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Induced pluripotent stem cells' self-renewal and pluripotency is maintained by a bovine granulosa cell line-conditioned medium

Claudia Solari [a,2], Noelia Losino [a,2], Carlos Luzzani [a,2], Ariel Waisman [a,2], Carolina Bluguermann [b,3], Maria Questa [b,3], Gustavo Sevlever [b,3], Santiago Miriuka [b,1,3], Lino Barañao [a,1,2], Alejandra Guberman [a,c,*,1,2]

[a] Laboratorio de Regulación de la Expresión Génica en el Crecimiento, Supervivencia y Diferenciación Celular, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

[b] Laboratorio de Biología del Desarrollo Celular, Fundación para la Lucha Contra las Enfermedades Neurológicas de la Infancia (FLENI), Buenos Aires, Argentina

[c] Departamento de Fisiología y Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

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ABSTRACT

Induced pluripotent stem cells (iPSCs) are a promising type of stem cells, comparable to embryonic stem cells (ESCs) in terms of self-renew and pluripotency, generated by reprogramming somatic cells. These cells are an attractive approach to supply patient-specific pluripotent cells, for producing *in vitro* models of disease, drug discovery, toxicology and potentially treating degenerative disease circumventing immune rejection. In spite of the great advance since iPSCs' establishment, their obtention and propagation is an increasing area of great interest.

In a recent work, we have shown that the conditioned medium from a bovine granulosa cell line (BGC-CM) is able to preserve the basic properties of mESCs. Therefore, based on our previous results and the reported resemblance between iPSCs and ESCs, we hypothesized that BGC-CM could provide a favorable context to culturing iPSCs. In this work, we have reprogrammed mouse embryonic fibroblasts obtaining iPSC lines, and showed that they can be propagated in BGC-CM while maintaining self-renewal and pluripotency, evidenced by expression of specific gene markers and capability of *in vitro* and *in vivo* differentiation to cell types from the three germ layers. We believe that these findings may provide a novel context to propagate iPSCs to study the molecular mechanisms involved in self-renewal and pluripotency.

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1. Introduction

Induced pluripotent stem cells (iPSCs) are generated by reprogramming somatic cells through ectopic expression of several combinations of transcription factors that originally included Oct4, Sox2, Klf4 and c-Myc. These cells are comparable to embryonic stem cells (ESCs) in terms of self-renew and pluripotency, i.e., the capability to differentiate into any cell lineage under appropriate conditions [1]. Since this technology was developed, cells from different species have been reprogrammed including mouse, rat, pig, monkey and human [2–6], and distinct cell types have been shown to be amenable to direct reprogramming including fibroblast cells, terminally differentiated B cells, neural precursors, and pancreatic β cells, among others [7–9]. Thus, iPSC make *in vitro* reprogramming an attractive approach to supply patient-specific pluripotent cells for producing *in vitro* models of disease, drug discovery, toxicology and potentially treating degenerative disease circumventing immune rejection. iPSCs are usually obtained using viral vectors, such as retroviruses and lentiviruses, encoding the reprogramming factors. Moreover, relevant improvements have been accomplished in iPSC production by combining transcription factors and small chemical molecules [10,11]. However, iPSCs can also be achieved without genetic modifications by transducing somatic cells with episomal expression vectors, proteins or mRNA for delivering the key factors [12–16].

* Corresponding author at: Laboratorio de Regulación de la Expresión Génica en el Crecimiento, Supervivencia y Diferenciación Celular Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Intendente Guiraldes 2160, Ciudad Universitaria, Pab. 2, 4to piso, QB-71W, Buenos Aires, Argentina. Fax: +54 11 4576 3342.

E-mail addresses: cmsolari@qb.fcen.uba.ar (C. Solari), nlosino@qb.fcen.uba.ar (N. Losino), carlosluzzani@qb.fcen.uba.ar (C. Luzzani), waisman@qb.fcen.uba.ar (A. Waisman), carobluguer@gmail.com (C. Bluguermann), mariaquesta@gmail.com (M. Questa), gsevlever@fleni.org.ar (G. Sevlever), smiriuka@fleni.org.ar (S. Miriuka), sbaranao@mincyt.gov.ar (L. Barañao), algub@qb.fcen.uba.ar (A. Guberman).

