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Characterization of encapsulated porcine cardiosphere-derived cells embedded in 3D alginate matrices

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ABSTRACT

Myocardial infarction is caused by an interruption of coronary blood flow, leading to one of the main death causes worldwide. Current therapeutic approaches are palliative and not able to solve the loss of cardiac tissue. Cardiosphere derived cells (CDCs) reduce scarring, and increase viable myocardium, with safety and adequate biodistribution, but show a low rate engraftment and survival after implantation. In order to solve the low retention, we propose the encapsulation of CDCs within three-dimensional alginate-poly-L-lysine-alginate matrix as therapy for cardiac regeneration. In this work, we demonstrate the encapsulation of CDCs in alginate matrix, with no decrease in viability over a month, and showing the preservation of CDCs phenotype, differentiation potential, gene expression profile and growth factor release after encapsulation, moving a step forward to clinical translation of CDCs therapy in regeneration in heart failure.

1. Introduction

More than 23 million people suffer from heart failure worldwide, leading to 32% deaths (Bui et al., 2011, Nichols et al., 2014). Myocardial infarction (MI) is an ischemic heart disease caused by deterioration and obstruction of the arteries of the heart (coronary arteriosclerosis). The accumulation of cholesterol plaques, lipids (fats) and inflammatory cells in the walls of these arteries, leads to the interruption of vascular flow to the heart muscle. After an acute MI, cardiac cells in the affected area die due to the lack of blood supply. The heart has limited capacity for self-renewal and, its regeneration potential by itself is unable to counteract the loss of heart tissue during MI.

Despite advances in cardiovascular biology and medical therapy over the last 30 years, the prognosis of patients hospitalized with heart failure remains poor, with a 5-year mortality that approaches 50% (Roger et al., 2011). Cardiac transplantation is the standard therapy to replace the injured heart, but donor supply is not sufficient to cover the demand of heart transplants (Lund et al., 2013). Moreover, this therapy is very

Abbreviations: APA, Alginate-poly-L-lysine-Alginate; $CaCl_2$, Calcium Chloride; CDCs, Cardiosphere Derived Cells; CCK-8, Cell Counting Kit-8; DMEM, Dulbecco's Modified Eagle's Medium; DPBS, Dulbecco's Phosphate-Buffered Saline (DPBS); FBS, Fetal Bovine Serum; FGFR2, Fibroblast Growth Factor Receptor 2; HGFL, Hepatocyte Growth Factor-like Protein; HLA, Human Leukocyte Antigens; IGF-1, Insulin-like Growth Factor 1; IGF-1R, Insulin-like Growth Factor 1 Receptor; ISCT, International Society for Cellular Therapy; IMDM, Iscove's Modified Dulbecco's Medium; MI, Myocardial Infarction; MSCs, Mesenchymal Stem Cells; MSP, Macrophage Stimulating protein; PBS, Phosphate-Buffered Saline; PLL, Poly-L-lysine; SLA, Swine Leukocyte Antigens; TGF-ß1, Transforming Growth Factor beta 1; UPLVG, Ultrapure low viscosity and high guluronic; VEGF, Vascular Endothelial Growth Factor.

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invasive and requires treatment with immunosuppressants for life. An alternative for this treatment is the development of regenerative therapeutic strategies to reverse the progression of advanced heart failure, focusing on the regeneration of the affected cells. In this regard, transplanted mesenchymal stem cells (MSCs) are able to engraft and differentiate into vascular cells and cardiomyocytes, secreting paracrine factors and recruiting endogenous cardiac stem cells (Williams and Hare, 2011, Sheng et al., 2013, Chiu, 2003). Intramyocardial injection of MSCs, therefore, is being investigated as a therapeutic option in the treatment of chronic myocardial ischemia (Van Ramshorst et al., 2011, Van Ramshorst et al., 2009, Mathiasen et al., 2015, Mathur et al., 2020). Thus, hypoxic MSCs conditioned culture medium shows to reduce apoptosis and necrosis of isolated rat cardiomyocytes exposed to low oxygen in-vivo (Gnecchi et al., 2005), reappearance of myocardial tissue and restoration of contractility is detected after MSC implantation (Amado et al., 2006).

Cardiosphere derived cells (CDCs) have also emerged as an effective cell type for cardiovascular cell therapy. CDCs are obtained from cardiac tissue explants of euthanized large white pig from endomyocardial biopsy (Blázquez et al., 2016, Makkar et al., 2012, Smith et al., 2007). These cells are clonogenic, multipotent stem cells that secrete growth factors able to promote revascularization and support cardiac recovery after MI. For example, CDCs secrete insulin-like growth factor 1 (IGF-1), whose administration leads to the activation of cardiac stem cells, increasing cardiomyogenesis, and significantly improving cardiac function (Ellison et al., 2011, Báez-Díaz et al., 2020). Moreover, they release vascular endothelial growth factor (VEGF), a specific mitogen for vascular endothelial cells, whose expression is potentiated in response to hypoxia, inducing endothelial cell proliferation, promoting cell migration and inhibiting apoptosis (NEUFELD et al., 1999). CDCs also release macrophage stimulating protein (MSP) with a fundamental role inhibiting apoptosis during regeneration of endothelial cells and cardiomyocytes potential after myocardial infarction (Frangogiannis, 2015, Cantaluppi et al., 2008). Finally, they also secrete transforming growth factor beta 1 (TGF-ß1) cytokine contributing in the regulation of inflammation, extracellular matrix deposition, cell proliferation, differentiation and growth. TGF-ß1 causes the transformation of cardiac fibroblasts to myofibroblasts, helping in the regeneration after myocardial infarction when fibrotic tissues are formed to replace the area lost following cardiomyocytes necrosis (Tarbit et al., 2019, Shi et al., 2011). The joint action of released factors by CDCs plays a central role in vasculogenesis regulation, inducing angiogenesis and permeabilization of blood vessels and achieving myocardial regeneration, features that can prevent and reverse remodel the ischemically injured ventricle.

CDCs can be safely delivered via the intracoronary route after myocardial infarction reducing scarring, increasing viable myocardium, and boosting cardiac function in preclinical and clinical models (Makkar et al., 2012). Although intrapericardially administration of CDCs has shown safety and adequate biodistribution (Blázquez et al., 2016), the low engraftment rate and survival of transplanted CDCs following implantation remains a major challenge. The constant heart contraction, contributing to mechanical lost by squeezing injected cells out the myocardium, has led to low retention of transplanted cells in current preclinical studies, independently of the delivery method (Fukushima et al., 2013, Van Ramshorst et al., 2011). Cell alginate microencapsulation represents an alternative strategy to increase retention, thanks to a higher adherence of microcapsules that could circumvent the wash out into the bloodstream caused by the contractile heart forces (Paul et al., 2009).

