

Efforts in the Stem Cell Biology Department are focussed to realize the clinical potential of embryonic stem cells (ESC). Adult stem cells including hematopoietic stem cells (HSC) and mesenchymal stem cells (MSC) derived from bone marrow, cord blood or any other source have restricted potential because of their inability to expand *in vitro*, limited plasticity, phenomenon of cell fusion post- transplantation and loss of stem-cell like properties after several population doublings of MSCs. ESCs are easily grown and expanded in culture, an ideal source of large number of cells for transplantation and exhibit maximum plasticity. Two major concerns regarding use of ESC to treat degenerative diseases are immunological rejection and teratoma formation. Immunological rejection can be avoided by developing patient specific ESC lines by somatic cell nuclear transfer (SCNT) viz. therapeutic cloning. Simultaneously protocols for lineage specific differentiation of ESC are being perfected and preliminary published literature indicates that concerns of teratoma formation may actually not exist. None the less it is mandatory that carefully planned pre- clinical studies in non- human primates are undertaken prior to clinical use of ES cells.

Various objectives which the department aims to achieve in next 5-6 years are (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

Recently it has been demonstrated that adult stem cells have minimal transdifferentiation potential and mesenchymal stem cells lose their stem-like nature after repeated doublings *in vitro*. However, human embryonic stem cells have tremendous potential as a source of cells to treat various age-related disorders. The objective is to grow human ES cells in xeno-free environment using human feeders. Feeder layers using human fetal fibroblasts have been made and inactivated using mitomycin. Human embryos at blastocyst stage were placed on the feeder layers, after pronase treatment, or laser manipulation of inner cell mass but minimal success was achieved. The number of clinical collaborators has now been increased to obtain more number of good quality human embryos for deriving cell lines.

Superovulation was carried out in Balb/c mice and blastocysts were collected from the uterine horns 5 days post coitum. These blastocysts were placed on mitomycin inactivated mouse embryonic feeder layers. They underwent natural hatching and got attached to the feeder layers, where they hatched embryo (comprising of trophoectoderm and inner cell mass) showed proliferation and growth on the feeder.

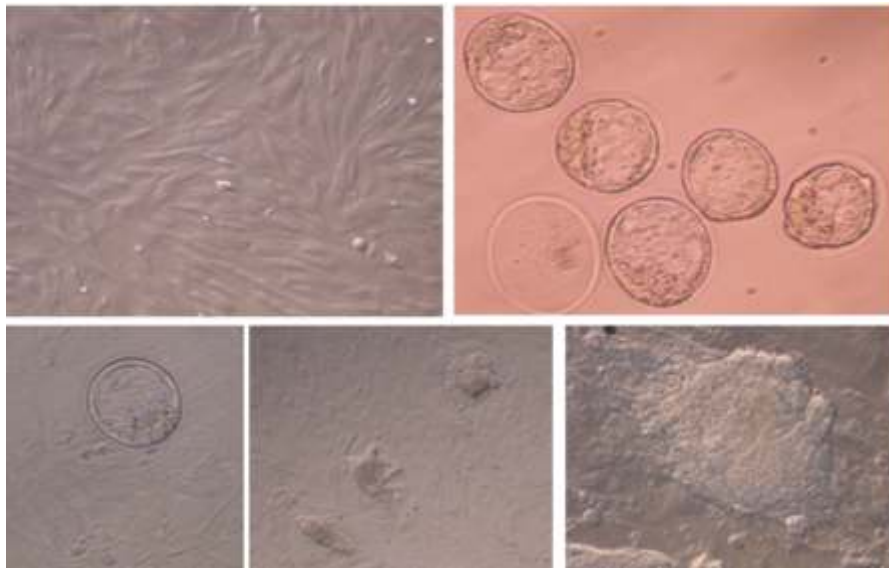


Fig.139: Attempts to grow mouse blastocysts on inactivated MEF layer. A. Mitomycin inactivated feeder layer B. in vivo created mouse blastocysts after superovulation C. Hatched blastocyst D. Attached blastocysts on feeder layer E. Growing blastocysts on feeder layer.

