Embryonic Stem Cells and iPS Cells: Sources and Characteristics

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KEYWORDS

• Embryonic stem cell • Induced pluripotent stem cell
• Differentiation • Tissue regeneration

By definition, embryonic stem (ES) cells are pluripotent, meaning they can form tissues from all 3 primary germ layers (ectoderm, endoderm, and mesoderm) of the embryo. This differs from totipotency in that pluripotent ES cells cannot form placental tissues, and therefore ES cells cannot form a viable embryo without contributions from other cell types. The mammalian embryo is totipotent up to the 16-cell stage, after which the cells of the morula begin to differentiate to defined fates. Multipotency defines cells that have the potential to form 2 or more differentiated tissues, but not necessarily form multiple germ layers.

Induced pluripotent stem (iPS) cells are somatic cells that have been reprogrammed to behave like an ES cell by artificially turning on expression of specific pluripotency genes. This reprogramming can be achieved using a number of techniques with varying efficiencies. Many iPS cell lines share gene expression patterns and epigenetic traits of ES cells; however, the exact relationship between ES and iPS cells is still poorly understood. Numerous differences have been identified between ES and iPS cells that may have significant impact on the future clinical use of each cell type. The methods to derive ES and iPS cell lines are distinct from each other and will be discussed separately. In contrast, the methods used to characterize these cell types are similar and will be discussed together.

ES CELLS

Tissue Sources and Isolation

ES cells are derived from the inner cell mass of the blastocyst stage embryo. In people, this stage occurs at 5 to 6 days after fertilization,[1] and the mouse at 3 to 4 days after fertilization.[2] There have been several reports describing the generation of ES-like

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cells from horses.[3–7] Embryo collection for equine ES cell isolation has been reported to range from 6 to 8 days after fertilization.[4,6,7] During this time period, the harvested equine embryo is in a blastocyst or an expanded blastocyst stage. To date, no reported equine ES cell lines have been proven to be pluripotent in any in vivo assay. The absence of any data verifying in vivo pluripotency prevents definitive classification of these cells as true ES cell lines.[8]

Two methods have been used to isolate the inner cell mass of the blastocyst for ES cell isolation. Across species, the most frequently used method is microsurgery. This procedure involves mechanical dissection under microscopic guidance and manual separation of the inner cell mass from trophoblastic lineage cells. The second method involves immunodissection using an antibody that targets trophoblast lineage cells of the blastocyst. Complement is added to the antibody-labeled blastocyst, leading to destruction of trophoblastic lineage cells while the inner cell mass remains unharmed. Both microsurgical dissection and immunosurgical dissection have been described for isolation of the equine inner cell mass.[4,6,7]

ES Cell Advantages

For clinical application, a fully validated ES cell product would have a number of advantages over currently available cell-based products. First, ES cells have the potential to replicate indefinitely under defined conditions without differentiation, making an off-the-shelf preparation possible. Next, ES cells are able to form many committed cell types for regenerative tissue repair when differentiated before implantation. Another significant advantage is that there is minimal genetic manipulation of ES cells, and there is a consequently decreased risk of aberrant tumor formation compared with iPS cell lines. ES cells have potential value in the treatment of genetic diseases through therapeutic cloning applications. ES cells have been used extensively in the production of transgenic mice, demonstrating proof of principle of their potential value with genetic manipulation.

