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Stem Cells and their Therapeutic Potential

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SUMMARY

Recent developments on embryonic stem cell lines from human blastocysts have the potential to revolutionize many of our approaches to study human biology and medicine. The current method of treating diseases will be revolutionized in the near future and medicine-based treatment will be replaced by cell-based therapy. Embryonic stem cells appear to have greater potential than adult stem cells because of their flexibility and genetic stability. Combined with therapeutic cloning, embryonic stem cells research has become the focus of intense research across the world.

Embryonic stem cells are pluripotent in nature and extremely valuable because they possess three properties not found together in other cell types. Firstly, they replicate indefinitely without undergoing senescence (aging and death) or mutation in their genetic material. They are thus a large-scale and invaluable source of cells. Secondly, embryonic stem cells are genetically stable. Thirdly, they exhibit marked plasticity i.e., can differentiate into variety of cell types in tissue culture.

There is an urgent need to generate well-characterized embryonic stem cell lines and make them available for further research in our country. Embryonic stem cell lines are derived from surplus cryopreserved embryos generated in IVF labs after taking proper informed consent. Embryonic stem cell programs need to be developed in non- human primates, as this will be essential to carry out pre-clinical evaluation of efficacy and efficiency of embryonic stem cell therapy. Methodologies to derive 'isogenic' embryonic stem cell lines after somatic cell nuclear transfer and therapeutic cloning need to be established since this will take care of problems associated with immune rejection during stem cell therapy to treat various disorders.

Studies have been initiated by us to develop human embryonic stem cell lines in xeno- free environment for future therapeutic purposes. Dedicated efforts, meticulous planning, appropriate ethical guidelines and uninterrupted financial support is required for successful fruition of embryonic stem cell research in our country.

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"Stem cells" are potentially immortal cells capable of self-renewal and also give rise to differentiated cells. No area of research except gene therapy has evoked so much enthusiasm and hope as stem cells. Most medical experts view stem cell research as the new frontier in medicine, a huge breakthrough that could save millions of lives.

Medicines today are based on drug therapy dominated by antibiotics, chemotherapy and other pharmaceuticals. Medicine of future will be based on cell therapies, focused on repair of tissues/ organs by cell transplants i.e. instead of drugs to prevent malfunction or death; diseased cells will be replaced by healthy differentiated stem cells.

Depending on their plasticity i.e. ability to differentiate into various cell types, stem cells may be: (a) **Totipotent** stem cells give rise to a fully functional organism as well as to every cell type of the body; (b) **Pluripotent** stem cells are capable of giving rise to virtually any tissue type, but not a fully formed organism; (c) **Multipotent** stem cells are more differentiated cells and give rise to a limited number of tissues or (d) **Unipotent** stem cells can differentiate into only one kind of cell e.g. germ cells.

Knowledge about stem cells and their potential applications has been known for the last 30 years when mouse embryonic stem cells were discovered. The development of embryonic stem (ES) cells can be traced from mouse teratocarcinomas, tumors in the gonads. It was found that teratocarcinomas produced differentiated embryonic carcinoma (EC) cells that contain a variety of cell types. However, these cells contain genetic mutations and scientists hoped to develop them directly from the blastocyst. This was accomplished in mice

Stem cells have been obtained from various body organs as shown in the following table:

Source	Types of Stem Cells	Potential
Adult	Cord blood stem cells, liver stem cells, epithelial stem cells, neuronal stem cells, pancreatic stem cells, spermatogonial stem cells etc.	Multipotent or unipotent
Embryo	Trophoectodermal stem cells, embryonic stem cells, morula stem cells	Totipotent or pluripotent
Fetus	Fetal germ stem cells, fetal germ carcinoma cells	Pluripotent

in 1981 by Gail Martin and Martin Evans (1, 2) independently. Later, in 1995, Thomson and his group (3) derived ES cells from rhesus monkeys. The major breakthrough came in 1998 when Thomson and his group (4) derived human ES cell lines, which were also shown to be pluripotent in nature. Simultaneously Shamblott and his group (5) derived pluripotent embryonic germ (EG) stem cell lines from primordial gonadal ridge from human abortuses. The potential of EG cells to differentiate into various cell types is relatively limited compared to ES cells, since they occur much further during development (5-9 weeks as compared ES cell derivation on day 5 post fertilization).

