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INFERTILITY MANAGEMENT

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INFERTILITY MANAGEMENT SERIES

BASIC LABORATORY PROCEDURE IN ART

Foreword
Nicolás Prados Dodd

JAYPEE

Contents

1. **Semen Analysis and Sperm Preparation** 1
   Hetal Shukla, Deven Patel, Preeti Shah
2. **Cryopreservation of Semen Samples** 26
   Pallavi Menon, Carol Rubina D'Souza
3. **New Techniques for Sperm Selection in the Andrology Laboratory** 47
   Cristina González-Ravina, Alberto Pacheco
4. **From the Gamete to the Zygote** 62
   Alberto Tejera, Belén Aparicio, Maria J de los Santos
5. **Embryo Culture** 77
   Deven Patel, Preeti Shah, Aditi Kotdawala
6. **Embryo Selection and Transfer** 96
   Anisha Uberoi, Himani Agnihotri
7. **Oocyte and Embryo Vitrification** 122
   Ana Cobo, Aila Coello
8. **Embryo Biopsy and Tubing** 138
   Rishina Bansal, Shridutt Gaitonde, Navin Desai
9. **Time-lapse Imaging: A New Technology for Embryo Development** 156
   Carmela Albert, Pilar Gámiz, Natalia Basile, Marcos Meseguer
10. **Design, Equipment and Personnel Management** 180
    Irene Rubio Palacios, Javier Herrero Zapata
11. **Future Perspectives: Developing Gametes from Stem Cells** 199
    José V Medrano, Carlos Simón

Chapter 11

Future Perspectives: Developing Gametes from Stem Cells

José V Medrano, Carlos Simón

INTRODUCTION

Regenerative medicine is an emerging multidisciplinary field that comprises knowledge from cellular biology, developmental biology, genetics, molecular biology and tissue engineering, aiming to the study of stem cells and their potential clinical application to regenerate tissues and organs.

Among the multiple translational applications of regenerative medicine, the study of stem cells for their use in reproductive medicine has achieved significant progress in the last years. In particular, the use of animal models for the study of germ cell development, together with a significant improvement of current knowledge of human germ cell biology, recently achieved thanks to the access to early human embryos for research purposes, has allowed an important step forward in the possibility of obtaining human functional gametes from different cell sources that convert this promise in a reality that is closer than ever (Flow chart 11.1).

Here, we will discuss the different approaches and applications that regenerative medicine is currently developing in order to offer a solution to infertile patients aiming to have genetically matched children.

