL or MSC-conditioned medium and placed as microdroplets in the petri-cover, which was inverted to allow cell re-aggregation and formation of pseudoislets in the hanging microdroplets. After 3 days, microdroplet medium was renewed and after 6 days microdroplets were collected. Cell clusters recovered from 10 microdroplets (10^5 islet cells) per well, alone or with MSC were used for the insulin secretion assay. To obtain MSC-conditioned medium, MSC were cultured in 75 cm^2 flasks at 80% confluence in complete CMRL and medium was recovered after 48 hrs. Experiments were performed in triplicates in a 24-well plate. Histology was performed after a 6 day culture.

Insulin secretion assays

A static incubation assay was used as previously described [28]. Islets alone (150 IEQ), islets co-cultured with MSC (15,000) in cell-cell contact, and islets co-cultured with MSC in permeable transwell plates were seeded (cell ratio 10:1) in triplicate in 24-well plates for 3 days in complete CMRL. For blocking experiments, 24h before performing the secretion assay, antibodies (25µg/ml) either low-endotoxin-azide-free (LEAF) purified anti-human CD325 (N-cadherin), ultra-LEAF purified mouse IgG1κ isotype control (Biolegend,San Diego, CA, USA) or the anti-human ICAM-1 antibody (CD54) (R&D Systems,Abingdon, UK) were added. Secreted insulin was measured by ELISA (Mercodia, Uppsala, Sweden), following the manufacturer's instructions. Values were normalized to the total amount of insulin measured, that was obtained after total cell lysis with acid ethanol. The values of insulin secretion were expressed as fold increase were the basal condition was set as 1. All experiments were performed in triplicates.

142 **Real-time RT – PCR**

143 Gene expression of adhesion molecules was analyzed by real-time reverse transcription PCR
144 (RT-PCR), as previously described [25]. After 72 hrs of separated culture of islets and MSC,
145 RNA was extracted using the Qiagen RNeasy Mini kit (Qiagen, San Diego, CA, USA) according
146 to the manufacturer's instructions. Primers listed in table 1 [see additional file 1] were designed
147 using Primer3 online software (http://primer3.ut.ee), tested with Primer Biosoft
148 (http://www.premierbiosoft.com) and blasted on BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

149 **Cell microencapsulation**

150 The polymer for cell microencapsulation consisted of 5% PEG-8-40 mixed in 1.5% sodium (Na)-
151 Alg, prepared as described previously [29]. Islets alone or with MSC were centrifuged at room
152 temperature (320x*g* for 2 min) and the pellet was gently mixed with Na-Alg/PEG-8-40 solution
153 (ratio 10-12 islet cells : 1 MSC). Microspheres were generated under sterile conditions using the
154 Encapsulator B-395 Pro (Büchi Labortechnik AG, Flawil, Switzerland): the solution in the
155 gelation bath comprised 10 mM MOPS, 100 mM $CaCl_2$ and 3 equivalents dithiothreitol.
156 Microsphere formation by ionotropic interaction occurred immediately after extrusion in the
157 gelation bath and was completed by covalent crosslinking during subsequent stirring for 30 min
158 in the gelation bath. Alg-PEG microspheres were collected by filtration and washed twice in
159 physiological saline (NaCl, 0.9%) for 10 minutes to eliminate remaining dithiothreitol.

160 **Islet transplantation**

161 Animal research was performed following protocols approved by the Geneva cantonal veterinary
162 authorities (license GE/34/13). Diabetes was induced in C57BL/6 male mice (Janvier, France) by

intraperitoneal injection of streptozotocin (Sigma, Buchs, Switzerland) at 220 mg/kg. Diabetes was defined as a blood glucose level >20 mmol/L. Three days after injection, diabetic mice were transplanted. Animals, anesthetized with isoflurane, were transplanted with 4500-5000 IEQ encapsulated islets, with or without MSC (cell ratio 10-12:1) into the peritoneum through a small incision. Blood glucose was measured 48 hrs after transplantation and thereafter twice weekly. Islet graft failure was concluded when glucose level was >20 mmol/L for 3 consecutive measurements.

Pseudoislets obtained after the *in vitro* formation, were transplanted in the presence or absence of MSC. Transplantation was performed in non-diabetic SCID mice, under the kidney capsule. Mouse kidneys were collected at day 15 after transplantation.

Experimental design of insulin secretion assays and transplantation of microcapsules

For insulin secretion assays using islets or islets with blocking antibodies: islets alone, islets/MSC in contact and islets/MSC without contact were compared.

For insulin secretion assays using pseudoislets: pseudoislets alone, pseudoislets/MSC, pseudoislets/conditioned medium (MSC) were compared.

For transplantation of microcapsules in mice: microcapsules containing islets alone and islets/MSC were compared.

In each experimental setting, islets and MSC derived from two distinct donors (allogenic) were used.

Intraperitoneal glucose tolerance test (IPGTT)

Fifteen days after transplantation, overnight fasted animals were subjected to IPGTT. Glucose (2 g/kg) was injected intraperitoneally and glucose measurements were performed on blood samples collected by tail excision at 0, 5, 15, 30, 60, and 120 minutes.

Histological analyses

Pseudoislets after 6 days culture were formalin fixed and paraffin embedded, using Histogel (Thermo Scientific, Waltham, MA, USA), following the manufacturer's recommendations. Four-µm sections were treated with 0.01 mol/l citrate for 15 min in a microwave, to unmask epitopes. To avoid nonspecific binding, slides were incubated with 0.5% BSA for 30 min at room temperature.

For detection of MSC, sections were stained with mouse anti-human vimentin antibody, diluted 1:50 (Dako, Glostrup, Denmark) and with Alexa Fluor 488 goat anti-mouse antibody (Life Technologies, CA, USA). For detection of islet cells, sections were stained with guinea pig anti-porcine insulin, diluted 1:500 (Dako), donkey anti-guinea pig coumarin AMCA, diluted 1:300 (Jackson Immunoresearch, West Grove, PA, USA), rabbit anti-human glucagon antibody, diluted 1:100 (Merck Millipore, Darmstadt, Germany) and Alexa Fluor 555 donkey anti-rabbit antibody (Life Technologies). Microscopic images were acquired using a fluorescence microscope (Mirax Midi, Zeiss, Germany) and Pannoramic Viewer (3DHISTECH, Hungary), and confocal laser scanning microscopy was performed using LSM700 equipment (Zeiss).

Statistical analysis

GraphPad Prism software was used. Mann-Whitney nonparametric test and Wilcoxon signed-rank test were used for *in vitro* test, nonparametric Kaplan-Meier survival curve and Mantel-Cox

tests were used to evaluate the statistical significance for *in vivo* graft survival data. For IPGTT the AUC was calculated and values were compared using the parametric t-test. Differences were considered significant when $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

Results

MSC improve insulin secretion by human islets in direct cell-cell contact

After 3 days of culture, islets alone (islets), islets in direct contact with MSC (islets-MSC contact), and islets and MSC without cell-cell contact (islets-MSC no contact) were subjected to the glucose-stimulated insulin secretion assay. We observed a significantly higher insulin release for islets cultured in contact with MSC than for islets cultured alone ($p<0.01$) and islets cultured with MSC without cell-cell contact ($p<0.05$) (Fig.1A). After maximal stimulation with theophylline, islets in contact with MSC showed a significantly larger increase in insulin secretion than islets alone or islets cultured with MSC without contact ($p<0.01$). These results show that insulin secretion by human islets is significantly enhanced upon culture with MSC in direct cell-cell contact.

This result prompted us to investigate whether increasing the contact opportunity between MSC and islet cells further increases insulin secretion. Therefore, we cultured dissociated islet cells in hanging drops for 6 days to induce formation of clusters of re-aggregated cells, called pseudoislets, without or with MSC. As control, islet cells were cultured in hanging drops in MSC-conditioned medium. All conditions showed similar insulin secretion after high glucose stimulation. However, upon maximal stimulation with theophylline, pseudoislets containing MSC

225 revealed a significantly higher insulin secretion than pseudoislets without MSC ($p<0.01$) or
226 pseudoislets cultured in MSC-conditioned medium ($p<0.001$) (Fig.1B): this indicates a beneficial
227 effect of direct cell-cell contact between MSC and islet cells within pseudoislets. Altogether,
228 these results show that insulin secretion is significantly higher when MSC are in direct cell-cell
229 contact with islets or islet cells in pseudoislets.

230 **MSC serve as a stromal structure for islets**

231 The architecture of native human islets comprises substructures, where centrally-located beta-
232 cells are surrounded by alpha-cells. Histological analysis of *in vitro* formed pseudoislets showed
233 that alpha- and beta-cells are oppositely arranged compared to native islets. However, according
234 to literature the native islet architecture reappears in pseudoislets after transplantation in mice
235 [27]. This data prompted us to assess whether MSC interfere with the structural organization of
236 islet cells in pseudoislets during *in vitro* culture, and after transplantation in mice. Pseudoislets
237 composed of islet cells formed islet-cell clusters composed of centrally-located alpha-cells,
238 surrounded by a layer of beta-cells (Fig.2A). Pseudoislets containing MSC showed a similar
239 organization of beta-cells surrounding alpha-cells, and presented single MSC intermingled within
240 alpha- and beta-cells. Areas, build exclusively of MSC, fit tightly to areas of islet cell clusters
241 (Fig.2B). Images acquired by confocal laser scanning microscopy revealed a predominant
242 localization of beta-cells beside MSC (Fig.2E).
243 To analyze the effect of MSC on the architecture of pseudoislets *in vivo*, pseudoislets comprised
244 of islet cells alone or with MSC, were transplanted under the kidney capsule of non-diabetic
245 SCID mice and analyzed 15 days later. As shown, pseudoislets without MSC (Fig.2C) and with
246 MSC (Fig.2D arrows) re-organized in centrally-located beta-cells surrounded by alpha-cells.

MSC in the pseudoislet grafts mostly localized between the substructures of the pseudoislets. In addition, MSC localize around the pseudoislet graft. These results demonstrated that MSC interact with islet cells *in vitro* and *in vivo*, further suggesting that MSC serve as a supportive stromal structure for islets.

Inhibition of N-cadherin decreases the enhanced insulin secretion

To investigate whether intercellular adhesion molecules are involved in the enhanced insulin secretion, we analyzed in islets and MSC separately the expression of E-cadherin, NCAM, EpCAM-1, VCAM-1, N-cadherin and ICAM-1. E-cadherin, NCAM and EpCAM were expressed in islets but not in MSC (Fig.3A-C). In contrast, VCAM-1 was expressed only in MSC (Fig.3D). Interestingly, N-cadherin and ICAM-1 were expressed both in islets and in MSC (Fig.3E-F). We therefore selected these molecules for blocking experiments in the glucose-stimulated insulin assay.

Islets with or without MSC were cultured for 24 hrs in the presence of blocking antibodies against N-cadherin or ICAM-1 and then stimulated to release insulin. Similar to data presented in Fig.1, islets in contact with MSC showed an increased insulin secretion after high glucose ($p<0.05$) and theophylline stimulation ($p<0.01$) when compared to islets alone or islets and MSC cultured separately. Upon culture in the presence of the blocking antibody against N-cadherin, this increase was significantly inhibited, both for glucose stimulation ($p<0.05$) and for glucose plus theophylline stimulation ($p=0.05$, Fig.4A). This inhibition was only observed for cultures in the presence of MSC in direct cell-cell contact with islets. The insulin secretion by islets cultured without MSC or without direct contact with MSC was not affected. The specificity of the observed effect was demonstrated in culture using an isotype control antibody: in this condition,

the enhanced insulin release upon glucose and theophylline stimulation was not affected (Fig.4A). Specific binding of the anti N-cadherin antibody to MSC was demonstrated by FACS analysis [see additional file 1, Fig.1]. Blocking of ICAM-1 did not inhibit the increased insulin secretion under stimulating conditions, irrespective of the presence of MSC in the culture (Fig.4B). These results demonstrate that intercellular interactions involving N-cadherin are relevant in enhancing insulin secretion during direct cell-cell contact between islets and MSC.

Islets microencapsulated with MSC maintain regulated insulin secretion

To evaluate the effect of MSC on the survival and function of microencapsulated islets after transplantation, islets with or without MSC were microencapsulated (Fig.5A-B). In the microspheres MSC were present in a scattered distribution (Fig.5B). Three days after microencapsulation, insulin secretion was similar for microencapsulated islets alone and microencapsulated islets with MSC (Fig.5C). These results show that islets maintained insulin secretion upon glucose stimulation, after microencapsulation. However, early after microencapsulation the enhanced effect on insulin secretion by MSC was not observed. To analyse whether the distribution of MSC inside the microcapsule changed with time, microencapsulated islets and MSC were retrieved 15 days after transplantation. Interestingly, inside the microcapsules MSC localized with islets [see additional file 1, Fig.2], contrary to MSC in microcapsules before transplantation, where MSC were present in a scattered distribution. This indicates that after transplantation, MSC localize to islets in microcapsules and suggests that this interaction fosters glucose-induced insulin secretion.

MSC prolong survival and function of microencapsulated human islets in diabetic mice

290 Free islets, microencapsulated islets and microencapsulated islets with MSC were transplanted
291 into the peritoneum of immunocompetent diabetic C57BL/6 mice. In the immediate post-
292 transplant period, all mice reversed diabetes [see additional file 1, Fig.3]. Mice transplanted with
293 free islets then rapidly lost normoglycemia and became diabetic: the median normoglycemia time
294 was 6 days. Mice transplanted with microencapsulated islets remained normoglycemic until day
295 18 and then progressively turned diabetic starting at day 20: the median survival time was 39
296 days. This difference in survival between free and microencapsulated islets was statistically
297 significant ($p<0.0001$). The period of normoglycemia was significantly longer in mice that
298 received microencapsulated islets with MSC, resulting in a survival period up to 88 days and a
299 median survival of 69 days ($p<0.01$ compared to microencapsulated islets, Fig.6A). After graft
300 failure, no insulin-positive cells were found in recovered beads originally containing islets alone,
301 [see additional file 1, Fig.4A]. However, insulin positive cells were still present in beads where
302 islets were co-encapsulated with MSC [see additional file 1, Fig.4B]. These results demonstrate
303 that MSC prolonged human islet graft survival *in vivo*.
304 To assess graft function *in vivo*, IPGTT was performed at day 15 after transplantation in
305 untreated healthy control mice and diabetic mice transplanted with microencapsulated islets.
306 Blood glucose levels raised 2.6 and 2.4 times over basal glucose levels at time 0, respectively,
307 and recovered to normoglycemia 2 hrs after injection (ratio 1.2 and 1.3 compared to the basal
308 concentration). Diabetic mice transplanted with microencapsulated islets with MSC manifested
309 lower blood glucose levels than healthy mice at time 0 (4.1 mmol/L compared to 9.1 mmol/L):
310 however, these mice did not show any manifestation of hypoglycemia (weight loss, decreased
311 movements, and erected haircoat). Moreover, after glucose stimulation, these mice showed a

312 lower increase in blood glucose levels (2.0 times compared to time 0) and also presented lower
313 levels after 2 hrs compared to time 0 (1.2 mmol/L, ratio 0.3). This lower glucose response in
314 comparison with mice transplanted with microencapsulated islets alone reached statistical
315 significance ($p<0.001$, Fig.6B). Altogether, these results indicate that human islets co-
316 encapsulated with human MSC display a more efficient glucose-induced insulin response *in vivo*
317 compared to islets alone.

318

319 **Discussion**

320 Transplantation of microencapsulated islets without chronic immunosuppression represents a
321 valuable therapeutic option for type 1 diabetic patients, potentially resolving current hurdles
322 associated with clinical transplantation of free islets, such as the adverse side effects of
323 immunosuppressive drugs. However, as for free islets, transplantation of microencapsulated islets
324 does not provide a permanent independence from exogenous insulin, and the microencapsulation
325 technology needs further improvements to achieve long-term graft function. Studies in diabetic
326 mice and rats have shown that MSC upon co-transplantation with islets are beneficial for islet
327 function and survival [30]. There are only a few studies in literature addressing the effect of
328 human MSC on human islets, which prompted us to assess whether bone marrow-derived human
329 MSC sustain human islet function *in vitro* and *in vivo*.

330 Human islets upon culture with MSC show enhanced insulin secretion in response to glucose and
331 theophylline stimulation (Fig.1). This potentiating effect of MSC was observed solely when islets
332 and MSC were co-cultured in direct cell-cell contact. This observation is supported by findings in

rodents, where the contact between MSC and rat islets maintained and increased glucose-induced insulin secretion [31, 32]. Based on the potentiating effect of MSC on insulin secretion, we assessed the direct cell-cell contact between human islet cells and MSC in pseudoislet formation. It has been described that alpha- and beta-cells in islets reorganize after transplantation of pseudoislets in SCID mice [27]. In the present study, MSC did not alter the arrangement of alpha- and beta-cells but located between the re-organized substructures suggesting the integration of MSC as a stromal tissue component in the pseudoislets (Fig.2).

In mice, optimal regulation of insulin secretion by beta-cells is dependent on N-cadherin as demonstrated for a pancreatic epithelium-specific knock-out of N-cadherin: N-cadherin controls insulin granule turnover and subsequent insulin secretion [33]. Further, N-cadherin promoted insulin secretion upon stimulation of human beta-cells [19]. N-cadherin is expressed in human islets and strongly in human MSC, which let us to investigate the potential role of N-cadherin in the enhanced insulin secretion in co-culture with MSC. Blocking N-cadherin interactions for 24 hrs, which did not affect insulin expression, abolished the enhanced insulin secretion by islets co-cultured with MSC in direct cell-cell contact (Fig.4A). We conclude that enhanced islet function induced in direct cell-cell contact with MSC requires N-cadherin interactions.

