Stem Cell Rev and Rep
DOI 10.1007/s12015-014-9521-4

Morphological and Molecular Changes of Human Granulosa Cells Exposed to 5-Azacytidine and Addressed Toward Muscular Differentiation

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Abstract Converting adult cells from one cell type to another is a particularly interesting idea for regenerative medicine. Terminally differentiated cells can be induced to de-differentiate in vitro to become multipotent progenitors. In mammals these changes do not occur naturally, however exposing differentiated adult cells to synthetic molecules capable of selectively reverting cells from their lineage commitment to a more plastic state makes it possible to re-address their fate. Only scattered information are available on the morphological changes and ultrastructural remodeling taking place when cells convert into a different and specific type. To better clarify these aspects, we derived human granulosa cell (GC) primary cultures and analyzed the morphological changes taking place in response to the exposure to the epigenetic modifier 5-azacytidine (5-aza-CR) and to the treatment with VEGF, as a stimulus for inducing differentiation into muscle cells. Ultrastructural modifications and molecular marker expression were analyzed at different intervals during the treatments. Our results indicate that the temporary up regulation of pluripotency markers is accompanied by the loss of GC-specific ultrastructural features, mainly through autophagocitosis, and is associated with a temporary chromatin decondensation. After exposure to VEGF the induction of muscle specific genes was combined with the appearance of multinucleated cells with a considerable quantity of non-spatially organized filaments. The detailed analysis of the morphological changes occurring in cells undergoing lineage re-addressing allows a better understanding of these process and may prove useful for refining the use of somatic cells in regenerative medicine and tissue replacement therapies.

Keywords Cell conversion · Ultrastructure · Autophagy

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Introduction

A primary goal of regenerative medicine is to produce new cells to repair or replace diseased and damaged tissues. Among the many innovative ideas proposed to achieve this goal, a particularly interesting one involves remaking existing adult cells into new ones, by converting them from one cell type to another. A clear challenge to remodeling adult cells is that mature cells of adult organisms are remarkably stable. Differentiated cells maintain their state for years and rarely, if ever, switch to a new state. It has been shown that cells arrive at a stable position by a progressive or sequential restriction in their options, beginning as a pluripotent embryonic cell and ending up at the mature differentiated state [1]. Once the differentiated state is acquired, cells stably retains it. In mammals neither trans-differentiation nor de-differentiation has yet been identified as a naturally occurring process. However a number of protocols have been developed in recent years that revealed the remarkable potential of cells to switch from a differentiated state to another less committed one.

Currently this is most commonly achieved by the forced expression of a small set of transcription factors that enable the reprogramming to pluripotency of a somatic cell (iPS) [2, 3]. This requires a process of de-differentiation which is gradually achieved through cell proliferation [4, 5]. Alternatively cell

Published online: 25 May 2014

conversion can be obtained without passing through a stable state of pluripotency by direct lineage transdifferentiation introducing a single [6] or a combination of transcription factors [7–9]. This process does not involve the transition through a de-differentiated state but requires the high expression of the specific transcription factors. It rapidly evolves towards the final phenotype in a matter of a few hours or days after overexpression of specific transcription factors [7–9] and does not require cell proliferation [10, 11].

Lineage conversion can also be accomplished creating a "flexible" epigenetic state by temporarily overexpressing the pluripotency transcription factors so that specific cell types can be generated by providing the suitable differentiation clues but without reversion to pluripotency [12, 13].

More recently we developed a conversion protocol based on the brief exposure of skin fibroblasts to the demethylating properties of the cytidine analog 5-azacytidine (5-aza-CR), a well-characterized DNA methyltransferase inhibitor, immediately followed by a specific differentiation protocol [14, 15]. This method was able to convert fibroblasts from both human and porcine species into cells of different lineage and has the substantial advantage to avoid the transfection of any exogenous transcription factor. However, many of the complex regulatory mechanisms involved are still to be understood. We know that the process involves a transient global reduction of DNA methylation which is consistent with a large body of information indicating the importance of substantial modifications of the methylation status of gene promoter regions [16] and the role of global hypomethylation in the transition to an undifferentiated state [17].

However only scattered information are available on the morphological changes and the ultrastructural modifications that take place in cells transiting along a conversion process in parallel with the epigenetic changes. Therefore, aim of the present study is to perform a thorough analysis of the morphological changes that take place in cells undergoing 5-aza-CR-mediated lineage re-addressing in order to reach a better understanding of the biology of this process, in view of its possible use in regenerative medicine and tissue replacement therapies.

To this purpose we selected human granulosa cell (GC) primary cultures obtained from pre-ovulatory follicles as a starting cell population because of their well described terminally differentiated state therefore excluding the casual presence of undifferentiated or precursor cells [18–20]. We exposed GCs to 5-aza-CR before we induced their conversion into muscle cells, growing them in a specifically formulated medium. We used this model because it implies extensive morphological changes.

We analyzed the morphological and ultrastructural modifications taking place during the conversion process in parallel with the expression of stage-specific differentiation markers.

Materials and Methods

All experiments were performed in three independent replicates. All chemicals were purchased from Sigma (Italy) unless otherwise indicated.

Collection and Culture of Human GC

GC were aspirated along with follicular fluid and oocytes from 3 patients participating in an IVF program. Informed patients were stimulated using a long protocol with a GnRH analogue (triptoreline, Decapeptyl 0.1; Ipsen S.p.A., Italy), or a protocol using a GnRH antagonist (Cetrotide; Serono, Italy). In both cases, ovarian hyperstimulation was achieved using recombinant FSH (rFSH, Gonal-F; Serono). Follicular development was monitored via ovarian ultrasonography and serum estradiol-17 assay. When at least three follicles with a mean diameter of 18 mm were present, patients were instructed to take 5,000 IU of hCG (Gonasi; AMSA, Italy).

Transvaginal follicular aspiration for oocyte retrieval was performed 36 h after hCG. During ovum pickup, oocyte cumulus complexes were immediately separated from follicular fluid which was processed to isolate GCs. Red blood cells were removed by centrifugation on a preparation of Percoll. After mechanical dispersion, cells were counted in a haemocytometer and plated (7.8×10^4 cells/cm^2) on 0.1 % gelatin pre-coating 4-wells culture dishes (Nunc, DK). After 24 h and before replacing the medium, three washings with PBS at 37 °C enabled the removal of dead GCs. Cells were then cultured in M199 (Gibco, Italy) supplemented with 10 % Fetal Bovine Serum (FBS, Gibco-Invitrogen, Italy), 200 mM glutamine, antibiotic-antimicotic, ITS 100X, 75UI FSH- 75 UI LH (Menogon, Serono, Italy) at 37 °C in a humidified environment with 5 % CO2 in air.