Efforts are ongoing to identify by 2-D approach, the proteins secreted from mouse embryonic fibroblast feeder layers that keep hES cells in undifferentiated state. We have successfully generated 2-D profiles of 13.5dpc, 17.5dpc and neonatal mouse fibroblasts. Many proteins were found differentially expressed and further analysis of these differentially expressed spots is in progress.

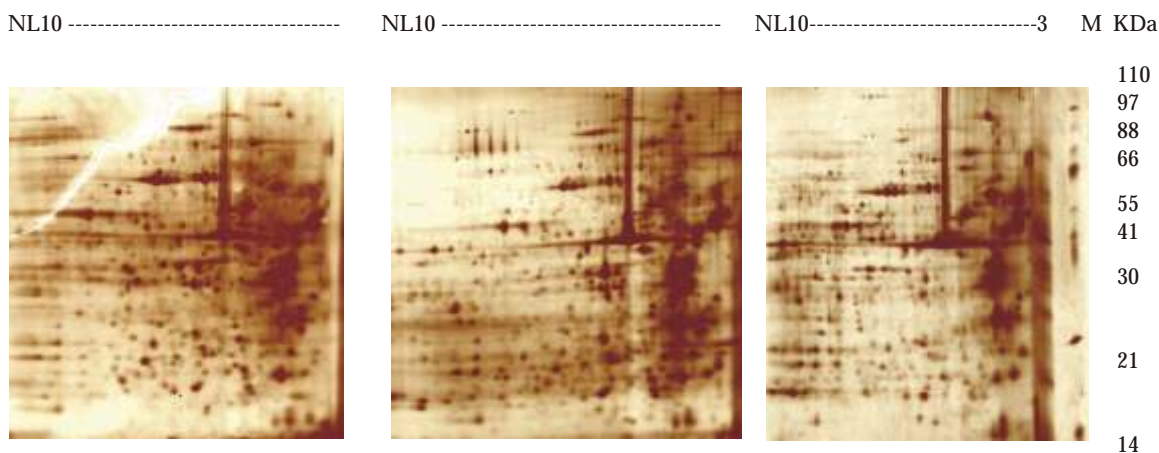


Fig.140: 2-D Profile of (A) 13.5dpc, (B) 17.5dpc, (C) neonatal mouse fibroblasts. 3-10 NL, 13 cm IPG strips were used in IEF followed by 10% SDS PAGE. Spots were visualized by silver staining.

Generation of non human primate ES cells

Work has been initiated to establish IVF in non- human primates to derive ES cell lines for preclinical studies. Appropriate clearance from IAEC and CPCSEA have been obtained to work on marmosets and Bonnet monkeys. Blastocysts will be obtained by *in vivo* flushing from normally fertilized animals as well as by IVF post superovulation sequentially with FSH and hCG. The inner cell mass of the blastocysts will be a source of ES cells.

The female marmosets have been successfully stimulated and 1012 eggs per animal have been obtained. Penile vibrator (FERTICARE) is being used for sperm collection.

Development of patient specific ES cell lines by somatic cell nuclear transfer

IAEC permission has been obtained to use mouse and sheep eggs to establish the procedures of somatic cell nuclear transfer. The guidelines and permission from National and Institute ethics committee to use human eggs for this project is being awaited. POLSCOPE with spindle view, a fully motorized inverted microscope, pilot guided laser and electrofusion apparatus have been procured to facilitate this project. Briefly, during SCNT, eggs have to be matured *in vitro*, enucleated by removing the spindle, a somatic cell has to be fused with the enucleated egg and then stimulated to form a blastocyst.