Further, we investigated the effect of human MSC on graft function of microencapsulated human islets in diabetic immunocompetent mice. For transplantation studies, we focused on non-dissociated islets since insulin secretion by pseudoislets containing MSC was equivalent compared to non-dissociated islets in contact with MSC. This demonstrated that increased contact between islet cells and MSC compared to non-dissociated islets did not further increase insulin

354 secretion. The microencapsulation itself prevented acute graft failure due to rejection, from a
355 median survival of 6 days for free islets to a median normoglycemia period of 39 days for
356 microencapsulated islets (Fig.6A). Co-encapsulation of islets and MSC resulted in a further
357 significant prolongation of survival up to 69 days. IPGTT showed lower blood glucose levels in
358 mice transplanted with islets co-encapsulated with MSC (Fig.6B), which might be attributed to an
359 increased insulin production by islets in the presence of MSC. This phenomenon could also be
360 related to the fact that the presence of MSC in microcapsules protects islets against pro-
361 inflammatory cytokines. This had been shown in co-culture studies, in which human MSC but
362 not human dermal fibroblasts preserved human islets exposed to pro-inflammatory cytokines
363 [34]. Interestingly, in a pig-to-nonhuman primate xenotransplantation model, the presence of pig
364 MSC in capsules containing pig islets improved oxygenation and neoangiogenesis but only a
365 minor improvement in long-term islet function was observed [35]. Others have shown that the
366 presence of kidney-derived MSC in microencapsulated mouse islets improved graft outcome,
367 even in the absence of MSC-mediated enhancement of revascularization and preservation of islet
368 morphology [30]. Recently, MSC have been described to serve as a "multifunctional islet
369 supportive carrier" for the housing of pancreatic islets in a three-dimensional co-culture [36].
370 Therefore, in order to establish standardized protocols for future application of MSC in clinics,
371 precise mechanisms leading to the supportive effect on islets, needs to be unravelled.

372 The strength of this work is based on the finding that molecular interactions of N-cadherins
373 between human islets and MSC are essential for the increased insulin response by islets co-
374 cultured with MSC. However, the transplantation setting using encapsulated islets and MSC has
375 some limitation. It remains to be investigated whether trophic factors released by MSC leading to

reduced cellular stress [37], may participate on the prolonged survival and function of encapsulated islets.

Conclusions

In conclusion, the present study is the first to show that MSC improved survival and function of microencapsulated human islets transplanted in mice. Altogether, our data suggest that MSC via N-cadherin interactions provide an optimal microenvironment for fine-tuning of insulin secretion and that N-cadherin-mediated interactions could be pivotal to support islet graft function in clinical islet transplantation.

Abbreviations

Alg	alginate
E-cadherin	epithelial
EpCAM-1	epithelial
ICAM-1	intercellular
IEQ	islet cell equivalents
IPGTT	intraperitoneal glucose tolerance test
MSC	multipotent mesenchymal stromal cells
NCAM	neural cell adhesion molecule

PEG	poly(ethylene glycol)
VCAM-1	vascular

Declarations:

Ethics approval and consent to participate

Human islets were provided to research only when considered not suitable for clinical transplantation, through the JDRF award 1-RSC-2014-100-1-X, ECIT Islet for Basic Research program. MSC were obtained from the femoral head of patients undergoing total hip replacement as previously described. Informed consent was given by all patients and the experimental procedure was approved by the local ethical committee of the University Hospitals of Geneva (NAC 01-015). Animal research was performed following protocols approved by the Geneva cantonal veterinary authorities (license GE/34/13).

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

EM designed and performed experiments, acquired and analyzed data, and wrote the manuscript; RPHM performed experiments and participated in research discussions; RM, CW and SGL developed and provided the polymeric materials; JDS participated in research discussions and editing; LHB participated in research discussions and editing; CGG designed and performed experiments, acquired and analyzed data, and participated in research discussions. CGG critically revised the manuscript and is the guarantor of this study. All authors read and approved the final version of the manuscript.

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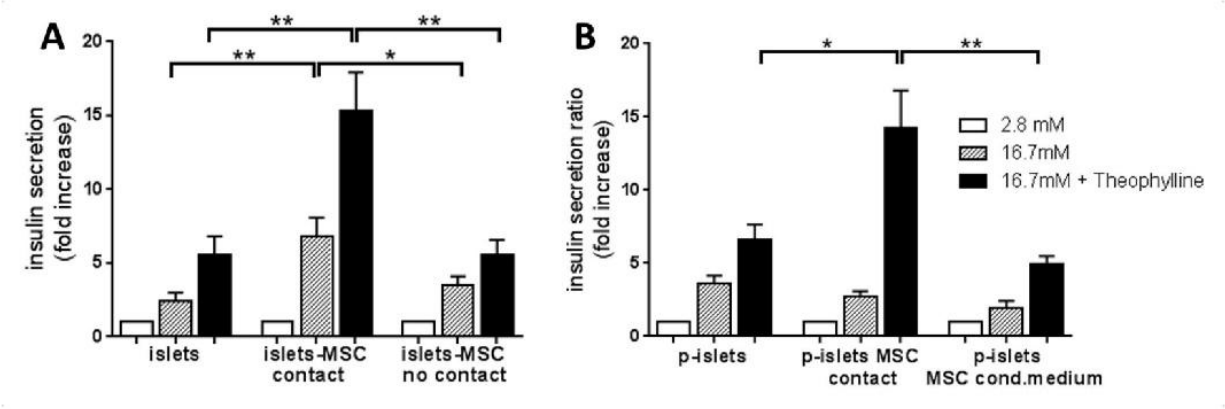
518

519 **Figures**

520



521

522 **Figure 1. Effect of MSC on insulin secretion by islets**. The insulin secretion assay was
523 performed by incubating islets (150 IEQ) or pseudoislets (10^5 islet cells) at basal glucose (2.8
524 mM, white bars), high glucose concentration (16.7 mM, grey bars) and high glucose plus
525 theophylline (16.7 and 5 mM, respectively, black bars) for 1 hr. **(A)** Islets alone, islets together
526 with $15x10^3$ MSC (ratio 1 IEQ:100 MSC) in direct cell-cell contact, and islets-MSC without cell-
527 cell contact separated in a transwell chamber, after a 3-day culture. **(B)** pseudoislets (p-islets),
528 pseudoislets cultured in direct cell-cell contact with $3x10^4$ MSC (ratio 3:1) (p-islets MSC contact)
529 and pseudoislets cultured in MSC-conditioned medium (p-islets MSC cond.medium) after a 6-
530 day culture. Values were normalized to the total amount of insulin, and insulin secretion is
531 expressed as fold increase were the basal level is set at 1. Data are presented as arithmetic mean ±
532 SEM of 4-5 independent islet donors respectively, and for each donor assays were performed in
533 triplicate. Mann-Whitney test; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

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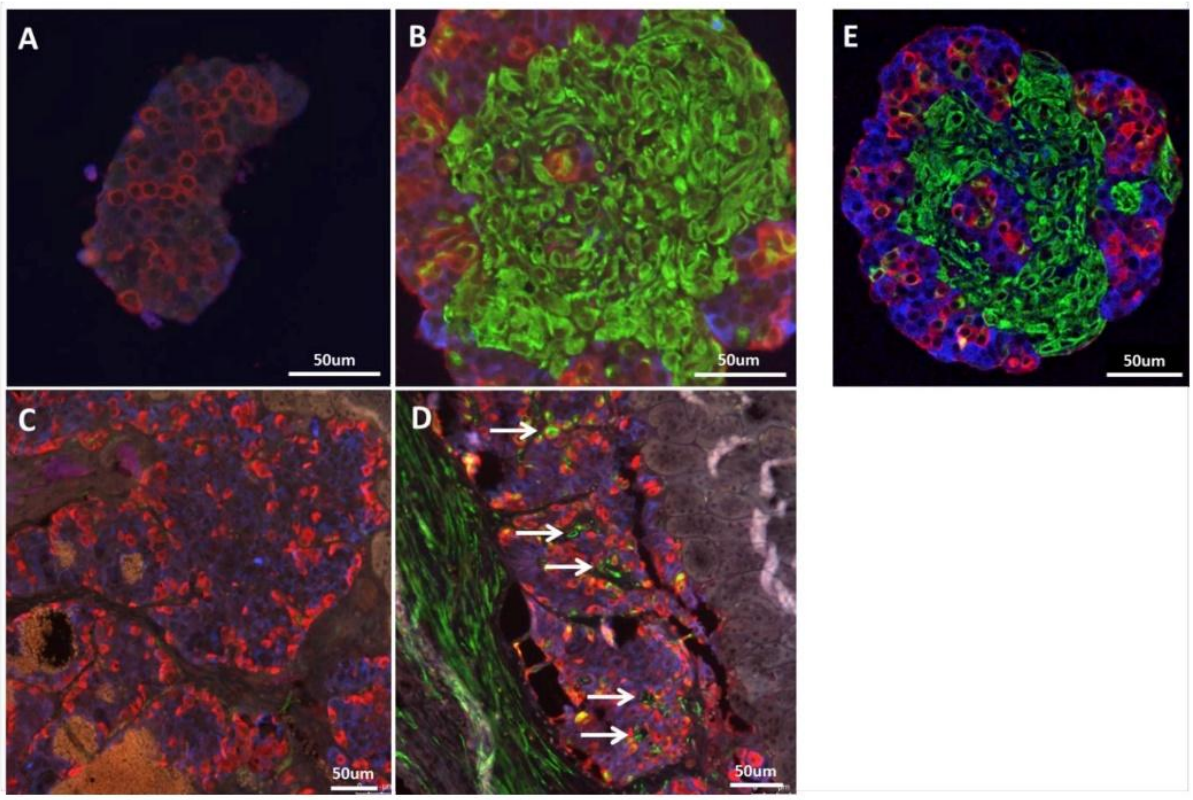


Figure 2. α- and β-cell arrangement in cultured pseudoislets and after transplantation in SCID mice. Islets were dissociated by enzymatic digestion, and 10^4 single cells were cultured in hanging drops for 6 days prior to *in vitro* immunofluorescence studies, and for 8 days prior to transplantation under the kidney capsule of SCID mice. β-cells were immunostained for insulin (blue), α-cells for glucagon (red) and MSC for vimentin (green). These pictures are representative images of 3 different experiments. **(A,B)** pseudoislets after *in vitro* formation: **(A)** islet cells alone, **(B)** islet cells and MSC. **(C,D)** pseudoislets 15 days after transplantation: **(C)** pseudoislets composed of islet cells alone, **(D)** pseudoislets composed of islet cells and MSC. White arrows indicate vimentin-positive cells within the islet graft located outside of the α and β cell arrangements (islets sub-structures). Right panel **E**: *in vitro* formed islets and MSC at day 6, confocal laser scanning microscopy.

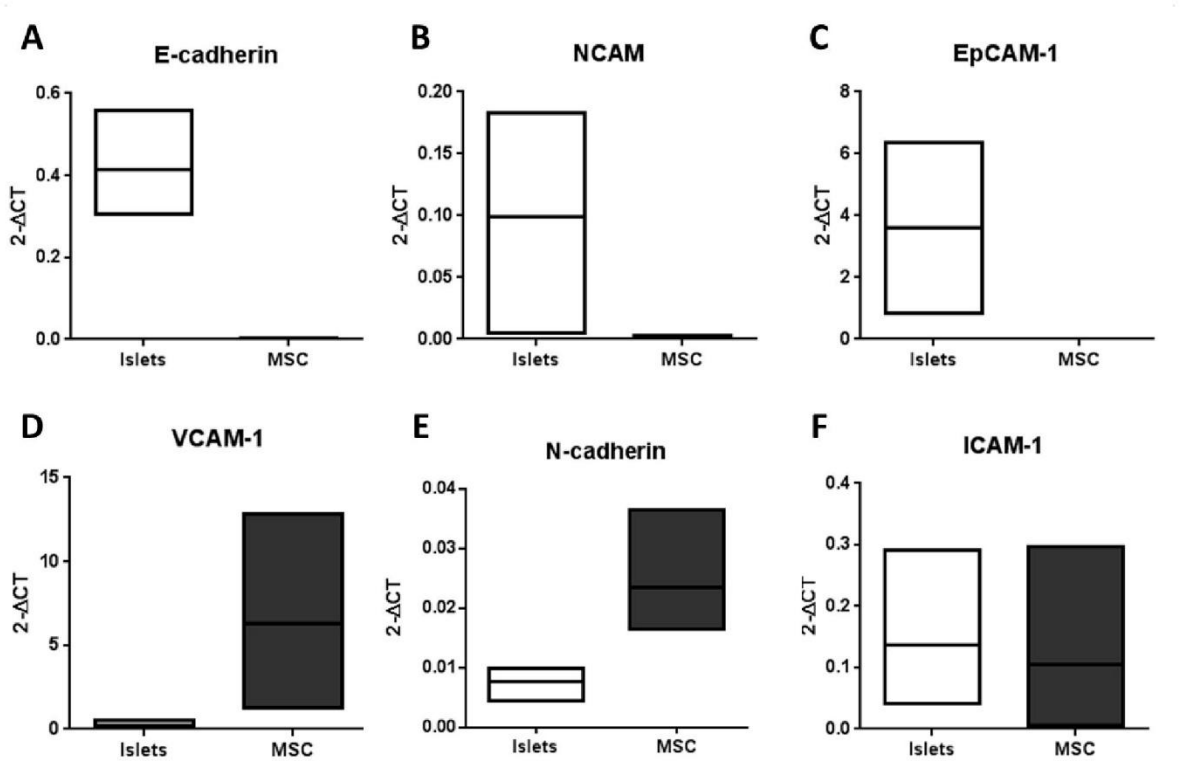


Figure 3. Gene expression of adhesion molecules in islets and MSC. Quantitative RT-PCR was performed to assess adhesion molecule expression, and values were normalized to the expression of housekeeping ribosomal protein large P0. Expression of epithelial (E)-cadherin **(A)**, neural cell adhesion molecule (NCAM) **(B)**, epithelial (Ep)CAM-1 **(C)**, vascular (V)CAM-1 **(D)**, N-cadherin **(E)** and intercellular (I)CAM-1 **(F)** were measured in islets (white boxes) and MSC (black boxes) after a 3-day culture period. Data from 3 islet donors are presented as floating bars (min to max) with the line presenting the mean value.

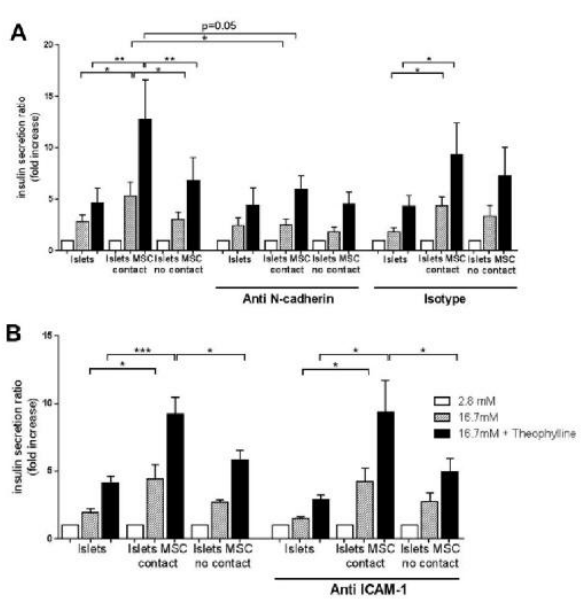
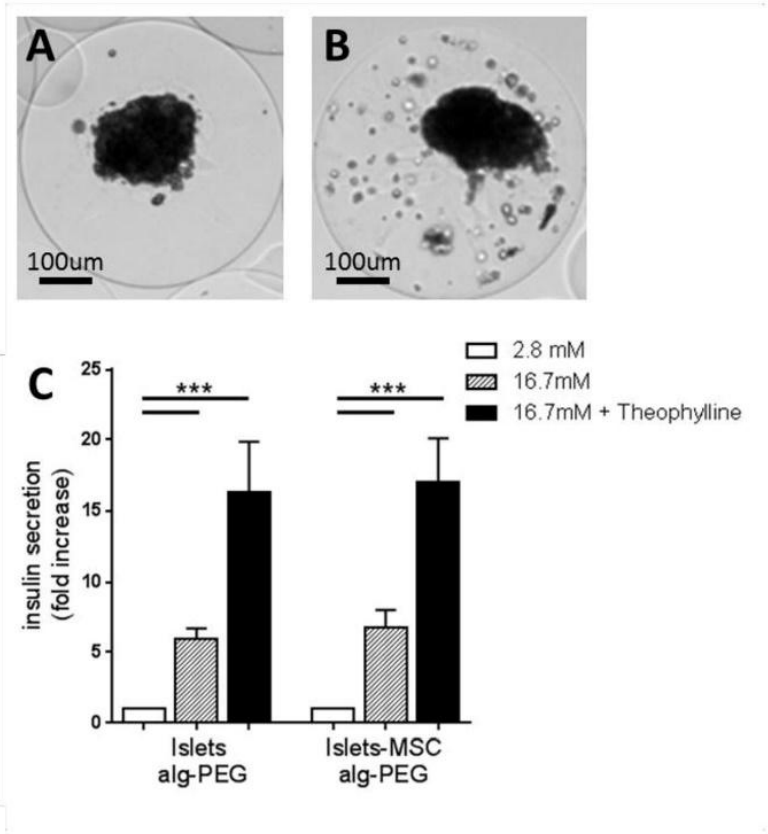
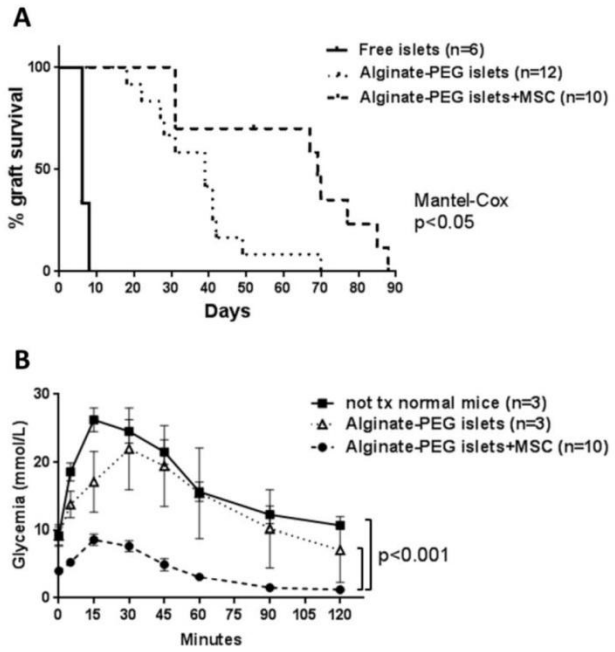


Figure 4. Blocking of N-cadherin but not of ICAM-1 decreased the MSC-enhanced insulin secretion. Antibodies against N-cadherin, ICAM-1 and the isotype control were added to the culture of islets (islets), islets and MSC in co-culture with cell-cell contact (islets MSC contact) and islets and MSC in culture without contact (islets MSC no contact) for 24 hrs prior to the insulin secretion assay. Cells were incubated in basal glucose (2.8 mM, white bars), high glucose concentration (16.7 mM, grey bars) and high glucose plus theophylline (16.7 and 5 mM, respectively, black bars) for 1 hr. Data are presented as in Figure 1. **(A)** Cells were cultured alone and with the addition of 25 μg/ml anti-N-cadherin antibody and isotype control. **(B)** Cells were cultured alone and with the addition of 25 μg/ml anti-ICAM-1 antibody. Values of secreted insulin were normalized to the total amount of insulin and expressed as fold increase where the basal level was set at 1. Human islets from 4 and 3 different donors were used for N-cadherin and ICAM-1 blocking experiments, respectively. For each donor the experiment was performed in triplicate. Each graph represents mean ± SEM. Mann-Whitney test; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.



