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REVIEW ARTICLE

Human Embryonic Stem Cells as a Powerful Tool for Studying Human Embryogenesis

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ABSTRACT: Human embryonic stem cells (HESC) are pluripotent stem cell lines derived from the inner cell mass (ICM) of human blastocyst-stage embryos. They are characterized by their unlimited capacity to self-renew in culture. In addition, they have a broad developmental potential, as demonstrated by their ability to form practically any cell type *in vivo* and *in vitro*. These two features have made HESC extremely important in basic and applied research. In addition, they may serve as a powerful tool for studying human development. HESC can recapitulate embryogenesis by expressing developmentally regulated genes and by activating molecular pathways as they occur *in vivo*. Moreover, they can be used to analyze the effect of specific mutations on particular developmental events and may enable us to identify critical factors that play a role in the processes of cell commitment, differentiation, and adult cell reprogramming. Thus, modeling human embryogenesis by the use of HESC may allow new insights into developmental processes, which would otherwise be inaccessible for research. **(*Pediatr Res* 60: 111–117, 2006)**

CURRENTLY AVAILABLE MODELS FOR HUMAN EMBRYO DEVELOPMENT

Human embryogenesis is one of the most exciting fields in biology and medicine. However, apart from the very early stages of preimplantation development, human embryos are inaccessible for research and are limited to section studies of diseased aborted fetuses. One approach to overcome this obstacle is to use animal models (Fig. 1), usually mice, taking advantage of their well-defined genetics and reproductive characteristics. The use of mice as a model for human development has been justified by the observation that there is strong conservation throughout evolution of developmental processes and control genes. Moreover, the relative ease by which their genome can be genetically manipulated and used for the introduction of specific mutations by homologous recombination has made them extremely important for studying specific genes and pathways that are involved in embryonic development. Yet, despite the similarities between mouse and human, there are still major differences between species in size, growth, and anatomy. In addition, critical developmental events vary between the two, both in time and morphology. All these are a product of variations in gene content and expression, leading to differences in biochemical pathways and phenotypes. These crucial discrepancies emphasize the need for a more powerful system for studying early human embryo development. Complementary to the usage of animal models are human cellular systems, which include either transformed or primary cultures (Fig. 1). However, these culture systems have only a limited potential due to the specific range of tissues from which cells can be obtained, and due to their transformed nature or their short life span, respectively. In this respect, HESC promise to be extremely useful. Because they are normal cells with a self-renewing potential, they have a capacity to differentiate into many different cell types *in vitro*, and can recapitulate early embryonic differentiation, as will be discussed in this review.

HUMAN EMBRYONIC STEM CELL CHARACTERIZATION AND DEVELOPMENTAL POTENTIAL

HESC are pluripotent cells derived from the ICM of blastocyst stage *in vitro* fertilized embryos, that have been donated for research purposes (1,2). These cell lines are unique in their unlimited life span and their broad developmental potential, *i.e.* the ability to differentiate to all cell types. In addition, they are characterized by the expression of a specific set of cell surface molecules [stage-specific embryonic antigens (SSEA)], expression of undifferentiated cell gene markers (OCT4, Nanog, Rex1), and activity of several enzymes (telomerase and alkaline phosphatase) [reviewed in (3)]. Not only do these cells remain undifferentiated and karyotypically stable during prolonged passages, they also readily differentiate *in vivo* and *in vitro* to cells representing the three germ layers (4). *In vivo*, when HESC are injected to nude mice, nonmalignant tumors

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Abbreviations: ES, embryonic stem; **HEB,** human embryoid body; **HESC,** human embryonic stem cell; **ICM,** inner cell mass; **IVF,** *in vitro* fertilization

