# Female Fertility Preservation through Stem Cell-based Ovarian Tissue Reconstitution In Vitro and Ovarian Regeneration In Vivo

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**ABSTRACT:** Historically, approaches designed to offer women diagnosed with cancer the prospects of having a genetically matched child after completion of their cytotoxic treatments focused on the existing oocyte population as the sole resource available for clinical management of infertility. In this regard, elective oocyte and embryo cryopreservation, as well as autologous ovarian cortical tissue grafting posttreatment, have gained widespread support as options for young girls and reproductive-age women who are faced with cancer to consider. In addition, the use of ovarian protective therapies, including gonadotropin-releasing hormone agonists and sphingosine-1-phosphate analogs, has been put forth as an alternative way to preserve fertility by shielding existing oocytes in the ovaries in vivo from the side-effect damage caused by radiotherapy and many chemotherapeutic regimens. This viewpoint changed with the publication of now numerous reports that adult ovaries of many mammalian species, including humans, contain a rare population of oocyte-producing germ cells—referred to as female germline or oogonial stem cells (OSCs). This new line of study has fueled research into the prospects of generating new oocytes, rather than working with existing oocytes, as a novel approach to sustain or restore fertility in female cancer survivors. Here, we overview the history of work from laboratories around the world focused on improving our understanding of the biology of OSCs and how these cells may be used to reconstitute "artificial" ovarian tissue in vitro or to regenerate damaged ovarian tissue in vivo as future fertility-preservation options.

**KEYWORDS:** ovary, oocyte, stem cell, oogenesis, folliculogenesis, fertility, chemotherapy

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## Introduction: Current Fertility-Preservation Strategies

In women, the incidence of cancer increases dramatically from about 1 in 10,000 shortly after birth to about 1 in 300 as women reach the end of reproductive life during their mid-forties.[1] It is well-documented that treatment of girls and of women before the age 45 for cancer with radiation, chemotherapeutic drugs or a combination of the two therapies can result in significant, and often irreversible, side-effect damage to the reproductive system.[2–6] Oocytes are highly sensitive to cytotoxic therapies, with premature depletion of the ovarian follicle reserve frequently reported as an unintended consequence of anticancer treatments, especially those that involve pelvic radiotherapy or alkylating agents.[7–11] At present, the cryopreservation of mature eggs and preimplantation embryos is the clinical standard of care for female cancer patients, although the patient's age (adolescents), type of cancer (estrogen-responsive), or urgency of treatment initiation (aggressive cancers) may preclude the use of these approaches for all patients. Alternative strategies that involve the collection of immature (germinal vesicle-stage) oocytes for subsequent in-vitro maturation (IVM) and in-vitro fertilization (IVF) followed by embryo transfer,[12–20] as well as the cryopreservation of ovarian cortical tissue strips for autologous transplantation after the anticancer treatments are completed,[21–27] have gained increased attention as potential fertility-preservation options and are in clinical study around the world.

In addition to these approaches, which rely almost entirely on the cryopreservation of either oocytes (or embryos) or ovarian tissue strips containing oocytes before the initiation of treatments for cancer, parallel work has focused on minimizing ovarian damage in female cancer patients in vivo through pharmacologic protection of existing oocytes from cytotoxic insults during treatment.[28–30] For example, building on early preclinical studies with rhesus monkeys showing that gonadotropin-releasing hormone (GnRH) agonists could reduce the loss of ovarian follicles caused by cyclophosphamide exposure,[31] promising results have been obtained in some clinical studies of GnRH agonist treatment for ovarian protection in female cancer patients.[32–40] However, other studies have questioned if use of GnRH analogs as a co-treatment in women receiving chemotherapy provides a clear benefit to maintaining specifically fertile potential in women.[41–44] Although the consensus appears to be that GnRH agonist treatment can minimize ovarian damage in female cancer patients such that a resumption of menses and normal endocrine profiles are observed, the field

remains divided over whether these outcomes actually lead to improvements in pregnancy success rates in women after cessation of treatment.