In mammals, the fertilized oocyte and the cells of morula stage embryos are totipotent, capable of giving rise to more than 220 cell types. The blastocyst is formed 5- 6 days after fertilization and is about 150 microns in diameter. The outer layer is the trophoblast and gives rise to the placenta whereas the cluster of about 30-50 cells inside the trophoblast is the inner cell mass

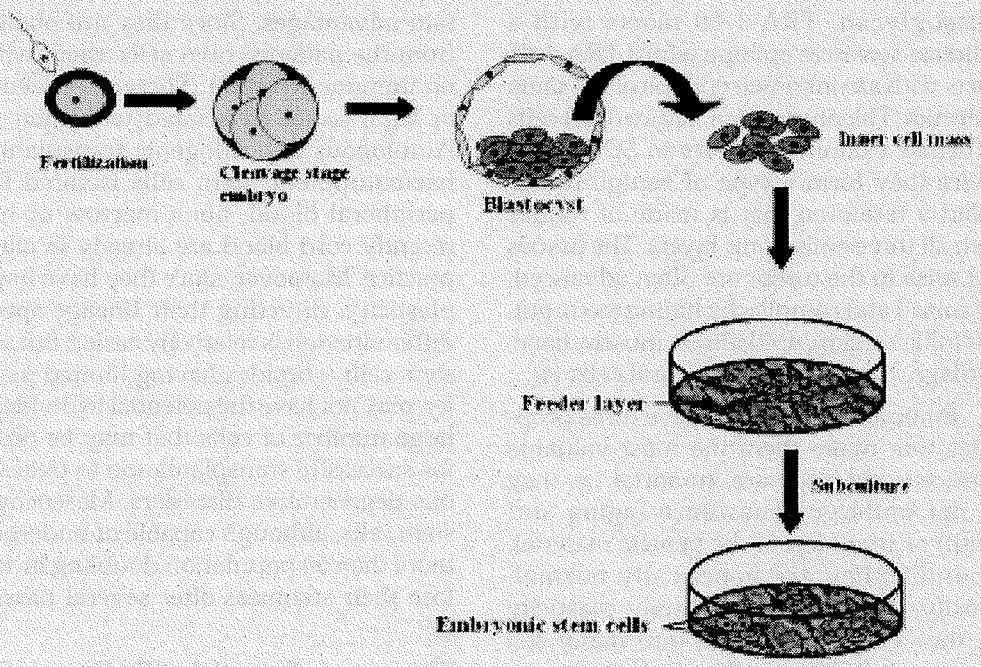

Fig 1. Schematic representation of embryonic stem cell line derivation

which in future will give rise to all cell types of the embryo viz. ectoderm, mesoderm and endoderm. When grown in vitro inner cell mass gives rise to ES cells that are pluripotent. These cells remain undifferentiated with unlimited self-renewal capacity as long as they are grown on feeder layer and spontaneously differentiate into somatic cells when feeder layer is removed (6). In suspension culture these cell aggregate in to balls of differentiated cells called 'embryoid bodies', which contain cells of all the three primary germ cell lineages (7).

Depending on the origin, stem cells express different kind of antigens on their cell surface commonly known as 'markers' which help in characterisation and identification of these cells (8). For example, haematopoetic stem cells express CD34 whereas pluripotent stem cells express stage specific embryonic antigens (SSEA 1, 3 & 4) and tumor recognition antigens (TRA-1-60, TRA-1-81). These antigens were originally identified by monoclonal antibodies recognising defined carbohydrate epitopes associated with lacto- and globo-series glycolipids. SSEA-1 is expressed on pre-implantation eight- cells stage murine embryos and teratocarcinoma stem cells. SSEA-3 and -4 are synthesised during oogenesis and are present in the membranes of oocytes, zygotes and early cleavage-stage embryos. Undifferentiated embryonic stem cells and carcinoma cells of primates and humans express SSEA-3 and SSEA-4, but not SSEA-1. TRA-1-81 and TRA-1-60 monoclonal antibodies (tumor rejection antigen) recognise antigens that are associated with a pericellular matrix

