9 STEM CELL BIOLOGY

Efforts of Stem Cell Biology Department are focused to realize the clinical potential of embryonic stem cells (ESC). Human embryonic stem cells represent an important resource for basic research and have great potential for cell replacement therapies. To derive and reproducibly generate the desired numbers of undifferentiated cells for therapy, it is important to develop reliable protocols to optimize their complex culture environment. These cells exhibit maximum plasticity, expand and grow in large numbers *in vitro* and directed differentiation of these cells into various lineages is being perfected. It is also mandatory to carry out pre-clinical studies in non-human primates prior to clinical use of ES cells based therapy.

The department aims to achieve the following (i) generation of human ES cell lines in xeno-free environment (ii) generation of non-human primate ES cell lines (iii) development of patient specific ES cell lines by SCNT (iv) develop an *in vitro* differentiation system for teratological screening using human ESC (v) develop animal models for human diseases (vi) transplantation studies to investigate the regenerative potential of ES cells in animal models (vii) establish a GMP facility to expand human embryonic stem (hES) cells for clinical use (viii) cryopreservation and maturation of germ stem cells to conserve fertility in individuals with gonadal insufficiency including cancer survivors.

9.1 To Establish Human Embryonic Stem Cell Lines in Xeno-free Environment

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Duration: 2003-2009

At present about 200 hES cell lines have been derived worldwide of which 78 hES cell lines have been registered on NIH registry. Only 10 lines of these 78 are available at present for research purpose. All the cell lines registered with NIH were grown on mouse embryonic feeder layers, using fetal calf serum

and also reagents for immuno-surgery were of animal origin, which are a potential source of animal pathogens and thus these cell lines are not optimal for clinical use. In general, human ES cell lines are extremely sensitive, have poor survival and revival rates, cryopreservation and thawing is a problem, undergo mutational changes and genetic drift in culture. Thus one can not depend on commercially available cell lines for use as a source to develop a national regenerative medicine program. Aim of this ongoing project is to develop the technology for hES cell lines derivation in a xeno- free environment using human feeders.

A total of 50 spare human embryos were used for the derivation of hES cell lines, of which first 32 were cryopreserved embryos, frozen at 4-6 cell stage. Only 8/32 formed blastocysts post- thaw and after removal of inner cell mass by laser micromanipulation, none of the inner cell mass attached to the feeder layer. Remaining 18 embryos were fresh, spare embryos. Ten of them developed into blastocysts, from which 7 cell lines were successfully obtained and maintained for more than 20 passages (Fig. 117). As evident in Fig. 118, the cells have large nucleocytoplasmic ration, characteristic morphology of stem cells.

