

# Maintenance of Murine Embryonic Stem Cells' Self-Renewal and Pluripotency with Increase in Proliferation Rate by a Bovine Granulosa Cell Line-Conditioned Medium

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Murine embryonic stem cells (mESCs) are pluripotent cells that can be propagated in an undifferentiated state in continuous culture on a feeder layer or without feeders in the presence of leukemia inhibitory factor (LIF). Although there has been a great advance since their establishment, ESC culture is still complex and expensive. Therefore, finding culture conditions that maintain the self-renewal of ESCs, preventing their differentiation and promoting their proliferation, is still an area of great interest. In this work, we studied the effects of the conditioned medium from a bovine granulosa cell line (BGC-CM) on the maintenance of self-renewal and pluripotency of mESCs. We found that this medium is able to maintain mESCs' self-renewal while preserving its critical properties without LIF addition. mESCs cultured in BGC-CM expressed the stem cell markers Oct4, Sox2, Nanog, SSEA-1, Klf4, Rex1, and ECAT1. Moreover, mESCs cultured in BGC-CM gave rise to embryoid bodies and teratomas that differentiated effectively to diverse cell populations from endoderm, mesoderm, and ectoderm. Further, we found that mESCs cultured in BGC-CM have an increased proliferation rate compared with cells grown in the mESC standard culture medium supplemented with LIF. These findings may provide a powerful tool to culture mESCs for long periods of time with high proliferation rate while preserving its basic characteristics, contributing to the application of these cells to assess potential tissue engineering and cellular therapy applications.

## Introduction

MURINE EMBRYONIC STEM CELLS (mESCs) are pluripotent cells derived from the inner cell mass of blastocysts. They were first generated from mice by culturing inner cell mass from day 3.5 preimplantation embryos on feeder layers [1,2]. Since then, multiple mESC lines have been generated and are widely used for research. The pluripotency of mESCs, combined with their ease of genetic manipulation and selection, has revolutionized gene function studies *in vivo* through the generation of transgenic, chimeric, and knockout mice. These cells provide a powerful system to investigate molecular and cellular processes involved in lineage specification and embryogenesis, to perform drug screening, and to assess potential tissue engineering and cellular therapy applications.

mESCs grow as colonies and can be cultured *in vitro* for extended periods, maintaining their capability to contribute to

all cell lineages when implanted into a blastocyst. Their pluripotency is demonstrated by their ability to generate chimeras and transgenic mice and to differentiate in culture into ectodermal, endodermal, and mesodermal derivatives [3].

mESCs can be propagated in an undifferentiated state in continuous culture on a feeder layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) or without feeders in the presence of leukemia inhibitory factor (LIF) or related cytokines [4]. Upon withdrawal of this factor, mESCs give rise to dimensional complex structures called embryoid bodies (EBs). These cell aggregates can be further induced to differentiate into a variety of specific cell types [5].

Although there has been a great advance since their establishment, ESC culture is still difficult and expensive. Therefore, finding conditions that maintain the stable self-renewal of ESCs, preventing their differentiation and promoting their proliferation, is still an area of great interest.

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Granulosa cells (GCs) are the primary cells in the ovary that provide the physical support and microenvironment required for the developing oocyte [6]. They are a major component of the ovarian follicles, surrounding the oocytes and playing a key role in follicle development [7]. Moreover, the GCs are an abundant source of numerous factors such as transforming growth factor  $\beta$  (TGF- $\beta$ ), insulin-like growth factors (IGF), IGF-binding proteins, activins, and inhibins [6,8].

Research performed by one of us (L.B.) led to the obtention of a bovine GC line (BGC-1) by spontaneous immortalization of primary cultures, which was originally selected for its maintenance of hormonally regulated fibronectin (FN) gene expression [9]. BGC-1 cells show some characteristics of primary cultures, such as the hormonal regulation of FN biosynthesis, similar responsiveness to growth factors, and a qualitatively similar protein secretion pattern [10,11]. In addition, conditioned medium from this cell line (BGC-CM) is mitogenic for both BGC-1 cells and primary cultures of BGCs [10]. The myriad of factors secreted by GCs and the mitogenic properties that BGC-CM showed led us to speculate that this cell line may provide a CM with valuable properties.

In the present work, we studied the effect of the CM from the BGC line, described above, on the maintenance of self-renewal and pluripotency of mESCs. We found that BGC-CM is able to maintain mESCs' self-renewal while preserving its critical properties without LIF addition. We have also found that mESCs cultured in this medium have an increased proliferation rate compared with cells grown in the mESC standard culture medium supplemented with LIF. These findings could provide a powerful tool to culture mESCs while preserving their basic properties and increasing their proliferation rate, both highly desirable features in an ESC culture medium.

## Materials and Methods

### Cell culture

Ainv15 and R1 mESC lines were obtained from ATCC. The mESCs were first expanded on an MEF feeder layer previously irradiated. Then, subculturing was carried out according to specifications. Briefly, subconfluent cultures were trypsinized and replated (1:3–1:6 split) every 48–72 h onto irradiated MEFs or 0.1% bovine gelatin-coated tissue culture plates. Cells were propagated either in mESC standard medium consisting of knockout Dulbecco's minimal essential medium (high-glucose DMEM) (Invitrogen/Gibco 10829-018) supplemented with 15% ES-qualified fetal bovine serum (FBS) (Invitrogen/Gibco 16141-061), 100  $\mu$ M MEM nonessential amino acids (Gibco), 0.5 mM beta-mercaptoethanol, 2 mM L-alanyl-L-glutamine (Gibco), and antibiotics [100 U/mL penicillin and 100  $\mu$ g/mL streptomycin (Gibco) and 0.5 mg/mL gentamicin (Sigma)] with the addition of 1,000 U/mL of LIF (Chemicon, ESG1106), in the same standard medium without the addition of LIF, or in BGC-CM.

### Cell differentiation

mESCs were differentiated using the hanging drop method. Briefly, cells were trypsinized, centrifuged, and suspended in differentiation medium (standard medium containing 20% FBS, without LIF) to a concentration of 25,000 cells/mL. Afterward, 25- $\mu$ L drops were placed on the

lid of a bacterial plate. After 48 h, EBs formed in the bottom of the drops were collected and placed in new bacterial plates with fresh differentiation medium, allowing them to grow for 3 more days. On the fifth day, EBs were collected and replated onto 0.1% gelatin-coated dishes. From the sixth day onward, adherent EBs were used as described.

### Conditioned media experiments

BGC-1 or HEK293T cells were grown in high-glucose DMEM supplemented with 10% FBS (Gibco), 1 mM sodium pyruvate (Sigma), and antibiotics to a 90% confluence. Then, the culture medium was replaced by fresh standard ES medium without FBS and beta-mercaptoethanol and incubated for 24 h. After incubation, CM was removed, centrifuged at 2,000  $g$  for 10 min, and supplemented with 15% FBS and 0.5 mM beta-mercaptoethanol before use.

