

The recent derivation of human embryonic stem cell lines has created excitement in the field of Stem Cell Biology, especially for its tremendous therapeutic potential. The use of stem cells to treat cellular degenerative diseases, as an *in vitro* model for drug toxicity testing and studying early stages of embryonic development are some of the benefits. Advantages of initiating work on embryonic stem cells include their greater plasticity and capability to proliferate into large numbers as compared to adult stem cells.

9.1 To Establish Human Embryonic Stem Cell Lines

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Duration: 2003-2006

Studies have been initiated in the field of stem cells with following two broad objectives: (i) to derive and characterize human embryonic stem (hES) cell lines using human feeder layers; and (ii) to identify proteins/factors secreted by mouse embryonic feeder layer that support hES cells proliferation *in vitro*.

To derive and characterize hES cell lines using human feeders

The project has received funding from Department of Biotechnology, New Delhi. Derivation of human ES cell lines involve the use of spare frozen human embryos from IVF clinic. Methods have been standardized to derive primary fibroblast culture from human fetal tissue (n=6) & foreskin (n=5) collected at the time of medical termination of pregnancy and circumcision respectively. The cells are grown in large numbers, gamma irradiated and cryopreserved for later use.

During the year, spare cryopreserved embryos at 4-6 cell stage (n=16) were revived, cultured to blastocyst stage (n=5) and placed on human fibroblast feeder layer (Fig. 119). Various methods were used to remove inner cell mass from the blastocyst viz. (i) complete zona removal by several laser shots; (ii) zona drilling and aspiration of inner cell mass by biopsy needle; (iii) zona drilling and squishing out of inner cell mass; and (iv) zona drilling followed by natural hatching. However, after being placed on gamma irradiated feeder layers, the inner cell mass failed to grow further, degenerated, did not attach and was infected in one case. Availability of spare embryos for research was restricted with only 20 percent of cryopreserved embryos maturing to blastocyst stage.

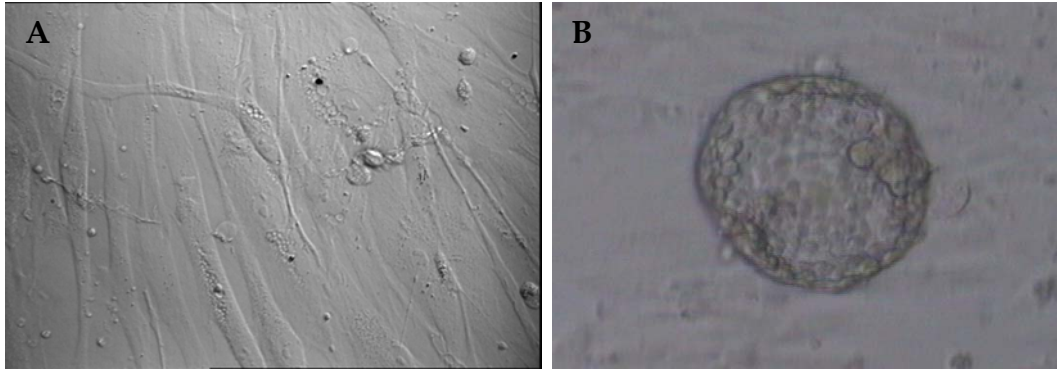


Fig. 119: (A): Human primary fibroblast cultures used as feeder layer. (B): A hatched blastocyst on the human fibroblast feeder layer

To identify proteins/factors secreted by mouse embryonic feeder layer that support hES cells proliferation

Mouse embryonic feeder (MEF) layer derived from embryos (13.5 dpc) support undifferentiated proliferation of hES cells. This suggests that there are factor(s) secreted in conditioned media, which are essential for the growth of human embryonic stem cells. The nature of these factors is still unknown. We have initiated a study with the objective to identify the factor(s) by employing proteomics approach. Mouse skin fibroblasts from embryos (13.5 dpc and 19.5 dpc) and neonates (Day 2) have been cultured. The protein from the fibroblast cells was extracted by whole cell lysis to carry out preliminary studies. Comparative SDS PAGE identified differences in protein profile (Fig. 120). Major difference were spotted in the region of 21kDa-45kDa and 66kDa-110kDa. The proteins were further separated by 2-D gel electrophoresis and several protein spots were identified (Fig. 121).