In parallel to studies of GnRH agonists, other work has explored the prospects of using anti-apoptotic small molecules as ovarian-protective agents in the face of radiotherapy and chemotherapy. This line of investigation arose from the observation that exposure of oocytes to chemotherapeutic drugs activates apoptosis or programmed cell death.[45] Extensive genetic studies in mice, designed to define the key pathways utilized by oocytes to die,[8] uncovered an indispensable role for ceramide generated by acid sphingomyelinase in triggering chemotherapy-induced oocyte apoptosis. In turn, a natural inhibitor of ceramide, termed sphingosine-1-phosphate (S1P), was shown in mice to prevent oocyte loss and infertility resulting from anticancer treatments.[46,47] These findings of a protective effect of S1P on fertile potential in vivo after radiotherapy exposure were eventually extended to nonhuman primates, with the birth of healthy offspring free of any evidence of propagated cytogenetic damage.[48]

Although clinical studies of S1P or S1P analogs have not yet, to our knowledge, been pursued, several reports have provided evidence supporting a similar cytoprotective function of S1P in human ovaries. For example, two separate studies have shown that S1P reduces primordial follicle loss in human ovarian tissue xenografted in mice and exposed to cyclophosphamide as an in-vivo model of chemotherapy-induced ovarian damage.[49,50] In addition and of direct relevance to clinical studies focused on restoration of fertility in women through orthotopic or heterotopic transplantation of cryopreserved-and-thawed ovarian cortical tissue strips,[21–27] S1P has also been reported to minimize the loss of primordial follicles that occurs during vitrification and thawing of mouse ovarian tissue grafts[51] as well as during the slow-freezing and thawing of human ovarian cortical tissue.[52]

These encouraging outcomes have prompted efforts to identify additional factors capable of minimizing ovarian damage caused by anticancer treatments, with one of the most recent being a preclinical study in rats reporting that injections of curcumin and capsaicin can offset cyclophosphamide-induced premature ovarian failure.[53] In other studies, co-administration of imatinib (Gleevec), a 2-phenyl amino pyrimidine derivative that inhibits activity of the tyrosine kinase domains of c-Abl, c-Kit and platelet-derived growth factor receptor, has been reported to attenuate follicle depletion in mice caused by cisplatin treatment,[54] the latter of which activates apoptosis in mouse oocytes through the TAp63 signaling pathway.[55] However, a subsequent study with two different strains of mice failed to show protective effects of imatinib on either oocyte apoptosis (follicle depletion) or infertility resulting from cisplatin treatment,[56] leaving open the question of whether small molecules such as imatinib, which specifically target receptor-associated tyrosine kinases, would be beneficial for fertility preservation in women exposed to cytotoxic agents. In fact, therapeutic strategies that interfere with c-Kit function may actually be counter-productive in efforts to maintain or restore female fertility, given the well-established importance of c-Kit to germ-cell development and survival.[57,58]

## Discovery of Mammalian Oogonial Stem Cells

While the use, in some manner, of existing oocytes or the embryos generated from these oocytes through IVF as the traditional approach to female fertility preservation has yielded positive clinical results, progress toward the development of future technologies aimed at sustaining or restoring fertility in female cancer survivors would be severely constrained if existing oocytes were the only resource to work with. In 2004, this constraint was lifted by a report that identified the existence of mitotically active germ cells in postnatal mouse ovaries capable of supporting de-novo oogenesis and folliculogenesis during adult life.[59–62] This report was met, not surprisingly, with a mix of cautious excitement along with outright disbelief.[63–66] In short, it defied one of the foundational tenets in the field of reproductive science—female mammals are endowed with all of the oocytes they will ever have at birth, and this pool is not subject to renewal during postnatal life.[67,68]

Amid debate, continued studies on this topic from multiple laboratories around the world eventually produced a now large body of evidence substantiating the occurrence of postnatal oogenesis in mammals,[69–76] as well as the characteristic features and functional properties of the germ cells responsible for continued oocyte formation.[77] These cells, termed female germline or oogonial stem cells (OSCs), have been identified in, and isolated for study from, adult ovarian tissue of mice,[75,76,78–102] rats,[103] cows,[104] pigs,[105,106] nonhuman primates,[107] and women.[82,85,102,108–111] In evaluating OSCs from a functional perspective, the identity of these cells as bona-fide oocyte-producing germ cells has been independently verified by several groups using intragonadal transplantation-based approaches in rodent models,[76,78,80,82,90,94,103] which are universally considered the litmus test for functional identity testing of the male-equivalent spermatogonial stem cells (SSCs) in the testis.[112–114] These studies have collectively shown OSCs, expressing a fluorescent reporter for cell fate tracking, transplanted into ovaries of adult wild-type recipients generate oocytes that develop into mature eggs which fertilize to produce viable embryos and offspring.[76,78,80,82,90,94,103] Extending these observations, two groups have also used genetic mouse models that enable inducible tracking of cell fate in vivo to discern the physiological significance, if any, of OSCs and postnatal oogenesis in adult females. These experiments confirmed the occurrence of germ cell meiotic entry and de-novo oogenesis in the ovaries during reproductive life,[75,76] and further showed that oocytes formed during adulthood are used to produce offspring in natural mating trials.[76] This considerable body of evidence, from multiple laboratories around the world, documenting the existence and functional properties of OSCs in adult mammalian ovaries across

species should erase any residual doubts that the long-standing paradigm of a fixed pool of oocytes being endowed in ovaries of female mammals at birth has repeatedly been proven incorrect.[77]