### Reverse transcription–polymerase chain reaction

ESCs were grown in BGC-CM, standard medium plus LIF, standard medium without LIF, or HEK293T-CM for at least three passages or 7 days on 0.1% bovine gelatin-coated tissue culture plates. Total cellular RNA was isolated from subconfluent cultures according to Chomczynsky and Sacchi [12]. The yield and purity of RNA samples were assessed by the absorbance at 260 and 260/280 nm ratio, respectively. One microgram of total RNA was retrotranscribed using MMLV reverse transcriptase (Promega) and 30 ng/ $\mu$ L Random Primers (Invitrogen). Conventional polymerase chain reaction (PCR) amplification of DNA was carried out using Taq DNA Polymerase (Invitrogen) and 12.5 pM specific oligonucleotides (Table 1) in a Peltier Thermal Cycler DNA engine (BioRad). Analysis of the PCR products was performed by electrophoresis in a 2% agarose gel stained with ethidium bromide.

TABLE 1. GENE SPECIFIC PRIMERS USED IN REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION

Gene name		Primer sequence (5'–3')
Oct4	Forward	TGACGGGAACAGAGGGAAAG
	Reverse	TCAGCTTGGGCTAGAGAAGG
Nanog	Forward	AGGGTCTGCTACTGAGATGCTCTG
	Reverse	CAACCACTGGTTTTTCTGCCACCG
Sox2	Forward	CACAACCTCGGAGATCAGCAA
	Reverse	CTCCGGGAAGCGTGTACTTA
$\beta$ -actin	Forward	GTGGGCCCTCTAGGCACCA
	Reverse	CGGTTGGCCTTAGGGTTCAGGG GGG
Klf4	Forward	GCGAACTCACACAGGCGAGAA ACC
	Reverse	TCGCTTCCTCTTCCTCCGACACA
Rex1	Forward	ACGAGTGGCAGTTTCTTCTTGGGA
	Reverse	TATGACTCACTTCCAGGGGGCACT
Ecat1	Forward	TGTGGGGCCCTGAAAGGCGAGCT GAGAT
	Reverse	ATGGGCCCGCCATACGACGACGCT CAACT
Miocardin	Forward	CATTGTTTCCCAAGGAGATTC
	Reverse	GCGATGTTACCCTCCTCAAAA

TABLE 2. SECONDARY AND PRIMARY SPECIFIC ANTIBODIES USED IN IMMUNOFLUORESCENCE AND IMMUNOHISTOCHEMISTRY

Antibody against	Working dilution	Provider
OCT3/4	1:200	Santa Cruz Biotechnology
SSEA-1	1:200	Santa Cruz Biotechnology
Alpha-fetoprotein	1:200	Santa Cruz Biotechnology
$\beta$ III Tubulin	1:200	Santa Cruz Biotechnology
Alpha smooth muscle actin	1:200	Dako
GATA4	1:200	Santa Cruz Biotechnology
Goat IgG-TR	1:200	Santa Cruz Biotechnology
Mouse IgG-FICT	1:100	Sigma

### Immunofluorescence

ESCs were seeded on Permanox chamber slides (Nunc) and grown in the indicated medium. The cells grown in each condition for at least three passages or 7 days on 0.1% bovine gelatin-coated tissue culture plates were fixed in 4% paraformaldehyde for 20 min. Fixed cells were permeabilized in 0.2% Triton X-100 for 30 min, blocked with 1% phosphate-buffered saline (PBS)-0.01% Tween normal goat serum, and incubated overnight with polyclonal primary antibodies listed in Table 2. Primary antibodies were detected with the secondary antibodies mentioned in Table 2. Nuclei were stained with DAPI according to the manufacturer's instructions (Santa Cruz Biotechnology).

### Teratoma formation and histological analysis

The ESCs that have been cultured in BGC-CM for at least three passages or 7 days on 0.1% bovine gelatin-coated tissue culture plates were trypsinized, centrifuged, and suspended at  $1 \times 10^7$  cells/mL in DMEM in the absence of FBS. Cells were kept at 37°C until the injection. Then, 100  $\mu$ L of the cell suspension ( $1 \times 10^6$  cells) were subcutaneously injected into

the dorsal flank of 6–8-week-old male nude (*nu/nu*) mice. Four weeks after the injection, animals were anesthetized in a CO<sub>2</sub> atmosphere chamber and sacrificed by cervical dislocation. Tumors were surgically dissected from the mice, fixed in PBS containing 4% formaldehyde, and embedded in paraffin. Sections (4  $\mu$ m) were stained with hematoxylin and eosin or immunostained. For immunohistochemistry, sections were blocked with 1% goat serum in PBS, washed, and incubated with the indicated primary antibodies (Table 2) for 1 h at room temperature. After rinsing in PBS, slides were incubated for 30 min with horseradish peroxidase-conjugated secondary antibodies, washed, and revealed (ABC Kit; Vector; Cat. No. PK-6200). Then, the samples were stained with hematoxylin. Mouse procedures were conducted under local ethical guidelines.

### Cell proliferation assay

ESCs were grown in the standard medium with LIF. Then, cells were trypsinized and replated in a flat-bottomed 96-well plate. For each experiment, 3,000 cells per well were seeded and allowed to grow in either BGC-CM or standard medium plus LIF prepared without phenol red. Cell proliferation was quantified by the sodium 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt (XTT) method (Sigma) according to manufacturer's instructions. Briefly, a volume of XTT solution equal to 20% of culture medium was added 3 h prior to measurement. Formation of colored product, the orange formazan derivative, was quantified by collecting the culture medium, reading its absorbance at 450 nm, and subtracting background contribution from the reactive sodium salt of XTT at 690 nm in a multiwell plate reader (Microplate Reader, BioRad model 680). An initial measurement was taken 3 h after seeding cells in the plate. Afterward, absorbance at 450 and 690 nm was measured once a day during at least 5 days. To assess cell proliferation rate, corrected absorbance vs. time was graphed and slopes for each culture medium were then compared.