Microencapsulation of cells allows their transplantation in absence of immunosuppression along a wide variety of diseases, with long retention in the engrafted tissue (Safley et al., 2008). In cell microencapsulation, immobilized foreign cells within a spherical polymeric matrix are protected from the host immune system by an artificial membrane, allowing nutrients to diffuse inside the capsule while releasing therapeutic products and waste outside (de Vos et al., 2006, Orive et al., 2010, Ciriza et al., 2015, Pérez-Luna and González-Reynoso, 2018). Microcapsules are three-dimensional hydrogels that can be delivered into the body via minimally invasive administration with good biocompatibility (El-Sherbiny and Yacoub, 2013, Lee and Kim, 2018). Alginate, a natural polymer isolated from brown algae (*Phaeophyceae*) that can form hydrogels in the presence of calcium ions with good biocompatibility and low toxicity (Lee and Mooney, 2012, Park et al., 2017), is a common biomaterial used in microencapsulation. Coating alginate microcapsules with poly-L-lysine (PLL) increase their stiffness, while a second coating with alginate, obtaining alginate-poly-L-lysine-alginate (APA) microcapsules, reduces immunological reaction after implantation.

We hypothesize that the administration of encapsulated CDCs will increase retention in the heart tissue after implantation, promoting the local and continuous release of biomolecules and growth factors, therefore promoting cardiac repair. However, microencapsulation can modify the nature of CDCs. In this paper, we demonstrate that cell encapsulation does not alter CDCs features, keeping a long viability and growth factor release, and providing a step forward to clinical translation of CDCs therapy in the regeneration of myocardial infarction.

2. Material and methods

2.1. Materials

Poly-L-lysine and calcium chloride ($CaCl_2$) were purchased from Sigma Aldrich (Madrid, Spain). Ultrapure low viscosity and high guluronic (UPLVG) alginate were purchased from NovaMatrix (FMC Biopolymer, Norway). Roswell Park, Dulbecco's Modified Eagle's Medium (DMEM) Ham's F12 with UltraGlutamina 3.996 mM, Iscove's Modified Dulbecco's Medium (IMDM), fetal bovine serum and penicillin–streptomycin solution were purchased from Lonza (Switzerland), phosphate-buffered saline (PBS), Dulbecco's phosphate-buffered saline (DPBS), trypsin-EDTA (0.25%), and LIVE/DEAD kit were purchased from Life Technologies (Invitrogen S.A., Spain). The ELISA Kits MST-1, TGF-ß1, IGF-1, and VEGF, were purchased from Elabscience, R&D Systems Inc., LifeSpain BioScience, Inc. and My BioSource, Inc. respectively.

2.2. Cell culture

CDCs were obtained from cardiac tissue explants of euthanized pigs. Tissues were subjected to three successive enzymatic digestions, culturing explants with complete explant medium composed by 2 mM L-glutamine (Lonza), 1% penicillin–streptomycin (Lonza), 0.2 mM 2-mercaptoethanol (Sigma), and 10% Fetal Bovine Serum (FBS) in IMDM (HyClone) at 37 °C and 5% CO_2. Three weeks after culture, fibroblast-like cells migrating from tissue explants were seeded. Suspended cells clusters, called cardiospheres, were formed, and cells migrating from those cardiospheres, called CDCs, were selected. CDCs were seeded again and expanded with complete explant medium at 37 °C and 5% CO_2. CDCs expanded as a monolayer cell culture were observed under optical microscopy.

Cardiosphere-derived cells were cultured in complete medium consisting of 65% DMEM Ham's F12 with UltraGlutamina a 3.996 mM and 35% IMDM supplemented with 10% of FBS, 1% Penicillin-Streptomycin, 2 mM L-glutamine (Invitrogen), and 0.1 mM 2-mercaptoethanol (Sigma Aldrich, United States). Cells were maintained at 37 °C in humidified 5% CO_2 atmosphere, and passaged every 2–3 days.

2.3. Cell encapsulation

UPLVG alginate was resuspended in 1% mannitol to obtain 1.5% (w/v) alginate and 0.1% alginate (FMC Biopolymer, Norway). Final solutions were filtered through a 0.22 μm syringe filter (Millipore, MA,

USA). Next, cardiosphere-derived cells were suspended at 5×10^6 cells/mL in alginate 1.5% solution and extruded with an electrostatic atomization generator (Nisco, Switzerland) and a peristaltic pump (flow rate: 5.9 mL/h). The resulting microcapsules were completely gelled by agitation for 15 min in a 55 mM $CaCl_2$ solution. Next, microcapsules were ionically linked with 0.05% (w/v) PLL by agitation for 5 min, followed by a second coating with 0.1% alginate for another 5 min. All the procedures were performed at room temperature, under aseptic conditions, and maintained with complete medium at 37 °C, in a humidified 5% CO_2 and 95% air atmosphere standard incubator. Microcapsules morphology and diameter were examined under an inverted optical microscopy (Nikon TSM, Japan).

2.4. Cell viability

Encapsulated CDCs viability was assessed by microscopy imaging after calcein ethidium staining. Briefly, 50 μL of microcapsules were rinsed twice in DPBS (Gibco, United States), next resuspended in 500 μL of 0.5 μM calcein AM (Life Technologies, United States) and 0.5 μM ethidium homodimer-1 (Life Technologies, United States). The samples were incubated in 24-well-plates for 45 min at room temperature, protecting from light. Samples were observed under a Nikon Eclipse TE2000-S confocal microscope with 470 nm wavelength for calcein AM and 575 nm wavelength for ethidium homodimer staining. Random images were analyzed with the NIS Elements AR software, version 4.51.00. At least three independent experiments were performed for each condition.

2.5. Metabolic activity

Metabolic activity was determined at day 1, 7, 21 and 42 of culture by Cell Counting Kit-8, CCK-8 (SigmaAldrich, United States), following the manufacturer recommendations. Briefly, 25 μL of microcapsules and 1.25×10^5 of non-encapsulated cells were rinsed, resuspended into 500 μL of culture medium supplemented with 50 μL of CCK-8 reagent, and plated in 5 wells of a 96-well plate. After incubation for 4 h at 37 °C, absorbance was read out on an Infinite M200 TECAN plate reader (TECAN Trading AG, Switzerland) at 450 nm and corrected at 650 nm.