## Characteristics of Human OSCs

The isolation of OSCs from ovaries of women by at least four independent groups to date has enabled detailed comparative studies of these cells with OSCs in animal models, and by all accounts, the human cells display the fundamental characteristics of oocyte-generating germline stem cells.[82,85,102,108–111] Although complete functional identity testing has not yet, at least to our knowledge, been performed with human OSCs, the cells have been tested for oocyte-forming capacity in a number of ways. For example, human OSCs maintained as pure germ cell cultures exhibit the same capacity as rodent OSCs to spontaneously generate in-vitro-derived (IVD) oocytes after passage and replating.[82,85] Importantly, these human IVD oocytes express expected patterns of oocyte-specific genes[82] and are able to complete meiotic progression to reach formal haploid status, as defined by flow cytometric analysis of DNA content per cell,[82] as well as by fluorescence in-situ hybridization of chromosome numbers per cell.[111] Building on these observations from cultured OSCs, introduction of enhanced green fluorescent protein (EGFP)-expressing human OSCs into adult human ovarian cortical tissue ex vivo has been used to trace the generation of new EGFP-positive oocytes that form primordial follicles capable of early development and growth.[82,108] Moreover, similar outcomes can be achieved by simple reaggregation of EGFP-expressing human OSCs with cellular dispersates prepared from adult[82] or fetal[110] human ovarian tissue, collectively supporting that OSCs are fully capable of differentiating into oocytes that interact with appropriate ovarian somatic cell partners to form new follicles.

Although still early, these exciting findings offer a strong foundation on which to push forward with additional models and testing, including incorporation of existing technologies for in-vitro follicle growth[115–119] along with those for IVM of germinal vesicle-stage oocytes for IVF and embryo transfer.[12–20] The ultimate goal will be to establish an in-vitro platform that reliably enables the generation of developmentally competent eggs derived from human OSCs recombined in some manner with autologous ovarian somatic cells to facilitate oogenesis and folliculogenesis.[77,120] Given the established ability of rodent OSCs to successfully differentiate into eggs that yield viable offspring,[76,78,80,82,90,94,103] we feel that, with the appropriate environmental support, human OSCs will indeed be capable of achieving the same outcomes as their mouse and rat counterparts.

## Reconstitution of Human Folliculogenesis in vitro

As discussed earlier, ovarian tissue cryopreservation and transplantation is now offered by some clinics as an option for fertility preservation in female cancer survivors.[21–27] However, the live-birth success rate of the approach, even 15 years after initial reports of its use, remains fairly low.[25] This limitation, coupled with the fact that the procedure presents significant risks associated with the multiple surgeries required to obtain and subsequently return the ovarian tissue used for cryopreservation and transplantation, highlights the need for additional technologies to adequately meet the family-planning hopes of these patients once their treatments are completed.[121] Cryopreservation of eggs and embryos are now well-established alternatives for women diagnosed with cancer to consider for a future chance at having a baby; however, these approaches are not a viable option for all patients, especially those who are adolescent or suffer from premature ovarian insufficiency. With just these few options available, efforts by several groups to design and test new in-vitro and in-vivo platforms rooted in the principles of stem cell-based regenerative medicine may offer additional fertility solutions for cancer survivors.

To that end, significant strides have been made over the past several years in the generation of functional eggs from stem cells entirely outside of the body, using mice as an exploratory model system. This line of investigation dates back to the early work of Hübner et al,[122] who first reported the in-vitro derivation of oocyte-like cells and follicle-like structures from mouse embryonic stem cells (ESCs) in culture. Almost a decade later, Hayashi et al[123] successfully specified primordial germ cell-like cells (PGC-LCs) from mouse ESCs and induced pluripotent stem cells (iPSCs). When the PGC-LCs were aggregated with fetal gonadal somatic cells and then grafted to ovaries of adult female recipients, the cells differentiated into immature oocytes that yielded viable offspring following IVM, IVF, and embryo transfer.[123] These experiments were further refined to eventually remove the need for in-vivo tissue grafting, ultimately providing a platform for complete reconstitution of female gametogenesis from ESCs and iPSCs entirely ex vivo.[124] Parallel studies of human ESCs and iPSCs have provided evidence that, like their murine counterparts, these cells are capable of generating PGC-LCs as well[125,126] and that ovarian follicle-like structures can be formed from human ESCs in culture.[127] Collectively, these studies have prompted widespread, but in our view quite premature, speculation that technologies involving ESCs and iPSCs will one day in the not-too-distant future offer women in need of fertility assistance the opportunity to produce essentially unlimited eggs for assisted reproduction.[128–130]