proteoglycan. TRA-1-60 reacts with a sialidase-sensitive epitope whilst TRA-1-81 reacts with an unknown epitope of the same molecule. The pluripotent nature of ES cells is further examined *in vivo* in SCID mice where they form a type of benign tumor called a teratoma that is made of tissues from all three embryonic layers. The tissues that arise in the tumor are often advanced, organized and complex including teeth, gut, hair follicles, skin, epithelium, muscle, bone, cartilage, lung tissue and neural cells (4).

Embryonic stem cells have three properties that make them the most valuable stem cells viz. they are immortal i.e. they do not undergo senescence (aging and death) or mutation of the genetic material. Secondly they are genetically normal. Thirdly, they exhibit maximum plasticity i.e. they can differentiate into many cell types in vitro. Human ES cells hold the potential to revolutionize therapies for many different diseases or disorders caused by cellular degeneration or damage. Only ES cells have the true capacity to develop into all the different cell types of the body and therefore represent the greatest potential for future cell therapies. As ES cells can proliferate indefinitely they represent an inexhaustible supply of cells, which can be cultured in quarantine conditions, free from the risk of other infectious agents.

Stem cells can also be derived from various developed body tissues in post-embryonic life of any organism and are termed as the adult stem cells e.g. hematopoietic stem cells in blood, bone marrow or umbilical cord blood and mesenchymal stem cells in bone, muscle, milk teeth etc. Adult stem cells are multipotent. Stem cell therapies using stem cells from adult organs will complement but cannot replace therapies that may eventually be achieved from ES cells. However adult stem cells have certain advantages. Since they are obtained from the patients' own cells, there will be no immune rejection. There are no ethical or legal issues associated with their use. Autologous and allogenic transplants of haematopoietic stem cells isolated from peripheral blood, bone marrow or more recently cord blood are already in clinical practice. Moreover, since they have limited plasticity, directing their lineage specific differentiation is relatively easier. But adult stem cells – besides having limited plasticity, may not have the potential to divide into large number of cells that may be needed for successful transplantation to treat various degenerative disorders. Mesenchymal stem cells, although capable of undergoing more than 50 population doubling in-vitro, lose their stemness after several passages (9).

Therapeutic Potential of Embryonic Stem Cells

Embryonic stem cells are a source of unlimited supply of specialized cells, which may not be possible with adult stem cells. Most of the age related cellular degenerative diseases cause functional disabilities and can be potentially cured by cell based therapies e.g. diabetes, neurodegenerative disorders like Parkinson's disease, Alzheimer's disease, heart diseases, stroke, spinal cord injuries, burns, immunodeficiency diseases, diseases of bones and cartilage like osteoarthritis, fractures, osteogenesis imperfecta, chondrodysplasias, cancer etc. For treating such type of diseases, large number of cells is required to resolve the function and ES cells are the only source that could generate such a large number of cells.

ES cells can be triggered to differentiate *in vitro* into fat cells, cells of brain and nervous system, insulin- producing cells of

pancreas, bone cells, hematopoietic cells, yolk sac, endothelial cells, endodermal cells, smooth and striated muscle cells including heart muscle cells. (10) Recently mouse ES cells have been reported to differentiate spontaneously into germ cells. (11, 12)