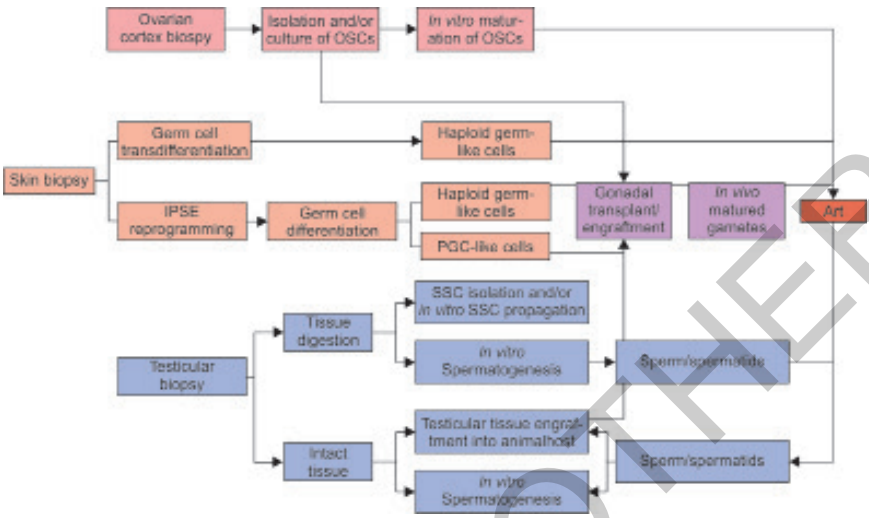
PLURIPOTENT STEM CELLS (ESCs AND IPSCs) AS A SOURCE OF HUMAN FUNCTIONAL GAMETES

The potential use of mouse embryonic stem cells (mESCs) as a source of gametes was first reported in 2003 when some oocyte-like structures able to achieve parthenogenetic activation and form pseudoblastocysts were spontaneously formed upon culture of mESCs without feeders nor leukemia inhibitory factor (LIF).[1] Upon this first probe of concept, a variety of strategies have been reported to achieve the formation of both male and female gametes from mESCs and induced pluripotent stem cells (iPSCs) with disparaging results.[2-7]

However, the real functionality of these *in vitro* generated gametes was not demonstrated until very recently when a Japanese group achieved for the first time the generation of viable offspring from sperm and oocytes

JAYPEE BROTHERS

Flow chart 1: Schematic diagram of the circuitry of different approaches that regenerative medicine offers for the development of gametes.



(ART, Assisted reproductive technology; iPSC, Induced pluripotent stem cell; OSCs, ovarian stem cells; PGC, Primordial germ cell; SSC, Spermatogonial stem cell).

derived from mESCs and iPSCs.[8,9] In their experiments, authors based in the unique potential of epiblastic cells to give rise to primordial germ cells (PGCs) in response to bone morphogenetic proteins 4 (BMPs4) and BMP8b secreted from the adjacent extraembryonic ectoderm (ExE) at embryonic day 6.25 (E6.25) *in vivo*.[10,11] Based on this, they designed an *in vitro* induction protocol to induce naive mESCs and iPSCs cultured in 2i + LIF conditions into epiblast-like cells (EpiLCs) resembling their *in vivo* counterparts by treating them with activin A and basic fibroblast growth factor (bFGF). Subsequently, in a second induction step, EpiLCs were exposed to LIF, stem cell factor (SCF), epidermal growth factor (EGF) and BMP4 or 8b to trigger the acquisition of a PGC-like cell (PGCLC). Resulting PGCLCs showed a phenotype resembling *in vivo* mouse PGCs, including the expression of key PGC markers, but also signs of epigenetic reprogramming including genome-wide demethylation, loss of H3K9me2 and gain of H3K27me3, as their *in vivo* counterparts. Moreover, PGCLCs were able to differentiate either into sperm or germinal vesicle oocytes when transplanted into the mouse testicular lumen or under the ovarian bursa in reconstituted mouse ovaries that produced healthy and fertile offspring upon intracytoplasmic sperm injection (ICSI). These works were complemented by a third report of the same group showing that the ectopic expression of the key transcription factors downstream of BMP signaling Prdm1, Prdm14 and Tfap2c over preinduced mouse EpiLCs resulted also in a PGCLC phenotype that formed sperm and gave rise to healthy offspring after transplantation into mouse testes.[12] In

JAYPEE BROTHERS

summary, these three complementary reports demonstrated for the first time the possibility to create functional gametes from pluripotent cells. At the same time, they described the regulatory signaling network of mouse PGC specification, where Prdm1, Prdm14 and AP2γ together constitute a transcriptional network necessary to enable the repression of somatic genes by Prdm1 and Prdm14 blocking of Hox enhancers, the expression of pluripotency and PGC-specific genes (executed mainly by Prdm14 and AP2γ) and the induction of epigenetic reprogramming by Prdm14 and Prdm1 repression of the de novo deoxyribonucleic acid (DNA) methyltransferase Dnmt3b and its cofactor Uhrf1; at the same time Prdm14 recruits 10–11 topoisomerases TET1 and TET2 to activate active hydroxylation of 5-methylcytosines and induce methylation marks erasure.