570

571 **Figure 5**. **Microencapsulated islets maintain regulated insulin secretion. (A,B)** Phase contrast
572 images of Alg-PEG microencapsulated islets (10^4 IEQ/ml Alg-PEG) **(A)**, and islets co-
573 encapsulated with $8x10^5$ MSC **(B)**. **(C)** Insulin secretion by microencapsulated islets (Islets Alg-
574 PEG) and islets co-encapsulated with MSC (Islets-MSC Alg-PEG) at 3 days after encapsulation.
575 Microencapsulated islets (150 IEQ) were incubated at basal glucose (2.8 mM, white bars), high
576 glucose concentration (16.7 mM, grey bars) and high glucose plus theophylline (16.7 and 5 mM,
577 respectively, black bars) for 1 hr. Values of secreted insulin were normalized to the total insulin
578 amount and expressed as fold increase where the basal level was set at 1. Data presented are
579 mean value ± SEM of 3 islet donors, and for each donor the assay was performed in triplicate.
580 Wilcoxon signed-rank test; *** $p<0.001$.



581
582 **Figure 6. Islet survival and function in diabetic mice. (A)** Islets (4.5-5x10^3 IEQ) were
583 transplanted into streptozotocin-induced diabetic mice. Mice were transplanted with free islets
584 (n=6, continuous line), with microencapsulated islets (n=12, dotted line), or with islets co-
585 encapsulated with MSC (n=10, dashed line) at a ratio IEQ:MSC 1:80. Glycemia was monitored 2
586 times per week and the graft was considered rejected after 3 glucose measurements > 20 mmol/L.
587 Mantel-Cox test was performed. **(B)** Intraperitoneal glucose tolerance test (IPGTT) was
588 performed at day 15 in non-transplanted healthy mice (n=3, continuous line), in diabetic mice
589 transplanted with alginate-PEG islets (n=3, dotted line) and in mice transplanted with alginate-
590 PEG islets+MSC (n=10, dashed line). A single intraperitoneal injection of glucose (2g/kg) was
591 performed at time 0 and glucose was monitored at 0, 5, 10, 15, 30, 45, 60, and 120 min thereafter.
592 Values are expressed as the mean (±SEM) of measurement from at least 3 mice. The area-under-
593 the-curve (AUC) was calculated to perform t-test statistical analysis.

Supplementary material

Supplementary table 1. Primers used in adhesion molecule assessment in islets and MSC

E-cadherin	Fwd 5'-AGA GAA ACA GGA TGG CTG AAG GTG-3' Rev 5'-ACT GCA TTC CCG TTG GAT GAC A-3'
NCAM	Fwd 5'-CCC TCT TCA CCA TCC ATC GA-3' Rev 5'-TTC ACC AAC TGC TCT CCA CT-3'
EpCAM-1	Fwd 5'-CAT GTG CTG GTG TGT GAA CA-3' Rev 5'-CCA GTA GGT TCT CAC TCG CT-3'
VCAM-1	Fwd 5'-GGG AAG ATG GTC GTC ATC CT-3' Rev 5'-GAT TCT GGG GTG GTC TCG AT-3'
N-cadherin	Fwd 5'-GAG CCT GAA GCC AAC CTT AAC TGA-3' Rev 5'-CTG GCA AGT TGA TTG GAG GGA TGA-3'
ICAM-1	Fwd 5'-CGT GGG GAG AAG GAG CTG AA-3' Rev 5'-CAG TGC GGC ACG AGA AAT TG-3'

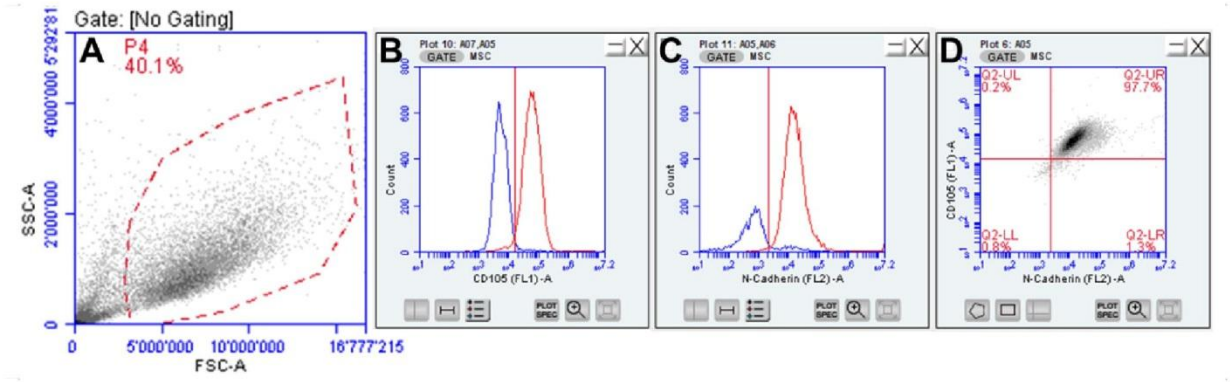
E-cadherin, epithelial-cadherin; NCAM, neural cell adhesion molecule; EpCAM-1, epithelial cell adhesion molecule; VCAM-1, vascular cell adhesion molecule-1; N-cadherin, neural-cadherin; ICAM-1, intercellular adhesion molecule-1.

Transplantation of microspheres under the kidney capsule

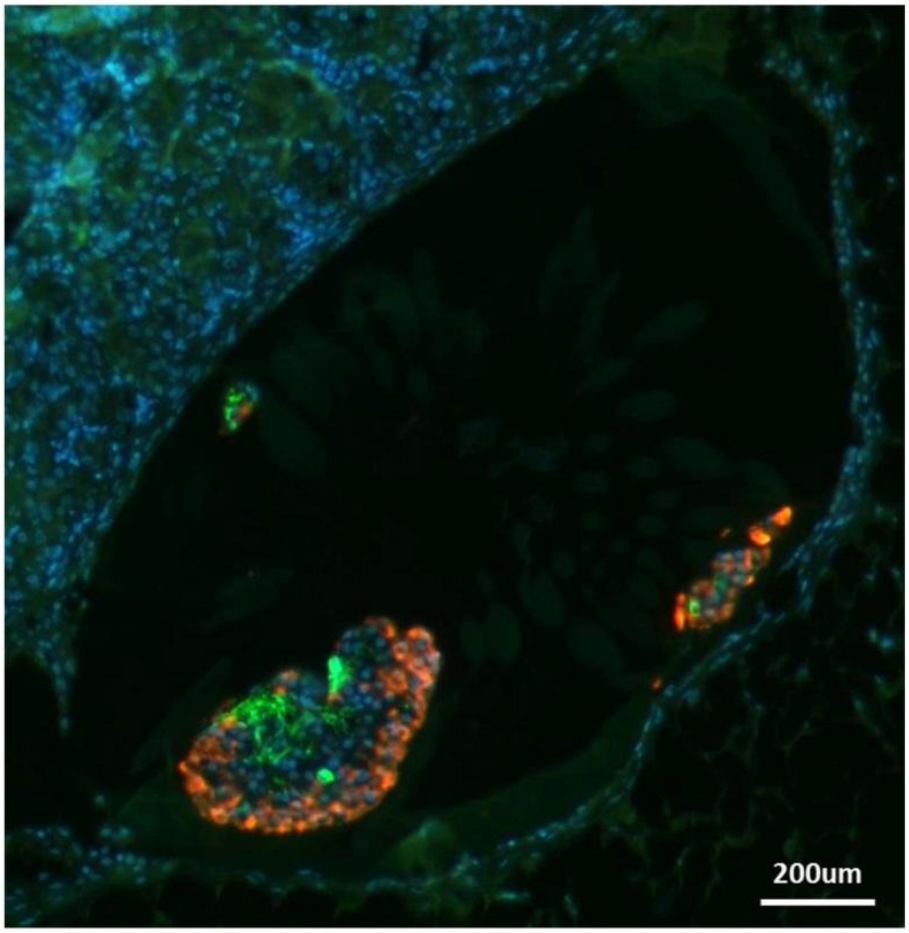
Mice were anesthetized with isoflurane and a side incision was performed to approach the kidney. Microspheres (50µl) were transplanted under the kidney capsule using an Abbocath-T 18G catheter (Hospira). Mouse kidneys were collected at day 15 after transplantation and paraffin embedded.

Histological analyses on microspheres from peritoneum and kidney

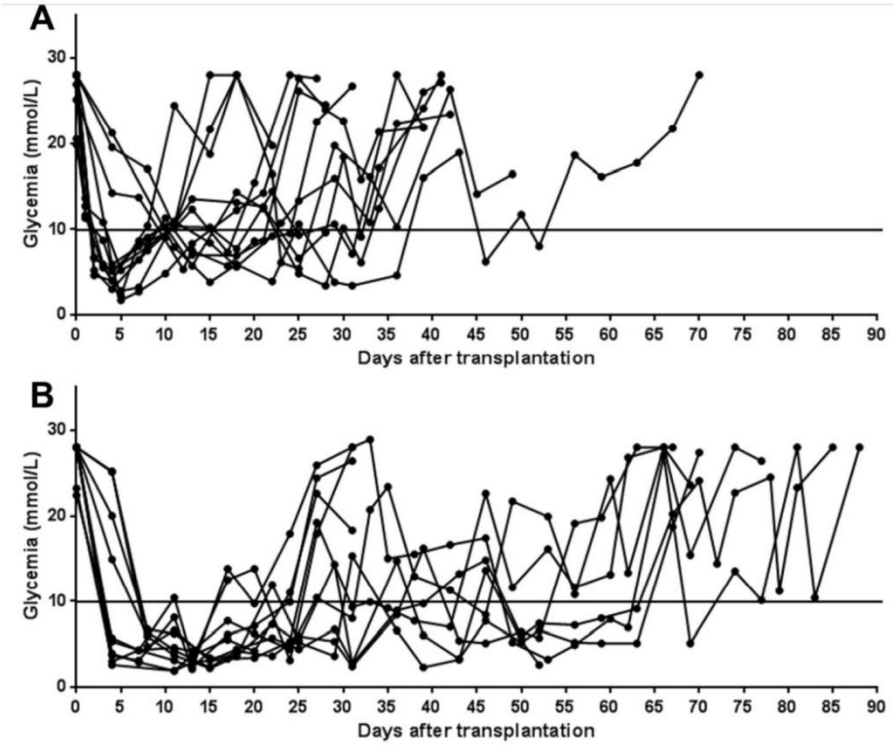
After islet graft failure, mice were euthanized and microspheres were retrieved from the peritoneum, formalin fixed in tubes and then paraffin embedded. Four-µm sections of paraffin embedded microcapsules and kidneys were treated with 0.01mol/l citrate for 15 min in a microwave, to unmask epitopes. To avoid nonspecific binding, slides were incubated with 0.5% BSA for 30 min at room temperature. MSC were stained with mouse anti-human vimentin antibody, diluted 1:50 (Dako, Glostrup, Denmark) and then with Alexa Fluor 488 goat anti-mouse antibody, diluted 1:1,000 (Life Technologies, CA, USA). Beta cells were stained with guinea pig anti-porcine insulin, diluted 1:500 (Dako) and Alexa Fluor 555 goat anti-guinea pig, diluted 1:1,000 (Life Technologies). Microscopic images were acquired using a fluorescence microscope (Leica DM 2000).



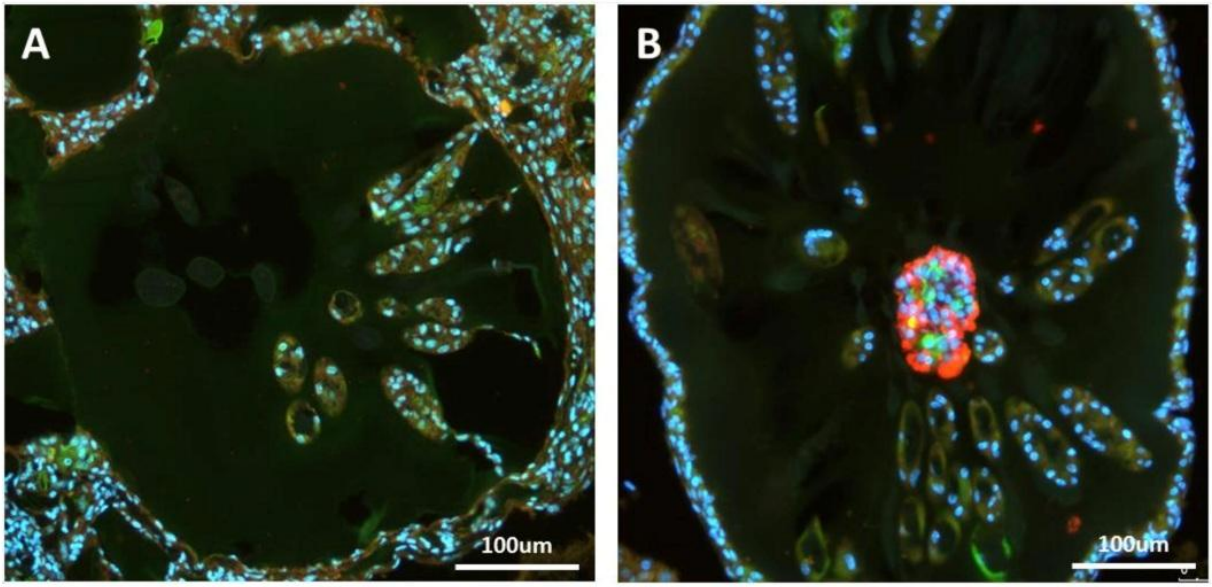
Supplemental Fig. 1. FACS analysis of anti-N-cadherin antibody-binding on MSC. (A) MSC were gated based on size and cell density. **(B)** MSC were stained by anti-CD105 antibody, a surface antigen expressed on bone marrow stromal cells. Blue line represents unstained MSC and red line represents CD105 positive MSC. **(C)** MSC stained by anti-N-cadherin antibody. Blue line indicates the isotype control and red line indicates N-cadherin positive cells. **(D)** Gated MSC double stained for CD105 and N-cadherin. 97.7% of gated MSC are double positive for CD105 and N-cadherin.



Supplemental Fig. 2. MSC co-localized with islets in microcapsules 15 days after transplantation. Microspheres transplanted under the kidney capsule were retrieved together with the kidney. Sections were immunostained for insulin (red) and vimentin (green) to stain islets and MSC, respectively. Cell nuclei were stained with Hoechst (blue).



Supplemental Fig. 3. Blood glucose measurements. Glycemia was measured 48 hrs after transplantation and thereafter twice weekly. Islet graft failure was concluded when glucose level was >20 mmol/L for 3 consecutive measurements. **(A)** Glucose measurements in 12 mice transplanted with encapsulated islets. **(B)** Glucose measurements in 10 mice transplanted with encapsulated islets and MSC.



Supplemental Fig. 4. Beads retrieved after loss of graft function. Beads were retrieved from the peritoneum when mice became diabetic. Sections were immunostained against insulin (red) and vimentin (green) for islets and MSC, respectively. Cell nuclei were stained with Hoechst (blue). **(A)** Beads recovered from mice transplanted with islets alone. Only empty beads were retrieved. **(B)** Beads recovered from mice transplanted with islets and MSC together still contained some islets.

5.3 MSCs and porcine hepatocytes

"Beneficial effects of Human Mesenchymal Stromal Cells on Porcine Hepatocyte Viability and Albumin Secretion"

E. Montanari et al. 2017 (manuscript in preparation for Journal of Immunology Research)

An optimized protocol for porcine hepatocytes isolation from 10-kilo pigs was established and a novel alginate-PEG-SH grafted hydrogel was adopted for hepatocyte encapsulation. Viability, functionality and diazepam metabolism capacities were maintained in free and encapsulated hepatocytes. Further, hepatocytes co-encapsulated and co-cultured with MSCs showed improved viability and albumin secretion, suggesting that MSCs provide a supportive environment comparable to stromal tissue.

Personal contribution:

In this work, I designed and performed experiments, analyzed the data and wrote the manuscript.