Besides the clinical potential, ES cells are also an ideal tool to understand basic developmental biology i.e. how various cell lineages become committed and differentiate into various lineages. They may also serve as tools to understand the cause of birth defects and may lead to their prevention in future. ES cells may be differentiated into various cell lineages which will serve as *in vitro* models for human diseases that are constrained by animal or culture models at present e.g. HIV and hepatitis C virus grow only in human or chimpanzee cells; only partial representation of Alzheimer's disease is possible in currently existing animal models. Recently human ES cells are also being used to create accurate human Lesch-Nyhan disease model (13), an ideal vehicle to carry the genetic material into diseased tissue that provides a necessary protein for therapeutic effect, as an ideal source of cells for drug screening, testing & toxicity and for functional genomics and proteomics. A new branch of science has emerged due to advances in stem cell research called tissue engineering which will result in making of tissues/ organs using artificial, biodegradable scaffolds for transplantation. Now it is possible to form three -dimensional organs by the use of biodegradable polymer scaffold, which allows proper alignment, and physical disposition of the cells and extracellular matrix to produce a reasonable organ (14) These organs along with stem cell technology allow the creation of biohybrid organs e.g. skin, cartilage, bone etc have been bioengineered *in vitro* for burns patients. Stem cells can also be used to deliver growth factors *in vivo* e.g. in the nervous system in the form of cell based drug delivery.

Availability of Human Embryonic Stem Cell Lines for Research: Till date more than 120 human ES cell line have been reported worldwide. Only a handful is available for research. United States has restricted its researchers to use existing 72 cell lines and no creation of new lines by destroying surplus frozen embryos is permitted. However, existing stem cell lines are gradually degrading and will soon be useless for research. Most of the lines have become useless; some of the lines are genetically identical to others; only 11 remain available for research. Research using embryonic stem cell lines has been authorised in Britain, U.S. private labs and in both government and private labs in the U.K., Japan, France, Australia and other countries. Till date no established human embryonic stem cell line has been reported in India.

Therapeutic Cloning: The embryonic stem cell lines to be used in future for therapeutic purposes are bound to have immunological differences. The impact of this difference will vary with different conditions – while using 'differentiated' stem cell transplantation to cure various degenerative disorders. In the brain, where immune rejection is less effective it may be possible that no immunosuppressive drugs would be required, or perhaps, only a low dose. When cells are transplanted to other sites, the patient would have to choose between the disadvantages of the initial condition and a lifetime of taking immunosuppressive drugs (and the resulting greater vulnerability to infections and cancer)

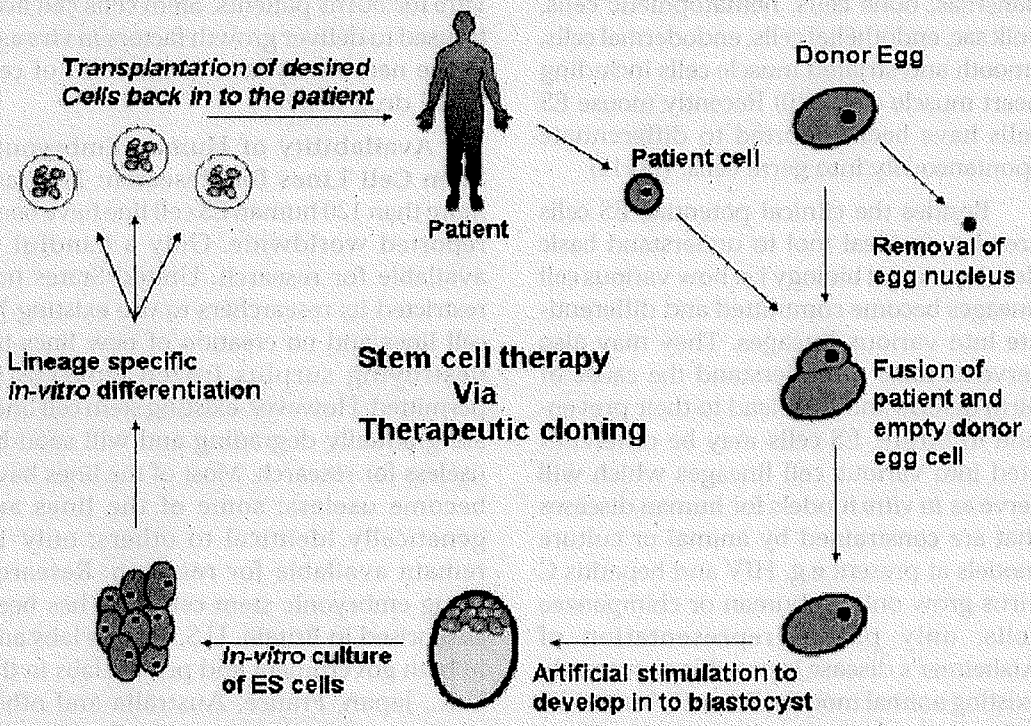

Fig 2. Schematic representation of derivation of stem cell line after "Somatic Cell Nuclear Transfer".