However, it is critical to emphasize several key points when evaluating the potential of ESCs or iPSCs to solve human female infertility in the future. The first of these is the apparent obligate need for PGC-LCs to interact with fetal gonadal somatic cells for differentiation into oocytes that can mature into competent eggs,[123,124] a concept reinforced by previous

studies with mice showing that PGC-LCs introduced in adult ovarian tissue generate only immature oocytes that arrest and degenerate at very early stages of follicle development.[128] It is therefore unclear, if only for practical reasons, how human fetal gonadal tissue would be obtained and made available for use in any type of clinical platform involving gametogenesis from pluripotent stem cells. Even if this major obstacle is overcome, the issue is further complicated by the fact that non-autologous fetal ovarian tissue would be needed to produce eggs from iPSC-derived germline cells of another individual. A second stumbling block to human translation of the mouse work with iPSCs pertains to regulatory issues surrounding genetic manipulation of the parental somatic cells to obtain iPSCs in the first place, whether or not one employs nonintegrating genetic approaches for nuclear reprogramming. Finally, it remains to be determined, even in mice, if nuclear reprogramming of differentiated somatic cells into iPSCs for subsequent generation of PGC-LCs generates germline cells that effectively carry out the process of maternal mitochondrial inheritance. In other words, studies must be performed to demonstrate that offspring produced from iPSC-derived eggs are not burdened from the outset with compromised mitochondrial genomes that are pre-existent in the parental somatic cells reprogrammed into iPSCs for germ cell specification.[131]

In comparison, OSCs represent an alternative source of oocyte-generating stem cells present in the ovaries of women which are free of these limitations and complications, but exhibit the same capacity as ESCs or iPSCs in mouse studies to generate fully functional eggs that fertilize and produce viable offspring.[76,78,80,83,90,94,103] Unlike iPSCs, however, the use of OSCs does not require any genetic manipulation or reprogramming since OSCs are already germline stem cells naturally wired to produce oocytes in adult ovaries.[76] Moreover, OSCs are capable of generating mature oocytes when introduced into adult ovarian tissue,[76,78,80,83,90,94,103] removing the need for fetal ovarian somatic cells that ESC- or iPSC-derived PGC-LCs depend on to achieve full functionality as egg precursor cells.[123,124] Despite these advantages, however, OSCs are not capable of differentiation into fully functional eggs alone, and any technology platform that uses these cells, like all other stem cells, will require parallel incorporation of appropriate somatic cell partners for success.[120]

Without question, the most important of these partners are primitive or undifferentiated granulosa cells (sometimes referred to as pregranulosa cells), which are capable of interacting with newly generated oocytes to form primordial follicle-like structures. While previous studies have reported the existence and functional characterization of ovarian somatic stem cells that can give rise to thecal-interstitial cells,[132,133] we are not aware of any publication to date that has definitively identified a putative granulosa stem cell population in postnatal ovarian tissue. The possibility that primitive granulosa cells differentiate from multi-potent stem cells present in the ovarian surface epithelium was proposed many years ago, based largely on indirect in-vitro studies of undefined (heterogeneous) ovarian cell cultures.[134,135] However, rigorous follow-up experiments that have functionally tested this hypothesized lineage relationship, or that have confirmed multi-potent stem cells in adult mammalian ovaries are a physiological source of pregranulosa cells for de-novo folliculogenesis in vivo, are lacking. In fact, it is still unclear if primitive granulosa cells can even be isolated from adult ovarian tissue and successfully expanded in vitro in an undifferentiated state for use in biomimetic stem cell-based platforms aimed at achieving folliculogenesis and, ultimately, ex-vivo egg generation.[120] By employing fluorescence-activated cell sorting (FACS), we have very recently isolated a distinct population of ovarian somatic cells that express stem cell-associated genes (e.g. *POU domain class 5 transcription factor 1* or *Pou5f1*, and *Nanog*), can be stably expanded in-vitro over many passages, and exhibit several key features of primitive granulosa cells. The latter includes expression of the *Forkhead box L2* (*Foxl2*) and *Wingless-type MMTV integration site family member 4* (*Wnt4*) genes, as well as inducible expression of follicle-stimulating hormone (FSH) receptor and the molecular machinery required for steroidogenesis (T. Akahori, D.C. Woods and J.L. Tilly, unpublished data). While we await the outcome of functional characterization studies that are underway in our laboratories to unequivocally establish the identity of these cells, we will turn our attention back to published studies on potential sources of primitive granulosa cells.

