Review Article

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Sourcing human embryos for embryonic stem cell lines: Problems & perspectives

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The ability to successfully derive human embryonic stem cells (hESC) lines from human embryos following *in vitro* fertilization (IVF) opened up a plethora of potential applications of this technique. These cell lines could have been successfully used to increase our understanding of human developmental biology, transplantation medicine and the emerging science of regenerative medicine. The main source for human embryos has been 'discarded' or 'spare' fresh or frozen human embryos following IVF. It is a common practice to stimulate the ovaries of women undergoing any of the assisted reproductive technologies (ART) and retrieve multiple oocytes which subsequently lead to multiple embryos. Of these, only two or maximum of three embryos are transferred while the rest are cryopreserved as per the decision of the couple. In case a couple does not desire to 'cryopreserve' their embryos then all the embryos remaining following embryo transfer can be considered 'spare' or if a couple is no longer in need of the 'cryopreserved' embryos then these also can be considered as 'spare'. But, the question raised by the ethicists is, "what about 'slightly' over-stimulating a woman to get a few extra eggs and embryos? The decision becomes more difficult when it comes to 'discarded' embryos. As of today, the quality of the embryos is primarily assessed based on morphology and the rate of development mainly judged by single point assessment. Despite many criteria described in the literature, the quality assessment is purely subjective. The question that arises is on the decision of 'discarding' embryos. What would be the criteria for discarding embryos and the potential 'use' of ESC derived from the 'abnormal appearing' embryos? This paper discusses some of the newer methods to procure embryos for the derivation of embryonic stem cell lines which will respect the ethical concerns but still provide the source material.

Key words Cryopreserved - embryonic stem cell lines - hESC - human embryos

Introduction

Embryonic stem cell lines are derived from cells of the early mammalian embryos. The two main characteristics of these cell lines are their capability of unlimited and undifferentiated proliferation *in vitro*. Even after four to five months of undifferentiated proliferation *in vitro*, these cells maintain the potential to differentiate into any of the three embryonic germ layers, including gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous epithelium (ectoderm). Thomson *et al*[1] were the first to report on the successful derivation of human embryonic stem cell (hESC) lines. They prophesised in

their path-breaking report on the potential application of this technique[1], "Many diseases, such as Parkinson's disease and juvenile-onset diabetes mellitus, result from the death or dysfunction of just one or a few cell types. The replacement of those cells could offer lifelong treatment"[1].

Potential applications and significance of human embryonic stem cells (hESCs)

The potential application of human ESC lines has generated tremendous interest not only in the scientific fraternity but also the general public. These cell lines provide new tools for the study of molecular mechanisms involved in normal human development, which cannot, for obvious ethical reasons, be studied *in utero*. This will aid our understanding of the formation of normal tissues from the embryonic stage to comprehend the development of organs and also to explore the oncogenesis and development of tumours of embryonic origin. In theory, by their capacity for self-renewal, hESCs can generate an unlimited number of specialized differentiated cells, providing a very interesting source of cells for cell therapy and thereby opening the doors to the science of regenerative medicine. One of the greatest potentials of the ESCs would be their ability to differentiate into cardiomyocytes among the other cell types. Cardiomyocytes become terminally-differentiated soon after birth and lose their ability to proliferate and there is no evidence that stem/progenitor cells derived from other sources, such as the bone marrow or the cord blood, are able to give rise to the contractile heart muscle cells following transplantation into the heart. The genetically stable human embryonic stem cells (hESCs) with their unlimited expansion ability and unrestricted plasticity, proffer a pluripotent reservoir for *in vitro* derivation of large supplies of human somatic cells that are restricted to the lineage in need of repair and regeneration[2].

To achieve this potential application of hESC, the Department of Biotechnology (DBT) and the Indian Council of Medical Research (ICMR) (2007) approved research for derivation of hESC lines for specific reasons[3]: *(i)* Develop methods to detect abnormalities in embryos before implantation. *(ii)* Advance knowledge, which can be used for infertility treatment or improving contraception techniques. *(iii)* Increase knowledge about causation of serious diseases and their treatment including tissue therapies. *(iv)* Developing methods of therapy for diseased or damaged tissues or organs.