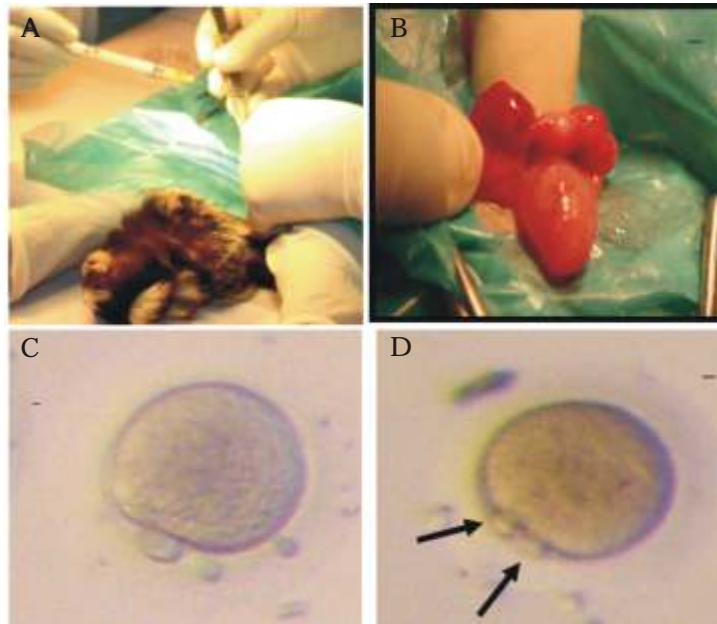


Fig. 141: Laprotomy, egg retrieval and IVF in marmosets. A. Laprotomy after superovulation B. Egg retrieval C. Marmoset egg with polar body D. Extrusion of second polar body after fertilization

Meiotic spindle has been observed in live mouse eggs by POLSCOPE technology which enables viewing spindle of live eggs without any fixation or fluorescent stain. Efforts are ongoing to standardize the methods of gamete micromanipulation and blastocyst culture using mouse and sheep oocytes after IVF or ICSI.

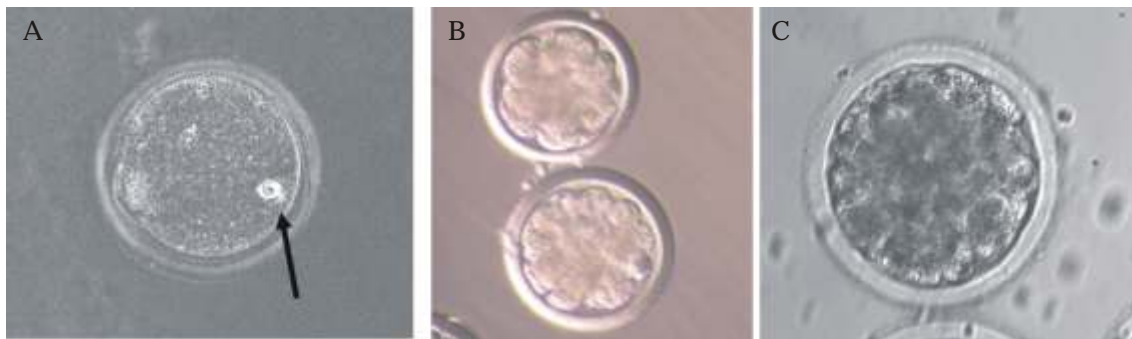


Fig. 142: A. POLSCOPE image of spindle (black arrow) in live mouse oocyte. B & C in vitro culture of sheep embryos

To develop an in vitro differentiation system for teratological screening using human ESCs:

Each year a number of new chemical entities are developed by pharmaceutical industries as new 'lead' drug molecule, however most of them have teratogenic or embryotoxic effects on target organism, and thus withdrawn. The embryo toxicity

testing is either performed *in vivo* using pregnant animals or *in vitro* on cultured embryos or embryonic tissues and cells from pregnant animals. In either case, pregnant animals have to be sacrificed. Whole animal test procedures are inadequate to screen a large number of chemicals already present as well as new ones being introduced in to market each year. These tests involve high cost, large number of animals is used and large amount of chemicals and time is required for each full-scale assessment. Apart from *in vivo* studies, there are many *in vitro* systems in which primary cultures or established cell lines are commonly used to analyze the mutagenic or embryotoxic potential of drugs and xenobiotics. However, these cellular systems do not include developmental processes from early embryonic stages up to terminally differentiated cell types.

An alternative approach to screen number of drugs is offered by pluripotent embryonic stem (ES) cells. The undifferentiated stem cell lines are characterized by unlimited self-renewal capacity and have been shown to differentiate *in vitro* into cells of all three primary germ layers. These cells recapitulate cellular developmental processes and gene expression patterns of early embryogenesis during *in vitro* differentiation.