Treatment of GC with 5-aza-CR

5-aza-CR was prepared following the manufacturer's instruction. Human GCs were then treated with 1 μM 5-aza-CR for 18 h as previously described [15].

Muscular Differentiation

Following exposure to 1 μM 5-aza-CR for 18 h, human GCs were washed twice with PBS and allowed a 3 h recovery period in human ESC medium [21] with no 5-aza-CR.. Muscular differentiation was induced by culturing the cells in DMEM High Glucose medium, supplemented with 10 % (FBS, Gibco-Invitrogen, Italy), 200 mM glutamine, antibiotic-antimicotic and 5 ng/ml Recombinant human $VEGF_{165}$ (Peprotech, USA). Differentiation culture was carried out for 15 days. Cell differentiation and morphological

changes were evaluated with molecular analysis, immunocytochemical technique and electron microscopy (see below).

Morphological Analysis

Cells were analyzed at three time points: immediately before exposure to 5-aza-CR, at the end of the recovery period after 5-aza-CR exposure and at the end of the differentiation culture.

Electron Microscopy

Samples were fixed for 1 h in 0.1 M cacodylate buffer pH 7.2, containing 2 % glutaraldehyde. Specimens were then washed in the same buffer and post-fixed for 1 h with 1 % osmic acid in cacodylate buffer. After standard serial ethanol dehydration, specimens were embedded in an Epon-Araldite 812 mixture. Sections were obtained with a Reichert Ultracut S ultratome (Leica, Austria). Semi-thin sections were stained by conventional methods (crystal violet and basic fuchsin) and subsequently observed under a light microscope (Olympus, Japan). Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 EX electron microscope (Jeol, Japan).

Immunocytochemistry

Markers of GCs and muscle cell differentiation were assessed by immunocytochemistry using the following primary antibodies: Cytokeratin-17 (1:100, Chemicon, USA); MyoD (1:40, SantaCruz, USA); Desmin (1:100, Chemicon, USA) and MHC (1:100, SantaCruz, USA). Staining conditions were as indicated by manufacturers. Incubation with suitable secondary antibodies (Alexafluor) was carried out for 30 min and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma, Italy). Samples were observed either under a TCS-NT laser confocal microscope (Leica Microsystems, Germany) or a Eclipse E600 microscope (Nikon, Japan).

Molecular Analysis

Reverse Transcription-Polymerase Chain Reaction

All chemicals were purchased from Invitrogen (Milan, Italy) unless otherwise indicated.

RNA was extracted using the acid-phenol method [22] and included a DNase I (1 U/μl) incubation. RNA was then immediately reverse transcribed, using Superscript™ II Reverse Transcriptase and following the manufacturer's instruction. Amplifications were carried out in an automated thermal cycler (iCycler, Biorad), using the conditions appropriate for each set of primers (Table 1). Expression of GAPDH was always examined as an internal control of the sample quality. RNA extracted from a human Skeletal Muscle Cell Line (HSkMC, Innoprot, Spain) was used as a positive control for muscle related genes. Amplification products were purified in Spin-X centrifuge tube filters (Corning, the Netherlands), sequenced (SEQLAB, Gottingen, Germany) and aligned using Clustal W 1.82 (EMBL-EBI service).

Karyotyping

At the end of an overnight incubation in 0.1 μg/ml Colcemid (Gibco, Italy) cells were dislodged with 0.25 % trypsin and centrifuged at 200*g* for 8 min. The cell pellet was gently resuspended in 0.075 M KCl solution and incubated for 30 min at 37 °C followed by fixation with methanol/glacial acetic acid (3:1) solution. Fixed cells were dropped on wet slides and air dried. Giemsa staining was carried out as indicated by manufacturer (Cariomax Giemsa, Gibco, Italy). A total of 493 metaphases were fully karyotyped under a Leica HC microscope. Images were captured with digital camera Leica DC250 and then analyzed using a Leica CW4000 Karyo software.

Results

GCs cultured in monolayers displayed the typical features of this cell population. As shown in Fig. 1, cells plated in culture dishes grew in tight patches of flattened, slightly rounded, epithelioid-like cells (panels A–B). Cell surface was covered with microvilli; nuclei were either deeply indented or more or less rounded, and the cytoplasm contained the organelles typical of this cell type such as mitochondria, smooth and rough reticulum, Golgi complex, granules and lipid droplets (panels C–D). Immunocytochemical characterization showed positivity for CYTOKERATIN17 (K-17) and CYCLOOXIGENASE2 (COX-2), while a negative immunophenotype was confirmed for muscular markers such as DESMIN, MYOSIN HEAVY CHAIN (MHC) and MYOD, indicating that neither muscle cells nor cells of muscular origin were present in the culture (Fig. 2 panel a). Molecular analysis demonstrated expression of transcripts that have been previously shown to be distinctive of granulosa cells, such as *CYTOKERATIN17*, *HYALURONIC ACID SYNTHASE2* (*HAS2*), *GREMLIN1* (*GREM1*) *and PENTRAXIN3* (*PTX3*). In agreement with immunocytochemical results, no transcription for *DESMIN*, *MYOD*, *MYOGENIN* and *MYOSIN HEAVY CHAIN* (MHC) was detected (Fig. 3 panel a).