ES Cell Disadvantages

Despite the tremendous potential ES cells hold for clinical benefit, several disadvantages need to be addressed. Routinely used methods for inner cell mass isolation necessitate destruction of an embryo, leading to ethical concerns across species, but especially in human ES cell research. Another concern for clinical application of ES cells is the potential for allogenic immunogenicity. Since ES cells are somewhat immunoprivileged, this may be less of a concern in therapeutic uses. One important disadvantage is the need for specific, complicated culture conditions to propagate and maintain ES cell lines in an undifferentiated state and the requirement for frequent monitoring of cultured cells for changes in genomic state to assure phenotypic stability.[9] Additionally, there is a risk for tumor formation if ES cells are not fully directed into a differentiated cell type before surgical implantation. For therapeutic applications in people, another major concern is the use of nonhuman materials such as fetal bovine serum and mouse feeder cells to derive ES cells. An added problem to address is that the genetic background of the blastocyst plays an important role in the efficiency of deriving ES cell lines, as has been clearly demonstrated in the mouse.[10] Horses have much more genetic variability than typical inbred mouse strains; therefore equine ES cell line generation may be complicated by their inconsistent genetic background. Horses have the additional disadvantages of low embryo numbers to harvest during blastocyst collection and lack of optimal culture conditions to promote equine ES cell expansion without differentiation.

iPS CELLS

Tissue Sources, Isolation, and Induction

Any nucleated somatic cell in the body can theoretically be reprogrammed using iPS techniques. Many human and murine studies have used skin cells and fibroblasts for the initial adult cell source. Adult neural stem cells have been reported as the initial cell type in cellular reprogramming studies.[11] Recently, a group has described the reprogramming of murine bone marrow mononuclear cells with higher efficiency than mouse embryonic fibroblasts.[12]

Numerous methods have been used to induce the reprogramming of non-ES cells. The initial techniques described used retroviral or lentiviral transfections to induce expression of oncogenes into the candidate cell following viral incorporation into the host cell's genome.[13–15] Later work demonstrated that adenoviral vectors could be used that would avoid viral incorporation into the host genome.[16] Use of adenoviral vectors may be a safer alternative compared with retroviral or lentiviral vectors; however, the gene expression induced by adenoviral vectors is not maintained long term in transduced cells, limiting their pluripotent longevity. Expression plasmids alone have been used to induce pluripotency genes in target cells, but generation of iPS colonies is extremely inefficient using this method. Concern about the use of viral vectors has led other groups to attempt reprogramming of human fibroblasts using direct delivery of proteins into cells, again with poor efficiency.[17] Recently, a significant advance was described using the introduction of modified mRNA to reprogram adult cells.[18] This group went on to demonstrate proof of concept that induced cells can be directed to a specific lineage using the modified RNA technique. The modified RNA reprogramming method may prove to be the safest and most efficient strategy to reprogram adult cells and promote subsequent differentiation into the desired cell product for eventual therapeutic use.

Although several groups are currently working to develop equine iPS cell lines, only 1 group has published a report of definitive iPS production in the horse.[19] The field of cellular reprogramming is changing rapidly, and adaptation of techniques developed for other species will likely prove useful to enhance the induction of equine cells to a pluripotent state in the near future. For example, a recent report describes surgical implantation of chemically induced putative ES-like cells in an equine model of superficial digital flexor tendonitis, leading to improved histologic repair in the ES-like cell treated versus control lesions.[20]

iPS Cell Advantages

iPS cells should be less prone to immunorejection, since they can be patient-derived or major histocompatibility complex (MHC) class 1-matched for compatibility. Production of iPS cell lines also avoids the ethical controversy of embryo destruction associated with ES cell generation. In the horse, abundant donor tissues (eg, dermal fibroblasts isolated from skin biopsies) are available to provide ample initial adult cells for reprogramming.

iPS Cell Disadvantages

The use of viral vectors (especially retroviruses or lentiviruses that randomly incorporate into the host genome) for iPS colony generation increases the risk of tumor formation and leads to concern for transplantation of reprogrammed cells in clinical trials. Overall, an increased risk of tumor formation has been demonstrated in human iPS cells compared with ES cells.[21] iPS cells can also show dramatic variability in the completeness of reprogramming and require extensive screening to select the most

ES-like cells. Similar to cultured ES cells, cultured iPS cells need to be frequently monitored for genomic abnormalities to ensure clinical safety.[9]

FACTORS TO INDUCE OR DEFINE PLURIPOTENCY

Many different combinations of pluripotency factors have been described for defining pluripotency or reprogramming cells with varying efficiencies in iPS cell colony generation or ES/iPS cell validation. All of the pluripotency induction genes have been previously linked to cancer, suggesting a connection between oncogenic transformation and pluripotency. The gene and protein expression pattern of fully reprogrammed cells and ES cells varies slightly by species; however, both types of cells must express the genes POU5F1, SOX2, NANOG, and TERT. ES/iPS cells also require a high level of telomerase activity to sustain self-renewal and proliferation. The following factors have been reported to contribute to iPS generation and are useful to evaluate ES and iPS pluripotency.