Beneficial effects of Human Mesenchymal Stromal Cells on Porcine Hepatocyte Viability and Albumin Secretion

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ABSTRACT

In acute liver failure, orthotropic liver transplantation is frequently needed, but cannot always be offered to patients due to the absence of suitable organs. The transplantation of encapsulated hepatocytes, to compensate for metabolic functions until the liver recovers, is under experimental investigation. Since the availability of human hepatocytes is limited, other potential sources for hepatocytes need to be evaluated. Our aim was to optimize the isolation process of high number of viable pig hepatocytes and establish the encapsulation procedure with different biocompounds. We further tested whether co-culture and co-encapsulation of hepatocytes with human mesenchymal stromal cells (MSCs), exert beneficial effects on hepatocyte function and viability. Livers from 10 kg pigs (n=9) were harvested and perfused with collagenase solution. Hepatocytes were isolated from the liver suspension and cells encapsulated using alginate and poly(ethylene-glycol)(PEG)-grafted alginate polymeric hydrogels, either alone or in combination with MSCs. Viability, albumin secretion and diazepam catabolism of hepatocytes were measured for one week. $9.2\pm3.6x10^9$ hepatocytes with 95.2±3.1% viability were obtained after cell isolation. At day 3, free hepatocytes displayed 99% viability, whereas alginate and PEG-grafted alginate encapsulated hepatocytes showed 62% and 48% viability, respectively. Albumin secretion and diazepam catabolism were maintained in free and encapsulated hepatocytes. Co-encapsulation of hepatocytes with MSCs significantly improved viability and albumin secretion on day 4 and 8 ($p<0.05$). Albumin secreted in co-culture with MSCs was significantly increased on day 3, 4 and 8, and was prolonged up to day 15. In conclusion, we developed a standardized protocol to isolate high numbers of viable pig hepatocytes and demonstrated that co-culture and co-encapsulation with human MSCs increased viability and albumin secretion from porcine hepatocytes.

1. Introduction

Hepatocyte cell transplantation is a potential solution to improve temporally acute liver failure in patients who are expecting a full liver transplant [1 448]. Nevertheless, in patients with acute liver failure, the availability of human liver cells remains a major problem. Transplantation of xenogeneic cells could be an adequate cell replacement treatment [2]. To circumvent the immune reaction toward the xeno-graft, encapsulation of cells in a semipermeable biocompatible polymer was developed [3]. Micropores permit exchanges of nutrients and oxygen as well as molecules of small sizes. Indeed, promising first transplantation studies with encapsulated hepatocytes showed that in animal models, such as pig to mouse, showed that encapsulated and transplanted hepatocytes were still functional [4-6]. In a baboon-to-pig model, 75% of transplanted pigs with encapsulated hepatocytes recovered from liver injury, previously induced by 75% hepatectomy and warm ischemia [7], further demonstrating the therapeutic potential of such a treatment.

Previously, our laboratory established a first protocol for isolation of primary pig and human hepatocytes and their encapsulation with poly-L-lysine (PLL) [5, 6]. However, hepatocytes, when encapsulated, showed reduced functionality, suggesting that polymers and micro-environmental conditions needed further optimization. Neonatal pig hepatocytes showed increased viability and functionality when previously re-aggregated, [4] or when heparin or hepatocyte growth factors were added during the encapsulation procedure [8]. The addition of PLL and collagen during encapsulation in alginate microcapsules also prolonged viability and functionality of human hepatocellular carcinoma cell line HepG2/C3A [9]. Furthermore, co-culture or co-encapsulation with cell types such as endothelial progenitor cells or fibroblast showed enhanced viability and function of rat hepatocytes [10, 11].

Multipotent mesenchymal stromal cells, also called mesenchymal stem cells (MSCs), support rodent hepatocyte growth by favoring cellular adhesion [12, 13]. Several studies in rodents reported that co-transplantation of hepatocytes together with rat MSCs prolonged graft function with the decrease of alanine transaminase (ALT), aspartate transaminase (AST) and bilirubin [14-16]. Further, co-culture of rodent hepatocytes together with MSCs improved albumin secretion and metabolic capacities of hepatocytes [14, 15, 17].

The aim of this study was to optimize hepatocyte isolation to reach high yields of viable porcine hepatocytes and to assess the viability, albumin secretory capacities and metabolic functions after *in vitro* culture and microencapsulation. Furthermore, we aimed to assess the effect of human MSCs, on free and co-encapsulated porcine hepatocytes, with particular attention to the effect on viability, albumin secretion and cell organization of pig hepatocytes.

2. Materials and Methods

2.1 Animals and liver harvesting

Ten kilos pigs were purchased from Stirnimann Markus (Apples, Switzerland) and were housed in the animal facility of the University of Geneva, following all cantonal dispositions. Animal research was performed following protocols approved by the Geneva cantonal veterinary authorities (license GE/79/15). The pigs were fasted overnight and premedicated with Azaperon (1 mg/mL) and Atropine (0.05 mg/mL) + Dormicum (0.5 mg/mL) by intramuscular injection, prior to anesthesia with isoflurane, Fentanyl (0.1 mg) and Atracurium (1 mg/mL). After heparin injection (5000 Ul) and extensive disinfection, an abdominal incision was performed, followed by gallbladder removal and perfusion of cold preservation medium, IGL-1 (3.5 l) (Institut Georges Lopez, Lissieu, France) through the portal vein and the hepatic artery. A total hepatectomy was performed and the liver was stored on ice prior to hepatocyte isolation.

2.2 Cell isolation and culture

Isolation of porcine hepatocyte was performed as previously described [5]. Briefly, liver was perfused *ex vivo* through the vena cava with liver perfusion medium during 15 minutes at 37 °C (Life technologies, Carlsbad, CA, USA). Thereafter, digestion media containing collagenase NB 4 Standard Grade (3 g/l) (Serva, Heidelberg, Germany) was infused during 25 minutes through the vena cava (Figure 1). Mechanical destruction and filtration of the liver through a 100 mm stainless steel mesh was performed to obtain hepatocyte suspension, as previously described [5]. Hepatocyte cell suspension was washed twice using hepatocyte wash medium (Life technologies). Cells were counted and cultured in DMEM/F12 (Life technologies), supplemented with Dexamethasone (0.4 µg/mL, Sigma-Aldrich, Buchs,

Switzerland), insulin (0.02 E/U/mL, Novo Nordisk, Plainsboro, NJ, USA), apo-transferrin (5 µg/mL, Sigma), Streptomycin (100 mg/mL), Penicillin (100 IU/mL) (P-S, Gibco-Thermo Fisher, Waltham, MA, USA) and 10% of autologous serum, obtained after high-speed centrifugation of porcine blood.

MSCs were obtained from the femur head of patients subjected to hip replacement and cultured as previously described [18, 19]. Briefly, after isolation, MSCs were cultured in Iscove's modified Dulbecco's medium (Gibco-Thermo Fisher) with 10% FCS (Gibco-Thermo Fisher), P-S and 10 ng/mL platelet-derived growth factor BB (PDGF-BB, PeproTech EC Ltd, London, UK). MSCs were used in between passages 2 to 5. All patients gave informed consent and the experimental procedure was approved by the local ethical committee of the University Hospitals of Geneva (NAC 01-015).

2.3 Polymer synthesis

Na-alg Kelton HV (lot no. 61650A, $[\eta]$ = 813 mL g^{-1} in 0.1 M NaCl, T = 25 °C, G/M = 0.6) was obtained from Kelco (San Diego, USA, CA). Commercial reagents (Fluka, Sigma, Switzerland; TCI Europe, Zwijndrecht, Belgium) were used without further purification. Unless special mention, all reactions were performed under argon atmosphere (1 atm.). Anhydrous solvents were obtained by filtration (Puresolv MD 5, Innovative Technology, Oldham, UK). Glassware was dried for 12 h in an oven (T > 100 °C) or under vacuum with a heat gun (T > 200 °C). NMR spectra were recorded on Bruker Avance III-400, Bruker Avance-400 or Bruker DRX-400 spectrometers at room temperature (rt) (400 MHz) (Bruker, Billerica, MA, USA). ^{1}H frequency is at 400.13 MHz. Chemical shifts are expressed in parts per million (ppm) and coupling constants (*J*) in hertz (Hz). Solvent used for NMR spectroscopy was deuterated water (D_2O).

A solution of TBA-Alg [20] (200 mg, 0.478 mmol) in DMSO (40 mL) was stirred for 12 hours at 22 °C. To this solution, CDI was added (77.4 mg, 0.478 mmol) and the mixture was stirred at 22 °C for 0.5 h. Acetone (80 mL) was added and the resulting precipitate was filtered and washed with acetone (20 mL, 3 times). The solid was dried for 15 minutes under vacuum at 40 °C and dissolved in distilled water (20 mL). A solution of α-amino-ω-azido PEG (H_2N-PEG-SH) (98.8 mg, 95.8 μmol) in distilled water (1 mL) was added and the solution was stirred for 2 hours at 22 °C. NaOH (0.05 M aqueous solution) was added until reaching pH 11.0 and the solution was transferred to a dialysis membrane and dialyzed against water. After one water change, TCEP (0.1 M, 1 ML) was added in the dialysis tube and the dialysis was continued against water for 3 days. An aqueous solution of $NaHCO_3$ was added until reaching pH 7. The solution was filtered (70 μm and 0.22 μm) and freeze-dried to afford Alg-PEG-SH as a white solid (106.7 mg) (Supplementary figure 1). The percentage of grafting, determined by H-NMR, was 30.3%. The viscosity of the polymer (measured in distilled water at 22 °C) was 204.6 mPa.s.

2.4 Hepatocyte encapsulation

Hepatocytes with or without MSCs (ratio 1:1) were gently mixed with calcium-alginate (Ca-Alg), under sterile conditions. Alg-PEG-SH was used to encapsulate hepatocytes. All polymers were provided by collaborators of the EPFL. Microbeads were produced using the Buchi Encapsulator B-395 Pro (Büchi Labortechnik AG, Flawil, Switzerland) and bead polymerization occurred after bead immersion into the gelation bath, constituted of 10 mM MOPS, 100 mM $CaCl_2$. Microspheres were collected by filtration, washed with NaCl, 0.9% and immediately cultured in complete DMEM/F12.

2.5 Viability of primary and encapsulated cells

Viability and cell death of free and microencapsulated cells were analyzed using fluorescein diacetate (FDA), and propidium iodide (PI) for the staining of viable and dead cells, respectively, as described previously [21]. Images were acquired using a florescent microscope and LAS V4.5 software (Leica Microsystem, Heerbrugg, Switzerland). Quantification of cell viability and mortality was performed 3 days after culture using ImageJ (https://imagej.nih.gov/ij) and expressed as a percentage of the total cell area positive for both, FDA and PI which was considered as 100%.

2.6 Measurement of albumin secretion

Hepatocytes (0.2×10^6 cells) alone or with MSCs (0.2×10^6 cells, ratio 1:1) and microencapsulated or not, were seeded with 1 mL complete DMEM/F12 medium in a 24 well Corning Primaria Cell Culture Multiwell Plates (Fisher Scientific, Hampton, NH, USA). Cells were starved to remove pig albumin 24 hours before collection of medium, from day 2 to day 8 and frozen until albumin measurement. Albumin was measured following manufacturer instructions using an albumin pig ELISA Kit (Abcam, Cambridge, UK).

2.7 Measurements of drug metabolism

One µg/mL of Diazepam was added to free and encapsulated hepatocytes for 6 hours at day 1, 3 and 7. The supernatant was collected and frozen until metabolite measurements. Prior to the quantitative analysis, protein precipitation was performed on the samples using a solution of methanol:ethanol at a ratio 1:1. Then samples were centrifuged at 14,000g for 15 minutes. Samples were lyophilized with a speedvac system, and reconstituted in 10% methanol. Quantitative analysis was performed by LC-MS/MS with selected reaction monitoring (SRM) mode. The UltiMate 3000 LC system from Dionex coupled to a triple quadripole 5500 QTRAP system from AB Sciex was used. The LC separation was conducted on a Kinetex

C18 (Phenomenex), 50x2.1 mm (i.d.) column. The mobile phases were made of A: H_2O with 0.1% formic acid and B: ACN + 0.1% formic acid. The flow rate was 0.6 mL/min. The SRM transitions used for the quantification of the diazepam, lidocaine and their respective metabolites are displayed in Supplementary table 1.

2.8 Histology

Hepatocytes (0.2 x 10^6 cells), alone or with MSCs (0.2 x 10^6 cells, ratio 1:1), were seeded on 12 mm coverslips in a 24 well plate in 1 mL of complete DMEM/F12 medium. After 3 days, cells were washed with PBS and fixed with Formalin solution 10% (Sigma-Aldrich, Buchs, Switzerland) for 12 minutes. Cells were then permeabilized with Triton-X 100 0.1% diluted in PBS during 15 minutes and epitopes were blocked using 0.5% BSA for 30 minutes. Hepatocytes were stained with anti-pig albumin antibody (Abcam), diluted 1/200, and a secondary Alexa Fluor 555 goat anti-rabbit antibody (Life Technologies), diluted 1/500. MSCs were stained with mouse anti-human vimentin antibody, diluted 1:50 (Dako, Glostrup, Denmark), and a secondary Alexa Fluor 488 goat anti-mouse antibody (Life Technologies). For Edu staining, Click-iT EdU Alexa Fluor 488 Imaging Kit (Thermo Fisher) was used, following manufacturer instructions. Coverslips were mounted using vectashield mounting medium with DAPI (Vector Laboratories, Cambridgeshire, UK). Images were acquired using a fluorescence microscope and LAS V4.5 (Leica Microsystem).

2.9 Statistical analysis

Results are expressed by mean ± standard error of the mean (SEM). Numbers of experiments are indicated in the legend of each figure. GraphPad Prism software was used. Values of hepatocytes alone or co-cultured with MSCs were compared using ratio paired T test, and the differences were considered significant when $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

3. Results

3.1 Hepatocyte isolation, cell yield and viability

After surgical recovery of the livers from 10 kg pigs (n = 12), $9.2x10^9 \pm 3.6$ total hepatocytes were isolated with a yield of $27.9x10^6 \pm 9.9$ cells/g (Table 1). Immediately after hepatocyte isolation and purification, viability was of 95.2 ± 3.1%, demonstrating that the isolation protocol allowed for high yield isolates of viable porcine hepatocytes from 10 kg pigs. In order to stock porcine hepatocytes for ulterior transplantation, we froze hepatocytes in 2%-10% DMSO and Cryostor cell cryopreservation media-CS2 (Sigma-Aldrich). The survival rate of Cryostor-frozen hepatocytes after thawing reached 90% (Figure 2A); no hepatocytes were viable when frozen with DMSO. Further thawed hepatocytes maintained albumin secretion at day 4, 8 and 11 (Figure 2B).

3.2 Hepatocyte viability is maintained after Ca-Alg and PEG microencapsulation

During the *in vitro* culture of free and encapsulated hepatocytes, we assessed hepatocyte viability and mortality using FDA-PI staining each day (Figure 2). *In vitro,* free hepatocytes maintained viability up to 10 days in adherent culture conditions *in vitro*; alginate and Alg-PEG-SH encapsulated hepatocytes remained viable up to 7 days. After 3 days of culture, viability and mortality was quantified. Free hepatocytes were 99% viable with minimal mortality at 1% (Figure 3A-B). After microencapsulation in beads of 500-600 µm of diameter, alginate-encapsulated hepatocytes maintained 62% viability and Alg-PEG-SH Alg-PEG-SH-encapsulated hepatocytes maintained 56% viability (Figure 3A-B). These results demonstrate that microencapsulation of porcine hepatocytes with both types of polymer allows survival of up to 50% of the encapsulated pig hepatocytes.

3.3 Albumin secretion is maintained in hepatocytes after encapsulation

To assess hepatocyte functionality after microencapsulation, albumin secretion was measured starting from day 2 to day 8 in free, alginate-encapsulated and Alg-PEG-SH-encapsulated hepatocytes. Twenty-four hour measurements showed that free hepatocytes secrete 10-12 µg/mL/24h albumin until day 6, and at day 7 and 8, albumin secretion was halved (Figure 4A). The total amount of albumin secreted during 8 days was around 46 µg/mL in free cultured hepatocytes (Figure 4A, grey bar). Albumin secretion from free hepatocytes isolated from 9 pig livers was comparable. Alginate-microencapsulated hepatocytes secreted lower amounts of albumin (2-1.5 µg/mL/24h), with a maximal amount measured at day 4 to day 6. Despite the decrease of the amount of albumin measured, secretion still occurred until day 8 (Figure 4B). Alg-PEG-SH-encapsulated hepatocytes secreted albumin to comparable amounts than free cultured hepatocytes until day 8 (Figure 4C), with highest secretion at day 6. These results shows that albumin secretion occurs from microencapsulated hepatocytes and is maintained up to 8 days in free, alginate-encapsulated and Alg-PEG-SH-encapsulated hepatocytes.

3.4 Microencapsulated hepatocytes maintain their capacity to metabolize drugs

To evaluate the metabolic capacities of free and encapsulated hepatocytes, diazepam was added at day 1, 3 and 7 to hepatocytes for 6 hours, and the metabolites nordiazepam and temazepam were measured. As shown in Figure 5, diazepam decreased in both free and alginate-encapsulated hepatocytes (white bars). At day 1, metabolic capacities were maximal; nordiazepam and temazepam were at 49 ng/mL and 93.4 ng/mL respectively, in free hepatocytes and 3.5 ng/mL and 5.6 ng/mL in encapsulated hepatocytes. The amounts of metabolites decreased progressively between day 3 and 7, in both conditions. These results show that hepatic metabolic functions are maintained in free and encapsulated hepatocytes during the first week *in vitro* culture.