After exposure to 5-aza-CR, cell phenotype changed (Fig. 1 panels e–h). Cells showed either a few short microvilli on the plasma membrane or a smooth cell surface (Fig. 1, panels e–f). Nuclei were large, round and showed a global chromatin decondensation (Fig. 1, panel f). In addition numerous

Table 1 Nucleotide sequence, annealing temperature, product size and accession number of the primers used for reverse transcription-PCR of human GCs

Genes	Primer sequences	Annealing temperature	Fragment size	NCBI accession n.
COX2	5′- CAGCACTTCACGCATCAGTT -3′ 3′- CAGCAAACCGTAGATGCTCA -5′	60 °C	253 bp	AY462100.1
CYTOKERATIN-17	5′-GGCCCGCCCGTGACTACA-3′ 3′-ATCTCCTCCTCGTGGTTCTTCTTC-5′	62 °C	301 bp	NM_000422
DESMIN	5′-CCTACTCTGCCCTCAACTTC-3′ 3′-AGTATCCCAACACCCTGCTC-5′	58 °C	519 bp	NM_001927
GAPDH	5′-CCCATCACCATCTTCCAGGA-3′ 3′-TTGTCATACCAGGAAATGAGC-5′	60 °C	731 bp	NM_002046
GREM1	5′-GTCACACTCAACTGCCCTGA -3′ 3′-GCACACGAACTACGCACAAG -5′	57 °C	251 bp	NM_013372
HAS2	5′-ATCATCCAAAGCCTGTTTGC -3′ 3′-GGACCCTTTTCGTGGAAGTT -5′	56 °C	301 bp	NM_005328
MYOGENIN	5′-GTCTTCCAAGCCGGGCATCCTTG-3′ 3′-GAGCTGGGGCATACACGAGGGG-5′	64 °C	279 bp	NM_002479
MYOD	5′-GGCCTTTGAGACACTCAAGC-3′ 3′-GGTATATCGGGTTGGGGTTC-5′	60 °C	572 bp	NM_002478
NANOG	5′- TTCCTTCCTCCATGGATCTG -3′ 3′- TCTGCTGGAGGCTGAGGTAT -5′	60 °C	213 bp	NM_024865.2
OCT4	5′- GAGGAGTCCCAGGACATCAA -3′ 3′- CTCCAGGTTGCCTCTCACTC -5′	60 °C	339 bp	NM_002701.5
PTX3	5′- ATTCAGAGGAAGGGCTCACA -3′ 3′-TGCTCCTCCGGTCTCTCTTA -5′	57 °C	241 bp	NM_002852
REX1	5′-TTGGAGTGCAATGGTGTGAT -3′ 3′-TCTGTTCACACAGGCTCCAG -5′	60 °C	200 bp	NM_174900.3
SOX2	5′- CATCACCCACAGCAAATGAC -3′ 3′- TTTTTCGTCGCTTGGAGACT -5′	60 °C	307 bp	NM_003106.3

lysosomes and autophagosomes occupied the cytoplasm previously filled with a large amount of granules and lipid droplets which became scarcely represented after 5-aza-CR treatment (Fig. 1, panels g–h). Simultaneously with the autophagic phenomena, a less dense peripheral organelle-free ring, became visible in the cytoplasm (Fig. 1, panels e–g). Cell immune reactivity for OCT4 and NANOG became evident with a specific nuclear localization of both molecules (Fig. 2, panel b). This positivity was also confirmed by PCR analysis that showed expression of the two pluripotency-related genes, as well as active transcription of *SOX2* and *REX1* (Fig. 3 panel b). These genes, originally undetectable in untreated human GC (lane 1), appeared to be induced by the exposure to 5-aza-CR (human GC + 5-aza-CR, lane 2) and were then switched off in response to VEGF (human GC + 5-aza-CR + VEGF, lane 3).

Treatment with human recombinant VEGF for 15 days addressed GCs exposed to 5-aza-CR towards muscular differentiation (Fig. 1, panels i–k). Efficiency of cell differentiation in response to VEGF treatment, resulted in 92.1±9.3 % cells positively staining for DESMIN, 94.3±7.7 % for MHC and 75±8.5 % for MYOD (Fig. 3, panel d), indicating that the differentiation process involved a large proportion of cells.

Cell phenotype showed a completely different spatial organization compared to the starting cell population. Electron microscopy evaluation demonstrated large elongated, multinucleated cells displaying a cytoplasm occupied by considerable quantity of non-spatially organized filaments (Fig. 1, panel k). Elongated spindle-shaped multinucleated cells (Fig. 1, panels i–j) were immunopositive for DESMIN, MHC and MYOD (Fig. 2, panel c). This was consistent with the results obtained by PCR that demonstrated expression of *DESMIN*, *MYOD*, *MYOGENIN* and *MHC* (Fig. 3, panel c). These genes, originally undetectable in untreated human GCs (lane 1), appeared to be induced by the exposure to VEGF (human GC + 5-aza-CR + VEGF, lane 2).

Muscular differentiation completely abolished both immune- and molecular-positivity for CYTOKERATIN17, which is distinctive of GCs (Figs. 2, panel a and 3, panel a). By contrast *COX2*, which is expressed in GCs as well as in muscular cells, was detected in GC primary cultures and persisted after muscular differentiation of the cells (Figs. 2, panels a, c and 3, panels a, c).

The presence of normal regulatory mechanisms that controlled the changes taking place in GCs transiting from an epithelial to a muscular phenotype was also confirmed by the karyotype analysis that showed maintenance of a normal chromosome set up in all cells analyzed (Table 2).

Discussion

The present paper describes the modifications taking place in human GC primary cultures while transiting from their original differentiated state to a higher plasticity condition–in