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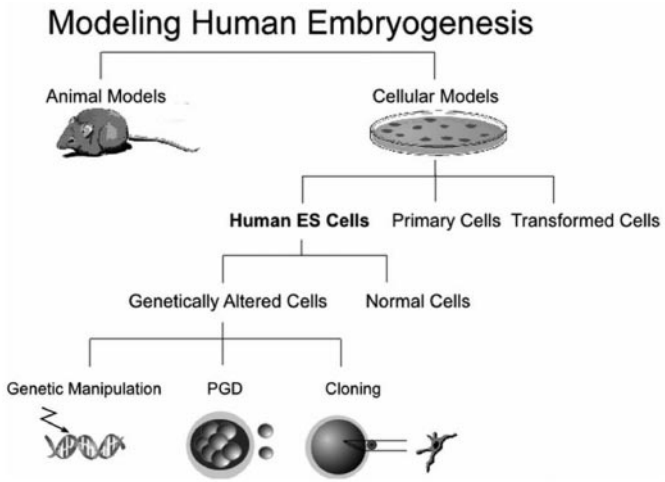

Figure 1. Modeling human embryogenesis. Human embryonic development can be modeled by the use of different systems. For *in vivo* developmental studies, laboratory animals are most commonly used. Direct study in human is limited to cellular systems: either primary cells, transformed tissue, or stem cells. *In vitro* differentiation of normal or genetically altered HESC can recapitulate embryogenesis. Cells containing a specific genetic mutation can be established by targeted mutagenesis. Alternatively, HESC containing naturally occurring mutations can be derived from affected embryos obtained either following PGD or embryo cloning.

named teratomas are formed. These tumors are composed of many different cell types including cartilage, squamous epithelium, primitive neuroectoderm, ganglionic structures, muscle, bone, and glandular epithelium (1,2,5). *In vitro*, they can be induced to spontaneously differentiate by growing in suspension culture. Under these conditions, HESC tend to aggregate, forming spherical structures termed HEB. HEB are dynamic structures that undergo extensive morphologic changes. Initially, the cells grow as densely packed cell aggregates, after which they cavitate and accumulate fluid to become cystic. While they grow, differentiation takes place spontaneously, resulting in the production of many different cell types including nerve, skin, adrenal, blood, endothelial, kidney, heart, bone, muscle, and liver, as determined by the expression of tissue-specific gene markers (6). Furthermore, in some cases it could be shown that the cells are functional, as in the formation of contracting cardiac muscle cells (6). Nevertheless, since differentiation in EB is largely disorganized, they are not suitable for the study of pattern formation *in vitro*. Moreover, since differentiation by the formation of EB results in the production of a mixture of many different cell types, it is impractical to use this system for obtaining large quantities of a specific cell type without the addition of inducing factors. Thus, much effort has been made to find protocols for efficiently directing the differentiation of HESC into specific cell types *in vitro*.

DIRECTED DIFFERENTIATION

Growth Factor–Induced Differentiation

Normal embryogenesis is dependent on the precise execution of a developmental program. Initially, the embryo is composed of a homogenous cell population. As embryogenesis proceeds, cells begin to acquire different fates according to their respective position and developmental timing. Consequently, cells in the developing embryo should be responsive to environmental signals. Indeed, HESC express a wide variety of receptors. Among them are members of protein-tyrosine phosphatase- (PTP), fibroblast growth factor- (FGF), insulin growth factor- (IGF), bone morphogenic protein- (BMP), activin-, and tumor necrosis factor- (TNF) receptor families, which are expressed at relatively high levels in HESC. These receptors are known to participate in a range of developmental pathways such as gastrulation, mesentoderm commitment, and neural maturation (7). Moreover, administration of growth factors such as activin A, nerve growth factor (NGF), and hepatocyte growth factor (HGF) modulate the expression of differentiated cell markers representing the three embryonic germ layers mesoderm, ectoderm, and endoderm, respectively (8).

Ectoderm Differentiation

Ectoderm differentiation of HESC can be achieved either by addition of growth factors that induce differentiation to cells expressing neuronal, adrenal or skin related genes, or by allowing spontaneous differentiation (8). Extensive work has been dedicated to neuronal differentiation, since derivation of various specialized neuronal cells is relatively easy, compared with other cell lineages, and could be of great value for the treatment of neurodegenerative disorders.

