

Embryonic Stem Cell Literatures

Mark H Smith

Queens, New York 11418, USA
mark20082009@gmail.com

Abstract: The definition of stem cell is “an unspecialized cell that gives rise to a specific specialized cell, such as a blood cell”. Stem Cell is the original of life. All cells come from stem cells. Serving as a repair system for the living body, the stem cells can divide without limit to replenish other cells as long as the living body is still alive. When a stem cell divides, each new cell has the potential to either remain a stem cell situation or become another type of cell with a more specialized function, such as a muscle cell, a red blood cell, a bone cell, a nerve cell, or a brain cell. Stem cell research is a typical and important topic of life science. This material collects some literatures on embryonic stem cell.

[Smith MH. **Embryonic Stem Cell Literatures.** *Stem Cell* 2011;2(1):105-169] (ISSN 1545-4570).
<http://www.sciencepub.net/stem>. 7

Key words: stem cell; life; gene; DNA; protein

Literatures

Abdelalim, E. M. and I. Tooyama (2009). "BNP signaling is crucial for embryonic stem cell proliferation." *PLoS One* 4(4): e5341.

BACKGROUND: Embryonic stem (ES) cells have unlimited proliferation potential, and can differentiate into several cell types, which represent ideal sources for cell-based therapy. This high-level proliferative ability is attributed to an unusual type of cell cycle. The Signaling pathways that regulate the proliferation of ES cells are of great interest. **METHODOLOGY/PRINCIPAL FINDINGS:** In this study, we show that murine ES cells specifically express brain natriuretic peptide (BNP), and its signaling is essential for ES cell proliferation. We found that BNP and its receptor (NPR-A, natriuretic peptide receptor-A) were highly expressed in self-renewing murine ES cells, whereas the levels were markedly reduced after ES cell differentiation by the withdrawal of LIF. Targeting of BNP with short interfering RNA (siRNA) resulted in the inhibition of ES cell proliferation, as indicated by a marked reduction in the cell number and colony size, a significant reduction in DNA synthesis, and decreased numbers of cells in S phase. BNP knockdown in ES cells led to the up-regulation of gamma-aminobutyric acid receptor A (GABA(A)R) genes, and activation of phosphorylated histone (gamma-H2AX), which negatively affects ES cell proliferation. In addition, knockdown of BNP increased the rate of apoptosis and reduced the expression of the transcription factor Ets-1.

CONCLUSIONS/SIGNIFICANCE: Appropriate BNP expression is essential for the maintenance of ES cell propagation. These findings establish BNP as a novel endogenous regulator of ES cell proliferation.

Adamson, E. D., S. Strickland, et al. (1985). "A teratocarcinoma-derived endoderm stem cell line (1H5) that can differentiate into extra-embryonic endoderm cell types." *Differentiation* 29(1): 68-76.

We investigated the ability of the teratocarcinoma-derived, epithelial-type cell line 1H5 to differentiate into either of the two pathways to primary endoderm, and tested the hypothesis that 1H5 represents a state similar to primitive endoderm in the late 4th-day blastocyst. Like other endodermal cell types, 1H5 cells mixed with embryonal-carcinoma cells sort out into "embryoid bodies" or structures that resemble 4th-day mouse embryos. The epithelial line conforms morphologically and biochemically to the few known characteristics typical of primitive endoderm. The present study demonstrates that the formation in vitro of overt visceral endoderm is readily achieved. The spontaneous arrangement of the cells into a cystic form is followed by the appearance of several markers of visceral endoderm, most notably alphafetoprotein, which is detected when 1H5 cells are cultured either in the presence of retinoic acid or when the cells interact with embryonal-carcinoma cells in a specific spatial arrangement after sorting out. However, some less specific properties of visceral endoderm are not expressed. Although 1H5 differentiates histologically into parietal-like endoderm in the tumor form, parietal cells cannot yet be identified with certainty in vitro because of the paucity of parietal-specific markers. The 1H5 cell line could provide a useful system for studying the characteristics and mechanisms underlying visceral-endoderm differentiation in vitro, since it has the distinct advantage that homogeneous cultures are produced, in contrast to other teratocarcinoma cell lines such as F9 which differentiate into a mixture of cell types.

Aejaz, H. M., A. K. Aleem, et al. (2007). "Stem cell therapy-present status." *Transplant Proc* **39**(3): 694-9.

