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Stem cells survive oncotherapy & can regenerate non-functional gonads: A paradigm shift for oncofertility

Deepa Bhartiya

Stem Cell Biology Department, ICMR-National Institute for Research in Reproductive Health, Mumbai, India

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A large proportion of patients who survive cancer are rendered infertile as an unwanted side effect of oncotherapy. Currently accepted approaches for fertility preservation involve banking eggs/sperm/embryos or ovarian/testicular tissue before oncotherapy for future use. Such approaches are invasive, expensive, technically challenging and depend on assisted reproductive technologies (ART). Establishing a gonadal tissue bank (for cancer patients) is also fraught with ethical, legal and safety issues. Most importantly, patients who find it difficult to meet expenses towards cancer treatment will find it difficult to meet expenses towards gonadal tissue banking and ART to achieve parenthood later on. In this review an alternative strategy to regenerate non-functional gonads in cancer survivors by targeting endogenous stem cells that survive oncotherapy is discussed. A novel population of pluripotent stem cells termed very small embryonic-like stem cells (VSELs), developmentally equivalent to late migratory primordial germ cells, exists in adult gonads and survives oncotherapy due to their quiescent nature. However, the stem-cell niche gets compromised by oncotherapy. Transplanting niche cells (Sertoli or mesenchymal cells) can regenerate the non-functional gonads. This approach is safe, has resulted in the birth of fertile offspring in mice and could restore gonadal function early in life to support proper growth and later serve as a source of gametes. This newly emerging understanding on stem cells biology can obviate the need to bank gonadal tissue and fertility may also be restored in existing cancer survivors who were earlier deprived of gonadal tissue banking before oncotherapy.

Key words Cancer - cryopreservation - fertility - gametes - ovary - stem cells - testis - transplantation - VSELs

An introduction to oncofertility

Oncofertility is a term coined by Teressa K. Woodruff from Northwestern University, Chicago, USA in 2006 which actually combines oncology with reproductive research to expand available options for cancer patients to preserve fertility to ensure biological parenthood later on in life (*http://oncofertility.northwestern.edu/about-oncofertility-consortium*). With advances in cancer treatment, survival rates in cancer patients have increased; however, infertility is one of the unwanted side effects of the treatment. A large fraction of cancer survivors are children and young adolescents. An international society named International Society for Fertility Preservation (ISFP) is active in this field (*http://www.isfp-fertility.org/*) and Teressa Woodruff's group has also established the Oncofertility Consortium at NW University, Chicago, USA (*http://oncofertility.northwestern.edu/about-oncofertility-consortium*). ISFP is governed by a

board of 18 Directors from America, Europe and Asia and has recently teamed up with American Society for Reproductive Medicine and European Society of Human Reproduction and Embryology to further advance the field. This development and the current status and future perspectives in the field were recently reviewed[1]. Because of excessive load of cancer patients, counselling patients regarding fertility options before oncotherapy is not streamlined in many parts of the world. However, awareness has increased, and Fertility Preservation Society of India (*www.fpsind.com*) has also been established[2].

In 2016, Fournier[3] discussed rights of cancer patients for oncofertility. Patient information describing fertility issues and cancer treatment was provided[4]. The role of oncologists to take care of fertility of cancer patients[5] and legal issues associated with cryopreserved embryos[6] were discussed. Bhartiya[7] pointed out that by targeting endogenous very small embryonic-like stem cells (VSELs) to restore fertility for cancer patients, one could avoid legal, ethical and safety issues associated with oncofertility. Gracia and Woodruff[8] responded to the concept of VSELs to tackle oncofertility issues and looked forward to ongoing research in the field. The aim of the present article was to provide an update on VSELs research and how these endogenous stem cells in adult ovary and testis could be targeted to restore fertility of cancer survivors.

Available options for fertility preservation before oncotherapy

Most important is the option to make 'artificial gametes' from embryonic and/or induced pluripotent [(ES)/iPS] stem cells. Other sex-specific available options for fertility preservation are discussed below and listed in Fig. 1.

***Males*:** The best option for males to preserve their fertility is sperm cryopreservation. If cryopreserved properly, sperm survive and remain viable for more than a decade and can be used for intra-cytoplasmic sperm injection (ICSI) to achieve biological parenthood. However, young boys who cannot produce semen samples are counselled for the preservation of testicular biopsies. The biopsies could be cryopreserved as such or germ cells are isolated and cryopreserved. The cryopreserved cells could later be used to either three-dimensional (3D) culture of tubular tissue to obtain sperm, germ cells differentiation into sperm *in vitro* or for the intra-tubular transplantation of germ cells in the azoospermic tubules[9].

***Females*:** Female cancer patients can possibly be subjected to ovarian stimulation, and eggs/embryos can be cryopreserved before oncotherapy. However, this option becomes unavailable at times when oncotherapy cannot be delayed and also when the cancer is hormone sensitive. In such cases and in young girls, where