Following this approach, an interesting report has recently demonstrated the BMP independent function of Nanog as a master regulator of PGCLC specification from EpiLCs *in vitro*.[13] In this study, ectopic expression of Nanog over EpiLCs resulted in the activation of Prdm1 and Prdm14 as well as the acquisition of a PGCLC phenotype *in vitro*. Thus, this new study highlighted the plasticity of PGC specification with the context-dependent role of a pluripotency-related transcription factor such as Nanog, acting as a member of the core pluripotency network in mESCs and iPSCs, but inducing PGCLC formation in EpiLCs.

Although there exist several reports on differentiation of human pluripotent stem cells into germ cells using different approaches,[14-21] the success of the reports described earlier in the mouse model inspired another recent works that adopted a very similar approach on human ESCs (hESCs) and iPSCs, demonstrating its reliability.[22-24] With slight differences in the cocktails employed to induce a mesodermic epiblast state from hESCs and human iPSCs (hiPSCs), in all three studies authors achieved the formation of a human PGCLC (hPGCLC) phenotype upon exposure of preinduced cells to BMPs, LIF, SCF and EGF. With similar conclusions, these works highlighted the mesodermic origin of hPGCLCs based on the need of creating a mesodermic epiblast phenotype expressing T (Brachyury) for their efficient derivation. Also, resulting human PGCLCs from all three studies displayed several differences compared to the ones obtained in mouse, such as null activation of SOX2, a delayed expression of PRDM14 and an upregulation of SOX17 and GATA4. Results from one of the studies also concluded that in contrast with mouse PGC specification, SOX17 might play an essential role in human germ line specification by acting upstream and synergistically with BLIMP1 or PRDM1 as the master regulators of PGC specification, at the same time that BLIMP or PRDM1 acts as a repressor of the somatic program in human PGCLCs.[22] However, compared with mouse studies were PGCLCs were matured into functional gametes by transplanting them into mouse gonadal hosts, further maturation of human PGCLCs obtained *in vitro* was not investigated.

JAYPEE BROTHERS

Related to the need of an animal host to achieve the complete maturation of human PGCLCs, it has recently been reported a new mouse study that based in the same approach has overcome this need and achieved the formation of functional gametes totally *in vitro*.[25] In this report, authors also followed the two-step induction of PGCLCs from EpiLCs. However, instead of transplanting them into a mouse gonad recipient, they used testicular somatic cells from KITW or KITW-V mice lacking germ cells to coculture with PGCLCs. After 2 weeks of PGCLC or testicular cells coculture with follicle-stimulating hormone (FSH), testosterone and bovine pituitary extract, around 14% of PGCLCs matured into spermatid-like haploid cells that were able to generate healthy offspring upon ICSI. This new achievement has supposed a new landmark since avoids totally the need of an animal host to generate gametes from pluripotent cells, offering an exceptional tool for the study of germ cell development and paves the way to its translation to humans.

Compared to the current knowledge of murine germ cell development, data regarding this process in humans has been scarce and based in classical histological studies.[26] However, the use of *in vitro* models of human germ cell derivation from pluripotent cells, together with very recent reports focused on the study of the germ cells development in early human embryos from a molecular point of view have supposed a new landmark.[27-29] Thanks to these last reports, now we have a better knowledge of the transcriptomic and epigenetic dynamics of human germ cells and indeed, the recent discovery of some evolutionary young transposable elements such as SINE-variable number of tandem repeats-Alu elements (SVAs) to resist epigenetic reprogramming of germ cell development *in vivo* opens new lines of study of the possibility of epigenetic inheritance with hPGCLCs obtained *in vitro*.[27]

THE MILLION-DOLLAR QUESTION: ARE THERE STEM CELLS IN THE HUMAN OVARY?