Stem cell research is a new field that is advancing at an incredible pace with new discoveries being reported from all over the world. Scientists have for years looked for ways to use stem cells to replace cells and tissues that are damaged or diseased. Stem cells are the foundation cells for every organ, tissue, and cell in the body. Stem cells are undifferentiated, "blank" cells that do not yet have a specific function. Under proper conditions, stem cells begin to develop into specialized tissues and organs. They are self-sustaining and can replicate themselves for long periods of time. Embryonic stem cells are pluripotent cells, isolated from the inner cell mass of the blastocyst-stage mammalian embryo. They have the ability to differentiate into several somatic or somatic-like functional cells such as neurons, hepatocytes, cardiomyocytes, and others. Adult stem cells are specialized cells found within many tissues of the body where they function in tissue homeostasis and repair. They are precursor cells capable of differentiation into several different cells. The knowledge of stem cells from various sources offered a new hope for the treatment of various diseases.

Ahuja, Y. R., V. Vijayalakshmi, et al. (2007). "Stem cell test: a practical tool in toxicogenomics." *Toxicology* **231**(1): 1-10.

During early embryonic development, at blastocyst stage, the embryo has an outer coat of cells and an inner cell mass (ICM). ICM is the reservoir of embryonic stem (ES) cells, which are pluripotent, i.e., have the potential to differentiate into all cell types of the body. Cell lines have been developed from ES cells. In addition, there are embryonic germ (EG) cell lines developed from progenitor germ cells, and embryonic carcinoma (EC) cell lines developed from teratomas. These cell lines are being used for the study of basic and applied aspects in medical therapeutics, and disease management. Another potential of these cell lines is in the field of environmental mutagenesis. In addition to ES cells, there are adult stem cells in and around different organs and tissues of the body. It is now possible to grow pure populations of specific cell types from these adult stem cells. Treating specific cell types with chemical or physical agents and measuring their response offers a shortcut to test the toxicity in various organ systems in the adult organism. For example, to evaluate the genotoxicity of a chemical (e.g., drug or pesticide) or a physical agent (e.g., ionizing radiation or non-ionizing electromagnetic radiation) during embryonic development, a large number of animals are being used. As an alternative,

use of stem cell lines would be a feasible proposition. Using stem cell lines, efforts are being made to standardize the protocols, which will not only be useful in testing the toxicity of a chemical or a physical agent, but also in the field of drug development, environmental mutagenesis, biomonitoring and other studies.

Aiba, K., M. G. Carter, et al. (2006). "Genomic approaches to early embryogenesis and stem cell biology." *Semin Reprod Med* **24**(5): 330-9.

Large-scale systematic gene expression analyses of early embryos and stem cells provide useful information to identify genes expressed differentially or uniquely in these cells. We review the current status of various approaches applied to preimplantation embryos and stem cells: expressed sequence tag, serial analysis of gene expression, differential display, massively parallel signature sequencing, DNA microarray (DNA chip) analysis, and chromatin-immunoprecipitation microarrays. We also discuss the biological questions that can only be addressed by the analysis of global gene expression patterns, such as so-called stemness and developmental potency. As the emphasis now shifts from expression profiling to functional studies, we review the genome-scale functional studies of genes: expression cloning, gene trapping, RNA interference, and gene disruptions. Finally, we discuss the future clinical application of such methodologies.

Amano, T., Y. Kato, et al. (2001). "Full-term development of enucleated mouse oocytes fused with embryonic stem cells from different cell lines." *Reproduction* **121**(5): 729-33.

The developmental potential of enucleated mouse oocytes receiving embryonic stem cells from ten lines with either the same or different genetic backgrounds using the cell fusion method was examined in vitro and in vivo. The development of nuclear-transferred oocytes into blastocysts was high (34-88%). However, there was no clear correlation between development into blastocysts after nuclear transfer and the chimaera formation rate of embryonic stem cells. The development into live young was low (1-3%) in all cell lines and 14 of 19 young died shortly after birth. Most of the live young had morphological abnormalities. Of the five remaining mice, two died at days 23 and 30 after birth, but the other three mice are still active at days 359 (mouse 1) and 338 (mice 4 and 5) after birth, with normal fertility. However, the reasons for the abnormalities and postnatal death of embryonic stem cell-derived mice are unknown.

Amit, M., M. K. Carpenter, et al. (2000). "Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture." *Dev Biol* **227**(2): 271-8.

