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Germ cell formation from embryonic stem cells and the use of somatic cell nuclei in oocytes

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Abstract

Embryonic stem cells (ESCs) have remarkable properties of pluripotency and self-renewal, along with the retention of chromosomal integrity. Germ cells function as a kind of “transgenerational stem cells”, transmitting genetic information from one generation to the next. The formation of putative primordial germ cells (PGCs) and germ cells from mouse and human ESCs has, in fact, been shown, and the apparent derivation of functional mouse male gametes has also been described. Additionally investigators have successfully reprogrammed somatic nuclei into a pluripotent state by inserting them into ESCs or oocytes. This would enable the generation of ESCs genetically identical to the somatic cell donor and their use in cell therapy. However, these methodologies are still inefficient and their mechanisms poorly understood. Until full comprehension of these processes is obtained, clinical applications remain remote. Nevertheless, they represent promising tools in the future, enhancing methods of therapeutic cloning and infertility treatment.

Keywords

ES cells; germ cells; somatic cell nuclear transfer; therapeutic cloning

Introduction

The existence of self-renewing cells, capable of originating multiple lineages, was postulated more than a century ago¹, but the realization of the character of stem cells and capitalization on their potential is very recent. Increasingly, intense interest is based on the findings that stem cells with varying degrees of pluripotency can be obtained not only from the embryo², but also from extra-embryonic tissues like the placenta³ and the umbilical cord⁴. “Adult” stem cells can also be obtained, from tissues including blood⁵, bone marrow⁶, and skin⁷. The efficient derivation of such stem cells into different cell lineages sustains hopes for possible interventive therapies for many diseases. For reproductive science in particular, a successful method for the production of germ cells from stem cells could translate into a dramatic improvement in the treatment of infertility. And for medicine on a larger scale, therapies are now progressively focusing on approaches adapted to the specific needs of the individual patient (“personalized medicine”). Individual genetic variation is gaining importance in assessing predisposition, diagnosis, and the prospects for successful therapy. From this perspective, the ability—through somatic cell nuclear transfer

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(SCNT) or production of induced pluripotent stem (iPS) cells—of creating stem cells genetically identical to a patient is especially promising. This review aims to summarize the current state of the art for the generation of germ cells from embryonic stem cells (ESCs) and the use of oocytes in SCNT technology.

Germ Cell Formation

Many molecular factors regulating germ cell formation have been identified, but knowledge of their action is still fragmentary. Primordial germ cells (PGCs) develop from a small population of cells that are specifically set aside in the extra-embryonic compartment very early during embryogenesis^{8,9}.

The first evidence of the presence of PGCs is indeed outside the embryo, in the endoderm of the dorsal wall of the yolk sac at the base of the developing allantois^{10,11}. In humans the first population of PGCs forms during the third week of development¹². The extra-embryonic localization of PGCs might be crucial for their specification, preserving the integrity of the germ cell lineage while the surrounding somatic cell program unfolds. During the 4th week of human development, PGCs start migrating into the developing embryo^{13,14} and reach the gonad about the sixth week¹⁴. This migration process, as well as the initial phase of colonization of the gonads, is accompanied by extensive proliferation of PGCs^{15,16,17}. Different from lower organisms like *Drosophila* and *C. elegans*, PGCs in mammals are indeed transcriptionally very active during specification and migration. The molecular program underlying these processes in mice models has been extensively investigated, and yet the mechanisms of PGCs formation, commitment, migration, and gonad colonization are still at a descriptive level. Specification of the first cluster of PGCs seems to be dependent on signalling by the bone morphogenetic protein (BMP) pathway from the extra-embryonic ectoderm¹⁸. In response to this event, a cohort of cells activates the expression of the *Alk2* receptor and *Smad1/5* signaling and commits to develop into PGCs¹⁹. Shortly thereafter, the expression of *Blimp1* seems to be critical to maintain PGC fate by inhibiting differentiation into the mesodermal cell lineage²⁰. Once specification has occurred, other factors are necessary to maintain germ cell competence, survival, and proliferation. Pluripotency-related genes *Oct4*, *Sox2*, and *Nanog*, for instance, play an important role in this process^{21,22}.

