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27 April 2024

UNIVERSITA DEGLI STUDI DI TORINO

Osteo-regenerative potential of ovarian granulosa cells: an *in vitro* and *in vivo* study

Mauro Mattioli[1]*, Alessia Gloria[1], Maura Turriani[1], Paolo Berardinelli[1], Valentina Russo[1],

Delia Nardinocchi[1], Valentina Curini[1], Mario Baratta[2], Eugenio Martignani[2], Barbara

Barboni[1]

'Corresponding author mattioli@unite.it, Department of Comparative Biomedical

Sciences, University of Teramo, Piazza A Moro 45, 64100 Teramo, Italy

Abstract

Background Granulosa cells (GC) express sternness markers and can differentiate into

n cell types not present within the follicles. Since different kinds of follicles populate the

ovary, we undertook this research in the pig model to identify the kind of follicle, growing or

luteinizing, from which GC with the best regenerative potential can be retrieved.

Methods Growing follicles were isolated form prepubertal gilts 50 h after eCG (1200 III)

administration. Luteinizing follicles were obtained from prepubertal gilts treated with eCG

(1200IU) followed,60h later, by hCG (500 IU). The follicles were isolated 30h after hCG.

GC isolated from growing (GGC) and from luteinizing (LGC) follicles were expanded *in

vitro* for 3 passages in aMEM based growth medium. Osteogenic medium (aMEM

supplemented with 50 |jM ascorbic acid, 10 mM (3-glicerol phosphate, 0.2 |jM

dexamethasone) was used to trigger differentiation. GC, cultured for 2 weeks in

osteogenic medium, were implanted s.c. in the dorsal region of SCID mice to assess their

osteogenic potential *in vivo.*

Results In addition to the typical granulosa cells characteristics (inhibin, progesterone and

estrogen production and FSH receptors), GGC and LGC showed a diffused expression of

the sternness markers Sox2, Nanog and TERT immediately after isolation. Expansion

caused in both cell types a rapid disappearance of granulosa cell characters while it did not modify sternness marker expression.

Osteogenic medium induced a marked extracellular matrix mineralization and alkaline phosphatase activation in LGC, clearly detectable after two weeks, while the process was much lighter in GGC, where it became evident after 3 weeks. Osteocalcin and Runx2 expressions were upregulated and sternness markers downregulated by osteogenic medium.

Implants retrieved 8 weeks after transplantation, had viable GC surrounding the several nodules of calcifications recorded. Similar effects were induced by GGC and LGC.

Conclusions These data confirm the sternness properties of GC, show that *in vitro* expansion cause a progressive de-differentiation of granulosa cells without affecting their staminality and demonstrate that both GGC and LGC have ostegenic potential, luteinizing cells being the more efficient. Transplanted in SCID mice, GC participate in new bone formation thus confirming their therapeutic potential.

Background

Adult stem cells are undifferentiated cells, located among specialized cells in a tissue, that can self renew an differentiate to yield some or all of the major specialized cell types of the same tissue or organ. The primary roles of adult stem cells are to maintain and repair the tissue in which they are found.

These cells have been found in many more tissues than expected and their ability to transdifferentiate towards cell types different from those of the tissue they are derived from has attracted the attention of researchers and clinicians.

If one considers that, in addition, these cells rarely have oncogenic deviations, as it occurs with a worrying frequency in transplanted embryonic stem cells [1,2], their therapeutic potential becomes even more robust.

Unfortunately only a small numbers of stem cells can be recovered from the body, their self-renewal capacity is limited and this makes generation of large quantities of stem cells difficult. Scientists in many laboratories are trying to find better ways to grow large quantities of adult stem cells *in vitro* still preserving their regenerative potential.

Moreover in most cases adult stem cells are retrieved through surgical procedures that provide heterogeneous pools of cells that must be purified before expansion *in vitro* [3-6]. This selection-further reduces the number of stem cells to work with.

Despite these technical limitations, adult stem cells are presently the type of stem cell that is more close to clinical application in a number of fields. The autologous transplantation of these cells allows to avoid immune reaction problems encountered with embryonic stem cell lines [7] and the increasing number of potential sources, from the bone marrow to the adipose tissue to the teeth pulp [8-16], that are being identified, partly compensate for the low number of cells that can be collected and foster the diffusion of these cells in regenerative medicine.

In this context ovarian granulosa granulosa cells may represent an interesting alternative source of adult stem cells [17]. Follicle derived granulosa cells express sternness markers and have been shown to differentiate into different cell types not present within the follicles [17], thus representing a new cell type to be used in cell therapy.

Granulosa cells are easy to collect, relaying on the well-established techniques for oocyte retrieval developed for assisted reproduction protocols, and the cell population isolated form one follicle is substantially homogeneous and therefore don't require any purification step. However, during the reproductive life, there is always a complex set of follicles, from growing to pre-ovulatory to regressing atresic follicles, that populate the ovary, each of them with granulosa cells in quite different functional conditions. The original investigation [17] refers to LGC recovered from the preovulatory follicles from patients treated for oocyte retrieval. The ovulatory surge of luteinising hormone (LH), or the administration of LH-like

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drugs, initiates the preovulatory phase culminating in the extrusion of a fertilizable oocyte and remodeling the follicle into a functional corpus luteum (CL). This differentiation of granulosa into luteal cells, known as luteinisation, is associated with the abrupt exit of granulosa cells from the cell cycle [18-21]. Therefore luteinising granulosa, at a final stage of differentiation and in a condition of cell cycle block, may not express the best staminality that these cells could offer. Alternatively granulosa cells could be retrieved from growing follicles before the pre-ovulatory gonadotropin surge and hence before luteinisation, e.g. in a condition of active proliferation and before the final differentiation.

The present research has been designed to address this issue and in particular to investigate whether granulosa cells isolated from pre and post gonadotropin surge follicles differ in the expression of sternness factors such as Sox2, Nanog and TERT, in their expansion potential and in their ability to undergo osteogenic differentiation. The results reported collectively confirm the staminality of these cells, show that granulosa cells progressively lose their original ovarian characterisitcisitcs during expansion *in vitro* while they keep on expressing sternness markers and demonstrate that they can promptly undergo osteogenic differentiation both *in vitro* and *in vivo.* Investigations *in vitro* revealed that granulosa cells derived from luteinising follicles have better osteogenic potential than those derived from growing follicles.