Embryonic stem (ES) cell lines derived from human blastocysts have the developmental potential to form derivatives of all three embryonic germ layers even after prolonged culture. Here we describe the clonal derivation of two human ES cell lines, H9.1 and H9.2. At the time of the clonal derivation of the H9.1 and H9.2 ES cell lines, the parental ES cell line, H9, had already been continuously cultured for 6 months. After an additional 8 months of culture, H9.1 and H9.2 ES cell lines continued to: (1) actively proliferate, (2) express high levels of telomerase, and (3) retain normal karyotypes. Telomere lengths, while somewhat variable, were maintained between 8 and 12 kb in high-passage H9.1 and H9.2 cells. High-passage H9.1 and H9.2 cells both formed teratomas in SCID-beige mice that included differentiated derivatives of all three embryonic germ layers. These results demonstrate the pluripotency of single human ES cells, the maintenance of pluripotency during an extended period of culture, and the long-term self-renewing properties of cultured human ES cells. The remarkable developmental potential, proliferative capacity, and karyotypic stability of human ES cells distinguish them from adult cells.

Andang, M., J. Hjerling-Leffler, et al. (2008). "Histone H2AX-dependent GABA(A) receptor regulation of stem cell proliferation." *Nature* **451**(7177): 460-4.

Stem cell self-renewal implies proliferation under continued maintenance of multipotency. Small changes in numbers of stem cells may lead to large differences in differentiated cell numbers, resulting in significant physiological consequences. Proliferation is typically regulated in the G1 phase, which is associated with differentiation and cell cycle arrest. However, embryonic stem (ES) cells may lack a G1 checkpoint. Regulation of proliferation in the 'DNA damage' S/G2 cell cycle checkpoint pathway is known for its role in the maintenance of chromatin structural integrity. Here we show that autocrine/paracrine gamma-aminobutyric acid (GABA) signalling by means of GABA(A) receptors negatively controls ES cell and peripheral neural crest stem (NCS) cell proliferation, preimplantation embryonic growth and proliferation in the boundary-cap stem cell niche, resulting in an attenuation of neuronal progenies from this stem cell niche. Activation of GABA(A) receptors leads to hyperpolarization, increased cell volume and accumulation of stem cells in S phase, thereby causing a rapid decrease in cell proliferation. GABA(A) receptors signal through S-phase checkpoint kinases

of the phosphatidylinositol-3-OH kinase-related kinase family and the histone variant H2AX. This signalling pathway critically regulates proliferation independently of differentiation, apoptosis and overt damage to DNA. These results indicate the presence of a fundamentally different mechanism of proliferation control in these stem cells, in comparison with most somatic cells, involving proteins in the DNA damage checkpoint pathway.

Araki, K., N. Takeda, et al. (2009). "Establishment of germline-competent embryonic stem cell lines from the MSM/Ms strain." *Mamm Genome* **20**(1): 14-20.

MSM/Ms is an inbred mouse strain established from the Japanese wild mouse, *Mus musculus molossinus*, which has been phylogenetically distinct from common laboratory mouse strains for about 1 million years. The nucleotide substitution rate between MSM/Ms and C57BL/6 is estimated to be 0.96%. MSM/Ms mice display unique characteristics not observed in the commonly used laboratory strains, including an extremely low incidence of tumor development, high locomotor activity, and resistance to high-fat-diet-induced diabetes. Thus, functional genomic analyses using MSM/Ms should provide a powerful tool for the identification of novel phenotypes and gene functions. We report here the derivation of germline-competent embryonic stem (ES) cell lines from MSM/Ms blastocysts, allowing genetic manipulation of the *M. m. molossinus* genome. Fifteen blastocysts were cultured in ES cell medium and three ES lines, Mol/MSM-1, -2, and -3, were established. They were tested for germline competency by aggregation with ICR morulae and germline chimeras were obtained from all three lines. We also injected Mol/MSM-1 ES cells into blastocysts of ICR or C57BL/6 x BDF1 mice and found that blastocyst injection resulted in a higher production rate of chimeric mice than did aggregation. Furthermore, Mol/MSM-1 subclones electroporated with a gene trap vector were also highly efficient at producing germline chimeras using C57BL/6 x BDF1 blastocyst injection. This Mol/MSM-1 ES line should provide an excellent new tool allowing the genetic manipulation of the MSM/Ms genome.

Auerbach, W., J. H. Dunmore, et al. (2000). "Establishment and chimera analysis of 129/SvEv- and C57BL/6-derived mouse embryonic stem cell lines." *Biotechniques* **29**(5): 1024-8, 1030, 1032.