2.6. Phenotypic analysis of non– encapsulated and encapsulated CDCs by flow cytometry

Non-encapsulated CDCs were detached from culture flasks with 0.25% trypsin solution, washed twice with PBS and suspended in culture medium. Encapsulated CDCs were previously incubated with 1 mg/mL alginate lyase (SigmaAldrich, United States) for 30 min at 37 °C. After washing twice with DPBS, the lysates were spun down and rinsed twice with DPBS. Cells extracted from microcapsules were treated following the same procedure than non-encapsulated CDCs. All samples were stained with FITC -conjugated porcine monoclonal antibodies against Mouse anti Pig CD31: FITC (LCI-4), Rat anti Pig wCD44: FITC (MAC329), Mouse anti Pig CD45: FITC (K252.1E4), Mouse anti Pig CD61: FITC (JM2E5), Mouse anti Pig SLA Class I: FITC (JM1E3) and Mouse anti Pig SLA Class II DR: FITC (2E9/13) purchased in Bio-Rad (United States), Mouse anti-CD90/Thy1: FITC (5E10) and Mouse anti-CD105: FITC (MEM-229) purchased in Abcam plc (London). The following isotype negative control antibodies were used as fluorescence minus one controls: Mouse IgG1 (FITC) - Isotype Control, Mouse IgG1 Negative Control: FITC, Rat IgG1 Negative Control: FITC, Mouse IgG1 Negative Control: FITC, Mouse IgG2a, kappa monoclonal - Isotype Control: FITC and Mouse IgG2b Negative Control: FITC (Bio-Rad, United States). DAPI (3.3 μM) was added before analysis to exclude dead cells. At least 10^6 cells were analyzed using a MacQuant Analyzer 10. Unstained cells were used to evaluate autofluorescence. In all cytometry experiments, at least three samples were analyzed for each condition. Flow cytometry analysis was performed by FlowJo software, version 8.8.7 (TreeStar, Ashland, OR).

2.7. Molecular characterization of cardiosphere-derived cells by RT-PCR

The expression of the following markers was analyzed by RT-PCR: *Kit, Nanog, Mef2c, Gata4, Cx43, Tnni3, Actc1, Vegf-a, Igf-1. Igf-1r, Hgfl, Fgfr2* and *Tgf-β1*. Cells were de-encapsulated by incubating with 1 mg/mL alginate lyase (Sigma Aldrich, United States) for 30 min at 37 °C. The lysates were spun down and rinsed twice with DPBS. After complete lysis of microcapsules, microcapsules pellets and non-encapsulated cells were resuspended in RLT buffer from the RNeasy Mini Kit (Qiagen, Germany). RNA was extracted following the manufacturer recommendations. Total extracted mRNA was quantified with a SimpliNano nanodrop (GE Healthcare Life Sciences, Iceland). cDNA synthesis was performed using Fast Gene Scriptase II, cDNA Synthesis Kit (Genetics, Nippon, Europe), following PCR amplification with the KAPA2G Robust PCR kit (Sigma Aldrich, United States) in a T100 Thermal Cycler (Bio-Rad, United States). Amplicons were run in 1.5% agarose gels in parallel to a 2-log ladder 1 kb Plus DNA Ladder (New England BioLabs), confirming their expected size. Beta-actin (*Actb*) was used as housekeeping gene. Gene expression was quantified by measuring the brightness intensity of each band with Bio-Rad-Image-Lab-Software-5.2.1, normalizing to housekeeping gene expression. At least three independent experiments were performed for each gene and condition.

2.8. Adipogenic, chondrogenic and osteogenic differentiation of porcine CDCs

To induce adipogenic and osteogenic differentiation $1x10^5$ cells/mL were seeded into a 6-well-plate. For osteogenic differentiation growth CDCs medium was supplemented with 100 nM dexamethasone, 20 mM ß-glycerolphosphate and 50 μM L-ascorbic acid. Adipogenic differentiation was induced culturing with growth CDCs medium supplemented with 0.5 μM dexamethasone, 0.5 μM 3-isobutyl-1-methylxanthine, and 50 μM of indomethacin. To induce chondrogenic differentiation, $2.5x10^5$ cells/mL were seeded in 15 mL conical tubes (Falcone) with 0.5 mL of growth CDCs medium supplemented with 10 ng/mL TGF-ß (Peprotech Inc.), 50 nM L-ascorbic acid and 6.25 μg/mL bovine insulin. Each differentiation medium was replaced every 2–3 days for 21 days. Cells were maintained at 37 °C in humidified 5% CO_2 atmosphere. For all the differentiation conditions the corresponding control, corresponded to CDCs growth in medium without supplements. For staining, after 21 days, non-encapsulated and encapsulated cells were washed twice with PBS and DPBS respectively, fixed with formaldehyde and stained with: Alizarin Red S for osteogenic differentiation, Oil Red O for adipogenic differentiation, and Alcian Blue 8GX for chondrogenic differentiation. Differentiated cells were observed by optical microscopy at day 21 and imaged.

2.9. Growth factors release quantifications

Supernatants after 24 h of complete medium change from cultures that began with a cell seeding density of $2.5x10^5$ CDCs or same density of encapsulated CDCs were collected at day 1, 7, 14, 21 and 42, and stored at −80 °C until further processing. Porcine cytokines levels of MSP, TGF-ß1, IGF-1 and VEGF were quantified by ELISA according to manufacturer's instructions. The recommended absorbance was read out on an Infinite M200 TECAN plate reader (TECAN Trading AG, Switzerland) at the recommended wavelengths. The concentration of each growth factor was calculated from the respective standard curve and expressed as pg/mL and ng/mL. At least three independent experiments were performed to collect supernatants and the read out for each growth factor was performed three times.

2.10. Statistical analysis

Statistical analysis was performed with IBM SPSS software, version 13.0. Data were expressed as means ± standard deviation, and differences were considered significant for comparison of groups using ANOVA, Tukey's Post Hoc Test when $p < 0.05$. Normality tests were performed to confirm a normal distribution.

3. Results and discussion

3.1. Long term-encapsulated CDCs metabolic activity and viability.

We chose to isolate the clonogenic porcine CDCs (Blázquez et al., 2016) in order to evaluate their regenerative capacity after encapsulation. Previous studies have shown that the percentage area of viable myocardium within the infarct from mice treated with CDCs for 20 days is higher than control group (Smith et al., 2007). Although CDCs express stem and endothelial progenitor cells markers, being capable of self-renewal and differentiation in vitro (Messina et al., 2004), their retention and survival after implantation is very low, and their exogenous origin can lead to immune response. Alginate microencapsulation, therefore, can represent a good alternative by increasing adherence in the tissue and reducing immunological reaction after implantation (Van Ramshorst et al., 2011, Paul et al., 2009). We used ultrapure low viscosity and high guluronic, since gels formed from alginate at high purity does not induce significant foreign body reaction after animal implantation (Orive et al., 2002, Lee et al., 2009).