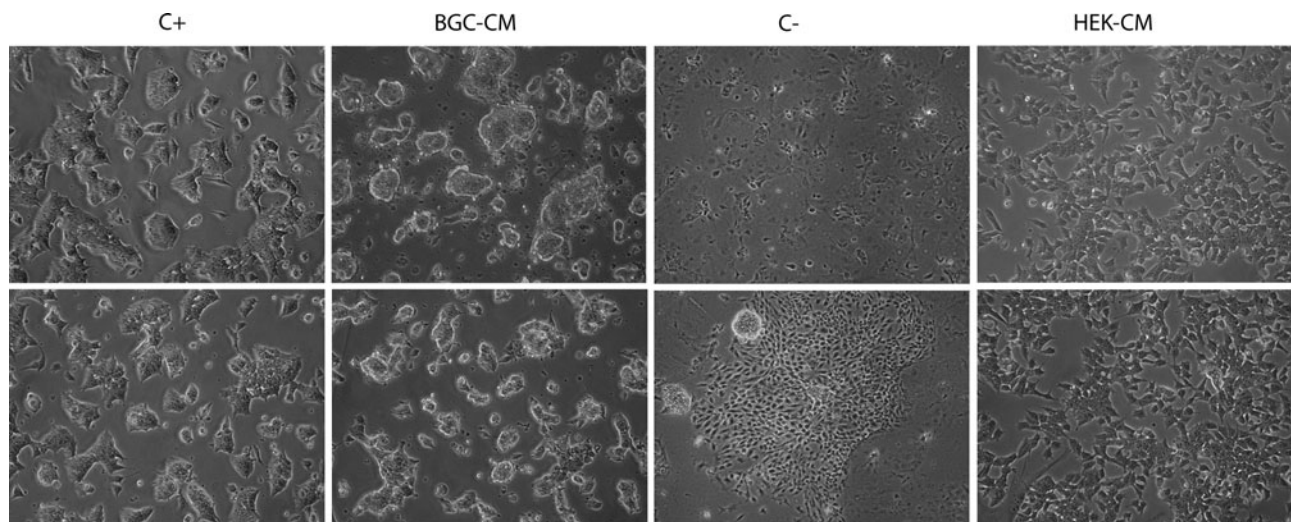
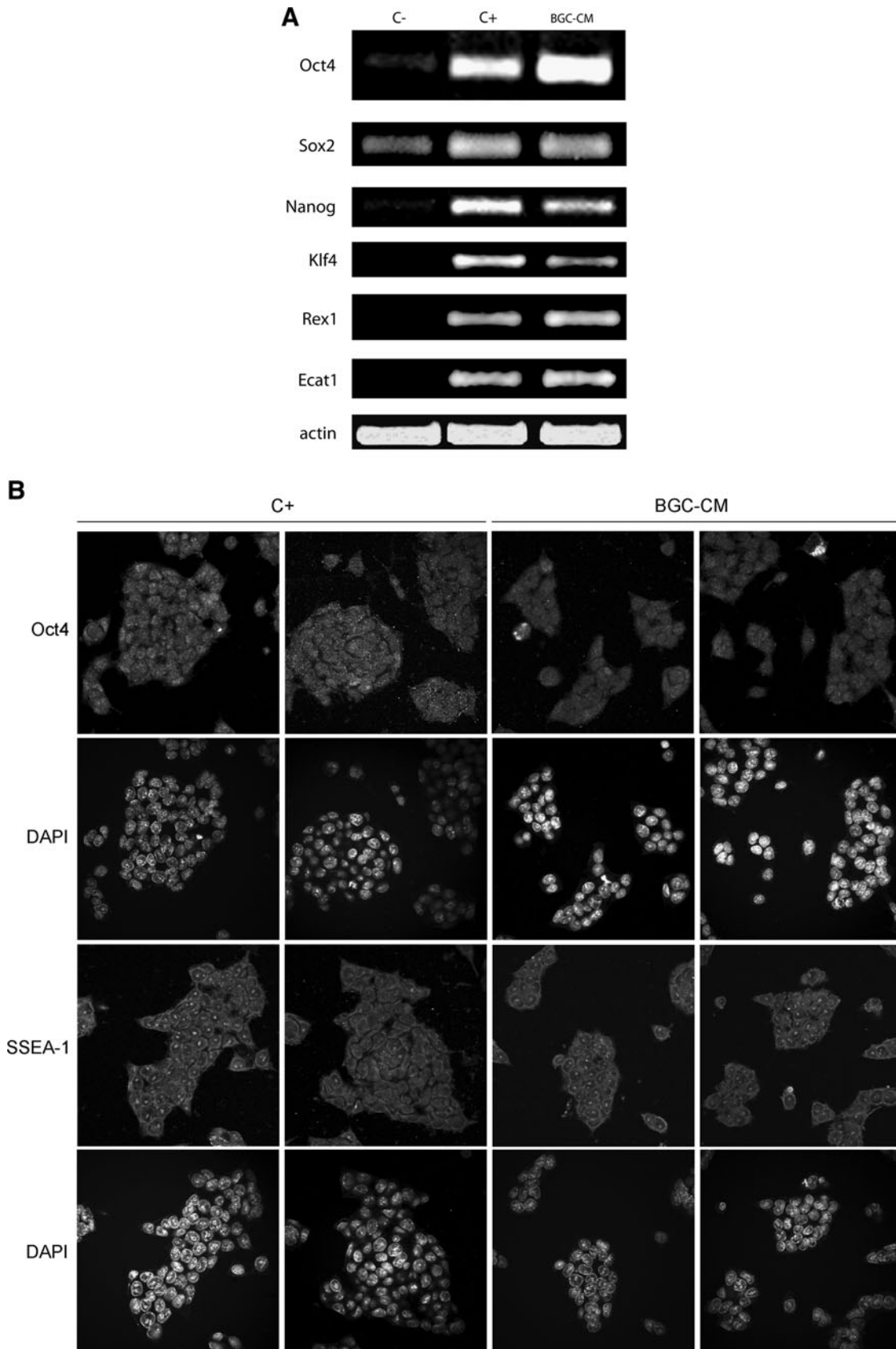


FIG. 1. mESCs cultured in BGC-CM show typical undifferentiated colony morphology. Ainv15 mESCs were cultured in the indicated medium for at least three passages or 7 days. Representative phase-contrast images of colonies cultured in BGC-CM, standard medium plus LIF (C+), standard medium without LIF (C-), or HEK293T-conditioned medium (HEK-CM). mESCs, murine embryonic stem cells; BGC, bovine granulosa cell; CM, conditioned medium; LIF, leukemia inhibitory factor.