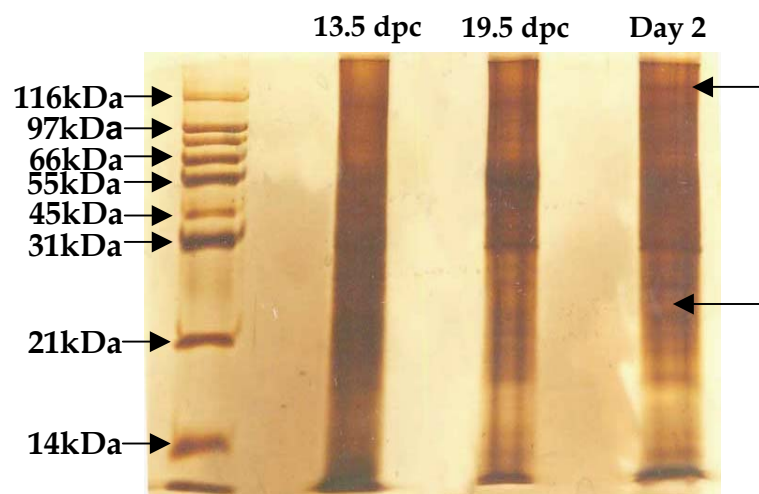


Fig. 120: Comparative SDS-PAGE protein profile of whole cell extracts from 13.5, 19.5 dpc mouse embryos and day 2 neonate mouse fibroblasts. Arrows indicate differentially expressed proteins.

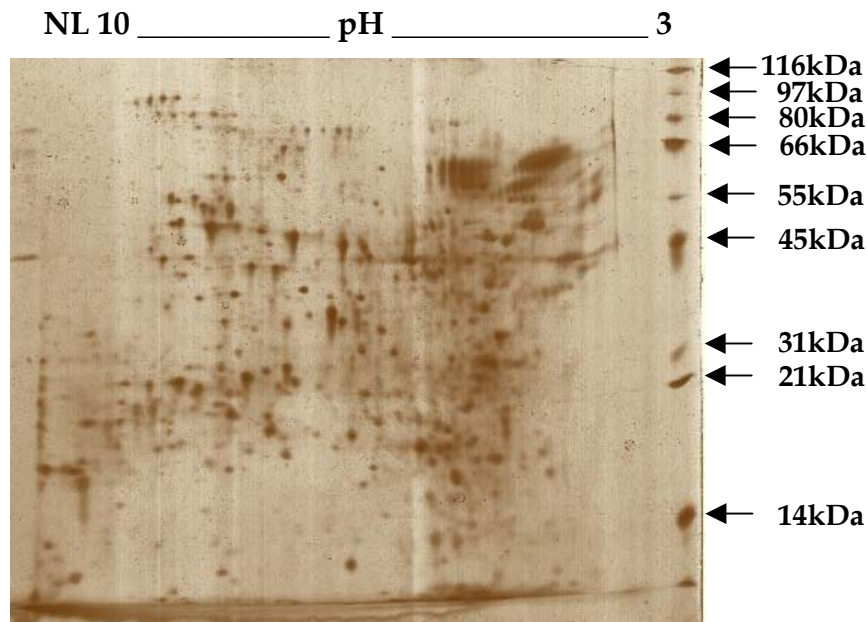


Fig. 121: 2-D gel electrophoresis of whole cell extract from 19.5 dpc mouse embryonic fibroblast. Iso electric focussing was performed using 13 cm 3-10 NL IPG strip.