CDCs expanded in a plastic-adherent monolayer cell culture when observed under optical microscopy (Fig. 1A). CDCs collected from these cultures were encapsulated in alginate-poly-L-lysine microcapsules at 4 × 10^6 cell/mL density. The physical appearance and morphology of the encapsulated CDCs were assessed under optical microscopy. Encapsulated cells were spherical, with an average diameter of 370 ± 10 μm and a smooth homogeneous surface, similar to other cell types encapsulated with the same conditions by our group, such as MSCs, myoblasts or fibroblasts (Cañibano-Hernández et al., 2019, Saenz Del Burgo et al., 2018, Ciriza et al., 2018, Ciriza et al., 2017, Ciriza et al., 2015). Metabolic activity of encapsulated cells progressively increased until day 21 of culture after encapsulation, reaching its maximum at this time point (Fig. 1B), similarly to the metabolic activity profile shown by encapsulated MSCs (Ciriza et al., 2018). Longer term culture up to 42 days was required to detect a significant metabolic activity decrease (Fig. 2B). CDCs, therefore, seem to adapt to the microenvironment provided by alginate matrix.

Metabolic activity profile was confirmed by the analysis of encapsulated CDCs viability under fluorescence microscopy after calcein ethidium staining during the same time of culture. Most of the encapsulated cells were stained with calcein, not showing almost ethidium staining, and indicating a lack of cell death during all the time points studied (Fig. 1C). We could also determine that the shape and volume of the capsules remain stable after 42-day culture period. The long-term stability of the capsules and the preservation of their morphology are requirements to preserve their biocompatibility, closely related to the quality of the biomaterial (Ponce et al., 2006). We consider, therefore, that the long term preservation of capsule integrity, their spherical and smooth shape without irregularities will prevent undesired host reactions in a future clinical application (Orive et al., 2003).

3.2. Encapsulated CDCs keep the same phenotype than non-encapsulated CDCs

We next proceeded to compare the encapsulated CDCs extracellular profile before and after encapsulation, in order to determine if encapsulation would modify their phenotype. We assessed the cell marker phenotype of encapsulated and non-encapsulated porcine CDCs by flow cytometry (Fig. 2), analyzing among others the expression of mesenchymal markers such as CD29, CD44, CD90, CD105 and CD117, since CDCs phenotype is described as similar to MSCs phenotype (Blázquez et al., 2016). Although marker panels for MSCs are rather heterogeneous, we also identified the expression of CD73, CD29, or CD44 and the lack of expression of CD45, since this profile is considered a criteria for expanded MSC in several clinical trials (Rojewski et al., 2008).

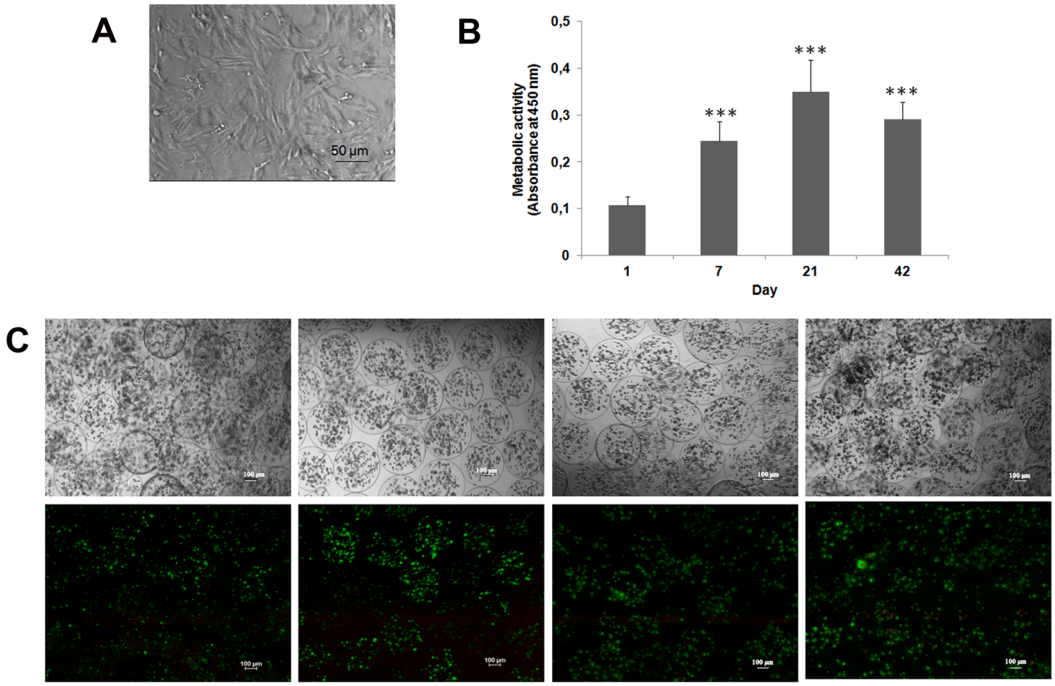

Fig. 1. Cell morphology and expansion, metabolic activity and cell viability. (A) CDCs morphology in monolayer cell culture. Scale bar: 50 μm. (B) Metabolic activity of the encapsulated CDCs throughout the 42-day period. Values represent mean ± SD. (C) Live-Dead assay (staining with calcein-ethidium) during 42-day period, cells stained in red are dead cells and the stained in green are living cells. Scale bar: 100 μm.