Building on earlier observations of Hübner et al[122] that follicle-like structures, capable of steroidogenesis and extrusion of oocyte-like cells, can be observed in cultures in mouse ESCs, other groups reported similar outcomes using mouse ESCs with some disagreement on "normalcy" of the oocyte-like cells contained within the follicle-like structures.[136–138] Moreover, all of these reports focused almost entirely on the germline side of the story, with comparatively little attention placed on the putative granulosa cells also present in these structures. In 2013, Woods et al[139] reported the first in-depth study of primitive granulosa cell specification from a dual-reporter mouse ESC line engineered to express EGFP driven by a modified *Pou5f1* gene promoter and red fluorescent protein (DsRed) under control of the *Foxl2* gene promoter to simultaneously track germ cell and granulosa cell formation, respectively. After confirming the generation of follicle-like structures containing EGFP-positive germ (oocyte-like) cells surrounded by DsRed-positive somatic (pre-granulosa) cells in ESC cultures (Figure 1), the DsRed-positive cells were isolated by FACS at various time points postspecification and analyzed by several approaches.

Of several interesting findings presented in this study, DsRed-expressing cells collected relatively early after specification from ESCs exhibited a gene expression profile consistent with an in-vivo pregranulosa cell phenotype, as defined by expression of *Foxl2, Wnt4, Follistatin* (*Fst*), and *Kit ligand*

