Steps toward the generation of ovarian somatic cells from pluripotent stem cells

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Abstract

The generation of functional gametes, both eggs and sperm, from murine pluripotent stem cell (PSC) sources, has set the stage for the eventual use of this emerging technology in other species. With the field enthusiastically embracing this eventuality, in particular for animal conservation efforts, there are a number of key factors to consider regarding the applicability of these methods across species, particularly with regard to the generation of eggs. To date, published studies point to the need for fetal somatic tissue and primitive granulosa cells to serve as a niche for the growth and maturation of oocytes generated from PSCs. In practice, the need for such tissue represents a major limitation when attempting to apply this to species in which access to fetal ovaries is limited or unethical. To circumvent this, we and others have derived methods to generate ovarian granulosa cells from PSCs, albeit with low yield. Herein we present an update on the status of generating early stage granulosa cells from PSCs, and provide evidence for improvements based on a stepwise, 2-dimensional protocol for the directed differentiation of human PSCs.

Keywords: Granulosa cell, ovarian soma, granulosa precursor cell, pluripotent stem cell

Introduction

The premise that oocytes, and potentially eggs, could be generated from pluripotent stem cells (PSCs) was first established in 2003,[1] with the initial characterization of oocyte-like cells that formed within primitive follicle-like structures. Subsequent attempts at the maturation and utilization of PSC-derived oocytes were largely incremental,[2] and it was not until 2012 that functional oocytes capable of maturation, fertilization, and the ability to give rise to live offspring were realized.[3] Notably, the protocol that was used to accomplish this feat, as well as a subsequent report by the same group, relied on the innate ability of PSC-derived primordial germ cell-like cells (PGCLCs) to interact with endogenous fetal ovarian tissue, providing the microenvironment and granulosa cells that enabled follicle growth and maturation, and with it oocyte development.[3-5] An additional recent report in which functional eggs were generated from induced PSCs (iPSCs) derived from granulosa cells similarly used fetal ovarian tissue.[6] Together, these reports highlighted the notion that the fetal ovarian somatic microenvironment is not only nurturing, but likely requisite for the development of functional eggs from PSC sources.

Albeit exciting and groundbreaking for the fields of stem cell and reproductive biology, adaptation of this technology to species outside of the easily obtained and manipulated laboratory mouse must overcome the reliance on fetal tissue. This is particularly relevant in instances where obtaining fetal tissue is unethical, which pertains to humans, or unpractical, as is the case for endangered species. Therefore, to circumvent this limitation, an alternative source of ovarian granulosa cells or their precursors that can be utilized in conjunction with PSC-derived female gametes should be identified.

Although much research effort has understandably centered on the female germline, efforts to progress the derivation of ovarian somatic cells from PSCs have not been entirely lacking. Soon after the discovery of embryonic stem cells, it was possible to direct embryonic stem cells into a steroidogenic lineage by inducing expression of nuclear receptor steroidogenic factor 1 (SF-1),[7] a protein involved in endocrine development and steroidogenesis. The steroidogenic cells produced only generated minimal amounts of progesterone, and were unresponsive to human chorionic gonadotropin; however, this work opened the door to the possibility of generating steroidogenic cells from pluripotent sources. Later, other groups verified the production of steroidogenic cells by retrovirus-mediated transfection to transduce human mesenchymal stem cells with SF-1, resulting in cells expressing various steroidogenic genes and producing progesterone. Additionally, mouse embryonic stem cells with inducible SF-1 also generated steroidogenic cells resembling adrenocortical cells.[8,9] In a study investigating epigenetic memory, induced pluripotent stem cells (iPSCs) were generated from mouse and human granulosa cells, and were then

subjected to undirected differentiation; these experiments yielded cells capable of producing more estrogen and progesterone than true embryonic stem cells or from fibroblast-derived iPSCs.[10] This work highlighted the many uses of pluripotent stem cells and could have useful implications in characterizing the differentiation stages required to obtain high efficiency for the differentiation of steroidogenic cells. Generating large numbers of granulosa cells by this method that are capable of hormone synthesis would require healthy granulosa cells to generate iPSCs, a lengthy and costly endeavor to complete on a per person basis. Furthermore, those methods would be impossible when functional granulosa cells are absent or not obtainable.