We propose to obtain hES cell lines from Professor Stephen Minger, King's College, UK and after maintaining and expanding the cells, we aim to develop this test. This will involve development of methods for lineage specific differentiation of hES cells into cardiac cells, hepatocytes and germ cells using different media compositions, growth factors and *in vitro* conditions. Embryotoxicity will be studied by a battery of cell based and molecular marker based tools.

9.2 Fertility Conservation in Individuals with Gonadal Insufficiency

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Duration: 2003-2009

The long term goal of this programme is to cryopreserve and bank gonadal tissue under liquid nitrogen, so that it can be subsequently used to achieve genetic parenthood. Studies have been carried out using rat gonadal tissue and recently the

project using human gonadal tissue to develop cryopreservation protocol and in vitro maturation of germ cells has been approved by the Institute Ethics Committee.

Cryopreservation

Studies have been initiated in rats to cryopreserve the gonadal tissue using different cryopreservative agents by slow rate cooler (PLANAR, UK). Viability post thaw, after 3- 12 months cryopreservation, is being checked using flow cytometry analysis. Of the four of cryoprotectants tested, dimethylsulfoxide (DMSO), propanediol sucrose (PROH) and ethylene glycol (EG) appear to be more effective for rat testicular tissue cryopreservation while DMSO and EG appeared to be more effective for rat ovarian tissue cryopreservation. The effect of different additives on the cryopreservation process is presently under study.

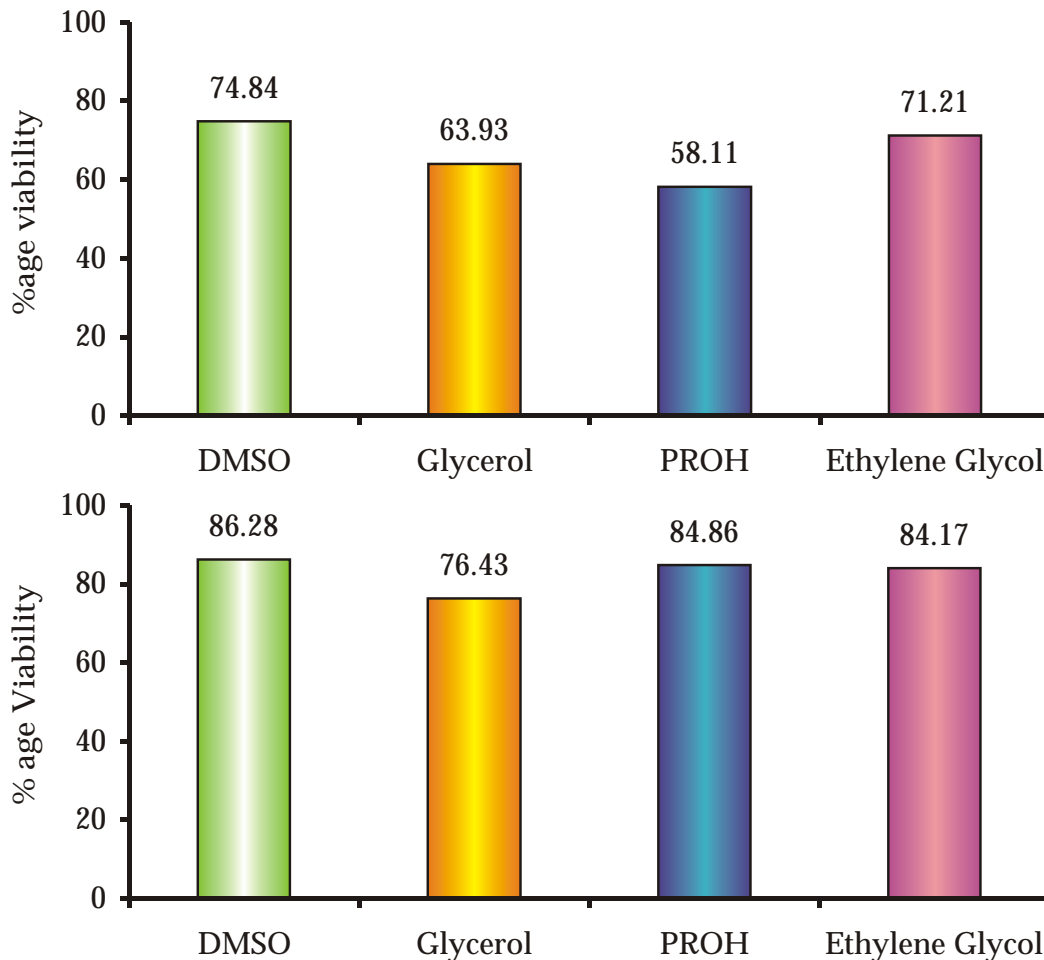


Fig.143: Percent viability of rat gonadal tissue post cryopreservation with different cryoprotectants by flow cytometric analysis