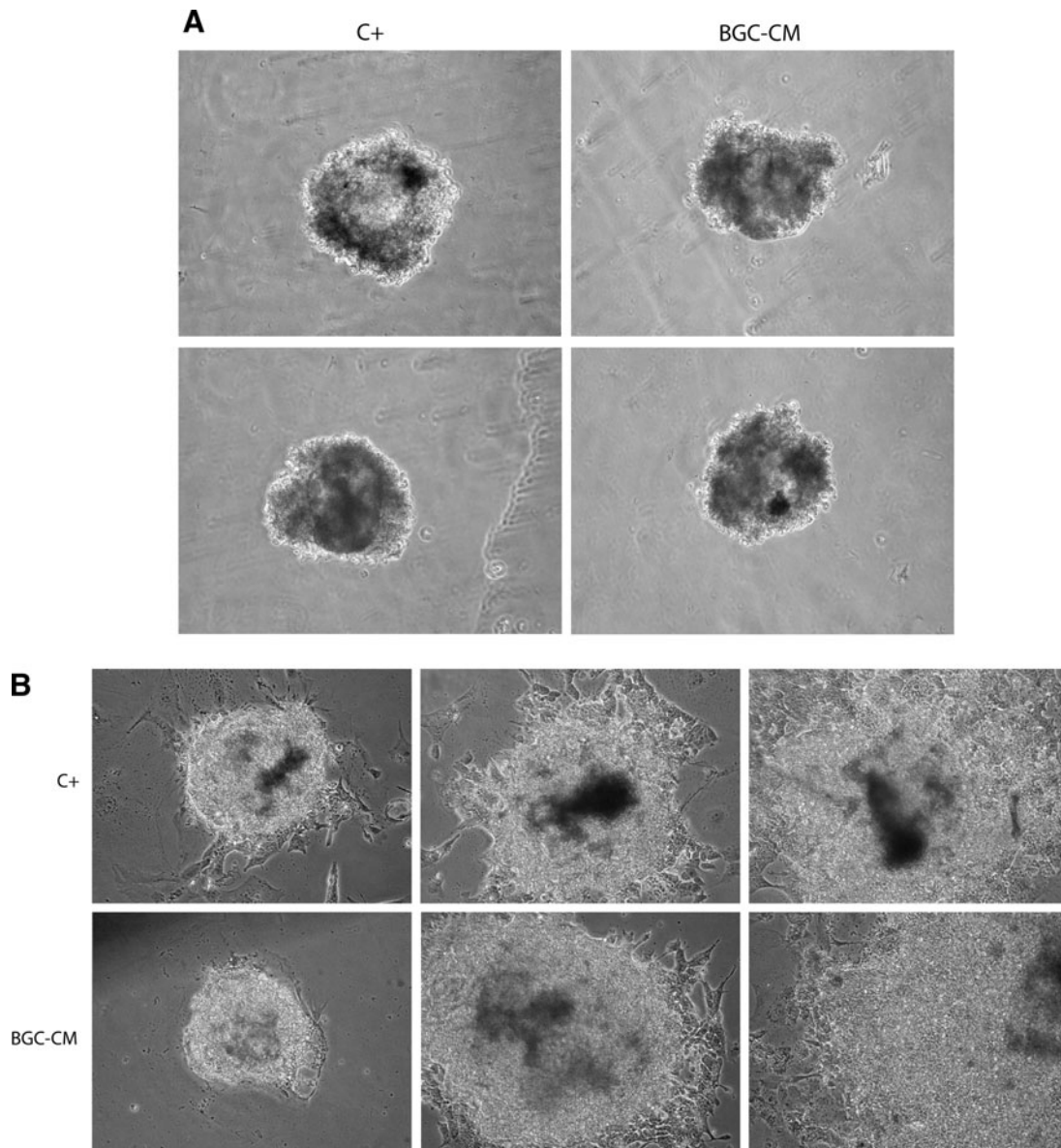


**FIG. 2.** mESCs cultured in BGC-conditioned medium express undifferentiated state marker genes. mESCs were cultured in BGC-CM, standard medium plus LIF (C+), or standard medium without LIF (C-) as indicated, for at least three passages or 7 days. **(A)** Reverse transcription-polymerase chain reaction (RT-PCR) analysis of RNA from Ainv15 mESCs of the stem cell marker genes Oct4, Sox2, Nanog, Rex1, Klf4, and Ecat1. The expression of the housekeeping  $\beta$ -actin genes was used as control. **(B)** Representative images from Ainv15 mESC immunostaining for stem marker genes Oct4 and SSEA-1 as indicated. Nuclei were stained with DAPI. RT-PCR, reverse transcription-polymerase chain reaction.

## Results

To determine if BGC-CM is capable to maintain mESCs' self-renewal, we cultured Ainv15 mESC line in the standard mESC medium that was previously conditioned by the BGC line, without LIF addition. We compared these mESCs with parallel cultures grown in the mESC standard medium supplemented with LIF or the standard medium without LIF. We evaluated the basic properties of stem cells cultured on gelatin-coated plates, such as colony morphology, expression of undifferentiated state-specific gene markers, and pluripotency.

A distinctive characteristic of mESCs is their defined colony morphology on culture. Changes in this morphology are highly notorious and clearly indicate whether the cells remain in an undifferentiated state or have undergone any differentiation process [3]. To have a broad view about the effect of BGC-CM on mESCs' self-renewal, we first examined the morphology of mESC colonies grown in the different experimental conditions for at least three passages by bright-field microscopy. As shown in Fig. 1, mESCs cultured in both the standard medium plus LIF and the BGC-CM presented a similar typical colony morphology, high refringence, and a



**FIG. 3.** mESCs propagated in BGC-CM remain pluripotent. Ainv15 mESCs were cultured in BGC-CM or standard medium plus LIF (C+) for at least three passages or 7 days. Then, the cells propagated in the indicated medium were subjected to the hanging drop protocol and gave rise to EBs. Representative phase-contrast images of EBs in suspension (**A**) or attached to gelatin-coated Petri dishes along the time (**B**) from mESCs cultured in BGC-CM (**A**, right panel) or in the standard medium plus LIF (C+, **A**, left panel). (**C**) Representative immunostaining images of attached EBs from the standard medium with LIF (C+) or BGC-CM. Alpha-fetoprotein (AFP) was stained as an endoderm gene marker, alpha smooth muscle actin ( $\alpha$ SMA) as a mesoderm gene marker, and  $\beta$ III tubulin ( $\beta$ 3T) as an ectoderm gene marker. Nuclei were stained with DAPI. (**D**) RT-PCR analysis of RNA from EBs of the marker genes alpha feto protein (AFP), Brachyury, Miocardin, and Nkx2.5. The expression of the housekeeping  $\beta$ -actin genes was used as control. EBs, embryoid bodies. (Figure continued  $\rightarrow$ )