[1] Established Investigators from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

[2] Address: Intendente Guiraldes 2160, Ciudad Universitaria, Pab. 2, 4to piso, QB-71, C1428EGA Buenos Aires, Argentina. Fax: +54 11 4576 3342.

[3] Address: Montañeses 2325, (1428) Buenos Aires, Argentina.

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Granulosa cells (GCs), are the primary cells that provide the physical support and microenvironment required for the developing oocyte [17]. They are the major component of the ovarian follicles, surrounding the oocytes and playing a key role in follicle development [18], and are an abundant source of numerous growth factors [17,19]. We have recently shown that the conditioned medium (CM) from a bovine granulosa cell line (BGC-CM) is able to maintain mESCs' self-renewal while preserving their critical properties without LIF addition. mESCs cultured in BGC-CM expressed stem cell markers and remained pluripotent, as they gave rise to embryoid bodies and teratomas that effectively differentiated to diverse cell populations from the three germ layers [20]. Moreover, we have also found that mESCs cultured in BGC-CM have an increased proliferation rate compared with cells cultured in ESC standard medium (SM). This cell line had been previously obtained by spontaneous immortalization of primary cultures [21] and its CM had been shown to be mitogenic for GC [22].

In spite of the great advance since Yamanaka's first report on iPSC establishment, their obtention and propagation is an increasing area of great interest. Therefore, based on our previous results and the reported resemblance between iPSCs and ESCs, we hypothesized that BGC-CM could provide a favorable context to culturing iPSCs. In this work, we have reprogrammed mouse embryonic fibroblasts (MEFs) obtaining IPSC lines, that could be propagated in BGC-CM while maintaining self-renewal and pluripotency.

This medium offers a novel context to propagate IPSCs to study the molecular mechanisms involved in self-renewal and pluripotency, and to compare them with the processes taking place in ESCs.

2. Materials and methods

2.1. Lentivirus production

Lentiviruses were produced using the pHAGE-EF1α-STEMCCA vector as previously described [23], with minor modifications. Briefly, the five-plasmid transfection system was introduced in 293T cells using Fugene 6 transfection reagent (Roche) according to the manufacturer's recommendations. Optimal transfection efficiency was obtained using a FuGENE 6 Reagent: DNA ratio of 6:1. Virus-containing supernatants were collected 48, 72 and 96 h after transfection.

2.2. Induction of pluripotent stem cells

Infection of MEFs was performed as described [23], making several modifications to the protocol. Briefly, MEFs from embryonic day 13.5 (passage 2) were seeded at 100,000 cells per well in gelatin-coated six-well plate. Cells were then transduced for 45 min at 750 g at room temperature with filtered supernatant supplemented with 7 μg/ml polybrene. Cells were then incubated overnight at 37 °C with 5% CO_2 and the medium was renewed 24 h after transduction. Forty-eight hours post-transduction, the medium was replaced with mESC standard medium in the presence of 1.9 mM valproic acid (VPA). Medium was changed every 2–3 days. The treatment with VPA lasted for up to 2 weeks. iPSC colonies were picked 17–22 days post-transduction based on colony morphology and reporter gene expression. Selected colonies were then expanded by plating on irradiated MEFs in ESC medium.

2.3. Cell culture

Ainv15 mESC line was obtained from ATCC. Its expansion and subculturing were carried out as previously described [20]. The established iPSC lines were similarly propagated.

2.4. Cell differentiation

iPSCs and mESCs were differentiated using the hanging drop *in vitro* differentiation protocol, as previously described [20].

2.5. Conditioned media experiments

BGC-1 culture and BGC-CM obtention were performed as previously described [20].

2.6. Teratoma obtention

Teratoma obtention was performed as previously described [20].

2.7. Reverse transcription-polymerase chain reaction

iPSC and ESCs were cultured in BGC-CM, standard medium or standard medium without LIF for at least three passages or 7 days on 0.1% bovine gelatin-coated tissue culture plates. Total cellular RNA was isolated and retrotranscribed as previously described [20]. Conventional polymerase chain reaction (PCR) amplification of DNA and analysis of the PCR products were also performed as described. Primer sequences were supplied in [20] except nestin [32].

2.8. Immunofluorescence

iPSC and ESCs were cultured in each condition for at least three passages or 7 days. Then, they were fixed, permeabilized and incubated with polyclonal primary antibodies that were detected with secondary antibodies as previously described [20]. Nuclei were stained with DAPI according to the manufacturer's instructions (Santa Cruz Biotechnology). Images were acquired with a confocal microcopy Olympus IX81/Fluoview FV 1000.