The limiting factors

It had been anticipated that it would be a long while before ESC would find clinical application. This was due to the current inadequate understanding on how to induce differentiation of these cell lines into the desired cell types and potential risk of tumour formation. Another important challenge for the clinical use of these cells is the issue of immunocompatibility which can lead to the rejection of these cells following hES cell transplantation. Such a problem may be dealt with by the establishment of hES cell banks to attend different populations. The ESC lines can be derived from only 8-13 per cent of the blastocysts[4] and the rate of formation of blastocysts *in vitro* necessitates a large number of embryos. The source and availability of human embryos for the derivation of hESC become a challenge in itself because of the immense ethical implications.

Ethical concerns with the use of hESC lines

The sourcing of human embryos for the derivation of human ESC line has been routed in controversy. hESC research has been shrouded by fears about human cloning, the commodification of human biological material, the mixing of human and animal species, and the hubristic quest for regenerative immortality[5]. Opponents of hES cell research have maintained that all preimplantation embryos have the potential to become full-fledged human beings and that it is always morally wrong to destroy this potential. All the ethical debates are pertaining to the moral status of the embryo and thereby intractable restricting research.

Sourcing of embryos for derivation of hESCs

The primary source of human embryos is the assisted reproductive technology (ART) programmes. This section reviews the common current sources of human embryos for the derivation of human ESC and their limitations and the some novel ideas being explored.

Good quality spare embryos: It is a common practice in ART programmess to subject the woman to ovarian stimulation so as to retrieve multiple oocytes. These oocytes following *in vitro* fertilization (IVF) lead to multiple embryos, of which a maximum of three embryos are transferred. 'Spare' embryos are obtained in most ART cycles. The good quality ones are cryopreserved for future use while the poor quality embryo are discarded. As treatment of infertility is the reason for couples to undergo ART most couples would not be comfortable with the idea of 'giving

away' their good embryos till their primary goal of achieving pregnancy is attained. A few couples do not prefer to cryopreserve embryos because of ethical or financial reasons and such embryos could be donated for research on ESC lines.

The questions and concerns that could be raised against the use of such 'spare' embryos are: *(i)* Could a woman be 'intentionally' over-stimulated so as to ensure a good number of 'spare' embryos?, and *(ii)* Would it be fair to subject embryos for research from couples who have been unable to cryopreserve their embryos for financial constraints?

Cryopreserved 'spare' embryos: As mentioned earlier, it is a common practice to cryopreserve the spare embryos following IVF as the cumulative pregnancy rates improve following fresh and cryopreserved embryo transferred after IVF. When a couple achieves pregnancy either with the transfer of fresh/ cryopreserved embryos, all the cryopeserved embryos may not be required by the couple. The first human embryonic stem cell line was derived from a frozen-thawed embryo[1]. This remains one of the main sources of human embryos for derivation of hESC lines. However, according to the ICMR Guidelines on ART[6] (2012), "Consent shall need to be taken from the couple for the use of their stored embryos by other couples or for research, in the event of their embryos not being used by themselves. This consent will not be required if the couple defaults in payment of maintenance charges after two reminders sent by registered post". That being the case, the question that arises is: can cryopreserved embryos from couples who default in making their payment of maintenance charges be used for the derivation of ESC lines even in the absence of a written consent?

The pro-life ethical concerns still dominate the use of such embryos. And, the earlier worry about intentionally subjecting women to over-stimulation so as to have 'spare' cryopreserved embryos still remains.

'Poor' quality discarded embryos: Embryos that are discarded during the IVF procedure because of poor morphology and a low likelihood for generating viable pregnancies or surviving the cryopreservation process are also a viable source of hES cells. It has been reported that poor quality day three embryos judged on the basis of morphological assessment have formed blastocysts and give rise to hES cell lines[7]. However, early-arrested or highly fragmented embryos only rarely yield cell lines, whereas those that have achieved blastocyst stage are a robust source of normal hES cells[8].

Arrested human embryos do not resume normal development during extended culture, yet most of them contain a substantial number of living cells on embryonic day six. Gavrilov *et al*[9] reported that 72 per cent of such embryos have <1 viable cell, 47 per cent have <5 viable cells. They suggested that this class of non-viable embryos could be a rich source of viable cells for derivation of hESC lines[9].