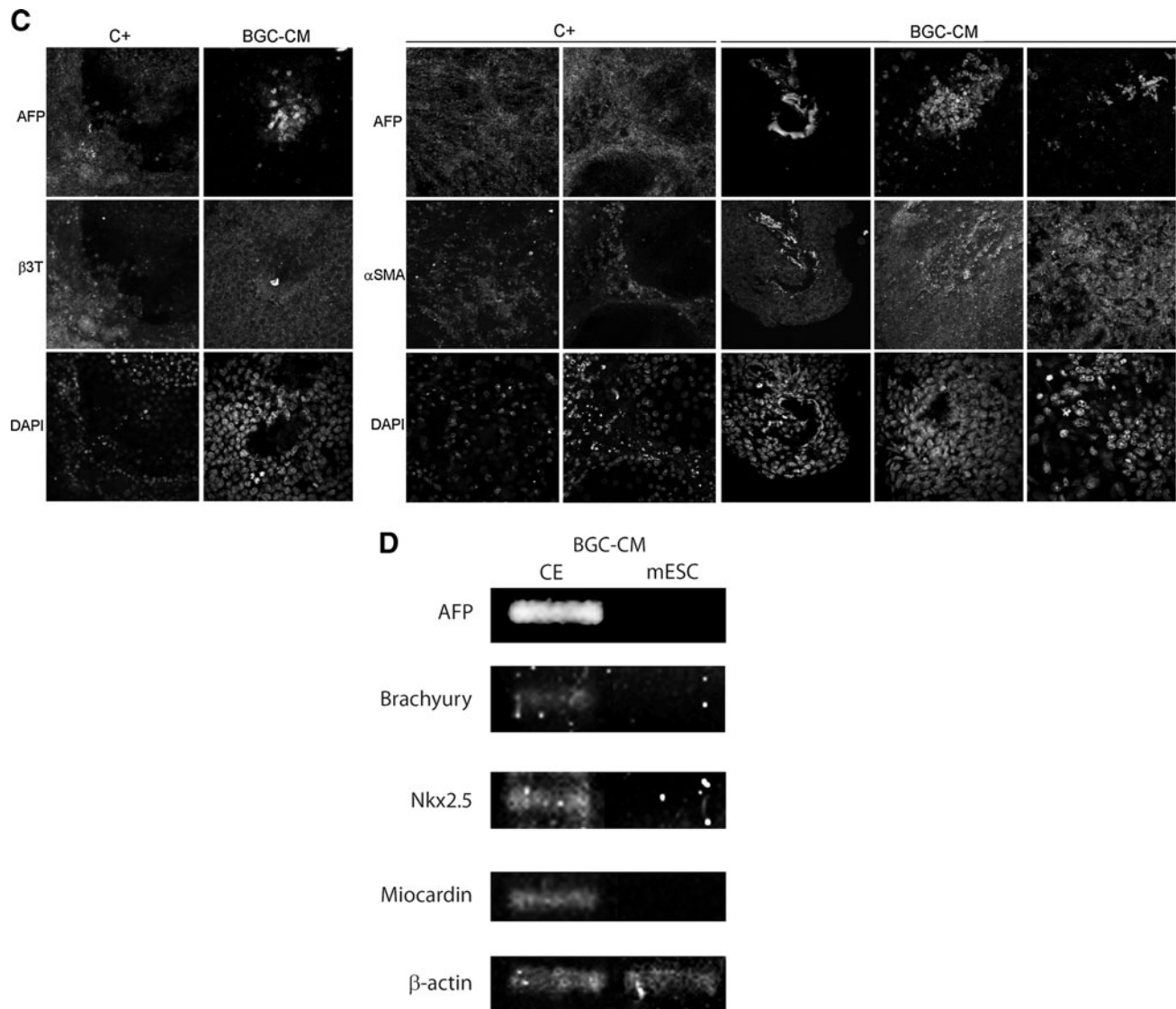


FIG. 3. (Continued).

low cytoplasm/nucleus ratio. On the other hand, both the negative control cultured in the standard medium without LIF and the mESCs cultured in a medium conditioned by a different cell line showed a clearly different aspect.

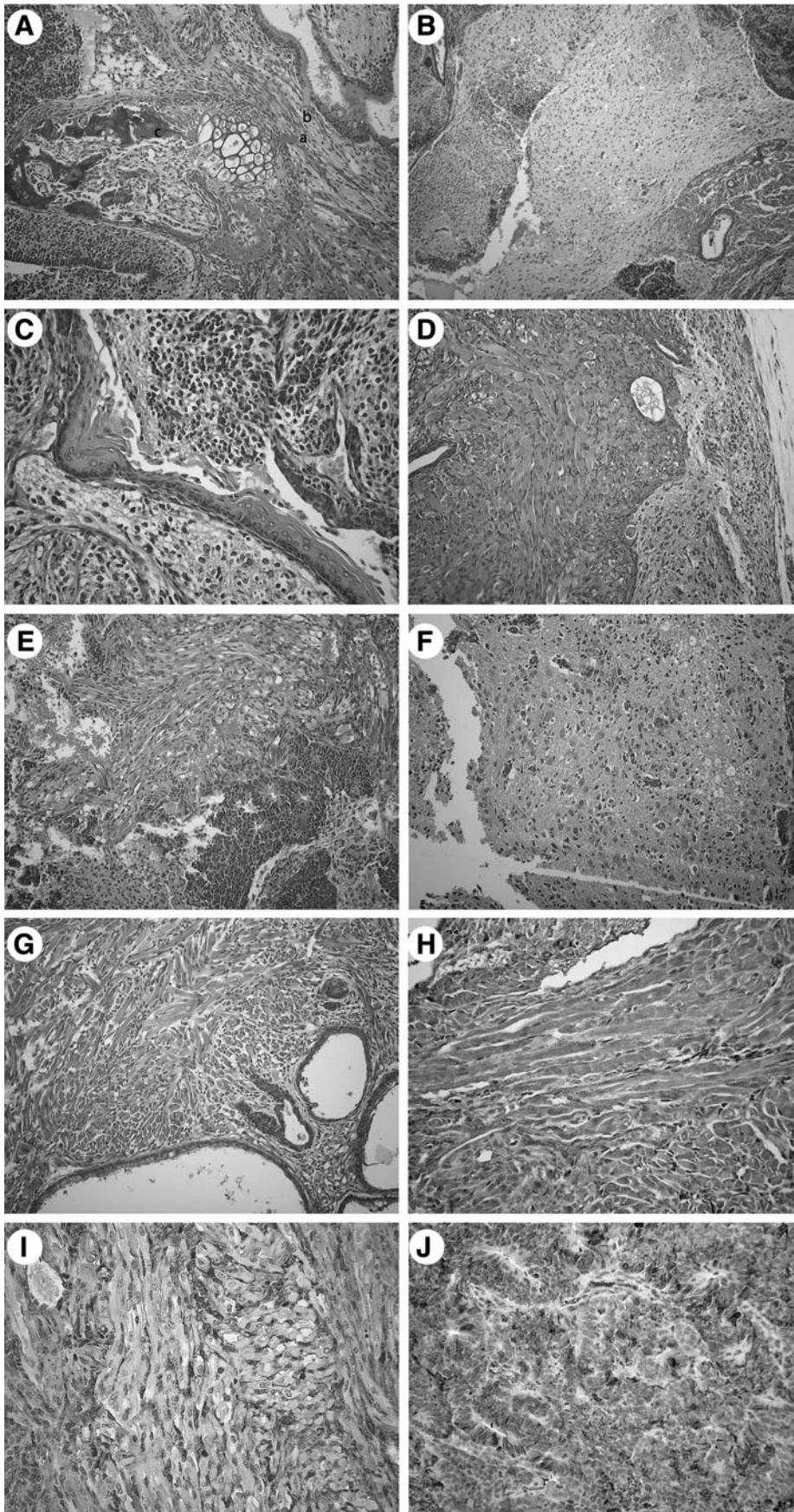
We next studied the expression of undifferentiated state-specific gene markers by immunofluorescence and RT-PCR. We first analyzed the expression of the stem cell markers Oct4, Sox2, Nanog, SSEA-1, and Klf4. Then, we also studied other genes expressed in mESCs, such as Rex1 and ECAT1 [13–15].