Neuronal Differentiation

HESC neuronal differentiation, *in vitro* or *in vivo*, seems to mimic the gradual restriction of cell fates observed in normal neuronal differentiation (neuronal stem cells, neuronal progenitors, and, finally, neurons and glial cells) (9). *In vitro*, HESC-derived neurons synthesize neurotransmitters, respond to neurotransmitters and are electrically active (10–13). Additionally, a neural-tube like structure is formed when HESC are induced to differentiate into neuronal progenitors in the presence of FGF2 (13). *In vivo*, when HESC-derived progenitor neurons were transplanted into a newborn rodent brain, they integrated into different locations in the brain (12,13). Furthermore, HESC acquire appropriate neuronal fates when transplanted in chick organogenesis-stage embryos (14). These studies demonstrate that HESC are able to respond to signals from neighboring cells and differentiate properly in response to their environment. Lately, HESC were assayed as a potential source of cells for replacement of degenerated neurons. It has been demonstrated that Parkinsonian rats that were transplanted with HESC-derived neuronal progenitors showed significant improvement in their pathologic state whereas only minor improvements were observed in rats transplanted with undifferentiated HESC. Accordingly, HESC might assist in recapitulating normal dopaminergic neuron function, although it was demonstrated that a pretransplantation differentiation process is needed to improve dopaminergic neurons' yield (15). In addition to dopaminergic neurons, differentiation *in vivo* to retinal cells (16) and *in vitro* into motor neurons (17) was also observed.

Mesoderm Differentiation

HESC differentiation into mesoderm lineage cell derivatives is demonstrated by expression of markers specific to cardiomyocytes (6,8,18–23), endothelial (24,25), bone (8,26), kidney (8), urogenital (8), muscle (8), and blood cells (6,8,27).

Cardiomyocyte differentiation. Pulsating HEB are the first indication of the presence of functional cardiomyocytes derived from HESC (6). Analysis of the contracting areas in the HEB indicates that they have physiologic characteristics similar to early cardiac tissue (18). Additionally, progressive stages of early cardiomyocyte differentiation are observed during HEB differentiation (19). It is also possible to obtain a variety of functional cardiac cells *in vitro* (nodal-like, embryonic atrial–like, and embryonic ventricular–like cardiomyocytes) (20). This is supported by the fact that HEB-derived cardiomyocytes form a functional electrical syncytium connected by gap junctions and activated by a focal pacemaker (21). Moreover, cardiomyocytes derived from HESC can respond to hormones of the vegetative nervous system (22), integrate *in vivo*, and pace the heart of pigs with complete heart block (23).

Endothelial differentiation. Endothelial cells were shown to appear during the spontaneous differentiation of HESC (24). The presence of endothelial cells within HEB is demonstrated not only by the expression of endothelial specific markers, but also by the successive morphologic progression from endothelial cell clusters to vascular-like structures and to a network-like organization (25). Additionally, isolation of endothelial cells from HEB yield vascular tubes both *in vitro* and *in vivo*.

Hematopoietic differentiation. Two differentiation methods were used to achieve hematopoietic differentiation of HESC: culturing HESC in the presence of stromal cells and the administration of growth factors such as stem cell factor (SCF), Fms-like tyrosine kinase 3 ligand (Flt3L), IL-3, IL-6, granulocyte colony-stimulating factor (G-CSF), and BMP-4 to HEB. These methods give rise to several types of hematopoietic lineages, including erythroid, myeloid, and lymphoid [reviewed in detail in (27)]. In contrast to HESC, hematopoietic stem cells (HSC) demonstrate a limited proliferative capacity. Thus, the ability to derive hematopoietic cells from HESC may offer a potential replacement for the currently used HSC for cell-based therapy.

Osteogenic differentiation. Recently, several protocols for bone differentiation were published. It was shown that addition of osteogenic supplements to HEB culture results in the expression of osteogenic markers (osteocalcin), mineralization of the culture and the appearance of a principle component of bone matrix (hydroxyapatite) (26). In another study, HESC were shown to respond in a similar way to a mouse ES cells osteogenic differentiation protocol, especially to the addition of dexamethasone to the culture (28). The resulting differentiated cells were transplanted into severe combined immunodeficient (SCID) mice on a scaffold and demonstrated the capacity to form mineralized tissue *in vivo*.

Endoderm Differentiation

Isolation and characterization of endoderm derivatives from HESC is of great interest inasmuch as these cells might serve as a source of cells for cell therapy in different pancreatic disorders and liver failure diseases. Recently, differentiation of HESC to definitive endoderm has been reported (29). Moreover, there are several reports of specific differentiation to hepatic- and pancreatic-like cells (8,30–32).

