CellR4 2017; 5 (6): e2467

Microencapsulated Umbilical Cord Wharton Jelly-Derived Human Mesenchymal Stem Cells for the Cell Therapy of Type 1 Diabetes Mellitus (T1d): Applications and Limits

P. Montanucci, T. Pescara, G. Basta, R. Calafiore

Department of Medicine, Section of Cardiovascular, Endocrine and Metabolic Clinical Physiology, Laboratory for Endocrine Cell Transplants and Biohybrid Organs, University of Perugia, Perugia, Italy

Giuseppe Basta and Riccardo Calafiore share senior co-authorship of this paper

Corresponding Author: Riccardo Calafiore, MD; e-mail: riccardo.calafiore@unipg.it

Keywords: Umbilical cord, Wharton jelly-derived human adult mesenchymal stem cells, Endotoxin-free sodium alginate, Microencapsulation, T1D.

Abstract

We aimed to blunt islet β-cell directed autoimmune aggression as a potential approach for the treatment of Type 1 Diabetes (T1D). For this purpose, we proposed the use of a particular type of Mesenchymal Stem Cells, retrieved from the post-partum umbilical cord Wharton Jelly (hUCMS). To maximize hUCMS immunomodulatory potential, that has been proven impaired by cell-to-cell contact, we encapsulated these cells within highly purified, alginate-based microcapsules. Encapsulation enabled physical isolation of the cells from the host's immune system.

Pilot *in vitro* experiments where microencapsulated hUCMS were co-incubated with PBMCs derived from T1D patients showed induction of Treg and rebalance of Th1/Th2 cells. *In vivo* studies in diabetic NOD mice showed that microencapsulated hUCMS yield long-term remission of hyperglycemia. Use of the encapsulated hUCMS showed that in cell-based immunomodulatory strategies, the transplanted cells can benefit from encapsulation. It is likely that such immune-therapeutic approach could have efficacy only if residual native β-cell mass is still present. Otherwise, hUCMS-induced rehabilitation of the immune system would be unable to grant for reversal of diabetes. The proposed method, employing hUCMS in alginate microcapsules, presents a good safety and efficacy profile. This could warrant initiation of pilot human clinical trials of microencapsulated hUCMS grafts patients with recent-onset T1D.

The disease

Diabetes mellitus is a chronic metabolic disease, consisting of uncontrolled high blood glucose levels, deriving from insufficient insulin production or action. Chronically elevated blood glucose may result in severe complications, associated with disabling outcomes, affecting the cardiovascular system, eye, nerves, and kidney. Diabetes mellitus may be considered a global epidemic with continuous, steep increase in prevalence and incidence worldwide[1,3]. Healthcare expenditure due to diabetes and its complications is climbing worldwide[1]. Type 1 diabetes (T1D), a form of diabetes mellitus characterized by autoimmune destruction of pancreatic islet β-cells, may occur at any age, but it frequently appears in the youth. T1D strictly requires immediate exogenous insulin supplementation. Unfortunately, though a life-saving therapy, exogenous insulin may delay but not eliminate the risk of developing secondary, chronic complications of the disease[2].

For the past 25 years, attempts to identify the underlying autoimmune mechanisms of T1D have been unsuccessful - due to either the polyclonal nature of the autoimmune response, or the immune dysregulation in T1D patients[4,5]. Combination of

approaches has often been proposed to address these challenges[1,6-8]. New strategies to treat or, desirably, cure the disease, are currently being sought.

Due to robust immunomodulatory properties, combined with low immunogenicity and multipotency, Mesenchymal Stem Cells (MSCs) have been regarded as a possible pro-regenerative and repair therapy to contrast the pathological changes occurring in T1D[9-12]. The ability of MSCs to modulate immune responses through paracrine mechanisms is well documented[13] and quite appealing for the treatment of T1D[14]. Among MSC, those deriving from the umbilical cord Wharton jelly have been associated with interesting effects on autoimmune disorders[15,16].

Umbilical Cord Wharton Jelly-Derived Human Adult Mesenchymal Stem Cells (hUCMS)

hUCMS do not pose ethical problems since they are derived from extra-embryonic tissue. These cells are also easy to purify by using either classic methods such as "chopping" procedures[17] or a faster and more efficient approach, consisting of an enzymatic digestion of the retrieved cord tissues[18]. hUCMS, generally identified as mesenchymal-like cells, express the surface markers CD10, CD13, CD29, CD44, and CD90. They also express, at lower levels, transcription factors that are primarily expressed by embryonic stem cells (OCT-4, SOX-2 and NANOG)[19,20], but they are not of hematopoietic nature (they do not express CD31, CD34, CD45)[21]. Noteworthy, hUCMS result negative for class II human leukocyte antigens (HLA)-DR. There are some discrepancies between data obtained by different research groups: some authors[22] reported on a stable expression of HLA-1 only through 5 culture passages, while Weiss et al[20] did not find any changes. These differences may relate to epigenetic factors induced by different culture conditions.