Studies to detect differentially expressed spots in the 2-D profiles, in fibroblasts derived from 13.5 days old embryos, which may be supporting the growth of human embryonic stem cells are in progress. The spots, which are differentially expressed, will be cored, in-gel tryptic digested followed by MALDI-TOF to generate peptide mass fingerprint. Once identified various approaches will be used to assess their functional ability.

9.2 Fertility Conservation in Individuals with Gonadal Insufficiency

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Duration: 2003-2006

Recent advances in clinical management have improved the survival rates of cancer patients. A new group of pediatric and adult patients is emerging who are cured of cancer but rendered infertile due to aggressive therapy. These patients will benefit enormously if their gonadal tissues are cryopreserved for

future use. Cryopreservation of their gonadal biopsy could help restore their fertility later during life and ensure genetic parenthood.

Broad objectives of the study initiated are to (i) set up protocols for cryopreservation/vitrification of gonadal tissue in rats, monkeys and humans; (ii) perform *in vivo* maturation of germ cells by subcutaneous transplantation of ovarian tissue and intratesticular transplantation of germ cells/tissue biopsy in male rats and (iii) study *in vitro* maturation of germ cells by organotypic cultures of testicular and ovarian tissue.

Cryopreservation of ovarian tissue and later maturation of follicles by *in vivo* and *in vitro* methods

Methodology for cryopreservation using slow rate cooler (PLANAR, UK), isolation of germ cells from immature/ mature rat ovaries and transplantation has been standardized earlier (Annual Report 2003-04, p 178).

A study to compare germ cell viability by cryopreservation of ovarian tissue using various cryoprotectants viz. dimethyl sulphoxide, ethylene glycol and glycerol was carried out. Viability of germ cells following cryopreservation with the above cryoprotectants was 80 percent, 90 percent and 70 percent respectively as judged by trypan blue method (Fig. 122). The use of propanediol-sucrose as a cryoprotectant is being evaluated. In addition the short term (3 months) and long term (12 months) effects of antioxidants such as ITS (Insulin, Transferrin, Selenium), Vitamin A and Vitamin E in combination with different cryoprotectants is also being studied. Standardization of flow cytometry method is being carried out to assess viability of germ cells post cryopreservation.

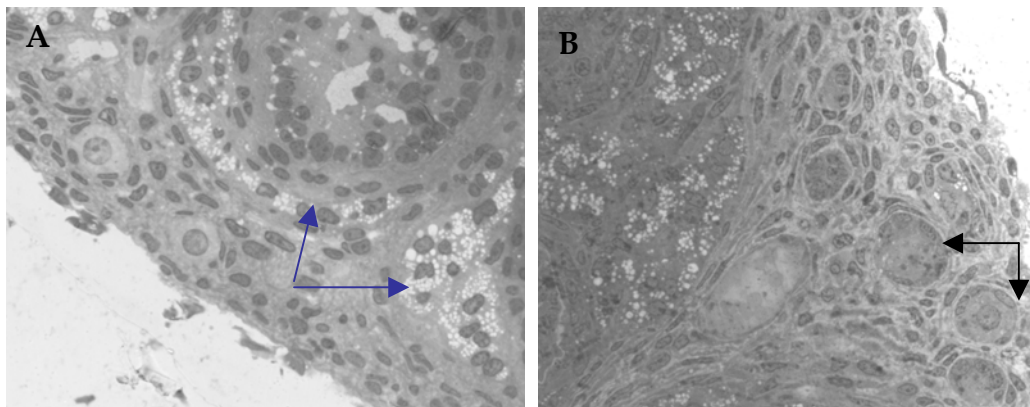


Fig. 122: Semithin sections of rat ovary showing, (A): extensive cryoinjury in larger follicles (blue arrows) while, (B): majority of the small primordial follicles (black arrows) are spared.