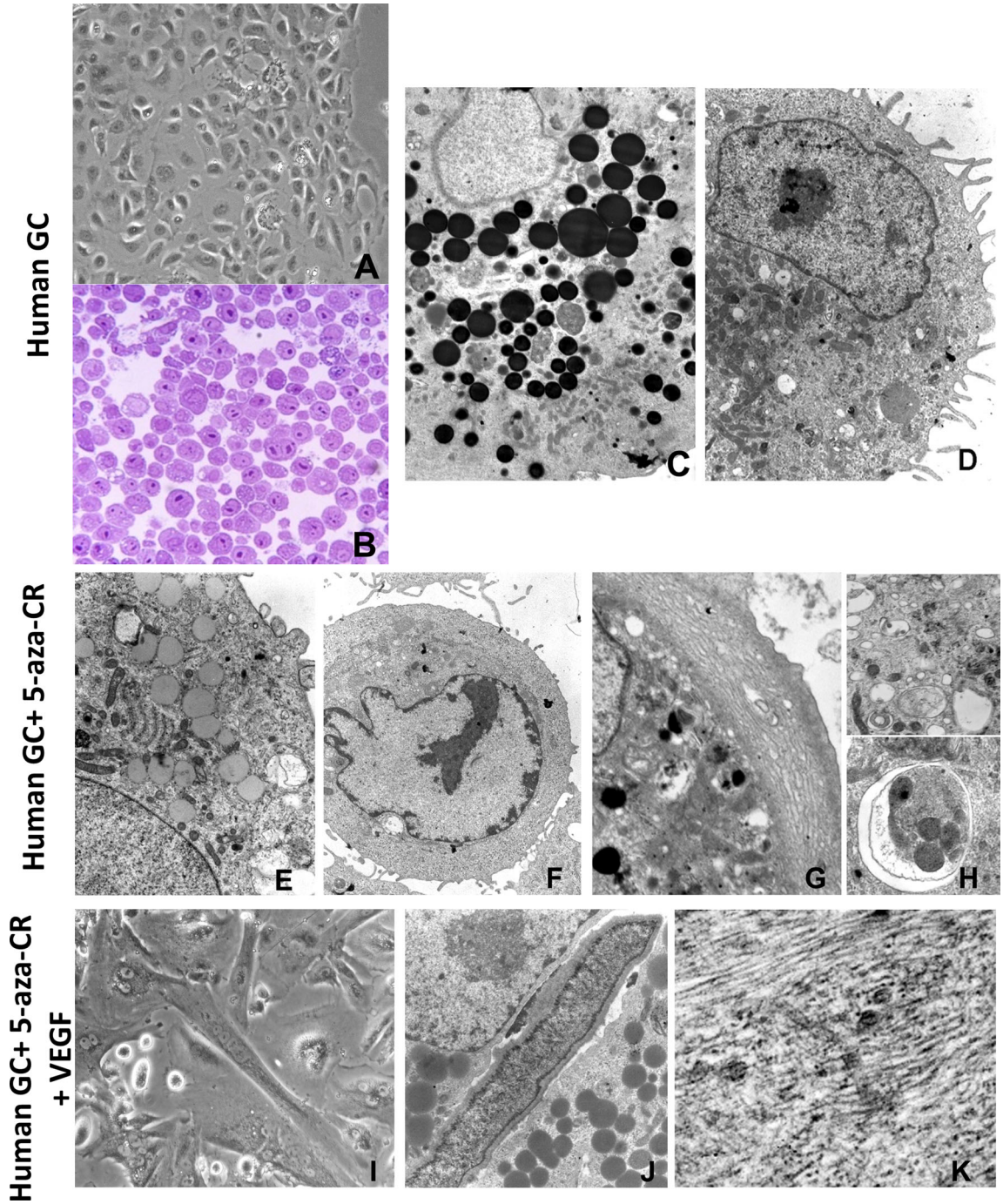

Fig. 1 Morphological changes of human granulosa cells exposed to 5-azacytidine and addressed toward muscular differentiation. GCs growing in tight patches of flattened, rounded, epithelioid-like cells (panels **a**–**b**). Cells presented a microvilled surface, rounded or deeply indented nuclei, and a cytoplasm containing cellular organelles such as mitochondria, smooth and rough reticulum, Golgi complex, granules and lipid droplets (panels **c**–**d**). Exposure of GCs to 5-aza-CR for 18 h, induced changes in cell phenotype (panel **e**–**h**). TEM analysis showed cell surface with very few short microvilli (panels **e**–**f**). Progressively lysosomes and autophagosomes (panel **h**) invaded the cytoplasm while granules and lipid droplets were scarcely represented after the treatment (panels **e**–**h**). A less dense cytoplasmic organelle-free peripheral ring was visible (panels **f**–**g**). VEGF treatment addressed 5-aza-CR-exposed GCs towards muscular differentiation. Electron microscopy examination (panels **i**–**k**) showed elongated, bi- or tetra-nucleated myotube-like cells (panel **i**) displaying a cytoplasm occupied by disarranged filaments (panels **k**). Spindle-shaped multinucleated cells (panels **i**–**j**) became visible

response to the treatment with the epigenetic modifier 5-aza-CR and after having been re-addressed towards muscular differentiation.

Despite GCs were obtained from patients participating in an IVF program and therefore were collected through the transvaginal aspiration of follicle walls, the primary cultures