Upon isolation and culture maintenance, hUCMS show a fibroblast-like appearance throughout 15 days along the initial culture passages. Some authors[22,23] found more than one cell phenotype in culture, identifiable also at later time. Doubling time (DT) ranges from 60 to 85 hours during the first passages, while it declines dramatically through the following passages, with variations between groups, down to 20-60 hours[20,24-26]. Subsequently, DT steadily increases until the cells reach senescence, with no karyotype changes. In light of this variability, stromal cells retrieved from the cord matrix likely contain more than one type of stem cells. Possibly a sub-group of primitive stem cells is present, with variations related to different isolation methods. hUCMS are peculiar, in that they present specific cytoskeleton filaments. This may support the idea that rather than fibroblasts or myocytes, they are in fact myofibroblasts - cells with characteristics of both smooth muscle cells and fibroblasts[27]. In particular, contractile proteins such as not muscular myosin, desmin, and alfa-actin of the smooth muscle (a marker of myofibroblasts) appear in these cells. On the contrary, muscle myosin is absent. Furthermore, hUCMS express vimentin, a protein of the intermediate filaments that is typical of cell of mesenchymal-origin, such as fibroblasts, but that is missing in the smooth muscle. Co-expression of vimentin and desmin indicates the myofibroblastic nature of these cells. Another intermediate filament expressed in stromal cells is cytokeratin, a typical marker of ectodermal and endodermal-derived epithelial cells. Co-expression of these markers make hUCMS a very attractive cellular model for their differentiation potential.

Notably, hUCMS are adult stem cells able to differentiate *in vitro* and *in vivo* into several cell phenotypes [18, 28-32]. hUCMS can also home to injured and inflamed tissue, a property that is probably related to the expression of a specific set of receptors for chemokines and adhesion molecules[33]. Over the past decade, growing evidence has shown that hUCMS are not only multipotent cells, with promising applications in regenerative medicine[34], but also a powerful tool to modulate the immune system (Table 1). Owing to their anatomical location, hUCMS express HLA-E, HLA-F, and HLA-G - the latter being involved in the tolerogenic process occurring at the fetal-maternal interface[35]. hUCMS can interact with the majority of immune cell types belonging to the adaptive or innate immune system. hUCMS can exert their functions via

TABLE 1. hUMSCs PROPERTIES

- Low immunogeneicity
- Immunoregulatory capacity
- Pro-regenerative properties
- Pancreatic tropism
- No ethical issues

cell to cell contact and via secretion of soluble mediators, including transforming growth factor β1 (TGF-β1) indoleamine 2,3-dioxygenase (IDO), nitric oxide (NO), interleukin-6 (IL6), prostaglandin E2 (PGE2), hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF). In this regard, the inhibition of effector T lymphocytes and the stimulation of regulatory T (Tregs) cells is one of their most relevant beneficial effects[9,36,37].

Immunomodulation of Autoimmune Diseases

Immunoregulatory properties, coupled with low immunogenicity, make hUCMS a potentially powerful tool for the cell therapy of T1D (Table 1). The suppression of immune cell activation would be of critical importance in the context of hyperactive immune conditions, such as transplant rejection, autoimmune and inflammatory diseases. Compelling preclinical results indicate that adoptive transfer of Tregs can prevent or reverse autoimmune diseases and allograft rejection, by restoring immune tolerance to self-antigens or alloantigens[38]. Tregs operate by both suppressing effector T-cells (CD4 and CD8) and regulating the activation of dendritic cells. They exert these functions by means of a microenvironment enriched in immunosuppressive cytokine IL-10 and TGF-β1, but possibly also by cell-to-cell interactions. Various $CD4^+$regulatory T-cell subsets have been extensively described[38], while here we specifically refer to a subset of $CD4^+$ T helper cells indicated as Tregs. Studies in preclinical models have indicated that polyclonal Tregs can prevent autoimmune diseases, whereas only self-antigen-specific Tregs can reverse active autoimmunity. In T1D, insulin-producing β-cells are destroyed by autoimmunity; several preclinical models of the disease have shown that the transfer of *ex vivo* primed Tregs can block the disease process[39-41]. Studies in NOD mice had previously shown that T1D is mainly a Th1-driven disease with a synergistic action of $CD4^+$ and $CD8^+$ cells on β-cell destruction pathways[42]. Moreover, the recent identification of Th17 cells and their role in multiple autoimmune diseases[43], prompted several authors to evaluate the involvement of this cell subset in T1D pathogenesis. It has been suggested that IL-17 and, therefore, Th17 cells, may be crucial for triggering autoimmunity in the early stages of the disease. Recent data demonstrated that Th17 cells are present among islet-infiltrating T lymphocytes at disease onset[44,45], thereby suggesting that IL-17 may be more important in the induction rather than the perpetuation of autoimmunity in T1D.