Hepatic differentiation. It has been suggested that culturing HESC with sodium butyrate results in cells with an epithelial morphology that express hepatic markers and possess metabolic activity similar to primary adult human hepatocytes (32). Furthermore, gene expression analysis of HESC throughout differentiation indicates that there are several hepatic-associated genes such as albumin, fibrinogen, and apolipoprotein, which are induced upon differentiation (30). These hepatic-like cells can be successfully isolated by introduction of a reporter gene regulated by a hepatic specific promoter (30). These cells express hepatic specific markers and are observed adjacent to cardiac mesodermal cells in teratomas, as observed in normal embryonic development.

Pancreatic differentiation. Pancreatic development is regulated by several factors such as Foxa2 and Pdx1 (33) and by epithelial-mesenchymal interactions. Yet, the initial steps in normal pancreatic development are still unknown. HESC have been shown to express pancreatic specific markers (8,31). However, so far, the generation of functional beta-like cells has been unsuccessful in that the differentiated cells failed to secrete insulin when stimulated with glucose, as expected from functional beta cells.

Extraembryonic Differentiation

The first differentiation event in mammalian embryogenesis is the appearance of the trophectodermal layer surrounding the blastocyst. This outer cell layer will yield the placenta and is extremely important for embryo implantation in the uterus. In contrast to mouse ES cells, which are unable to differentiate into extraembryonic tissues, HESC can successfully differentiate to trophoblast cells spontaneously (1,2), or in the presence of BMP-4 (34). BMP-4 treatment can induce trophoblast-expressed markers and placental hormones in HESC cultures (34). Moreover, when plated at low density, the BMP-4–treated HESC form syncytia and produce chorionic gonadotrophin. HESC may therefore serve as the primary model for extraembryonic tissue development.

Limitations of HESC *In Vitro* Differentiation

We have described the wide developmental potential of HESC. However, certain limitations of this system should be addressed. Firstly, there are still certain cell types that could not be obtained by *in vitro* differentiation of HESC. A prominent example for this is the inability to obtain insulin secreting pancreatic β-cells in culture. Furthermore, some of the differentiated cell products derived from HESC do not express the full repertoire of markers that characterize their *in vivo* counterparts, as is illustrated in the case of the HES hepatic-like derived cells, in which adult liver transcripts are undetectable (30). Finally, it is unclear whether all the differentiated derivatives are indeed functional, an issue that could be

addressed only by *in vivo* transplantation experiments, which are limited to animal models for obvious reasons.

MODELING HUMAN EMBRYOGENESIS *IN VITRO*

There is by now substantial evidence to show that *in vitro* differentiation of mouse and HESC can recapitulate, at least to some extent, early embryonic development. In an attempt to determine how well differentiation of developing EB correlates with embryogenesis, the expression of several developmentally regulated genes was examined. Genes that are known to be expressed *in vivo*, either very early (germ layer formation) or late (lineage specification) during embryogenesis, demonstrate that the temporal and spatial expression mode of these markers is maintained in mouse EB (35). This has been further supported in human, by profiling growing HEB for gene expression at different stages of their differentiation using cDNA microarrays (7). This large-scale cDNA analysis has allowed the identification of several sets of temporally expressed genes, which can be associated with sequential stages of embryonic development. By comparing gene expression of early, cavitated, and cystic HEB, five transiently expressed gene clusters were identified. These corresponded to different stages in embryo development including blastocyst, primitive ectoderm, gastrulation, and early and late organogenesis. The power of EB formation as an *in vitro* model system to study early lineage determination and organogenesis was further confirmed by examining the temporal expression of specific gene cascades that are known to be involved in a given developmentally regulated pathway and are active in succession. One such example is the expression of the α- and β-globin gene cluster, which includes a group of several highly conserved genes that are expressed in a stage-specific pattern during development, from embryo to adulthood, in the erythroid compartment. By analyzing the transient expression of globin genes in growing EB, it was shown that the full complement of mouse embryonic globin genes are expressed in the correct order (Fig. 2). In addition, upon further differentiation, the switch in expression from embryonic to fetal/adult genes takes place as occurs *in vivo* (36).