Hundreds of new mutant mouse lines are being produced annually using gene targeting and gene trap approaches in embryonic stem (ES) cells, and the number is expected to continue to grow as the human and mouse genome projects progress. The

availability of robust ES cell lines and a simple technology for making chimeras is more attractive now than ever before. We established several new ES cell lines from 129/SvEv and C57BL/6 mice and tested their ability to contribute to the germline following blastocyst injections and/or the less expensive and easier method of morula-ES cell aggregation. Using morula aggregation to produce chimeras, five newly derived 129/SvEv and two C57BL/6 ES cell lines tested at early passages were found to contribute extensively to chimeras and produce germline-transmitting male chimeras. Furthermore, the two 129S/vEv ES cell lines that were tested and one of the C57BL/6 ES cell lines were able to maintain these characteristics after many passages in vitro. Our results indicate that the ability of ES cells to contribute strongly to chimeras following aggregation with outbred embryos is a general property of early passage ES cells and can be maintained for many passages. C56BL/6-derived ES cell lines, however, have a greater tendency than 129-derived ES cell lines to lose their ability to colonize the germline.

Baharvand, H., S. K. Ashtiani, et al. (2004). "Establishment and in vitro differentiation of a new embryonic stem cell line from human blastocyst." Differentiation **72**(5): 224-9.

Embryonic stem cells have the ability to remain undifferentiated and proliferate indefinitely in vitro while maintaining the potential to differentiate into derivatives of all three embryonic germ layers. These cells have, therefore, potential for in vitro differentiation studies, gene function, and so on. The aim of this study was to produce a human embryonic stem cell line. An inner cell mass of a human blastocyst was separated and cultured on mouse embryonic fibroblasts in embryonic stem cell medium with related additives. The established line was evaluated by morphology; passaging; freezing and thawing; alkaline phosphatase; Oct-4 expression; anti-surface markers including Tra-1-60 and Tra-1-81; and karyotype and spontaneous differentiation. Differentiated cardiomyocytes and neurons were evaluated by transmission electron microscopy and immunocytochemistry. Here, we report the derivation of a new embryonic stem cell line (Royan H1) from a human blastocyst that remains undifferentiated in morphology during continuous passaging for more than 30 passages, maintains a normal XX karyotype, is viable after freezing and thawing, and expresses alkaline phosphatase, Oct-4, Tra-1-60, and Tra-1-81. These cells remain undifferentiated when grown on mouse embryonic fibroblast feeder layers in the presence or absence of recombinant human leukemia inhibitory factor. Royan H1 cells can differentiate in

vitro in the absence of feeder cells and can produce embryoid bodies that can further differentiate into beating cardiomyocytes as well as neurons. These results define Royan H1 cells as a new human embryonic stem cell line.

Baharvand, H. and K. I. Matthaee (2004). "Culture condition difference for establishment of new embryonic stem cell lines from the C57BL/6 and BALB/c mouse strains." In Vitro Cell Dev Biol Anim **40**(3-4): 76-81.

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of blastocysts. These cells are appropriate for creation of animal models of human genetic diseases, the study of gene function in vivo and differentiation into specific types as potential therapeutic agents for several human diseases. We describe here, the production of new ES cell lines from blastocysts recovered from the C57BL/6 and BALB/c mouse strains by changing the concentration of leukemia inhibitory factor (LIF) and primary culture conditions. The established cell lines were analyzed by simple karyotype, C banding, alkaline phosphatase activity, and Oct-4 expression as well as for the presence of the SRY gene. Two ES cell lines from C57BL/6 and three from the BALB/c were produced. The two C57BL/6 ES cell lines were established with either 1000 or 5000 IU LIF, whereas the BALB/c ES cell lines required 5000 IU LIF. Four of the ES cell lines had a normal karyotype. C banding and sex-determining region of Y chromosome-polymerase chain reaction showed that all cell lines had an XY sex chromosome composition. All five of the cell lines expressed alkaline phosphatase activity and Oct-4. One of the BALB/c ES cell lines, when injected into C57BL/6 blastocysts, produced high rates of chimerism as assessed by coat color, and the male chimera produced germ-line offspring when mated with BALB/c females. These results indicate that ES cells from inbred strains can be isolated using commercially available reagents and that the establishment of BALB/c ES cell lines may require different culture conditions to the 129 or C57BL/6 strains.

Bendall, S. C., M. H. Stewart, et al. (2008). "Human embryonic stem cells: lessons from stem cell niches in vivo." Regen Med **3**(3): 365-76.