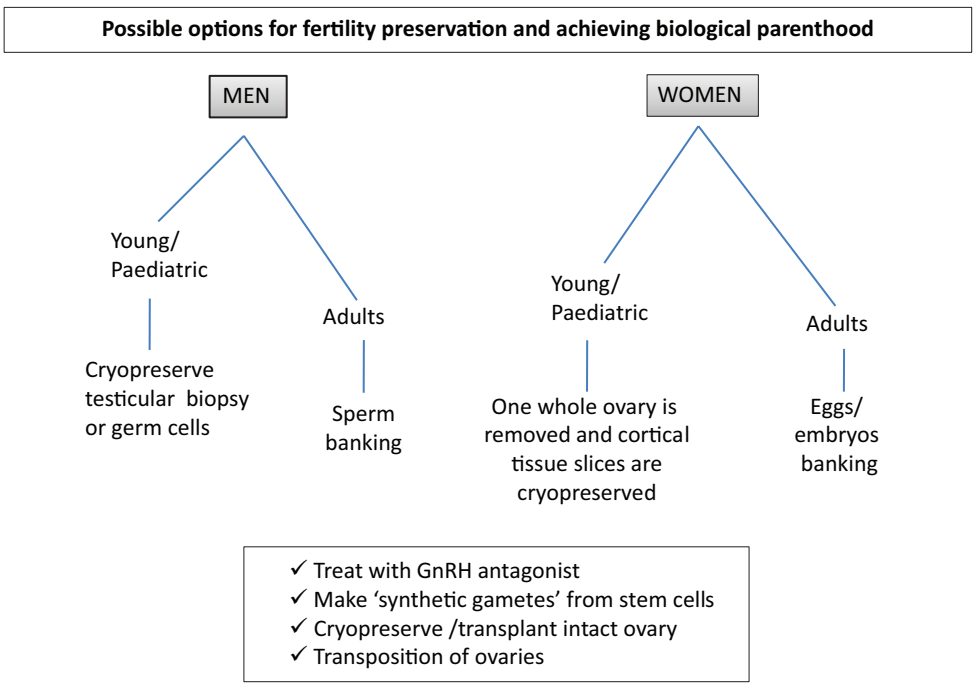

Fig. 1. Possible options of fertility preservation for cancer patients before oncotherapy.

oocytes/embryos cannot be preserved, a whole ovary is removed, and cortical tissue slices are cryopreserved as a source of large numbers of primordial and primary follicles for future use. Attempts are also being made to generate 'artificial ovary' and 3D/2D culture to mature primordial follicles *in vitro*[10].

Current worldwide usage of available options to restore fertility in cancer survivors

Infertility clinics offer facilities such as sperm; oocyte/embryos cryopreservation and cancer patients are referred to *in vitro* fertilization (IVF) clinics to avail such facilities. Ovarian cortical tissue transplantation (OCT) has resulted in remarkable success; 130 live births have been reported worldwide after transplanting frozen-thawed ovarian cortical tissue slices on the surface of the non-functional ovary[11], however the procedure is still considered experimental[12,13]. Donnez *et al*[14] were the first group to combine ovarian tissue cryopreservation and orthotopic transplantation resulting in a live birth, and this is considered as a landmark in the history of human reproductive medicine. Basically, OCT involves transplantation of ovarian cortical tissue slices at either orthotopic or heterotopic sites. Fig. 2 shows a comparison of the two approaches. Babies were born after orthotopic transplantation of the ovarian tissue whereas birth of babies after heterotopic transplantation was rare. Kristensen *et al*[15] reported live birth after heterotopic OCT followed by IVF. Other options to restore fertility include making gametes from stem cells, whole ovary transplantation, maturation of follicles in 2D/3D culture and use of testicular tissue to restore spermatogenesis. An update on these varied approaches and current status are available in recent reviews[16-21].

Making 'artificial gametes' using ES/iPS cells is still in the research phase. Success was recently achieved to derive mouse gametes and birth of pups[22]. However, the process (rate of pregnancy outcome) remained low and fraught with safety issues possibly due to inappropriate epigenetic reprogramming[22,23]. Deriving human gametes from ES/iPS stem cells is a long way to reach the clinics[24]. The following issues need to be resolved (*i*) hES cells need to be derived by somatic cell nuclear transfer for providing biological parenthood, (*ii*) iPS cells have inherent drawbacks including high chances that they may harbour genomic as well as mitochondrial mutations and also at times they may remain partially reprogrammed and retain the epigenetic marks of the somatic cells from which they are derived, (*iii*) methods need to be evolved to convert hES/iPS cells into primordial germ cells-like cells (PGCLCs) since PGCs are natural precursors for gametes, and (*iv*) hES cells are in a 'primed' state and need to be converted to 'naïve' state to improve differentiation into gametes. This may expand their differentiation/regenerative ability[17,18,25-28].

Our research efforts have led to the identification of a novel population of pluripotent stem cells in adult tissues termed very small embryonic-like stem cells (VSELs) in the gonads. VSELs are developmentally equivalent to late migratory PGCs, are quiescent in nature, survive oncotherapy, spontaneously differentiate into gametes and can be targeted to regenerate the ablated, non-functional gonads[29].

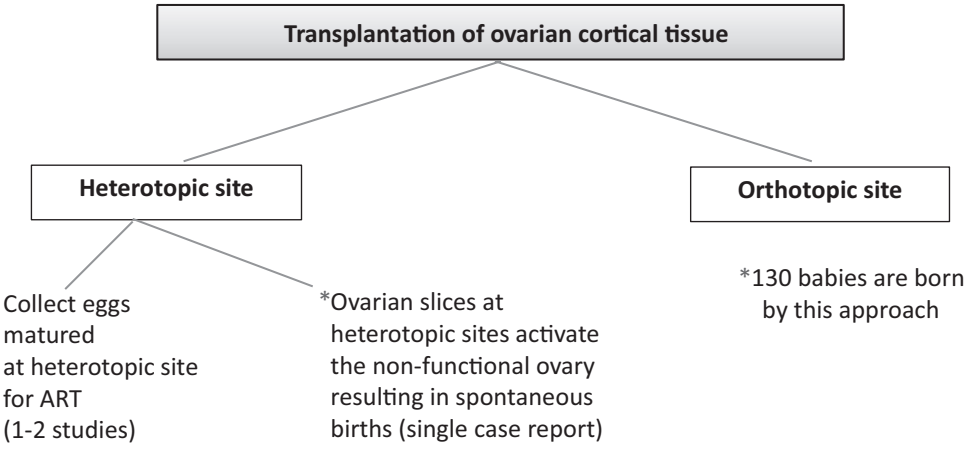

Fig. 2. Various strategies to transplant cryopreserved ovarian slices and their outcome. *The transplanted cortical tissue could act as a source of growth factors/cytokines to the non-functional ovary resulting in its regeneration