It is generally accepted that follicular regeneration occurs in most non-vertebrate animals[30] and in some vertebrates.[31] However, one of the central dogmas in reproductive medicine is that female mammals are born with a finite number of germ cells (follicular reserve) that decreases along individual's lifespan until become completely depleted at the start of menopause.[32] However, this view was questioned few years ago when John Tilly's group at Harvard University postulated the existence of ovarian stem cells in postnatal mammalian ovaries.[33] After counting the amount of atretic follicles in mouse ovaries, Tilly's group hypothesized that the ovarian reserve should become depleted at a much faster rate than what actually occurs, suggesting the presence of some kind of germinal stem cells within mammalian ovaries that replace atretic follicles by neofolliculogenesis. In order to test

JAYPEE BROTHERS

this hypothesis, ovarian fragments from wild-type mice were transplanted into the ovarian bursal cavity of transgenic green fluorescent protein (GFP) mice, resulting in the appearance of newly formed chimeric follicles with GFP positive oocytes surrounded by non-GFP expressing granulosa cells.[33] This first report on neofolliculogenesis caused great criticism since many argued against the methods employed to calculate follicle atresia and the ovarian graft results.[34,35]

In response to these criticisms, only 1 year later, based on the observation that stage-specific embryonic antigen-1 (SSEA-1) positive cells isolated from either disassociated mouse ovaries or bone marrow shared the expression of early germ line markers, Tilly's group suggested the bone marrow as the putative source of germ stem cells for adult neofolliculogenesis in mammals.[36] Supporting this idea, transplantations of both bone marrow and peripheral blood from GFP transgenic mice into chemotherapy-ablated wild-type females resulted in the formation of new chimeric follicles with GFP-positive oocytes.

In response to the intense discussion derived to these reports,[37] a new study comprising parabiosis experiments in which the circulatory systems of wild-type and GFP transgenic female mice were joined for up to 6 months demonstrated the presence of some GFP-positive granulosa cells associated with ovulated oocytes in wild-type mice, but no evidence of GFP-positive oocytes derived from GFP donors in wild-type females. In contrast to Tilly's results, transplantation of bone marrow from a GFP donor into wild-type females treated with cyclophosphamide or busulfan also failed to demonstrate the presence of oocyte chimerism, suggesting that the recovery of ovulation in chemically sterilized mice could be explained by incomplete depletion of endogenous follicles by the alquilant drugs, whereas the presence of some GFP-positive cells within the granulosa cell layer could be a consequence of the infiltration of white cells from the GFP donor.

After the publication of several reports supporting[38-42] or arguing against[43-45] the neofolliculogenesis hypothesis in mammalian ovaries, a new report in 2009[46] established germ stem cell lines *in vitro* from mouse vasa homolog (Mvh)-positive cells isolated from mouse ovaries. *In vitro* cultured Mvh cells expressed pluripotency-associated markers as well as early germ cell markers. Since Mvh is a ribonucleic acid (RNA) helicase that localizes in the cytoplasm of all germ cells, the use of an antibody for Mvh to isolate germ cells based on its surface membrane localization generated scepticism about these results.

More recently, Tilly's group published a new report in 2012 describing a similar approach to isolate Mvh or VASA germ cells from both mouse and human ovaries and establish germ cell line cultures *in vitro*.[47] Additionally, this report also demonstrated the spontaneous formation of oocyte-like cells (OLCs) able to complete meiosis in ovarian germ cell cultures. As a

JAYPEE BROTHERS

functional assay, *in vitro* cultures were labeled with GFP and either transplanted into mouse ovaries or cocultured with disassociated adult human cortical tissues and transplanted into immunosuppressed mice, resulting in the formation of GFP-positive oocytes that were able to form healthy embryos upon ICSI fertilization in the case of mouse oocytes. However, another recent report from a consortium of independent groups replicating the same experimental approach did not support the presence of VASA positive cells in the human ovary.[48]

With the exciting controversy upon the existence of neofolliculogenesis in the mammalian ovaries, some authors proposed an ovarian cell dedifferentiation process that, under specific *in vitro* conditions or stress may produce cells with certain potential to develop into germ-like cells in the same way as previously described for other cell types,[49-52] as one possible explanation for the existence of germinal stem cells in mammalian ovaries. Related to this hypothesis, some authors have even postulated a possible relationship between the existence of stem cells in the ovarian surface epithelium (OSE) and the origin of ovarian cancer of epithelial cells.[53] Alternatively, others also propose the presence of very small embryonic-like (VSEL) stem cells resident in the ovaries which have the potential to differentiate into both germ and somatic cells.[54]