The expansion of pathogenic T cells and the reduction of Treg cells appear to be the most likely scenario for the development of T1D, similarly to other autoimmune disorders[46-48].

The inhibition of effector T lymphocytes and the stimulation of Tregs cells are among the most relevant beneficial effects induced by hUCMS[49]. Therefore, several studies attempted to determine the therapeutic potential of hUCMS in a wide array of autoimmune diseases. Despite promising results in experimental arthritis and in *in vitro* studies using immune cells from patients with rheumatoid arthritis (RA)[50], it has become evident that the physical interaction between hUCMS and immune cells is pivotal in many other conditions[51]. In fact, we have demonstrated that if hUCMS are co-incubated with lymphocytes isolated from patients with primary Sjögren's syndrome[15] or with T1D[16], their immunomodulatory function fades away.

Hence, physical separation between hUCMS and effector T cells appears to be necessary. We have addressed this issue by using microcapsules.

Alginate-Bases Microcapsules: Application TO hUCMS

Microencapsulation consists of entrapping live cells within polymeric and non-cytotoxic artificial membranes that constitute immunoprotective barriers. Based on this concept, a wide spectrum of cells, including pancreatic islets, have been encapsulated within microcapsules. The applicability of the encapsulation strategy is broad and extends beyond T1D. It was recently postulated that the success of this approach might require a detailed focus on multiple issues concerning biocompatibility and bio-performance of the microcapsules[52-54].

Since the time of the first successful reports from Lim and Sun[55], alginates that are highly purified and almost endotoxin- and protein-free have gained popularity for the preparation of transplantable microcapsules[56]. Microcapsules proved to promote better growth, differentiation and maturation of different cell types, including mesenchymal stem cells, mESCs, hESCs, neural stem cells and hepatocytes[57-59]. Biocompatibility and chemical composition of the alginate-based biomembranes are critical parameters for the maintenance of encapsulated cells. These properties are mainly related to protein absence, in conjunction with very low endotoxin

levels[60,61]. Since alginate is the major component of microcapsules, endotoxin and pyrogen-free criteria have to be thoroughly fulfilled to make this compound suitable for clinical application[62].

In our studies, microencapsulation was performed by mixing hUCMS pellets with 1.8% (*v/v*) ultrapurified alginate solution, to make a final homogeneous alginate/cell suspension. The employed ratio was 1.3 ml alginate/2.5x10^6 cells, with this suspension being subsequently extruded through a microdroplet generator. The alginate microdroplets were collected on 100 mM $CaCl_2$ solution, which immediately turned the microdroplets into gel microbeads. These were sequentially coated with 0.12% (*w/v*), and 0.06% (*w/v*) poly-l-ornithine (Sigma-Aldrich, St: Louis, MO, USA), the beads were then de-gelled with 55 mM sodium citrate at pH = 7, and finally, coated with 0.1% (*v/v*) ultrapurified alginate. The final preparation was incubated for additional 24 h, for sterility assay[62]. Microencapsulated hUCMS were incubated overnight with 300 U/ml IFN-γ (Sigma-Aldrich), that was removed at the end of the treatment, before setting-up the coculture with Peripheral blood mononuclear cells (PBMCs), or before transplantation. Viability testing, with ethidium bromide and fluorescein diacetate (Sigma-Aldrich) was performed after microencapsulation, upon treatment with IFN-γ, and before TX (Fig. 1).

Encapsulation overcomes the need for host's immunosuppression, hence offering an additional benefit in terms of immunosuppressive drug-sparing approach. Microcapsules permit access to spatial cell distribution, within a three-dimensional architecture, which is favorable, since it mimics an extracellular matrix-like microenvironment, resulting very helpful for cell survival and function[58,59]. Moreover, alginate microcapsules themselves may interact with activated PBMCs whose proliferation is blunted[16]. This observation appears in striking contrast with several other studies, reporting that alginate capsules may trigger immune reactivity per se. This may be due to the fact that while unpurified alginates can induce immune responses due to their high protein and endotoxin content, highly purified alginates can inhibit lymphocyte reactivity, due to exposure of saturated alginic acid radicals (guluronate/mannuronate) on the capsular membrane[63]. The latter concept has been confirmed by our Center, with the demonstration of modulatory effects by empty capsules[16].