The production of steroidogenic cells provided proof of concept for the use of pluripotent stem cells to generate gonadal lineages. Our previous studies built upon this and demonstrated that in mouse models, embryonic stem cells are capable of generating functional granulosa cells.[11] Mouse embryonic stem cells (mESCs) containing a dual-fluorescence reporter for germ cell specific Pou5f1-driven GFP reporter and forkhead box L2 (Foxl2)-driven Discosoma sp. red (DsRed) to label granulosa cells were allowed to spontaneously differentiate. Foxl2-DsRed labeled cells localized nearly exclusively with Pou5f1-GFP positive cells, and demonstrated a progressive differentiation profile, first expressing primitive markers associated with granulosa cell precursors (including Foxl2; wingless-type MMTV integration site family, member 4, Wnt4; follistatin, Fst; and kit ligand Kitl), followed by the acquisition of later granulosa cell makers, such as follicle stimulating hormone receptor (Fshr), Cyp19a1, and steroid acute regulatory protein (Star) and were capable of producing progesterone and estradiol in vitro. When the mESC derived Foxl2-DsRed positive cells were injected into neonatal mouse ovaries, follicles were generated incorporating the labeled cells. This study not only showed that mESCs are capable of generating steroidogenic cells, but that given the appropriate cues and environment, they are capable of participating in folliculogenesis and steroidogenesis. Unfortunately, the spontaneous differentiation rate of mESCs into Foxl2 expressing cells is low and unpredictable, and more robust methods are needed for eventual utility as a substitute for fetal ovarian tissue.

Here, we propose and test a strategy for the directed differentiation of granulosa cells from human embryonic stem cells. By mimicking developmental pathways in a stepwise, 2-D differentiation utilizing small molecules and ligands to target specific pathways, we have established a framework for identifying human, granulosa-like cells from a pluripotent stem cell source. These cells will form the groundwork for establishing the hormone producing capabilities of stem cell derived granulosa cells, and will enable in vitro studies for human follicle formation, for which there is currently no model. This strategy might also help advise work in other species that have similar limitations in background knowledge regarding organogenesis of the ovary.

Materials and methods

Cell culture and differentiation

Human embryonic stem cells (hESC) lines WA09 (WiCell) and ESI051 (ESIBio) were maintained in feeder-free culture conditions in Essential 8 Media (ThermoFisher Scientific, Waltham, MA) on tissue culture grade plates coated with hESC-qualified Matrigel (Corning, Corning, NY). Cells were routinely passaged by EDTA (0.5 mM) dissociation and all cells were used prior to passage 55. Prior to differentiation, cells were dispersed by brief incubation in 0.05% Trypsin-EDTA and were washed prior to plating at 45,000 cells/well in 12-well plates in Essential 8 media supplemented with RevitaCell™ Supplement (ThermoFisher Scientific) to aid in single cell seeding and consistent plating density. When cells reached 50% confluent, differentiations were begun by transition to differentiation media (DMEM/F12 [ThermoFisher Scientific], 1X Nonessential Amino Acids [ThermoFisher Scientific], 20% KnockOut ™ Serum Replacement [ThermoFisher Scientific], 0.11 mM beta mercaptoethanol) supplemented as described with the following compounds: 10 μM CHIR99021 (Sigma, St. Louis, MO), 1 μM retinoic acid (Sigma), 100 ng/ml human recombinant bFGF (ThermoFisher Scientific), 1 μg/ml Jagged 1 (StemRD, Burlingame, CA), or 50 μM resveratrol (MP Biomedical, Santa Ana, CA).

Gene expression

RNA was isolated by ReliaPrep RNA Mini Kit (Promega, Madison, WI) and reverse transcription was carried out using the Revertaid First Strand cDNA Synthesis Kit (Thermo, ThermoFisher Scientific). Quantitative RT-PCR was performed with Fast SYBR Green (Thermo, ThermoFisher Scientific) on a StepOnePlus Thermocycler (Applied Biosystems, Foster City, CA), using the following primers: B2M F-GGCATTCCTGAAGCTGACAG, R-TGGATGACGTGAGTAAACCTG, FOXL2 F- ACGGCCTGTACTCGGTTTAC, R- TCCACCGATCTTTCAAACAA, and AMHR2 F-TGTGTTTCTCCCAGGTAATCCG, R- AATGTGGTCGTGCTGTAGGC. Fold change in gene expression was calculated using the ΔΔCt method, with B2M as the reference.