Besides the great interests on demonstrating the existence of germ stem cells in the mammalian ovary that may lead to neofolliculogenesis and thus the design of strategies to restore the fertility of infertile women without follicular reserve, as can be the case of patients suffering premature follicular reserve depletion due to cancer treatments, their existence is not fully supported and further research is needed in order to solve this scientific debate. Nevertheless, with independence of the existence of stem cells in the ovary, other methods for fertility preservation such as oocyte or embryo vitrification currently offered to this kind of patients is a worldwide employed technique with excellent results. Alternatively, in prepubertal patients or patients without possibility of obtaining oocytes before the initiation of their oncological treatment, ovarian cortex cryopreservation for its reimplantation after the disease has been overpassed is an experimental technique that has already probed its efficiency to restore fertility of oncological patients.[55]

SPERMATOGONIAL STEM CELLS: TAKING ADVANTAGE OF PLASTICITY OF SPERMATOGENESIS FOR FERTILITY PRESERVATION

Spermatogonial stem cells (SSCs) are the resident stem cells in the mammalian testes and the origin of the process of spermatogenesis during adulthood. The SSC pool is comprised by a subset of spermatogonia attached to

JAYPEE BROTHERS

the basal layer of the seminiferous tubules.[56] SSCs were first described in 1994 by Brinster's laboratory using a murine germ cell transplant model, and were defined as "the spermatogonial subpopulation of spermatogenic stem cells with the ability to colonize the seminiferous epithelium and fully reconstitute spermatogenesis".[57,58]

Due to their ability to potentially restore full spermatogenesis, there is great interest in the study and use of SSCs as a tool for fertility restoration. Specifically, patients unable to generate sperm such as prepubertal boys in risk of acquired permanent azoospermia due to the gonadotoxic effect of oncologic treatments can be potential beneficiaries from biotechnology derived of the study of SSCs.[59] Indeed, having in mind the use of SSCs to restore spermatogenesis, based in the Oncofertility Consortium of Northwestern University database, there are currently more than 120 health centers around the world offering the cryopreservation of testicular biopsies to prepubertal oncological patients in risk of permanent azoospermia as a consequence of their oncologic treatment.[60-65]

Among the strategies designed to restore spermatogenesis in azoospermic patients, the SSC transplantation (SSCT) is probably the most interesting.[66,67] Described for the first time by Brinster's laboratory in mice, transplantation of testicular cell suspensions containing spermatogonial cells into the germ cell depleted seminiferous lumen of mice results in its colonization and fully restoration of spermatogenesis, producing sperm able to give rise to healthy offspring.[57,58] Moreover, this technique has been applied to other animal models including nonhuman primates with successful results.[68] However, despite these encouraging results, there are three main issues to solve before the use of SSCT in humans. First, since transplantation of human SSCs (hSSCs) into germ cell depleted mouse testes results in colonization of the seminiferous epithelium, but fails to restore spermatogenesis because of phylogenetic discrepancies,[69] there is a need for appropriate models for spermatogenic restoration in humans beyond the technical trials performed in human cadaveric testes.[70] Second, the lack of appropriate markers to isolate SSCs has hampered their study, leaving their ability to colonize mouse testes is the best approach to identify them. By using this approach as a read out, several surface markers such as GFRA1,[71] CD9,[72] fibroblast growth factor receptor 3 (FGFR3),[73,74] epithelial cell adhesion molecule (EpCAM),[75] integrin alpha 6 (ITGA6)[75-77] and THY-1,[76] as well as intracellular markers such as PLZF, Sal-like protein 4 (SALL4), UTF1 and UCHL1[76] have been identified as putative hSSCs markers. The isolation of pure SSC populations is not only necessary for their study, but also as a technique to prevent the transplantation of malignant cells back into the testes of cancer survivors.[75] Finally, due to the limited size of testicular biopsies, *in vitro* propagation to increase the number of SSCs to transplant is a necessary step prior to perform SSCT in humans. However, although long-term