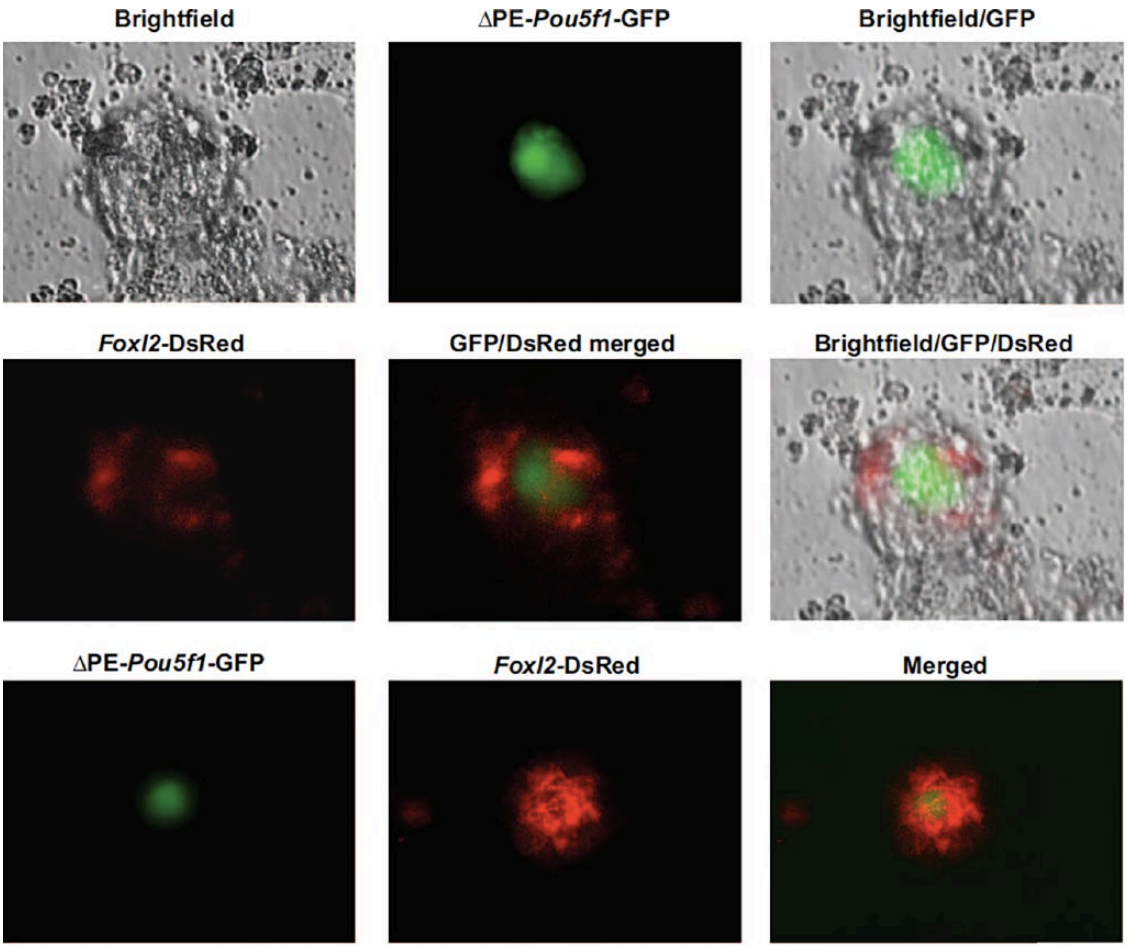


**Figure 1.** Specification of primitive ovarian granulosa cells from ESCs in vitro, which are capable of interacting with early germ cells to initiate folliculogenesis. Differentiation of mouse ESCs engineered to express green fluorescent protein (GFP) driven by a ΔPE-*Pou5f1* gene promoter (germ cell marker) and DsRed driven by a *Foxl2* gene promoter (primitive granulosa cell marker) leads to the in-vitro formation of ovarian follicle-like structures containing GFP-positive oocytes surrounded by DsRed-expressing granulosa cells. Reproduced from Woods et al.[139]

(*Kitl*), among other genes.[139] As the culture time was extended, the gene expression profile in the DsRed-positive cells changed to one more in line with granulosa cells at an early stage of differentiation, as demonstrated by the activation of *FSH receptor* (*Fshr*), *anti-Müllerian hormone* (*Amh*), *cytochrome P450 family 19 subfamily a polypeptide 1* (*Cyp19a1*), and *steroidogenic acute regulatory protein* (*Star*) gene expression. Perhaps the most convincing evidence of a granulosa cell identity, however, was derived from in-vivo transplantation studies of DsRed-positive cells isolated from these ESC cultures, in which the fate of the cells in ovaries was traced to incorporation in the granulosa cell layer of immature follicles.[139] The findings reported in this study, which showed that Foxl2-expressing somatic cells formed from differentiating ESCs express granulosa cell markers, actively associate with germ cells in vitro, synthesize steroids, respond to FSH, and participate in folliculogenesis in vivo,[139] have been repeated and extended by others.[140–142]

Collectively, these observations offer a strong impetus to consider the use of patient-derived iPSCs to generate autologous primitive or pregranulosa cells for aggregation with OSCs to successfully reconstitute the process of oogenesis and folliculogenesis in vitro[143] (Figure 2). It is also worth mentioning that several studies have reported the generation of steroidogenic cells from iPSCs derived from reprogrammed granulosa cells.[144–146] In one of these studies, it was further concluded that generation of iPSCs from the cell type that one seeks to ultimately specify in vitro may prove advantageous due to epigenetic memory of the parental cells being carried through the reprogramming and lineage specification process.[145] In any case, the prospect of designing an in-vitro platform for the generation of mature human eggs through stem cell-based bioengineering, while still early in development, is clearly inching closer to reality.

## Human Ovarian Regeneration in vivo

In parallel to the efforts outlined above for in-vitro reconstitution of human oogenesis and folliculogenesis from stem cells, a growing body of evidence supports that a reintroduction of OSCs back into ovarian tissue following chemotherapy may enable at least some recovery of normal ovarian function and fertility. The first observations made in this regard came from mouse studies a decade ago, in which