Matching embryonic stem cells to potential recipients may not be ethical, since it will require destruction of a large number of in vitro fertilized embryos. The immunogenicity of a cell depends on its expression of Class I major histocompatability antigens (MHC), which allows the body to distinguish its own cells from foreign. The immunological rejection of human ES-derived cells might be avoided by genetically engineering the ES cells to express the MHC antigens of the transplant recipient or by using somatic nuclear transfer technology.

During therapeutic cloning, somatic cell nuclear transplantation (SCNT) is carried out, with no goal of attempting to implant the resulting blastocyst in a uterus. An egg has its own nucleus removed and replaced by a nucleus taken from a somatic (e.g. skin) cell. The oocyte, thus "fertilized," could be cultured *in vitro* to the blastocyst stage. ES cells could then be derived from its inner cell mass, and directed to lineage specific differentiation. The result would be differentiated (or partly differentiated) ES-derived cells that match exactly the immunological profile of the person who donated the somatic cell nucleus, and who is also the intended recipient of the transplant-a labor intensive, but truly personalized therapy.

Clones have been produced from somatic cells of various animal species viz. sheep, cattle, goats, rabbit, pigs and recently dogs (15-20) However, despite considerable efforts by experienced labs, the same

method has failed to work in non- human primates and humans. Simerly and co-workers reported defective mitotic spindles after SCNT in nonhuman primate embryos and reported that therapeutic cloning may not be possible in primates (21, 22). They hypothesized that this may perhaps be due to the depletion of microtubule motor and centrosome proteins lost to the meiotic spindle after enucleation.

Ethical Issues Involved in ES Cells Research

Embryonic stem cell research along with therapeutic cloning is a highly sensitive area for research as it is associated with several ethical issues. There are three main issues viz. availability of embryos for stem cell research, creation of embryos specifically for research and source of eggs for therapeutic cloning.

A. It is known that very few cryopreserved embryos are available for research and their quality is compromised as the best ones are always implanted. In general, freeze thaw rate of cryopreserved embryos is about 65%, only about 25% of thawed embryos develop to blastocyst stage and the chances to derive successful stem cell lines is only 15%. This means that about 50 cryopreserved embryos are needed to hopefully derive one good cell line.

B. Use of embryos for research is a sensitive issue but it is a condition of "no harms and many benefits". If our society accepts abortions, then embryo research should not be a controversial issue. It needs to be decided the stage up to which embryonic development should be permitted for research and whether we could create embryos specifically for research. In general, it is believed that embryos up to 14 days have no relevant physical properties neither a stable identity since twinning and reabsorption of twins is still possible. Thus embryonic research, which involves the use of blastocyst cultured only up to Day 5, as such is not an issue but the issue is whether embryo could be created specifically for research as a part of therapeutic cloning protocol.

By definition an embryo results from fusion of male and female pronuclei i.e. fertilization of egg by sperm. However, during somatic cell nuclear transfer, there is no fusion of gametes – it is just an adult nucleus transferred into an enucleated egg. The embryo is cultured only up to day 5 to obtain blastocyst from which 'patient specific' cell lines are derived.

C. The other major issue that is extremely sensitive is the source of eggs for somatic cell nuclear transfer. Risks involved in egg donation are minimal but do exist such as bleeding, scarring, pelvic swelling, unintentional pregnancy etc. Donors may experience premenstrual syndrome - like symptoms, could get pregnant since due to hormonal treatment they become highly fertile, ovaries may get hyper-stimulated, could become infertile or might get ovarian cancer. But these risks exist even for patients who undergo IVF cycles. In a typical IVF cycle, the female partner always undergoes hyperstimulation of ovaries to collect eggs for *in vitro* fertilization with the sperm.

