Cellular Reprogramming
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Cellular Reprogramming

DOES THE BOVINE PRE-OVULATORY FOLLICLE HARBOUR PROGENITOR STEM CELLS?

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3 **Running head:** characterization of progenitor granulosa cells in bovine
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8 DOES THE *BOVINE PRE-OVULATORY FOLLICLE HARBOUR PROGENITOR STEM CELLS?*
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ABSTRACT

Recent studies have revealed the presence of a MSC population in human and in gilt granulosa membrane, thus increasing the interest in identifying the same population in the bovine species. We first isolated granulose cells (GCs) by scraping from bovine pre-ovulatory follicles and then tested several different media to define the ideal conditions to select granulosa-derived stem cells. Although expressing MSC-associated markers, none of the media tested proven to be efficient in selecting MSC-like cells, able to differentiate into mesodermic or ectodermic lineages. Then, we performed another experimental approach exposing cells to a chemical stress, such as lowering of pH, as a system to select a more plastic population. Following the treatment, granulosa specific granulose markers (*FSH-R, FST* and *LIF-R*) were lost in bovine granulosa cells, whereas an increase in multi- (*CD29*, *CD44*, *CD73*) and pluripotent (*Oct-4* and *c-Myc*) genes was noticed. The stress allowed up-regulation of TNF-α and IL-1β expression and the de-differentiation of GCs that was demonstrated by differentiation studies. Indeed, pH-treated cells were able to differentiate into the mesodermic and ectodermic lineages, thus suggesting that the chemical stress allows for the selection of cells that are more prone to adjust and respond to the environmental changes.

INTRODUCTION

As in humans, also in veterinary medicine, mesenchymal stem cells (MSCs) have been harvested successfully from a wide range of adult tissues as bone marrow, adipose tissue, endometrial polyps, menses blood, etc. [1]. Adult stem cells are currently the most used stem cell type for clinical applications [2] but, usually, their isolation requires invasive techniques for sampling, not devoid of complications. Moreover, their culture and expansion *in vitro* is time-consuming and cells obtained display an ability to proliferate and differentiate that is inversely proportional to the age of the donor and to the number of *in vitro* passages [3]. Adult tissues are not satisfactory also for the low yield of stem cells that can be obtained, thus representing a strong limitation for the therapeutic application of these cells in veterinary and human medicine.

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3 Based on these considerations, the interest to the clinicians to find alternative sources of multipotent
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5 cells has increased over the past years. MSCs from the fetal adnexa have been reported as a
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7 potential tool to overcome some of these limitations, opening up new prospects for the development
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9 of regenerative medicine [4]. Recently, other research groups have suggested the granulosa
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11 membrane as a promising adult source of MSCs in human [5] and in gilt [6]. The investigation of
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13 Kossowska-Tomaszczuk et al. [5] refers to luteinizing granulosa cells (GCs) recovered from pre-
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15 ovulatory follicles of patients treated for oocyte retrieval. Mattioli et al. [6], instead, compared GCs
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17 retrieved from growing follicles before the pre-ovulatory gonadotropin surge, - and hence before
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19 luteinisation (e.g., in a condition of active proliferation and before the final differentiation)-, with
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21 those obtained from pre-ovulatory follicles. In both studies, cells expressed MSC-associated
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23 markers and were able to differentiate into different cell types not present within the follicles, thus
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25 suggesting their potential use in cell-based therapy. Moreover, Mattioli et al. [6] demonstrated that
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27 luteinizing GCs have more efficient osteogenic potential compared to GCs isolated from growing
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29 follicles.
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31 Granulosa membrane represents a readily available source to easily isolate cells from, relying on the
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33 well-established techniques for oocyte retrieval used in assisted reproduction. This increased our
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35 interest in identifying a MSC-like population in the bovine species considering that ovaries can be
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37 easily collected in slaughterhouses due to the absence of their commercial value, and cells obtained
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39 from them could have a high value in regenerative medicine for repair of ovarian function in bovine
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41 with fertility problems. In this context, the present research has been designed to define a suitable
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43 protocol to select from bovine pre-ovulatory follicles GCs with MSC-associated features, in terms
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45 of morphology, specific markers and differentiative potential.
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49 **MATERIAL AND METHODS**
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51 Samples were collected from bovine slaughtered in a slaughterhouse (INALCA, Ospedaletto
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53 Lodigiano, Lodi, Italy) under legal regulations. Chemicals were obtained from Sigma-Aldrich
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Chemical (Milan, Italy) unless otherwise specified, and tissue culture plastic dishes were purchased from Euroclone (Milan, Italy).

Collection of ovaries and cells isolation

Bovine GCs employed in this study were isolated from ovaries collected from slaughterhouses. The age, genealogy, physiological status and race were unknown for the animals. The transport of the gonads to the laboratory occurred in a portable thermos maintained at the temperature of 30°C in physiological saline solution (0.9% NaCl) supplemented with kanamycin (150 mg/l). This temperature was maintained constant from the beginning of the ovary collection and during the time needed to reach the laboratory. In laboratory, the ovaries were washed repeatedly in physiological saline solution supplemented with antibiotic then they were maintained in a thermos for the period of sampling to avoid thermal shock. GCs were isolated from the bovine ovaries by scraping [6]. Briefly, follicles with diameter between 0.8-1.2 cm were opened using scalpel blade and GCs were gently scraped away from the internal face of the follicle wall. The material collected by scraping was deposited in 50 mL tubes containing TCM-Hepes medium supplemented with 1 mM pyruvic acidic, 2.2 g/l of sodium bicarbonate, 100x penicillin/streptomycin and 10% fetal calf serum (FCS). The tubes were left at room temperature until the oocytes were deposited at the bottom of the tubes. The deposited portion was aspirated and plated in a gridded Petri plate (100x15 mm) to select and discharge oocytes. This procedure was performed using a stereomicroscope with a 40X enlargement equipped with a heating plate set at 38°C (Olympus SZX-ILLK200). Then, GCs were pooled, washed in TCM-Hepes through two successive centrifugations (200xg for 5 min at room temperature), counted in a Burker chamber and used in the following experiments.