JAYPEE BROTHERS

in vitro propagation of mouse SSCs was first reported in 2003,[77] *in vitro* propagation of hSSCs[78,79] has been difficult to replicate.[80-83]

An alternative promising strategy to restore spermatogenesis is the testicular tissue grafting.[84] This approach consists of the engraftment of small pieces of immature (prepubertal) testicular tissue under the skin of immunosuppressed mice previously castrated in order to prevent the feedback inhibition of gonadotropins by their own testes. In this way, the host animals serve as bioreactors that activate spermatogenesis of engrafted tissue after few weeks, producing sperm that can be retrieved for ICSI and production of healthy offspring. As happened with the SSCT, this technique has also demonstrated its feasibility in other donor species, using always mice as hosts,[85,86] as well as slight improvement of results by modifying the engraftment in the scrotum[60] or even in intratesticular orthotopic grafts.[87] However, xenografts from other species with higher than average daily sperm production such as pig, goat and human donors show limited survival of the engrafted tissues and fibrosis probably due to insufficient vascularization causing ischemia.[60,61,85,88,89] Thus although data suggests that grafts from immature tissue show better resistance to ischemia due to the quiescent nature of tissue, so far human grafts designed to preserve spermatogenesis have not been able to develop beyond the spermatocyte stage,[61] highlighting once again that probably the mice are not the most appropriate hosts for human testicular grafts. Thus, despite its success in other animal models, this approach still needs further research to prevent ischemia of engrafted tissue as well as improvements to the technique to support full maturation of sperm cells before an eventual possible clinical application to produce functional human sperm in azoospermic patients.

In vitro spermatogenesis is a third approach that has gained interest in the last years as a possible source of sperm from SSCs. Despite the achievement of a recent report describing the production of haploid human spermatids able to fertilize mouse oocytes from SSCs obtained from cryptorchid patients and treated with SCF and retinoic acid (RA),[90] due to the three-dimensional (3D) structure of the spermatogenesis with several maturation stages of germ cells tightly dependent of sertoli cells regulation, most efficient strategies to achieve the production of sperm from mouse SSCs comprise 3D cocultures of both somatic and immature germ cells.[91] In a similar way to these 3D models of *in vitro* spermatogenesis, in 2011 a Japanese group obtained for the first time sperm able to be used in ICSI in a model of mouse 3D testis tissue culture.[92,93] In their work, authors used immature testicular pieces from newborn mice carrying GFP gene expression under the control of the postmeiotic markers Gsg2 (haspin) and acrosin, and were placed them in a liquid-gas interphase onto agarose rafts soaked in a medium containing knockout serum replacement and were placed at 34°C to mimic *in vivo* testicular conditions. After around 3 weeks, GFP spermatids and sperm

JAYPEE BROTHERS

were retrieved and used for ICSI, giving rise to healthy offspring. Although this technique has been reproduced in further reports,[94,95] no data regarding the feasibility of this technique with human tissue has been reported so far. However, the use of cytocompatible scaffolds from decellularized human testicular tissue may be an interesting approach to test the potential of hSSCs to mature *in vitro*.[96]

With an experience of more than 15 years of cryopreserving immature testicular biopsies from prepubertal patients, it seems a matter of time that the first reports on fertility restoration in azoospermic men appear.[97] However, even the success of different techniques of spermatogenesis restoration in animal models, as described earlier, there are still important issues to solve before the application of SSCs as a clinical cell therapy in the next years, making this possibility still an experimental procedure that should be offered only to patients with a high-risk of acquired azoospermia as the unique via to preserve their fertility.