Methods

Animals and granulosa cells isolation

Prepubertal large white gilts with an average weight of 90 kg were used. Growing and luteinising follicles were obtained as previously described with a combination of eCG and hCG administrations [22,23]. Follicular growth was induced by subcutaneous injections of 1250 I.U. of eCG (Folligon; Intervet, Boxmeer, The Netherlands) followed 60 h later by 750

I.U. of hCG, that mimic the LH preovulatory surge, to triggers the ovulatory phase that eventually leads to ovulation in 40-44 h.

Growing follicles were isolated from the ovaries of 3 gilts slaughtered 50 h after eCG while luteinising follicles, committed to ovulation, were isolated from the ovaries of 3 gilts slaughtered 30 after hCG. The follicles were individually isolated with the aid of the stereomicroscope. Healthy follicles, as indicated by the transparent and vascularized wall, with a diameter > 4mm for eCG and eCG-hCG treated gilts were selected, opened using 110watchmaker forceps, turned inside out [24] and granulosa cells were gently scraped away in from the internal face of follicle wall. When the follicles were opened, care was taken to recover cumulus oocyte complexes in order to evaluate the condition of the cumulus mass and the meiotic maturation of the oocyte. For this purpose the oocytes were denuded by repeated aspirations through small bore glass pipettes and stained with lacmoid (Sigma Chemical Co. St. Louis, MO) for the identification of the stage of meiosis. Growing follicles used in the following experiments contained compact cumulus-oocyte complexes with the oocytes in a condition of complete meiotic arrest at the germinal vesicle stage. This allowed us to exclude that these follicles had not been exposed to an undesired endogenous preovulatory gonadotropin surge eventually released in response to the increased steroidogenesis caused by the treatment with eCG. On the contrary, luteinising follicles isolated after hCG injections, contained cumulus-oocyte complexes with marked signs of expansion and all the oocytes analysed had resumed meiotic maturation, the vast majority of them (27 out of 33, 82%) being at the metaphase I stage. This confirms that these follicles had been committed to ovulate.

Granulosa cells from growing follicles , then defined as GGC, and granulosa cells from luteinising follicles, defined as LGC, were pooled, washed in PBS-BSA through two successive centrifugations and used in the following experiments.

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Granulosa cell expansion

Granulosa cells were cultured in growth medium consisting of aMEM supplemented with 20% FCS, 1% Ultraglutamine, 1% Penicillin/Streptomycin and 10 ng/ml EGF (Sigma Chemical Co. St. Louis, MO). Cells were seeded in 50 ml flasks at a concentration of 3 x 10^3 cells/cm^2 and incubated at 39°C in 95-5% air-CO_2 atmosphere. At 80% confluence the flasks were washed to remove dead cells and debris, plated cells were dissociated by 0.05% trypsin EDTA and plated again at 3 x 10^3/cm^2 for 3 consecutive expansion passages.

Differentiation *in vitro*

The plasticity of granulosa cells was assessed by evaluating osteogenic differentiation following culture in standard osteogenic medium. Granulosa cells were seeded at the concentration of 2 x 10^4 in 35 mm wells and cultured in 2ml of growth medium for 2-3 days. At this time of culture, when the cells were approaching confluence, the growth medium was replaced by differentiation medium (aMEM supplemented with 50 |jM ascorbic acid, 10 mM (3-glicerol phosphate, 0.2 u.M dexamethasone and FCS reduced to 10% [25-26]. The culture medium was replaced three times a week and the degree of osteogenic differentiation was tested after 14 and 21 days of culture. Cells cultured in growth medium were taken as controls.

Differentiation *in vivo*

The differentiation capacity of granulosa cells *in vivo* was assessed by subcutaneous implant of scaffolds loaded with granulosa cells on the back of immuno-deficient mice. For this purpose GCs, previously stained with the fluorescent cell linker dye PKH26 (Sigma Chemical Co. St. Louis, MO), were incorporated in porous poly-lactic-co-glycolic acid (PLGA) porous scaffolds by culturing cubic fragments of the scaffolds of 2x2x2 mm,

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with granulosa cell suspensions at the concentration of 1×10^6 cells/5ml, under gentle agitation for 24 h. Cell loaded scaffolds were then inserted in multi well plates whose bottom had previously been covered by a layer 2% agarose in order to prevent the cells from leaving the scaffold and migrating on to the vessel surface. Cell loaded scaffold were then collected after 14 days of culture in osteogenic medium and then transferred subcutaneously in NOD/SCID mice.

Mice. Male NOD/SCID mice were bred and housed at the animal facility of the Faculty of Veterinary Medicine of the University of Turin according to the procedures and guidelines approved by the Italian Ministry of Health. Animal handling described in this study has been reviewed and approved by the Italian Ministry of Health and by the Bioethics Committee of the University of Turin. Mice were used at 8 to 10 weeks of age.

Surgical procedure. After inducing general anaesthesia, the back of the mice was shaved and washed with 70% ethanol. A midline longitudinal incision of approximately 1 cm was made on the dorsal surface of the lumbar regionof each mouse and subcutaneous pockets were created by gently separating the skin from the body wall. Cell loaded scaffolds were inserted in these pockets. Control implants consisted of scaffold fragments without cells. The skin was closed with interrupted 5-0 polyglycolic acid absorbable sutures (PGA Resorba®, RESORBA Wundversorgung GmbH & Co. KG, Germany). At 8 weeks post-treatment, the mice were sacrificed by an overdose injection of anesthetic and the skin including the implant was retrieved, fixed overnight in 10% buffered formalin at 4°C, embedded in paraffin, and sectioned for histological examination.