As for the migration of PGCs into the gonad, it seems to be strictly dependent on the interaction of PGCs with the surrounding somatic environment. *c-Kit* signalling between PGCs and hindgut cells is indispensable for survival, migration, and proliferation²³. In addition, PGCs express the *Cxcr4* receptor, which binds to the chemokine ligand *Sdf1*, expressed along the migratory route to the gonad²⁴. β 1/2 *integrins* also have an important role, interacting with the extracellular matrix²⁵, though the motive force and presumptive pattern of “make-and-break” interactions of migrating cells remains conjectural.

Finally, upon entry into the gonad, PGCs begin to express *Dazl*, *Vasa*, and *Gcna26* and interact with gonadal somatic cells that will later determine their commitment to a sex-specific fate. In females, PGCs differentiate into oogonia and begin to arrest in prophase I of meiosis¹¹. During this process, retinoic acid (RA) induces the initiation of meiosis by upregulating the expression of *Stra8*²⁷. In males, by contrast, the expression of *Cyp26b1* causes the degradation of RA, preventing PGCs from entering meiosis²⁷; and in the male gonads PGCs arrest in the G0/G1 stage of cell cycle and mature into prespermatogonia²⁸.

Derivation of PGCs from ESCs

Although the gene expression profile of PGCs and somatic cells is distinct, many genes expressed by PGCs are also expressed by mouse and human embryonic stem cells (mESCs

and hESCs respectively)^{29,30}. They include the pluripotency gene OCT431, as well as NANOG, STELLAR and GDF3, whose level in hESCs decreases with differentiation³⁰. In addition, DAZL, NANOS, PUM2 and cKit expression is shared by these two cell types³². A comparable gene program shared between PGCs and ESCs can also be found in the mouse, however it is important noting that hESCs display some differences from mESCs (see below). The resemblance of gene expression profiles in PGCs and ESCs makes it difficult to identify and isolate PGCs after ESC differentiation *in vitro*. ESCs can be maintained in culture indefinitely, though they can be induced to differentiate by withdrawal from the medium of leukaemia inhibitory factor (LIF), a chemokine associated with the maintenance of self-renewal. Two strategies can be used for the differentiation of ESCs: 1) adherent monolayer cultures, or 2) embryoid bodies, which are three-dimensional structures in suspension³³ (Fig. 1). In both cultures, ESCs can differentiate into cells of all three primitive germ layers, endoderm, mesoderm, and ectoderm. In order to direct the differentiation towards a particular cell type, specific factors such as cytokines or morphogens are usually added to the culture medium. Several methods have been described for the derivation of PGCs from ESCs, but the exact composition of such media is often unidentified (see below).

Once PGCs have been obtained in culture, it is crucial to be able to identify and isolate them from ESCs in order to successfully produce germ cells of more differentiated stages. Usually these steps are improved by using culture enrichment methods, through the addition of factors that drive the proliferation of PGCs to improve their isolation from the rest of ESCs. Derivation techniques still lack reproducibility, consistent with the sparse information about the molecular mechanisms underlying germline formation. Reports describing the derivation of germ cells from ESCs are few, and fewer used human ESCs.