In vivo the stem cell niche is an essential component in controlling and maintaining the stem cells' ability to survive and respond to injury. Human embryonic stem cells (hESCs) appear to be an exception to this rule as they can be removed from their blastocytic microenvironment and maintained indefinitely in vitro. However, recent observations reveal the existence of an autonomously derived in

in vitro hESC niche. This provides a previously unappreciated mechanism to control hESC expansion and differentiation. Recognizing this, it may now be possible to take aspects of in vivo stem cell niches, namely extracellular matrices, paracrine signals and accessory cell types, and exploit them in order to gain fidelity in directed hESC differentiation. In doing so, routine customization of hESC lines and their application in regenerative therapies may be further enhanced using unique hESC niche-based approaches.

Ben-Yosef, D., M. Malcov, et al. (2008). "PGD-derived human embryonic stem cell lines as a powerful tool for the study of human genetic disorders." *Mol Cell Endocrinol* **282**(1-2): 153-8.

Human embryonic stem (ES) cells are derived from the inner cell mass (ICM) of blastocyst embryos. They are established from spare embryos that have been obtained by in vitro fertilization (IVF) and donated for research purposes. The ICM-derived cell lines have two unique properties, they can be propagated indefinitely in culture and have the potential to develop into practically any cell type in vitro and in vivo. Human embryonic stem (hES) cells carrying specific mutations can be used as a valuable tool for studying genetic disorders in human. One favorable approach to obtain such mutant ES cell lines is their derivation from affected preimplantation genetic diagnosed (PGD) embryos. This review focuses on the importance of deriving human ES cell lines from genetically abnormal embryos, especially in cases where no good cellular and/or animal models exist.

Berthier, R., M. H. Prandini, et al. (1997). "The MS-5 murine stromal cell line and hematopoietic growth factors synergize to support the megakaryocytic differentiation of embryonic stem cells." *Exp Hematol* **25**(6): 481-90.

Murine embryonic stem (ES) cells are able to differentiate into erythroid, mast, and granulomonocytic cells by using appropriate culture conditions. Because we were interested in the regulation of tissue-specific expression of the platelet glycoprotein IIb gene, we studied the culture conditions, aiming at the reproducible production of myeloid cells that included megakaryocytes (MKs) from ES cells. We showed that even a complex cocktail of HGFs (stem cell factor, interleukin 3, IL6, IL11, granulocyte colony-stimulating factor, and erythropoietin) is unable to induce significant myeloid differentiation in day 12 embryoid bodies. Cocultures of MS-5 stromal cells with ES cells were slightly more productive than HGFs. A strong synergistic effect was observed on the growth of myeloid colonies and MKs when we used a combination of

MS-5 cells plus the HGF cocktail. Conditioned medium from MS-5 cells also synergized with the HGF cocktail to produce a substantial number of mixed colonies containing MKs. The addition of fibroblast growth factor-2 (FGF-2) to the HGF cocktail plus MS-5 nearly doubled the number of myeloid progenitors, including those with MKs. Thrombopoietin (TPO) alone or in any combination with MS-5 or HGFs, did not increase the number of MK-containing colonies. However, when TPO was added to the HGF cocktail + FGF-2 + MS-5, the number of MKs in liquid cultures and mixed colonies increased, and many exhibited a "hairy" appearance resembling pseudopodial proplatelet formation. Having defined the culture conditions of ES cells that allow the production of all the myeloid lineages including MKs, we conclude that the hematopoietic differentiation model of ES cells is especially useful for studying the regulation of expression of any gene important in early hematopoiesis.

Beyhan, Z., A. E. Iager, et al. (2007). "Interspecies nuclear transfer: implications for embryonic stem cell biology." *Cell Stem Cell* **1**(5): 502-12.

Accessibility of human oocytes for research poses a serious ethical challenge to society. This fact categorically holds true when pursuing some of the most promising areas of research, such as somatic cell nuclear transfer and embryonic stem cell studies. One approach to overcoming this limitation is to use an oocyte from one species and a somatic cell from another. Recently, several attempts to capture the promises of this approach have met with varying success, ranging from establishing human embryonic stem cells to obtaining live offspring in animals. This review focuses on the challenges and opportunities presented by the formidable task of overcoming biological differences among species.

Boiani, M., L. Gentile, et al. (2005). "Variable reprogramming of the pluripotent stem cell marker Oct4 in mouse clones: distinct developmental potentials in different culture environments." *Stem Cells* **23**(8): 1089-104.