Analysis of cell characteristics

Follicular cell characteristics

The production of progesterone, estradiol 17(3, the expression of FSH receptors and the incidence of inhibin secreting cells were investigated in granulosa cell preparations

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immediately after collection, after expansion *in vitro* and after differentiation *in vitro* in order to evaluate whether these activities, typical of granulosa cells, were maintained throughout the expansion and the successive differentiation phases.

For the analysis of steroid production, granulosa cells were incubated in growth medium at the concentration of 1 x 10^6 cells/0.5 ml in 1.5 ml eppendorf tubes under gentle agitation. After 3 hours the tubes were centrifuged at 1000 g X 10 min and the supernatant was collected for the assessment of estradiol 17(3 or of progesterone released in the medium. Estradiol 17(3 and Progesterone levels were measured in triplicate using commercial assay kits (Progesterone ELISA kit, DRG Diagnostics, Mountainside, N.J. USA and -17(3-Estradiol EIA Kit, BioQuant Diagnostic; San Diego California CA). Steroid production is expressed as amount of hormone produced/10^6 cells.

Cell secreting Inhibin were identified by immunohistochemistry using an anti-Human Monoclonal (R1) Antibody (Alpha, INHA, LifeSpan BioSciences) displaying cross reactivity with sheep inhibin.

The expression of FSH receptors was evaluated by RT-PCR using specific primers listed in Table 1.

Assessment of stemness markers

Granulosa cells immediately after collection, after expansion and after differentiation *in vitro* were investigated for the expression of sternness markers by RT-PCR using the primers summarized in Table 1. The evolution of sternness markers production in granulosa cell preparations throughout the different experimental phases was assessed by immunocytochemistry using species specific antibodies. For immunocytochemistry the cells were cultured on glass coverslips positioned in 35 mm wells for 24 h and then fixed in 4% paraformaldehyde for 10 min. The cells were then washed with PBS 0.05% Tween-20 (wash solution) and incubated in a blocking solution (PBS with 1% BSA) for 1 h.

Fixed cells were incubated overnight at 4°C with the following primary antibodies: anti-Sox2 1:200 purchased from Abeam (Cambridge, UK), anti-TERT 1:250 purchased from Calbiochem (Gibbstown, NJ) and anti-NANOG 1:1000 purchased from Millipore (Milano). After three washings of 5 min under gentle agitation, the secondary antibody was added (Cy3 or Alexa fluor 488 anti-rabbit 1:500, purchased from Sigma) and incubated for 1 h at room temperature. The samples were rinsed twice in wash solution and incubated in DAPI (Vectastain, Vector laboratories, Bulingame) 1:5000 for 10 min for nuclear identification. Coverslips were finally mounted with Fluoromonut (Sigma Chemical Co. St. Louis, MO) and evaluated with Axiovision Cam Zeiss in order to confirm the presence of the stem cell markers and describe their subcellular distribution.

Assessment of osteogenic differentiation

The differentiation capacity of granulosa cells isolated from growing or luteinising follicles was evaluated after 14 and 21 days of culture by analysing extracellular matrix mineralisation, alkaline phosphatase (ALP) activity and the expression of the bone related genes osteocalcin and RUNX2.

Matrix mineralisation. At 14 and 21 days of culture under osteogenic conditions the cells, plated on glass coverslips, were incubated in osteogenic medium supplemented with calcein 1 u.g/ml (Sigma Chemical Co. St. Louis, MO) for 48 h [25]. At the end of this incubation plated cells were washed twice with PBS and the coverslips were mounted on glass slide. Calcein incorporation in the extracellular matrix was evaluated by fluorescent microscopy using a Ziess inverted microscope at 20 and 40x.

Samples of plated cells were stained in parallel with Alizarin Red S to confirm culture mineralisation. In brief plated cells were fixed in cold 4% paraformaldehyde for 15 min after a preliminary rinse with PBS. Following a rinse with distilled water the cells were incubated in 40 mM Alizarin Red S (Sigma Chemical Co. St. Louis, MO) for 30 min at room temperature and then quickly washed with 80% acetone. Glass coverslips were finally

rinsed in PBS mounted on glass slide and analysed on a phase contrast microscope (Nikon Eclipse 600).

Histochemical detection of ALP. Alkaline phosphatase (ALP) was revealed by cytochemical staining with BCIP/NTB (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) Liquid Sistem (Sigma Chemical Co. St. Louis, MO) [27]. Plated cells were fixed in pre-cooled 4% paraformaldehyde for 10 min, rinsed with deionized water, then incubated with BCIP/NTB liquid system for 2 hours at 37°C temperature in the dark. The reaction was stopped by removing the substrate solution, the coverslips were washed in distilled water mounted on glass slide and the cells were analyzed with a phase contrast microscope (Nikon Eclipse 600).

RT-PCR Analysis

Total RNA was extracted from 1 x 10^6 granulosa cells using TRI Reagent (Sigma Chemical Co. St. Louis, MO) according to the manufacturer's instructions. RNA concentrations were measured by absorbance at 260 nm with a spectrophotometer; integrity and size distribution were evaluated by agarose gel electrophoresis and ethidium bromide staining. The eventual contaminating genomic DNA was eliminated by DNaseI digestion (Sigma Chemical Co. St. Louis, MO) for 15 min at room temperature. 1 ug of total RNA of each sample was used for reverse transcription reaction with Oligo dT primer and BioScript™ (Bioline). 2X Ready mix™ Taq PCR Reaction mix (Sigma Chemical Co. St. Louis, MO) was used for PCR reaction using 3 ul of cDNA and 0.5 |jM of each primer, in a final volume of 25 ul. The primer sequences, Genebank number of reference mRNA sequence and product length are shown in Table 1. The reaction mixtures were incubated for 5 min at 95 °C, followed by 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s up to 40 cycles and then 72 °C for 7 min. For each gene, a reaction mixture with water instead of cDNA template was run at the same time as a negative control. RT-PCR was normalized by the

transcriptional levels of GAPDH. The PCR products were separated on agarose gel (2%) stained with ethidium bromide, visualized on a Gel Doc 2000 (Biorad) and analyzed with Quantity One 1-D Analysis software (Biorad). Each PCR reaction was carried out in triplicate.