As shown in Fig. 2, we detected the expression of all the mESC markers evaluated in mESC cultures maintained in BGC-CM. Moreover, we were able to culture mESCs up to 20 passages in this medium, and we did not detect changes in the pattern of gene expression or the colony morphology (data not shown). As expected, in the negative control, the expression levels of the stem cell markers evaluated were clearly lower than in the other experimental conditions (Fig. 2A and data not shown).

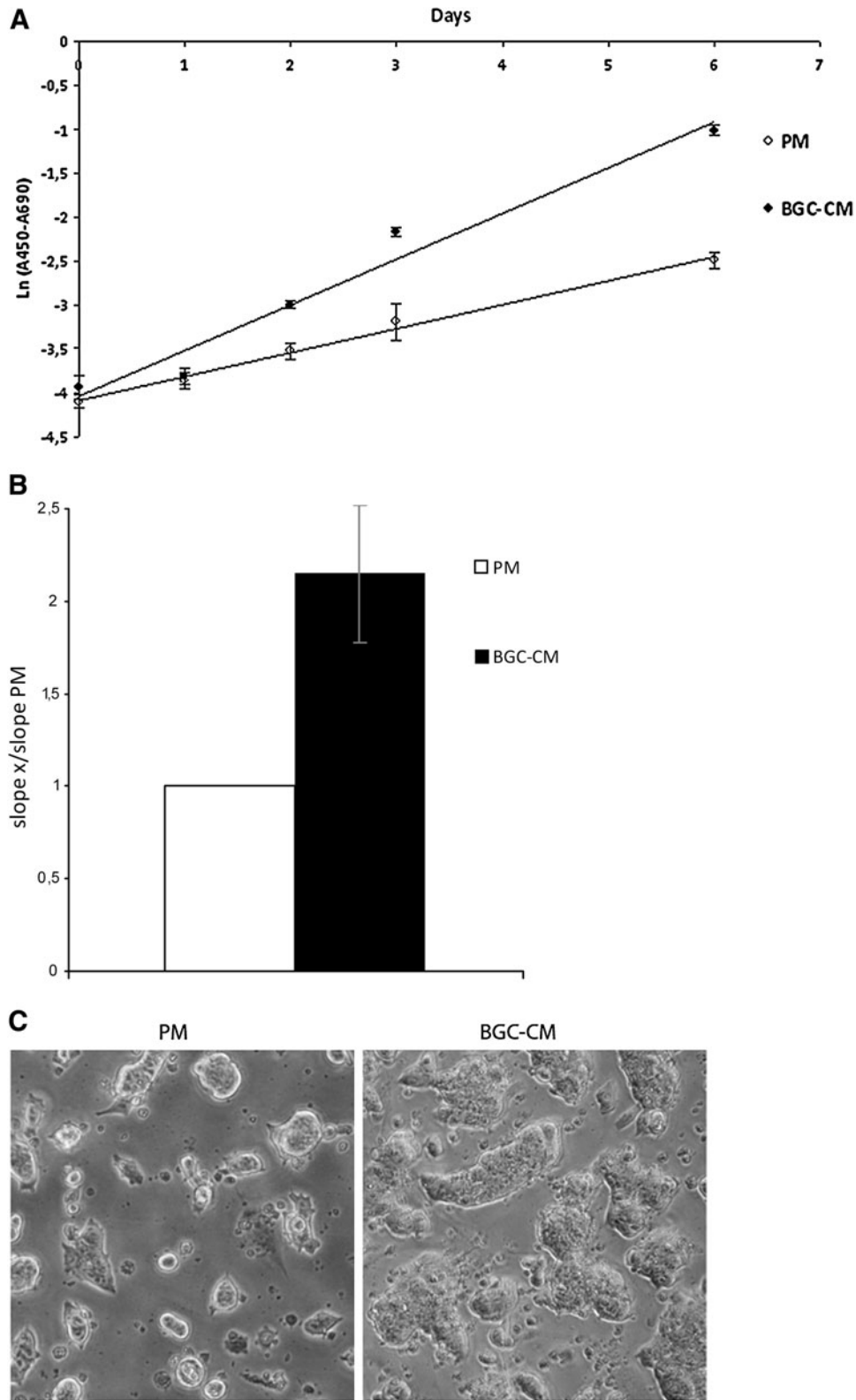
Further, we have also studied BGC-CM effect on different mESC lines, such as CGR8, R1, and NKx2.5eGFP ESCs. In all

cases, we were able to propagate them in BGC-CM for several passages while preserving their morphology and the expression of stem cell marker genes (data not shown).

Having established that mESCs cultured in BGC-CM preserve their undifferentiated state, we wondered whether the cells that have been cultured in this medium remain pluripotent, maintaining their capability to originate in vitro, cells from endoderm, mesoderm, and ectoderm. Following the previously described hanging drop protocol [16], we attempted to differentiate them. Both mESCs maintained in BGC-CM or standard proliferation medium gave rise to EBs (Fig. 3A). When plated on gelatin-coated Petri dishes, these EBs differentiated effectively to diverse cell populations (Fig. 3B). By immunofluorescence or RT-PCR, we analyzed the expression of alpha-fetoprotein, as an endoderm gene marker; Brachyury or alpha smooth muscle actin, as mesoderm gene markers; and  $\beta$ III tubulin, as an ectoderm marker [13,14]. We found that EBs derived from ESCs cultured in BGC-CM expressed gene markers from cell types derived from the three embryonic layers (Fig. 3C, D).



**FIG. 4.** mESCs propagated in BGC-CM gave rise to teratomas. Four-micrometer sections from teratoma tissue were stained with hematoxylin and eosin or immunostained. (A–G) Representative histology of teratoma. (A) Cartilage (a, arrow), glandular epithelium (b, arrow), and bone trabeculae (c, arrow). (B) Brain and primitive neuroectodermal component (upper right). (C) Squamous cells intermixed with neuroectodermal epithelium. (D) Skeletal muscle on the left and brain tissue on the right. (E) Skeletal muscle on the left and neuroectodermal epithelium on the right with neuroblastic rosette (arrow). (F) Brain tissue. (G) Muscle (upper right) and glandular epithelium (lower left). (H–J) Immunohistochemistry. (H) Muscle tissue immunopositive for  $\alpha$ SMA. (I) Muscle tissue immunopositive for GATA4. (J) Primitive neuroectodermal epithelium immunopositive for  $\beta$ 3T.