Further support for the use of *in vitro* differentiated HESC as a model for human embryogenesis is illustrated by the study of the Nodal signaling pathway, which plays a major role in the determination of embryonic axes (right-left, dorsal-ventral, anteroposterior) as well as in mesoderm induction during early gastrulation (37). By comparing the expression level of *NODAL* and its downstream targets, *LEFTY A*, *LEFTY B*, and *PITX2*, between early, mid-, and fully matured HEB, a transient expression pattern is observed. All four genes are expressed at different time points during differentiation, in keeping with the conserved pathway as it occurs in the embryo (7).

X inactivation is another biologic phenomenon that is developmentally regulated and is tightly linked with cell differentiation. In this process, a single X chromosome undergoes transcriptional silencing in every XX cell of the female during early development in mammals. It occurs randomly, where either the maternal or the paternal X chromosome is inactivated, and involves the induction of several epigenetic modifications that appear in a stepwise and apparently hierarchical manner (Fig. 3) (38). X inactivation is initiated by the up-regulation and cis accumulation of a nonprotein-coding RNA molecule, termed XIST, on the chosen chromosome to be inactivated. Cis accumulation of XIST recruits silencing complexes that establish a heritably repressed chromatin conformation along the entire chromosome. Analyzing differentiating XX ES cells in mice and human, has allowed us to define the developmental time window during which cells have the capacity to inactivate the chromosome as a response to *XIST* RNA expression (39,40). In addition, it was possible to study the molecular events that are involved and occur in succession. For instance, examining mouse XX ES cells at different time points has allowed us to characterize the different steps

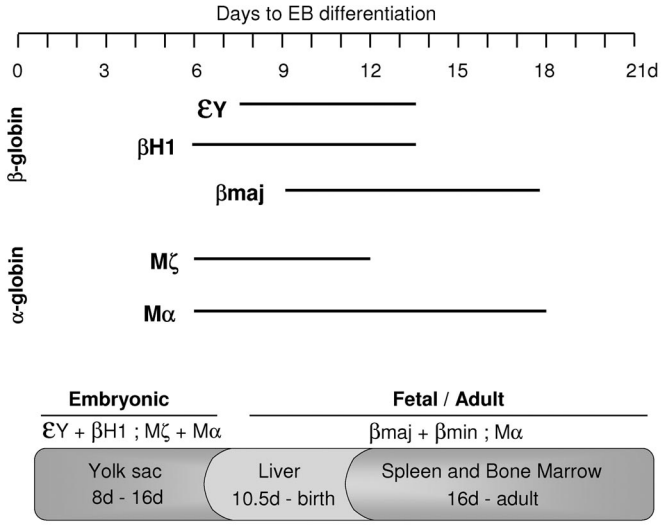

Figure 2. Globin gene switching during mouse ES cell differentiation. α- and β-globin genes are expressed in a stage-specific pattern during development, from embryo to adulthood, at different sites of erythropoiesis. ESC-derived EB express the full complement of mouse embryonic β- and α-globin genes (εY, βH1, Mζ, and Mα) in the correct temporal order, as they appear *in vivo*. In addition, upon further differentiation, the switch in expression from embryonic to fetal/adult genes (βmaj and Mα) takes place, as occurs *in vivo*.

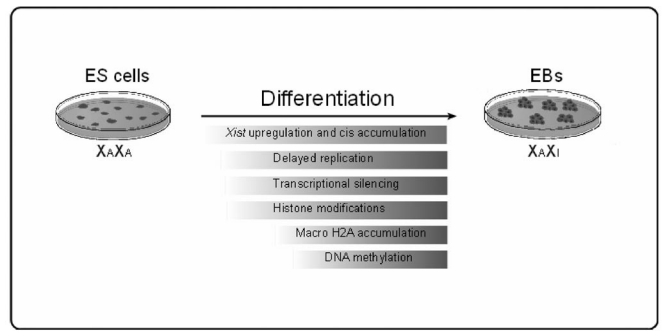

Figure 3. X inactivation in differentiating XX embryonic stem cells. *In vitro* differentiation of XX ESC has allowed the dissection of molecular events that lead to X inactivation and appear in a stepwise fashion. The latter include up-regulation and cis accumulation of Xist transcript followed by delayed replication, inactivation of gene transcription, and histone modifications (hypoacetylation of H3- and H4-tail histones and methylation of H3K9 and H3K27). Thereafter, histone macro-H2A1.2 incorporates and DNA methylation of CpG sequences takes place.

that are required for establishing the inactive state of the X chromosome in females and to identify developmentally regulated factors that are necessary to induce the full pathway that leads to stable inactivation (41–47).