Attempts are also being made to develop strategies for harvesting of live hESC from dead embryos[10]. The inner cell mass (ICM) could be produced in 13.9 per cent of poor quality embryos cultured[11]. Of the good-quality ICM, 15.4 per cent of those used in hESC derivation attempts resulted in a novel line[11].

Modifying culture medium to obtain cell lines from poor quality embryos

Culturing poor-quality embryos in modified medium containing human recombinant leukaemia inhibitory factor and human basic fibroblast growth factor resulted in a two-fold increase in the blastocyst formation rate and a seven-fold increase over the derivation efficiency in conventional medium[12]. All cell lines shared typical human pluripotent stem cell features including similar morphology, normal karyotypes, expression of alkaline phosphatase, pluripotency genes, the ability to form teratomas in SCID mice, and the ability to differentiate into cells of three embryonic germ layers *in vitro*. These data suggest that poor-quality embryos that have reached the blastocyst stage in this modified culture medium are a robust source for normal HESC line derivation[12].

Two new sibling human embryonic stem cell lines BJNhem19 and BJNhem20, have been derived and characterized from discarded grade III embryos of Indian origin. The cells from these two sibling cell lines showed normal diploid karyotype and continued to express all pluripotency markers after long-term continuous culture for over two years and passaged over 200 times. However, BJNhem19 was unable to generate teratomas (a unique characteristic of ESCs) in nude or SCID mice or differentiate into beating cardiomyocytes while the cardiac differentiation capacity of BJNhem20 was greatly increased, and it could generate beating cardiomyocytes[13].

These reports indicate that some poor quality embryos may develop into good ESCs but others may

not. But, these embryos remain a good potential, ethical source for embryonic stem cell derivation.

Abnormally fertilized oocytes and aneuploid embryos

Abnormally fertilized human oocytes depicted by the presence of more than two pronuclei can develop into normal appearing embryos. Such embryos are discarded because of aneuploidy. Therefore, their use, for generating ESC lines, would not conflict with the interests of the donor as these in any case have to be discarded. Many ART clinics offer embryo biopsy for the removal of a single blastomere for genetic diagnosis or screening for chromosomal anomalies. Aneuploid embryos are not transferred and discarded. Initially, human ESC lines were generated from aneuploid embryos as these created a unique repository of cell lines. The spectrum of aneuploidies in these ESC lines reflects the range of common embryonic chromosomal aberrations. Such aneuploid human ESC lines represent an excellent model to study human chromosomal abnormalities especially in the early stages of development[14].

Recent studies have demonstrated that genetically normal ESC lines have been derived from aneuploid embryos. Eleven stem cell lines were obtained from 41 embryos in 36 cultures[15]. The resulting stem cell lines were karyotyped, and surprisingly, six of the nine lines from aneuploid embryos were karyotypically normal. Three lines from pre-implantation genetic diagnosis (PGD) embryos were aneuploid exhibiting trisomy 5, trisomy 16, and an iso chromosome 13, respectively. None of the aneuploid lines presented the same anomaly as the original PGD analysis. This study demonstrated that normal stem cell lines could be derived from biopsied developmentally abnormal embryos offering specialty stem cell lines for research into the clinically important aneuploidies. Mosacism in human embryos is well known and this study further demonstrates the emerging dominance of the stem cell line by karyotypically normal cell[15]. In another study, karyotype analysis of ESC lines derived from aneurploid embryos showed a diploid female karyotype[16]. Harkness *et al*[4] also managed to derive seven normal euploid ESC lines from 74 fresh PGS screened aneuploid embryos. Thus, aneuploid embryos detected after preimplantation genetic screening can lead to the development of genetically normal ESC lines.

From unfertilized oocytes

One hESC line (RCM1) was obtained from a failed-to-fertilize inseminated egg recovered by parthenogenetic activation. Standard *in vitro* and *in vivo* characterization revealed this line to possess all of the properties attributed to a normal euploid hESC line. Whole-genome single-nucleotide polymorphism analysis further revealed that the line was biparental, indicating that sperm penetration had occurred, although parthenogenetic stimulation was required for activation[17].