Experimental design

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GCs were collected from bovine pre-ovulatory follicles by scraping and cultured in different media or exposed to acid conditions (pH 5.7), then induced to differentiate in mesodermic and ectodermic lineages and analyzed by qualitative and quantitative PCR.

GCs isolation and culture

At first, GCs isolated by scraping were randomly allocated to five different culture media with the basic medium (MB) consisted of high glucose-Dulbecco modified Eagle medium (HG-DMEM) supplemented with 10% FCS, 100 UI/mL penicillin-100 µg/mL streptomycin, 0.25 µg/mL amphotericin B, 2 mM L-glutamine. This medium was chosen because it is the most commonly used for the isolation of mesenchymal stem cells (Wang et al., 2004). The other conditions included: MB supplemented with 0.1% epidermal growth factor (MB+EGF) [6], MB supplemented with 0.02% leukemia inhibitory factor (MB+LIF) [5], MB supplemented with only 2% FCS (2%-MB) as reported by Solmesky et al. [7] to perform *in vitro* isolation of stem cells, and MB supplemented with 0.02% leukaemia inhibitory factor (MB+LIF) and 0.014 µl/mL β-mercaptoethanol (MB+LIF+BME) as reported by Marshall et al. [8] for culturing *in vitro* embryonic stem cells. In our study this protocol was modified because BME was maintained only for passage (P) 1 and then removed from the culture medium. For each condition, cells cultures were established in an atmosphere of 5% CO_2 and 90% humidity, and at a temperature of 38.5°C. Medium was replaced after 72 h after isolation to remove non-adherent cells. Cells were analysed for the expression of specific markers by qualitative and quantitative PCR and at P3 were tested for multi-differentiative potential.

In a second step, GCs isolated by scraping were divided into two portions: one was cryopreserved and used as control (day 0, d0). The second one was exposed to acidic conditions (pH: 5.7) and was constantly observed for 7 days to assess the viability and morphological changes (d7). The acidic treatment was performed for 25 minutes at pH 5.7 and at 37°C [9]. After that, cells were centrifuged

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3 and the pellet was resuspended in a DMEM/F12 medium supplemented with LIF and B27. The
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5 cellular suspension was seeded at a density of $1x10^5$ cell/cm^2 in T25 flasks.
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10 ***In vitro* differentiation**
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12 GCs from each condition were seeded at the density of $3x10^3$ cells/cm^2 for differentiation in
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14 adipogenic, osteogenic and neurogenic lineages. Cells plated at the density of $1.5x10^3$ cells/cm^2
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16 were used as control. For the first 3-4 days, the cells were incubated with basic medium to allow the
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18 adhesion and at 60-70% of confluence they were induced to differentiate.
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21 *Osteogenic differentiation* was assessed by incubating cells for up to 3 weeks at 38.5°C under 5%
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23 CO_2 in medium composed of HG-DMEM medium supplemented with 10% FBS, 100 UI/mL
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25 penicillin-100 mg/mL streptomycin, 0.25 mg/mL amphotericin B, 2mML-glutamine, 10mM b-
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27 glycerophosphate, 0.1 mM dexamethasone and 250 mM ascorbic acidic. Non-induced control cells
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29 were cultured for the same time in standard control medium (HG-DMEM supplemented with 10%
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31 FCS, 100 UI/mL penicillin-100 mg/mL streptomycin, 0.25 mg/mL amphotericin B, 2mML-
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33 glutamine). Osteogenesis was assessed by conventional Von Kossa staining, using 1% silver nitrate
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35 and 5% sodium thiosulphate, which allowed detection of calcium deposits.
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38 *Adipogenic differentiation*. Near-confluent cells were cultured through three cycles of
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40 induction/maintenance to stimulate adipogenic differentiation. Each cycle consisted of feeding the
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42 GCs for three days with supplemented adipogenesis induction medium, followed by culture for
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44 other 3 days (38.5 °C, 5% CO_2) in supplemented adipogenic maintenance medium. The induction
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46 medium consisted of HG-DMEM supplemented with 10% FCS, 100 UI/mL penicillin-100 mg/mL
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48 streptomycin, 0.25 mg/mL amphotericin B, 2mML-glutamine, 10 mg/mL insulin, 150 mM
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50 indomethacin, 1 mM dexamethasone and 500 mM 3-isobuty-l-methyl-xanthine. The maintenance
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52 medium consisted of HG-DMEM supplemented with 10% FCS and 10 mg/mL insulin. Non-
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54 induced control cells were cultured for the same time in standard control medium. Adipogenesis
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was assessed using conventional Oil red O staining (0.1% in 60% isopropanol) to detect lipid droplets.

Neurogenic induction was performed by culturing cells for 24 h in pre-induction medium consisting of HG-DMEM, 20% FCS and 1mM b-mercaptoethanol [10,11], then neural induction was performed by switching to a medium composed of DMEM plus 2% FCS, 2% dimethylsulphoxide and 200 mM butylated hydroxyanisole for 3 days [12]. Non-induced control cells were cultured for the same time in standard medium. Neurogenic differentiation was demonstrated by conventional Nissl staining (0.1% cresyl violet solution), to detect increase of Nissl bodies.

Molecular biology study

Qualitative PCR analysis was performed to evaluate the expression of specific granulosa-, MSC-, pluripotent-, histocompatibility- and haematopoiesis-associated markers, to confirm the occurred differentiation and the stress induced by the acidic treatment. RNA was isolated using TRIZOL ® Reagent (Invitrogen, Carlsbad, CA) according to the protocol indicated by the manufacturer. RNA concentration and purity were measured using a NanoDrop Spectrophotometer (NanoDrop ND1000, Wilmington, DE, USA). Complementary DNA (cDNA) was synthesized from 300 ng of total RNA using the PrimeScript RT reagent kit (Takara Bio). Gene expression evaluation was performed using specific sequences. Bovine-specific oligonucleotide primers were designed using open source PerlPrimer software v. 1.1.17, based on available NCBI Bos Taurus sequences or on Mammal multi-aligned sequences. Primers were designed across an exon–exon junction in order to avoid DNA amplification. Primers sequences and characteristics are reported in Table 1. Bovine glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was employed as a reference gene in each sample in order to standardize the results by eliminating variation in mRNA and cDNA quantity and quality.