Assessment of new bone formation in transplanted cell-loaded scaffolds

For a general overview, implant sections were stained with hematoxylin and eosin (H&E). To identify mineralization, a staining according to von Kossa in 5% AgNO for 1 h, exposed to a 60 W bulb, was performed.

Statistical analysis

All data were expressed as mean (± S.D.). The results of immunocytochemical, gene analysis and the effect of differentiation were compared by one-way ANOVA followed, when required, by Tukey's test (Orion Software Development, Version 2.0, Longmont CO). Differences were considered significant for $P < 0.05$ values.

Results

Granulosa cells from growing and luteinising follicles

A large number of granulosa cells could be isolated from each follicle (ranging from 4 to 5 x 10^6 cells/follicle), independently of the hormonal treatment.

LGC produced higher levels of progesterone than GGC as shown in Table 2 (80.12±5.99 vs 14.47±3.6 ng/10^6 cells, p<0.01) confirming the luteinisation process undertaken by these cells in response to hCG administration. By contrast, similar estradiol productions *in vitro* were recorded in the two cell types (1090.92±36.05 vs 1044.23±45.07 pg/10^6 cells). Freshly isolated granulosa cells showed a widespread production of inhibin that involved about 70% of the cells regardless of the treatment (Table 2).

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Granulosa cells typically expressed FSH receptors. The semi-quantitative analysis of RT-PCR data revealed higher levels of expression in LGC than in GGC although the difference did not achieve statistical significance (Table 2).

RT-PCR showed that granulosa cell preparations expressed Sox2, Nanog and TERT (Figure 1) immediately after isolation. The analysis of the sternness marker production at the single cell level revealed that the vast majority of the two granulosa cells types expressed the sternness markers Nanog and TERT (Figure 2) while a slightly lower proportion of cells, ranging from 60 to 70%, expressed Sox2 (Table 2).

Effect of *in vitro* expansion

Granulosa cells expansion *in vitro* caused an overall reduction of granulosa cell characteristics without affecting sternness markers expression as shown in Table 2.

The steroidogenic activity of both preparations progressively decreased with estradiol levels falling more rapidly than progesterone ones (Table 2). After three passages (corresponding to about 15 doublings in 24 days of culture) the estradiol and progesterone levels have dropped to approximately 1/100 and 1/10 of initial values, respectively.

The progressive reduction in steroidogenic activity of granulosa cells is paralleled by the rapid reduction of inhibin positive cells that, in fact, at the end of the third passage, were virtually absent (Table 2) in all the slides analysed.

Analogously, the expression of FSH receptors dropped throughout the expansion; at the first passage the cells had reduced their receptor concentration to about 1/3 of the level recorded immediately after isolation (data not shown) in both cell types and by the third passage the levels of expression were only barely detectable as shown in Table 2.

On the contrary, expansion did not substantially affect the expression of sternness markers: Nanog and TERT were expressed in more than 95% of the cells analyzed after three passages and Sox2 expression, although at lower levels, was retained on nearly

constant values (Table 2). No different behaviours were recorded in the two cell preparations throughout expansion *in vitro.*

Osteogenic differentiation *in vitro*

The results of *in vitro* experiments demonstrate that LGC cultured under inducing conditions display clear signs of osteogenic differentiation. Calcein is in fact consistently deposited in the extracellular matrix of LGC (Figure 3) after two weeks of culture under osteogneic conditions while this reaction is only barely detectable in the same cells cultured in growth medium. The fluorescent dye deposition in GGC at the same phase of expansion was much lighter, although the fluorescent signal was always stronger than that recorded in growth medium (Figure 3). The superior calcein deposition in luteinising cells compared with growing cells was retained also after 3 weeks of incubation although the difference between the two cell types was progressively reduced.

The analysis of extracellular matrix mineralization carried out by Alizarin red staining gave superimposable results (Figure 4): marked mineralized areas were recorded at 14 and 21 days of culture with a more evident calcium deposition in LGC compared to GGC.

The biochemical evolution of these cells, involved an increased alkaline phosphatase activity. Cells cultured in osteogenic medium had an alkaline phosphatase activity markedly higher than cells cultured in growth medium as shown in Figure 4. Moreover, this adaptation to the osteogenic function was more evident in luteinising than in growing cells, consistent with the mineralization analyses previously described.

The analysis of bone-related genes showed that the expression of osteocalcin and Runx2 recorded after 3 weeks of *in vitro* differentiation significantly increased in both cell types, (Figure 5), further confirming the osteogenic transformation of granulosa cells cultured in the presence of the inducing factors dexamethasone, ascorbic acid and (3-glicerol phosphate. In parallel with this functional transformation, the expression of the sternness

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factors, that had remained fairly constant throughout the *in vitro* expansion, markedly dropped in granulosa cells exposed to the above inducing agents, positive cells ranging from 10 to 20% (Table 2).

New bone formation *in vivo*

After 8 weeks of implantation, all implants were easily identified and retrieved for histological analysis. The implants, all without any sign of infection, were incompletely encapsulated by loose fibrogranulomatous connective tissue and permeated with host blood vessels. Examinations under fluorescent microscopy revealed the presence of PKH26 positive cells spread in the implant (Figure 6). H&E and Von Kossa staining demonstrate the extensive deposition of hydroxyapatite salts in granulosa cell loaded scaffolds. This bone matrix appeared after HE staining as a not organized woven bones deposited within circle cavity surrounded by flattened cells. Interestingly enough a significant proportion of these cells were PKH26 positive as shown in Figure 6. No signs of new bone formation were recorded in implants without cells.

Discussion

This paper clearly shows three fundamental features that make granulosa cells a promising candidate for the development of regenerative medicine: granulosa cells express sternness markers; they can be expanded *in vitro* preserving their plasticity and they can rapidly undergo osteogenic differentiation both *in vitro* and *in vivo.* Moreover, this investigation indicates that luteinising follicles, committed to ovulate by gonadotropin stimulation, represent the source of granulosa cells with the highest osteo-regenerative potential.

In detail, the present investigation demonstrates that high numbers of granulosa cells can be isolated from antral follicles with an average of about 5×10^6 cells per follicle.