Cell sorting and analysis

Cells were dissociated in 0.25% Trypsin-EDTA, washed in media, and blocked for 20 minutes on ice-cold blocking buffer (2% bovine serum albumin and 2% normal goat serum in PBS). Cells were stained in mouse antiAMHR2 (ab64762, 0.1 µg/ml) on ice for 30 minutes, washed, and labeled with goat antimouse Alexa Fluor 488 (1:500) on ice for 45 minutes. Cells were washed and re-suspended in cold FACS Buffer (1% fetal bovine serum, 25 mM HEPES, 1 mM EDTA in PBS). Samples were analyzed on a BD FACS Aria III special order flow cytometer equipped with the following lasers and filter sets: Blue laser (488 nm) equipped with FSC and SSC detectors and a FITC filter (505LP; 530/40 nm) and a UV laser (355 nm) equipped with a DAPI filter (450/50 nm). DAPI (Sigma, 0.2 µg/ml) was added to cells immediately prior to sorting. Cells were gated from debris by FSC versus SSC plots, followed by gating for singlet events and lastly, gating for DAPI negative events for dead cell exclusion. Population gating was performed in FlowJo (Ashland, OR) for statistical analyses.

Results

We presented a method to differentiate cells from PSCs with molecular signatures of human granulosa-like cells. To differentiate granulosa cells from hESCs, undifferentiated cells were exposed to a stepwise differentiation via small molecules to induce signaling cascades proposed for each stage and RT-qPCR was performed to detect increases in marker gene expression at each stage (Figure 1). Because human pre-granulosa cells are not well characterized, we analyzed our previously reported comprehensive

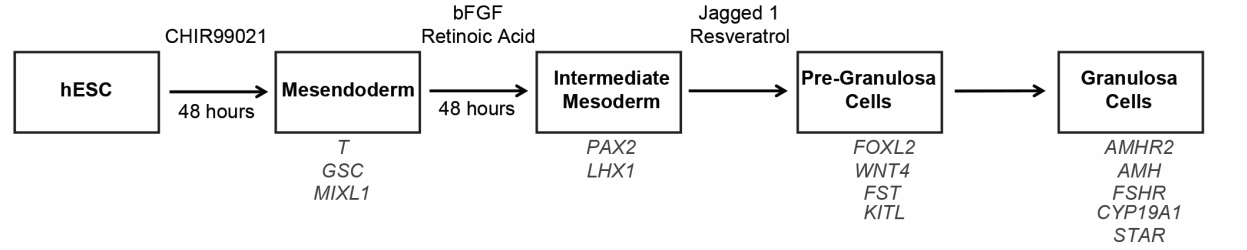

Figure 1. Differentiation strategy for the production of human pregranulosa cells from pluripotent stem cells. Human embryonic stem cells (WA09, WiCell) were maintained in feeder free conditions and subjected to 2-D directed differentiation. Cells were plated and treated with GSK3 inhibitor (GSK3i, CHIR99021, 10 µM) to induce mesendodermal lineage via WNT signaling. Further treatment with fetal growth factor (bFGF, 100 ng/ml) and retinoic acid (1 µM) was utilized to induce cells of a gonadal/renal lineage, and subsequent treatment with Notch activators, Jagged 1 (1 µg/ml), a ligand for notch receptors, and resveratrol (50 µM), a compound that induces notch signaling, to increase the incidence of potential granulosa-like cells. Cells were subject to quantitative RT-PCR to assess gene expression throughout differentiation (genes chosen for analysis shown in gray).

quantitative mass spectrometric proteomics dataset of human fetal ovarian tissue across developmental time points[12] to determine that Forkhead box L2 (FOXL2), a transcriptional regulator required for granulosa cell formation in mice, is expressed just prior to and during folliculogenesis in humans (Figure 2A). To confirm that FOXL2 expression was specific to pre-granulosa cells, immunohistochemistry (IHC) verified that FOXL2 was not detected in tissue from 56 days of development prior to follicle formation, and as germ cell cyst breakdown occurred, was localized specifically to somatic cells surrounding germ cells and was primarily nuclear (Figure 2B).