3. Results and discussion

In order to determine if BGC-CM is capable to maintain mouse iPSC's self-renewal and pluripotency, we first set out to reprogram MEF to establish several iPSC lines. We transduced primary MEF with lentiviruses that were produced using the pHAGE-EF1α-STEMCCA vector [23], which contains the three mouse factors Oct4, Sox2, Klf4 and the gene reporter Cherry instead of c-Myc. As shown in Fig. 1A–C, we obtained colonies that presented similar mESC morphology and expressed the undifferentiated state-specific gene markers Oct4, Sox2, Nanog, and SSEA-1, assessed by RT-PCR or immunofluorescense. We also detected other genes that are expressed in mESC, such as Rex1 and Ecat1 [1,24,25]. As expected, we did not find expression of these genes when we analyzed MEFs. Furthermore, the obtained cells showed to be pluripotent, as they were capable to originate embryoid bodies (EBs) that presented cells from endoderm, mesoderm and ectoderm, when they were induced to differentiate *in vitro* by the hanging drop protocol [26]. iPSC-derived EBs showed expression of α-Fetoprotein, as endoderm gene marker; Brachyury or α-Smooth Muscle Actin, as mesoderm gene markers; and βIII tubulin or Nestin as ectoderm markers [1,24] evaluated by RT-PCR or immunofluorescense. (Figs. 1D and E). Taking together, these results indicate that MEF were successfully reprogrammed into iPSCs.

In order to study if BGC-CM could enable iPSC propagation, we cultured iPSC lines in standard mESC medium or in the medium previously conditioned by the BGC line, without LIF addition. We evaluated self-renewal and pluripotency after culturing the cells in both conditions for at least three passages or 7 days.