3.5 Hepatocytes show increased viability and albumin secretion when co-encapsulated with MSCs

Trophic molecules secreted by MSCs have been described to be beneficial for cell function in acute liver failure [22]. To assess whether MSCs could provide a beneficial effect in microencapsulated hepatocytes, both were co-encapsulated in alginate polymer. To assess viability, FDA-PI staining was performed at day 3. The results show that viability was higher in hepatocytes encapsulated with MSCs, compared to hepatocytes alone. Hepatocyte survival reached 87%, while hepatocytes alone reached 40% (Figure 6A-B). Further, to assess the functionality of hepatocytes co-encapsulated with MSC, albumin secretion was measured each day starting at day 2 until day 8. In the presence of MSCs (grey bars), albumin secretion was significantly improved at day 4, 5 and 8 ($p < 0.05$, Figure 7A), compared to encapsulated hepatocytes alone (white bars). To assess the importance of cellular contact between hepatocytes and MSC, co-culture was performed and albumin secretion was measured in the supernatants. As shown, at day 3 and 4 albumin secretion was significantly higher in the presence of MSCs (grey bars, $p < 0.001$) compared to hepatocytes alone (white bars, Figure 7B). MSCs alone secreted only limited amounts of albumin (black bar). Further, the presence of MSCs allowed the prolonging of albumin secretion up to day 15 (data not shown). All together, these results show that co-encapsulation of hepatocytes with MSCs improve and significantly prolong albumin secretion from porcine hepatocytes.

3.6 Human MSCs and pig hepatocytes distribution in co-culture

Co-cultures of hepatocytes and MSCs were used to analyze the cell distribution after 3 days of culture. MSCs were stained for the cytoskeleton protein, vimentin (in green) and hepatocytes for albumin (in red) (Figure 8A). Hepatocytes alone formed a typical adherent epithelial cell

layer (Figure 8A). In co-culture with MSCs, hepatocytes appeared in small cell-cluster (hepatocyte doublets or triplets), with the presence of vimentin positive cells intermingled throughout the hepatocyte culture (Figure 8B-C).

4. Discussion

Acute liver failure has high mortality rate. Liver transplantation, which has to occur in the few days after liver destruction, remains the only treatment with lifelong immunosuppression thereafter [1]. Additionally, the disposability of an adequate human liver is not guaranteed, demonstrating the need for new therapeutic options. Porcine hepatocyte cell transplantation might present a solution to overcome the acute liver failure by replacing the metabolic function of the liver until its recovery. Earlier studies in mice showed that transplantation of encapsulated human or pig hepatocytes with acetaminophen- and hepatectomy-induced liver failure increased the survival rates of mice [5, 6], and the first protocols for porcine hepatocyte isolation and encapsulation with poly-L-lysine alginate capsules had been developed [5, 6].

Here, we developed an optimized high-yield porcine hepatocyte isolation protocol from 10 kg pigs. Hepatocytes were encapsulated in a recently developed polymer, which showed an improved biocompatibility compared to previous polymers. Currently, new biocompatible polymers which allow the production of long-term stable microcapsules for hepatocyte encapsulation, are being investigated. Long-term stability remains an important issue to prevent immunoreaction due to microcapsule disaggregation. Several types of polymers are under investigation. Durkut and collaborators showed that rat hepatocytes maintained comparable viability in free and encapsulated conditions in alginate-chitosan-alginate microcapsules [23]. However, this study did not focus on the stability of microbeads. Furthermore, another study reports that hepatocytes isolated from rats and encapsulated in PEG did not survived after encapsulation [11]. Therefore, improvements are needed to maintain the viability of primary hepatocytes which do not resist harsh conditions. Our data shows that using alginate and the new Alg-PEG-SH polymer for encapsulation allows the

maintenance of a 56% viability of encapsulated hepatocytes, compared to hepatocytes alone. The diminished hepatocyte viability was reflected in decreased albumin secretion and diazepam metabolization, but function was partially maintained after encapsulation when compared to free cultured hepatocytes. In fact, looking closely at the results, the initial amount of diazepam decreased in free and encapsulated conditions; however, the quantity of metabolites did not significantly increase in the supernatants of encapsulated hepatocytes. An issue to be considered is that encapsulation could decelerate molecule diffusion: albumin, diazepam and its metabolites could spend more time passing through the capsule; however cell viability and molecule dispersion are maintained reliably.

Studies have been conducted to improve hepatocyte viability and functionality upon encapsulation. The supplementation of adjuvants like heparin or collagen during encapsulation improved albumin and urea synthesis and increased hepatocyte viability [8, 9]. Further co-encapsulation with other cell types, such as endothelial progenitor cells, also improved albumin and urea secretion of rat hepatocytes [10]. MSCs have anti-fibrotic properties [19] and have been successfully used to treat liver failure [24]. We explored the effect of human MSCs on porcine hepatocyte survival and function. We demonstrated that MSCs significantly improve hepatocyte viability and albumin secretion in co-culture and co-encapsulated conditions. However, others reported that 3D cultures of human MSCs and human hepatocytes did not affect albumin secretion, but rather compacted hepatocyte morphology and provided phenotypic stability [25]. Also, human MSCs potentiated hepatotrophic and anti-apoptotic genes in human primary hepatocytes [26], with the accumulation of hepatocytes in the G2/S phase of the cell cycle, meaning that they are prone to proliferation [17]. In line with these results, we show here that hepatocytes in co-culture with MSCs tend to survive more compared to hepatocyte alone.

The mechanism using which MSCs exert their beneficial effects needs further investigation, and might not be solely due to paracrine effects. Interestingly, the systemic injection of extracellular vesicles, derived from bone marrow MSCs after *in vitro* culture, reduced hepatic injury and improved mice survival [22], suggesting that such vesicles contained molecules acting either directly on liver cells or modulating the immune system. Moreover, the evaluation of the secretome of human MSCs evidenced a correlation between vascular endothelial growth factor and cell proliferation, development processes and immune system processes. Also, the systemic injection of conditioned medium of MSCs in liver-injured mice improved survival [27]. In our experimental conditions, we found increased albumin secretion when hepatocyte and MSCs were cultured together; this might not exclusively be due to cellular interactions, but could also include the effect of secreted molecules. In particular, in encapsulated conditions, hepatocytes and MSCs are distributed throughout the microcapsules (figure 6A); both improved microenvironment and paracrine signaling through secreted molecules might contribute to the increased viability and functionality of hepatocytes. Further studies using MSC-conditioned mediums are needed to identify factors implicated in this beneficial effect.

5. Conclusion

In conclusion, we performed high-yield porcine hepatocyte isolations that allow us to obtain high quantities of viable hepatocytes. Furthermore, we used a newly developed polymer which allowed the maintenance of hepatocyte viability, albumin secretory and drug metabolic functions for up to 8 days. Hepatocyte co-encapsulation with MSCs also increased further and prolonged hepatocyte viability, suggesting that cell-to-cell contact and paracrine effects are beneficial for hepatocyte function and survival. This standardized and optimized protocol of porcine hepatocyte isolation and encapsulation can now be used for further experimental

research in liver diseases to evaluate the clinical potential in treating acute liver failure in humans.

Competing Interests

The authors declare that there is no conflict of interest regarding the publication of this article.

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Figures

Total hepatocytes	$9.2 \times 10^9 \pm 3.6$
Cells par gram	$27.9 \times 10^6 \pm 9.9$
Viability %	95.2 ± 3.1

Table 1. Hepatocyte isolation yield

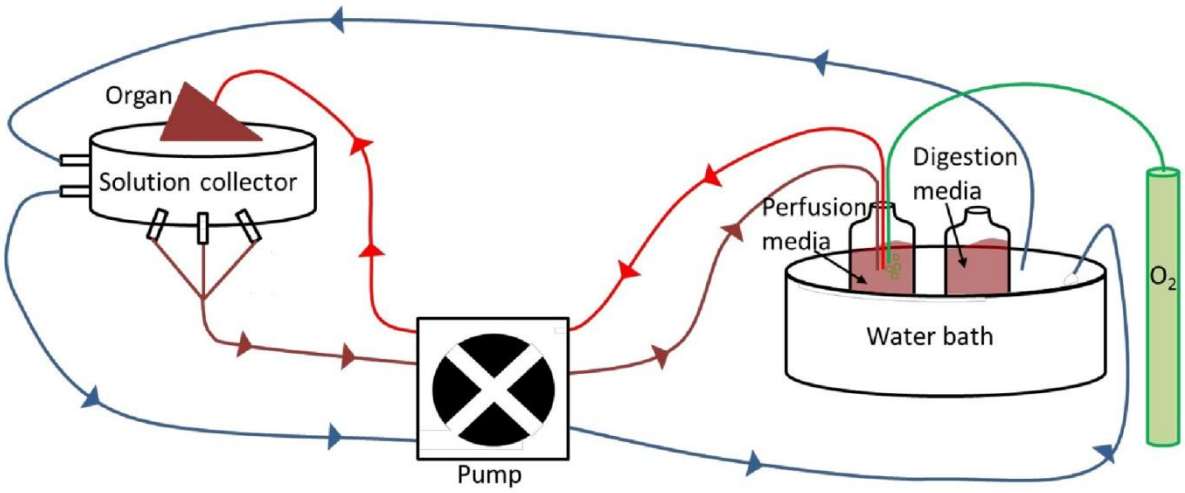


FIGURE 1. Liver digestion perfusion method. Liver temperature is maintained at 37°C by blue tubes derivate from the water bath. Perfusion media is maintained at 37°C and oxygenated through the green tube; thereafter it is infused into the liver through vena cava by the red tube and collected by the brown tube. Then, digestion media is infused and hepatocytes are collected after mechanical destruction.

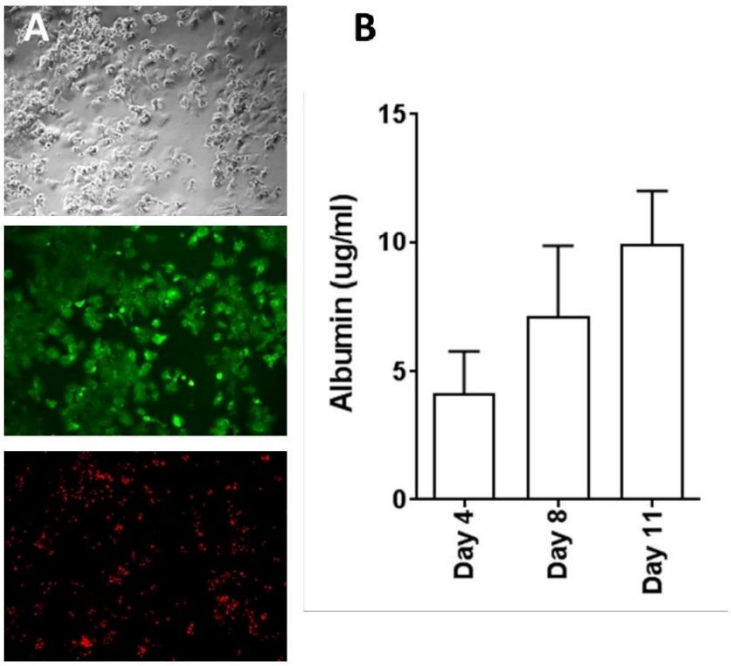


FIGURE 2. Hepatocyte viability and functionality after thawing. A. First line are brightfield pictures, second line are viable cells stained with FDA, third line are dead cells stained with PI. **B.** Albumin was measured by ELISA in the supernatant of cultured hepatocytes at day 4, 8 and 11. Experiment is performed in duplicate in 1 pig.

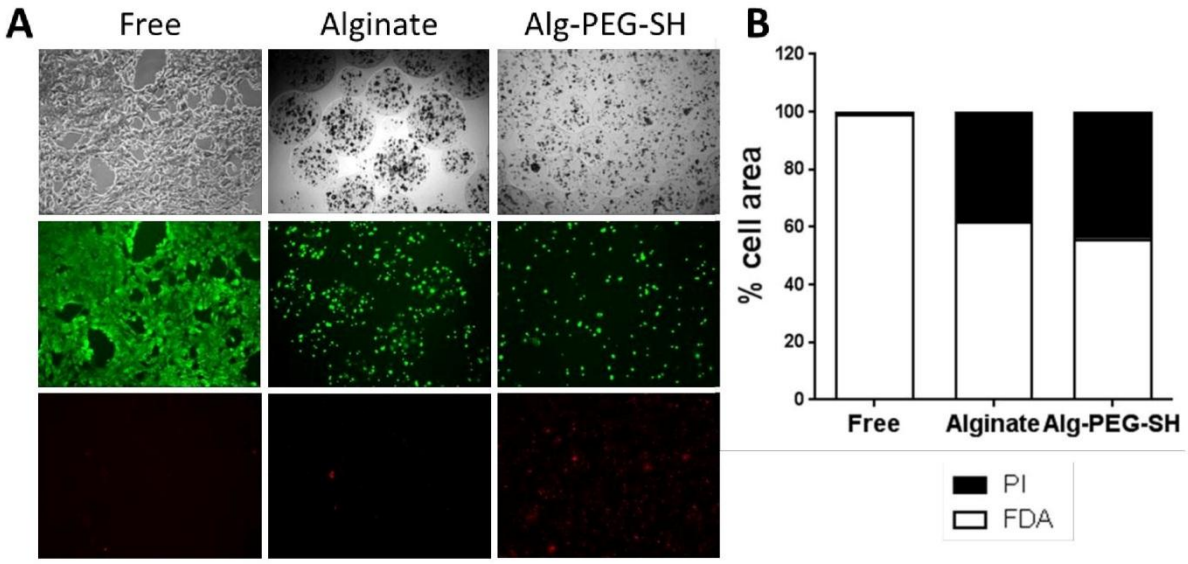


FIGURE 3: Viability of free and microencapsulated hepatocytes. A. Representative images at day 3 for free, alg-encapsulated and Alg-PEG-SH-encapsulated hepatocytes. First line are brightfield pictures, second line are viable cells stained with FDA, third line are dead cells stained with PI. **B.** Quantification at day 3, values are the % of the total cell area (n=6). Quantification has been performed using Image J.

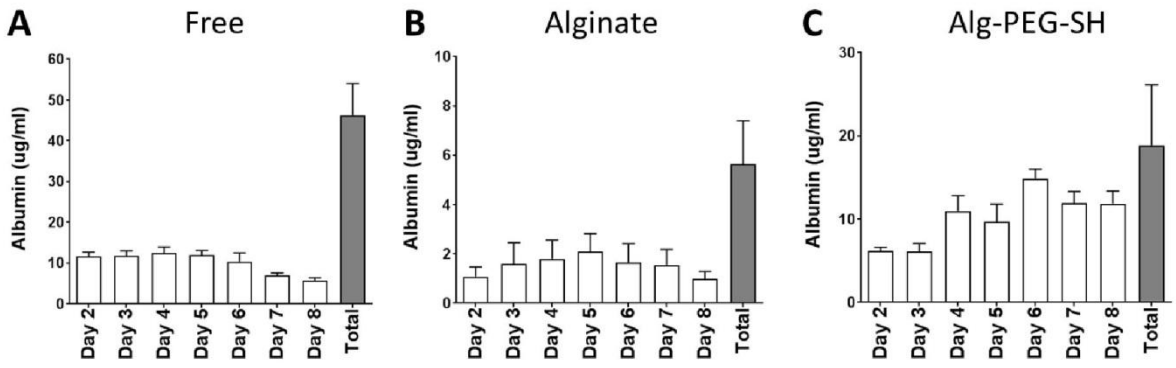


FIGURE 4: Albumin secretion from free and microencapsulated hepatocytes. Albumin was measured by ELISA in the supernatant of free (n=9) **(A)**, alginate-encapsulated (n=3) **(B)** and Alg-PEG-SH-encapsulated (n=3) **(C)** hepatocytes. White bars represent 24h albumin secretion from day 2 to day 8, grey bars represent the total albumin secreted during 8 days.

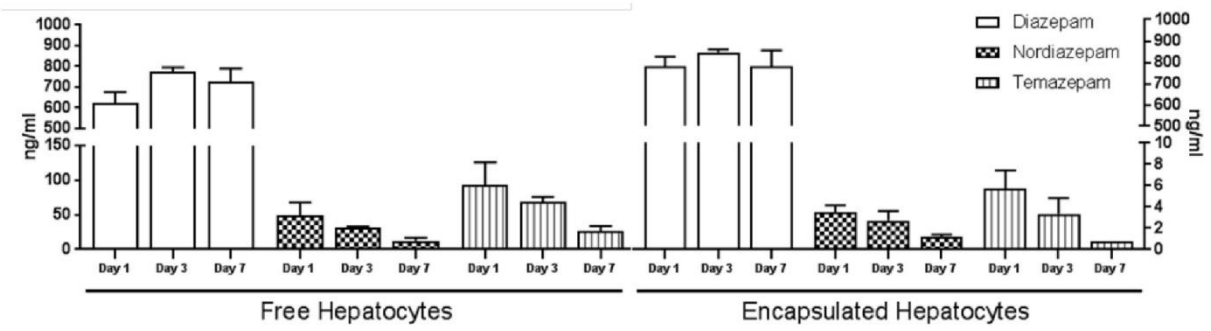


FIGURE 5: Diazepam metabolism in free and microencapsulated hepatocytes. Diazepam was added at day 1, 3 and 7 and supernatant was retrieved after 6 hours. Diazepam and their metabolites Nordiazepam and Temazepam were measured by LC-MS/MS in 4 independent experiments.