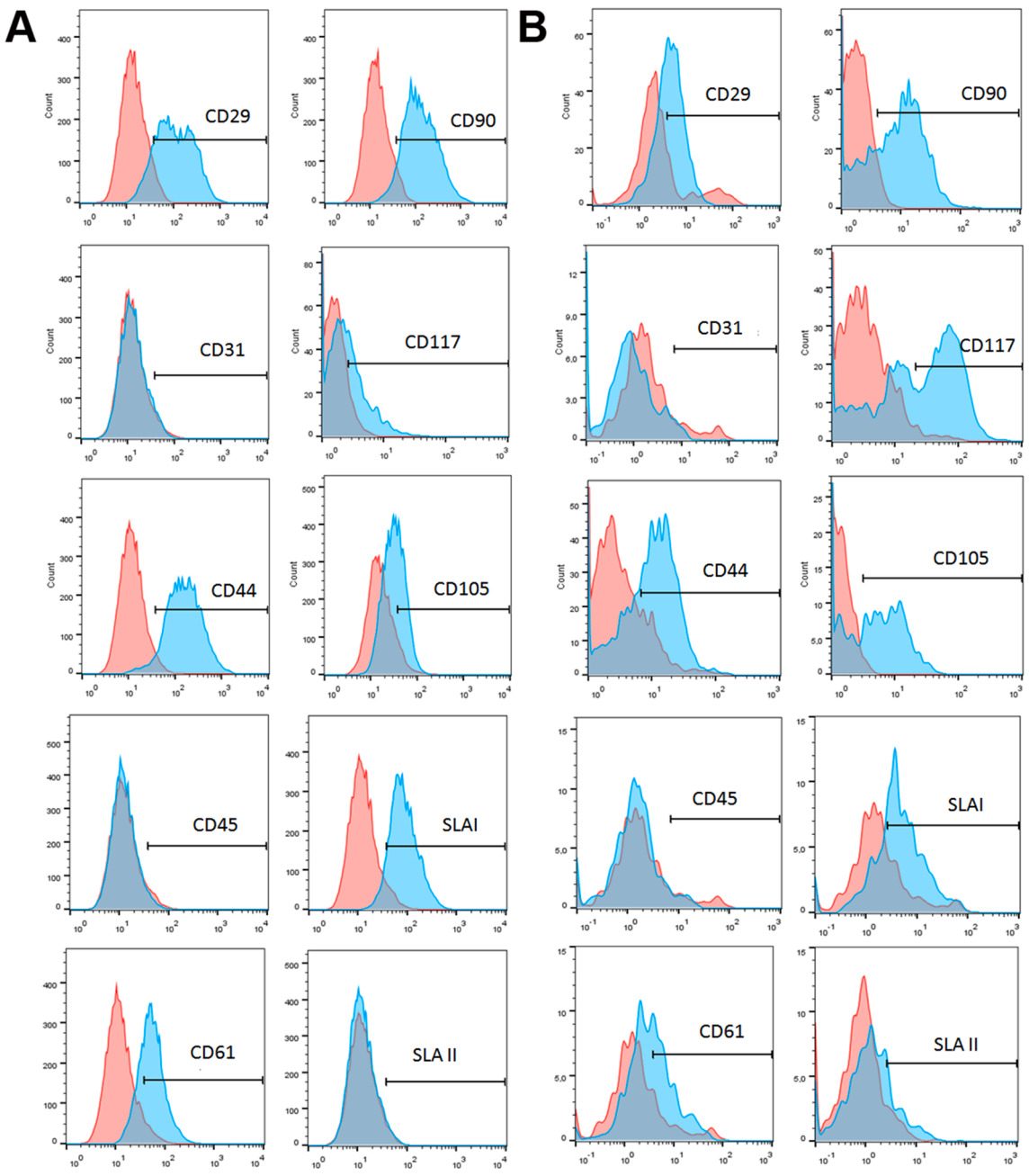

Fig. 2. Phenotype characterization of encapsulated and non-encapsulated CDCs. Dark and light histograms show isotype control and specific antibody stained sample respectively. Values represent mean ± SD for the positive stained cell population. (A) Phenotype characterization of non-encapsulated cells. (B) Phenotype characterization of encapsulated cells.

Thus, encapsulated CDCs, similarly to non-encapsulated CDCs, expressed CD29 and CD90 (Fig. 2). CD29 (ß1 integrin), expressed by adipocyte progenitors (Rodeheffer et al., 2008) and human epidermal stem cells (Jones and Watt, 1993), is observed in combination with other cluster of differentiation markers for the identification of MSCs, such as CD90, also known as thymocyte antigen 1, commonly associated with osteoprogenitor cells (Chung et al., 2013). Encapsulated CDCs kept expression of CD44, involved in the interaction between stem cells and the niche for stemness maintenance (Wagner et al., 2008), commonly used as marker of MSCs in combination with other markers, such as CD29 (Rojewski et al., 2008). A small increment of CD105 expression was detected in encapsulated CDCs compared to non-encapsulated CDCs. Differences in expression of CD105 has also been reported when comparing MSCs from different sources, with high expression in bone marrow derived MSCs and lower in amnion membrane-derived MSCs (Rojewski et al., 2008). We consider that the interaction with alginate could activate the expression of CD105. The expression of CD117, known as cKit, was also increased in encapsulated CDCs. The expression of CD117 in MSCs is controversial since some authors have described that MSCs derived from bone marrow do not express CD117 (Jones et al., 2002), while other have shown an expression in 50% of the MSC population (Titorencu et al., 2007). Other authors have also described the expression of CD117 in MSCs in gelatin-alginate hydrogels (Joddar et al., 2018), and it could suggest that alginate activate the expression of the stemness related marker CD117.

As expected, a lack of the hematopoietic marker CD45 expression was shown by CDCs, either encapsulated or non-encapsulated, accomplishing with the criteria stablished by the International Society for

Cellular Therapy, ISCT (Dominici et al., 2006). Similarly, no expression of platelet endothelial cell adhesion molecule (PECAM-1 or CD31) was observed in CDCs, either encapsulated or non-encapsulated. In contrast, CDCs expressed CD61, either in non-encapsulated or encapsulated CDCs, as previously reported for MSCs (Duggal et al., 2009). Finally, swine leukocyte antigens (SLA), SLA I and SLA II, the homologs to the human leukocyte antigens (HLA) HLAI and HLA II respectively, were also assessed (Ladowski et al., 2018). Non-encapsulated CDCs expressed SLA I but not SLA II (Fig. 2), with similar pattern in encapsulated CDCs, accomplishing with ISCT criteria (Dominici et al., 2006). Although microcapsules can evade immune cells after implantation based on the restriction of the diffusion of immune mediators through the pores of the microcapsule (Ashimova et al., 2019), the lack of SLAII expression, in combination with encapsulation, can lead to circumvent the rejection after transplantation of encapsulated CDCs (Essler et al., 2013), avoiding the presentation of non-self-antigens by HLA and, therefore, the activation of $CD4^+$ cells (Zakrzewski et al., 2014).

3.3. *Cdcs resemble MSCs differentiation with higher potential to differentiate into osteocytes after cell encapsulation.*

Another criterion to define human MSC is its ability to differentiate to osteoblasts, adipocytes and chondrocytes in vitro according to ISCT (Dominici et al., 2006). Therefore, we characterized the differentiation potential of CDCs into osteoblasts, adipocytes and chondrocytes before and after cell encapsulation. After 21 days of differentiation, the presence of a calcified extracellular matrix in the samples confirmed the potential of CDCs to be differentiated into osteocytes (Fig. 3A). More intense staining was detected in differentiated encapsulated CDCs (Fig. 3B), compared to non-encapsulated CDCs, indicating an enhancement in the potential of differentiation into osteocytes. This differentiation potential increment could be related to the lack of three-dimensional architecture of natural tissues in non-encapsulated CDCs (two-dimensional culture). Two-dimensional cultures could lead to the deterioration of MSCs' progenitor properties (Fehrer and Lepperdinger, 2005), while encapsulation would improve osteogenic differentiation of the CDC (Bae et al., 2017).