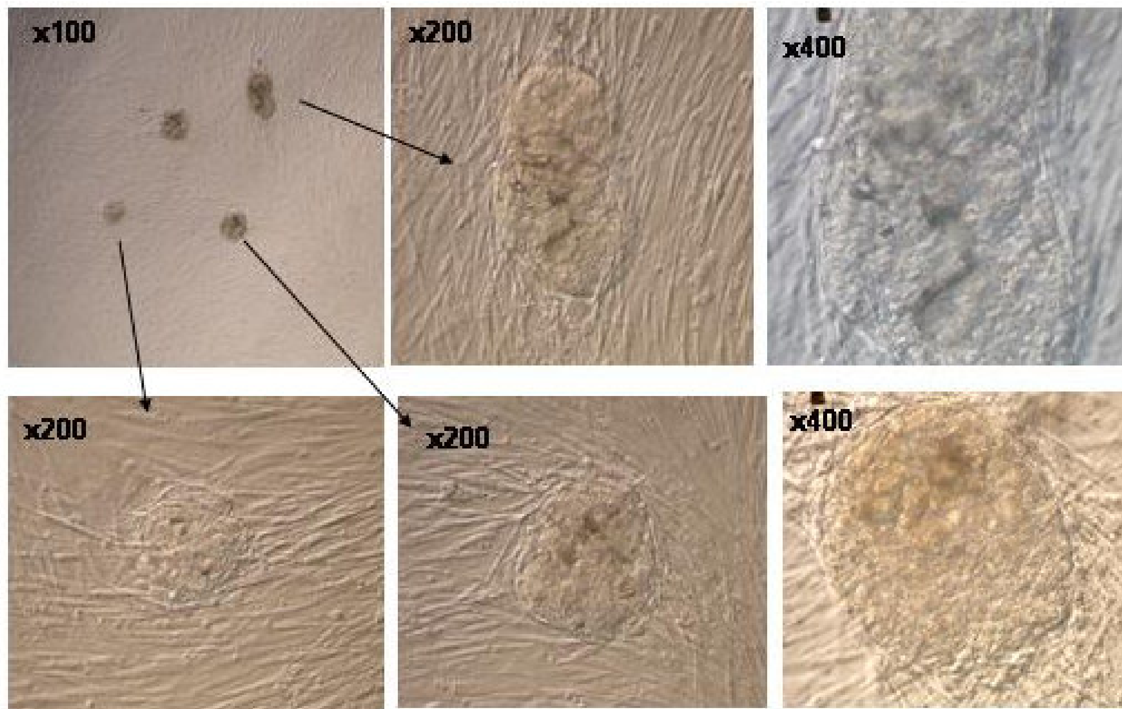

Fig.117: Cut and paste method used to expand the colonies. Note the 4 pieces cut with a needle and placed on feeder layer. They attach to the feeders overnight and also appear to dislodge the feeder fibroblasts at times.

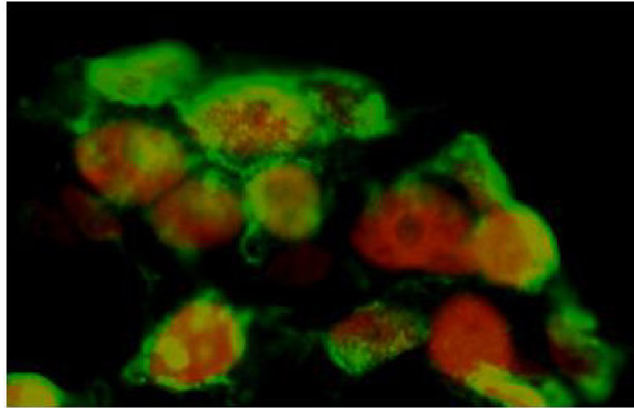

Fig.118: A clump of cells after trypsin dissociation exhibiting a high nucleo-cytoplasmic ratio. Note the presence of a large nucleus surrounded by a thin rim of cytoplasm. The cells are stained for TRA-1-81 and counterstained with propidium iodide used as a counter stain.

9.2 Fertility Conservation in Individuals with Gonadal Insufficiency

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Duration: 2003-2009

The long term goals of this program's are to cryopreserve gonadal tissue under liquid nitrogen for individuals with gonadal insufficiency including cancer survivors. The tissue could later be thawed and germ cells matured *in vivo* by transplantation or *in vitro* maturation to achieve genetic parenthood.

Cryopreservation of Gonadal Tissue

Studies have been initiated to cryopreserve gonadal tissues using different cryopreservative agents by slow rate cooler (PLANAR, UK) as reported earlier (Annual Report 2005-06, p 201-204). Ovarian and testicular tissues are being cryopreserved using four cryoprotectants namely, DMSO, glycerol, ethylene glycol, and propanediol sucrose in concentrations ranging from 0 M to 2.5 M. To gauge the toxic/ cryoprotective effect exerted by different concentration of each cryoprotectant, viability is being assessed by flow cytometry pre and

post thaw. In addition to identifying the most effective CPA, the optimal concentration at which it exerts its protective effect, is also being ascertained.

Culture of Human Testicular Tissue

It has recently been reported that pluripotent spermatogonial stem cells can be isolated from adult mouse testis. These cells have embryonic stem cell properties and can spontaneously differentiate into three embryonic germ layers *in vitro* and generate teratomas in immunodeficient mice. Isolation of similar cells from human testicular biopsies may allow individual cell- based therapy without any ethical or immunological problems.