An introduction to pluripotent stem cells in adult tissues (VSELs)

Pluripotent stem cells including human ES cells derived from inner cell mass of blastocyst stage embryo[30] and embryonic germ (EG) cells derived from PGCs[31] were reported in 1998. Being equivalent to PGCs, VSELs are pluripotent and yet relatively mature compared to the human ES cells and closer to human EG cells. Similar to EG cells which do not divide readily *in vitro*, neither form teratomas but form spheres readily[31,32], VSELs do not divide in culture and have been reviewed[33-35].

After having worked on human ES cells for more than a decade, we have now shifted gears on VSELs as these possibly have better regenerative potential[36]. These stem cells have been labelled differently by various investigators and have remained elusive over decades due to their small size and presence in very few numbers[37]. VSELs are the most primitive, pluripotent stem cells in the adult organs and give rise to tissue-specific stem cells by undergoing rare asymmetrical cell divisions to self-renew and give rise to slightly bigger tissue-committed adult stem cells 'progenitors'[38]. The tissue committed adult stem cells or 'progenitors' in turn undergo symmetrical cell divisions, clonal expansion and further differentiation into tissue-specific cell types.

Being developmentally equivalent to the late migratory PGCs, VSELs express pluripotent and also PGCs-specific markers. Scaldaferri *et al*[39] have reported hematopoietic activity in putative mouse PGCs that were also found to co-express several markers of hematopoietic precursors. Virant-Klun[40] have described VSELs representing a potential developmental link between germinal lineage and haematopoiesis. Being pluripotent, VSELs have euchromatin and show biallelic expression of various imprinted genes including *IGF2* and *H19*. *H19* expression is high due to biallelic expression and *IGF2* is not expressed - resulting in their quiescent nature[41,42]. Ratajczak *et al*[34] have been successful to achieve expansion of VSELs *in vitro* by treating them with valproic acid and nicotinamide. Another group could expand them *in vitro* by treating with a small molecule pyrimidoindole derivative (UM171) in a feeder-free condition while retaining their pluripotent state[43]. Tripathi *et al*[44] reported increased expression of pluripotent markers in peripheral blood on treating with a highly active nano-formulation of resveratrol. Ratajczak's group[45] reported these stem cells in mouse bone marrow for the first time in 2006 and showed their ability to differentiate into three germ layers. Havens *et al*[46] reported that human cord blood VSELs had the ability to differentiate into three germ layers. Our group has also shown differentiation of mouse bone marrow VSELs into three germ layers, hematopoietic stem cells and male germ cells on providing proper cues[47] and Monti *et al*[48] reported the presence of pluripotent VSELs in human cord blood using a novel strategy and also their ability to differentiate into three germ layers.

VSELs in mammalian testis

The studies on testicular VSELs (Fig. 3) and effect of transplanting niche cells (sertoli or mesenchymal cells) to restore spermatogenesis in chemoablated mouse testis (Fig. 4) are discussed in details below. Immuno-localization studies of OCT-4 led to the identification of VSELs in addition to spermatogonial stem cells (SSCs) in the human testes[49] (Fig. 3A). Three different antibodies were used for OCT-4 immuno-expression on testicular cell smears, sections and by Western blotting. Specific primers/probes were designed to evaluate two distinct isoforms of OCT-4 by quantitative reverse transcription-polymerase chain reaction and *in situ* hybridization studies. The two isoforms of OCT-4 include OCT-4A (nuclear expression and specific to pluripotent state of a stem cell) and OCT-4B (cytoplasmic expression and possibly represents differentiated state of the stem cells). Our findings suggested the presence of small-sized VSELs expressing nuclear OCT-4A and slightly bigger SSCs with cytoplasmic OCT-4B. Results suggested that VSELs were the most primitive stem cells that differentiated into SSCs in the testis. VSELs were found in very few numbers whereas the cells expressing cytoplasmic OCT-4B were in abundance. Later, we studied these stem cells in mouse testis (Fig. 3B) and the following results have emerged: (*i*) VSELs survive busulfan treatment (25 mg/kg) in mouse testis. They initially increased in numbers on day 15 after busulfan treatment and later could be detected on day 30 whereas germ cells/sperm were lost after chemoablation[50,51]. (*ii*) Testicular VSELs were found to express receptors for follicle-stimulating hormone (FSHR) including both alternatively spliced isoforms FSHR1 and FSHR3[54]. VSELs in chemoablated mouse testis increased in numbers after FSH treatment and this action was possibly mediated through FSHR3. (*iii*) VSELs were found to undergo self-renewal and asymmetric cell division and considered the most primitive stem cells in the testis[38,54]. Thus, the most primitive stem cells in the