Immediately after isolation, granulosa cells displayed the typical characteristics of constituents of ovarian follicle walls: they were steroidogenically active, with a progesterone/estradiol ratio reflecting the stage of the follicle they were derived from, they synthetized inhibin and showed a wide expression of FSH receptors. At the same time, granulosa cells expressed typical sternness markers, such as Nanog, Sox2 and TERT, consistent with what previously reported [17]. In this study, that originally described the staminality of granulosa cells [17], the cells were first FACS sorted by the presence of FSH receptors. By contrast we decided not to carry out any purification step because none of the parameters generally used to divide granulosa cells in sub-populations (e.g. steroid production, receptors etc) has any known relationship with the potential staminality of the cells. Moreover the majority of these markers, as shown in our own paper, are rapidly switched of in culture. Therefore, based on the sternness marker expression and the widespread differentiation potential, we considered granulosa cells homogeneous cell population from the point of view of their staminality.

Expansion in vitro had profound effects on granulosa cell characteristics. Steroid and inhibin production were rapidly switched off during expansion and the expression of FSH receptors was quickly down-regulated in both kinds of cells investigated, showing that *in vitro* granulosa cells modify their original functional conditions. Apart from the study of steroidogensis as a mean to characterize these cells, we investigated estradiol production for the favourable implications that this steroid might have on the process of osteogenesis [28-30]. If a significant production of estradiol had been retained by the cells, it could have locally potentiated osteogenic differentiation and new bone formation thus improving the regenerative capacity of granulosa cells. Unfortunately this was not the case; steroid production ceased *in vitro* and the block was even more complete after inducing osteogenic differentiation.

By contrast, expansion did not substantially affect the expression of sternness markers. After expansion most of the cells expressed Nanog, TERT and Sox2 and a detailed cytological examination confirmed that the factors were selectively localised within the nucleus, condition required for these factors to express their function [31]. This demonstrates that granulosa cells not only can be collected in large numbers but that they can also be amplified *in vitro* without losing their regenerative potential.

This somehow contrasts with the reduced expansion capacity reported for human purified LGC [17]. In this investigation the Authors found that granulosa cell, preliminarily purified on the basis of FSH receptor expression, a parameter unrelated to the sternness properties of these cells, had a limited survival *in vitro,* not exceeding 10 days, but this limit could be overcame by the addition of LIF to the culture medium. In our culture systems, characterised by the presence of EGF and by 20% FCS the cells retained their proliferation at a constant rate for the 20-25 days of the expansion.

Beyond confirming the expression of sternness markers in granulosa cells isolated from the pig model, this work was designed to identify the kind of follicle that may provide the granulosa cells with higher osteo-regenerative potential in order to provide useful information for translating the use of these cells from the bench to the clinics.

This question was raised by the consideration that oocyte retrieval for ART, which offers a precious opportunity to collect granulosa cells, is frequently carried out from preovulatory follicles few hours before ovulation [32,33]. In these cases, as reported for human LGC [17], the follicles are punctured well after the gonadotropin treatment that mimic the LH pre-ovulatory surge, i.e. shortly before ovulation. Preovulatory gonadotropin surge causes a dramatic change in the overall follicular function and, in particular, triggers the final differentiation phase of granulosa cells that culminates with their transition into luteal cells [32,33]. Since the rationale underlying this research is to take advantage of the ability of granulosa cells to transdifferentiate into a different cell type" we were wondering whether

granulosa cells taken before the surge, i.e. before starting this final step of differentiation, might have better transdifferentiation capacity. In addition LGC might have an impaired expansion potential since the gonadotropin surge causes the abrupt exit of granulosa cells from the cell cycle [32,33]. Therefore luteinising granulosa, at a final stage of differentiation and in a condition of cell cycle block, may not express the best staminality that these cells could offer.

To this aim we gave particular emphasis to the definition of the two phases of follicle development that we were interested in, namely antral follicle before and after the gonadotropin surge, i.e. growing healthy follicles and luteinising follicles. The high progesterone production and the presence of cumulus oophorus expansion as well as the resumption of meiotic maturation by the enclosed oocytes, allowed to confirm that hCG injected in eCG primed gilts, did mimic the preovulatory gonadotropin surge and produced luteinising follicles, while, on the other hand, the absence of these indicators allowed us to exclude that animal treated only with eCG had had an undesired premature endogenous gonadotropin surge triggered by increasing oestrogen levels and, therefore, that their follicles were true pre-gonadotropin surge follicles.

Interestingly enough, once isolated and maintained *in vitro,* granulosa cells from luteinising follicles had a proliferating activity similar to that of granulosa cells before hCG treatment and, despite their advanced differentiation status, these cells turned out to be more efficient to undergo osteogenic differentiation *in vitro.* All the parameters studied, that collectively document the ability of the cells to activate osteogenic genes, to produce extracellular matrix and to create the chemical conditions for its mineralisation, demonstrate that, contrarily to the expectations, LGC have greater plasticity showing an osteogenic evolution that was both more intensive on each single cell and more extensively diffused amongst the cultured cells than in cells recovered from growing follicles, before the LH surge.

Granulosa cells are known to undergo spontaneous luteinisation *in vitro* [34,35] and therefore similar behaviours could have been expected as expansion *in vitro* might have brought the two cell preparations to a similar condition of luteinisation but, on the contrary, LGC maintained higher differentiation potentials, indicating that the endocrine conditions experienced by the cells before isolation influence cell function also after prolonged intervals *in vitro.*

The different potential of the two cells types could not be confirmed *in vivo* where both cell types had stimulated similar nodules formation. The *in vitro* outcomes do not always reflect cell behaviour *in vivo* but, as far as the present research is concerned, the *in vitro* experiments, analysed through a complex set of investigations, are likely to be more sensitive and therefore to allow to reveal differences that could not be picked up *in vivo.* Nonetheless bone formation recorded in granulosa cell loaded scaffolds, definitely strengthen the regenerative potential of granulosa cells whenever osteogenesis is required. In addition to bone nodule formation, it was interesting to find that cells loaded into the scaffolds survived for 4 weeks after transplantation in host SCID mice thus providing the essential basic condition for the cells to exert any regenerative influence in host tissues. It is generally difficult to establish whether stem cells enhance tissue regeneration by stimulating local cells to proliferate and heal lesions or by directly taking part in the healing process. The histological examinations, showing bone nodules formation only in transplanted scaffolds loaded with granulosa cells, generically demonstrate that these cells can indeed stimulate bone formation but their localisation, concentrated around the nodules, suggests that they may actively participate in the process of new bone formation. Researches are currently under way to address this question.