THE ANSWER OF REGENERATIVE MEDICINE TO REPRODUCTIVE NEEDS: THE PROMISE MAY NOT BE SO FAR TO BE ACCOMPLISHED

Apart from the important advances of regenerative medicine about the potential use of stem cells as a source of functional gametes for human reproduction, their study itself has provided key insights for the better understanding of gamete-related diseases, as well as the design of new strategies to help patients affected by them.

Based in the expression of germ cell markers such as DAZL in hESCs, suggesting that this pluripotent stem cell stage could be formed by a heterogeneous population in which some cells may be primed toward a germ cell fate,[14] further investigation on the role of evolutionary conserved germ cell RNA-binding proteins such as the DAZL family[17,18] or VASA[21] suggested their role in the initiation of meiosis of *in vitro* differentiated germ cells from hESCs upon their exogenous expression. In the same line, another report demonstrated that DAZL may function in limiting pluripotency as well as differentiation to control apoptosis in PGCs through a robust interactive network. If this network is broken or DAZL expression is lost, the consequence is teratoma formation.[98] Moreover, by the employment of xenotransplantation of *in vitro* derived human germ cell-like cells into seminiferous tubules of immunosuppressed mice, one study demonstrated that ectopic expression of VASA in hiPSCs improved their colonization efficiency, highlighting the role that VASA plays in controlling the pluripotency state in a combined *in vitro* or *in vivo* model.[99] In the same line, recent reports have also designed a potential cell therapy for azoospermic men with microdeletions of the Y chromosome (AZFα), demonstrating the decreased ability

JAYPEE BROTHERS

of iPSCs derived from this kind of patients to colonize the seminiferous tubules of mice, and how the complementation via ectopic expression of DDX3Y can be a potential genetic therapy to rescue their ability to colonize this niche and form germ cell-like cells *in vivo*.[100,101] This same approach has also been used recently to demonstrate that germ cell-like cells can be formed *in vivo* independently of X chromosomes genetic profile.[102] In this case, hiPSCs from infertile Turner syndrome patients (46, XO) were derived and xenotransplanted into mice testes, resulting in proper colonization and demonstrating that the presence of a second X chromosome is not required for human germ cell formation, although is necessary for their maintenance until adulthood.

Finally, a recent study performed by our group opened a new interesting strategy for the obtention of human gametes by direct reprogramming of somatic cells.[103] Based in the success of direct transdifferentiation of somatic cells into other somatic cell fates,[104-108] human male somatic cells were directly transdifferentiated into a meiotic germ cell-like phenotype by the ectopic expression of PRDM1, PRDM14, LIN28A, DAZL, VASA and SYCP3 *in vitro*. Induced germ cell-like cells were able to colonize the seminiferous tubules of germ cell depleted mouse testes, showed their ability to form haploid cells and evidences of epigenetic reprograming toward an oocyte-like expected epigenetic profile. Despite the low efficiency of the germ cell-like transdifferentiation, it can represent a step forward to the creation of human functional gametes and serve as an *in vitro* model to test the role of critical key factors in human germ cell development.

CONCLUSION

The study of germ cells is critical for our society not only because of their potential use in human reproduction but because they have the extremely important task of transmitting genetic and epigenetic information to the next generation. In summary, all the advances discussed earlier represent significant improvements to our current knowledge of germ cell biology. Those may have important implications in reproductive medicine from the point of view of the future clinical use of the *in vitro* obtained gametes, but also for the information that they provide about germ cell biology for the design of new strategies to offer to infertile patients. Ultimately, with the proper ethical precautions, the combination of the study of germ cells with the new molecular techniques of gene editing can even be the next scientific landmark for the eradication of genetic diseases, as demonstrated by some preliminary reports.[109-111]

Taking together, for the first time in recent history it seems that the promise of regenerative medicine as a tool for treating all kind of diseases, including reproductive diseases, may not be so far to be accomplished.