No differences were detected 21 days after differentiation into adipocytes, with presence of fat vacuoles in encapsulated and non-encapsulated CDCs, indicating their ability to differentiate to adipose tissue (Fig. 3A), without differences when encapsulated CDCs were differentiated (Fig. 3B). When differentiation into chondrocytes was assessed after 21 days, sulfated proteoglycan deposits were observed in either non-encapsulated and encapsulated CDCs (Fig. 3A-B). Considering the described potential of encapsulated CDCs, we were able to confirm the mesenchymal potential differentiation maintenance in CDCs after encapsulation, with an enhancement of differentiation into osteocytes after encapsulation.

3.4. *Cardiac progenitor gene expression of CDCs is maintained after encapsulation*

We next quantified the RNA expression from encapsulated and non-encapsulated CDCs of several markers related to stemness, early cardiac differentiation and mature cardiomyocytes, as well as the expression of several growth factors and their receptors, since they represent specific roles in cardiac regeneration after myocardial infarction. To analyze the expression of those markers, total RNA from encapsulated and not encapsulated CDCs was isolated, and RT-PCR analysis performed (Fig. 4). The results showed a positive expression of the stemness related genes markers (*Kit, Nanog*), early cardiac differentiation related genes markers (*Mef2c* and *Gata-4*), mature cardiomyocytes related genes markers (*Cx43*, *Tnni3* and *Actc1*), as well as different growth factors and their receptors (*Vegf-A, Igf-1*, *Igf-1r*, *Hgfl*, *Fgfr2* and *Tfg-β1*).

Significant differences ($p < 0.05$) were observed between encapsulated and non-encapsulated CDCs *Kit* expression (Fig. 4), with an increment of expression after encapsulation of CDCs. This increment of *Kit* expression is correlated with data observed by flow cytometry, showing $CD117^+$ encapsulated CDCs and $CD117^{low}$ in non-encapsulated CDCs (Fig. 2). The glucoprotein CD117 is related to apoptosis regulation,

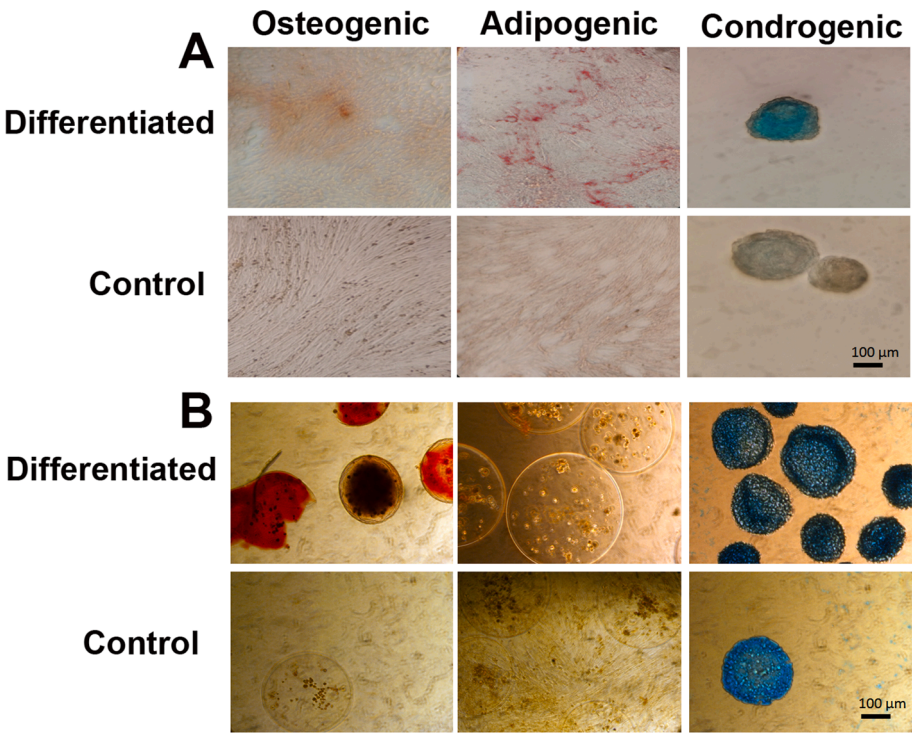

Fig. 3. Differentiation of non-encapsulated and encapsulated CDCs with their corresponding controls. (A) Micrographs after 3 weeks of osteogenic, adipogenic and chondrogenic non-encapsulated CDCs differentiation. (B) Micrographs after 3 weeks of osteogenic, adipogenic and chondrogenic encapsulated CDCs differentiation. Scale bar: 100 µm.

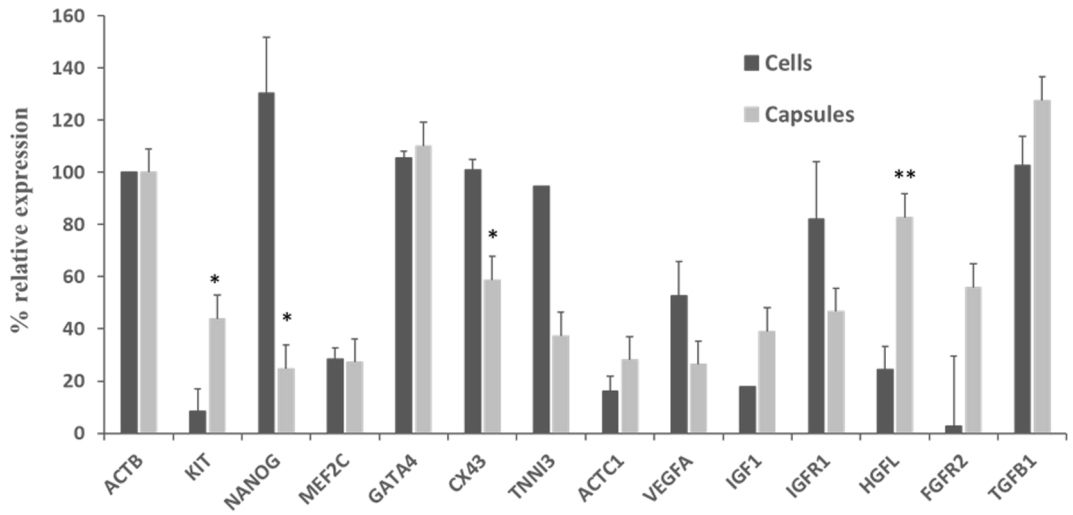

Fig. 4. Gene expression of non-encapsulated and encapsulated CDCs. Data represent the fold regulation between the gene of interest and the housekeeping. Note: ***: $p < 0.001$; **: $p < 0.01$ and *: $p < 0.05$ comparing non-encapsulated CDC to encapsulated CDC gene expression of each gene.