A prevailing view of cloning by somatic-cell nuclear transfer is that reprogramming of gene expression occurs during the first few hours after injection of the nucleus into an oocyte, that the process is stochastic, and that the type of reprogramming needed for cloning success is foreign and unlikely to be readily achieved in the ooplasm. Here, we present evidence that the release of reprogramming capacity is contingent on the culture environment of the clone while the contribution of aneuploidy to altered gene expression is marginal. In particular, the rate of blastocyst formation in clones

and the regional distribution of mRNA for the pluripotent stem cell marker Oct4 in clonal blastocysts was highly dependent on the culture environment after cumulus cell nuclear transfer, unlike that in genetically equivalent zygotes. Epigenetic modifications of genetically identical somatic nuclei continue after the first cell division of the clones and are amenable to a degree of experimental control, and their development to the blastocyst stage and appropriate expression of Oct4 predict further outcome, such as derivation of embryonic stem (ES) cells, but not fetal development. This observation indicates that development to the blastocyst stage is not equivalent to full reprogramming and lends support to the novel concept that ES cells are not the equivalent of the inner cell mass, hence the discrepancy between ES cell derivability and fetal development of clones.

Bongso, A. and M. Richards (2004). "History and perspective of stem cell research." Best Pract Res Clin Obstet Gynaecol **18**(6): 827-42.

Several types of stem cell have been discovered from germ cells, the embryo, fetus and adult. Each of these has promised to revolutionize the future of regenerative medicine through the provision of cell-replacement therapies to treat a variety of debilitating diseases. Stem cell research is politically charged, receives considerable media coverage, raises many ethical and religious debates and generates a great deal of public interest. The tremendous versatility of embryonic stem cells versus the unprecedented reports describing adult stem cell plasticity have ignited debates as to the choice of one cell type over another for future application. However, the biology of these mysterious cells have yet to be understood and a lot more basic research is needed before new therapies using stem-cell-differentiated derivatives can be applied. Stem cell research opens up the new field of 'cell-based therapies' and, as such, several safety measures have also to be evaluated.

Bongso, A. and S. Tan (2005). "Human blastocyst culture and derivation of embryonic stem cell lines." Stem Cell Rev **1**(2): 87-98.

Human embryonic stem cell (hESC) biology is expected to revolutionize the future of medicine by the provision of cell-based therapies for the treatment of a variety of debilitating diseases. The tremendous versatility of hESCs has reinforced this hope. To understand the biology of these mysterious cells and attempt to differentiate them into desirable tissues, bona fide hESCs that maintain their stability with time are required for research and clinical application. This review discusses the various protocols to derive and propagate hESCs from high quality embryos. The

nature and properties of hESCs are also described together with unanswered questions that need to be addressed if this science is to be taken to the bedside.

Boyd, A. S., Y. Higashi, et al. (2005). "Transplanting stem cells: potential targets for immune attack. Modulating the immune response against embryonic stem cell transplantation." Adv Drug Deliv Rev **57**(13): 1944-69.

The curative promise of stem cells and their descendants for tissue regeneration and repair is currently the subject of an intense research effort worldwide. If it proves feasible to differentiate stem cells into specific tissues reliably and safely, this approach will be invaluable in the treatment of diseases that lead to organ degeneration or failure, providing an alternative or supplementary source of tissue for transplantation. Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of a pre-implantation blastocyst that can produce all cells and tissues of the foetus. In recent years, several laboratories have described the directed differentiation of ES cells into multiple mature cell types including: cardiomyocytes; haemopoietic cells; hepatocytes; neurones; muscle cells and both endocrine and exocrine cells of the pancreas. How the immune system of the host will respond when these ES cell-derived mature cells are transplanted is ill defined. This review will focus on the potential mechanisms that the immune system could use to target ES cell-derived transplants and how unwanted responses might be prevented.

Brachvogel, B., H. Moch, et al. (2005). "Perivascular cells expressing annexin A5 define a novel mesenchymal stem cell-like population with the capacity to differentiate into multiple mesenchymal lineages." Development **132**(11): 2657-68.