Derivation of oocytes from mouse ESCs

Hubner *et al.*³⁴ used a monolayer culture system in which ESCs expressed a GFP construct under the control of the germ cell-specific *Oct4* promoter. The expression of GFP, along with that of stage-specific markers *c-kit* and *Vasa*, was used to identify premigratory and migratory PGCs (GFP+/c-kit+), and postmigratory PGCs (GFP+/c-kit-/Vasa+). The authors also observed GFP-/c-kit-/Vasa+ cells that detached from the monolayer and formed aggregates in the supernatant, representing meiotic germ cells. Nuclear SCP3 was detected in these putative oocyte-like cells, but chromosomal alignment was not reported. Interestingly, further differentiation of these aggregates led to the formation of follicle-like structures which released oocyte-like cells capable of undergoing parthenogenetic activation. Using the same protocol, Novak *et al.*³⁵ described a similar formation of follicle-like structures accompanied by the release of estrogen in the supernatant. Once again, SCP3 was detected, but the alignment of chromosome was abnormal, showing only short filaments. Furthermore, the expression of SCP1, SCP2, and other meiotic markers was not detected, so that the extent and fidelity of the process was not completely clear.

Another method employed a GFP construct, in this case driven by the promoter of *Gdf9*³⁶. The authors described the formation of GFP+ oocyte-like cells after culture on feeder cells or in suspension without LIF. Although withdrawal of LIF is commonly used to induce the differentiation of ESCs, surprisingly, the addition of LIF to the medium increased the number of GFP+ cells serving as a specification factor. The oocyte-like cells also expressed *Gdf9* and *Zp3*, but degenerated quickly, probably due to their inability to enter or progress through meiosis.

Lacham-Kaplan *et al.*³⁷ reported the formation of follicle-like structures from EB cultures, using testicular conditioned medium as a specification method. The follicular structures formed at the borders of the EBs and contained oocyte-like cells that expressed the germ cell

markers *Stra8*, *Figla* and *Zp3*. A different protocol used treatment with RA to enrich PGCs from ESCs, followed by co-culture with ovarian granulosa cells and RA conditioned medium to promote oocyte maturation³⁸. EB cultured with granulosa cells expressed late stage germ cell markers like *Figla*, *Gdf9*, *Zp1-3*, *Vasa* and *Scp3*. However, no formation of follicular structures was detected.

In conclusion, the production of oocyte-like cells and even oocytes from ESCs seems possible. However the above reports did not assess the meiotic capability, functionality, or competence in fertilization of the germ cell-like cells produced.

Derivation of sperm from mouse ESCs

For the production of oocytes, several approaches have been utilized to generate sperm cells from ESCs. For example, an intriguing study by Kerkis *et al.*³⁹ reported the formation of both sperm-like and oocyte-like cells from XY ESCs. ESCs were differentiated to EB using RA to induce enrichment in PGCs. Cells in the EB were positive for several germ cell markers (*Oct4*, *Dazl*, *Ssea1*, *Stra8*, *Vasa*, *Scp1-3*, and *Zp3*). These genes are also expressed by ESCs, but during culture the expression of the later stage germ cell markers *Gdf9* and *Acrosin* increased, consistent with progressive differentiation. Notably, cells from the surface of the EB showed spermatid-like morphology, with the formation of acrosome, midpiece and axoneme. In addition, from the centre of the EB oocyte-like cells developed, and the formation of blastocyst-like structures was described. Speculation of possible fertilization between the two cell types is exciting, but parthenogenetic activation cannot be ruled out.

Transgenic ESCs containing the Lac-Z gene driven by the *Vasa* promoter were used in EB cultures to generate germ cells⁴⁰. EB were co-cultured with BMP-producing cells and the formation of putative *Vasa*+ cells was reported. To induce maturation into male gametes, these cells were isolated by cell sorting, co-cultured *in vitro* with fetal gonadal cells, and finally transplanted into the testis capsule of recipient mice. Strikingly, these ESCs-derived PGCs were able to generate seminiferous tubules and sperm-like cells, but no evidence of functionality of these putative gametes was provided. Geijsen *et al.*⁴¹ used RA to enrich EB cultures for PGCs. The expression of SSEA1 was then monitored to isolate the putative PGCs. SSEA1 is expressed by migrating PGCs, but also by ESCs. To proceed further, the EB-derived SSEA1+ cells were cultured in the presence of RA to induce proliferation of PGCs. After treatment with RA, undifferentiated ESCs became SSEA1 negative, whereas PGCs expressed other germ cell markers such as *Oct4* and *Dazl* in addition to maintaining SSEA1 expression. Furthermore, DNA methylation analysis demonstrated imprint erasure at the H19 and *Igf2r* loci, consistent with the notion that EB-derived SSEA1+ cells were indeed PGCs. The authors were able to produce haploid male germ cells that were further tested for functionality by injection into oocytes. A significant number of injected oocytes progressed to the blastocyst stage, but live offspring were not obtained.