Octamer-binding protein (Oct 3/4) has been used in nearly all cellular reprogramming strategies. It is a homeodomain transcription factor encoded by the gene POU5F1. A precise quantity of Oct 3/4 protein is needed to control cellular differentiation or maintenance of pluripotency. An excess of Oct 3/4 leads to spontaneous differentiation of ES cells into either primitive endoderm or mesoderm, while a deficiency in protein expression leads to a trophoectoderm phenotype.[22] One study has demonstrated POU5F1gene transfection can be used as a single factor (although with extremely low efficiency) to reprogram adult neural stem cells.[23] Oct 3/4 is considered to be one of the master regulators of pluripotency in conjunction with Sox2 and Nanog.

Sex-determining region Y-box 2 (Sox2) is a less-specific transcription factor that plays a role in pluripotency induction and other differentiation processes. For example, Sox2 is expressed in large quantities in developing and adult neural tissue[24] and plays an important role in the regulation of Oct 3/4 expression.[25] The Sox2 and Oct 3/4 proteins form a heterodimer that binds DNA and regulates the expression of many other genes involved in embryonic development.

Nanog is a transcription factor important to the maintenance of undifferentiated ES cells and is a key gene in the regulation of pluripotency.[26] Although not absolutely required as a transduction factor, Nanog expression has been shown to be upregulated in iPS cells and is a good marker of cellular reprogramming.[27] Overexpression of Nanog permits pluripotent self-renewal in human ES cells without the use of feeder cells or specialized media supplements.[28] In addition, it has been shown that the tumor suppressor p53 binds to the promoter of Nanog, resulting in down-regulation of gene expression following DNA damage, and leading to differentiation of ES cells.[29]

c-Myc is a proto-oncogene commonly linked to neoplastic transformation. In addition to functioning as a transcription factor, c-Myc and the other Myc family members also play a role in modification of chromatin structure through recruitment of histone acetyltransferases.[30] Although c-Myc improves the efficiency of iPS cell generation, about 20% of chimeric mice produced from c-Myc induced iPS cells developed cancer.[13] Ultimately, iPS production strategies to be used clinically should avoid the use of Myc family members as reprogramming factors.

Kruppel-like factor 4 (Klf4) is a member of a family of transcription factors with important roles in cell proliferation, differentiation, and survival. It was one of the key factors used by Yamanaka's group in the first description of murine iPS generation.[13] Subsequently, other Klf family members have been used to generate iPS cells. In contrast, other research groups have demonstrated human iPS cells can be generated without addition of a Klf family member.[14]

Lin28 serves as an mRNA-binding protein and enhances the efficiency of iPS cell generation.[14] Its main function is to enhance the translation of specific proteins, leading to more efficient protein synthesis. Lin28 is also expressed during fetal liver development and in cells undergoing muscle or neural differentiation and is therefore not a specific marker of pluripotency.

CULTURE OF ES/iPS CELLS

Propagation of pluripotent cells follows the basic principles of routine tissue culture, requiring a humidified, temperature-controlled environment with supplemental carbon dioxide. However, specific culture conditions are recommended to enhance the propagation of pluripotent cells. For example, FGF2 treatment in low oxygen conditions has been shown to increase the expression of pluripotency induction genes and may improve iPS cell generation.[31] To maintain pluripotency, ES/iPS cells must be kept isolated from differentiating cells in the colony to prevent cell signaling that can stimulate further differentiation. Conditions for propagation of pluripotent cells can vary widely by species, and have not yet been optimized for the horse, but the following specialized components have been used.