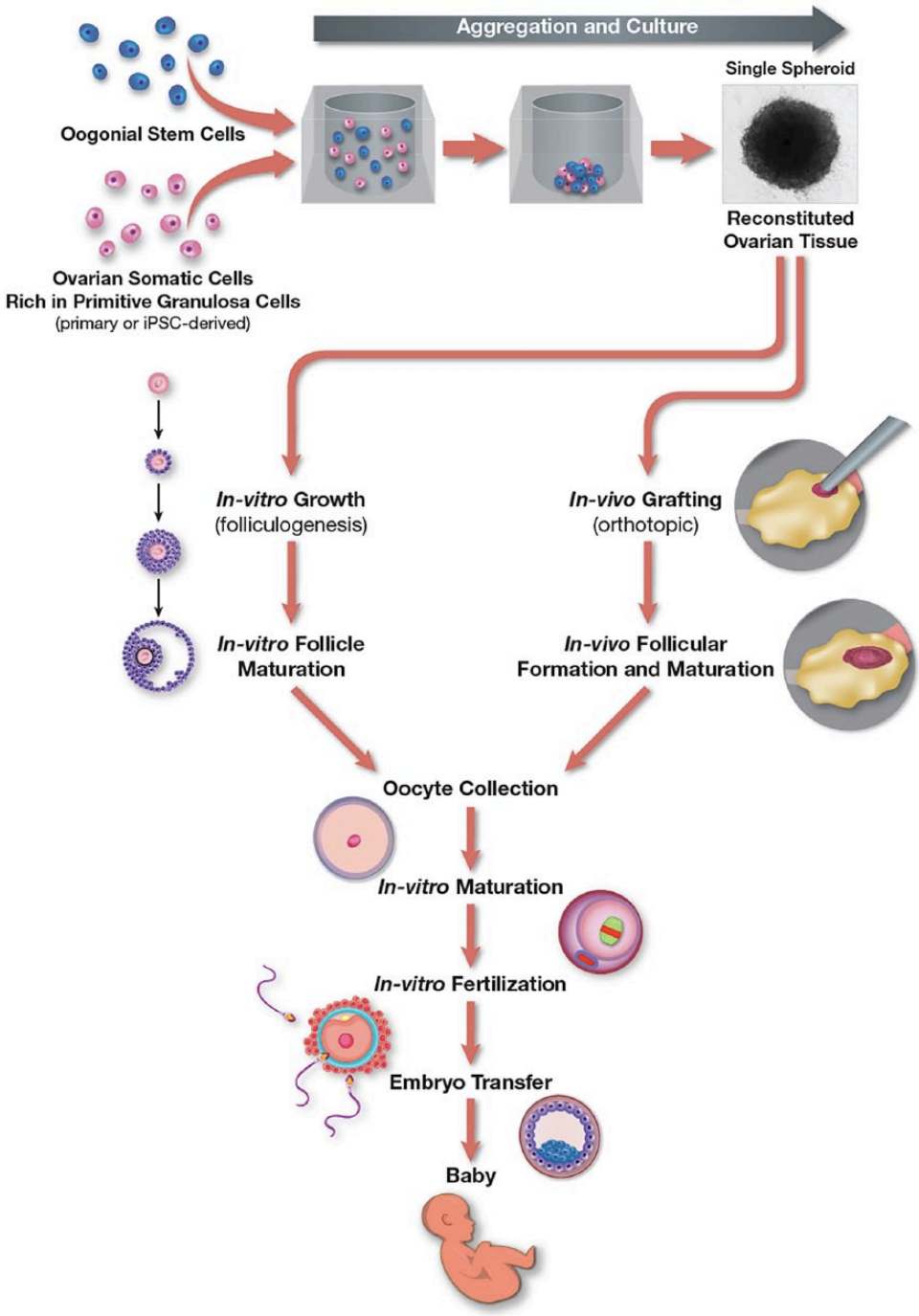


**Figure 2.** Working model for ex-vivo reconstitution of autologous human ovarian tissue. Aggregation of OSCs with primitive granulosa cells, specified from iPSCs or isolated from ovarian tissue during OSC purification, enables de-novo oogenesis and folliculogenesis in the reconstituted tissue in vitro. The tissue containing new follicles is then used for orthotopic grafting to the ovaries for in-vivo growth to produce maturing follicles for oocyte aspiration or for in-vitro follicle culture to generate oocytes. Oocytes obtained from either approach are subjected to in-vitro maturation and in-vitro fertilization to generate blastocysts for embryo transfer and establishment of successful pregnancies.

transplantation of EGFP-expressing OSCs into ovaries of adult wild-type female mice previously conditioned with chemotherapy was used to demonstrate that the cells can successfully differentiate into GFP-positive oocytes which complete maturation and produce viable offspring in natural mating trials.[78] These observations from OSC transplantation have been independently confirmed by others using chemotherapy-treated mice as a model[90] and are in keeping with the well-established ability of SSC transplantation to restore fertility in male mice conditioned with busulfan.[114]

A different way to look at the question of ovarian regeneration in vivo could entail therapeutic "reactivation" of resident OSCs, which perhaps undergo some type of protective dormancy in the face of cytotoxic insults, to resume normal function once the anticancer treatments are completed.

However, such an approach to female fertility preservation or restoration would depend on several factors to realize success. One of these would be the identification and validation of deliverable "oogenic" factors capable of driving OSC differentiation into oocytes. Unfortunately, the list of these factors is quite small at present and includes histone deacetylase inhibitors,[73] bone morphogenetic protein 4 (BMP4),[84] Hippo signaling pathway components,[95] and as-yet unidentified factors present in the peripheral circulation of males.[72] Nevertheless, the ability of OSCs to generate IVD oocytes in culture[76,82,84,85,102,111] provides a firm foundation on which to perform high-throughput screening of candidate oogenic factors that can then be rigorously tested for their ability to expand the ovarian reserve in vivo.[120]