cell differentiation, proliferation, chemotaxis, cell adhesion and also stemness (Miettinen and Lasota, 2005). In fact, $CD117^{+}CD45^{dim/moderate}$ mast cells have been proposed as putative cardiac progenitor cells. We believe that the expression of CD117 (Fig. 2) indicate that CDCs could differentiate to more mature cardiac progenitors after encapsulation, acquiring the phenotype of the aforementioned cardiac progenitor-mast cells (Zhou et al., 2010). In contrast, *Nanog* expression significantly decreased after encapsulation (Fig. 4). *Nanog* is a pluripotency factor specifically expressed in stem cells, but rarely in their differentiated derivatives. Its expression in stem cells-differentiated derivatives is related to form teratomas after transplantation (Rong et al., 2012), reducing the appearance of teratomas by inhibiting *Nanog* (Heaney et al., 2012, Rong et al., 2012). The down-regulation of *Nanog* in encapsulated CDCs, therefore, indicates a lower risk in teratoma formation, maybe related with the differentiation of CDCs into more mature cardiac progenitors, suggesting a safe clinical transplantation of encapsulated CDCs in terms of teratoma formation.

We did not detect significant differences in the expression of *Gata4* and *Mef2c* transcription factors when comparing encapsulated and non-encapsulated CDCs. *Gata4* was highly expressed in both samples, corroborating the cardiomyocyte phenotype of CDCs (Fig. 4), since *Gata4* is highly expressed in cardiomyocytes after a trauma throughout the subepicardial ventricular layer (Kikuchi et al., 2010), and fibroblasts can be directly reprogrammed to cardiomyocytes by overexpressing a combination of the cardiac specific transcription factors *Gata4* and *Mef2c* (Tani et al., 2018). When analyzing the expression of *Cx43*, a mature cardiomyocytes related gene marker, significant lower expression was quantified in encapsulated CDCs (Fig. 4). *Cx43* is the major gap junction protein expressed in the heart, altered substantially after myocardial infarction (Fontes et al., 2012). We consider that the reduction of *Cx43* expression in encapsulated CDCs reflect the lower interaction between cells in the 3D scaffold compared to 2D cultures, not indicating a lack of mature cardiac marker. Moreover, *Cx43* expression enhancement to reduce susceptibility to arrhythmias is still unclear, since its reduced expression attenuates ventricular remodeling after myocardial infarction via impaired *Tgf-β1* signaling (Zhang et al., 2010). No significant differences were detected between encapsulated and non-encapsulated CDCs when *Tnni3* or *Actc1* were compared, codifying troponin I and actin alpha cardiac muscle 1 respectively. Both are normally expressed in cardiac muscle, indicating that the cardiac phenotype of CDCs is kept after encapsulation.

The expression of the pro-angiogenic vascular endothelial growth factor receptor (*Vegf-a),* did not show significant differences between non-encapsulated and encapsulated CDCs (Fig. 4), indicating that their implantation *in vivo* will lead to an increase in the number of endothelial cells and shorter extravascular distances in the infarct zone, improving vascularization and oxygen diffusion (Zak et al., 2019). No significant differences were also observed in the expression of insulin-like growth factor 1 (*Igf-1)* and insulin-like growth factor 1 receptor (*Igf-1r)* between encapsulated and non-encapsulated CDCs. We also quantified significant higher expression of *Hgfl* in encapsulated CDCs compared to non-encapsulated. Since, hepatocyte growth factor-like protein (*Hgfl*) is a potential candidate for boosting migration, engraftment and commitment of resident cardiac stem cells (Madonna et al., 2010), similarly to its behavior in liver regeneration (Bezerra et al., 1994), these results point out to encapsulated CDCs as a putative tool for cardiac regeneration (Madonna et al., 2012).

When analyzing the expression of *Fgfr2*, the fibroblast growth factor receptor 2 involved in diverse biological and cellular processes, such as cell proliferation, apoptotic resistance, angiogenesis, differentiation and tissue regeneration (Katoh and Nakagama, 2014), non-significant higher expression was quantified in encapsulated CDCs (Fig. 4). We also did not detect a significant difference in the expression of *Tgf-β1*, tumour growth factor beta 1, comparing encapsulated and non-encapsulated CDCs. Overall, we can conclude that cardiac progenitor gene expression of CDCs is maintained after encapsulation with slight beneficial modifications after encapsulation, such as decrease in *Nanog* or an increment in *cKit* or *Hgfl* expression.

3.5. Sustained release of growth factors from encapsulated CDCs

Next, we quantified the release of growth factors involved in cardiac regeneration. We collected supernatants from encapsulated and non-encapsulated CDCs during 42-days, quantifying several growth factors. Thus, we identified a sustained and gradual release of VEGF, in either encapsulated or non-encapsulated CDCs during 42 days without significant differences. Moreover, no significant differences were identified between encapsulated and non-encapsulated CDCs VEGF release (Fig. 5. A), indicating that encapsulation does not affect the release of VEGF. Since VEGF induce vasculogenesis and angiogenesis, stimulating the formation of blood vessels to providing more effective muscle regeneration (Frey et al., 2012) and restoring the oxygen supply into tissues when blood circulation is inadequate in hypoxic conditions, we can conclude that the implantation of encapsulated CDCs into cardiac infarcted tissues would promote blood vessels formation and oxygen supply, enhancing cardiac tissue regeneration.