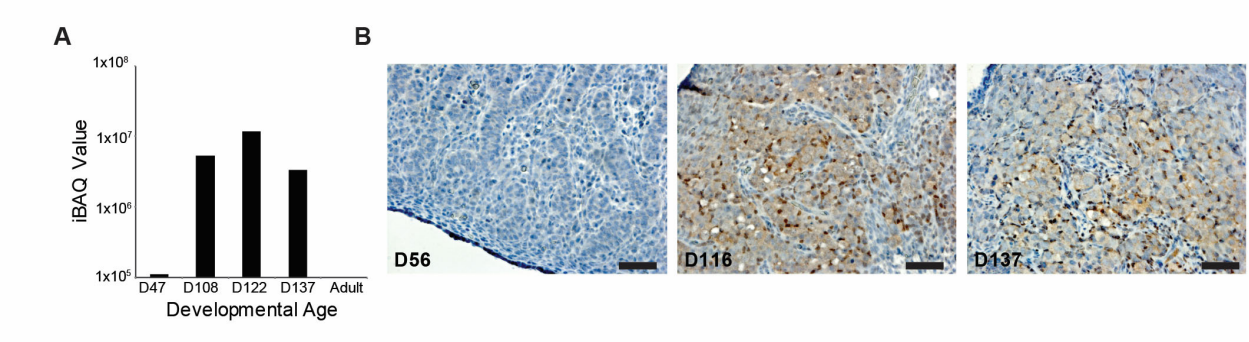

Figure 2. FOXL2 as a marker for human pre-granulosa cells based on proteomics and IHC confirmation of expression and localization. (A) Quantitative mass spectrometry detection of pre-granulosa cell protein FOXL2 at four stages of fetal development (days 47, 108, 122, and 137) and in a representative adult tissue sample. (B) Representative IHC images of FOXL2 immunostaining in human ovarian tissue from 56, 116, or 137 days of development. Primary antibodies were labeled and detected with DAB substrate (brown) and nuclei were counterstained with hematoxylin (blue). Scale bar = 100 µm.

During the first stage of in vitro differentiation, cells were treated with CHIR99021, a glycogen synthase kinase 3 (GSK3) inhibitor that activates WNT signaling, for 48 hours to induce differentiation to the mesendoderm. CHIR99021 treatment resulted in a 520-fold increase in brachyury (TBXT gene) expression at 48 hours ($p = 0.0005$) (Figure 3A). Following mesendoderm induction, cells were treated with bFGF and retinoic acid for 48 hours, followed by treatment with either Jagged 1 or resveratrol to induce notch signaling (Figure 1). In comparison to vehicle controls, FOXL2 expression increased 3.1- or 4.5-fold in response to bFGF and retinoic acid, followed by Jagged 1 or resveratrol, respectively ($p > 0.05$) (Figure 3B).

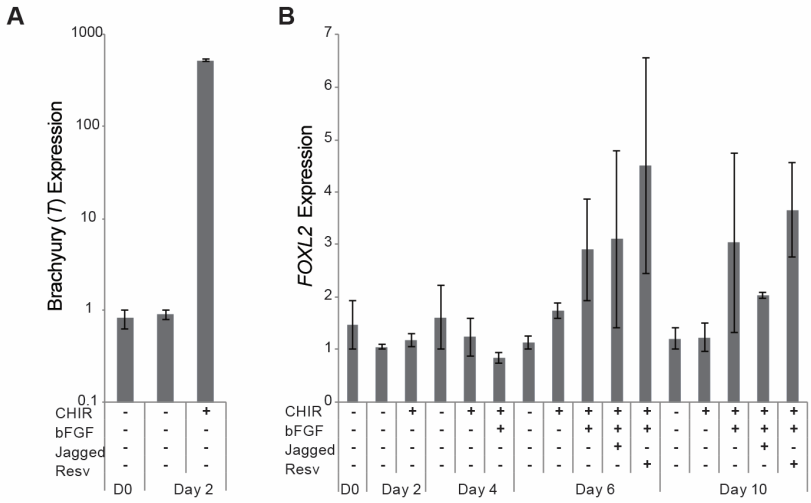

Figure 3. Directed differentiation results in an increase in mesoderm marker T at 48 hours, followed by an increase in *FOXL2* at day 6. (A) Inducing WNT signaling from 0 - 8 hours resulted in a 521-fold increase in T expression at 48 hours ($p = 0.0005$). (B) Subsequent treatment with bFGF/retinoic acid followed by notch activators Jagged 1 or resveratrol resulted in a 3.1- or 4.5-fold increase in pregranulosa cell marker FOXL2. Fold change calculated against B2M and compared to vehicle controls at each timepoint.