The annexin A5 gene (*Anxa5*) was recently found to be expressed in the developing and adult vascular system as well as the skeletal system. In this paper, the expression of an *Anxa5-lacZ* fusion gene was used to define the onset of expression in the vasculature and to characterize these *Anxa5-lacZ*-expressing vasculature-associated cells. After blastocyst implantation, *Anxa5-lacZ*-positive cells were first detected in extra-embryonic tissues and in angioblast progenitors forming the primary vascular plexus. Later, expression is highly restricted to perivascular cells in most blood vessels resembling pericytes or vascular smooth muscle cells. Viable *Anxa5-lacZ*⁺ perivascular cells were isolated from embryos as well as adult brain meninges by specific staining with fluorescent X-gal substrates and cell-sorting. These purified *lacZ*⁺ cells specifically express known markers of pericytes, but also markers

characteristic for stem cell populations. In vitro and in vivo differentiation experiments show that this cell pool expresses early markers of chondrogenesis, is capable of forming a calcified matrix and differentiates into adipocytes. Hence, Anxa5 expression in perivascular cells from mouse defines a novel population of cells with a distinct developmental potential.

Brehany, J. (2005). "Nontraditional sources of pluripotent stem cells: a new chapter in the debate about embryonic stem cell research." Health Care Ethics USA **13**(2): E3.

Recent efforts to resolve the political impasse over human embryonic stem cells (ESC) have generated proposals for obtaining ESC while avoiding the destruction of human embryos. This new chapter in the scientific and ethical debate provides an important opportunity to introduce additional ethical considerations to enhance public discourse.

Chang, J. C., L. Ye, et al. (2006). "Correction of the sickle cell mutation in embryonic stem cells." Proc Natl Acad Sci U S A **103**(4): 1036-40.

Sickle cell anemia is one of the most common genetic diseases worldwide. Patients often suffer from anemia, painful crises, infections, strokes, and cardiopulmonary complications. Although current management has improved the quality of life and survival of patients, cure can be achieved only with bone marrow transplantation when histocompatible donors are available. The ES cell technology suggests that a therapeutic cloning approach may be feasible for treatment of this disease. Using a transgenic/knockout sickle cell anemia mouse model, which harbors 240 kb of human DNA sequences containing the beta(S)-globin gene, we prepared ES cells from blastocysts that had the sickle cells anemia genotype and carried out homologous recombination with DNA constructs that contained the beta(A)-globin gene. We obtained ES cells in which the beta(S) was corrected to the beta(A) sequence. Hematopoietic cells differentiated from these ES cells produced both hemoglobin A and hemoglobin S. This approach can be applied to human ES cells to correct the sickle mutation as well as beta-thalassemia mutations.

Chen, H., K. Qian, et al. (2005). "The derivation of two additional human embryonic stem cell lines from day 3 embryos with low morphological scores." Hum Reprod **20**(8): 2201-6.

BACKGROUND: Immune rejection can lead to the failure of human embryonic stem cell (hES cell) transplantation. One approach to address the problem is to establish hES cell line banks. Due to the limited

source of human embryos and to ethical reasons, the hES cell lines are not readily available. This study was undertaken to determine whether discarded day 3 embryos with low morphological scores could develop into blastocysts and produce hES cell lines. **METHODS:** A total of 130 day 3 embryos with low morphological scores were cultured to blastocyst stage, and inner cell masses (ICM) were isolated by immunosurgery. Colonies derived from the ICM were passaged every 4-7 days and evaluated for cell surface markers, differentiation potentials and karyotypes. **RESULTS:** A total of 19 blastocysts were obtained from 130 embryos (quality score <16), which resulted in the formation of 10 ICM, and two cell lines. Both cell lines satisfied the criteria that characterize pluripotent hES cells. **CONCLUSION:** Our results suggest that a subset with poor quality day 3 embryos judged on the basis of morphological assessment can form blastocysts and give rise to hES cell lines.

Chen, U., M. Kosco, et al. (1992). "Establishment and characterization of lymphoid and myeloid mixed-cell populations from mouse late embryoid bodies, "embryonic-stem-cell fetuses"." Proc Natl Acad Sci U S A **89**(7): 2541-5.

Mouse embryonic stem (ES) cells have the potential to differentiate into embryoid bodies in vitro and mimic normal embryonic development. The "ES fetus" is a specific development at a late stage seen under our culture conditions. We have established several mixed populations from ES fetuses by using combinations of retroviruses carrying different oncogenes (v-abl, v-raf, c-myc), interleukins 2 and 3, and Con A. Six groups of mixed populations were characterized by immunophenotyping. For some groups, transfer of cells into sublethally irradiated mice resulted in the development of macrophages, mature T and B lymphocytes, and plasma cells of donor origin. Thus, these mixed populations may contain immortalized precursors of hematopoietic lineages. These mixed populations should be valuable for defining hematopoietic stem cells and their committed progenitors.