We also quantified TGF-ß1 release from encapsulated and non-encapsulated samples for 42 days. A gradual increment in TGF-ß1

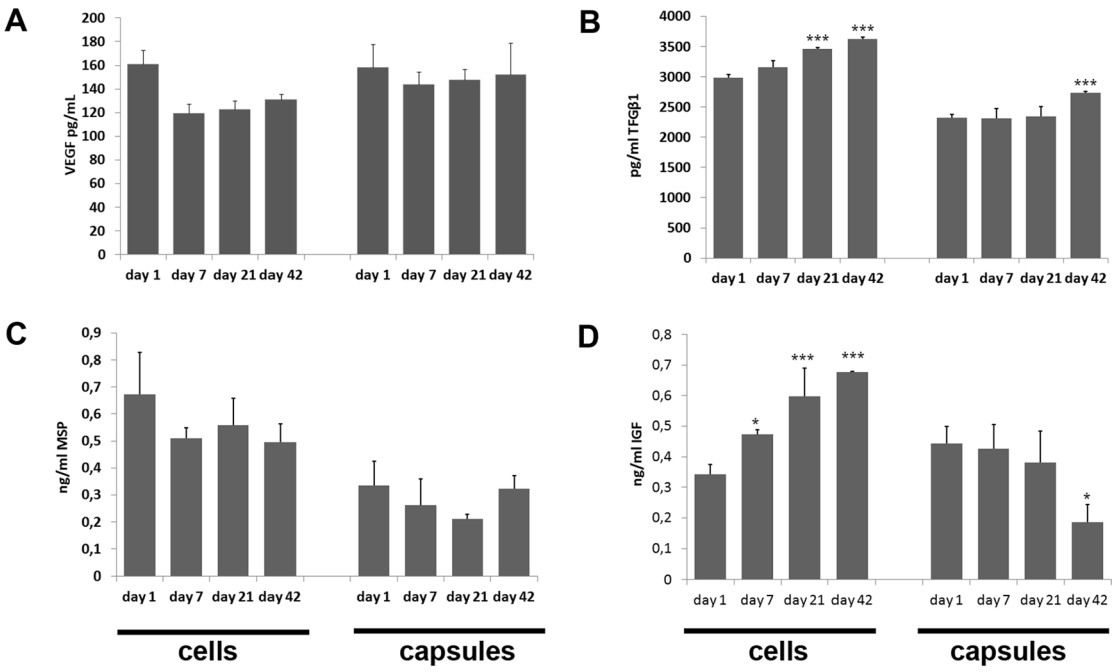

Fig. 5. Release profile of different growth factors in non-encapsulated and encapsulated CDCs throughout 42-days period. (A) VEGF (pg/mL). (B) TFGß1 (pg/mL). (C) MSP (ng/mL). (D) IGF (ng/mL). Note: ***: $p < 0.001$; **: $p < 0.01$ and *: $p < 0.05$. compared to day 1.

release was quantified in non-encapsulated CDCs, with significant differences at day 21 and 42 compared to day 1. In contrast, in encapsulated CDCs, a significant TGF-ß1 release increment was only quantified at day 42 of collection. Moreover, a significant lower TGF-ß1 release was quantified at each time point when comparing non-encapsulated CDC to its encapsulated counterpart (Fig. 5.B). Interestingly, the quantification of TGF-ß1 gene expression was non-significantly higher in encapsulated CDCs, indicating that there is partial retention of the growth factor in the microcapsules that precludes its release. TGF-ß1 release is primordial in regeneration after myocardial infarction, since promotes the transformation of cardiac fibroblasts to myofibroblasts, (Tarbit et al., 2019, Chen et al., 2016) and regulates the inflammatory and reparative response following infarction, with a wide range of actions on vascular cells, cardiomyocytes, fibroblasts and immune cells (Hanna and Frangogiannis, 2019, Frangogiannis, 2017). However, TGF-β1 overexpression in the mouse heart is associated with fibrosis and hypertrophy (Rosenkranz et al., 2002, Huntgeburth et al., 2011). Therefore, a sustained TGF-β1 release without gradual increment overtime could result more convenient if CDCs are implanted in cardiac infarcted models, pointing out to encapsulated CDCs as a more reliable therapy for clinical cardiac regeneration.

We also quantified a progressive decrease in MSP release overtime in either non-encapsulated or encapsulated CDCs, with a slight increment at day 42 in encapsulated CDCs (Fig. 5.C). No significant differences were identified between non-encapsulated and encapsulated CDCs, indicating that encapsulation does affect the MSP release, although a slight retention is detected by alginate microcapsules. MSP is a factor related to hepatocyte growth factor (Brunelleschi et al., 2001), that exerts proliferative and antiapoptotic effects, being upregulated during regeneration of injured tubular cells, and exerting biological effects that may aid recovery from acute kidney injury (Cantaluppi et al., 2008). In the heart, infiltrating macrophages can be primed to acquire a cardioprotective phenotype in ischemic heart, exerting a proactive effect through activation of an antiapoptotic program in cardiomyocytes (Frangogiannis, 2015). Although the release of encapsulated CDCs is lower than non-encapsulated CDCs, we envision that the sustained released of this factor could exert a protective effect against apoptosis after infarction.

Finally, we quantified IGF-1 release from encapsulated and non-encapsulated CDCs. A significant progressive IGF-1 release increment overtime was quantified in non-encapsulated CDCs (Fig. 5.D). In contrast, encapsulated CDCs showed a sustained IGF-1 release with a significant decrease at day 42 after encapsulation, indicating again a partial retention of the growth factor in the alginate microcapsules, supported by the higher *Igf-1* expression quantified in encapsulated CDCs (Fig. 4). Transplantation of MSCs secreting IGF-1 after a myocardial infarction significantly increases neovascularization, inhibiting apoptosis of cardiomyocytes after third day of transplantation (Enoki et al., 2010). Similar to MSP, we consider that, even with a lower IGF-1 release compared to non-encapsulated CDCs, the sustained release of IGF-1 would benefit in cardiac regeneration during the first month of transplantation. Overall, we can conclude that the implantation of encapsulated CDCs will promote infarcted tissue regeneration, by a sustained release of the aforementioned factors, giving rise to the formation of new blood vessels, allowing the maturation of cardiac fibroblasts into myofibroblasts, and inhibiting apoptosis.

4. Conclusions

We have shown that porcine CDCs encapsulation does not alter CDCs features, while maintaining a long viability up to a month. In fact, encapsulated CDCs keep the same phenotype than non-encapsulated CDCs, with a phenotype similar to MSCs, resembling also MSCs differentiation potential into osteoblasts, adipocytes and chondrocytes, with a slight increment in differentiation into osteoblasts. However, our results show that encapsulation activates the expression of the stemness related marker CD117, correlating the gene expression with the surface protein expression. These results, the quantified decrease of *Nanog* expression after encapsulation and a gene expression profile similar to cardiac progenitor cells, suggest that encapsulated CDCs are committed cardiac progenitor cells, with low risk of teratoma formation if encapsulated CDCs would be implanted. Interestingly, we have also quantified a sustained release of the growth factors VEGF, TGF-ß1, MSP and IGF-1 from encapsulated CDCs, suggesting that the implantation of encapsulated CDCs would promote the formation of new blood vessels and the regeneration of infarcted cardiac tissue. Altogether, we can conclude

that encapsulated CDCs represent an alternative in cardiac regenerative medicine of myocardial infarction, providing a step forward to their clinical translation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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