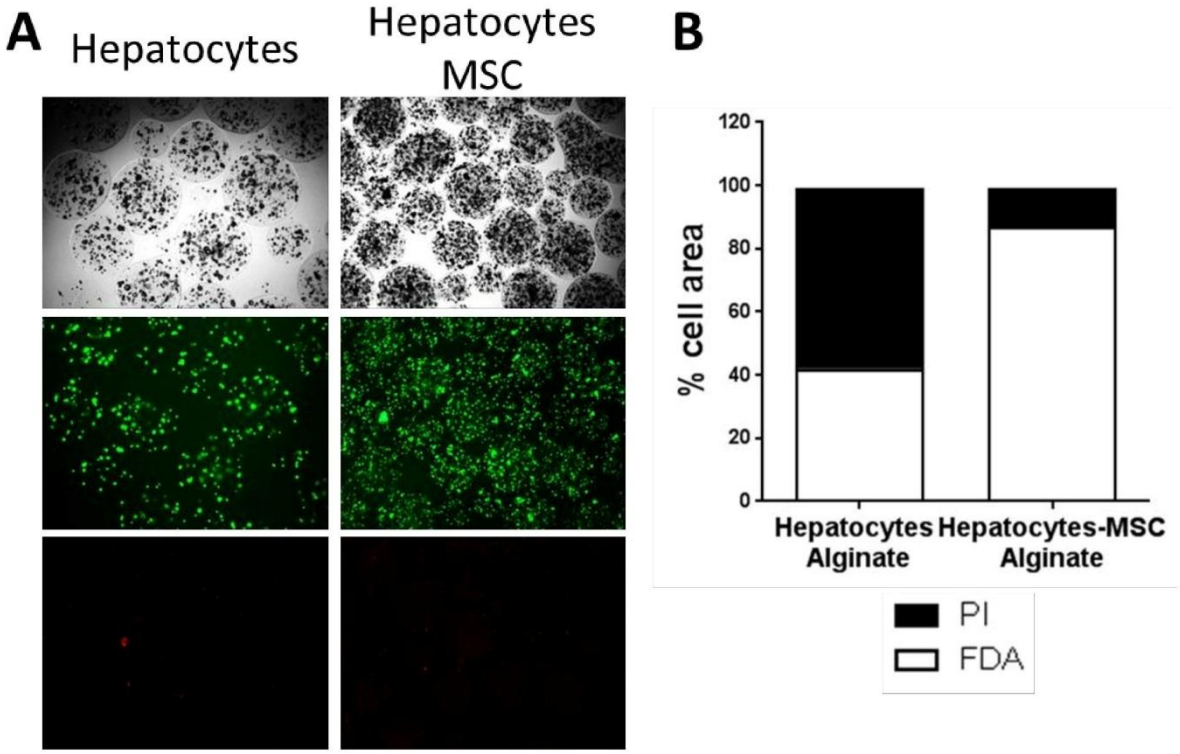


FIGURE 6: Viability of co-encapsulated hepatocytes and MSC. A. Representative images at day 3 for alg-encapsulated hepatocytes alone or with MSC. First line are bright field pictures, second line are viable cells stained with FDA, third line are dead cells stained with PI. **B.** Quantification at day 3, values are expressed as % of the total cell area (n=2). Quantification has been performed using Image J.

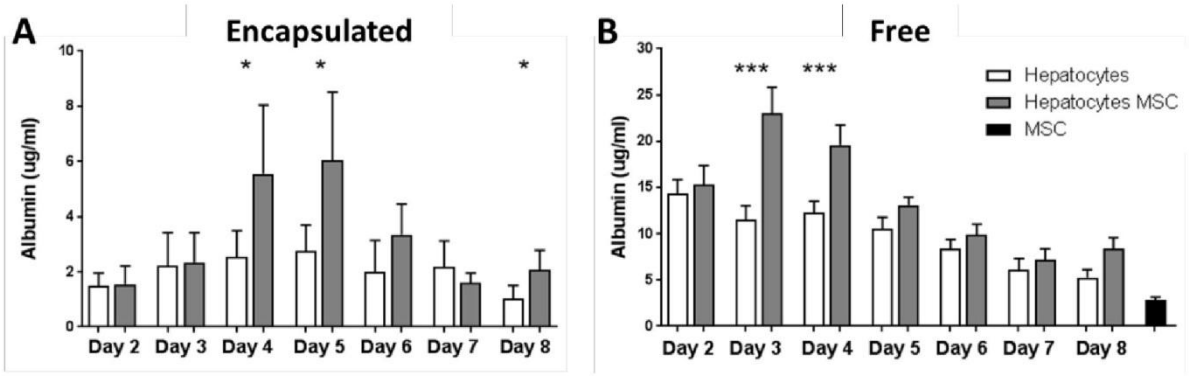


FIGURE 7: Albumin secretion of hepatocytes co-cultured and co-encapsulated with MSC. Albumin secretion was measured by ELISA in the supernatant of cell culture from day 2 to day 8. **A.** Alginate-encapsulated hepatocyte alone (white bars) or with MSC (grey bars), measured in 2 independent experiments. **B.** Free hepatocytes alone (white bars) or with MSC (grey bars), measured in 5 independent experiments.

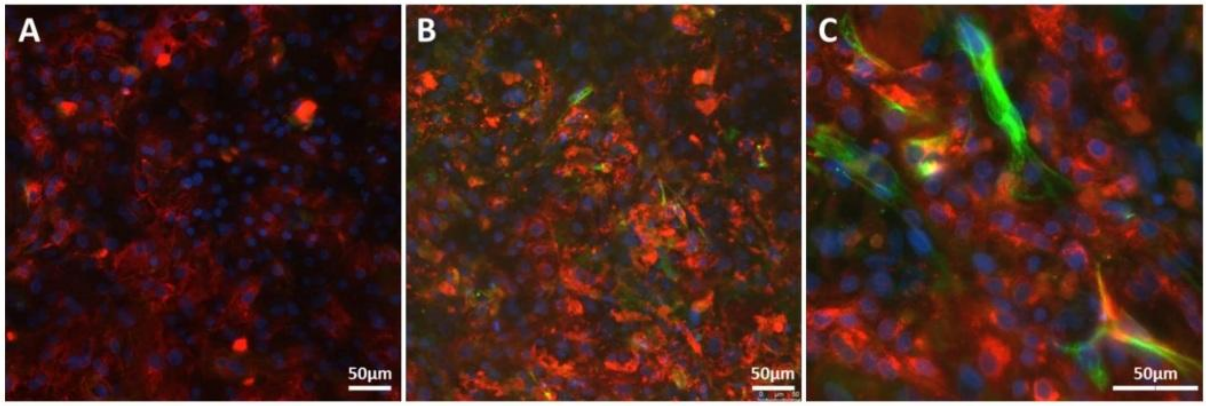
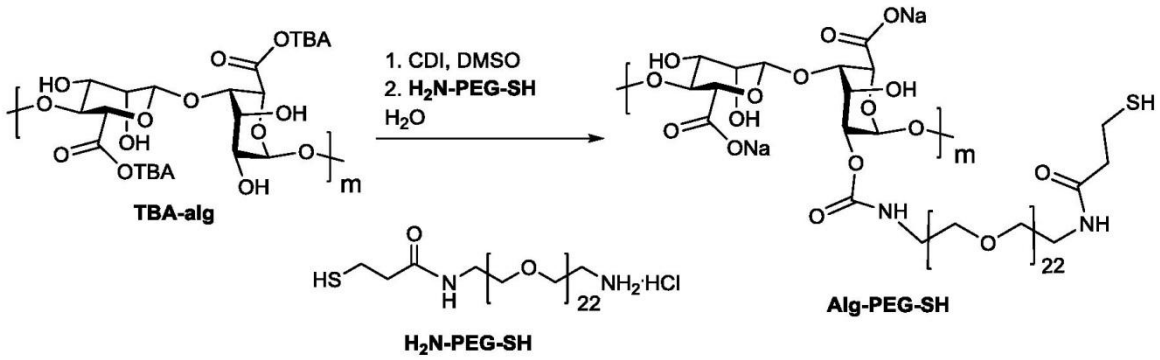


FIGURE 8: Distribution of pig hepatocytes and human MSC in co-culture. Cells were cultured for 2 days and then serum starved to avoid non-specific staining. Immunofluorescence was performed after 3 days on fixed cells. **A.** Porcine albumin was stained in red, to evidence hepatocytes. **B.** Co-culture of hepatocytes and MSC. Human vimentin in MSC is stained in green. **C.** Co-culture of hepatocytes and MSC in higher magnification. Images are representative of 2 independent pig hepatocyte isolations.



Supplementary figure 1. Polymer synthesis

Supplementary table 1

Analytes name	Q1 (*m/z*)	Q3 (*m/z*)	Collision energy (CE)
diazepam	285.0	241.0	55
nodiazepam	271.0	140.0	55
oxazepam	287.0	241.0	55
Temazepam	301.0	255.0	55
oxazepam-glucuronide	463.2	287.1	18
temazepam-glucuronide	477.2	283.3	30
Lidocaine	235.0	86.0	30
GX	179.0	122.0	30
MEGX	207.0	58.0	30
EMGX	221.0	72.0	30

6. DISCUSSION AND PERSPECTIVES

MSCs are multipotent cells that exhibit regenerative and immunomodulatory properties; MSCs have the capacity to differentiate into chondrocytes, osteocytes and adipocytes. These capacities are currently exploited for cell therapies; in clinics, MSCs have been used for cell regeneration and for the modulation of immune reactions, like GVHD (129, 131 , 132-134). Further, MSCs are investigated for the use as supportive cells, which may allow the reduction of cell mass for cell therapies in limited accessible tissues (like islets and hepatocytes). The supportive effects i.e. enhancement of cell survival, can occur through paracrine action or by direct contact (4). Here, we describe strategies to enhance islet and hepatocyte function through the use of MSCs in co-encapsulation, which represent promising approaches for future cell treatments of T1D and acute liver failure. As described in the literature, other strategies are under investigation for patients with T1D and T2D, including infusion of MSCs which lead to ameliorations in blood glucose levels and in organs affected secondarily by diabetes, such as renal failure (17, 139, 144, 145). Furthermore, MSCs have been infused to treat liver damage after viral or parasite infection or in acute liver failure (31, 149, 150, 153). However, an efficient clinical application for the treatment of T1D or acute liver failure that takes advantage of the potential of MSCs has not yet been successfully established.

The aim of this study was to analyze the potential of MSCs co-cultured with human islets of Langerhans and with porcine hepatocytes to improve their viability and function. We were interested in investigating the cellular mechanisms involved in the beneficial effects observed in view also of pre-clinical studies. Our experimental approach for the treatment of T1D was the transplantation of human islets of Langerhans and human MSCs encapsulated into streptozotocin-induced diabetic mice, which allowed us to observe a prolonged function when islets were co-encapsulated together with MSCs. We further elaborated an optimized and

standardized protocol for the isolation of high amounts of viable porcine hepatocytes, whose functions were improved after encapsulation with MSCs.

6.1 MSCs and islets of Langerhans

In this work, we co-cultured human MSCs in contact with human islets of Langerhans. In the presence of MSCs, insulin secretion by islets increased. In line with our results, others have shown that contact between MSCs and rat islets increased insulin secretion after glucose stimulation (174, 175). The effect of MSCs on insulin secretion by islets was not observed when islets and MSCs were co-cultured without direct cell-to-cell contact; however, MSCs' release of soluble factors that sustain insulin secretion might also be necessary. In our experimental setting, it remains to be investigated whether higher concentrations of soluble factors, which are probably achieved locally when MSCs are cultured in close vicinity (i.e. direct contact) to islets, are absent when islets are separated from MSCs by a transwell system. Effectively, it has been suggested that the beneficial effects of MSCs on islets, implicate the release of trophic factors and the down regulation of inflammatory cytokine production, and also expression of PDX-1, a transcription factor essential for pancreatic development and β cell maturation, thereby positively affecting β cell mass (176).

Since MSCs elicited boosting effects on insulin secretion by islets cultured in cell-to-cell contact, we used pseudoislets (islet cell aggregates) to increase the possible cell-to-cell contact with islets cells. MSCs were ineffective on insulin secretion by pseudoislets after high glucose stimulation, but still efficient after maximal stimulation with theophylline. Theophylline is a weak non-selective inhibitor of phosphodiesterases, which breaks down cell cyclic nucleotides, leading to an increase in intracellular cyclic 3'5' adenosine monophosphate (AMP) and cyclic 3',5' guanosine monophosphate (254). It has to be explored whether

theophylline acts also on MSCs. Moreover, immunostaining *in vitro* on such pseudoislets revealed that MSCs preferentially interacted together and formed cell structures with insulin and glucagon secreting cells perched around, suggesting that MSCs serve as stromal cells. Confirming our result, others have shown that the presence of MSCs improved islet cell adhesion and sprout formation *in vitro* (173).

It is known that N-cadherin interactions are important for insulin granule turnover and also for insulin secretion. Insulin secretion was decreased in a model of pancreatic epithelium specific knock-out of N-cadherin (255). We found that N-cadherin was involved in the MSC-induced increased insulin secretion. Thus, in our model, the effect on insulin secretion involves a molecular mechanism implicating N-cadherin interactions.

Further, we detected the expression of ICAM-1 in islets and MSCs. The adhesion molecule ICAM-1, known to be implicated in T-cell recognition in mice (256) and humans (257), is mainly expressed in leukocytes and endothelial cells, but also in human islets and MSCs. The ligand of ICAM-1, integrin α-2 (CD18), is expressed in MSCs and is involved in the adhesion to endothelial cells (258). Also, in the setting of islet transplantation, the blockade of ICAM-1 reduced inflammation and improved islet function (256). However, blocking ICAM-1 interaction with its counterpart did not affect MSC-induced insulin secretion. Therefore, in contrast to the role described in host cell and islet cell interaction, ICAM-1 does not appear to be involved in the enhanced insulin secretion induced by MSCs.

Several soluble factors released by MSCs were noted to play a role in the enhanced insulin secretion by MSCs. Recent work has shown that Annexin A1, a calcium and phospholipid binding protein expressed and released by mouse MSCs, enhanced insulin secretion by mouse islets *in vitro*. However, culturing islets with Annexin A1 before transplantation did not improve the ability of islets to regulate blood glucose levels in diabetic mice, suggesting that

other factors secreted by MSCs are required, in addition, for the beneficial effect on islets *in vivo* (206).

Furthermore, we demonstrated that MSCs exert beneficial effects on human islets also in a model of co-encapsulation and transplantation in diabetic mice. MSCs co-encapsulated with islets allowed to achieve and maintain normoglycemia in diabetic non immunosuppressed mice for up to 90 days, compared with encapsulated islets, which were rejected after 39 days. Moreover, an intraperitoneal glucose tolerance test in mice transplanted with co-encapsulated MSCs and islets presented decreased levels of glycemia. Whether this is related to an increased insulin secretion needs to be clarified. Our data are in line with other reports describing the beneficial effects of MSCs in various species, such as mouse (192, 194, 198, 234), rat (185, 191) and non-human primate (193). Graft survival and normoglycemia in diabetic rats was sustained upon transplantation of rat islets at marginal doses, in the case that islets were co-cultured with rat MSCs before transplantation, both in syngeneic and allogeneic combinations (259). In allogeneic islet transplantation in non-human primates, the systemic infusion of donor or third-party MSCs resulted in reversal of rejection (193) whereas others showed that MSCs failed to prevent islet rejection when injected systemically (182). Mouse MSCs as well as MSC-conditioned media, upon systemic injection, improved normoglycemia in STZ-induced diabetic mice in syngeneic model, which was mainly ascribed to enhance β cell regeneration in islets (204). Others report that MSCs acted concomitantly via cell-to-cell contact and via trophic factors regulating islet regeneration (204), facilitating islet survival (180) and modulating the immune system (259).

The beneficial effect of MSCs is not only due to an effect on islets and their secretory function, as it has been shown that MSCs protect islets from proinflammatory cytokines. This seems to be specific to MSCs as it cannot be achieved by other stromal cells, such as dermal fibroblasts (177). Furthermore, MSCs, in a pig-to-non-human primate transplantation model,

improved neoangiogenesis, as demonstrated by increased presence of VEGF expressing cells around the graft (260). Also, effects on endothelial cell migration and vascularization on islets has been shown (192, 198). However, in the present study, analysis of pseudoislets composed of islet cells and MSCs transplanted under the kidney capsule in SCID mice did not show an increased number of human CD31 positive endothelial cells. We, therefore, concluded that under such conditions, the vascularization process was not increased (unpublished data). This might be related to the different experimental approach, such as the site of transplantation (muscle versus renal capsule) and the xenogeneic condition of human islets and human MSCs in a mouse recipient.

6.2 MSCs and porcine hepatocytes

In this work we standardized an efficient protocol for porcine hepatocyte isolation, and optimized co-encapsulation together with MSCs for future transplantations. We used a new polymer developed by our collaborators at the Ecole Polytechnique Fédérale de Lausanne – EPFL (224). This new biocompatible polymer is mechanically highly resistant compared to alginate, and is therefore more suitable for future clinical applications. Encapsulation of primary hepatocytes is challenging since hepatocyte viability is severely reduced through the encapsulation procedure (245). Indeed, the survival of hepatocytes depends on the rapidity of the procedure, chemical composition, oxygen and nutrient supply (221). To find optimal conditions, we investigated the effect of MSCs on porcine hepatocyte function and survival. We showed that encapsulation with the new polymer allowed the preservation viability of 50% of hepatocytes, and that MSCs improved further hepatocyte viability when compared to hepatocytes encapsulated alone. We further demonstrated that the presence of MSCs increased albumin secretion from porcine hepatocytes in co-culture conditions, confirming a

previous study that showed increased albumin and urea secretion by cryopreserved human hepatocytes co-cultured with MSCs (216). Further, rat hepatocytes encapsulated in alginate-poly-L-lysine microcapsules secreted increased levels of albumin in the presence of MSCs (16, 210).

Several other studies reported that MSCs exert beneficial effects on hepatocytes, as well as in liver fibrosis, through paracrine secretion or MSC-derived vesicles. Indeed, the injection of MSC-conditioned medium or MSC-derived vesicles in animals with liver injury, improved mice survival, strongly suggesting that MSCs exert beneficial effects through secreted molecules (41, 161). In our encapsulation settings, paracrine signaling might contribute to the beneficial effects on hepatocytes. In fact, co-encapsulated hepatocytes and MSCs are closely entrapped into the polymer; therefore, it is possible that both cell-to-cell contact and paracrine signaling contribute to the increased albumin secretion detected by hepatocytes encapsulated with MSCs.

Studies searching for improved culture conditions for isolated hepatocytes showed that proliferation and function of hepatocytes are increased when cells are cultured on scaffolds such as polymers, conditioned by collagen, heparin or other stromal cells, like endothelial progenitor cells or MSCs (242, 247, 261). The use of MSCs in a 3D culture model improved hepatocyte stability, as revealed by the analysis of cell morphology and phenotype, through scanning electron microscopy (209). In accordance to this, the co-culture of porcine hepatocytes and MSCs showed a homogenous distribution of MSCs around small hepatocyte clusters. We concluded that that MSCs were able to interact with hepatocytes since they did not clearly segregate in cultures. Therefore, this observation suggests that MSCs serve as stromal cells sustaining hepatocyte function and proliferation through the improvement of their microenvironment *in vitro*.