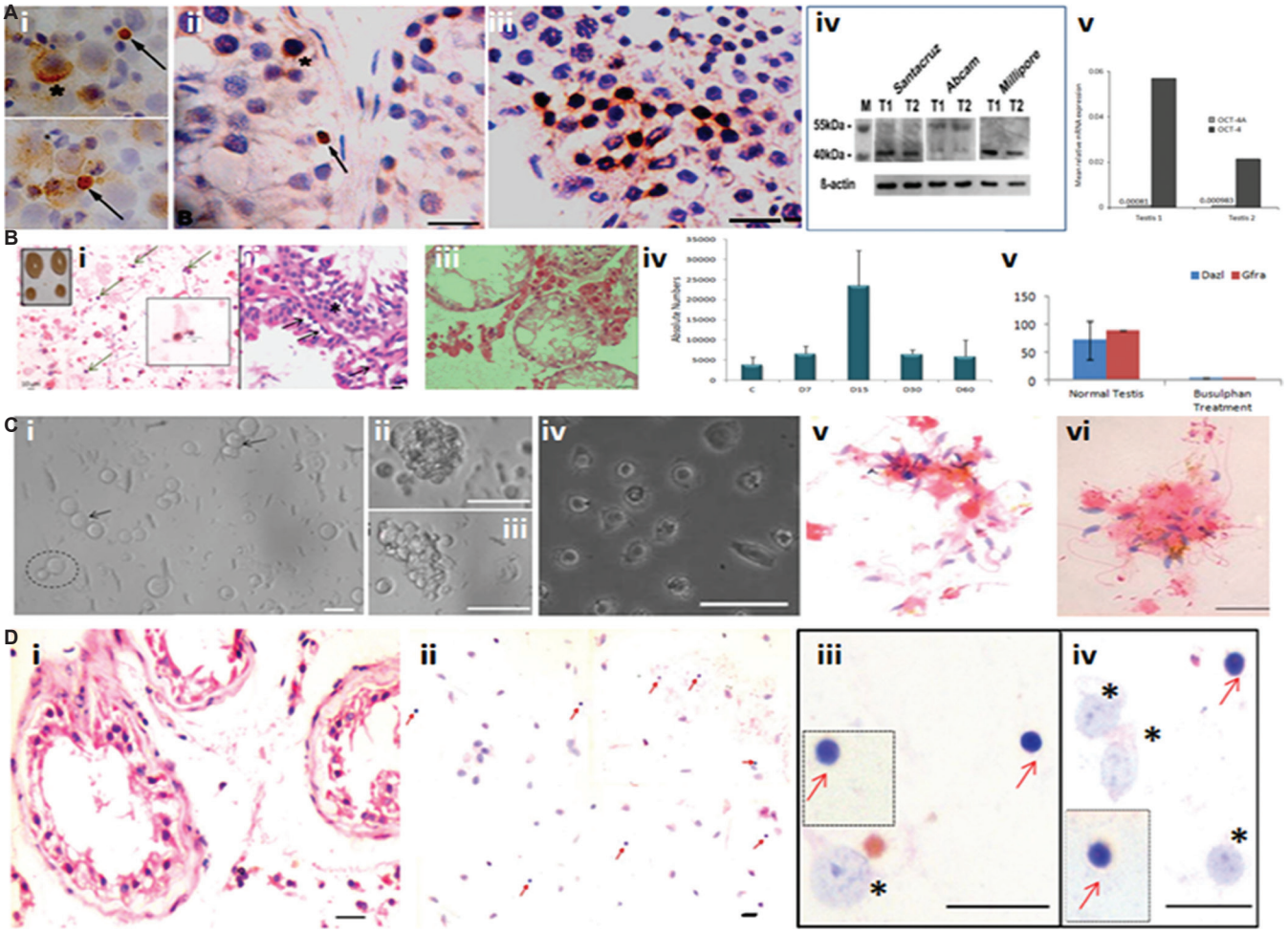

Fig. 3. Testicular stem cells, effect of oncotherapy and *in vitro* culture. (**A**) Nuclear octamer-binding transcription-4 (OCT-4) expressing small-sized spherical stem cells (SSCs) (arrow) exist along with slightly bigger cells with cytoplasmic OCT-4 (asterix) in (*i*) testicular cell smears, and (*ii*) testicular sections, (*iii*) the bigger sized SSCs undergo clonal expansion implying rapid cell division with incomplete cytokinesis, (*iv*) testicular tissue was studied for OCT-4 expression by Western blotting using three different commercial antibodies. Santa Cruz and Millipore antibodies were specific to OCT-4A whereas Abcam antibody detected both alternatively spliced isoforms OCT-4A and B. (*v*) relative expression of Oct-4A and Oct-4B (alternately spliced isoforms of OCT-4 of which OCT-4A is alone responsible for pluripotent state whereas OCT-4B is suggestive of cells entering differentiation) was studied by quantitative reverse transcription-polymerase chain reaction for relative mRNA expression normalized to 18S RNA. As evident, Oct-4A is very less compared to OCT-4 (Oct-4A and B). T1 and T2 are two different testicular tissue samples obtained from men undergoing orchidectomy to manage prostate cancer. *Source*: Reproduced with permission from Ref. 49. (**B**) (*i*) Effect of busulfan treatment (25 mg/kg) on mouse testis. Inset shows the effect of treatment on testes which appear to shrink in size and reduce in weight by 4 wk after chemoablation; H&E stained cell smear on day 15 after treatment shows presence of small sized spherical putative stem cells (<5 µm) with high nucleo-cytoplasmic ratio marked by arrows, (*ii*) testis sections prepared on day 15 after busulfan treatment show the presence of small spherical stem cells along the basement membrane of the seminiferous tubules marked by arrows, (*iii*) by day 30, the seminiferous tubules get devoid of germ cells, (*iv*) flow cytometry data enumerating absolute number of VSELs on different days after busulfan treatment show that initially, the number of VSELs increases on day 15 and later reduce but they do survive till day 30. (*v*) Reverse transcription-polymerase chain reaction for germ cell markers - Dazl and Gfra shows marked reduction of these transcripts on day 30 in agreement with histological data shown in (*iii*). *Source*: Reproduced with permission from Refs 50, 51. (**C**) *In vitro* culture of cells isolated from chemoablated seminiferous tubules. (*i*) Testicular stem cells that survive chemotherapy remain non-adherent and semi-attached on top of a feeder made by the Sertoli cells that get attached to the culture surface. The smaller VSEL divide and give rise to a slightly bigger SSC (broken circle) whereas the SSC divides rapidly and forms chains (arrow), and (*ii* & *iii*) clusters. These cells undergo differentiation to form (*iv*) spermatids, and (*v* & *vi*) sperm. *Source*: Reproduced with permission from Ref. 52. (**D**) Azoospermic human testicular biopsies of cancer survivors of childhood cancer harbour stem cells. (*i*) H&E stained testicular sections show azoospermia and complete lack of germ cells, (*ii*) testicular smears show presence of small, spherical putative stem cells, (*iii* & *iv*) higher magnification of smears showing Sertoli cells (*) and VSELs (red arrow) that survive oncotherapy in seminiferous tubules. VSELs are spherical in shape with minimal cytoplasm whereas Sertoli cells are characterized by a large nucleus with prominent nucleoli. Insets include VSELs captured from different microscopic fields. *Source*: Reproduced with permission from Ref. 53.