More recently, Nayernia *et al.*⁴² generated male gametes from ESCs in a monolayer culture system in the presence of RA. The ESCs were modified with two constructs: a *Stra8* promoter driving GFP, and a *Prm1* promoter driving red fluorescent protein (RFP). *Stra8*-GFP served as a marker for premeiotic germ cells in the initial differentiation of PGCs from ESCs, and *Prm1*-RFP was used as a marker for postmeiotic germ cells after further maturation, once again in the presence of RA. Motile male haploid germ cells were produced and were able to generate live offspring when injected into oocytes. However, the methylation pattern of imprinted loci in these gametes was abnormal and all the offspring died prematurely. Nonetheless, this is the first and most complete proof to date of functionality of germ cells derived by ESCs.

Derivation of germ cells from human ESCs

The formation of germ cells from human ESCs (hESCs) has proven more challenging than from mESCs. This may be due to the nature of hESCs, which may not be identical to the population of inner cell mass (ICM) from which they are derived. Consistent with this hypothesis, it has been found that hESCs have a gene expression profile different from ICM43. Undifferentiated hESCs do not express early germ cell markers like VASA, GCNA and SSEA1, but as in mouse, they express DAZL, NANOS, c-KIT and PUM232. hESCs seem rather more similar to mouse epiblast-derived stem cells, and might therefore have a developmental potential different from mESCs44 (Yu J, 2008).

Clark *et al.*³⁰ differentiated hESCs into EB, showing a progressive reduction of pluripotent markers like OCT4, STELLAR, GDF3 and NANOS concomitant with an increase in expression of the later stage germ cell markers VASA, GDF9 (oocyte) and TEK1 (sperm). The meiotic markers SCP1 and SPC3 were detected, but the chromosomal alignment was not reported. Other studies reported similar results, with the expression of VASA and SCP3 using BMP factors as a culture specification method⁴⁵, or the detection of VASA, c-KIT, and GDF9 along with the formation of follicle-like structures – which, however, degenerated after 10 days in culture⁴⁶. The use of a monolayer culture system along with feeder cells and BFGF as an enrichment method produced similar results, with the expression of later-stage germ cells (PIWIL2), spermatogonia (DAZ1-4, PUM2, NANOS1) and meiotic markers (MHL1 and SCP3)⁴⁷. However, once again the alignment of chromosomes was not reported.

In another monolayer culture system, SSEA1-positive cells were isolated from hESCs and expressed germ cell markers VASA, SCP1 and SCP3⁴⁸. Histone methylation analyses at the promoter of several germ and somatic cell markers was also performed, and imprint methylation erasure at one of two loci was shown in the isolated SSEA1-positive cells. Notably, these results conform with an initiation of the epigenetic reprogramming process of these cells. However, the report that a subpopulation of these putative PGCs may be haploid is unexpected, as they were derived from XX hESCs and oocytes do not complete meiosis II until fertilization.

Bucay *et al.*⁴⁹ recently proposed a different culture protocol, based on the reduction of hESC colony size and less frequent passages. The authors observed an increase in number of cells expressing the germ cell marker CXCR4. These cells were isolated and shown to express BLIMP1 and c-KIT, gene expressions consistent with early or migratory PGCs^{50,51}. After further differentiation, CXCR4-positive cells were found to express the later stage germ cells markers DAZL, VASA, and ACROSIN. Also of interest is the formation in culture of Sertoli-like cells, which seemed to cluster with the germ-like cells, though the nature and extent of interaction between the two cell types remains unexplored.