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Conventional qualitative PCR was performed using 1µl of the obtained cDNA in 25µl final volume with Taq DNA Polymerase, recombinant (Invitrogen, Life Technologies, Monza, Italy). Amplified PCR products were run in electrophoresis on a 1.8% agarose gel with ethidium bromide.

For quantitative PCR, one single representative gene per set of markers (*CD73, FSH-R,* and *Oct4*) was chosen to evaluate the selection efficiency of any culture condition used in this study. In addition, the expression of TNF-α and IL-1β were evaluated to confirm cellular stress induced by acidic treatment. Analyses were carried out with SYBR (a fluorescent intercalating agent, able to bind the DNA in double strand conformation) method, in MyiQTM single-color Real-Time PCR Detection System (BioRad). Triplicate PCR reactions were carried out for each analyzed sample. Reactions were set on a strip in a final volume of 25µl by mixing, for each sample, 1µl of cDNA, 12,5µl of 2X concentrated SYBR® Select Master Mix (Applied Biosystems), 1µM forward primer and 1µM of reverse primer and MQ water.

Statistical Analysis

For quantitative PCR data, non-parametric tests were used. The Mann-Whitney U-test was employed to compare two groups (treated vs untreated). Results were considered statistically significant if the value of P was < 0.05.

RESULTS

Granulosa cells yield and morphology

From each ovary, about 2 million GCs were isolated with a 80% viability. Cells were plated and selected based on their ability to adhere to plastic. Microscopic observation revealed the presence of cells with epithelial morphology when cultured in MB (Figure 1 a**)**, with atypical morphology in LIF+BME (Figure 1 b), whereas when cultured in other conditions they displayed fibroblast-like morphology (Figure 1.c,d,e). After pH treatment, in the next 7 days of culture, cells displayed

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morphological changes compared to initial epithelial morphology and a progressive vacuolization (Figure 2). After acidic treatment, the number of viable cells decreased of 50% and viability rate was of 40%.

Molecular analysis of granulosa cells

Table 2 shows expression of GCs studied in different culture conditions. These cells expressed mesenchymal- (*CD29*, *CD44*, *CD166*, and *CD73*), and pluripotent- (*Oct-4* and *c-Myc*) markers and lacked of *CD34* marker expression. For these markers, no differences induced by the culture conditions were observed compared to P0, except for *CD166* whose expression was not detected at P0. On the other hand, changes in the granulosa-associated markers were detected in the different culture conditions. Specifically, *FSH-R* was expressed only at P1 when cells were cultured in MB, MB+EGF, MB+LIF and MB+LIF+BME. Cells cultured in 2%-MB expressed *FSH-R* over the passages studied. *FST* expression was observed in all the conditions and passages, but in MB+LIF+BME disappeared at P3.

qPCR results highlighted differences in the expression of *FSH-R* between cells cultured in different conditions (Figure 3**)**. In MB+LIF and MB+EGF *FSH-R* expression was found about 10 times less expressed compared to the baseline (P0). *Oct4* expression reached the maximum expression in MB+LIF medium (157.8±65.07) and the minimum expression in 2%-MB and MB (0.83±0.38 and 1.73±0.27, respectively). A relatively high value was recorded also in MB+EGF and MB+LIF+BME (4.1±1.1 and 39.7±13.6**)**. The expression level of *CD73* was found higher in MB (49.7±23.19) and MB+LIF+BME (29.75±19.87). The lowest expression of *CD73* was registered in 2%-MB, showing a 1.37(±0.44)-fold increase**.**

GCs before and after acidic treatment expressed *Oct4, C-Myc, CD73, CD29, CD44, MHC-I, MHC-II* and granulosa-associated markers *(FST* and *LIF-R)* but not *CD34*. The main difference found between d0 and d7 is the loss of *FSH-R* expression (Figure 4a).

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Considerable differences in gene expression were observed in GCs before and after acidic treatment (Figure 4b). In particular, *FSH-R* expression was found significantly decreased in treated cells d7. Further indications supporting the efficacy of the treatment were provided by the loss of other granulosa markers (i.e *FST* and *LIF-R*) and the increase of *Oct4* and *CD29* compared to untreated cells (d0). A decreased expression of *MHC-I* and *MHC-II* in d7 was also observed.

In addition, as response to the acidic treatment, in bovine granulosa cells expression of inflammatory markers, such as *TNF-α* and *IL-1β,* was found significantly up-regulated in d7 compared to d0, with a 7.02 (±0.2) and 21.26 (±0.25)- fold increase (Figure 5; $P<0.05$ and $P<0.001$, respectively).

***In vitro* differentiation**

GCs cultured in different conditions did not show any ability to differentiate. On the other hand, GCs treated with pH acidic were easily induced into the adipogenic, osteogenic and neurogenic lineages. After 18 days of induction, the presence of intracellular lipid vacuoles was determined by Oil red O staining (Figure 6a). After 21 days in osteogenic media, extracellular mineral deposits were demonstrated by Von Kossa staining (Figure 6b). Interestingly, cells induced to differentiate toward the neurogenic lineage demonstrated acquired the typical neuronal morphology with axon- and dendrite-like processes and were positive for Nissl staining Nissl bodies (Figure 6c). Uninduced cells were maintained in culture for the same period of each differentiation protocol time and used as negative control. They resulted negative for all the staining performed. Differentiation was confirmed by molecular analysis through the use of specific markers, including *LEP* and *PPARγ* for adipogenic differentiation, *BGLAP, SPP1* and *SPARC* for osteogenic differentiation, and *GFAP* for neurogenic differentiation.

In cells induced to undergo adipogenesis the expression of PPAR-y but not *LEP* was revealed in induced cells while control cells did not express the genes tested. Following osteogenesis induction, *BGLAP* was not expressed in induced cells. Qualitative PCR did not allow for the discrimination

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between induced and uninduced cells when the expression of *SSP1* and *SPARC* was assessed. For this reason, qPCR was performed to determine the levels of expression of these two markers in induced cells compared to their respective uninduced controls. The expression of osteogenesis-associated genes quantitatively confirmed the induction. *SPP1* expression increased 2.17 (±0.09)-fold (P<0.05), whereas a slight but statistically significant (P<0.05) increase (1.45±0.085) in *SPARC* expression was found compared to the uninduced counterparts (Figure 7).