A second point worth considering here is the type of anticancer treatment employed, since those regimens known to present a high risk for premature ovarian failure—such as radiotherapy or alkylating agents, may destroy resident OSCs. However, other chemotherapeutic agents, such as doxorubicin (Adriamycin), may not kill off OSCs and thus might allow for a spontaneous recovery of oocyte numbers and ovarian function, depending on dose, mode of delivery and duration of the treatment.[69] Likewise, a recent study of age-matched ovarian tissue collected from women without and with previous treatment with an adriamycin, bleomycin, vinblastine, and dacarbazine (ABVD)-based chemotherapeutic regimen uncovered a significant increase in nongrowing follicle density in ABVD-exposed ovaries.[147] Although the mechanistic basis of this surprising observation remains to be determined, it was postulated that OSCs were possibly recruited into action following the initial damage to the ovaries, leading to enhanced de-novo oogenesis and folliculogenesis after the ABVD treatments were ceased.[147]

Any approach designed to achieve ovarian regeneration in vivo should also take into account methods that entail delivery of various somatic cell preparations capable of broadly repairing ovaries damaged by anticancer treatments, possibly enabling resident stem cells to resume normal function. For example, early studies by Johnson et al[69] demonstrated that systemic bone marrow transplantation (BMT) into adult female mice conditioned beforehand with a combination of cyclophosphamide and busulfan could partially restore oocyte numbers and ovarian function compromised by the chemotherapy regimen. Evaluation of the ovaries of the wild type mice receiving BMT using EGFP-expressing transgenic female mice as donors further indicated the presence of EGFP-positive oocytes contained in immature follicles.[69] However, like the developmental arrest observed in oocytes generated from ESC-derived PGC-LCs transplanted into adult ovaries,[128] bone marrow stem cell-derived oocytes exhibit a similar developmental arrest and do not contribute to the pool of ovulated eggs used for reproduction.[148,149]

Nonetheless, in rodent models, BMT has a clear beneficial effect on fertility preservation in chemotherapy-treated adult females,[149] and these restorative outcomes are also observed in untreated female mice that exhibit natural aging-associated impairments in fertility and reproductive outcomes in natural mating trials.[150] Although it remains unresolved if BMT exerts similar protective effects in women subjected to chemotherapy or radiotherapy,[151] recent studies using human bone marrow-derived stem cells infused into immunodeficient female mice treated with chemotherapy to induce ovarian damage indicate that human bone marrow cells can, like their mouse counterparts, significantly improve follicle development and fertility.[152] These types of studies support that stem cell-based ovarian regeneration in women is, in all likelihood, possible, but that the approach will need to encompass a restoration of oogenesis and follicular development[69,149,152] along with repair of the ovarian stroma and microvascular beds which are known to be negatively impacted by chemotherapy.[153]

## Concluding Remarks and Future Directions

The field of fertility preservation has made tremendous strides over the years in bringing new hope to survivors of cancer that they can have genetically matched children once their treatments are completed. Progress in human oocyte, embryo, and ovarian tissue cryopreservation, coupled with continued improvements in IVM of human oocytes and in efforts to therapeutically protect ovarian function in vivo during the course of cytotoxic treatments aimed at killing cancer cells, offers several options for women to consider. However, many limitations exist, both in technology and in application, which support the need for development of additional approaches to achieve fertility preservation or restoration. Of the various scientific directions currently being pursued, the relatively recent introduction of regenerative medicine-based technologies into efforts designed to improve natural and assisted reproduction is one of the most exciting, and potentially the most high-impact, areas of investigation.[77,120,143,154,155]

In this regard, it is important to emphasize that the successful reconstitution of female gametogenesis in mice from mitotically active germ cells to functional eggs entirely in vitro[123,124]—an accomplishment that only a decade ago was incomprehensible to most scientists in the field of reproductive biology, conceptually epitomizes the power of stem cell biology for potentially addressing unmet needs in reproductive medicine. Likewise, the existence of oocyte-producing stem cells in the ovaries of mammals,[59–62] which are fully capable of supporting de-novo oogenesis in adult life,[76,78,80,82,90,94,103] was not even considered a possibility 15 years ago because of five decades of previous dogmatic thinking arguing in support of fixed endowment of oocytes at birth.[67,68] With human OSCs now identified and under rigorous investigation by several groups,[82,85,102,108–111] scientists have tools to work with today that have the inherent capacity to forever change the landscape of human reproduction and infertility. These types of research

investigations, coupled with those in other exciting arenas of reproductive medicine and health, combine to offer great hope for the future of female fertility preservation.[77,120,143,154–157]

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## Author Contributions

All authors contributed to the writing and preparation of this article, and approved its final submission.

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