The major research interest is on primordial follicles in the ovary as they tolerate cryopreservation and transplantation procedures better compared to mature follicles. Primordial follicles will determine the success of ovarian preservation and future maturation to achieve genetic parenthood. It has been reported that almost 50-60 percent of primordial follicles are lost during transplantation due to hypoxic environment and ischaemia. Time required for revascularization of transplanted ovary varies from 48-72 hours in rats to about 8 days in women. The life of the ovarian graft after transplantation could be prolonged by better understanding the mechanism of primordial follicle loss post transplantation. The role of exogenous treatment of vascular endothelial growth factor in preventing primordial follicle loss will also be explored.

Experiments were conducted to understand cellular changes post transplantation at various time intervals (Fig. 123). As evident there was a massive infiltration of blood cells in the transplant, neovascularization and atretic changes in large follicles. However primordial follicles were intact and located below the surface epithelium.

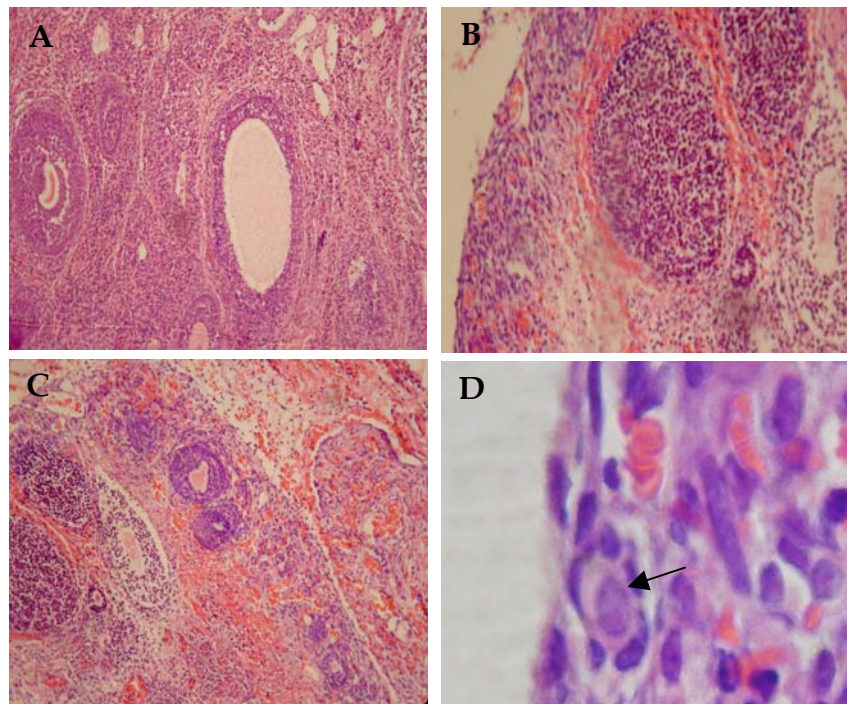


Fig. 123: Histological sections of ovary depicting kinetic cellular changes, after subcutaneous ovary transplantation. A: After 12 hours, B: After 24 hours, C: After 48 hours, note the infiltration of blood cells in the transplant. Magnification (20X). D: Healthy primordial follicle (black arrow) Magnification (100X).

Studies have been initiated in rabbits to explore the possibility of conserving fertility by intraovarian transplantation of ovarian germinative epithelium rich in stem cells rather than ovarian tissue biopsies.

Testicular germ cell cryopreservation and maturation by *in vivo* and *in vitro* methods for fertility conservation

Spermatogonial stem cells are reported to be the only cells in the postnatal animal that undergo self-renewal throughout life and transmit genes to subsequent generations. Thus the ability to cryopreserve, transplant and culture these unique cells provides a powerful system to study stem cell biology, preserve individual genomes and help restore fertility in cancer patients post chemo/radiotherapy (Annual Report 2003-04, p 180). In addition, repeated testicular biopsies could also be avoided if a small portion of the biopsy is cryopreserved when men are being investigated for cause of infertility. Germ cell transplantation has resulted in successful testicular recolonization and sperm production in male cancer patients in remission.

Methodologies for creation of germ cell aplasia by busulfan treatment, BrDU incorporation to be used as a marker to identify donor germ cells during intratesticular transplantation and procedure of transplantation via rete testis have been standardized (Annual Report 2003-04, p 180).