Earlier studies showed that MSCs differentiate into hepatocyte-like cells, expressing typical hepatocyte features, such as cytokeratins and albumin, only when cultured in a specific medium containing fibroblast growth factor-4 and HGF (80). In our experimental setting, MSCs were cultured for 8 days in a hepatocyte medium and adapted for primary porcine hepatocytes, without supplementation of growth factors. Therefore, we exclude trans-differentiation of MSCs into hepatocyte-like cells and attribute the effect of MSCs to a bystander action rather than trans-differentiation of MSCs.

In conclusion, encapsulation of porcine hepatocytes together with MSCs could represent a possible approach to protect primary hepatocytes in view of a clinical application (245). Further, large animal models with acute liver failure are needed to investigate whether transplantation of microcapsules with functional hepatocytes represent a potential new treatment for patients suffering from acute liver failure.

6.3 Perspectives

We demonstrated that MSCs improved insulin secretion by islets through cell-to-cell contact, implicating the adhesion molecule N-cadherin. However, other adhesion molecules could be involved in cell-to-cell interactions that lead to improved insulin secretion and they need to be investigated.

To analyze further adhesion molecules involved in the contact between MSCs with islets, we would like to screen for the expression of integrins (258, 262), cadherins (200, 255) and CAMs (263).The potential adhesion molecules should be expressed in both MSCs and islets. The involvement of a potential protein will be analyzed using siRNA (264), to specifically

silence its expression and evaluate the consequences of its absence. Further, the effect of increased expression of such an adhesion molecule could be investigated by viral transfection.

To explore the beneficial effects of MSC interaction on islets on their survival *in vivo,* transplantation in diabetic mice could be addressed by different settings and relative hypothesis:

A. MSCs, co-transplanted with islets, will improve graft survival compared with mice transplanted without MSCs.
B. MSCs, co-transplanted with islets that are silenced for an adhesion molecule through siRNA, will manifest a decreased graft survival.
C. MSCs, co-transplanted with islets that overexpress an adhesion molecule by viral transfection, will manifest improved MSC interactions, resulting in a prolonged islet graft survival.

These studies will help to elucidate the functional importance of the cellular microenvironment and its fine-tuning through cellular interactions implicating adhesion molecules.

In this work, we showed that MSCs exerted beneficial effect when co-cultured with islets or hepatocytes. To use MSCs in future clinical trials, we would like to develop standardized tests for the characterization of MSCs. Human MSCs from each donor need to be expanded and characterized. Therefore, we need to develop genetic assays that evaluate gene expression of surface molecules and extra cellular matrix proteins to allow the selection of suitable MSCs. Moreover, such characterized MSCs need to be freeze-stocked and maintained ready-to-use for the impending patient to be transplanted. A standard operating procedure (SOP) needs to be prepared, with controlled material for encapsulation and transplantation.

6.4 Conclusions

This work evidenced the beneficial effects of MSCs on isolated human islets of Langerhans and isolated porcine hepatocytes.

We show that MSCs co-cultured with human islets improved insulin secretion, implicating the adhesion molecule N-cadherin. Further, transplantation of human MSCs and islets in immunocompetent diabetic mice, using cell encapsulation, increased islet graft survival in the presence of MSCs, suggesting that paracrine signaling also plays a role.

MSCs also improved hepatocyte survival and function; albumin secretion improved when MSCs were co-cultured or co-encapsulated with hepatocytes. We also optimized a protocol for hepatocyte isolation and encapsulation with high yield and viability.

In conclusion, these results suggest that MSCs represent a valuable cell, useful to improve islet and hepatocyte function for future applications in the treatment of T1D and acute liver failure respectively. A better understanding of the molecular events leading to the beneficial action of MSCs will be necessary to control and apply MSCs in future clinical applications.

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8. APPENDIX

8.1 "Microencapsulation of hepatocytes and mesenchymal stem cells for therapeutic applications"

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Chapter 18

Microencapsulation of Hepatocytes and Mesenchymal Stem Cells for Therapeutic Applications

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Abstract

Encapsulated hepatocyte transplantation and encapsulated mesenchymal stem cell transplantation are newly developed potential treatments for acute and chronic liver diseases, respectively. Cells are microencapsulated in biocompatible semipermeable alginate-based hydrogels. Microspheres protect cells against antibodies and immune cells, while allowing nutrients, small/medium size proteins and drugs to diffuse inside and outside the polymer matrix. Microencapsulated cells are assessed in vitro and designed for experimental transplantation and for future clinical applications.

Here, we describe the protocol for microencapsulation of hepatocytes and mesenchymal stem cells within hybrid poly(ethylene glycol)-alginate hydrogels.

Key words Encapsulation, Capsule, Microspheres, Microbeads, Alginate, Hydrogels, Hepatocyte, Mesenchymal stem cells, Liver failure, Hepatitis, Fibrosis, Cirrhosis

1 Introduction

The treatment of end-stage acute and chronic liver failure comprises optimal clinical care and in most of the cases liver transplantation. Currently, its application is severely limited by organ shortage. Hepatocyte and mesenchymal stem cell (MSC) transplantation are promising strategies to support liver function in acute and chronic liver failure, respectively, with the aim to bridge the patient to transplantation. Microencapsulated xenogeneic hepatocytes (e.g., porcine cells) and allo-/autologous MSCs represent an unlimited source that could function as a bioreactor requiring minimal or no immunosuppression [1]. Microspheres (also called (micro-)capsules or microbeads) are composed of hydrogels with adjustable permeability between 70 and 140 kDa. Hence, microspheres protect cells against larger compounds such as circulating antibodies (~150 kDa) and immune and inflammatory cells,

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while allowing nutrients such as O_2 (32 Da) and glucose (180 Da) to diffuse inside the cells and desired low-molecular weight products and cellular waste to diffuse out. Therefore, immunosuppression can be reduced or eliminated. Hydrogels are commonly composed of biocompatible macromolecules that are linked together by ionotropic interactions and/or covalent crosslinking. Alginate-divalent cations hydrogels and alginate-poly(L-lysine)-alginate are the most frequently used polymer combinations for cell microencapsulation. Unlike poly(L-lysine)-based hydrogels, alginate-divalent cations hydrogels created upon ionotropic gelation have an excellent biological acceptance and high permeability [2–5]. Nevertheless, limitations include a relatively low physical resistance and stability [6]. In this context we developed an original combination of sodium alginate with poly(ethylene glycol) (PEG) that confers mechanical resistance and stability [6–8]. Hence, the microspheres are formed in a one-step process, using a sterile cell encapsulator, by an interpenetrating network of ionotropic interactions of calcium alginate molecules and covalent crosslinking from vinyl sulfone-terminated multi-arm PEG molecules. The physical properties such as permeability and swelling are adjustable, offering a balance between biocompatibility, mechanical resistance, and stability [7]. The method described below is based on previously published results that allowed our team to transplant microencapsulated hepatocytes or MSCs to treat acute [9, 10] and chronic [11, 12] liver failure, respectively, in small animal experimental and xenogeneic settings.

In this method chapter we describe the step-by-step process to generate biocompatible and resistant microspheres for human hepatocyte and human MSC microencapsulation. We further outline the in vitro culture, viability and functionality testing, transplantation procedure, and the assessment of microencapsulated cells in vivo.

2 Materials

Prepare all solutions using ultrapure water. All chemicals are of analytical grade and are used as supplied, unless otherwise indicated. Store all reagents at 4 °C and prepare solutions at room temperature unless indicated otherwise. All solutions and material that are in contact with cells before and during cell culture are prepared under sterile conditions.

2.1 Gelation Bath and Cell Encapsulation

1. Sodium-alginate (Na-alg) (PRONOVA UP LVM) is obtained from FMC BioPolymer, Novamatrix, Norway.
2. 8-arm PEG (PEG-8-20, i.e., 20 kg/mol, Shearwater Polymers, Huntsville, AL, USA). This PEG consists of a poly(glycerol)

backbone with multiple PEG arms attached through an ether bond (PEG-OH) designed at EPFL [7].

3. Mix 8-arm PEG (e.g., 0.5 g, molar mass 20 kg/mol) with Na-Alg (e.g., 10 mL) to obtain a final concentration of 5 wt%. Mix overnight. Filtrate with a 0.22 μm sterile disposable filter.
4. Prepare the gelation bath according to the total polymer volume that was previously determined. For 1 mL of 8-arm PEG/alginate, prepare 10 mL of gelation bath. To do so, mix 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS) in distilled water: 4.2 g for 2 L. Adjust the pH with 10% NaOH to pH 7.4. Add 100 mM calcium chloride dihydrate ($CaCl_2 \times 2H_2O$): 29.4 g for 2 L to the MOPS solution. Finally, just before use: add 2.31 mg of dithiothreitol (DTT) for 10 mL of gelation bath. Filtrate with a 0.22 μm sterile disposable filter.
5. Co-axial air-flow droplet generator (e.g., Sterile Cell Encapsulator (B chi, Flawil, Switzerland)). Reactor, stirrer and receiving vessel are washed and sterilized.
6. 0.025% trypsin–EDTA.
7. Cell medium supplemented with 10% fetal calf serum (FCS) (v/v).
8. Phosphate-buffered saline (PBS).
9. 10 mL syringe.
10. Needle 1.2 × 40 mm 18G.
11. 70 μm cell strainer.
12. 50 mL capped conical tubes.
13. 0.9% sodium chloride (NaCl).
14. 10 cm petri dish.

2.2 Hepatocyte Culture Medium

1. F12 medium.
2. 10% (v/v) FCS.
3. 100 IU/mL penicillin.
4. 100 μg/mL streptomycin.
5. 0.1 μM insulin (Huminsulin, Lilly France S.A.S).
6. 1 μM dexamethasone.
7. 25 ng/mL EGF.
8. 5 μg/mL apo-transferrin.
9. 0.01 μM L-TT.

2.3 MSC Culture Medium

1. Iscove's modified Dulbecco's Medium (IMDM).
2. 10% (v/v) FCS.
3. 100 IU/mL penicillin.

4. 100 μg/mL streptomycin.
5. 10 ng/mL platelet derived growth factor BB (PDGF-BB).

2.4 Assessment of Cell Viability In Vitro

1. Conical tube.
2. 3 mL petri dish.
3. Living cell staining: 2.5 mg fluorescein diacetate (FDA) in 250 mL acetone.
4. Dead cell staining: 10 mg propidium iodide (PI) in 158 mL PBS.
5. FDA/PI solution: 280 μL FDA and 420 μL PI in 9.3 mL PBS in a 15 mL tube. The tube should be protected from light using an aluminum sheet.
6. 1× PBS.
7. Fluorescence optical microscope.
8. P1000 pipette.

2.5 Assessment of Cell Proliferation In Vitro

1. 3 mL petri dish.
2. 5-ethynyl-2′-deoxyuridine (EdU).
3. 4% paraformaldehyde (PAF).
4. 0.5% Triton X-100.
5. Click-iT EdU Cell Proliferation Assays (Thermo Fischer Scientific Invitrogen).
6. Hoechst stain: 1 μL of stock solution in 999 μL PBS.
7. 1× PBS.
8. Fluorescence optical microscope.

2.6 Assessment of Hepatocyte Functionality In Vitro

1. 3 mL petri dish.
2. 0.56 mM ammonium sulfate.
3. 1 mg/mL lidocaine.
4. 1 μg/mL diazepam.
5. Cobas INTEGRA 400 (Roche Diagnostic, Basel, Switzerland) measurement of albumin, urea, lidocaine.
6. D×C 800 system (Beckman Coulter Inc, Brea, California, USA) measurement of diazepam.

2.7 MSC Differentiation

1. 3 mL petri dish.
2. Adipogenic differentiation media: IMDM base with 10% rabbit serum, 0.5 mM 3-isobutyl-1-methylxanthin (IBMX), 1 mM hydrocortisone, 0.1 mM indomethacin, and 100 IU/mL penicillin/ 100 μg/mL streptomycin. Osteogenic differentiation media: IMDM base supplemented with 0.1 mM dexamethasone, 10 mM β-glycerolphosphate, ascorbic acid 200 mM, and 100 IU/mL penicillin/100 μg/mL streptomycin.

3. Chondrogenic differentiation media: DMEM base (25 mmol/L of glucose) supplemented with 0.1 mM dexamethasone, 50 mg/mL ascorbic acid, insulin-transferrin-selenium-premix (10 mg/L insulin, 5.5 mg/L transferrin, 5 μg/L selenium), 40 mg/mL L-proline, 10 ng/mL TGF-β3 (PeproTech EC Ltd, London, UK), 100 IU/mL penicillin/100 μg/mL streptomycin.
4. 10% formalin.
5. Oil-red-O solution.
6. 2% Alizarin Red S.
7. 10% cetylpyridinium chloride monohydrate.
8. Goldner's trichrome staining. Fixation and washing: 2% acetic acid; Stain: hematoxylin, phosphomolybdic acid light green.
9. Optical microscope.

2.8 Transplantation of Microencapsulated Cells into Mice

1. Isoflurane.
2. Buprenorphine.
3. Surgical instruments including: scissor, forceps, and a needle holder.
4. Abbocath-T 18G catheter (Hospira, Lake Forest, IL, USA).
5. Resorbable sutures.
6. Cotton swab.
7. P1000 pipette.

3 Methods

All procedures are performed at room temperature (RT) and under sterile conditions unless otherwise specified.

3.1 Formation of Microspheres

1. First, it is important to determine the amount of polymer needed for the encapsulation process. Usually, this ranges between 1 and 8 mL of polymer. This amount is dictated by the experimental design, encapsulator capacity, the amount of polymer at disposal, and the number of cells available for the experiment. 1 mL of 8-arm PEG/alginate is required to encapsulate between 0.5×10^6 and 2×10^6 hepatocytes or MSCs.
2. Then, fill the reactor with the gelation bath and add the stirrer.
3. Once the encapsulator, the polymer, and the gelation bath are ready, the first step consists of cell preparation. Detach hepatocytes or MSCs from the plastic petri dish using 0.25% trypsin–EDTA for about 30 s–2 min and wash twice (*see* **Note 1**).

4. Block the reaction adding the medium with 10% (v/v) FCS and put cells in a conical tube. Centrifuge (1200 rpm (300×*g*) for MSCs, 500 rpm (60×*g*) for hepatocytes, 5 min) the cell suspension and discard the supernatant.
5. Wash with PBS, centrifuge and discard the supernatant (*see* **Note 2**).
6. Once the cells are ready and freed from the supernatant, resuspend the pellet in 8-arm PEG/alginate.
7. Homogenize carefully and take the cell-polymer mixture carefully in a 10 mL syringe with an 18G needle. It is important to carefully remove all bubbles present in the cell-polymer mixture.
8. At this point, PEG/alginate-cell solution is ready to be processed by the encapsulator to form microspheres (Fig. 1). The solution is extruded through a 400 μm needle using a pump and a co-axial air-flow; this will form droplets that fall and immediately polymerize in the gelation bath.
9. To do so, connect the syringe without needle containing the cell-polymer mixture to the liquid connector located on the lid of the reactor.
10. Extrude the mixture into the sterile gelation bath through a 400 μm needle, using the co-axial air-flow droplet generator at 2 mL/min, the pump set at 3 mL/min and the stirrer at 70%.
11. Once the solution has fully passed through, stop the pump and incubate microspheres in the gelation bath for 30 min with 70% stirring to allow polymerization.

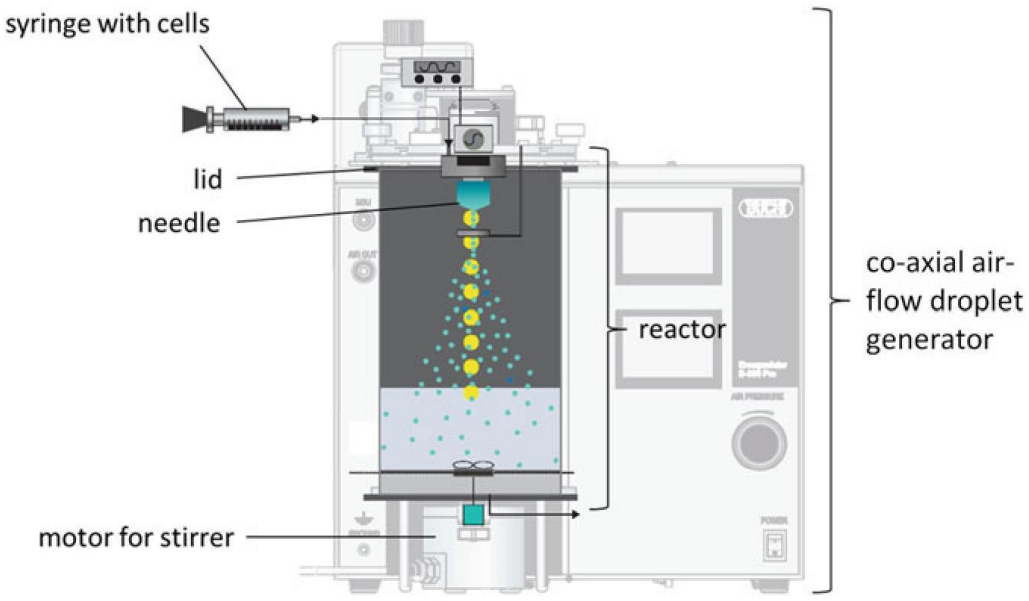

Fig. 1 Schematic representation of the co-axial air-flow droplet generator (B chi encapsulator is represented). 8-arm PEG/alginate mixed with hepatocytes or MSCs ($0.5–2 \times 10^6$/mL) is extruded through a 400 μm needle using a pump and a co-axial air-flow. Droplets are formed and polymerize in the gelation bath composed of MOPS at pH 7.4, $CaCl_2 \times 2H_2O$, and dithiothreitol

12. This process will produce microspheres measuring on average about 550 μm in diameter. Increasing or decreasing the co-axial air-flow will respectively decrease and increase the size of the microspheres.
13. Collect microspheres from the gelation bath by filtration through the 70 μm cell strainer.
14. Incubate microspheres in petri dish with 0.9% NaCl for 10 min to remove all DTT (repeat twice).
15. Microencapsulated cells are placed in culture (*see* Subheading **3.2** and **Notes 3** and **4**). Empty microspheres are prepared using the same protocol.