The expression of *GFAP* confirmed the neurogenic differentiation.

DISCUSSION

The purpose of this study was to identify a new source of stem cells easy to collect and able to comply with the requirements of regenerative medicine on a large scale. Based on recently published studies in humans [5] and gilts [6], the granulosa membrane represents an alternative source of MSCs.

To reach our goal, we tested different culture conditions and evaluated their efficacy in selecting MSCs from cell population obtained from the follicle. Once isolated, GCs have been characterized based on the minimal criteria defined by the “International Society for Stem Cell Therapy” to define mesenchymal stem cells [13]. According to results obtained in human [5] and gilt [6], cells adhered to plastic dish and expressed a pattern of mesenchymal (*CD44*, *CD29*, *CD166*, *CD73*), pluripotency (*Oct4*, *c-Myc*) genes with no expression of the hematopoietic *CD34* and the functional marker *FSH-R*. The levels of expression of *CD73*, *Oct4* and *FSH-R* were also confirmed quantitatively showing the reduction of specific granulosa markers and the up-regulation of MSC-associated genes.

Contrarily to data reported for human [5] and gilts [6], however, despite inducing a MSC-like phenotype in bovine granulosa GCs, none of the culture conditions tested was efficient in selecting a plastic cell population. In fact, cells exposed to each condition were not able to differentiate, as a consequence of the fact that cells maintained a strong epigenetic imprinting that prevented them from differentiating. To date, the expression of the follistatin (*FST*), a protein secreted only by

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granulosa cells in the ovary, was never found down-regulated, as we would expect it to be for cells retaining/acquiring a stem cell-associated phenotype. Based on our findings, it is reasonable to assume that GCs isolated in our study and maintained in different conditions, with the only exception of those kept in MB+LIF+BME that lost the expression of *CD44* and *CD166*, can be considered progenitors of GCs. The discrepancy in the differentiation outcome observed between the present study and that reported by Mattioli et al. [6] and Kossowska-Tomaszczuk et al. (2009) is difficult to explain considering that GCs were isolated from the internal side of the pre-ovulatory follicle and, thus, from periantral layer, as reported by Mattioli et al. [6]. Also Erickson et al. [14] postulated that stem cells could be located in the periantral layer of granulosa. As such, this method could be the most appropriate for the collection of multipotent stem cells from granulose compared to the aspiration protocol performed by Kossowska-Tomaszczuk et al. [5].

Based on our negative results to detect in identifying stem cells in pre-ovulatory follicles, we decided to expose GCs to chemical stress, by lowering the pH in culture, to elucidate whether acidic stress could influence the phenotype and, eventually, help the selection of more plastic cells. In plants, drastic environmental changes have been reported to convert mature somatic cells (for example, dissociated carrot cells) into immature blastema cells, from which a whole plant structure, including stalks and roots, develops in the presence of auxins [15]. In our study, molecular analysis revealed significant differences between the cells before and after the acidic treatment. In particular, 7d cells showed a significant decrease of expression of granulosa-specific markers (*FSH-R, FST, LIF-R*), with a concomitant increase in the pluripotency-associated marker *Oct4*. Chiou et al. [16] also reported the up-regulation of *Oct4* in these conditions. The loss of *FSH-R* could be explained with the lack of its ligand (FSH) in the culture media, which is in agreement with the findings obtained by Kossowska et al. [5], focusing on human granulosa cells isolated from the ovarian follicles of infertile patients and cultured in the presence of LIF. Changes in gene expression can be, further, justified hypothesizing that the chemical stress is able to induce either a cell de-differentiation or a strong selection of progenitor cells. Changes in the external environment

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(including the pH reduction) have been previously associated to a specific phenotype acquired by cells exposed to them, as in the case of ovary cells and cancer cells [17]. Moreover, when cultured in acidic conditions, cells lose the expression of the immunogenic markers (*MHC-I* and *MHC-II*), confirming their more undifferentiated state as MSCs have been reported to be immuno-privileged cells, with no or low expression of those markers [18]. Differentiation studies, further, corroborated such observations. pH-treated cells were induced toward the mesodermic (adipogenic and osteogenic) and ectodermic (neurogenic) lineages. Oil Red O, Von Kossa and Nissl staining respectively, demonstrated the occurred differentiation, which was further confirmed by molecular analysis. For adipogenesis, we investigated the expression of *PPARγ* and *LEP*, however, only the first one was expressed in differentiated cells. The expression of *PPARγ* suggests a pre-adipocytes commitment of cells [19], which is further confirmed by the lack of *LEP*, a marker is regarded as an intermediate and late marker. The expression of *GFAP* in induced cells suggests astrocyte differentiation occurred, as previously reported for bovine and equine amniotic-derived cells [4,19,20]. Osteogenesis was assessed investigating the osteogenic-specific markers *BGLAP, SPP1* and *SPARC*. All of these markers were expressed in induced cells, with the only exception of *BGLAP*. This might be explained considering that *BGLAP* is expressed in terminally differentiated osteoblasts [21]. Surprisingly, the expression of *SPP1* and *SPARC* was detected also in the negative controls (7d uninduced cells) although with a lower expression compared to induced cells. We hypothesized that the expression of these markers (*SPP1* and *SPARC*) in negative controls can be due to the role they play in inflammation. Indeed, these markers are mainly involved in the immune response to an inflamed environment [22,23], as it the case of the acidic treatment. The up-regulation of *TNF-α* and *IL-1β* expression confirmed our hypothesis. In particular *SPARC* levels are significantly correlated with inflammation [22] and *SPP1* is strikingly up-regulated at sites of inflammation and tissue remodelling, as it promotes the migration of inflammatory cells to the wound site and functions as a pro-inflammatory cytokine [23].