Efforts are underway to establish culture conditions for proliferation and differentiation of rat (Annual Report 2005-06, 201-204) and human testicular germ cells. Human germ cells (Fig 119) or testicular tissue explants (Fig 120) were cultured on inserts at 34^0C and could be maintained for a period of approximately two weeks *in vitro*. Marker based identification of different cell types by immunoflourescence and immunocytochemistry is being standardized. The role of growth factors such as GDNF, LIF and EGF and hormones such as testosterone and FSH in promoting the proliferation and differentiation of germ cells is being studied. The spermatogonial stem cells enriched in organotypic cultures by Day 6 will be isolated and attempts will be made to expand them *in vitro* to obtain pluripotent stem cells with ES cell characteristics.

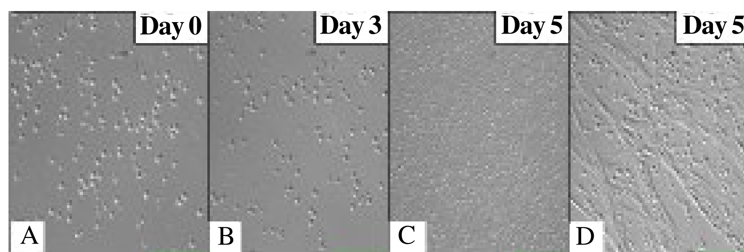

Fig. 119: Human germ cells culture *in vitro* (A) Germ cells after isolation. (B) Cells attach and grow in clusters (C & D) Somatic cells form feeder; germ cells loosely adhere on feeder

Culture of Human Ovarian Tissue

Available literature indicates that perhaps stem cells reside in ovarian surface epithelium and new follicles are formed continuously throughout the lifecycle of female contrary to earlier reports that a female is born with fixed number of eggs. We are keen to grow follicles *in vitro* for fertility conservation as well as an alternative source of human eggs for therapeutic cloning.

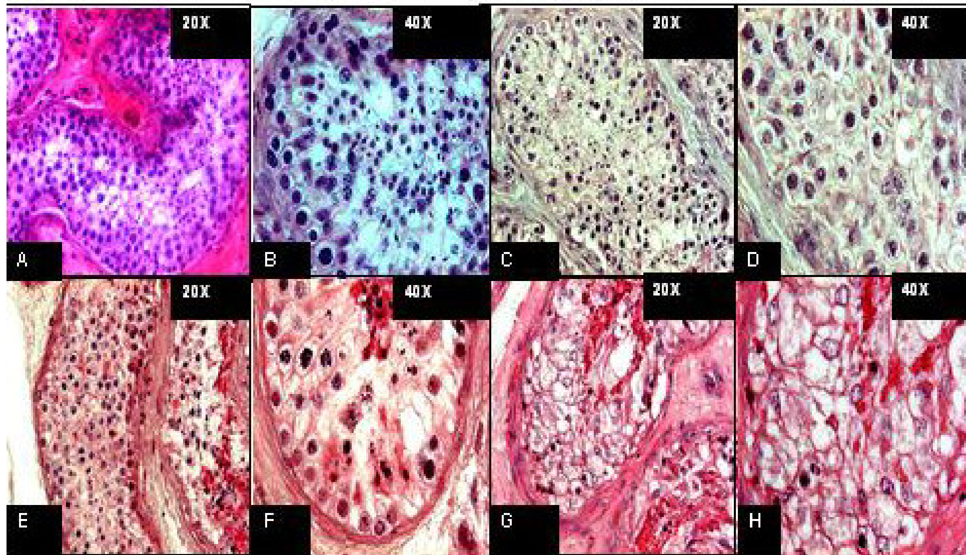

Fig.120 : (A & B) Day 0, normal architecture of human seminiferous tubule. (C & D) Day 4, presence of healthy spermatogonia(*) and pachytene spermatocytes(+) noticed. (E & F) Day 6, spermatogonia(*) and pachytene spermatocytes(+) noticed, beginning of vacuolization observed. (G & H) Day 11, tubule degeneration noticed.