JAYPEE BROTHERS

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AUTHOR CONTRIBUTIONS

José V Medrano and Carlos Simón conceived this work and José V Medrano wrote the manuscript.

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JAYPEE BROTHERS

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Juan A García-Velasco MD PhD is Director of IVI Madrid. He is also Professor of Obstetrics and Gynecology at Rey Juan Carlos University, Madrid, Spain, where he is Director of their Master's Degree Program in Human Reproduction. Professor García-Velasco graduated from University Medical School, Madrid, in 1990 and received his obstetrics and gynecology certification from La Paz Hospital, Madrid, in 1995. He completed his PhD in Medicine at Autonoma University, Madrid, in 1995, and from 1997 to 1998 studied at Yale University, New Haven, Connecticut, USA, under a Reproductive Endocrinology and Infertility Fellowship. Professor García-Velasco's main research interests have been in IVF and endometriosis. He is the Principal Investigator of projects funded by the Ministry of Education and Ministry of Health in Spain, and has received awards from the Spanish Fertility Society, Spanish Society of Obstetrics and Gynecology, and the European Society of Human Reproduction and Embryology. He has published over 150 peer-reviewed articles and 22 book chapters on human reproduction, endometriosis and hypo- and hyper-ovarian stimulation response.



Manish Banker MD DGO is an infertility specialist and the Director of Nova IVI Fertility Clinics, India. He has been the President of the ISAR (2012–2014). He sits on the International Affairs Committee of ASRM and on the Board of the PSRM, is a Member of the Draft Committee for ART Bill of India, ICMR, Ministry of Health and is the Regional representative for ICMART. He has also been a Founder Board Member of ASPIRE and the Chairman of Infertility Committee of FOGSI (2008–2011). He is on the editorial board of Journal of Human Reproductive Sciences (JHRS), Clinical and Experimental Reproductive Medicine (CERM) and Global Reproductive Health.



Irene Rubio Palacios PhD is Co-Head of the IVF Unit of Nova IVI Fertility Clinics, India. She is also Clinical Embryologist of the General Medical Management Unit of Equipo IVI, Spain. Dr Rubio graduated in Biology at Autonoma University, Madrid, in 2007, pursuing both specialties: Evolutive Biology and Diversity and Molecular Biology. She completed her Master Degree in Biotechnology of Assisted Human Reproduction at Instituto Valenciano de Infertilidad, IVI-Valencia (Valencia, Spain) in 2009, where she started her clinical practice as embryologist. The primary areas of her research are morphokinetics and new technologies applied to embryo selection and culture. Dr Rubio's review on Fertility & Sterility and European Obstetrics and Gynecology, published in International Scientific Reviews, took part in reviews or book chapters and has more than 20 accepted abstracts at national and international congresses as ASEBIR, SEF, ESHRE, ASRM, ALPHA, and CFAS.



Javier Herrero Zapata PhD is Co-Head of the IVF Unit of Nova IVI Fertility Clinics, India. He is also Clinical Embryologist of the General Medical Management Unit of Equipo IVI, Spain. Dr Herrero received his Biological Sciences Degree from the Complutense University of Madrid, Spain, in 2007. He completed his Master Degree in Biotechnology of Human Assisted Reproduction from the Universidad de Valencia, Spain, in 2009 and joined the Instituto Valenciano de Infertilidad (IVI), where he developed his professional career as Embryologist. He has coordinated fellowship courses from the Teaching Area of the IVI group and is Professor of the postgraduate Master in Biotechnology of the Human Reproduction from Instituto Universitario IVI, Valencia University. The primary areas of his research are the application of new technologies to embryo selection and culture, genetic diagnosis and quality control and quality assurance in the IVF laboratories. He has published over 10 articles at international journals and several reviews or book chapters, has more than 30 accepted abstracts at national and international congresses, and presented more than 10 lectures at national and international congresses and symposia. In 2010, he received the research award from the Spanish Society of Embryology (ASEBIR).

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