Immunoregulatory Properties Of CPS/hUCMS

Use of a microencapsulation technology with biocompatible materials provides for a dynamic and immunoisolating microenvironment, where molecules secreted by hUCMS can reach and modulate immune cells of the host, while immune cells cannot physically get in contact with hUCMS. Our *in vitro* studies suggest that microencapsulated hUCMS exhibit at least two major effects on T cells, effects that could be relevant for the treatment of T1D: i. reduction of effector Th1 cells; ii. expansion of Tregs which leads, at least partially, to rebalance of the Teff/Treg ratio. The lack of suppressive action on T1D-Th17 cells may indicate that this cell subset is insensitive to hUCMS immunomodulatory functions. On the other hand, effector immune cells may be themselves resistant to the modulatory/suppressive action of cell-based therapies. In this context, it was recently demonstrated that in systemic lupus erythematosus (SLE)[64], Tregs are defective and T effector cells are deranged, as they cannot be suppressed by Tregs from SLE patients or normal individuals. Based on these observations, the i.v. administration of free hUCMS for therapeutic purposes may not be an optimal solution for autoimmune disorders, and failure to achieve efficacy does not necessarily mean that hUCMS are not effective *per se*. In fact, we demonstrated that prevention of hUCMS contact with the patient's immune cells, by microcapsules, can overcome this problem and provide an efficient modulation of the immune cells via soluble mediators[15,16]. These *in vitro* observations seem to be confirmed by our ongoing *in vivo* experiments in NOD mice with spontaneous DM[65]. Microencapsulated hUCMS were transplanted into the peritoneum of NOD mice with glycemia of 250 to 300 mg/dl and abnormal intraperitoneal glucose tolerance test (IPGTT). The transplantation restored normoglycemia throughout 7 months post transplantation. 7 months after transplantation, T cell immunophenotyping from retrieved lymphoid organs showed that the levels of $CD4^+FOXP3^+CD25^{high}$ Treg cells were comparable to normal, but not to overtly diabetic control NOD mice (where no Tregs were detectable). Hence, induction of acquired central tolerance towards autoreactive T cell clones is made possible by administration of microencapsulated hUCMS. Moreover, *in vitro*, the inverse correlation between the suppressive effects on T cells and the number of hUCMSC cells encapsulated, supports the operative use of very low dosages of encapsulated hUCMS to obtain therapeutic effects[15,16].