Transplantation studies using ovarian and testicular tissue

These studies have been carried out in immature Holtzman rats to mature germ cells *in vivo* by subcutaneous transplantation of ovaries and germ cells transplantation via rete testis in males.

Briefly, female rats were bilaterally ovariectomized and the ovaries were transplanted subcutaneously on the ventral side. The ovarian graft was studied at various intervals post-transplantation. The graft was functionally active as reported earlier (Annual Report 2004-05, p 181). In the reporting year, morphometric studies were carried out to quantitate the primordial follicles in the graft and it was observed that a loss of almost 50 percent of follicles occurred by 72 hrs post-transplantation due to hypoxia and ischemia. VEGF was administered to induce early neo-angiogenesis with the hypothesis that this may help reduce the loss of primordial follicles (PF) thereby increasing the life of the ovarian graft. At 72 hrs post transplantation, there was no significant difference in the count of the primordial follicles between the two groups (Fig. 144). Studies are in progress to evaluate the effect of VEGF one-week post transplantation.

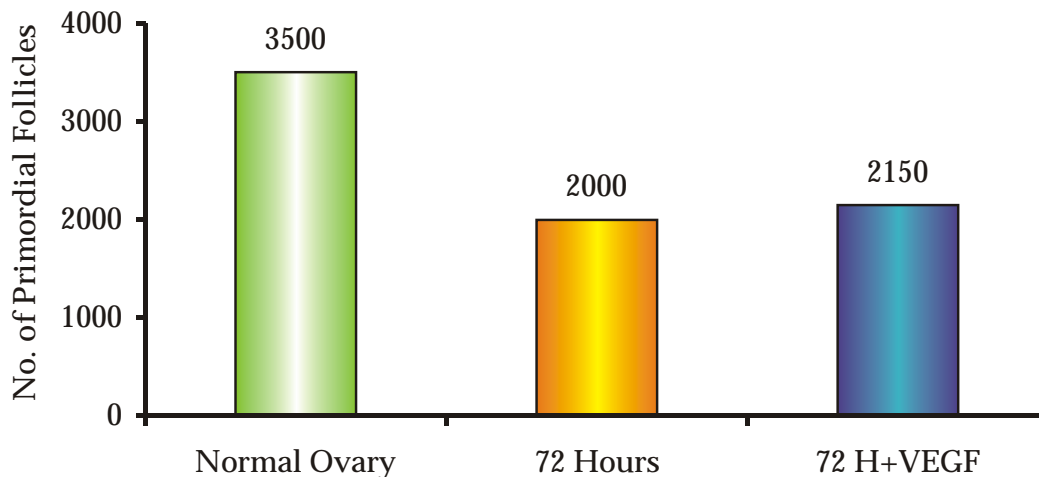


Fig. 144: Effect of subcutaneous transplantation and VEGF treatment on primordial follicle count in sc transplanted rat ovary

A marked hypertrophy of ovarian surface epithelial (SE) cells was observed after VEGF treatment. This layer has recently been described in literature as a source of 'stem cells', which continuously replenish the primordial follicle pool in the ovary in contrast to the earlier held hypothesis of fixed number of follicles in ovary since birth.