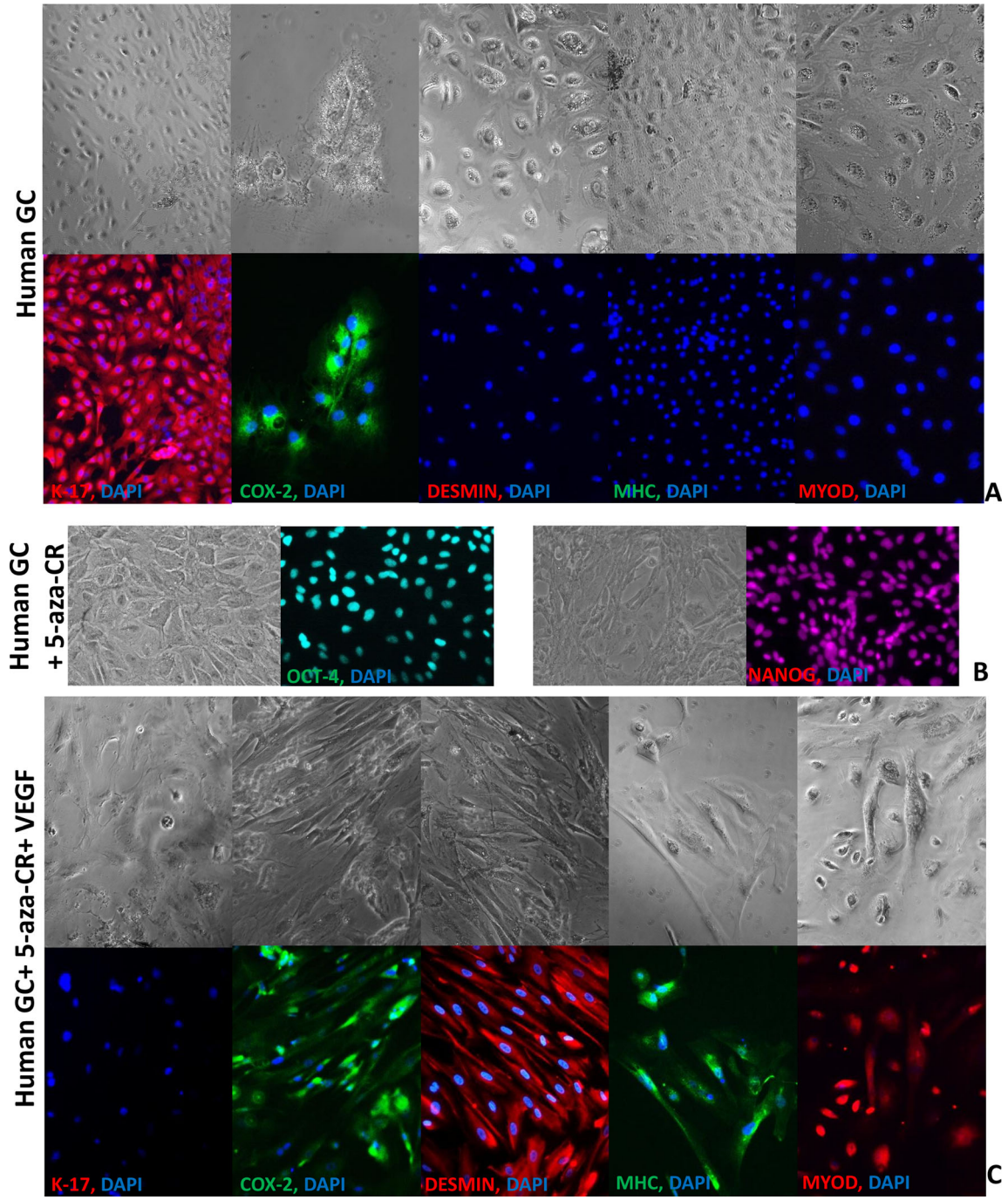

Fig. 2 Immunocytochemical changes of human granulosa cells exposed to 5-azacytidine and addressed toward muscular differentiation. Immunocytochemical characterization of GCs showed positivity for K17 and COX-2, while a negative immunophenotype was confirmed for muscular markers such as DESMIN, MHC and MYOD (panel **a**). After 5-aza-CR exposure, cell immune reactivity for Oct-4 and Nanog became evident and both molecules displayed a specific nuclear localization (panel **b**). 5-aza-CR treated cells exposed to VEGF were immunopositive for COX-2, DESMIN, MHC and MYOD (panel **c**)

were highly purified and homogeneous, as demonstrated by their morphology, the even immunocytochemical localization of CYTOKERATIN17 *and* COX2, which are typical of GC population. At the same time the complete absence of muscular markers such as DESMIN, MYOD, and MHC indicated that cultures were not accidentally contaminated by muscle cells or cells of muscular origin (Figs. 1, panel a, 2, panel a and 3, panel a). GC monolayer high purity was further supported by the expression of specific transcripts such as *HAS2*, *GREM1* and *PTX3* (Fig. 3, panel a).

A number of observations indicate that inhibition of DNA methylation, can coax cells into achieving more easily