3.2 Culture of Microencapsulated Cells

1. The microencapsulated cells are cultured under normal culture condition, i.e., in petri dishes at 37 °C with 5% CO_2. The microspheres are seeded in a 10 mL petri dish in order to obtain one or two layers or capsules (Fig. 2). The present protocol allows obtaining an average of approximately 500–1000 cells per microsphere.
2. The medium is changed every 2–3 days, in the same way as for free cells (*see* **Note 4**).

3.3 Assessment of Cell Viability

1. The assessment of cell viability and death can be performed at selected time points including the point immediately after isolation (*see* **Notes 5** and **6**). This procedure is performed using FDA/PI fluorescent staining: Harvest a small number of microspheres (typically 100–200 μL) using a P1000 pipette (*see* **Note 3**) and place on a 3 mL petri dish.

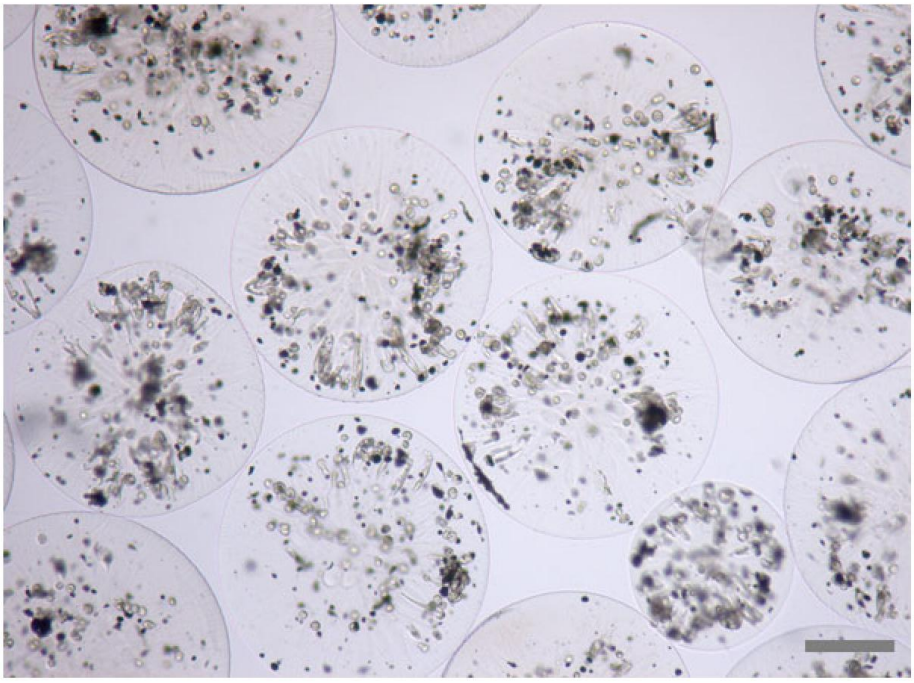

Fig. 2 Microencapsulated MSCs in culture. The size of microsphere is 550 μm in diameter on average. *Scale bar*: 200 μm

2. Add 3 mL of FDA/PI solution and wait for 2 min. Inspect cells under a fluorescence microscope with the appropriate wavelength.
3. The same procedure can be applied to determine the viability of free cells.
4. Take immediately pictures of both FDA and PI and bright field (*see* **Note 7**).

3.4 Assessment of Cell Proliferation

Cell proliferation inside the microspheres can be analyzed by measuring EdU incorporation. This method is similar to that used for free cells; the aim is to determine whether microencapsulated cells proliferate.

1. Prepare microencapsulated and free cells in 3 mL petri dish: Collect 20–30 microspheres (typically 200–400 μL) using a P1000 pipette (*see* **Note 3**) and place on a 3 mL petri dish.
2. Add 1 μL of EdU to the culture medium and culture cells for 24 h at 37 °C with 5 % CO_2.
3. After this culture, remove supernatant, wash the capsules with PBS.
4. Fix cells with 4 % paraformaldehyde for 15 min.
5. Then, permeabilize cells using 0.5 % Triton X-100 for 5 min (*see* **Note 8**).
6. The Edu-positive cells are detected using small fluorescent molecules (Click-iT EdU Cell Proliferation Assays).
7. After a washing step using PBS, the nuclei are stained using Hoechst stain and expose the cells for 5 min.
8. Wash in PBS.
9. Take pictures and count EdU-positive and Hoechst-positive cells.

3.5 Assessment of Hepatocyte Functionality

The determination of hepatocyte metabolic function in vitro is typically performed during the first week of culture. This method is based on the measurement of albumin or urea synthesis (a metabolite of ammonium catabolism) and the detoxification of drugs such as lidocaine or diazepam (*see* **Note 9**). To achieve this, the supernatant of hepatocyte culture is collected at various time points, typically at day 1, 3, and 7.

1. The measure of albumin is performed directly on the collected hepatocyte culture medium. To measure catabolic activity, add either 0.56 mM ammonium sulfate or 1 mg/mL lidocaine or 1 μg/ml diazepam to the hepatocyte culture medium of 5×10^4 hepatocytes. Incubate for 6 h under normal culture condition (i.e., 37 °C, 5 % CO_2) and collect supernatants.

2. The concentrations of albumin, urea, lidocaine and diazepam are measured using automated systems (e.g., Cobas INTEGRA 400 and D×C 800 system). The less lidocaine and diazepam remain in the hepatocyte culture medium, the more efficient is the detoxification.

3.6 MSC Differentiation

The functionality of MSC after microencapsulation can be assessed by testing their capacity to differentiate into cells of the adipocyte, osteocyte, and chondrocyte lineage within the capsules.

1. The first step is to prepare the three differentiation media.
2. Wash microencapsulated MSCs and cover them with the specific culture medium (*see* Subheading 2.7). Culture for 3 weeks and change the media every 3 days.
3. To detect adipocyte differentiation, fix microencapsulated MSCs with cold 10% formalin for 1 h (*see* **Note 8**), and wash twice with tap water. Then, cytoplasmic triglyceride droplets are stained with Oil-red-O solution for 2 h at RT, followed by washing twice with tap water and inspection under an optical microscope. Lipid droplets appears in red.
4. To detect osteogenic differentiation, fix microencapsulated MSCs with cold 10% formalin for 1 h into the petri dish (*see* **Note 8**), and wash twice with tap water. Then, stain with 2% Alizarin Red S for 20 min at RT to reveal calcific depositions, followed by washing with tap water, destain in 10% cetylpyridinium chloride monohydrate for 30 min and inspect under an optical microscope. Calcific depositions are stained in red.
5. To detect chondrogenic differentiation, fix microencapsulated MSCs with cold 10% formalin for 1 h, and wash twice with tap water. Microencapsulated MSCs are dehydrated and embedded in paraffin (*see* **Note 10**), and 5 μm-thick sections performed. Detect collagen using Goldner's trichrome staining. Wash slides or fixed cells in a petri dish with 2% acetic acid, stain successively with hematoxylin, phosphomolybdic acid, and light green, wash with 2% acetic acid in between and inspect under an optical microscope. Collagen fibers appear in green.

3.7 Transplantation of Microencapsulated Cells

After 24 h of culture, microencapsulated cells can be transplanted in mice under the kidney capsule or in the peritoneal cavity, to analyze their in vivo function. A large volume of microencapsulated cells requires the peritoneal cavity as implantation site (*see* **Note 11**).

1. Anesthetize mice with isoflurane and give appropriate analgesia (i.e., buprenorphine).
2. For the transplantation under the kidney capsule (allowing an easier recovery of the microspheres, e.g., for histological analysis), mice are placed in ventral position.

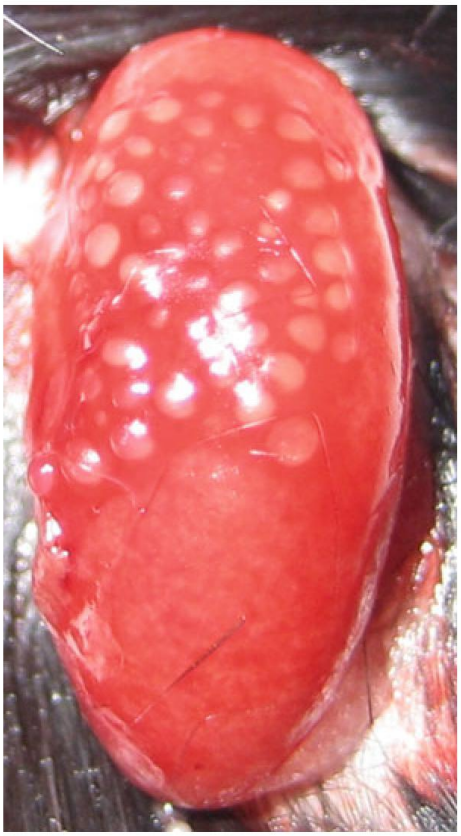

Fig. 3 Microencapsulated cells transplanted under the kidney capsule in mice. The left mice kidney (*red*) is exposed through a side incision. The small transparent droplets seen through the kidney capsule at the surface are microcapsules containing cells. The size of microspheres is 550 μm in diameter on average

3. Perform a side incision and dissect muscles to reach the kidney, exteriorize the kidney using cotton swab, create a 0.5 mm hole into the kidney capsule using a 18G needle.
4. Use an abbocath-T 18G catheter to place the microspheres under the kidney capsule (Fig. 3) (*see* **Note 12**).
5. Close the muscle layer and skin with resorbable sutures.
6. For the transplantation of microspheres in the peritoneal cavity, open the skin and peritoneum; inject the microspheres using a P1000 pipette (*see* **Note 3**) (Fig. 4), and close in a two-layer fashion with resorbable sutures.

3.8 Analyses After Transplantation

1. Analyze mice survival.
2. Collect blood samples to detect aspartate transaminase, alanine transaminase, bilirubin and human and murine cytokines using appropriate detection tests.
3. Collect tissue samples and place in 10% formalin (tissue sections), in embedding medium for tissue freezing (placed in cold methylbutanol, frozen using liquid nitrogen) (frozen tissue sections), protein extraction buffer (protein detection) or in tubes and put tubes directly into liquid nitrogen (RNA extraction).

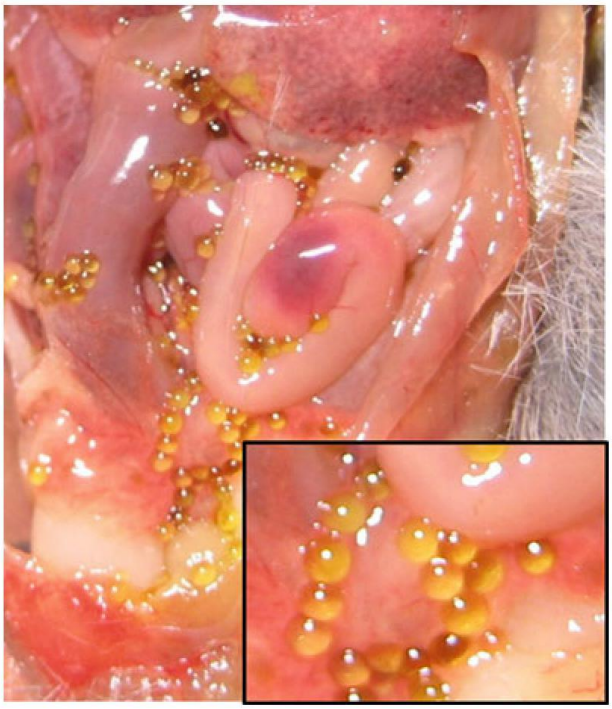

Fig. 4 Microencapsulated cells transplanted into the peritoneal cavity of mice after bile duct ligation: this model is used to create chronic liver failure and fibrosis and cause severe hyperbilirubinemia. The capsules are thereby *yellow-colored*

4. Proceed with histo-morphological and immunochemistry analyses on tissue sections.
5. Analyze protein expression using western blot analysis.
6. Extract RNA in order to proceed with gene expression analysis using RT-PCR.

4 Notes

1. First collect the culture medium and keep it to block trypsin reaction later on. Wash cells three times with PBS. Add 1.5 mL of trypsin 0.025 % for a 175 cm^2 flask; make the trypsin cover the entire surface changing orientation and "hit" the flask to help cell detachment. Incubate cells with trypsin for 2 min.
2. This step can be repeated twice or trice. Balance between the fact that cells are fragile and need to stay as short as possible outside ideal conditions of culture and the fact that cells need to be rinsed properly before microencapsulation.
3. Microspheres can be carefully collected using a P1000 pipet; however, it is recommended to cut the end of the pipette tip using a sterile heated scalpel blade or to use a 10 mL pipette in order to allow the microsphere flow without any mechanical stress.

4. Microspheres cannot be centrifuged. To change the media, incline the petri dish, wait for 15–20 s to allow the microspheres to settle down, or, if microspheres are at a conical tube, wait for 2 min to allow them to settle at the bottom of the tube. Carefully remove the supernatant using a P1000 pipette.
5. Primary hepatocytes remain viable in vitro up to 1 week [9, 10] and MSCs can be maintained viable for extended periods of time (more than 6 months) [11].
6. The same technique can be used for the assessment of viability on non-encapsulated cells as control. Cells are kept at RT during the microencapsulation process and assessed at the same time as microencapsulated cells.
7. The fluorescence will disappear within 5–10 min. Since microspheres may be floating in the medium, it is worth to diminish the quantity of liquid and avoid shaking the petri dish.
8. Fixation (PAF) and permeabilisation (Triton) steps can be shortened. This is important especially for the fixation step because it can disrupt the microspheres. EdU assay uses small molecule-based detection and therefore allows mild fixation and detergent permeabilization steps.
9. Other assays exist to test hepatocyte functionality, i.e., synthetic function assays: clotting factors, complement, and lipid synthesis. Storage for capacity assays, including assays of glycogen and fat soluble vitamins [12, 13].
10. It is possible to place the microencapsulated cells under the kidney capsule of mice immediately before fixation to create a substrate for the microspheres. Indeed, fixing and embed microcapsule in paraffin can be challenging because these processes tend to disrupt the hydrogel. Kidneys with microcapsules can be embedded following a standard protocol. Microcapsules are then better preserved and easier to retrieve during sample cutting step.
11. For example, 1.5×10^6 cells microencapsulated in 1 mL of PEG/alginate will represent approximately 2.5 mL to be transplanted in one animal (considering fluid around microspheres).
12. 18G is the minimal size that should be used. It is recommended to cut the tip of the catheter because microspheres may block inside if it is too long.

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8.2 List of publications

1) "Beneficial effects of Human Mesenchymal Stromal Cells on Porcine Hepatocyte Viability and Albumin Secretion"

   Montanari, J. Pimenta, S. Passemard, L. Szabo', F. Noverraz, R.P.H. Meier, J. Meyer, A. Balaphas, J. Sidibe, A. Thomas, H. Schuurman, S. Gerber-Lemaire, C. Gonelle-Gispert, L.H. Buhler.

   Manuscript in preparation for Journal of Immunology Research 2017

2) "Multipotent mesenchymal stromal cells enhance insulin secretion from human islets via N-Cadherin interaction and prolong function of transplanted encapsulated islets in mice"

   Montanari, R.P.H. Meier, R. Mahou, J.D. Seebach, C. Wandrey, S. Gerber-Lemaire, L.H. Buhler, C. Gonelle-Gispert.

   Under revision in Stem Cell Research and Therapy 2017

3) "Prolongation of rat-to-mouse islet xenograft survival by co-transplantation of autologous tolerogenic IL-10 dendritic cells"

   N. Madelon, E. Montanari, Y.D. Muller, J. Pimenta, L. Gruaz, L. Buhler, G. Puga Yung, J.D. Seebach.

   Manuscript in preparation

4) “Synthesis strategies to extend the variety of alginate-based hybrid hydrogels for cell microencapsulation”

S. Passemard, L. Szabó, F. Noverraz, E. Montanari, C. Gonelle-Gispert, L. Bühler, C. Wandrey, S Gerber-Lemaire.

Submitted to Biomacromolecules 2017

5) “Microencapsulation of Hepatocytes and Mesenchymal Stem Cells for Therapeutic Applications”

R.P.H. Meier, E. Montanari, P. Morel, J. Pimenta, H. Schuurman, C. Wandrey, S. Gerber-Lemaire, R. Mahou, L.H. Buhler.

Methods in molecular biology 2016

6) “Cell rearrangement in transplanted human islets”

Lavallard, M. Armanet, G. Parnaud, J. Meyer, C. Barbieux, E. Montanari, R. Meier, P. Morel, T. Berney, D. Bosco.

FASEB journal 2016

7) “Tuning the properties of hydrogel microspheres by adding chemical cross-linking functionality to sodium alginate”

R. Mahou, F. Borcard, V. Crivelli, E. Montanari, S. Passemard, F. Noverraz, S. Gerber-Lemaire, L.H. Buhler, C. Wandrey.

Chemistry of materials 2015

8) “Microencapsulated human mesenchymal stem cells decrease liver fibrosis in mice”

R.P.H. Meier, R. Mahou, P. Morel, J. Meyer, E. Montanari, Y.D. Muller, P. Christofilopoulos, C. Wandrey, C. Gonelle-Gispert, L.H. Buhler.

Journal of hepatology 2015

9) “Survival of free and encapsulated human and rat islet xenografts transplanted into the mouse bone marrow”

R.P.H. Meier, J.D. Seebach, P. Morel, R. Mahou, S. Borot, L. Giovannoni, G. Parnaud, E. Montanari, D. Bosco, C. Wandrey, T. Berney, L.H. Buhler, Y.D. Muller.

Plos One 2014

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