Collectively the data obtained *in vitro* and *in vivo* demonstrate a remarkable osteogenic potential of granulosa cells. The reasons for this peculiar attitude is presently unclear but

the genetic arrangement of these cells may support their plasticity. RUNX2 has been identified as fundamental factor in osteogenic differentiation. It directly induces osteocalcin gene expression [36] and its deletion causes a lack of ossification [37,38]. Interestingly the up-regulation of the same RUNX2 gene characterisitcises the process of luteinisation that granulosa cells undergo after exposure to gonadotropins. Maybe the involvement of RUNX2 in the process of granulosa cell luteinisation may create a favourable starting condition for these cells once addressed toward osteogenic differentiation. This might explain the best osteogenic performances recorded in LGC although, even if to a lesser extent, GGC may have undergone spontaneous luteinization *in vitro* creating, also in this case, a favorable genetic condition for ostegenesis.

CONCLUSION

In conclusion, these data reported confirm the potential of granulosa cells for the development of new osteogenic regenerative medicine and contribute to further enlarge the list of adult cells, apparently fully differentiated, that in response to a variety of specific environmental cues and signals, may resume an unsuspected plasticity and, either by dedifferentiating or by directly transdifferentiating, originate cell types different from those present in the tissue they are derived from thus creating new options for cell therapies.

List of abbreviations used

GC: granulosa cells; LGC: granulosa cells from luteinising follicles; GGC: granulosa cells from growing follicles; LH: luteinising hormone; PBS: phosphate buffer saline; FCS: fetal calf serum; MeM: minimal essential medium; EGF: epithelial growth factor; ALP: alkaline phosphatase; eCG: equine chorionic gonadotropin; hCG: human chorionic gonadotropin.

Authors' contribution

MM participated in the conception of the research and in the cell cultures, AG participated in cell culture and analysis, MT participated in tissue cultures, PB, VR and DN carried out histological investigations, VC carried out the molecular genetic studies, MB and EM conducted in vivo tests, BB participated in the conception of the research and in cell isolation.

Authors' information

[1] Department of Comparative Biomedical Sciences, University of Teramo, 64100 Teramo, Italy

department of Veterinary Morphophysiology, University of Turin, 10095 Grugliasco (TO), Italy

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Figure legends

Figure 1 RT-PCR analysis of LGC. Expression of Sox2, Nanog and TERT in granulosa cells immediately after isolation (GAPDH internal control).

Figure 2 Immunohistochemical detection of inhibin, TERT, Sox2 and Nanog in granulosa cells immediately after isolation.
A, C, E, G are specific immunostaining for inhibin, TERT, Sox2 and Nanog. B, D, F, H are immunostainings merged with Dapi nuclear staining. Note the clear nuclear localization of sternness markers exception for few cases like the arrowed cell. By contrast inhibin is clearly localized in the cytoplasm.

Figure 3 Calcein deposition in cultures of granulosa cells.
Calcein deposition in GGC and LGC cultured for 14 and 21 days under osteogenic conditions (A, C, E, G) or in control growth medium (B, D, F, H). Calcein uptake appears as a fluorescent green particularly evident in LGC and absent in both cell types cultured in growth medium.

Figure 4 Alkaline phosphatase activity and matrix mineralization in cultures of granulosa cells.

Alkaline phosphatase activity (ALP) and matrix mineralization revealed by alizarin red (AR) in LGC and GGC cultured in osteogenic (DM) or control growth medium (CTR) for 14 or 21 days. ALP activity and matrix mineralization are more intense in LGC than in GGC, they increase with the progression of the culture. Both reactions remain on baseline levels in control medium.

Figure 5 Expression of Osteocalcin and RUNX2 in cultured granulosa cells

Semiquantitative expression of Osteocalcin and RUNX2 (relative to a GAPDH internal control) in LGC and GGC before differentiation (TO) and after 3 weeks of culture under osteogenic conditions (D3). Values are average +/-1 SD of 3 replicates.

Figure 6 Mineralization of granulosa cell - loaded implants

Left - Von Kossa staining of a granulosa cell loaded scaffold retrieved 8 weeks after sc implantation in a SCID mouse. The black nodules represent calcified extracellular matrix. (Magnification 20x).

Right - HE staining of a granulosa cell loaded scaffold merged with the corresponding fluorescent image showing in blu cell nuclei (stained with DAPI) and in red granulosa cells (marked with the membrane marker PHK26). (magnification 40x).

Table 1. Primer sequences used for RT-PCR analysis of granulosa cells

| Target gene	GeneBank no.	Primer sequence	Product length (bp)
S0X2	NM_001123197.1 Sus Scrofa	F: GCAGCAGGGCACCCCTGGC R: TGTGAGAGAGAGGCAGTGTACC	266
NANOG	NM_001129971.1 Sus Scrofa	F: CCCAGAGTAAAACCACTGCCC R: GAATAGAAGCCCGGGTATTCTG	323
TERT	AY785158.1 Sus Scrofa	F: CTTAGATCGTGTGCGGCTTCG R: CTCGTAGTTCAGCACAGCAAAC	230
FSHR	AF025377.1 Sus Scrofa	F:GGAGAGCAAGGTGACAGAGAT R: ATATCGGAGGTTGGGAAGGTT	274
OCN	NM_001164004.1 Sus Scrofa	F: CCACCAACAGACGCACCATG R: TTGAGCTCACACACCTCCCT	247
RUNX2	XM_001924306.1 Sus Scrofa	F: GGA CGAG GCAAGA GTTTCAC R: GTGGATTAAAAGGACTTGGTGC	264
GAPDH	AF069649.1 Sus Scrofa	F: CACTGCCACCCAGAAGACTG R: TGCTTCACCACCTTCTTGATG	251