These experimental findings support the notion that HESC can indeed model, to a certain degree, early embryonic development. This has been demonstrated by specific gene expression and developmentally regulated pathways, activated during HESC differentiation, as they would be *in vivo*.

STUDY OF DEVELOPMENTALLY REGULATED PROCESSES AND DISEASES BY ANALYZING THE EFFECT OF SPECIFIC MUTATIONS

HESC promise to be invaluable in the study of human genetic disorders, especially in cases where animal models fail to fully reproduce the human phenotype. In particular, they can be used for investigating developmentally regulated disorders, which have no rodent counterparts. One such example is the group of unstable triplet repeat expansion disorders (*e.g.* fragile X syndrome). Thus, having a HESC-based cellular system that complements other available models would be extremely useful.

HESC can be used for modeling human disorders either by targeted mutagenesis or by the use of cell lines, which already carry naturally occurring mutations (Fig. 1). Indeed, it has been previously demonstrated that genetic manipulation of HESC is feasible (48) and that specific genes can be targeted by homologous recombination (49,50). The use of targeted mutagenesis has allowed us to model disorders for which animal and other cellular models have been proved to be inadequate (49). For example, Lesch-Nyhan disease is caused by a mutation in the HPRT1 gene causing overproduction of uric acid. However, knockout mice lacking HPRT1 expression do not display the Lesch-Nyhan phenotype, possibly because they do not accumulate uric acid due to biochemical differences between rodents and humans. Disruption of the HPRT1 in HESC by homologous recombination has allowed us to recapitulate some of the characteristics of Lesch-Nyhan syndrome, including the accumulation of uric acid (49). Such mutated cells can help researchers to further investigate the genetic disease and to analyze drugs that will prevent the onset of its symptoms. An alternative approach for modeling human inherited disorders is the derivation of HESC that have naturally inherited mutations. This can be achieved by the procedure of preimplantation genetic diagnosis (PGD) (Fig 1). This technique is offered to couples at high risk of transmitting a genetic defect, eliminating the need for therapeutic abortion. In PGD, embryos diagnosed to be free of the genetic mutation are selectively transferred for implantation and the affected embryos are discarded. The affected embryos, which carry specific mutations that are associated with particular inherited disorders, can be used for the establishment of new ES cell lines (51,52). Such cell lines have recently been established for Thalassemia, Duchenne muscular dystrophy, Huntington disease, Fanconi anemia, and several other diseases (51,52). A different approach to obtain HESC with naturally occurring mutations would be to establish new cell lines from nuclear transfer (NT)-derived blastocysts (Fig. 1). In this method, a nucleus from a somatic cell of an adult is introduced into an enucleated oocyte, resulting in a cloned embryo. The NT-derived embryo can then be used for the establishment of ES cells (Fig. 4). By introducing the nucleus of a somatic cell from a genetically affected patient, an ES cell line with the specific mutation will be produced. This approach, which involves embryo cloning, has been demonstrated to be feasible in mouse (53,54). By transferring a somatic cell nucleus from skin cells of patients into enucleated oocytes, it might be possible in the future to generate genetically tailored HESC for different disorders with reasonable efficiency. ES cell lines containing a specific genetic alteration, either by targeted mutagenesis, PGD, or embryo cloning, may thus serve as a model system for specific diseases, allowing us to improve our understanding of the pathology by studying the abnormal phenotype at the cellular and molecular levels.