testes[55] that undergo self-renewal and give rise to SSCs might be VSELs. (*iv*) Microarray studies on Sertoli cells (niche cells for testicular stem cells) isolated from normal and chemoablated testis showed marked changes in the transcriptome after busulfan treatment[51] suggesting that although stem cells survive, their niche gets compromised by chemotherapy. (*v*) Transplanting healthy niche cells including Sertoli cells from syngeneic mice or bone marrow mesenchymal cells through inter-tubular route could regenerate chemoablated testis[51]. Spermatogenesis was restored from the VSELs that survived chemotherapy when paracrine support was provided by the transplanted healthy niche cells (Sertoli or mesenchymal stromal cells) (Fig. 4). This was further discussed in depth[56]. (*vi*) Chemoablated testicular tubules collected on day 60 after busulfan treatment when cultured on a Sertoli cells bed and in the presence of Sertoli cells conditioned medium and FSH revealed that the surviving stem cells spontaneously differentiated into sperm *in vitro*[52] (Fig. 3C).

These results were intriguing since ES/iPS cells require very sophisticated *in vitro* system with sequential addition of hormones and growth factors

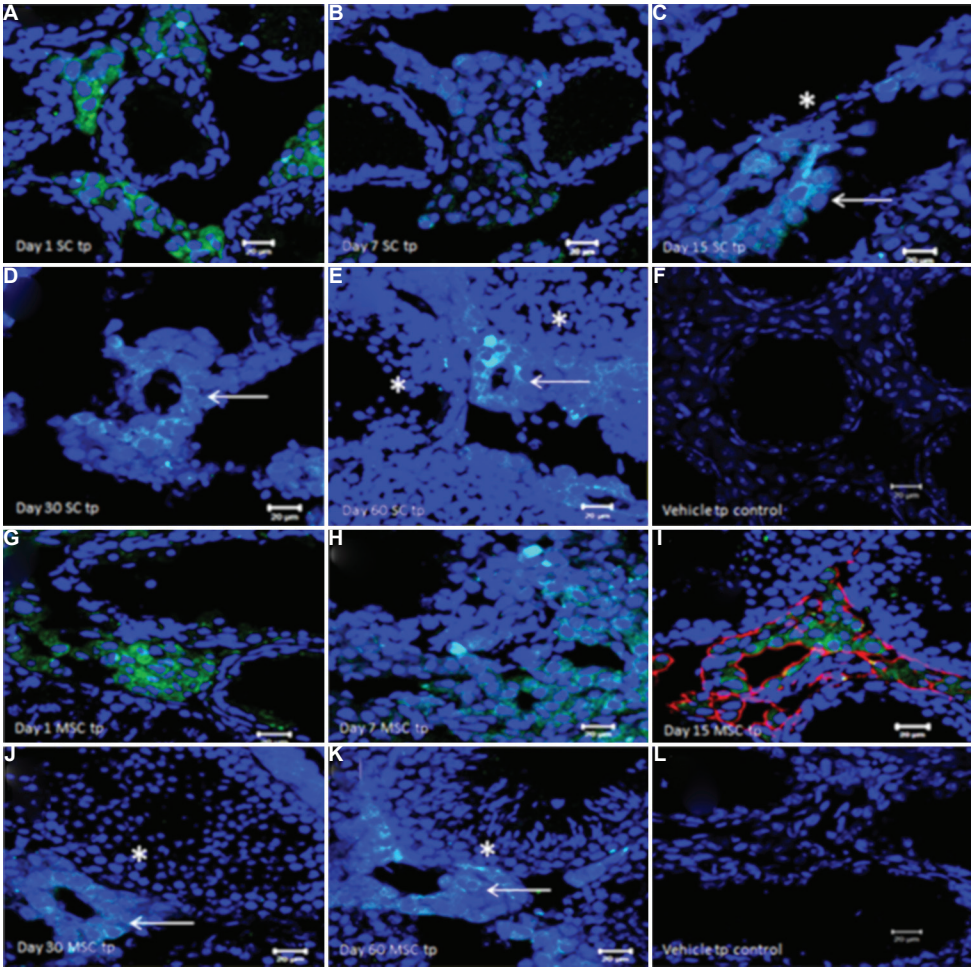

Fig. 4. Fate of green fluorescent protein (GFP)-positive Sertoli/mesenchymal cells on transplantation in chemoablated mice testis, studied by immuno-expression of GFP on cryosections. (**A** and **B**) GFP+ve Sertoli cells were observed in the interstitium on days 1 and 7 after transplantation, (**C**) on day 15, GFP+ve cells start to align as neo-tubule-like structures (arrow) in the vicinity of germ cells depleted 'native' tubules (*), (**D** and **E**) the neo-tubules are retained through days 30 and 60 after transplantation (arrows). Note resumption of spermatogenesis in 'native' non-GFP tubules (*) on day 60. (**F**) No GFP staining in is detected in busulfan-treated controls corresponding to day 30. (**G** and **H**) GFP+ve mesenchymal cells were detected in the interstitium on days 1 and 7 after transplantation, (**I**) GFP+ve cells aligned themselves as neotubule-like structures by day 15 and also colocalized vimentin (red staining) confirming their mesenchymal nature, (**J** and **K**) On day 30 and 60 GFP+ve neo-tubules (arrows) were clearly visualized in the vicinity of native tubules showing spermatogenesis (*). (**L**) No GFP expression was observed in busulfan-treated controls corresponding to day 30. Neo-tubules formed after transplantation of both Sertoli and mesenchymal stromal cells do not differentiate into sperm and only provide paracrine support to the stem cells in the native tubules that survived chemotherapy and undergo spermatogenesis. *Source*: Reproduced with permission from Ref. 51.

for their induction into gametes. The main reason for our success was because VSELs are developmentally equivalent to PGCs which are also natural precursors to gametes. We further isolated bone marrow VSELs and cultured in a manner similar to that described above. Male germ cells were detected in culture after 14 days[47]. Shirazi *et al*[57] purified stage-specific embryonic antigen 1 (SSEA-1)-positive cells (SSEA-1 is a specific marker for pluripotent stem cells and is also expressed on VSELs) and reported their differentiation into PGCs, SSCs and spermatogonial cells.

Similar to our findings in mouse testes, Kurkure *et al*[57] and Virant-Klun group[58,59] reported the presence of VSELs in azoospermic human testicular biopsies of cancer survivors and other clinical conditions (Fig. 3D). A recent systematic review[60] has compiled data published by several groups reporting beneficial effects of transplanting MSCs in chemoablated mouse testes. However, none of these studies acknowledge presence of VSELs or throw any light on how transplanting MSCs could restore testicular function.

This understanding of testicular stem cells biology has significant implications in the field of oncofertility. Since VSELs survive oncotherapy in human testes, there may be no need to cryopreserve/bank testicular germ cells/biopsies. Azoospermic testes of cancer survivors are expected to harbour VSELs and a simple transplantation of niche cells - mesenchymal cells