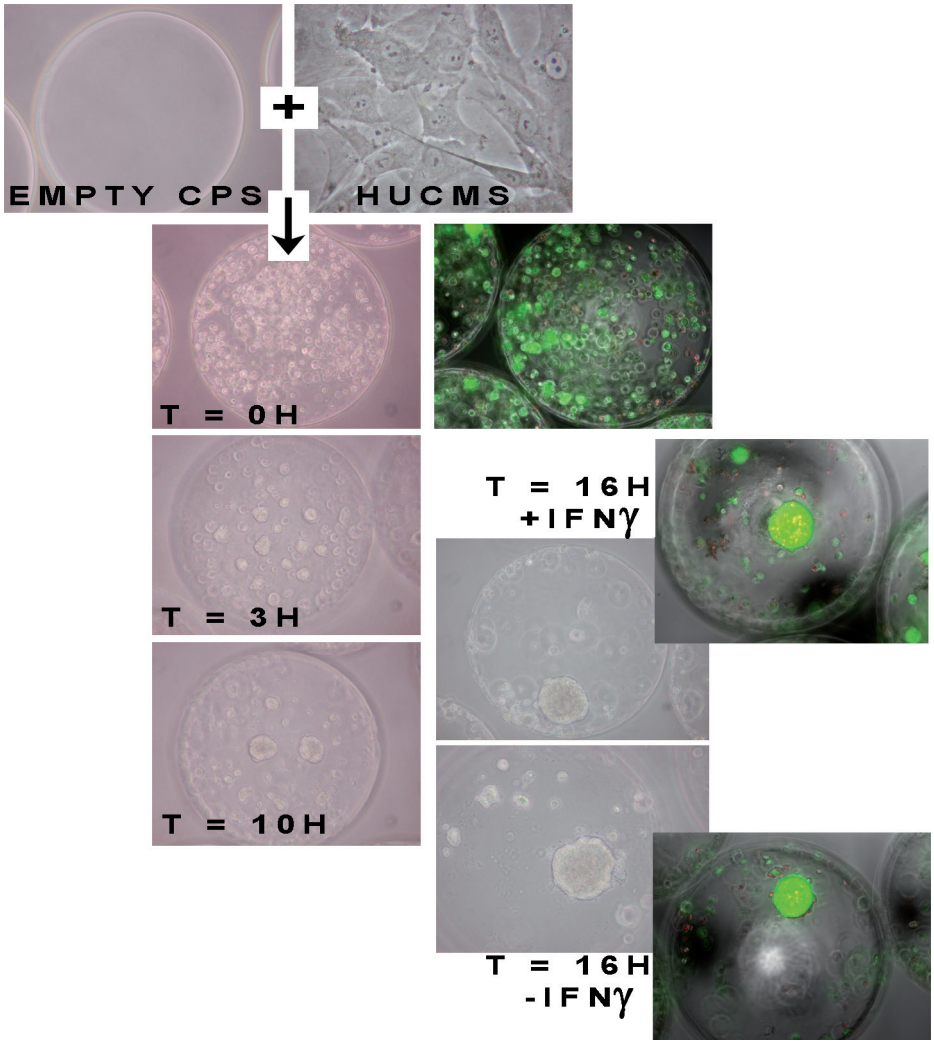

Fig. 1. Process of encapsulation of isolated hUCMS within alginate-based microcapsules. After encapsulation, hUCMS tend to form spheroids that fully retain viability, whether or not IFNγ is added to culture.

Critique and Outlook

We have demonstrated that hUCMSC are endowed with robust immunomodulatory functions, which may interrupt the disease process of autoimmune disorders such as T1D. From what above reported, the best way to exploit such properties relies on microencapsulation. In several pre-clinical, as well as pilot clinical studies, it emerged that naked hUCMS hold more limited immunoregulatory potential, and may not perform as expected. On the contrary, as shown by our *in vitro*, and ongoing *in vivo* pre-clinical studies in diabetic models, microencapsulated hUCMS are extremely powerful in modulating the host's immune system. This resulted in blocking the autoimmune β-cell directed immune destruction, with preservation of the residual β-cell mass.

At least two possible of limitations for the use of this approach in T1D are envisionable: 1) hUCMSC have not yet been proven to induce the regeneration of β-cells; 2) encapsulated-hUCMSC cell therapy for T1D may have predominantly immunomodulatory functions, which could partially limit application to the early stages of the disease - when β-cell destruction is not complete, and marginal numbers of β cells are still viable. The observations in NOD mice, corroborating the *in vitro* findings, are paving the way toward pilot clinical trials of microencapsulated hUCMS transplantation into patients with recent onset of T1D. This form of cell therapy appears feasible and could spare initiation of exogenous insulin supplementation therapy regimens.

Conflict of Interests:

The Authors declare that they have no conflict of interests.