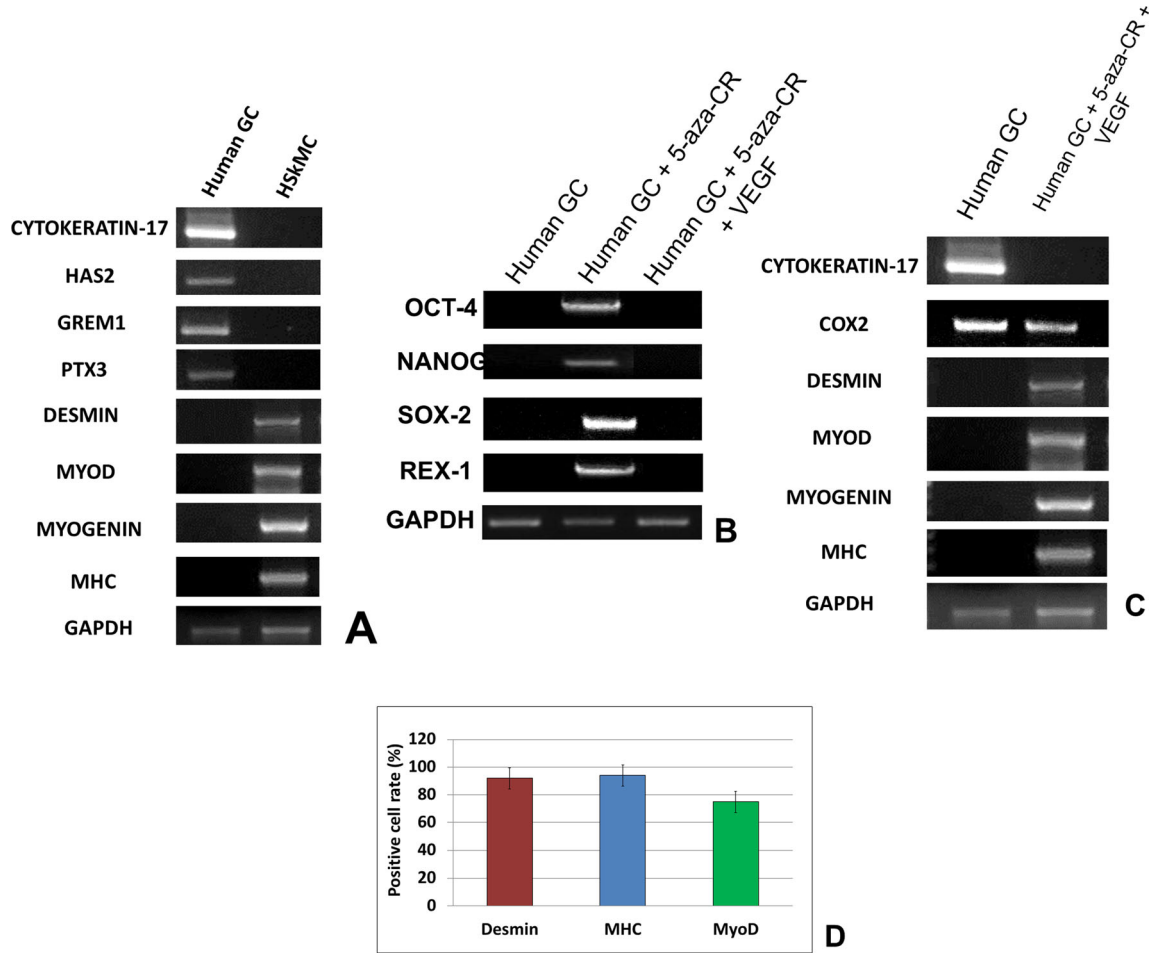

Fig. 3 Molecular changes and efficiency differentiation of human granulosa cells exposed to 5-azacytidine and addressed toward muscular differentiation. Molecular analysis of GCs demonstrated expression of *K17*, *HAS2*, *GREM1* and *PTX3*. No signal was detected for *DESMIN*, *MYOD*, *MYOGENIN* and *MHC* (panel **a**, lane 1: human GC). Lane 2: human Skeletal Muscle Cells (HSkMC). GCs exposed to 5-aza-CR for 18 h showed expression of *OCT4*, *NANOG*, *SOX2* and *REX1* (panel **b**). Originally undetectable in untreated human GC (lane 1), these genes appeared after exposure to 5-aza-CR (human GC + 5-aza-CR, lane 2) and were then absent in cells treated with VEGF (human GC + 5-aza-CR + VEGF, lane 3). After muscular induction with VEGF cells expressed *DESMIN*, *MYOD*, *MYOGENIN* and *MHC* (panel **c**). These genes, originally undetectable in untreated human GC (lane 1), appear to be induced by the exposure to VEGF (human GC + 5-aza-CR + VEGF, lane 2). Along muscular differentiation induction, molecular-positivity for K17–which is distinctive of GCs–was lost (panels **c**). By contrast *COX2*, which is expressed in GCs as well as in muscular cells, persisted after muscular induction with VEGF treatment (panels **c**). Differentiation efficiency in response to VEGF treatment, resulted in 92.1±9.3 % cells positively staining for DESMIN, 94.3±7.7 % for MHC and 75±8.5 % for MYOD (panel **d**), indicating that the differentiation process involved a large proportion of cells

pluripotency albeit in conjunction with the transfection of transcription factors [23]. Indeed it has been demonstrated that DNA de-methylation is an inefficient step in the transition to pluripotency and treatment with DNA methyltransferase inhibitors, like 5-aza-CR, improves the efficiency of the reprogramming process [24, 25]. In our study an 18-h exposure to 5-aza-CR was required to induce the appearance of morphological and molecular markers of de-differentiation. Preliminary experiments indicated that shorter exposure times were ineffective and did not succeed in generating modifications related to a higher plasticity state (data not shown). This is consistent with the notion that 5-aza-CR is a direct inhibitor of methyltransferase activity, as well as of methylation process in newly synthesized DNA. Therefore exposure time must be proportional to the cell-cycle length of the cell type under study.

Table 2 Karyotype analysis of GC primary cultures and after different culture treatment

	Total n° metaphases analysed	2n (rate ± S.E.)
Human GC	165	46, XX (99,3±0,1)
Human GC + 5-aza-CR	172	46, XX (96,7±0,4)
Human GC + 5-aza-CR + VEGF	156	46, XX (95,9±0,3)

In our experiments we showed that 5-aza-CR is able to induce immune-positivity for OCT4 and NANOG that showed a specific nuclear localization (Fig. 2, panel b). Their expression was confirmed by PCR analysis together with that of *SOX2* and *REX1*, all of which are transcription factors expressed by pluripotent cells (Fig. 3, panel b). This is consistent with the ability of 5-aza-CR to reactivate previously silent genes and to alter differentiation states of eukaryotic cells [26]. Furthermore 5-aza-CR has been shown to improve reprogramming efficiency by 10-fold [24] and can be used to facilitate the induction of *OCT4* gene expression in neurosphere cells [27]. However expression of these genes is not an exclusive property of pluripotent cells since expression of *OCT4* or *NANOG* has been detected in mesenchymal stem cells (MSC) [28] and recent data demonstrate the important roles of OCT4 and NANOG in maintaining MSC properties [29]. Furthermore the transcription factor SOX2 is required to sustain self-renewal and the undifferentiated state not only in MSC but also in the osteoblastic lineage [30, 31]. This is consistent with the fact that expression of *OCT4*, *REX1* and *GATA4* in human MSCs was correlated with an increase in differentiation efficiency towards osteogenic and adipogenic phenotypes [32]. Altogether these data suggest that after the exposure to 5-aza-CR somatic cells reach a less committed state similar to a number of stem cells.

It is also important to note that GCs tended to revert to their original phenotype after removal of 5-aza-CR and completely down-regulated the expression of the pluripotency-related factors *OCT4*, *NANOG*, *SOX2* and *REX1* within 4 days. (data not shown). This confirms previous data showing that the expression of these genes is transient and becomes undetectable within 7 days if cells are addressed toward a specific fate or within 4 days if cells are allowed to return to their original phenotype when skin fibroblasts are exposed to 5-aza-CR [14, 15]. All these data indicate that the poised state acquired in response to the compound is transient and is unlikely to exert toxic effects on the cells, since it appears to be completely reversible and is supported by the results obtained through karyotype analysis that confirmed maintenance of a normal chromosome set up.

Particularly interesting are the changes in cell morphology that followed the exposure to 5-aza-CR: cells lost the thick layer of microvilli, typical of GCs. Microvilli are now few and short or almost absent. Cells presented reduced dimensions and an increased nuclear volume, which appeared larger than that of differentiated cells (Fig. 1, panels e, f). This nuclear enlargement has been described as correlated to a relaxed chromatin structure [33]. Indeed, 5-aza-CR treated cells exhibited clear evidence of chromatin decondensation, suggesting the acquisition of a more permissive, less committed state in response to the epigenetic modifier. This is in agreement with the notion that chromatin decondensation is one of the hallmarks of nuclear reprogramming [34]. According to Tamada et al. [34], undifferentiated cells contain more loosely packed chromatin than their differentiated counterparts, to maintain many genes in a potentially open state and prepare them for future expression. This suggests a transition from full differentiation to a poised state receptive to differentiation clues. Such transition was accompanied by the disappearance of GC typical features like large amount of smooth endoplasmic reticulum, granules and lipid droplets, replaced by numerous lysosomes and autophagosomes (Fig. 1, panels g,h). This suggests an increment in autophagic activity which is consistent with the role described for autophagy in vertebrate cell differentiation/de-differentiation process [35]. Autophagy, which mediates the turnover of bulk proteins, cytosol portions and organelles, helps the cell to rapidly adapt and respond to changing conditions, allowing for a profound renovation of its appearance and content, in agreement with the complex rearrangements involved in changes of differentiation state [36].