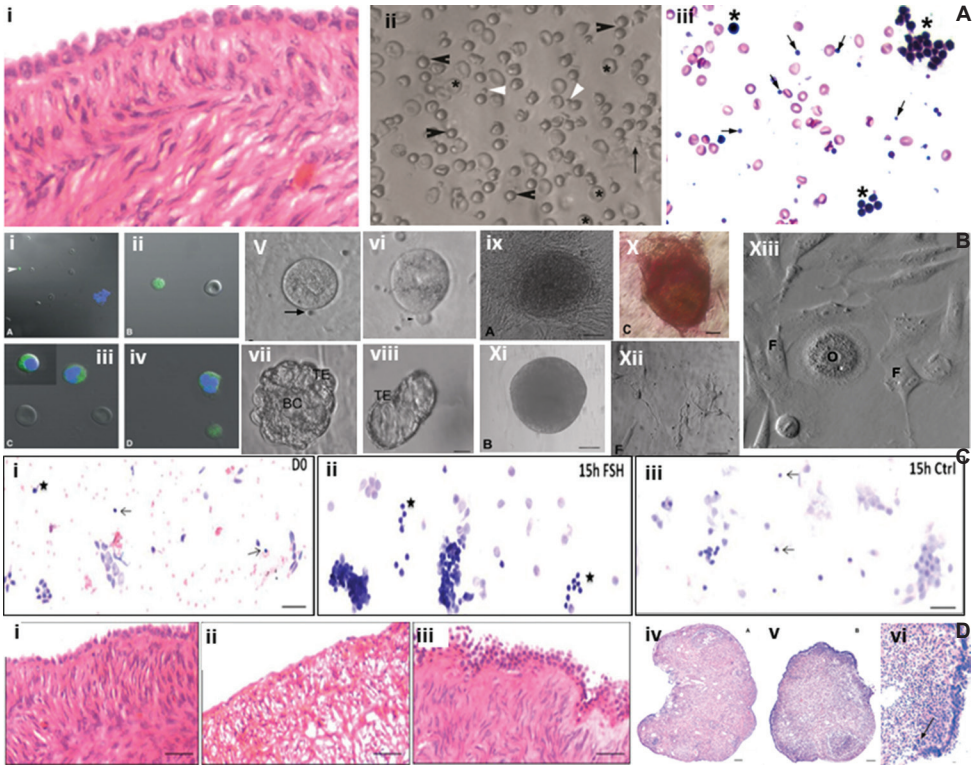

Fig. 5. Stem cells in adult mammalian ovary. (**A**) (*i*) Adult aged human ovary showing prominent ovary surface epithelial (OSE) cells with no follicles in the ovarian cortex, (*ii*) gentle scraping of ovary surface epithelial cells show the presence of spherical stem cells of two distinct sizes including very small embryonic-like stem cells (white arrow) and slightly bigger OSCs (black arrow) along with red blood cells, (*iii*) after H&E staining, very small embryonic-like stem cells (arrow) and OSCs (*) along with germ cell nest are clearly visualized. *Source*: Reproduced with permission from Refs 63, 64. (**B**) (*i-iv*) Confocal images of stem cells expressing OCT-4, (*v-vi*) OSCs in culture spontaneously differentiate into oocyte-like structures, polar body extrusion is also observed, (*vii-viii*) Parthenote embryo and hatching blastocyst in culture, (*ix-x*) embryonic stem-like colony, positive for alkaline phosphatise, (*xi*) embryoid body-like structure, (*xii*) neuronal-like structures, (*xiii*) at places, somatic fibroblasts surround an oocyte-like structure giving an impression of primordial follicle-like structure. *Source*: Reproduced with permission from Ref. 63. (**C**) Sheep ovary surface epithelial cells smear shows the presence of VSELs (arrow) and OSCs (*) along with large number of epithelial cells, (*ii*) 15 h of follicle-stimulating hormone treatment resulted in extensive proliferation of stem cells and formation of germ cell nests whereas, (*iii*) untreated control did not show any change compared to initial culture. *Source*: Reproduced with permission from Ref. 65. (**D**) (*i*) Prominent single layer of ovary surface epithelial and stroma devoid of follicles in peri-menopausal ovarian tissue, (*ii*) loss of epithelial cells and disorganized stroma evident after three days in culture, (*iii*) follicle-stimulating hormone treatment results in proliferation and multi-layered appearance of ovary surface epithelial cells, (*iv*) chemoablated mouse ovary devoid of follicles, (*v-vi*) prominent ovary surface epithelial after follicle-stimulating hormone treatment to chemoablated mouse. *Source*: Reproduced with permission from Refs 66,67.

through intertubular route could enable restoration of spermatogenesis - thus ensuring biological parenthood.

VSELs in mammalian ovary

It is generally believed that mammalian ovary has fixed number of follicles which deplete with age and their sudden loss results in menopause. However, stem cells have been reported in the ovary surface epithelium (OSE) but are still debated[61]. There exist two distinct populations of stem cells in adult mammalian ovary including VSELs and OSCs similar to VSELs and SSCs in the testis[62]. Our group reported the presence of two populations of stem cells (Fig. 5) in rabbit, sheep, marmoset and human OSE cells[63].