References

1. Bluestone JA, Herold K, Eisenbarth G. Genetics, pathogenesis and clinical interventions in type 1 diabetes. Nature 2010; 464: 1293-1300.
2. Jensen RA, Agardh E, Lernmark A, Gudbjörnsdottir S, Smith NL, Siscovick DS. HLA genes, islet auto antibodies and residual C-peptide at the clinical onset of type 1 diabetes mellitus and the risk of retinopathy 15 years later. PLoS One 2011; 6: e17569.
3. IDF Diabetes Atlas Group. Update of mortality attributable to diabetes for the IDF diabetes atlas: estimates for the year 2013. Diabetes Res Clin Pract 2013; 109: 461-465.
4. Couzin-Frankel J. Trying to reset the clock on type 1 diabetes. Science 2011; 333: 819-821.
5. Mathieu C, Gillard P. Arresting type 1 diabetes after diagnosis: GAD is not enough. Lancet 2011; 378: 291-292.
6. van Belle TL, Coppieters KT, von Herrath MG. Type 1 diabetes: etiology, immunology, and therapeutic strategies. Physiol Rev 2011; 91: 79-118.
7. Chatenoud L. Immune therapy for type 1 diabetes mellitus-what is unique about anti-CD3 antibodies? Nat Rev Endocrinol 2010; 6: 149-157.
8. Atkinson MA, Bluestone JA, Eisenbarth GS, Hebrok M, Herold KC, Accili D, Pietropaolo M, Arvan PR, Von Herrath M, Markel DS, Rhodes CJ. How does Type 1 diabetes develop?: the notion of homicide or {beta}-cell suicide revisited. Diabetes 2011; 60: 1370-1379.
9. Selmani Z, Naji A, Zidi I, Favier B, Gaiffe E, Obert L, Borg C, Saas P, Tiberghien P, Rouas-Freiss N, Carosella ED, Deschaseaux F. Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+CD25highFOXP3+ regulatory T cells. Stem Cells 2008; 26: 212-222.
10. Sordi V, Pellegrini S, Krampera M, Marchetti P, Pessina A, Ciardelli G, Fadini G, Pintus C, Pantè G, Piemonti L. Stem cells to restore insulin production and cure diabetes. Nutr Metab Cardiovasc Dis 2017; 27: 583-600.
11. Domínguez-Bendala J, Lanzoni G, Inverardi L, Ricordi C. Concise review: Mesenchymal Stem Cells for Diabetes. Stem Cells Trans Med 2012; 1: 59-63.
12. Xv J, Ming Q, Wang Q, Zhang W, Li W, Wang S, Li Y, Li Y. Mesenchymal stem cells moderate immune response of type 1 diabetes. Cell Tissue Res 2017; 368: 239-248.
13. Wang J, Liao L, Tan J. Mesenchymal-stemcell- based experimental and clinical trials: current status and open questions. Expert Opin Biol Ther 2011; 11: 893-909.
14. Carlsson PO, Schwarcz E, Korsgren O, Le Blanc K. Preserved b-cell function in type 1 diabetes by mesenchymal stromal cells. Diabetes 2015; 64: 587-592.
15. Alunno A, Montanucci P, Bistoni O, Basta G, Caterbi S, Pescara T, Pennoni I, Bini V, Bartoloni E, Gerli R, Calafiore R. In vitro immunomodulatory effects of microencapsulated umbilical cord Wharton jelly-derived mesenchymal stem cells in primary Sjögren's syndrome. Rheumatology (Oxford) 2015; 54: 163-168.
16. Montanucci P, Alunno A, Basta G, Bistoni O, Pescara T, Caterbi S, Pennoni I, Bini V, Gerli R, Calafiore R. Restoration of t cell substes of patients with type 1 diabetes mellitus by microencapsulated human umbilical cord Wharton jelly-derived mesenchymal stem cells: an in vitro study. Clin Immunol 2016; 163: 34-41.
17. Mitchell KE, Weiss ML, Mitchell BM, Martin P, Davis D, Morales L, Helwig B, Beerenstrauch M, Abou-Easa K, Hildreth T, Troyer D. Matrix cells from Wharton's jelly form neurons and glia. Stem Cells 2003; 21: 50-60.
18. Montanucci P, Basta G, Pescara T, Pennoni I, Di Giovanni F, Calafiore R. New simple and rapid method for purification of mesenchymal stem cells from the human umbilical cord Wharton jelly. Tissue Eng Part A 2011; 17: 2651-2661.
19. Wang HS, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ, Fu YS, Lai MC, Chen CC. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. Stem Cells 2004; 22, 1330-1337.
20. Weiss ML, Medicetty S, Bledsoe AR, Rachakatla RS, Choi M, Merchav S, Luo, Y, Rao MS, Velagaleti G, Troyer D. Human umbilical cord matrix stem cells: preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease. Stem Cells 2006; 24, 781-792.
21. Horwitz EM, Le Blanc K, Dominici M, Mueller I, Slaper-Cortenbach I, Marini FC, Deans RJ, Krause DS, Keating A. International Society for Cellular Therapy. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. Cytotherapy 2005; 7, 393-395.
22. Sarugaser R, Lickorish D, Baksh D, Hosseini MM, Davies JE. Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. Stem Cells 2005; 23: 220-209
23. Can A, Karahuseyinoglu S. Concise review: human umbilical cord stroma with regard to the Source of fetus-derived stem cells. Stem Cells 2007; 25: 2886-2895.
24. Maurya DK, Doi C, Pyle M, Rachakatla RS, Davis D, Tamura M, Troyer D. Non-random tissue distribution of human naïve umbilical cord matrix stem cells. World J Stem Cells 2011; 3: 34-42.
25. Conconi MT, Burra P, Di Liddo R, Calore C, Turetta M, Bellini S, Bo P, Nussdorfer GG, Parnigotto PP. CD105(+) cells from Wharton's jelly show in vitro and in vivo myogenic differentiative potential. Int J Mol Med 2006; 18: 1089-1096.
26. Montanucci P, Pescara T, Pennoni I, Alunno A, Bistoni O, Torlone E, Luca G, Gerli R, Basta G, Calafiore R. Functional profiles of human umbilical cord-derived adult mesenchymal stem cells in obese/diabetic versus healthy women. Curr Diabetes Rev; 2016 Jun 28. [Epub ahead of print]
27. Majno G, Gabbiani G, Hirschel BJ Ryan GB, Statkov PR. Contraction of granulation tissue in vitro: Similarity to smooth muscle. Science 1971; 173: 548-550.
28. Montanucci P, di Pasquali C, Ferri I, Pescara T, Pennoni I, Siccu P, Sidoni A, Cervelli V, Basta G, Calafiore R. Human umbilical cord wharton jelly-derived adult mesenchymal stem cells, in biohybrid scaffolds, for experimental skin regeneration. Stem Cell Int 2017. In Press

29. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284: 143-147.
30. Baksh D, Song L, Tuan RS. Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. J Cell Mol Med 2004; 8: 301-308.
31. Mitchell KE, Weiss ML, Mitchell BM, Martin P, Davis D, Morales L, Helwig B, Beerenstrauch M, Abou-Easa K, Hildreth T, Troyer D. Matrix cells from Wharton's jelly form neurons and glia. Stem Cells 2003; 21: 50-56.
32. Jiang J, Au M, Lu K, Eshpeter A, Korbutt G, Fisk G, Majumdar AS. Generation of insulin-producing islet-like clusters from human embryonic stem cells. Stem Cells 2007; 8: 1940-1945.
33. Ganta C, Chiyo D, Ayuzawa R, Rachakatla R, Pyle M, Andrews G, Weiss M, Tamura M, Troyer D. Rat umbilical cord stem cells completely abolish rat mammary carcinomas with no evidence of metastasis or recurrence 100 days post-tumor cell inoculation. Cancer Res 2009; 69: 1815-1820.
34. Barry F, Murphy M. Mesenchymal stem cells in joint disease and repair. Nat Rev Rheumatol 2013; 9 :584-594.
35. La Rocca G, Corrao S, Lo Iacono M, Corsello T, Farina F, Anzalone R. Novel immunomodulatory markers expressed by human WJ-MSC: an updated review in regenerative and reparative medicine. The Open Tissue Engineering and Regenerative Medicine Journal 2012; 5: 50-58.
36. Sato K, Ozaki K, Oh I, Meguro A, Hatanaka K, Nagai T, Muroi K, Ozawa K. Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. Blood 2007; 109: 228-234.
37. Djouad F, Charbonnier LM, Bouffi C, Louis-Plence P, Bony C, Apparailly F. Mesenchymal stem cells inhibit the differentiation of dendritic cells through an interleukin-6-dependent mechanism. Stem Cells 2007; 25: 2025-2032.
38. Esensten JH, Wofsy D, Bluestone JA. Regulatory T cells as therapeutic targets in rheumatoid arthritis. Nat Rev Rheumatol. 2009; 5: 560-565.
39. Tarbell KV, Petit L, Zuo X, Toy P, Luo X, Mqadmi A, Yang H, Suthanthiran M, Mojsov S, Steinman RM. Dendritic cell-expanded, islet-specific CD4+ CD25+ CD62L+ regulatory T cells restore normoglycemia in diabetic NOD mice. J Exp Med 2007; 204: 191-201.
40. Roncarolo MG, Battaglia M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. Nat Rev Immunol 2007; 7: 585-598.
41. Graham JG, Zhang X, Goodman A, Pothoven K, Houlihan J, Wang S, Gower RM, Luo X, Shea LD. PLG scaffold delivered antigen-specific regulatory T cells induce systemic tolerance in autoimmune diabetes. Tissue Eng Part A 2013; 19: 1465-1475.
42. Phillips JM. Type 1 diabetes development requires both CD4+ and CD8+ T cells and can be reversed by non-depleting antibodies targeting both T cell populations. Rev Diabet Stud 2009; 6: 97-103.
43. Kennedy Bedoya S, Lam B, Lau K, Larkin J. Th17 Cells in immunity and autoimmunity. Clin Dev Immunol 2013; 2013: 986789.
44. Kuriya G, Uchida T, Akazawa S, Kobayashi M, Nakamura K, Satoh T, Horie I, Kawasaki E, Yamasaki H, Yu L, Iwakura Y, Sasaki H, Nagayama Y, Kawakami A, Abiru N. Double deficiency in IL-17 and IFN-γ signalling significantly suppresses the development of diabetes in the NOD mouse. Diabetologia 2013; 56: 1773-1780.
45. Li CR, Mueller EE, Bradley LM. Islet antigen-specific Th17 cells can induce TNF-α-dependent autoimmune diabetes. J Immunol 2014; 192: 1425-1432.
46. Lehuen A, Diana J, Zaccone P, Cooke A. Immune cell crosstalk in type 1 diabetes. Nat Rev Immunol 2010; 10: 501-513.
47. Ryba-Stanisławowska M, Skrzypkowska M, Myśliwiec M, Myśliwska J. Loss of the balance between CD4(+) Foxp3(+) regulatory T cells and CD4(+)IL17A(+) Th17 cells in patients with type 1 diabetes. Hum Immunol 2013; 74: 701-707.
48. Alunno A, Bartoloni E, Bistoni O, Nocentini G, Ronchetti S, Caterbi S, Valentini V, Riccardi C, Gerli R. Balance between regulatory T and Th17 cells in systemic lupus erythematosus: the old and the new. Clin Dev Immunol 2012; 2012: 823085.
49. Mattar P, Bieback K. Comparing the immunomodulatory properties of bone marrow, adipose tissue, and birth-associated tissue mesenchymal stromal cells. Front Immunol 2015; 6: 560-565.
50. Liu Y, Mu R, Wang S, Long L, Liu X, Li R, Sun J, Guo J, Zhang X, Guo J, Yu P, Li C, Liu X, Huang Z, Wang D, Li H, Gu Z, Liu B, Li Z. Therapeutic potential of human umbilical cord mesenchymal stem cells in the treatment of rheumatoid arthritis. Arthritis Res Ther 2010; 12: R210.
51. Alunno A, Bistoni O, Montanucci P, Basta G, Calafiore R, Gerli R. Umbilical cord mesenchymal stem cells for the treatment of autoimmune diseases: beware of cell-to-cell contact. Ann Rheum Dis 2017 Jun 13. pii: annrheumdis-2017-211790. doi: 10.1136/annrheumdis-2017-211790. [Epub ahead of print]
52. Murua A, Portero A, Orive G, Hernández RM, de Castro M, Pedraz JL. Cell microencapsulation technology: towards clinical application. J Control Release 2008; 132: 76-83.
53. de Vos P, Andersson A, Tam SK, Faas MM, Hallé JP. Advances and barriers in mammalian cell encapsulation for treatment of diabetes. Immunol Endocr Metabol Agents Med Chem 2006; 6: 139-153.
54. Thanos CG, Calafiore R, Basta G, Bintz BE, Bell WJ, Hudak J. Formulating the alginate-polyornithine biocapsule for prolonged stability: evaluation of composition and manufacturing technique. J Biomed Mater Res A 2007; 83: 216-224.
55. Lim F, Sun AM. Microencapsulated islets as bioartificial endocrine pancreas. Science 1980; 210: 908-910.
56. Basta G, Montanucci P, Luca G, Boselli C, Noya G, Barbaro B, Qi M, Kinzer KP, Oberholzer J, Calafiore R. Long-term metabolic and immunological follow-up of nonimmunosuppressed patients with type 1 diabetes treated with microencapsulated islet allografts: four cases. Diabetes Care 2011; 34: 2406-2409.
57. Dean SK, Yulyana Y, Williams G, Sidhu KS, Tuch BE. Differentiation of encapsulated embryonic stem cells after transplantation. Transplantation 2006; 82: 1175-1184.