Table 2. Sternness markers and granulosa cell characteristics

	TO		T3		D3	
	GGC	LGC	GGC	LGC	GGC	LGC
	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.
Sox2 (%)	53.04 ± 3.4^{A}	47.45 ± 6.77^{A}	53.8 ± 3.6^{A}	56.6 ± 4.35^{A}	23.13 ± 6.11^{B}	18.36 ± 3.02^{B}
Nanog (%)	99.33 ± 1.15^{A}	96.58 ± 4.34^{A}	98.33 ± 1.52^{A}	93.57 ± 3.7^{A}	21.33 ± 3.5^{B}	15.1 ± 2.01^{B}
TERT (%)	97.33 ± 1^{A}	94.07 ± 2.67^{A}	98.33 ± 1.53^{A}	98.9 ± 3.32^{A}	9.6 ± 1.5^{B}	8.07 ± 1.6^{B}
Inhibin (%)	65 ± 3.45^{A}	71.56 ± 3.8^{A}	5.03 ± 2.25^{aB}	3.67 ± 2.21^{aB}	1.38 ± 0.93^{DB}	1.56 ± 0.42^{DB}
FSH-R	1.42 ± 0.43^{A}	1.83 ± 0.43^{A}	0.25 ± 0.04^{aB}	0.09 ± 0.02^{aB}	0.02 ± 0.06^{D}	0.1 ± 0.02^{aB}
P4 (ng/ml)	14.47 ± 3.6^{A}	80.12 ± 5.99^{B}	$2.6610.64^{\wedge}$	7.08 ± 1.07^{u}	0.26 ± 0.05^{b}	0.83 ± 0.28^{b}
E2 (pg/ml)	1044.23 ± 45.07^{A}	1090.92 ± 36.05^{A}	10.31 ± 2^{A}	11.61 ± 0.1^{B}	10.66 ± 1.57^{B}	9.33 ± 1.5^{B}

Percentages of granulosa cells positive for Sox2, Nanog, TERT and inhibin; expression of FSH receptors and steroid production in GGC and LGC immediately after isolation (TO), after three passages of expansion in vitro (T3) and after 3 weeks of differentiation in vitro (D3). In the same row, values with different letters in superscripts differ significantly (a/b, $P < 0.05$; A/B/C/D/E, $P < 0.01$)
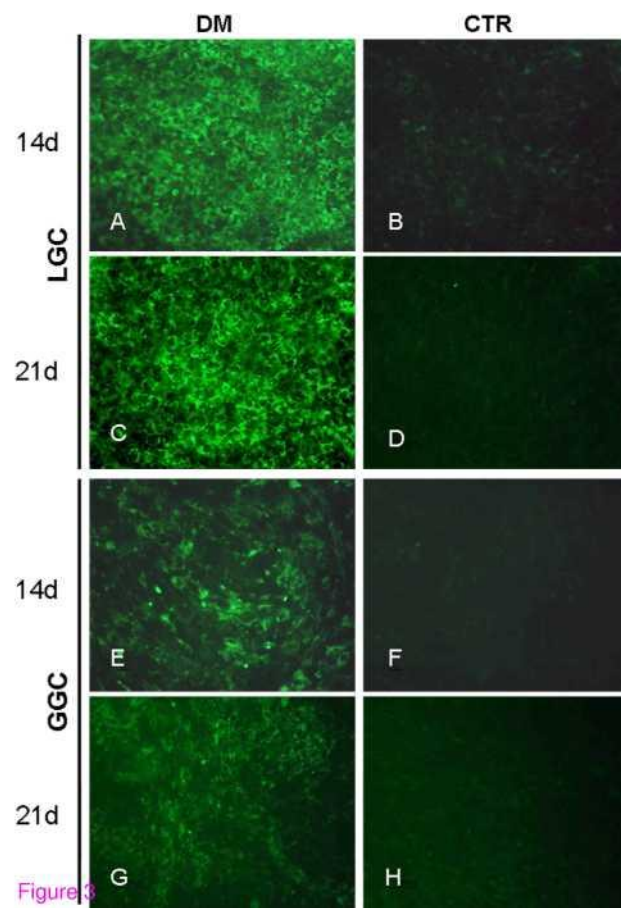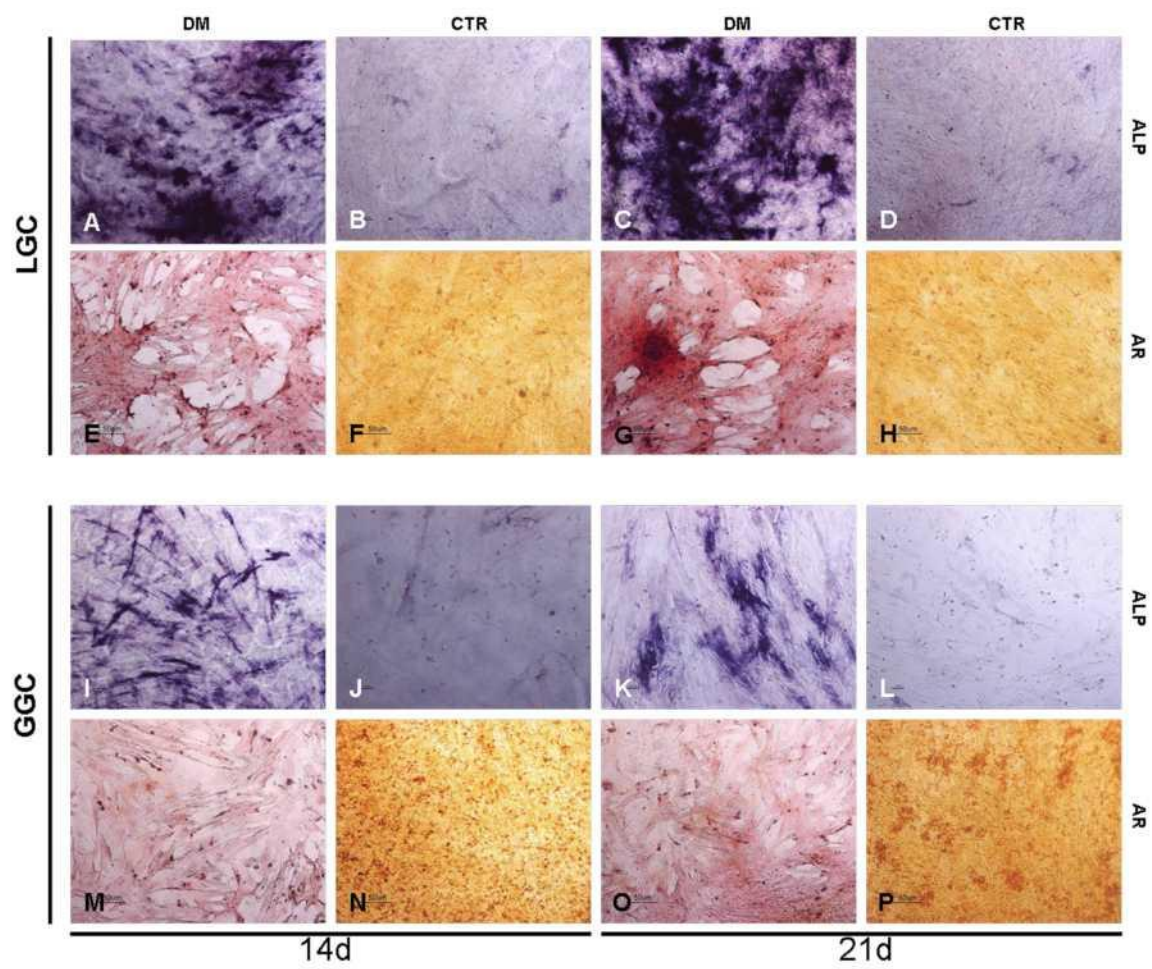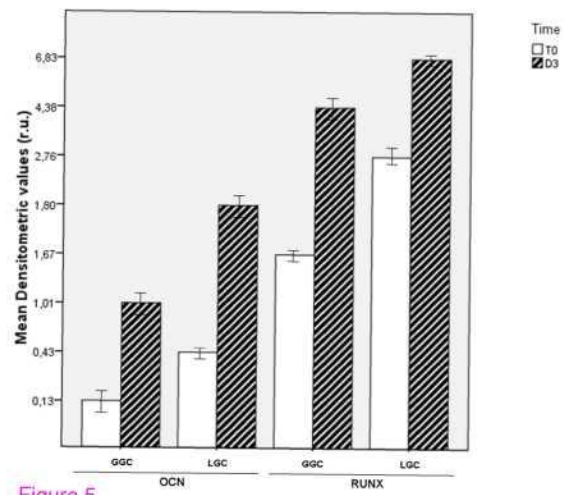
Figure 1