We have published several observations on ovarian stem cells biology. Initially we observed a direct action of pregnant mare's serum gonadotropin (PMSG) on adult mouse OSE. Treatment resulted in upregulation of pluripotent and meiotic markers along with increased numbers of primordial follicles below the OSE. It was concluded that PMSG activated the pluripotent VSELs and also appeared to augment neo-oogenesis and PF assembly in adult mouse ovaries[68]. OSE cells gently scraped from the ovaries of rabbit, sheep, marmoset and humans and enriched for the stem cells were found to have the ability to spontaneously differentiate into oocyte-like structures[63]. The epithelial cells attach to the bottom of the culture dish and provide feeder support whereas the stem cells spontaneously differentiate into oocyte-like structures, extrude polar bodies and parthenotes *in vitro*[63]. Further studies showed that events such as germ cell nest formation, Balbiani body and cytoplasmic streaming - which are characteristic of foetal ovaries are replicated while ovarian stem cells differentiate *in vitro*[69] (Fig. 5A and B) and that this process is modulated by FSH[66] (Fig. 5C). Contradictory results were published in a study[70] which failed to detect any stem cell activity in adult ovary including formation of 'germline cysts' by lineage tracing approach and thus concluded that ovaries lacked stem cells. Our group suggested that absence of evidence was not necessarily evidence for absence for ovarian stem cells[64].

In another experiment, sheep ovarian stem cells, enriched by gentle scraping of OSE were cultured in the presence of FSH resulted in activation of stem cells to undergo self-renewal and clonal expansion resulting in the formation of germ cell nests (Fig. 5C). Evidence was further generated that FSH action on ovarian stem cells (Fig. 5D, i-iii) was mediated through alternatively spliced FSHR3 isoform[65].

Chemoablation of mouse ovaries by treating with busulfan+cyclophosphamide resulted in complete depletion of follicles whereas the stem cells survived in the OSE[67] (Fig. 5D, iv-vi). Treating chemoablated mice with PMSG (FSH analogue, 5 IU subcutaneously), after 48 h, resulted in increased numbers of VSELs with a cell surface phenotype of LIN-CD45-SCA-1+ (intact ovary have 0.02+0.01%; after chemoablation 0.03+0.017% and 0.08+0.03% after PMSG treatment to chemoablated mice) along with increased uptake of BrdU (Fig. 6). PMSG treatment during culture of chemoablated mouse ovaries resulted in stem cells' proliferation and differentiation into pre-meiotic germ cell clusters (nests)[67]. These results provided evidence that VSELs survived chemotherapy in mice ovaries, were modulated by FSH, retained the ability to undergo oocyte-specific differentiation and could be the ideal endogenous stem cells to regenerate non-functional ovaries[67]. Initial evidence was provided to show primordial follicle assembly below the OSE[71].

Esmaeilian *et al*[72] reported the presence of pluripotent stem cells in the mouse OSE with the ability to differentiate into the three germ layers and oocyte-like cells. Silvestris *et al*[73] also confirmed the presence of two populations of stem cells in the human ovary and we discussed their findings[74]. VSELs and OSCs exist in ovary similar to VSELs and SSCs in the testis[62,74]. Thus, it is evident that similar populations of pluripotent stem cells exist in ovary and testis which are developmentally equivalent to the PGCs, and being quiescent in nature, they survive oncotherapy. The stem cell-niche apparently gets compromised by oncotherapy and is unable to support stem cells' differentiation into germ cells. This is true for both the testis and the ovaries. A simple replacement of the niche cells may allow surviving stem cells to regenerate non-functional ovaries.