Simultaneously with the autophagic phenomena, a less dense, organelle-free cytoplasmic peripheral ring, became visible (Fig. 1, panels f, g). Recent evidence led to the idea that a perinuclear clustering that result in a peripheral cytoplasmic ring, devoid of organelles, might be a cellular marker for stemness. This has been reported in detail by several Authors that described a specific arrangement of mitochondria in adult and embryonic stem cells [37–39] and has been indicated as a potential marker to monitor the maintenance of "plasticity" in adult stem cell lines [40]. The possible functional significance of this arrangement is open to question and a more efficient manner to provide energy for all cellular process or a buffering system to protect the nucleus from large fluctuations in ion levels may be involved [41, 42].

Treatment with human recombinant VEGF for 15 days after removal of 5-aza-CR addressed GCs towards muscular differentiation (Fig. 1, panels i–k). Cell morphology was completely different and spatial organization appeared markedly changed in these cells, when compared to those distinctive of the starting population. The elongated spindle shape, the presence of multiple nuclei, the cytoplasm occupied by a considerable quantity of non-spatially organized filaments and the expression of DESMIN, MHC and MYOD (Figs. 2, panel c and 3, panel c), are the typical morphological features described during the early phase of the mammalian myoblast fusion process [43]. These genes, that were originally undetectable in untreated human GC (Fig. 3, panel c, lane 1), appeared to be induced by the exposure to VEGF (human GC + 5-aza-CR + VEGF, lane 2) and showed that the conversion to a diverse differentiation state was not taking place only at the morphological level but was reflected, and most probably driven, by molecular signaling. The high efficiency of cell differentiation in response to a 15 day VEGF treatment is consistent and comparable with previous work that showed the ability of human ESCs to assume a smooth muscle-like morphological appearance and express the early smooth

muscle-marker gene, SM α-actin with an efficiency >94 % [44]. Notably cells expressing vascular and mesenchymal markers were also detected (data not shown) and further investigations will be of interest to better elucidate this aspect.

It is interesting to note that the effect of 5-aza-CR is very specific. In fact whereas cells that were addressed towards muscular differentiation down-regulated immune- and molecular-positivity for cytokeratin-17, which is distinctive of GCs, COX-2, a gene expressed in GCs and in muscular tissues, was not down-regulated and persisted in both cell population (Figs. 2, panels a, c and 3, panels a,c). This supports the notion that 5-aza-CR is an effective molecule for transforming cell fate with no obvious a specific effects.

In conclusion our results indicate that exposure to 5-aza-CR induces a temporary poised state characterized by chromatin decondensation and the autophagic removal of specialized cell structures. These events clear the way for the change of expression pattern and the acquisition of a new specialized phenotype that takes place during the new differentiation process.

We think these findings will be useful for a better understanding of the biology of cell conversion obtained by epigenetic modification without the forced expression of transcription factors.

Acknowledgments This work was funded by Carraresi Foundation and GP is supported by a post-doc fellowship of the University of Milan. The authors are members of the COST Action FA1201 Epiconcept: Epigenetics and Periconception environment.

Disclosures The authors indicate no potential conflicts of interest.

References

1. Zhou, Q., & Melton, D. A. (2008). Extreme makeover: converting one cell into another. *Cell Stem Cell, 3*, 382–388.
2. Takahashi, K., & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell, 126*, 663–676.
3. Takahashi, K., Tanabe, K., Ohnuki, M., et al. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell, 131*, 861–872.
4. Papp, B., & Plath, K. (2011). Reprogramming to pluripotency: stepwise resetting of the epigenetic landscape. *Cell Research, 21*, 486–501.
5. Ruiz, S., Panopoulos, A. D., Herrerias, A., et al. (2011). A high proliferation rate is required for cell reprogramming and maintenance of human embryonic stem cell identity. *Current Biology, 21*, 45–52.
6. Davis, R. L., Weintraub, H., & Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell, 51*, 987–1000.
7. Vierbuchen, T. (2010). Direct conversion of fibroblasts to functional neurons by defined factors. *Nature, 463*, 1035–1041.
8. Caiazzo, M., Dell'Anno, M. T., Dvoretskova, E., et al. (2011). Direct generation of functional dopaminergic neurons from mouse and human fibroblasts. *Nature, 476*, 224–227.
9. Huang, P., He, Z., Ji, S., et al. (2011). Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. *Nature, 475*, 386–389.
10. Pfisterer, U., Kirkeby, A., Torper, O., et al. (2011). Direct conversion of human fibroblasts to dopaminergic neurons. *Proceedings of the National Academy of Sciences of the United States of America, 108*, 10343–10348.
11. Marro, S., Pang Zhiping, P., Yang, N., et al. (2011). Direct lineage conversion of terminally differentiated hepatocytes to functional neurons. *Cell Stem Cell, 9*, 374–382.
12. Efe, J. A., Hilcove, S., Kim, J., et al. (2011). Conversion of mouse fibroblasts into cardiomyocytes using a direct reprogramming strategy. *Nature Cell Biology, 13*, 215–222.
13. Kim, J., Efe, J. A., Zhu, S., et al. (2011). Direct reprogramming of mouse fibroblasts to neural progenitors. *Proceedings of the National Academy of Sciences of the United States of America, 108*, 7838–7843.
14. Pennarossa, G., Maffei, S., Campagnol, M., Rahman, M. M., Brevini, T. A., & Gandolfi, F. (2014). Reprogramming of pig dermal fibroblast into insulin secreting cells by a brief exposure to 5-aza-cytidine. *Stem Cell Reviews, 10*, 31–43.
15. Pennarossa, G., Maffei, S., Campagnol, M., Tarantini, L., Gandolfi, F., & Brevini, T. A. (2013). Brief demethylation step allows the conversion of adult human skin fibroblasts into insulin-secreting cells. *Proceedings of the National Academy of Sciences of the United States of America, 110*, 8948–8953.
16. Dean, W., Santos, F., Stojkovic, M., et al. (2001). Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. *Proceedings of the National Academy of Sciences of the United States of America, 98*, 13734–13738.
17. Zvetkova, I., Apedaile, A., Ramsahoye, B., et al. (2005). Global hypomethylation of the genome in XX embryonic stem cells. *Nature Genetics, 37*, 1274–1279.
18. Myers, M., van den Driesche, S., McNeilly, A. S., & Duncan, W. C. (2008). Activin A reduces luteinisation of human luteinised granulosa cells and has opposing effects to human chorionic gonadotropin in vitro. *Journal of Endocrinology, 199*, 201–212.
19. Pepin, D., Paradis, F., Perez-Iratxeta, C., Picketts, D. J., & Vanderhyden, B. C. (2013). The imitation switch ATPase Snf2l is required for superovulation and regulates Fgl2 in differentiating mouse granulosa cells. *Biology of Reproduction, 88*, 142.
20. Woods, D. C., Haugen, M. J., & Johnson, A. L. (2007). Actions of epidermal growth factor receptor/mitogen-activated protein kinase and protein kinase C signaling in granulosa cells from Gallus gallus are dependent upon stage of differentiation. *Biology of Reproduction, 77*, 61–70.
21. Brevini, T. A., Pennarossa, G., Antonini, S., et al. (2009). Cell lines derived from human parthenogenetic embryos can display aberrant centriole distribution and altered expression levels of mitotic spindle check-point transcripts. *Stem Cell Reviews, 5*, 340–352.
22. Chomczynski, P., & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry, 162*, 156–159.
23. Wu, S. C., & Zhang, Y. (2010). Active DNA demethylation: many roads lead to Rome. *Nature Reviews Molecular Cell Biology, 11*, 607–620.
24. Huangfu, D. (2008). Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nature Biotechnology, 26*, 795–797.
25. Mikkelsen, T. S., Hanna, J., Zhang, X., et al. (2008). Dissecting direct reprogramming through integrative genomic analysis. *Nature, 454*, 49–55.
26. Glover, T. W., Coyle-Morris, J., Pearce-Birge, L., Berger, C., & Gemmill, R. M. (1986). DNA demethylation induced by 5-azacytidine does not affect fragile X expression. *American Journal of Human Genetics, 38*, 309–318.