Human ovarian tissue was scrapped mechanically to release surface epithelial cells and both epithelial as well as cortical cells were cultured in twenty fourwell plates. Two distinct cell types are observed after 3 weeks in culture (Fig 121). Further characterization of cultured cells is underway. Organotypic cultures of human ovarian tissue will be initiated shortly.

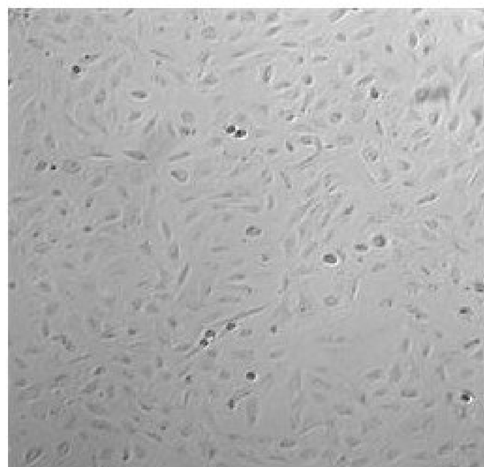

Fig.121: Primary culture of human ovarian surface epithelium and cortical cells

Transplantation Studies

Heterotypic transplantation of cryopreserved-thawed ovarian cortical strips, as a source of eggs for assisted conception, will help such individuals to achieve genetic parenthood, get over the stigma of infertility induced by aggressive chemotherapy and radiotherapy and lead a quality life. Massive loss of primordial follicles occurs prior to neovascularization in the transplanted ovaries. Present study was aimed to conserve the primordial follicles (PF) reserve in the transplanted ovaries by treating with VEGF; since VEGF is known to induce early angiogenesis thereby may reduce the loss of follicles due to hypoxia and ischemic insult. Various strategies have been used to expedite angiogenesis in ovarian grafts with the objective to lengthen the life of the transplanted tissue. Antioxidants like vitamin C, vitamin E and recently transplanting the ovarian xenografts into a granulation tissue bed appear to have a beneficial effect.

Bilateral ovariectomy in immature, 20-21 days old Holtzmann rats were carried out and the removed intact ovaries were immediately autotransplanted subcutaneously on the ventral surface of the animals. VEGF (200ng) was injected subcutaneously at the site of transplant. A gvp that was not treated with VEGF saved as control. The rats were sacrificed 3 and 7 days after transplantation (n=6 per group), with and without VEGF treatment.

Results indicate that VEGF not only helped to prevent PF loss, but also induced *de novo* assembly of PF in the ovarian surface epithelium (Fig. 122). The study has further provided direct evidence in support of the earlier reports in literature, that postnatal mammalian ovary is a dynamic organ and is not endowed with fixed number of eggs since birth.

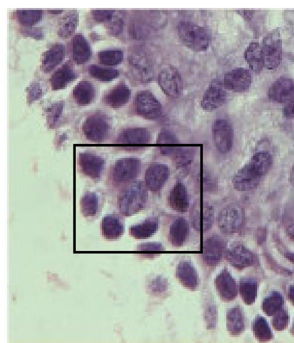

Fig. 122: Higher magnification of ovarian section by day 7 post- transplantation reveals prominent, multi- layered and highly convoluted ovarian surface epithelium composed of two distinct cell types viz. cells with large nucleus, abundant euchromatin and prominent nucleoli surrounded by very thin rim of cytoplasm. The other prominent cells are with darkly stained nucleus and

abundant heterochromatin. *de novo* assembly of primordial follicles in ovarian surface epithelium is evident in the picture. Note pale germ 'stem' cells are surrounded by darkly stained granulosa - like cells; resembling primordial follicles. Pale germ stem cells and darkly stained cells are also abundant in the ovarian stroma.