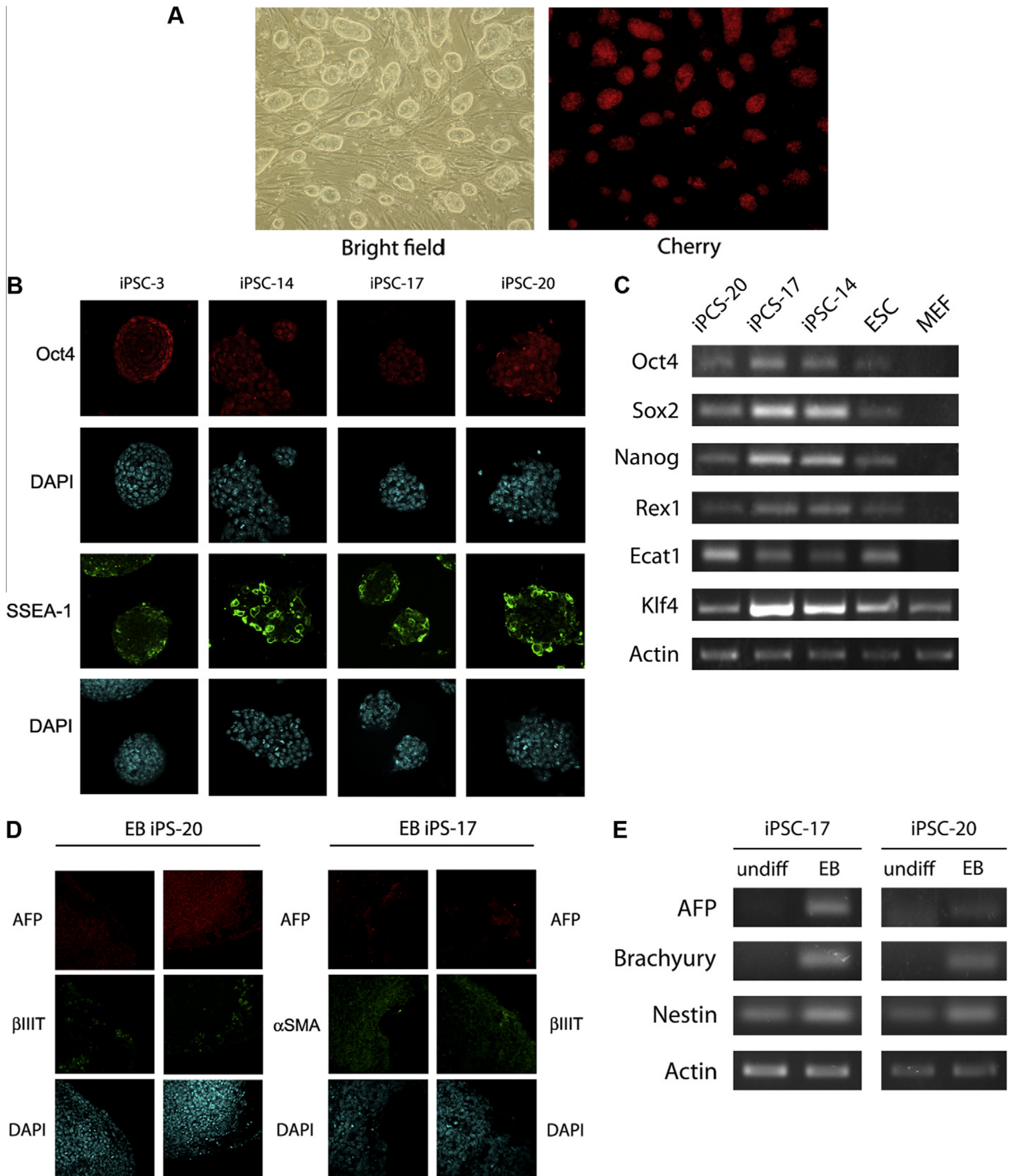

Fig. 1. Induction of pluripotent stem cells. (A) Representative phase contrast images of colonies from iPSC exhibiting typical ESC morphology (left panel) and expression of Cherry reporter gene (right panel) (B) Representative images from iPSC immunostaining for the undifferentiated state marker genes Oct4 and SSEA-1, as indicated. Nuclei were stained with DAPI. (C) RT-PCR analysis of RNA from iPSC lines, Ainv 15 mESC (ESC) and MEF of the undifferentiated state marker genes Oct4, Sox2, Nanog, Rex1, Ecat1 and Klf4. The expression of the housekeeping β-*actin* gene was used as control. (D and E) Following, the iPSCs propagated in standard medium (SM) for at least three passages or 7 days were subjected to the hanging drop protocol and gave rise to EBs. (D) Representative immunostaining images of attached iPSC-derived EBs. Alpha-fetoprotein (AFP, first row) was stained as an endoderm gene marker, Alpha Smooth Muscle Actin (αSMA, second row, second and third columns) as a mesoderm gene marker, and βIII tubulin (βIIIT, second row, first and fourth columns) as an ectoderm gene marker. Nuclei were stained with DAPI. (E) RT-PCR analysis of RNA from iPSC-derived EBs of the marker genes AFP, Brachyury and Nestin. The expression of the housekeeping β-*actin* gene was used as control.

Interestingly, iPSCs cultured both in standard medium and in BGC-CM showed typical mESC colony morphology and expressed undifferentiated state-specific gene markers, evaluated by immunofluorescence and RT-PCR. On the contrary, a negative control cultured in standard medium in absence of LIF looked clearly different, showing signals of spontaneous differentiation and absence of expression of undifferentiated state-specific gene markers (Fig. 2 and data not shown). Taking into account these results, we conclude that iPSC lines can be propagated in BGC-CM while maintaining self-renewal.

We next studied the pluripotency of iPSCs propagated in BGC-CM. Reprogrammed cells that had been cultured in BGC-CM for three passages or 7 days were subjected to the *in vitro* hanging drop differentiation protocol. As shown in Fig. 3, both iPSC cultured in BGC-CM or in proliferation standard medium gave rise to EBs that could differentiate to cell populations that expressed markers of the three germ layers, detected by immunofluorescense or RT-PCR.