**FIG. 5.** BGC-CM increases the proliferation rate of mESCs. Ainv15 mESCs were cultured in BGC-CM or the standard medium plus LIF (PM) for at least 5 days. The proliferation was evaluated once a day in the indicated medium by XTT assay. **(A)** Representative plots and linear regression slope of proliferation rate from mESCs cultured in BGC-CM (●) or PM (○) day by day. The results correspond to one experiment representative of three biological replicates performed in quadruplicate, all of them with similar results. Error bars indicate standard deviation. **(B)** The white bar represents the mean of PM slope/PM slope ratio; the black bar represents the mean of CM slope/PM slope ratio of the proliferation plot of four independent experiments each performed in quadruplicate. Error bars indicate standard deviation. **(C)** Representative bright-field pictures of the second passage on gelatin-coated Petri dish of colonies cultured in PM or BGC-CM. XTT, sodium 2,3,-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt.



Up to this point, we have found out that mESCs maintained in BGC-CM preserve their basic properties, so we sought to evaluate whether these cells were able to differentiate to a cell type with possible therapeutic applications. The regeneration of cardiac tissue being an important target of stem cell-based therapy, we evaluated the expression of cardiomyogenic mesoderm gene markers Nkx2.5 and Myocardin [17,18]. We detected expression of these markers in Ainv15 (Fig. 2D) and in other mESC lines (data not shown), suggesting that mESCs cultured in BGC-CM preserved their ability to differentiate to cardiomyocytes.

We next examined the pluripotency of mESCs cultured in BGC-CM by teratoma formation. As shown in Fig. 4, mESCs that have been propagated in BGC-CM for at least three passages or 7 days remain pluripotent as they were able to differentiate *in vivo* into tissues from all the three germ layers. We confirmed differentiation into endoderm, mesoderm, and ectoderm by immunostaining with antibodies against GATA4, alpha smooth muscle actin, and  $\beta$ III tubulin, respectively (Fig. 4H–J). As a whole, these data demonstrate that mESCs cultured in BGC-CM preserve its critical properties: self-renewal and pluripotency.

As mentioned earlier, one of the properties of BGC-CM is its mitogenic activity on both GC primary cultures and BGC-1 cell line. Based on this evidence and on the fact that the colonies of mESCs cultured in BGC-CM looked bigger than those cultured in standard conditions, we considered important to study whether this CM had a mitogenic effect on mESC lines. To test our hypothesis, we measured culture proliferation rates by XTT assay for 5 days. As shown in Fig. 5, the proliferation rate of mESCs cultured in BGC-CM was clearly higher than the rate of the cells cultured in standard conditions. This feature of BGC-CM together with its ability to preserve mESCs' basic properties makes this medium a suitable and inexpensive alternative to propagate mESCs in culture for long periods of time.

## Discussion

In this work, we have explored the possibility of culturing mESCs in a novel CM. mESCs propagated in this context maintained its critical properties and showed an increased proliferation rate.

The medium was conditioned by a BGC line previously established by one of us (L.B.) [9] and selected by its mitogenic properties on the same cell line and on granulosa primary cultures [10].

GCs express a myriad of secreted factors that could be involved in both the maintenance of mESCs' essential properties and the increase of mESC proliferation rate, such as fibroblast growth factors (FGFs), IGF, TGF- $\beta$  family members, for example, Activin A [8,10,19], and LIF [20]. Moreover, Activin A [21] and LIF expression has been detected (unpublished results) in this BGC line. Originally, the mESC requirement of a feeder layer was substituted with the addition of LIF to the culture medium [22,23] and fetal serum was replaced by a TGF- $\beta$  family member, the bone morphogenetic protein 4 [24]. In the last few years, multiple defined conditions that enable to culture of ESCs free of feeder layers and serum had been established. These conditions include combinations of inhibitors of pathways involved in differentiation, such as the FGF/Mek/Erk, and of

glycogen synthase kinase 3 [25,26]. Although the precise components of BGC-CM have not been addressed in this work, the expression of LIF in GCs led us to speculate that this cytokine could be the main factor responsible for the maintenance of mESC features. Taking into account the multiple factors that are present in the CM, we cannot be sure that LIF is the sole factor responsible for this property. Therefore, we believe that the balance of the multiple signals in it preserves ESCs' self-renewal and pluripotency.

On the other hand, several candidates could be responsible for the increased proliferation rate of mESCs cultured in this medium. Although Activin A was reported to be a negative regulator of proliferation in some cell types, such as pancreatic acinar cells [27] and renal tubular cells [28], this TGF- $\beta$  family member has mitogenic effects on multiple cell types [29], such as placenta [30], granulosa [21], and mESCs [31,32]. In contrast, although LIF induces proliferation in diverse cell types [33–35], it has been reported that it is not related to proliferation in mESCs [36,37]. Conversely, it was reported that an isoform of FN present in the mentioned CM could be responsible for its mitogenic effect observed on GCs [11]. Moreover, it has been suggested that FN expression induced in mESCs by high glucose concentrations was responsible for an augmented proliferation of these cells [38]. Based on these evidences, we are studying whether Activin A and/or FN expressed by BGC line would be responsible for the mitogenic effect on mESCs. Moreover, we are evaluating whether there is an induction of FN expression on mESCs cultured in BGC-CM that could also be involved in this effect. Currently, we are analyzing the signaling pathways that are activated in mESCs cultured in the different conditions, followed by blocking the different candidate pathways to unravel which of them and how are they involved. Last but not least, as it is well known that bFGF is involved in human ESC pluripotency maintenance [39], and BGC line expresses this factor (unpublished results), we are evaluating whether BGC-CM preserves human ESCs' self-renewal and pluripotency.

Hereby, we propose that BGC-CM offers an inexpensive way for culturing these cells for long periods of time with a high proliferation rate, while preserving its basic characteristics. Moreover, we think that this medium would be a powerful research tool, because it provides a novel culture context to study the involved molecular mechanisms in self-renewal and pluripotency maintenance. We are confident that, in the near future, novel factors identified from this kind of culture contexts will contribute to produce improved therapy-suitable defined ESC culture medium.

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## Author Disclosure Statement

No competing financial interests exist.