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CONCLUSION

Results obtained from this work demonstrated that none of the culture conditions employed in this study allowed for the selection of the stem cell population within granulosa cells isolated by scraping. The stress induced by the acidic treatment on bovine granulosa cells endorsed the selection of the more plastic cells, which were the only ones able to respond to stimuli and adjust to a more rigid environment. According to this, compared to the freshly isolated cells, selected cells showed an increased expression in the pluripotent marker Oct-4 and were able to differentiate into mesodermic and ectodermic lineages. The acquired phenotype of those cells can be also explained as a consequence of the activation of an inflammatory process, able to determine de-differentiation or nuclear reprogramming in GCs [24]. Further studies are required to better understand the effect of the acidic treatment and the consequent stress induced by it at molecular level. Moreover, different approaches will be required to discover a possible stem cell niche in bovine pre-ovulatory follicle.

Disclosure of potential conflicts of interest

The authors indicated no potential conflicts of interest

ACKNOWLEDGEMENTS

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Figure 1. Morphology of cells cultured in different culture conditions: a) MB; b) MB with LIF and BME; c) MB with EGF; d) MB with LIF; e) BM with 2% of FCS. Scale bars: 20 µm. Magnification 20x.

Figure 2. Morphology of cells isolated from bovine pre-ovulatory follicles and exposed to a chemical stress (d7) display a progressive vacuolization. Scale bars: 20 µm. Magnification 20x.

Figure 3. Quantitative RT–PCR analysis for the expression of multi- (*CD73*), pluripotent (*Oct-4*) and granulosa specific (*FSH-R*)- markers in cells cultured in different conditions (MB, 2%MB, MB+EGF, MB+LIF, MB+LIF+BME) at P3. Expression levels normalized to the reference gene (*Gapdh*). Data are represented as fold-change compared with expression observed in P0. Values are mean ±SD (n=3). Asterisks depict highly significant (**; $P<0.01$) differences.

Figure 4. Qualitative (a) and quantitative (b) RT-PCR analysis for the expression of specific granulosa (*LIF-R, FSH-R, FST*)-, MSC (*CD73, CD29, CD44*)-, pluripotent (*Oct-4, c-Myc*)-, histocompatibility (*MHC-I* and *MHC-II*)- and haematopoiesis (*CD34*)-associated markers in cells before (d0) and after (d7) acidic treatment. b) Quantitative data are reorganized in heatmap. Colors represent the intensity of the gene expression: red refers to the higher level of expression while green to the lowest level of expression.

Figure 5. Quantitative RT–PCR analysis for the expression of inflammatory markers such as *TNF-α* and *IL-1β* in GCs before (d0) and after acidic treatment (d7). Expression levels normalized to the reference gene (*GAPDH*). Values are mean ±SD (n=3). Asterisks depict highly significant (**; $P<0.01$) and significant (*; $P<0.05$) differences compared to d0.

Figure 6. Differentiative potential. Adipogenic differentiation: Oil red O staining in induced and control cells at d7, and adipogenesis-associated markers expression (a). Osteogenic differentiation: Von Kossa staining on induced and control cells d7, and osteogenesis-associated markers

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3 expression (b). Neurogenic differentiation: Nissl staining in induced and control cells d7, and
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5 neurogenic marker expression (c). Scale bars: 20 µm. 20x Magnification. Panel on the right shows
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7 specific gene expression on induced and controls cells. *BGLAP*, *SPP1* and *SPARC* mRNA were
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9 investigated for osteogenesis, *PPARY* and *LEP* for adipogenesis, and *NES* and *GFAP* for
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11 neurogenesis. *Gapdh* was employed as reference gene. Bone, adipose tissue, and spinal cord were
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13 used as positive control.
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19 **Figure 7.** Quantitative RT–PCR analysis for the expression of osteogenesis-associated genes such
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21 as *SPP1* and *SPARC.* Data are represented as fold-change compared with expression observed in
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23 P0. Values are mean ±SD (n=3). Asterisks depict highly significant (**; P<0.01) or significant (*;
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25 P<0.05) differences compared to uninduced cells.
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Table 1. Oligonucleotide sequences used for the molecular analysis.

	GENES	FORWARD	REVERSE	Annealing Temperature	size cDNA bp
Markers of Pluripotency	***Oct4***	CACACTAGGATAT ACCCAGGC	GGAGATATGCAAGGC AGAGA	60°C	177
	c-Myc	GCGCCGCATTCGC GAAACTT	TGAGGGGCATCGCTG CAAGC	58°C	214
Markers of Multipotency	***CD73***	AAGGTTCCTGTGG TCCAGGCCT	TGCATTCTCGAAAGC GGCAGGA	68°C	260
	CD29	GTTGGTTCTGCAG TTACGATCAG	AACCAAACCCAATTC GGAAGTC	52°C	203
	CD44	AACAGTAGGAGA AGGTGTGG	TCATGAACTGGTCTT GGGTC	61°C	166
	MHC-I	GATCTCACTGACC TGGCA	CTGAGGAGGTTCCCA TCTC	60°C	199
	MHC-II	CCTCGCTTGCCTG AATTTGC	ACAGGTGCCGACTGA TGC	53°C	266
Hematopietic Marker	***CD34***	CCTGAAGCTAAAT GAGACCT	AACTTTCTGTCCTGTT GGTC	58°C	173
Markers of Granulosa Cells	***FSHR***	TGGTCCTGTTCTA CCCCATCA	GAAGAAATCCCTGCG GAAGTT	58°C	83
	FST	CTCTGCCAGTTCA TGGAGGACC	GGCCAATCCAATAGA TCTGCCC	63°C	651
	LIFR	TGGCAGTACACAT TGTCCCC	TCCCGCAAAAACAAC CGTTC	60°C	145
Markers of differentiation	***LEP***	CAATGACATCTCA CACACGCAG	CGGCCAGCAGGTGGA GAAG	55°C	212
	PPARγ	CGCACTGGAATTA GATGACAGC	CACAATCTGTCTGAG GTCTGTC	55°C	214
	BGLAP	TCGGGCAAAGGC GCAGCCTTC	GCAGGGCTGCAAGCT CTAGACG	55°C	231
	SPP1	CGCCGATCTAACG TTCAGAGTC	GACTCTCAATCAGAT TGGAATGC	55°C	199
	SPARC	CTGGTCACGCTGT ACGAGAG	CGGTGTGAGACAGGT ACCCGT	55°C	232
	GFAP	GGCACCTTGAGGC AGAAGCTC	CTCCTGGAGCTCCCG CACCT	60°C	195
Markers of inflammation	***TNF-α***	ACATACCCTGCCA CAAGGC	TGGGGACTGCTCTTC CCTCT	60°C	259
	IL-1β	TGCAGCTGGAGG AAGTAGAC	GTCGGGCATGGATCA GACAA	60°C	338
Housekeeping gene	***GAPDH***	ATGAGATCAAGA AGGTGGTG	CCAAATTCATTGTCGT ACCAG	60°C	190