58. Montanucci P, Pennoni I, Pescara T, Blasi P, Bistoni G, Basta G, Calafiore R. The functional performance of microencapsulated human pancreatic islet-derived precursor cells. Biomaterials 2011; 32: 9254-9262.
59. Montanucci P, Pennoni I, Pescara T, Basta G, Calafiore R. Treatment of diabetes mellitus with microencapsulated fetal human liver (FH-B-TPN) engineered cells. Biomaterials 2013; 34: 4002-4012.
60. Orive G, Tam SK, Pedraz JL, Hallé JP. Biocompatibility of alginate-poly-L-lysine microcapsules for cell therapy. Biomaterials 2006; 27: 3691-3670.
61. Dusseault J, Tam SK, Ménard M, Polizu S, Jourdan G, Yahia L. Evaluation of alginate purification methods: effect on polyphenol, endotoxin, and protein contamination. J Biomed Mater Res A 2006; 76: 243-251.
62. Calafiore R, Basta G, Luca G, Lemmi A, Racanicchi L, Mancuso F, Montanucci MP, Brunetti P. Standard technical procedures for microencapsulation of human islets for graft into nonimmunosuppressed patients with type 1 diabetes mellitus. Transplant Proc 2006; 38: 1156-1157.
63. Paredes-Juarez GA, de Haan BJ, Faas MM, de Vos P. A technology platform to test the efficacy of purification of alginate. Materials (Basel) 2014; 7: 2087-2103.
64. Nocentini G, Alunno A, Petrillo MG, Bistoni O, Bartoloni E, Caterbi S, Ronchetti S, Migliorati G, Riccardi C, Gerli R. Expansion of regulatory GITR+CD25 low/-CD4+ T cells in systemic lupus erythematosus patients. Arthritis Res Ther 2014; 16: 444-450.
65. Montanucci P, Pescara T, Cavallo M, Parrettini S, Basta G, Calafiore R. Reversal of Hyperglycemia by Microencapsulated Human Umbilical Cord Wharton Jelly-Derived Adult Mesenchymal Stem Cells (hUCMS) Graft (TX) in Spontaneously Nonobese Diabetic (NOD) Mice. Diabetes 2017; 66: A481.