Regenerating chemoablated testis and ovaries

It has been discussed that regenerating ovaries is a better approach rather than rejuvenating individual eggs by transplanting young mitochondria isolated from stem cells that exist in the ovarian cortex[75]. Also there is no need to transplant germ cells since stem cells survive in chemoablated testis. A simple transplantation of niche providing cells - Sertoli or bone marrow-derived mesenchymal cells through intertubular route could restore spermatogenesis in the chemoablated testes. A novel population of stem cells exists in the gonads and survives oncotherapy.

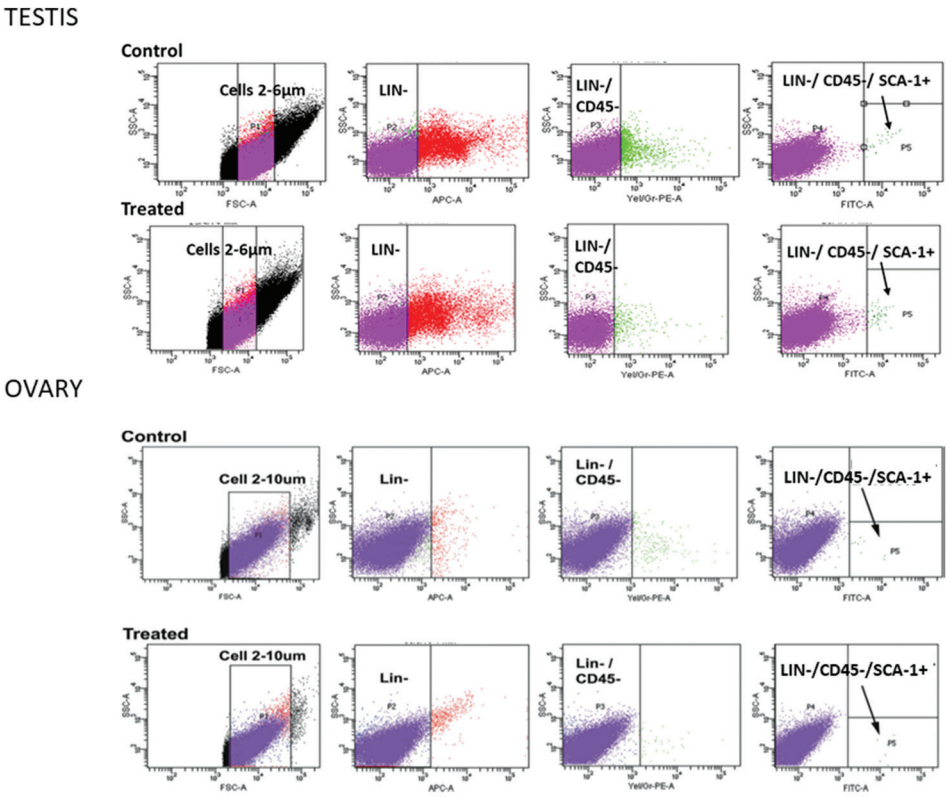

Fig. 6. Flow cytometry analysis of VSELs in adult intact and chemoablated mouse testis and ovary. VSELs are small in size (2-6 µm) and have a surface phenotype of LIN-CD45-SCA-1+. Their numbers are increased in the chemoablated gonads. VSELs were found to be 0.03 per cent in normal and 0.06 per cent in chemoablated testis and 0.02 per cent in normal and 0.03 per cent in chemoablated ovary of total events analyzed. *Source*: Reproduced with permission from Refs 51, 67.

This has been shown in both mouse testis and ovary by multiple techniques including flow cytometry (Fig. 6). A simple and direct transplantation of mesenchymal cells in the non-functional gonads may suffice to regenerate them.

Oktay *et al*[76] reported four spontaneous pregnancies and three live births following subcutaneous transplantation of frozen-banked ovarian tissue at heterotopic site in a female survivor of Hodgkin lymphoma. The woman was earlier rendered menopausal due to preconditioning chemotherapy before bone marrow transplantation. They discussed that possibly the microenvironment of the non-functional ovary was destroyed by chemotherapy and paracrine/ endocrine signals provided by the transplanted cortical tissue (at a heterotypic site) resulted in regeneration of the intact, non-functional ovary possibly by the stem cells from the bone marrow or resident stem cells. It has been suggested that ovarian stem-cell niche gets disrupted by chemotherapy and also with age[77-79]. Aged, nonfunctional mouse ovaries were made functional on transplanting in a young host[77] stressing on the fact that the stem cells niche gets compromised with age and that a healthy, young niche is crucial for stem cells function and oocyte development[80]. Birth of a child has been reported to a woman with premature ovarian failure on transplanting autologous bone marrow-derived mesenchymal cells in the ovary[81]. This concept was discussed as a possible ray of hope for women with premature ovarian failure (POF)[82].

To conclude, pilot clinical studies need to be undertaken to regenerate non-functional gonads of cancer survivors. In future, it would be possible to restore fertility in cancer survivors obviating the need to cryopreserve gonadal tissues before oncotherapy.

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For correspondence: Dr Deepa Bhartiya, Stem Cell Biology Department, ICMR-National Institute for Research in Reproductive Health, Jehangir Merwanji Street, Parel, Mumbai 400 012, Maharashtra, India
e-mail: bhartiyad@nirrh.res.in