Somatic cell nuclear transfer

The generation of gametes from ESCs represents an eventual future route for the clinical management of infertility, but has even greater potential when coupled with somatic cell nuclear transfer (SCNT) for a wide variety of therapeutic applications. Therapeutic cloning is the derivation of stem cells from the product of SCNT, and is central to proposals for regenerative medicine. Candidate sources of stem cells include tissue stem cells, ESCs, and reprogrammed stem cells, which are generated by means of SCNT. Tissue stem cells are usually few in number, have a limited capacity to be maintained in an undifferentiated state *in vitro*, and tend to be multipotent rather than pluripotent. In contrast, ESCs are pluripotent and easily maintained in culture, but are often rejected by the immunological response to allogeneic grafts. Somatic cells can be reprogrammed to a pluripotent state when injected

into enucleated oocytes, giving rise to stem cells immunocompatible with the donor of the somatic cells.

Thus, these cells might represent the solution to the problem of rejection in clinical applications like diabetes, neurodegenerative conditions and blood disorders. This property accounts for much of the special interest in SCNT. SCNT relies on the ability of the enucleated oocyte environment to reprogram a somatic cell nucleus to become totipotent. Insertion (through fusion or microinjection) of an isolated somatic nucleus into an enucleated oocyte is sufficient to drive the initiation of the embryonic developmental program⁵²⁻⁵³. Oocytes generated by ESCs could produce stem cells that are genetically identical to the patient while avoiding the use of human oocytes harvested from ovaries, and therefore can be used in tissue repair and transplantation medicine⁵⁴ (Fig. 2). ESCs produced by SCNT have the same potential as ESCs derived from normally fertilized embryos⁵⁵. Indeed, it has been shown that SCNT-derived ESCs were capable of differentiating into all three germ layers *in vitro* and into gametes in chimeric mice⁵⁵. However, when further compared to those from fertilized embryos, ESCs produced by SCNT revealed some deviations in DNA methylation and gene expression profiles. These differences were described as far less than those found in previous reports, and the authors reaffirmed the potential use of these cells in therapy^{56,57}. SCNT has been used in many animal models^{54,58,59}, but reproductive cloning in primates has proven elusive^{60,61}, raising the question whether therapeutic cloning in human could be feasible.

Recently Byrne *et al.*⁶² used a modified approach for spindle detection and removal, and were able for the first time to demonstrate the successful reprogramming of somatic cells from rhesus macaque into pluripotent ES cells. The efficiency of the methodology was low and the epigenetic status of the ES cells was not reported. But this generation of ESCs from primates by means of SCNT encourages further considerations. Recently, an intriguing study showed a dramatic improvement in the efficiency of ESC line derivation following SCNT⁶³. Instead of using enucleated oocytes, the authors injected somatic cells into intact MII oocytes and confirmed the reprogramming capability of these cells. The reported efficiency of ESCs generation per injected oocyte was 30%, and the pluripotent ESCs were shown to have undergone epigenetic reprogramming at the Oct4 promoter. The established ESC lines were, however, tetraploid, a condition obviously not compatible with clinical use. Nevertheless, this methodology represents an interesting therapeutic strategy if the maternal chromosomes can be efficiently targeted for elimination. Matsumura *et al.* proposed a possible chromosome elimination system⁶⁴ that might be adapted, based on the use of a chromosome elimination cassette (CEC) bearing a fluorescent reporter and a drug-resistance gene between oppositely oriented loxP sites. Cre-mediated sister-chromatid recombination would generate dicentric and nullicentric chromosomes, which are spontaneously deleted during cell division. Cells are then sorted for the presence of the fluorescent cassette, permitting the enrichment of chromosome-eliminated cells.