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Table 2: gene expression analysis using RT-PCR in GCs in different culture conditions

		GAPDH	*OCT4*	*C-myc*	*CD44*	*CD29*	*CD166*	*CD34*	*CD73*	*FSHR*	*FST*
BASELINE	P0	✔	✔	✔	✔	✔	x	x	✔	✔	✔
MB	P1	✔	✔	✔	✔	✔	✔	x	✔	✔	✔
	P3	✔	✔	✔	✔	✔	✔	x	✔	x	✔
	P5	✔	✔	✔	✔	✔	✔	x	✔	x	✔
2%-MB	P1	✔	✔	✔	✔	✔	✔	x	✔	✔	✔
	P3	✔	✔	✔	✔	✔	✔	x	✔	✔	✔
	P5	✔	✔	✔	✔	✔	✔	x	✔	✔	✔
MB+ EGF	P1	✔	✔	✔	✔	✔	✔	x	✔	✔	✔
	P3	✔	✔	✔	✔	✔	✔	x	✔	x	✔
	P5	✔	✔	✔	✔	✔	✔	x	✔	x	✔
MB + LIF	P1	✔	✔	✔	✔	✔	✔	x	✔	✔	✔
	P3	✔	✔	✔	✔	✔	✔	x	✔	x	✔
	P5	✔	✔	✔	✔	✔	✔	x	✔	x	✔
MB + LIF + BME	P1	✔	✔	✔	✔	✔	✔	x	✔	✔	✔
	P3	✔	✔	✔	x	✔	x	x	✔	x	x
		190 bp	*177 bp*	*214 bp*	*166 bp*	*203 bp*	*755 bp*	*173 bp*	*260 bp*	*83 bp*	*651 bp*

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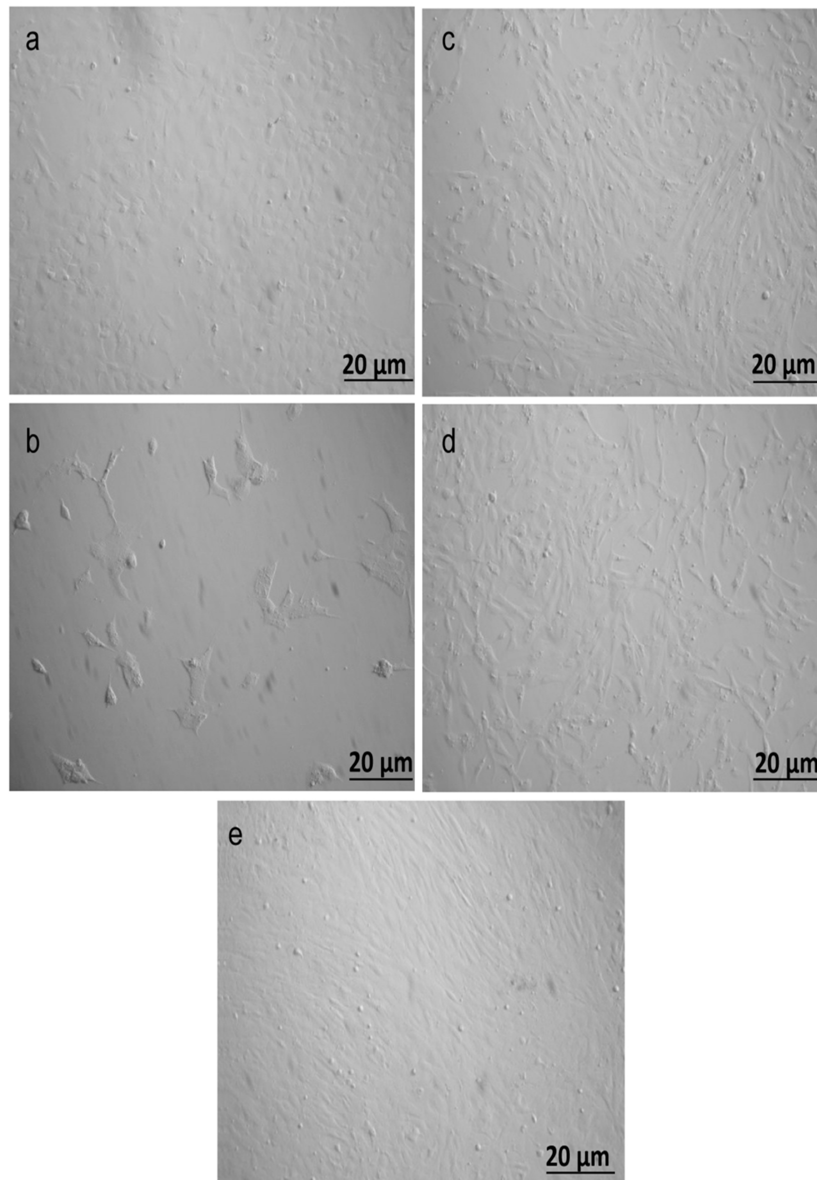