9.3 Establishing IVF Program in Marmosets and Sheep as Study Models for ES Cell Lines Derivation, Parthenogenesis and Somatic Cell Nuclear Transfer

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Duration: 2006-2009

Studies in Marmosets

The main objectives of this project is to derive ES cell lines from marmosets for pre-clinical evaluation of ES cell therapy in marmoset models for human diseases like myocardial infarcts, diabetes and infertility.

Four attempts were made to carry out timed mating in marmosets followed by flushing of blastocysts on Day 7-10 post-fertilization. As described in literature, micro-clamps were used to block the oviducts, a 26 G needle was used to flush the uterus and the blastocysts were collected from the fundus region with a 20 G needle. However, no blastocyst could be collected, although the ovaries showed signs of recent ovulation. Whether mating occured successfully or not could not be confirmed. Experiments involving superovulation, *in vitro* maturation, *in vitro* fertilization and *in vitro* cleavage to grow blastocysts are under progress. Blastocysts will serve as source of a inner cell mass for deriving the ES cell lines.

Studies in Sheep

Gonadal tissue of sheep was used to establish the IVF program. The isolation of follicles from the ovaries, *in vitro* maturation, caudal sperm capacitation and *in vitro* fertilization were carried out. The zygotes grew well to

morula stage and efforts are ongoing to obtain blastocysts Fig. 123. Different media composition, growth factors and also hypoxic culture condition of 5 percent CO2 and 5percent oxygen will be tried with an aim to obtain 50- 60percent blastocysts from the *in vitro* fertilized sheep eggs. Standardization of this system has given us a strong grip of the various culture methods and will be extrapolated for stem cell derivation program in marmosets.

Once established, this will also be an excellent system to establish the procedures of parthenogenesis and somatic cell nuclear tranfer. Parthenogenesis will enable us to obtain bioidentical stem cells from unfertilized eggs. Since sheep eggs are large and available in plenty, these will be an excellent starting material for establishing the procedures of SCNT. Both the procedures of SCNT and parthenogenesis will help to produce histocompatible cells for cell- based therapy in future.

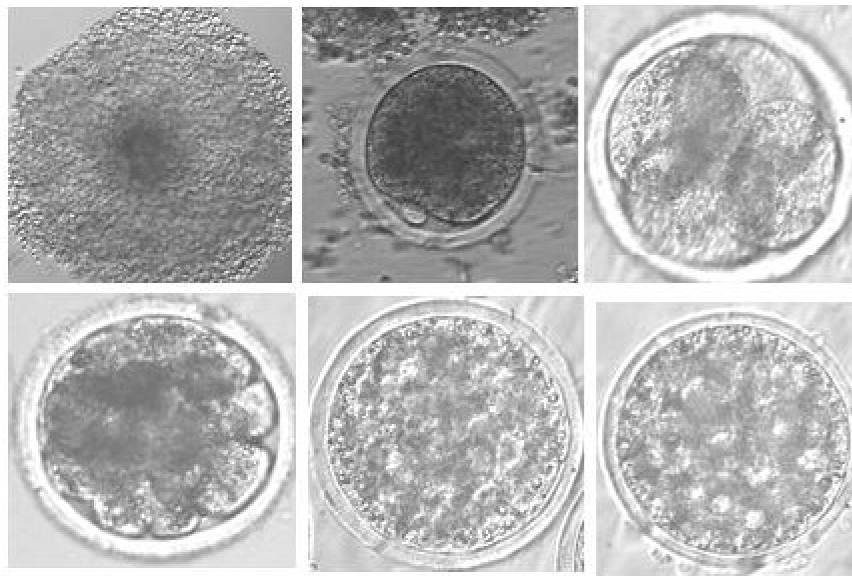

Fig. 123: *in vitro* matured sheep oocyte surrounded by several layers of cumulus cells (A); *in vitro* fertilization of mature egg with a prominent polar body (B); *in vitro* cleavage of sheep zygote at various developmental stage after 2- 8 days in culture (C- F).