Will it be ethical to give financial incentive to egg donors and how much? Most egg donors are fertile young women

in 20s or early 30s and usually earn about $ 3000 to 5000 in USA. The other alternative is to request couples who attend IVF clinics to donate few of their eggs for research or thirdly the scientists need to establish methods to *in vitro* mature human follicles from ovaries removed due to various clinical indications. In the near future it may be also possible to differentiate ES cells into germ cells, which could be used as a source of eggs for therapeutic cloning.

Therapeutic cloning is permitted in our country. However, creation of embryos is not yet permitted by the policy makers. Stem cell research and therapeutic cloning warrants focussed research efforts rather than legal restrictions.

Paradigm Shifts that have occurred due to Stem Cell Research

Cancer Stem Cells: Recently it has been shown that all kinds of cancers are stem cell diseases. Even after extensive research, it appears that current approaches to cure cancer are targeting wrong cells. New treatment modalities need to target cancer stem cells, which form a very small fraction of cells within the tumor. This has resulted in a conceptual paradigm shift of how tumors are formed, spread and are treated. Recurrence of cancer is now understood to be because conventional cancer therapies target majority of cells in the tumor and the cancer stem cells escape the treatment. These immature stem cells are more resistant to chemotherapy and radiotherapy than the mature cancer cells and result in recurrence.

Studies have shown that cancer stem cells are the only cells present in many different kind of cancers, including solid tumours (breast cancer, brain tumours), that have the capacity to keep the tumours growing e.g. in leukaemia cases it is only one in a million cells that has the ability to sustain the disease. To cure cancer it will be ideal to devise therapies that will target cancer stem cells (23).

Stem Cells in Ovaries: An underlying principle of female reproductive biology appears to have been challenged by the compelling evidence reported by Johnson and co-workers (24, 25) that proliferative germ cells exist in the surface epithelial cell layer of mice ovary which keep replenishing the oocytes pool in the postnatal life. He demonstrated the presence of cells in the surface epithelium which stained positive for 5- bromodeoxyuridine and mouse vasa homologue (MVH), a gene expressed exclusively in germ cells. Cells in the surface epithelium are also positive for synaptonemal complex protein 3 in juvenile and adult ovaries. When the wild-type ovaries were grafted into transgenic mice expressing green fluorescent protein (GFP) showed appearance of preantral follicles with GFP negative granulosa cells and GFP positive oocytes. Finally, the germ cell toxicant busulphan is found to eliminate primordial follicle reserves by early adulthood without inducing follicular atresia, indicating the presence of proliferative germ cells between postnatal days 25 and 40. Similar evidence has also been generated in human ovaries. These results have significant clinical implications related to the therapeutic expansion of the follicular reserve as a means to postpone normal and premature ovarian failure.

Future Outlook of Stem Cells

Stem cells have come a long way and are with us to stay. But lot of hurdles need to be overcome and methodologies need to be refined before they can be incorporated in clinical settings. We have to develop technologies to control their differentiation into

pure cell populations in large numbers. The availability of a homogenous cell population will enable stem cell transplantation to replace diseased cells, possible in future. Secondly we need to achieve 100% differentiation of these cells since even a single undifferentiated cell may lead to teratoma formation. Therapeutic cloning protocols need to be developed with fair success rate to avoid immune rejection at the time of cell therapy. Finally we need to produce large number of embryonic stem cell lines and make them available for research purposes immediately. Extensive research needs to be carried out to elucidate the mechanisms of stem cell lineage specific differentiation. They are like a wild horse, which has to be tamed before being used to its full potential.

In future, it might be possible to "reprogram" an adult cell - even one as specialised as a skin cell, for example - to become any other cell type in the body. Extensive work on embryos will help to understand some of the secrets of how early cells are controlled. What makes a cell committed or how they get out of control to form tumor are some basic questions that will be answered in near future. Success to realize full potential of stem cells and to develop effective and safe therapies requires time, money, perseverance and an urgent need to lay down appropriate national ethical guidelines

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