Leukemia inhibitory factor (LIF) is an interleukin class 6 cytokine that prevents ES/iPS cells from differentiating. LIF functions through activation of the JAK-STAT pathway. In the early embryo, LIF is produced by trophectoderm cells, while the LIF receptor is expressed by the inner cell mass of the blastocyst. Isolation of the inner cell mass removes ES cells from their endogenous source, necessitating supplementation of LIF through either recombinant protein or another LIF-expressing cell type. Media supplementation with recombinant LIF has been used in some, but not all, putative equine ES/iPS cell expansion culture systems to maintain cells in an undifferentiated state.

Feeder cells provide an alternate source of LIF for maintenance of pluripotent cells. Feeder cells can be derived from a variety of sources including mouse embryonic fibroblasts (MEF), equine embryonic fibroblasts (EEF), JK1 feeder cells, SNL76/7 cells, and STO cells. Feeder cells need to be irradiated or treated with mitomycin-c to inhibit their replication before use. Different types of feeder cells have been used for propagation of equine ES/iPS cells, but conditions are not yet optimized.

Bone morphogenetic proteins (BMP) are a family of growth factor proteins that contribute to propagation of pluripotent cells without differentiation and are very important in embryonic patterning and skeletal development. BMPs function through activation of the SMAD pathway. Serum supplies endogenous BMPs or media can be supplemented with recombinant BMP proteins if serum-free conditions are used. Serum is an important media component of all equine ES cell culture protocols to date.

CHARACTERIZATION OF ES/iPS CELLS AND EVALUATION OF PLURIPOTENCY

Cellular Morphology

Pluripotent cells have a unique appearance when grown in culture that can be used as an initial screen during colony selection. ES/iPS cells typically have a round shape, with large nucleolus and scant cytoplasm (**Fig. 1**). Colonies of human ES cells have sharp edges and are flat with tightly packed cells. In contrast, mouse colonies are less flat and tend to aggregate and become 3-dimensional. iPS colonies appear morphologically similar to the phenotype of ES cells from their respective species. Equine pluripotent cells are similar in morphologic appearance to human ES cells.[19] ES/iPS cells are mitotically active, leading to self-renewal and spontaneous differentiation, necessitating frequent passage to maintain the cells in a pluripotent state.

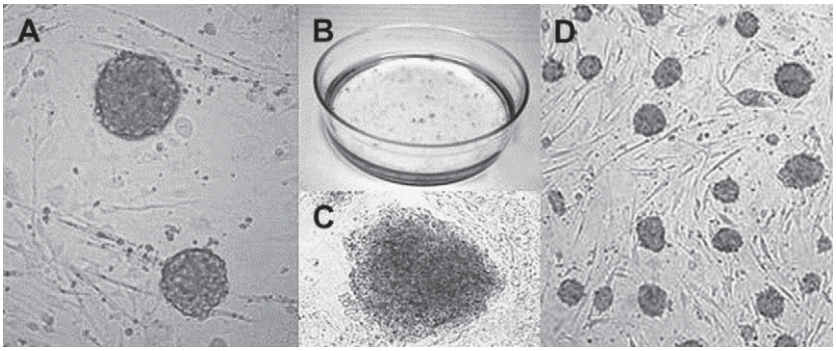

Fig. 1. Putative equine induced pluripotent stem (iPS) cells on a primary transformation plate (*A*–*C*) and after culture for 13 passages (*D*). The putative equine iPS colonies (*A*, *D*) are morphologically similar to human iPS cell colonies and are positive for alkaline phosphatase staining (*B*, *C*). (*Courtesy of* Lauren V. Schnabel, DVM, Ithaca, NY.)