Additionally, we assessed the expression of AMHR2 gene expression to determine the eventual utility of using AMHR2 as a live cell marker for FACS sorting of granulosa-like cells, using the stepwise directed differentiation protocol. Following the directed differentiation approach described and using resveratrol specifically for the final treatment, AMHR2 expression increased at day 10 of differentiation in both WA09 (21.5-fold, $p = 0.005$) and ESI051 cells (58.4-fold, $p = 0.001$) over vehicle control

differentiations (Figure 4A). When differentiated cells were labeled with antiAMHR2 and analyzed by FACS, there was an increase in AMHR2+ cells from day 0 to day 10 ($p = 0.005$), and no difference in percent of AMHR2+ cells at day 10 between vehicle and directed differentiations for WA09 ($p > 0.05$). However, there was an increase in AMHR2+ cells with directed differentiation at day 10 for ESI051 (2.6-fold increase, $p = 0.008$), indicating a difference between the 2 cell types with regards to surface marker expression (Figure 4B).

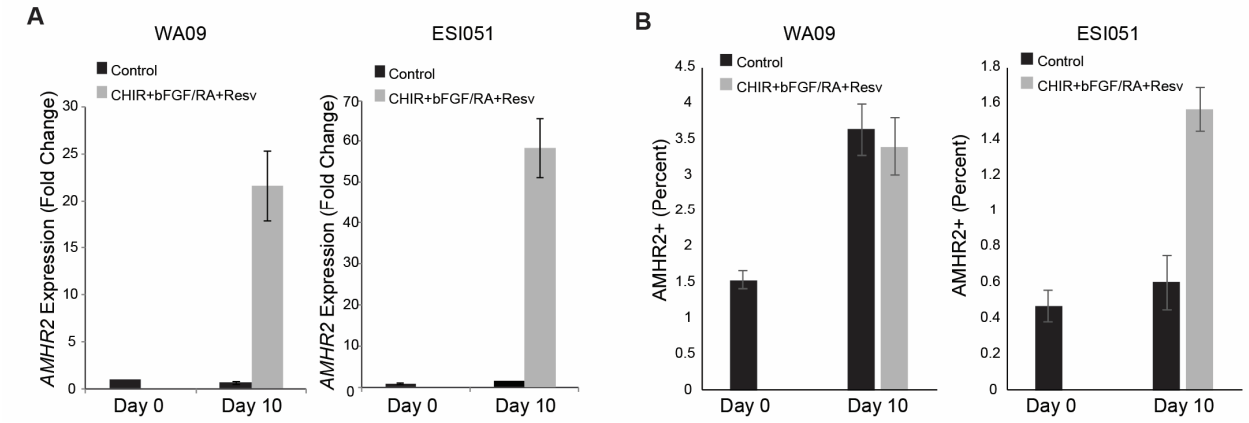

Figure 4. Directed differentiation results in an increase in granulosa cell marker AMHR2 at day 10 of differentiation and live cells can be sorted by FACS labeling for AMHR2. (A) Inducing WNT signaling from 0-48 hours, followed by subsequent treatment with bFGF/retinoic acid and resveratrol, resulted in a 21.5- or 58.4-fold (WA09 $p = 0.005$ and ESI051 $p = 0.001$) increase in granulosa cell marker AMHR2 at day 10. Fold change calculated against B2M and compared to vehicle controls at each timepoint, $n = 3$. (B) FACS analysis of AMHR2-labeled cells in undifferentiated cells and at day 10 of a differentiation under vehicle control conditions or under directed differentiation conditions. The population of AMHR2+ cells was unchanged for WA09 cells at day 10 of differentiation ($p > 0.05$) and increased over vehicle for ESI051 cells ($p = 0.008$).