We next examined the pluripotency of iPSCs cultured in BGC-CM by their ability to differentiate *in vivo* through teratoma

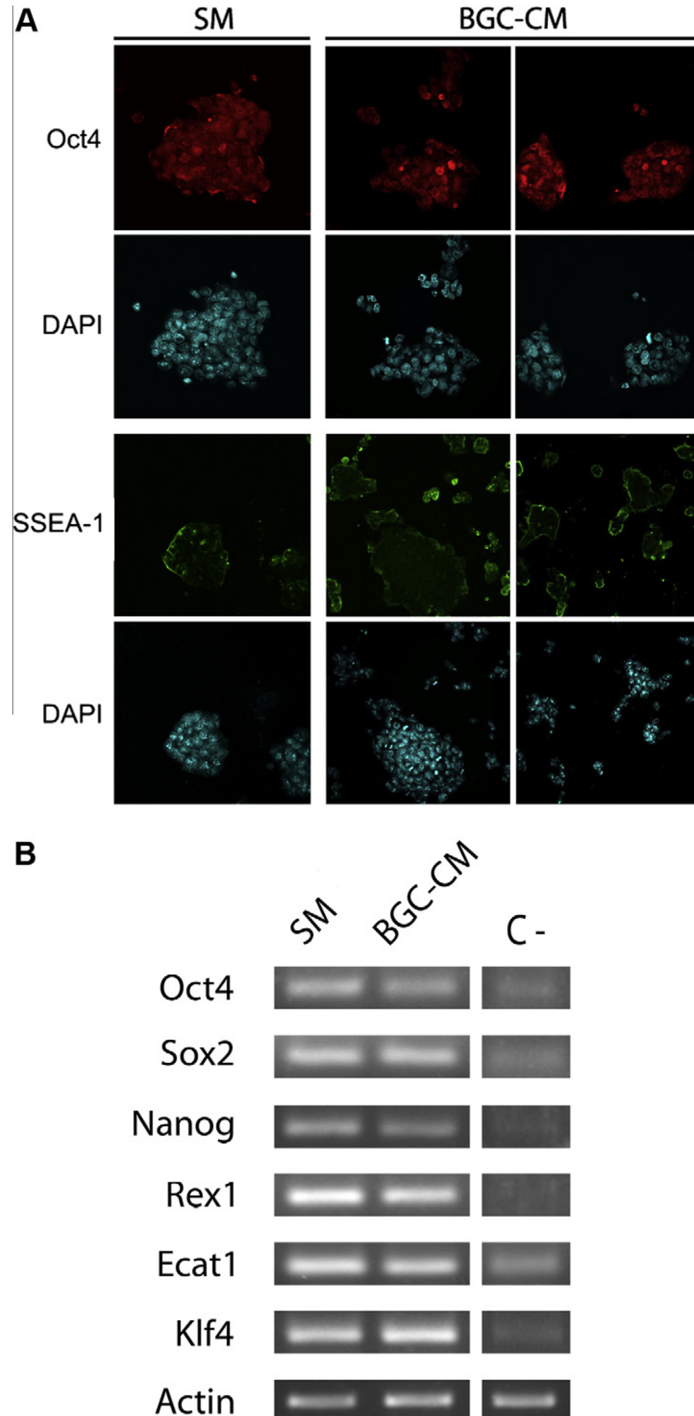

Fig. 2. iPSCs cultured in BGC-conditioned medium express undifferentiated state marker genes. iPSCs were cultured in standard medium (SM), BGC-CM (BGC-CM), or standard medium without LIF (C-) as indicated, for at least three passages or 7 days. (A) Representative images from iPSC-20 immunostaining for the undifferentiated state marker genes Oct4 and SSEA-1, as indicated. Nuclei were stained with DAPI. (B) RT-PCR analysis of RNA from iPSC-20 of the undifferentiated state marker genes Oct4, Sox2, Nanog, Rex1, Ecat1 and Klf4. The expression of the housekeeping β-*actin* gene was used as control.

Fig. 3. iPSCs propagated in BGC-CM remain pluripotent. iPSC were cultured in BGC-CM or standard medium (SM) for at least three passages or 7 days. Following, the cells propagated in the indicated medium were subjected to the hanging drop protocol and gave rise to EBs. (A) Representative immunostaining images of iPSC-derived attached EBs from iPSC-20 cultured in standard medium (SM) or BGC-CM. Alpha-fetoprotein (AFP) was stained as an endoderm gene marker, Alpha Smooth Muscle Actin (αSMA) as a mesoderm gene marker, and βIII tubulin (βIIIT) as an ectoderm gene marker. Nuclei were stained with DAPI. (B) RT-PCR analysis of RNA from EBs of the marker genes Alpha-fetoprotein (AFP), Brachyury and Nestin. The expression of the housekeeping β-*actin* gene was used as control.

formation when injected subcutaneously in immunosuppressed mice. As shown in Fig. 4, iPSC lines propagated in BGC-CM gave rise to multiple tissues. We could observe neural tissue, multiple blood vessels and lacunae, smooth muscle and epithelial tissue, among others. Moreover, we found smooth muscle surrounding ciliated epithelial tissue around a lumen, resembling an upper airway duct. Considering these evidences, we conclude that these reprogrammed cells remained pluripotent as they were able to differentiate *in vivo* into tissues from the three germ layers.