Morphology of cells cultured in different culture conditions: a) MB; b) MB with LIF and BME; c) MB with EGF; d) MB with LIF; e) BM with 2% of FCS. Scale bars: 20 µm. Magnification 20x.
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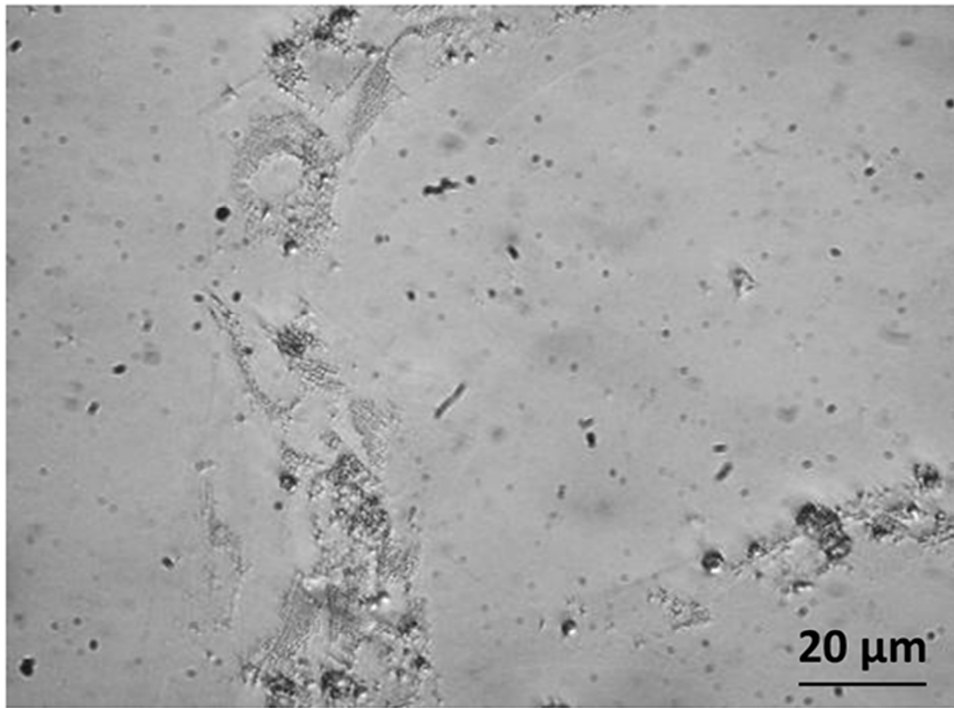

Morphology of cells isolated from bovine pre-ovulatory follicles and exposed to a chemical stress (d7) display a progressive vacuolization. Scale bars: 20 µm. Magnification 20x.
54x39mm (300 x 300 DPI)

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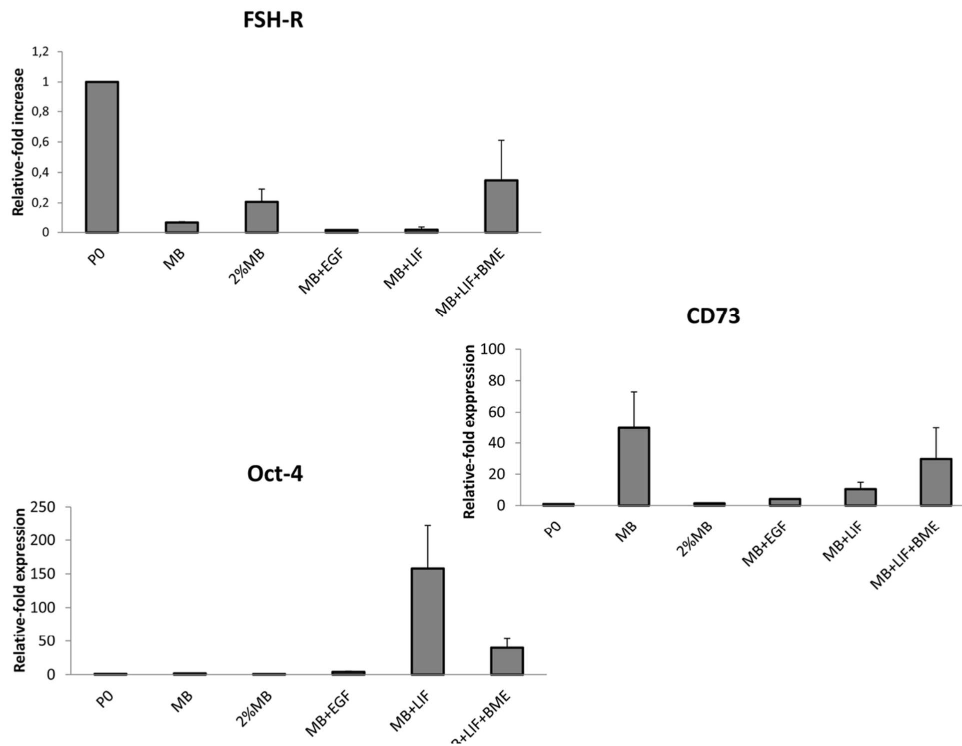

Quantitative RT–PCR analysis for the expression of multi- (CD73), pluripotent (Oct-4) and granulosa specific (FSH-R)- markers in cells cultured in different conditions (MB, 2%MB, MB+EGF, MB+LIF, MB+LIF+BME) at P3. Expression levels normalized to the reference gene (Gapdh). Data are represented as fold-change compared with expression observed in P0. Values are mean ±SD (n=3). Asterisks depict highly significant (**; P<0.01) differences.
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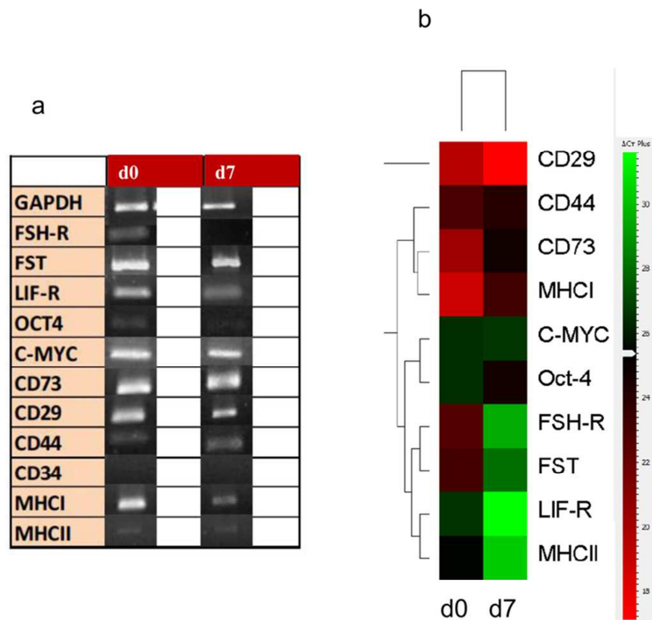