Embryoid body (EB) formation is one assay that has been used as evidence of pluripotency in putative ES/iPS cells. Embryoid bodies are spherical cell aggregates that contain a variety of differentiated cell types on the outer surface with a core of mitotically active ES cells. EBs can be induced to form in culture by suspending ES/iPS cells within a hanging drop, by the use of nonadherent plasticware, or through use of spinner flasks that reduce normal ES cellular attachment. Although not definitive for ES/iPS validation, EB formation suggests that cells have the potential to form multiple tissues.

In vitro differentiation assays previously described for evaluating pluripotency in human and murine ES cells have been used in the validation of putative equine ES/iPS cells. Evidence of in vitro differentiation of equine-derived cells into all 3 primary germ layers has been reported.[4,7] However, without evidence of in vivo differentiation, the value of in vitro assays to demonstrate pluripotency is questionable.

Antibody reaction is one of the greatest challenges in characterizing putative ES/iPS cells. Reagents need to be developed that will accurately detect specific proteins required for validation of pluripotency in the species of interest. This is particularly true in the horse. Antibodies that are not specifically designed to identify the equine epitope need to be carefully validated for cross-species reactivity and specificity for the target equine protein. Since only about 4% of human antibodies react with their equivalent equine protein, most antihuman antibodies are not suitable for use in validation of cells derived from horses.[32] Further, cross-species variability in pluripotency markers makes definitive validation of putative equine ES/iPS cells using antibody profiling difficult.[8] Many of the nonequine antibodies that have been tried for equine ES/iPS cell immunohistochemical analysis include antibodies directed against Oct4, SSEA1, SSEA4, SSEA4, TRA-1-60, and TRA-1-81 proteins.[4] In this particular study, antibodies reacted not only with the equine inner cell mass, but also with equine trophoblast cells, suggesting these antibodies are not specific to proteins found exclusively on ES cells. Given this information, it is safe to conclude that currently available nonequine antibodies are not adequate to demonstrate pluripotency in equine cells.

Histologic assays that employ stains such as alkaline phosphatase are not specific for pluripotency. Most tissues in the body have alkaline phosphatase activity, and some tissues such as kidney, liver, and bone have increased levels of alkaline phosphatase. Previous studies have reported reactivity of antibodies directed against the specific alkaline phosphatase found in embryonic stem cells with many adult cell

types, demonstrating the nonspecific nature of this assay.[33] Although alkaline phosphatase staining can provide supportive evidence that cells are in a pluripotent state, it is not specific or definitive.

Teratoma formation is a more stringent assay for assessment of cellular pluripotency. Teratomas are tumors containing cell types representative of all 3 primary germ layers. Unlike most tumors that contain only one cell type, teratomas can contain a variety of tissue types including cartilage, bone, skin, hair, and glandular tissue. The assay is typically performed by injection of putative ES/iPS cells into immunodeficient mice. In species other than mice, teratoma formation is the definitive test to validate that ES/iPS cell lines are pluripotent.

Recently, a report described generation of equine iPS cells derived from fetal fibroblasts that were able to form complex teratomas in NOD/SCID mice.[19] This group used many novel techniques including a nonviral, *piggyBac* transposon-based method to deliver transgenes containing the reprogramming factors Oct4, Sox2, Klf4 and c-Myc into horse cells. Reprogrammed equine iPS cells were under the influence of a tetracycline-inducible promoter and were identifiable by constitutive green fluorescent protein expression. Following transplantation into NOD/SCID mice, the researchers continued to feed recipient mice doxycycline for several weeks to prolong pluripotency gene expression in the iPS cell transplants, enhancing teratoma formation. The authors readily discuss the pitfalls of their study, but this report represents a major advance in the field of equine ES/iPS research.

Prior to this publication, there had been no reports of equine ES or iPS cells forming teratomas in immunocopromised mice. Some groups suggested that the horse was a species with a unique ability to avoid teratoma formation when ES cells are injected, making equine ES-like cells a potentially suitable source of cells for therapeutic use.[3,4,7] An alternate explanation was that a true equine ES/iPS cell line had not yet successfully been isolated. It was difficult to reconcile the argument that horse ES/iPS cell lines were unique when ES/iPS cells isolated from a number of mammalian species including cattle, swine, mice, and people all yielded teratomas under similar conditions.[8] It is now known that teratoma formation can occur in equine pluripotent cells.