Discussion

The need for a PSC-derived ovarian microenvironment containing granulosa cell precursors to complement strategies for the generation of functional eggs across species is apparent, as future applications cannot rely on a steady source of fetal gonads to serve in this capacity. Although mice have served as an important model for understanding mammalian ovarian organogenesis, key differences in the timing of differentiation processes, including folliculogenesis, across species call for an assessment of the developmental timeline, when possible. To this end, we previously completed a comprehensive proteomic analysis of human fetal ovarian tissue[12] to inform us in the selection of our differentiation pathway, including the significance of WNT signaling components.[13]

Additionally, research has shed light on the origin of granulosa cells, using various animal model systems to lineage trace cells and estimate the dynamics of follicle formation. Granulosa cells were once assumed to be derived from either the mesonephros, from which ovarian somatic tissues arise during development, or the ovarian surface epithelium.[14-16] In mice, granulosa cells originate from the surface epithelium and may constitute several waves of differentiation.[17] In bovine models, precursors to these cells, termed gonadal ridge epithelial (GREL) cells, express LGR5 and appear to act similar to other epithelial stem cell niches, in which cells are differentiated and migrate into the ovary to form primordial follicles.[18] Although these experiments have begun to elucidate the origin and differentiation pathway of granulosa cells, none have identified whether these pathways are directly paralleled in human ovarian organogenesis, and efforts to find conserved pathways between species have been sporadic.

Here, building upon previously established protocols we demonstrated a protocol for the stepwise directed differentiation of granulosa precursor cells from human PSCs. First, mesendoderm was differentiated from hESCs by activating WNT signaling via GSK3 inhibition with the small molecule, CHIR99021, which dramatically increased the expression of mesendoderm marker T within 24 - 48 hours of culture. This was consistent with previously established protocols, in which treatment with CHIR99021 significantly and reliably induced T expression within the first 2 days of treatment.[19] Subsequently, bFGF

and retinoic acid were simultaneously applied[20] to induce cells of the intermediate mesoderm, from which the gonads are derived and prior to formation of pregranulosa cells. Following successful differentiation to intermediate mesoderm, induction of NOTCH signaling, a requisite for the initiation of granulosa cell specification and the onset of folliculogenesis, was applied through addition of compounds Jagged 1 ligand, or the compound resveratrol.[21,22] This stepwise differentiation was successful in generating cells with increased expression of FOXL2 and AMHR2, markers of pregranulosa and early granulosa cells, respectively. Additionally, AMHR2 is a viable target for live cell antibody tagging and subsequent FACS sorting for culture and analysis. This approach has several important benefits; primarily, the differentiation time is relatively brief (days) and does not require embryoid body formation, reducing the labor associated with embryoid bodies and spheroid formation; second, the sorting of AMHR2-labeled cells does not require genetic manipulation to generate a reporter line for identification and isolation, making this strategy widely applicable.

Finally, whereas the mammalian ovary functions to develop and mature oocytes for reproduction, it is a vital component of the tightly regulated hypothalamic-pituitary-gonadal axis that maintains hormonal stasis throughout the female reproductive lifespan in mammals. Granulosa cells are at the crux of steroid production within the ovary, and, in concert with theca cells, generate estradiol and progesterone in response to luteinizing hormone and follicle stimulating hormone surges during reproductive years. This finely tuned steroid biosynthesis pathway is disrupted due to follicle loss in the case of polycystic ovarian syndrome, in response to chemotherapeutic agents, exposure to toxicants, and inevitably with age. The development of synthetic sources of granulosa cells or their precursors from pluripotent stem cells is not only key to the reproductive success of these artificial gametes, but also represents a putative way to address the endocrine disruption associated with ovarian failure.

Conclusion

We previously established a framework for identifying human, granulosa-like cells from a pluripotent stem cell source. Building upon established protocols, we demonstrated a protocol for the stepwise directed differentiation of granulosa precursor cells from human PSCs. We presented a method to differentiate cells from pluripotent stem cells with molecular signatures of human granulosa-like cells and we have provided evidence for improvements, based on a stepwise, 2-dimensional protocol for the directed differentiation of human PSCs.

Conflict of interest

None to declare.

Acknowledgement

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