Qualitative (a) and quantitative (b) RT-PCR analysis for the expression of specific granulosa (LIF-R, FSH-R, FST)-, MSC (CD73, CD29, CD44)-, pluripotent (Oct-4, c-Myc)-, histocompatibility (MHC-I and MHC-II)- and haematopoiesis (CD34)-associated markers in cells before (d0) and after (d7) acidic treatment. b) Quantitative data are reorganized in heatmap. Colors represent the intensity of the gene expression: red refers to the higher level of expression while green to the lowest level of expression.
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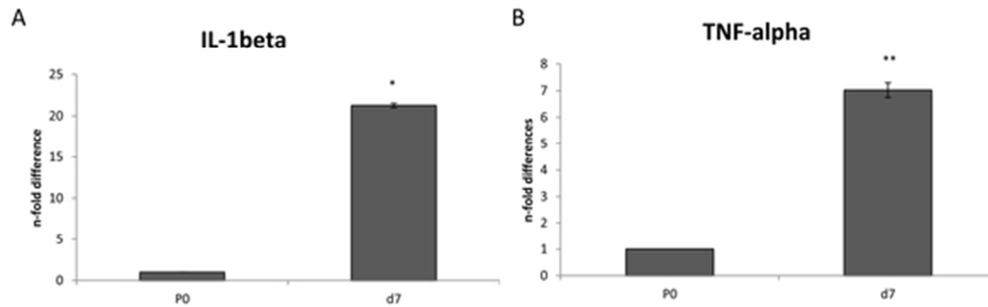

Quantitative RT–PCR analysis for the expression of inflammatory markers such as TNF-α and IL-1β in GCs before (d0) and after acidic treatment (d7). Expression levels normalized to the reference gene (GAPDH). Values are mean ±SD (n=3). Asterisks depict highly significant (**; $P<0.01$) and significant (*; $P<0.05$) differences compared to d0.
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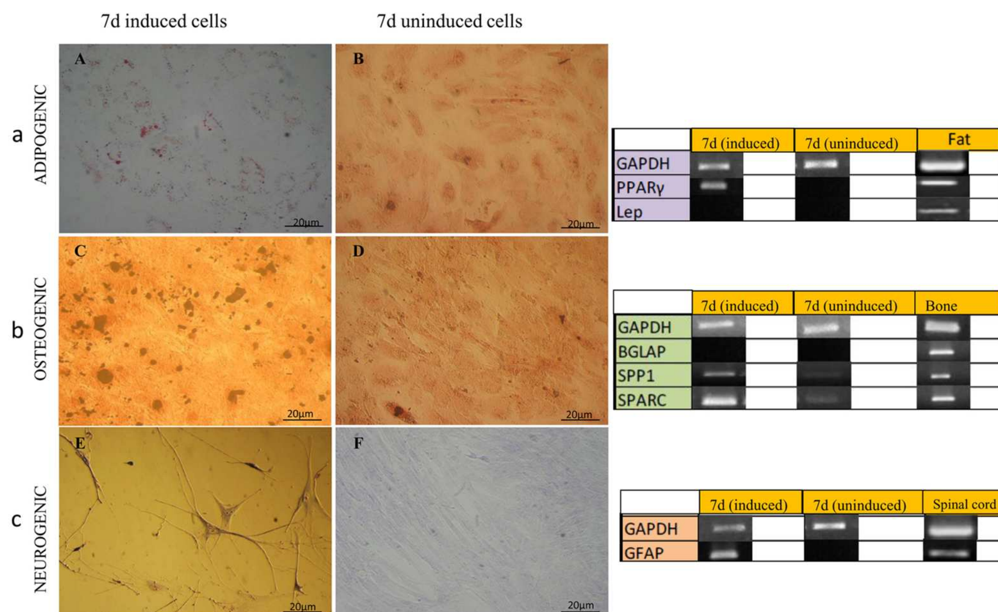

Differentiative potential. Adipogenic differentiation: Oil red O staining in induced and control cells at d7, and adipogenesis-associated markers expression (a). Osteogenic differentiation: Von Kossa staining on induced and control cells d7, and osteogenesis-associated markers expression (b). Neurogenic differentiation: Nissl staining in induced and control cells d7, and neurogenic marker expression (c). Scale bars: 20 μm. 20x Magnification. Panel on the right shows specific gene expression on induced and controls cells. BGLAP, SPP1 and SPARC mRNA were investigated for osteogenesis, PPARY and LEP for adipogenesis, and NES and GFAP for neurogenesis. Gapdh was employed as reference gene. Bone, adipose tissue, and spinal cord were used as positive control.
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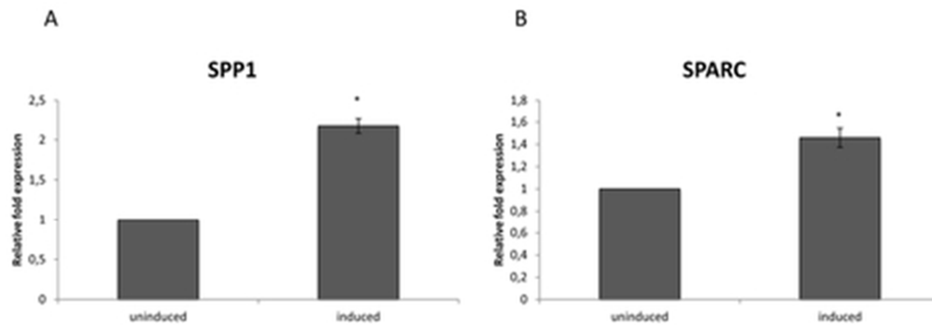

Quantitative RT–PCR analysis for the expression of osteogenesis-associated genes such as SPP1 and SPARC. Data are represented as fold-change compared with expression observed in P0. Values are mean ±SD (n=3). Asterisks depict highly significant (**; $P<0.01$) or significant (*; $P<0.05$) differences compared to uninduced cells.
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