Taken all together, these data demonstrate that the iPSC lines that we obtained could be propagated in BGC-CM while preserving pluripotency.

In regard to the BGC-CM mitogenic activity [20], we did not observe this effect on iPSC as we did on mESC. However, iPS colonies cultured in this medium looked similar than those cultured in the standard proliferation medium. Since we are still studying the BGC-CM in order to identify the factors responsible for the mitogenic effect, and given the increasing evidence that supports iPSC

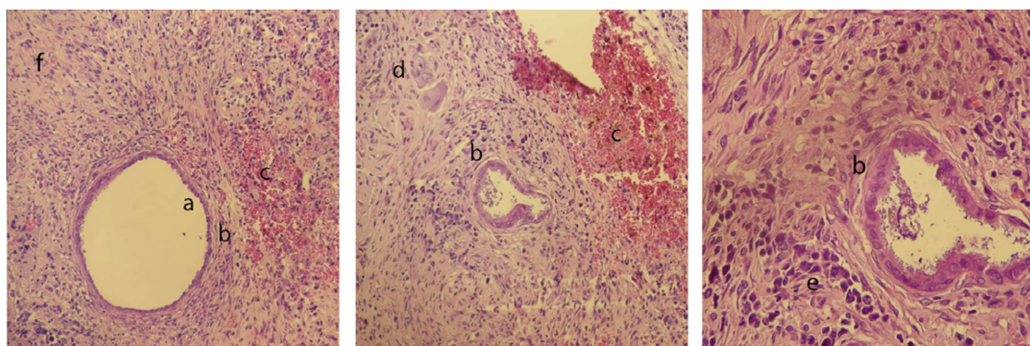

Fig. 4. IPSCs propagated in BGC-CM gave rise to teratomas. iPSC-20 were cultured for three passages or 7 days in BGC-CM previously to be injected into nude mice. Two weeks after tumors detection, they were surgically dissected, fixed and stained with hematoxylin and eosin, as previously described [20]. Representative histology of teratoma showed differentiation of iPS cells to different tissues from three germ layers, a: ciliated epithelial tissue, b: smooth muscle, c: blood cells, d: neural tissue, e: primitive neural tissue, f: blood vessels. The third panel shows a more amplified image of a different slice from the same region as the middle panel.

are similar but not identical to ESC [27], further research should be conducted to elucidate the precise differences between them. Particularly, studying their dissimilar behavior in BGC-CM, could contribute to the comprehension of the mechanisms involved in iPS cells proliferation.

Summing up, in the present work we have demonstrated that iPSC lines developed by our group can be cultured in a novel conditioned medium. We have recently shown that this BGC-CM is capable to preserve mESC, and here we have found that it is also a useful tool to propagate iPSC preserving their basic properties: self-renewal and pluripotency.

Future studies must be developed to unravel the molecular mechanism involved in the maintenance of mESC and iPSC properties cultured in BGC-CM. We are now studying candidate factors like fibroblast growth factors, LIF, and TGF-β that are well known to be involved in ESC self-renewal and pluripotency [28–30], and are expressed by this granulosa cell line [22] (unpublished results). Nevertheless, we presume that there are multiple factors responsible of this BGC-CM property, working together in an orchestrated way.

Moreover, as BGC-1 line expresses bFGF (unpublished results), a well known factor involved in human ESC (hESC) pluripotency maintenance [31], and BGC-CM seems to preserve hESC self-renewal and pluripotency (unpublished results), it would be interesting to evaluate if this medium could also be useful to propagate human iPSC.

Last but not least, BGC-CM could also provide a good context for cell reprogramming since it contains multiple factors that could influence proliferation, stemness and dedifferentiation, which have to be controlled to improve reprogramming efficiency. Currently, we are studying iPSC obtention in this medium to find out if there is a condition that improves reprogramming efficiency.

Hence, we propose that BGC-CM offers an inexpensive way for culturing iPSC preserving their basic characteristics.

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