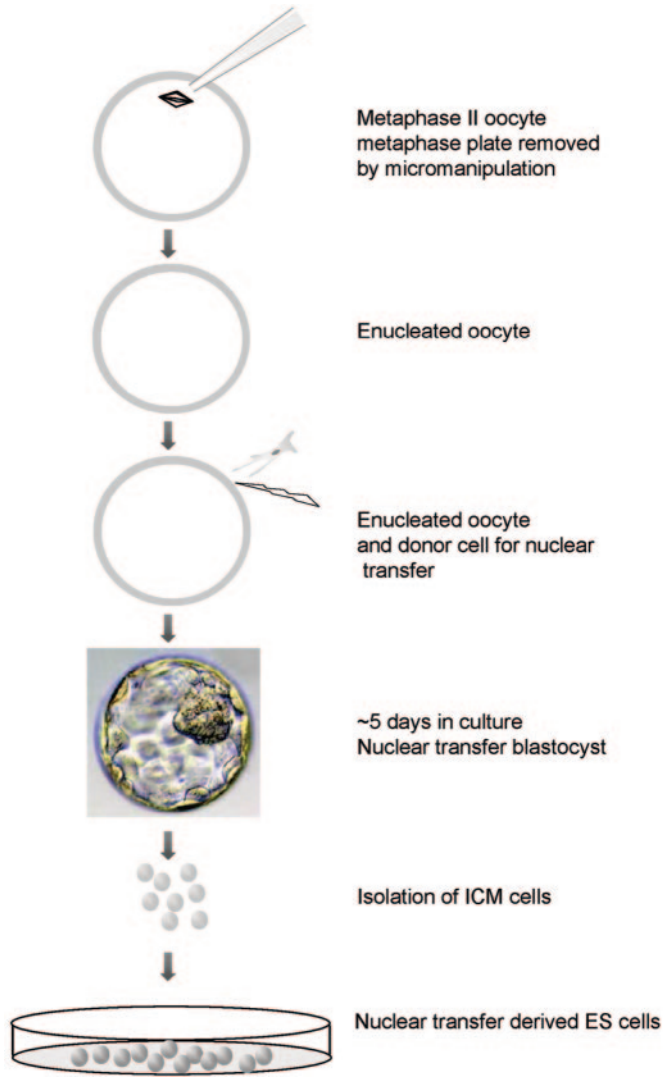

Figure 4. Somatic nuclear transfer (NT). A procedure that involves the introduction of a nucleus from a somatic cell into a metaphase II stage enucleated oocyte. The NT-derived zygote can be left to develop into blastocyst *in vitro*. Isolation and further propagation of the ICM cells of the cloned blastocyst results in the establishment of a new HESC line.

CELL REPROGRAMMING BY CELL FUSION

The recent advances in the derivation of HESC and the success in mammalian embryo cloning by somatic cell nuclear transfer (NT), provide an attractive possibility for restoring tissue function by cell transplantation through therapeutic cloning. An alternative approach for dedifferentiating somatic cells, to produce autologous ES cell lines that would bypass the problematic issue of embryo cloning, is to produce hybrid cells that are a product of fusion between a somatic cell and an existing ES cell line. Such hybrid cells have been obtained by fusing human fibroblasts with HESC (55). Some of these hybrids are tetraploids and resemble the phenotype of ES cells in terms of morphology, growth rate, antigen expression, and immortality. They also have a wide differentiation potential, as determined by their ability to form EB *in vitro* and teratomas *in vivo*. Genome-wide transcriptional activity, reporter gene activation, allele-specific gene expression, and DNA methylation showed that the fibroblast genome has been reprogrammed by the ES cell to an embryonic state (55). Thus, fusion between ES and somatic cells may serve as a powerful tool for the identification of critical factors and underlying mechanisms by which adult cells can be reprogrammed. Furthermore, they may teach us more about cell commitment and embryonic differentiation.

SUMMARY

The recent derivation of HESC has generated much interest, mainly due to their therapeutic potential. Yet, another crucial role that these cells will probably have in the near future will be as a model system for basic developmental research. HESC provide a new approach to the study human embryo development, which has so far been limited by the inadequacy of available models. Investigation of HESC differentiation will open new opportunities for observing and studying early human embryonic development and disease in a straightforward fashion. In this review, we have summarized the methods by which HESC can be used for this purpose.

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