A study to compare germ cell viability by cryopreservation of isolated germ cells using various cryoprotective agents like dimethyl sulphoxide, ethylene glycol, glycerol and propanediol-sucrose was carried out and more than 90 percent viability of germ cells was observed by trypan blue exclusion method. Flow cytometry method to evaluate viability is being standardized. This method will allow more unbiased evaluation of germ cell status and enable examination of larger number of cells. In addition the short term (3 months), long term (12 months) effects of antioxidants such as ITS, Vitamin A and Vitamin E in combination with different cryoprotectants on improving the viability is under progress.

Experiments have been designed in rats to mimic the clinical situation where young boys suffering from cancer have their germ cells cryopreserved and transplanted in adult life to study recolonization pattern and germ cell maturation *in vivo*. Immature rats were unilaterally castrated and germ cell cryopreservation with and without BrDU labeling was carried out. The castrated animals were treated with busulfan to induce germ cell aplasia. The cryopreserved germ cells will be later transplanted via rete testis and recolonization will be studied.

In vitro maturation of germ cells will be studied by setting up an organotypic culture system of seminiferous tubules on cell culture inserts. The temporal appearance/disappearance of testis specific proteins Phosphoprotein p19, Testis-specific Histone TH2B, Transition Proteins TP1 and TP2 during the meiotic prophase and/or the mRNAs encoding them, make them valuable biochemical markers for monitoring spermatogonial differentiation.

In the testis, immunoreactive p19 first appears in late zygotene spermatocytes, is abundant throughout the pachytene stage, and decreases to undetectable levels in maturing spermatids. One of the biochemical events that occur during the meiotic process is the replacement of somatic histones (H2B) by testis-specific histone variants (TH2B) that in turn are replaced by transition proteins (TP1, TP2), whose synthesis has been shown to occur in haploid spermatids. TP1 and TP2 appear in the nucleus essentially simultaneously in association with the beginning of chromatin condensation.

The maturation of germ cells in culture will be monitored by examination of these genes specifically expressed during meiosis (p19, TH2B) and after meiosis (TP1 and TP2). The relative changes in mRNA levels of p19, TH2B, TP1 and TP2 in the testis of young rats will be related to sequential appearance of different types of spermatogenic cells in culture which will be monitored by RT-PCR. Standardization of multiplex PCR with gene specific primers p19 and TP1 is under progress (Fig. 124).

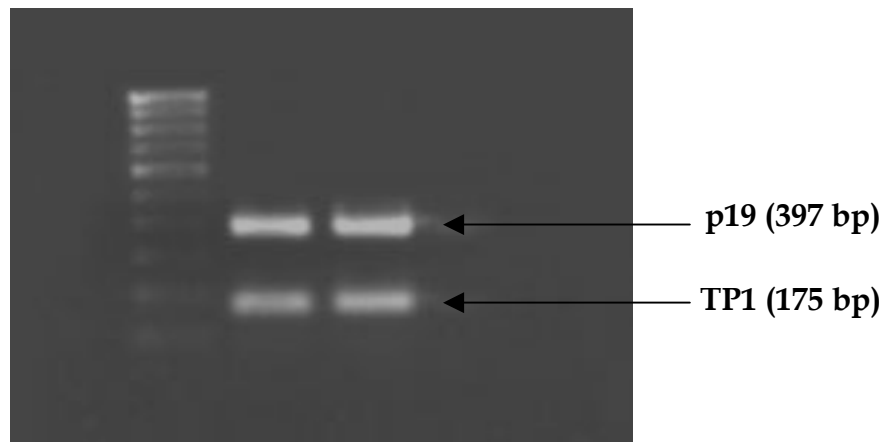


Fig. 124: Multiplex PCR with gene specific primers p19 and TP1. Lane M: 100 bp marker, Lane 1: Annealing temperature 62°C. Lane 2: Annealing temperature 64°C.