27. Do, J. T., & Scholer, H. R. (2004). Nuclei of embryonic stem cells reprogram somatic cells. *Stem Cells, 22*, 941–949.
28. Greco, S. J., Liu, K., & Rameshwar, P. (2007). Functional similarities among genes regulated by Oct4 in human mesenchymal and embryonic stem cells. *Stem Cells, 25*, 3143–3154.
29. Tsai, C.-C., Su, P.-F., Huang, Y.-F., Yew, T.-L., & Hung, S.-C. (2012). Oct4 and Nanog directly regulate Dnmt1 to maintain self-renewal and undifferentiated state in mesenchymal stem cells. *Molecular Cell, 47*, 169–182.
30. Basu-Roy, U., Ambrosetti, D., Favaro, R., Nicolis, S. K., Mansukhani, A., & Basilico, C. (2010). The transcription factor Sox2 is required for osteoblast self-renewal. *Cell Death and Differentiation, 17*, 1345–1353.
31. Park, S. B., Seo, K. W., So, A. Y., et al. (2012). SOX2 has a crucial role in the lineage determination and proliferation of mesenchymal stem cells through Dickkopf-1 and c-MYC. *Cell Death and Differentiation, 19*, 534–545.
32. Roche, S., Richard, M.-J., & Favrot, M.-C. (2007). Oct-4, Rex-1, and Gata-4 expression in human MSC increase the differentiation efficiency but not hTERT expression. *Journal of Cellular Biochemistry, 101*, 271–280.
33. Niwa, H. (2007). How is pluripotency determined and maintained? *Development, 134*, 635–646.
34. Tamada, H., Van Thuan, N., Reed, P., et al. (2006). Chromatin decondensation and nuclear reprogramming by nucleoplasmin. *Molecular and Cell Biology, 26*, 1259–1271.
35. Cecconi, F., & Levine, B. (2008). The role of autophagy in mammalian development: cell makeover rather than cell death. *Developmental Cell, 15*, 344–357.
36. Pan, H., Cai, N., Li, M., Liu, G. H., & Izpisua Belmonte, J. C. (2013). Autophagic control of cell 'stemness'. *EMBO Molecular Medicine, 5*, 327–331.
37. Piccoli, C., Ria, R., Scrima, R., et al. (2005). Characterization of mitochondrial and extra-mitochondrial oxygen consuming reactions in human hematopoietic stem cells. Novel evidence of the occurrence of NAD(P)H oxidase activity. *Journal of Biological Chemistry, 280*, 26467–26476.
38. St John, J. C., Ramalho-Santos, J., Gray, H. L., et al. (2005). The expression of mitochondrial DNA transcription factors during early cardiomyocyte in vitro differentiation from human embryonic stem cells. *Cloning and Stem Cells, 7*, 141–153.
39. Cho, Y. M., Kwon, S., Pak, Y. K., et al. (2006). Dynamic changes in mitochondrial biogenesis and antioxidant enzymes during the spontaneous differentiation of human embryonic stem cells. *Biochemical and Biophysical Research Communications, 348*, 1472–1478.
40. Lonergan, T., Bavister, B., & Brenner, C. (2007). Mitochondria in stem cells. *Mitochondrion, 7*, 289–296.
41. Park, M. K., Ashby, M. C., Erdemli, G., Petersen, O. H., & Tepikin, A. V. (2001). Perinuclear, perigranular and sub-plasmalemmal mitochondria have distinct functions in the regulation of cellular calcium transport. *EMBO Journal, 20*, 1863–1874.
42. Bruce, J. I., Giovannucci, D. R., Blinder, G., Shuttleworth, T. J., & Yule, D. I. (2004). Modulation of [Ca2+]i signaling dynamics and metabolism by perinuclear mitochondria in mouse parotid acinar cells. *Journal of Biological Chemistry, 279*, 12909–12917.
43. Duan, R., & Gallagher, P. J. (2009). Dependence of myoblast fusion on a cortical actin wall and nonmuscle myosin IIA. *Developments in Biologicals, 325*, 374–385.
44. Huang, H., Zhao, X., Chen, L., et al. (2006). Differentiation of human embryonic stem cells into smooth muscle cells in adherent monolayer culture. *Biochemical and Biophysical Research Communications, 351*, 321–327.