## References

- Evans MJ and MH Kaufman. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292:154–156.
- Martin GR. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA* 78:7634–7638.
- Ginis I, Y Luo, T Miura, S Thies, R Brandenberger, S Gerecht-Nir, M Amit, A Hoke, MK Carpenter, J Itskovitz-Eldor and MS Rao. (2004). Differences between human and mouse embryonic stem cells. *Dev Biol* 269:360–380.
- Niwa H. (2007). How is pluripotency determined and maintained? *Development* 134:635–646.
- Nichols J, B Zevnik, K Anastasiadis, H Niwa, D Klewe-Nebenius, I Chambers, H Scholer and A Smith. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* 95:379–391.
- Skinner MK. (2005). Regulation of primordial follicle assembly and development. *Hum Reprod Update* 11:461–471.
- Buccione R, AC Schroeder and JJ Eppig. (1990). Interactions between somatic cells and germ cells throughout mammalian oogenesis. *Biol Reprod* 43:543–547.
- Knight PG and C Glistler. (2001). Potential local regulatory functions of inhibins, activins and follistatin in the ovary. *Reproduction* 121:503–512.
- Bernath VA, AF Muro, AD Vitullo, MA Bley, JL Baranao and AR Kornblihtt. (1990). Cyclic AMP inhibits fibronectin gene expression in a newly developed granulosa cell line by a mechanism that suppresses cAMP-responsive element-dependent transcriptional activation. *J Biol Chem* 265:18219–18226.
- Lerner AA, DF Salamone, ME Chiappe and JL Baranao. (1995). Comparative studies between freshly isolated and spontaneously immortalized bovine granulosa cells: protein secretion, steroid metabolism, and responsiveness to growth factors. *J Cell Physiol* 164:395–403.
- Colman-Lerner A, ML Fischman, GM Lanuza, DM Bissell, AR Kornblihtt and JL Baranao. (1999). Evidence for a role of the alternatively spliced ED-I sequence of fibronectin during ovarian follicular development. *Endocrinology* 140:2541–2548.
- Chomczynski P and N Sacchi. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159.
- Niwa H, J Miyazaki and AG Smith. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* 24:372–376.
- Takahashi K and S Yamanaka. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663–676.
- Shi W, H Wang, G Pan, Y Geng, Y Guo and D Pei. (2006). Regulation of the pluripotency marker Rex-1 by Nanog and Sox2. *J Biol Chem* 281:23319–23325.
- Boheler KR, J Czyz, D Tweedie, HT Yang, SV Anisimov and AM Wobus. (2002). Differentiation of pluripotent embryonic stem cells into cardiomyocytes. *Circ Res* 91:189–201.
- Koike M, S Sakaki, Y Amano and H Kurosawa. (2007). Characterization of embryoid bodies of mouse embryonic stem cells formed under various culture conditions and estimation of differentiation status of such bodies. *J Biosci Bioeng* 104:294–299.
- Creemers EE, LB Sutherland, J McAnally, JA Richardson and EN Olson. (2006). Myocardin is a direct transcriptional target of Mef2, Tead and Foxo proteins during cardiovascular development. *Development* 133:4245–4256.
- Alak BM, S Coskun, CI Friedman, EA Kennard, MH Kim and DB Seifer. (1998). Activin A stimulates meiotic maturation of human oocytes and modulates granulosa cell steroidogenesis in vitro. *Fertil Steril* 70:1126–1130.
- Arici A, E Oral, O Bahtiyar, O Engin, E Seli and EE Jones. (1997). Leukaemia inhibitory factor expression in human follicular fluid and ovarian cells. *Hum Reprod* 12:1233–1239.
- Fazzini M, G Vallejo, A Colman-Lerner, R Trigo, S Campo, JL Baranao and PE Saragueta. (2006). Transforming growth factor beta1 regulates follistatin mRNA expression during in vitro bovine granulosa cell differentiation. *J Cell Physiol* 207:40–48.
- Smith AG, JK Heath, DD Donaldson, GG Wong, J Moreau, M Stahl and D Rogers. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* 336:688–690.
- Williams RL, DJ Hilton, S Pease, TA Willson, CL Stewart, DP Gearing, EF Wagner, D Metcalf, NA Nicola and NM Gough. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* 336:684–687.
- Ying QL, J Nichols, I Chambers and A Smith. (2003). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* 115:281–292.
- Ying QL, J Wray, J Nichols, L Battle-Morera, B Doble, J Woodgett, P Cohen and A Smith. (2008). The ground state of embryonic stem cell self-renewal. *Nature* 453:519–523.
- Hall J, G Guo, J Wray, I Eyres, J Nichols, L Grotewold, S Morfopoulou, P Humphreys, W Mansfield, R Walker, S Tomlinson and A Smith. (2009). Oct4 and LIF/Stat3 additively induce Kruppel factors to sustain embryonic stem cell self-renewal. *Cell Stem Cell* 5:597–609.
- Yasuda H, S Tanaka, H Ohnishi, H Mashima, N Ogushi, T Mine and I Kojima. (1994). Activin A: negative regulator of amylase secretion and cell proliferation in rat pancreatic acinar AR42J cells. *Am J Physiol* 267:G220–G226.
- Maeshima A, Y Nojima and I Kojima. (2002). Activin A: an autocrine regulator of cell growth and differentiation in renal proximal tubular cells. *Kidney Int* 62:446–454.
- Shi Y and J Massague. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 113:685–700.
- Lafontaine L, P Chaudhry, MJ Lafleur, C Van Themsche, MJ Soares and E Asselin. (2010). Transforming growth factor-beta regulates proliferation and invasion of rat placental cell lines. *Biol Reprod* Oct 6. DOI:10.1095/biolreprod.110.086348
- Ogawa K, A Saito, H Matsui, H Suzuki, S Ohtsuka, D Shimosato, Y Morishita, T Watabe, H Niwa and K Miyazono. (2007). Activin-Nodal signaling is involved in propagation of mouse embryonic stem cells. *J Cell Sci* 120:55–65.
- Watabe T and K Miyazono. (2009). Roles of TGF-beta family signaling in stem cell renewal and differentiation. *Cell Res* 19:103–115.
- Austin L and AW Burgess. (1991). Stimulation of myoblast proliferation in culture by leukaemia inhibitory factor and other cytokines. *J Neurol Sci* 101:193–197.
- Kurek J, J Bower, M Romanella and L Austin. (1996). Leukaemia inhibitory factor treatment stimulates muscle regeneration in the mdx mouse. *Neurosci Lett* 212:167–170.

35. Kamohara H, K Sakamoto, T Ishiko, Y Masuda, T Abe and M Ogawa. (1997). Leukemia inhibitory factor induces apoptosis and proliferation of human carcinoma cells through different oncogene pathways. *Int J Cancer* 72:687–695.
36. Zandstra PW, HV Le, GQ Daley, LG Griffith and DA Lauffenburger. (2000). Leukemia inhibitory factor (LIF) concentration modulates embryonic stem cell self-renewal and differentiation independently of proliferation. *Bio-technol Bioeng* 69:607–617.
37. Viswanathan S, T Benatar, S Rose-John, DA Lauffenburger and PW Zandstra. (2002). Ligand/receptor signaling threshold (LIST) model accounts for gp130-mediated embryonic stem cell self-renewal responses to LIF and HIL-6. *Stem Cells* 20:119–138.
38. Kim YH, JM Ryu, YJ Lee and HJ Han. Fibronectin synthesis by high glucose level mediated proliferation of mouse embryonic stem cells: involvement of ANG II and TGF-beta1. *J Cell Physiol* 223:397–407.
39. Vallier L, M Alexander and RA Pedersen. (2005). Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. *J Cell Sci* 118: 4495–4509.

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