Generation of offspring through chimera/tetraploid complementation assays is an even more rigorous test of pluripotency than teratoma formation. Murine studies have a distinct advantage here, with accurate methods of tracking cell fate in vivo. Through direct injection of putative ES/iPS cells into mouse trophoblasts, viable chimeric pups can be produced with 10% to 90% contribution to the chimeric pups' tissues. Germ-line transmission is possible in the chimera and can be tracked in subsequent generations using coat color analysis.

In mice, tetraploid complementation is the gold standard for validation of ES cell pluripotency. Tetraploid complementation is performed using blastocysts with twice the normal number of chromosomes (tetraploid). Putative ES/iPS cells are then injected into the tetraploid blastocysts, which then form whole, nonchimeric, fertile mice. The offspring are entirely derived from the injected ES/iPS cells. The tetraploid cells of the blastocysts contribute only to the formation of extraembryonic tissues such as the fetal portion of the placenta.

DNA/chromatin methylation patterns and the role of epigenetics have become important areas of research in studies related to cellular differentiation. In general, chromatin structure and organization have important effects on the expression of genes as cells pass through differentiation or reprogramming. Decreased cytosine methylation is often noted in the DNA promoter regions of genes that are transcriptionally active. Similarly, the H3 histones may also be demethylated when genes become

active, opening the chromatin and making the DNA more accessible to transcriptional machinery. The addition of agents that alter chromatin structure and DNA methylation states have been used as media supplements to enhance the reprogramming of iPS cells.[34]

CLINICAL TRIALS

Much excitement has been generated about the transition of ES/iPS cell research into both human and equine clinical applications. The Geron Company (Menlo Park, CA, USA) has just received US Food and Drug Administration (FDA) approval for phase 1 safety trials in people using oligodendrocyte precursor cells (GRNOPC1), derived from human ES cells. Transplantation studies have begun in clinical patients. The original FDA approval was delayed when small numbers of microscopic cysts were found in the spinal cords of experimental rats treated with the human GRNOPC1cells. Initial safety studies will be conducted in paraplegic human patients with complete loss of sensory and neurologic function following subacute (<2 weeks) spinal cord injuries, before significant scar tissue has formed.

In horses, there is a published report of injection of undifferentiated equine ES-like cells into experimentally induced tendon lesions.[3] In that study, ES-like cells displayed longer survival and more extensive migration throughout areas of tendon damage than the adult bone marrow-derived cell treatment group. However, the study reported no significant histologic differences in tendon architecture between the serum control group and both cell (adult bone marrow and ES-like) therapy groups, suggesting the contribution of implanted cells to the repair was minimal at the time of histologic analysis.

Several companies are in the development process of commercializing equine ES/iPS therapies. The Celavet Company (Oxnard, CA, USA) has developed a line of putative ES-like cells that have been implanted in an equine experimental model of collagenase-induced superficial digital flexor tendonitis.[20] A multicenter, blinded, randomized controlled study using the Celavet cell lines in clinical cases of equine tendonitis is awaiting final FDA approval. ViaGen (Austin, TX, USA), Incorporated, along with researchers from the Monash Institute of Medical Research, is also developing commercial lines of reprogrammed equine cells (ViaGen.com) for clinical use.

Although little information is currently available on the translation of ES/iPS cells to the clinics, the rapid advances being made in basic research will likely lead to effective, regenerative cell-based products in the near future. The equine patient stands to gain tremendous benefit from ES/iPS applications, when safe, fully validated products become available for enhanced repair of musculoskeletal injuries. However, care needs to be exercised in preclinical analysis to ensure accurate classification of ES/iPS cell phenotype and subsequent differentiation into the desired cell product before implantation.

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