At present, SCNT remains inefficient, and there is unfortunately not yet a basis for understanding its limitations. Possibly the presence of the chromosome-spindle complex or other unidentified nuclear factors in the intact oocyte may enhance reprogramming or may assist normal cell division in the developing embryo. But among many less physiological factors, mere trauma to the oocyte during the enucleation procedure may impair its subsequent functionality.

Whatever the cause, it is notable that normal fertilized embryos and SCNT-derived embryos are epigenetically different, and have differential expression profiles for several pluripotency and developmental genes⁶⁵. Those differences could themselves be a proximal cause of low efficiency of derivation from ESCs and SCNT. In this regard, it was recently pointed out

that non-random events like the ectopic expression of *Xist*, a noncoding RNA that inactivates one of the two X chromosomes in females, and the persistence of specific histone modifications, might be a cause for SCNT inefficiency⁶⁶.

Clearly, in order to improve efficiency, both the changes in the oocyte nucleus and the corresponding reprogramming mechanisms in the reconstructed embryo must be analyzed much more.

Conclusions

In recent years, technological advancements, and manipulation of ESCs in particular, have allowed the planning of new strategies to understand fundamental aspects in cell lineage specification and cell reprogramming, with further relevance to clinical intervention. The generation of germ cells from ESCs could remedy many cases of infertility as well as open up the possibilities for personalized regenerative medicine.

However, efficiency in germ cell derivation from ESCs is still low, and the functionality of such gametes has only been achieved in one case thus far⁴². The mechanisms of derivation are poorly understood, and the establishment of the epigenetic signature is obscure. Especially in humans, only the generation of PGCs has been described, with many issues remaining to be solved.

Oocytes produced by ESCs could provide a continuous supply for SCNT, but naturally clinical application of SCNT-derived ESCs in human would require great caution. The same SCNT technology has been largely and successfully employed in cloning animals (mice^{53,67}, sheep⁵², pigs⁶⁸, goats⁶⁹, and cows⁷⁰). However, even in those cases, the efficiency of this technique is still low due to embryonic lethality, malformations, and diseases developed by the offspring. Cloned embryos often exhibit abnormalities like inappropriate X chromosome inactivation in bovine clones⁷¹, shorter telomeres⁷², excessive growth⁷³, obesity⁷⁴, and immunodeficiency⁷⁵. Inactivation of the X chromosome was shown to occur properly in SCNT-derived cloned mice in a different study⁷⁶, but it remains to be seen how complete and reproducible these dynamics are. Epigenetic abnormalities and alterations in the expression of several imprinted genes were reported in mice produced by SCNT⁷⁷ even when offspring survived to adulthood. One possibility is that somatic cells – or at least some lineages of somatic cells – are incapable of reactivating fundamental embryonic genes such as the pluripotent marker Oct4 during preimplantation and postimplantation development, resulting in the failure to form embryonic lineages⁷⁸. However, because therapeutic cloning does not require full-term embryonic development, it may be possible to bypass this block. But whatever the course of the field, it is vital to understand how pluripotency is maintained, lost, and then restored during cell lineage specification and development; and correspondingly, how epigenetic reprogramming by SCNT occurs. Thus ambitious clinical perspectives remain tightly linked to the progress of fundamental research in gonadal differentiation and early embryonic development.

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Figure 1.

In vitro derivation of germ cells from ESCs. PGCs are produced using specific specification and enhancement methods, starting either from adherent monolayers (top left) or from embryoid bodies (bottom left). PGCs are then further cultured to produce mature germ cells (sperm and oocytes).

Figure 2.

Strategy for therapeutic cloning from ESC-derived oocytes. Oocytes generated from ESCs are enucleated and subsequently injected with a somatic nucleus from a patient donor. SCNT-ESCs are then isolated from the derived blastocyst and differentiated in vitro to produce immunocompatible lineage-specific cells that can be used in therapy for the same somatic cell nucleus donor.