Metaphase II stage oocytes have been parthenogenetically activated or reinseminated with donor sperm, then allowed to develop up to the blastocyst stage. Fertilization occurred in 65 per cent of the activated or reinseminated oocytes, which resulted in a blastocyst formation rate of 8 per cent. Evaluation of a number of developmentally important genes in those embryos exhibiting normal development revealed profile and levels of expression similar to control embryos[18]. The use of artificial parthenogenesis represents an alternative ethical source for pluripotent cell lines[19]. Pluripotent stem cells (PSCs) derived from parthenogenetically activated human oocytes demonstrate the typical characteristics displayed by human embryonic stem cells (hESCs) including infinite division and *in vitro* and *in vivo* differentiation into cells of all germ lineages[20].

The use of unfertilized oocytes does not involve the destruction of or harm to human embryos[5] and, therefore, does not face the ethical dilemma associated with the use of embryos. As these technologies are far from therapeutic, concerns over the morality of embryonic stem cell derivation should not hinder their advancement[21].

From single blastomeres

Human embryonic stem cell lines have been derived from biopsied single blastomeres. Chung and colleagues[22] succeeded in deriving mouse embryonic stem cells from single blastomeres separated from eight-cell-stage mouse embryos. Since this technique sought to preserve the ability of the embryo to implant and develop to birth, it theoretically could allow for the banking of autologous hES cell lines for children born from biopsied ex-corporeal embryos. Blastomeres are removed from morula stage embryos and cultured until multicell aggregates are formed. These blastomere-

derived cell aggregates are plated into microdrops seeded with mitotically inactivated feeder cells, and then connected with neighbouring microdrops seeded with green fluorescent protein-positive hES cells. The resulting blastomere-derived outgrowths are cultured in the same manner as blastocyst-derived hES cells[23].

Chung *et al*[24] have demonstrated that a single blastomere can be removed to generate a human embryonic stem cell line without prejudicing the development of the biopsied embryo. Their method stimulates new ideas about hESC formation, but ethico-political concerns remain.

Autologous nuclear transfer

Markoulaki *et al*[25] have developed in mice a variation of somatic cell nuclear transfer (SCNT), a technique whereby the DNA of an unfertilized egg is replaced by the DNA of a somatic cell, by blocking the action of a gene [caudal type homeobox 2 (*Cdx2*)] that enables the developing embryo to implant into the uterus. By introducing this genetic defect in mouse somatic cells prior to nuclear transfer, they created cloned mouse embryos that generated pluripotent stem cells just before arresting developmentally.

Another concept called altered nuclear transfer (ANT) was proposed by William Hurlbut. Research with mouse embryos carrying a mutation in the *Cdx2* gene showed that these embryos failed to form a trophectoderm and thus died at the blastocyst stage, but not before giving rise to mouse embryonic stem cells. Extrapolating from this mouse study, Hurlbut reasoned that a *Cdx2* genetic mutation introduced into a human somatic cell prior to nuclear transfer might produce a blastocyst that could produce human pluripotent stem cells but lacked the biologic potential to develop into a complete human being. He also suggested that these possible ANT products should be viewed as complex tissue cultures (*i.e.*, bioengineered embryo-like artifacts) rather than viable human embryos because of their limited cellular systems[26]. Unfortunately, there were many uncertainties surrounding ANT as a possible source for human pluripotent stem cells. Meissner and Jaenisch[27] pointed out that it was unknown whether *Cdx2*-deficient human embryos would behave just like their mouse embryo counterparts, yielding pluripotent human stem cells just before arresting at the late blastocyst stage. To determine whether ANT was feasible, efficient, and effective for research and clinical applications in humans would require significant amounts of time-consuming research and a considerable diversion of resources that could be used toward known methods for deriving hES cells.

Conclusions

Embryonic stem cells have great potential application in transplantation and regenerative medicine as well as improving our understanding of developmental biology. There is a need for regularly sourcing human embryos to develop a genetic bank of embryonic stem cells. With an estimation of nearly 85000 ART cycles being done in India every year, it should be possible to source human embryos either by the conventional or novel means which can be used for derivation of embryonic stem cells lines without compromising on any of the ethical concerns.

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