For transplantation studies with testicular tissue, immature male rats were unilaterally castrated. Germ cells from the excised testis were cryopreserved. The unilaterally castrated animal was treated with busulphan to induce germ cell aplasia. After one month, the cryopreserved germ cells were transplanted via the rete. Rats

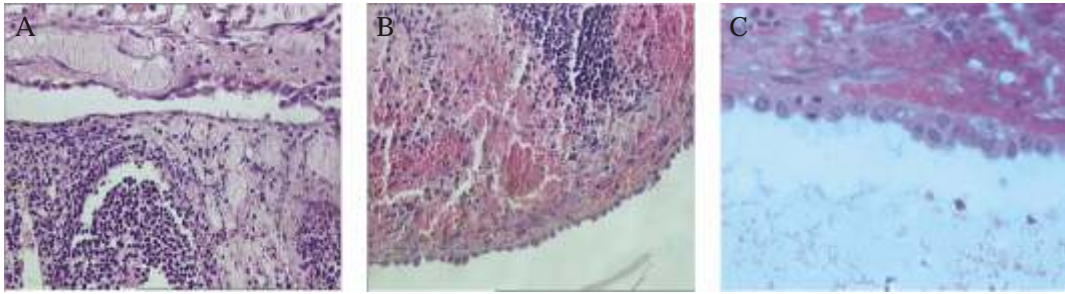


Fig. 145: Histological sections of ovarian tissue after 72 hours of subcutaneous transplantation using fresh tissue. A. Control ovary B & C. VEGF treatment. Note the difference in the cells of the surface epithelium (arrows)

were sacrificed a month later and colonization of transplanted germ cells was studied.

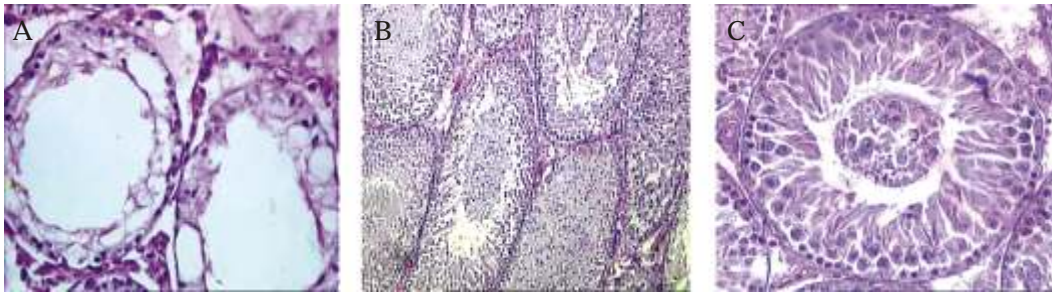


Fig. 146: Histological sections of rat seminiferous tubules. (A) Germ cells aplasia due to busulphan treatment B & C Recolonization of seminiferous tubules by transplanted germ cells.

Organotypic cultures using rat ovarian and testicular tissue were maintained for varying periods of time and further studies are planned using human tissue.

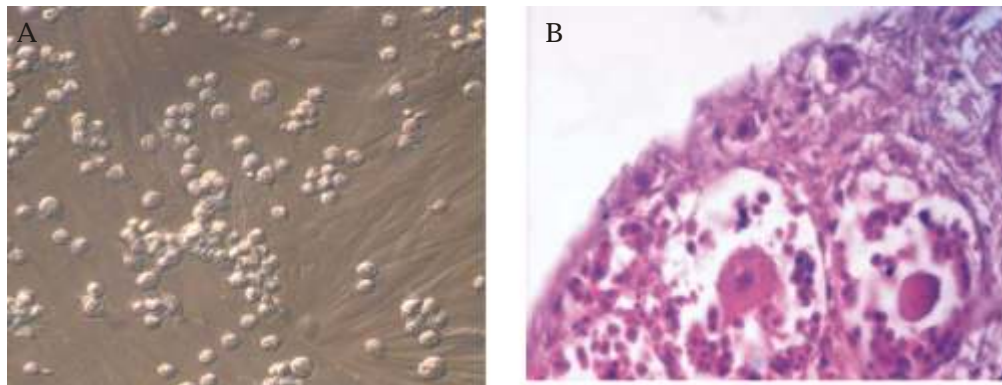


Fig 147: Testicular germ cells were maintained in minimal media for a period of 6 weeks at 34 C. The somatic cells spread out and form a feeder layer on which the germ cells attach and form small colonies (A) Rat primordial follicles appear healthy after 6 days in paraffin sections of organotypic culture of small ovarian tissue pieces. The larger follicles are disorganized. At this stage PF need to be dissected & cultured separately (B)