Nanog | SOX 2 | TERT | Inhibin alpha
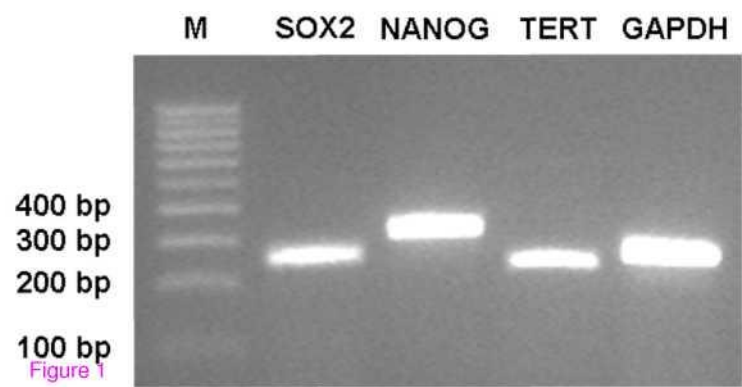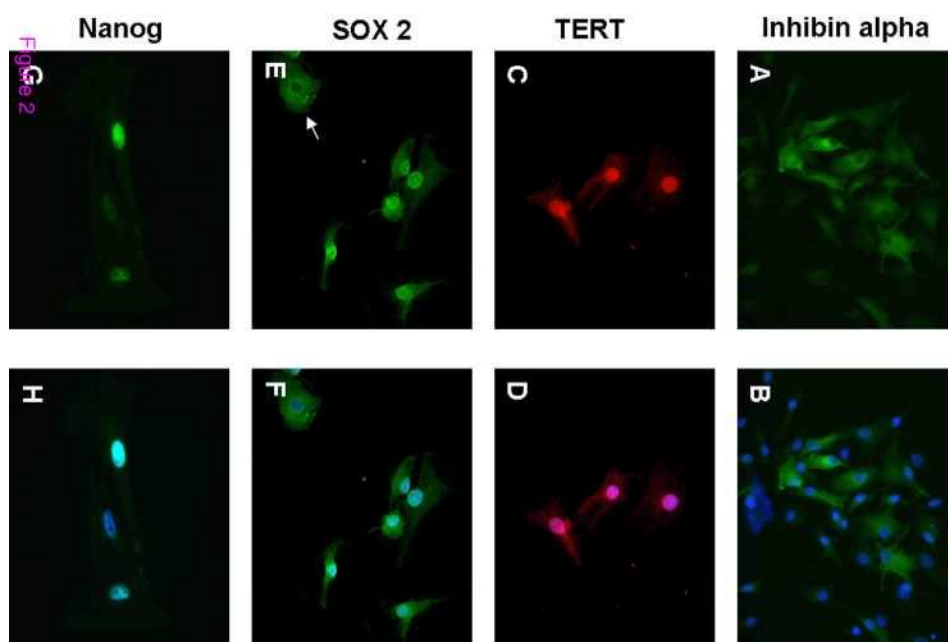
Figure 2

Figure 3

Figure 4

Figure 5

A   B