Chen, Y. M., Z. W. Du, et al. (2005). "Molecular cloning and functional analysis of ESGP, an embryonic stem cell and germ cell specific protein." Acta Biochim Biophys Sin (Shanghai) **37**(12): 789-96.

Several putative Oct-4 downstream genes from mouse embryonic stem (ES) cells have been identified using the suppression-subtractive hybridization method. In this study, one of the novel genes encoding an ES cell and germ cell specific protein (ESGP) was cloned by rapid amplification of cDNA ends. ESGP contains 801 bp encoding an 84

amino acid small protein and has no significant homology to any known genes. There is a signal peptide at the N-terminal of ESGP protein as predicted by SeqWeb (GCG) (SeqWeb version 2.0.2, <http://gcg.biosino.org:8080/>). The result of immunofluorescence assay suggested that ESGP might encode a secretory protein. The expression pattern of ESGP is consistent with the expression of Oct-4 during embryonic development. ESGP protein was detected in fertilized oocyte, from 3.5 day postcoital (dpc) blastocyst to 17.5 dpc embryo, and was only detected in testis and ovary tissues in adult. In vitro, ESGP was only expressed in pluripotent cell lines, such as embryonic stem cells, embryonic caoma cells and embryonic germ cells, but not in their differentiated progenies. Despite its specific expression, forced expression of ESGP is not indispensable for the effect of Oct-4 on ES cell self-renewal, and does not affect the differentiation to three germ layers.

Cho, M., M. Jang, et al. (2006). "An alternative method of deriving embryonic stem cell-like clones by aggregation of diploid cells with tetraploid embryos." *Fertil Steril* **85 Suppl 1**: 1103-10.

OBJECTIVE: To assess whether embryonic stem (ES) cells could be derived from the aggregation of diploid cells with tetraploid embryos. **DESIGN:** Randomized, prospective study. **SETTING:** University embryology and gamete biotechnology laboratory. **ANIMAL(S):** F1 (C57BL6/DBA2) mice. **INTERVENTION(S):** Four- to eight-cell F1 tetraploid embryos were aggregated with 10 to 15 donor E14 ES cells. **MAIN OUTCOME MEASURE(S):** Embryogenesis and ES cell establishment. **RESULT(S):** No difference (78% to 89%) in blastocyst formation was detected between the aggregated tetraploid and the control diploid embryos. In a total of 27 transfers, pregnancy was detected in three tetraploid (23.1%) and five diploid (35.7%) cases, and three live births developed from the aggregated tetraploid embryos. The tetraploid blastocysts without aggregation were plated, but no ES cell-like colony was formed. Six of eight aggregated blastocysts derived well-proliferated colonies, which were positive for anti-stage-specific embryonic antigen (SSEA)-1 antibody, Oct-4, and alkaline phosphatase. The microsatellite assay confirmed the homogenous makeup among the donor E14 cells and live-birth and ES-like cells derived from the E14-aggregated, tetraploid embryo. **CONCLUSION(S):** The aggregation of pluripotent diploid cells with tetraploid embryos yielded live births and ES-like cells that were homogenous to the donor diploid cells.

Choudhary, M., X. Zhang, et al. (2007). "Putative role of hyaluronan and its related genes, HAS2 and RHAMM, in human early preimplantation embryogenesis and embryonic stem cell characterization." *Stem Cells* **25**(12): 3045-57.

Human embryonic stem cells (hESC) promise tremendous potential as a developmental and cell therapeutic tool. The combined effort of stimulatory and inhibitory signals regulating gene expression, which drives the tissue differentiation and morphogenetic processes during early embryogenesis, is still very poorly understood. With the scarcity of availability of human embryos for research, hESC can be used as an alternative source to study the early human embryogenesis. Hyaluronan (HA), a simple hydrating sugar, is present abundantly in the female reproductive tract during fertilization, embryo growth, and implantation and plays an important role in early development of the mammalian embryo. HA and its binding protein RHAMM regulate various cellular and hydrodynamic processes from cell migration, proliferation, and signaling to regulation of gene expression, cell differentiation, morphogenesis, and metastasis via both extracellular and intracellular pathways. In this study, we show for the first time that HA synthase gene HAS2 and its binding receptor RHAMM are differentially expressed during all stages of preimplantation human embryos and hESC. RHAMM expression is significantly downregulated during differentiation of hESC, in contrast to HAS2, which is significantly upregulated. Most importantly, RHAMM knockdown results in downregulation of several pluripotency markers in hESC, induction of early extraembryonic lineages, loss of cell viability, and changes in hESC cycle. These data therefore highlight an important role for RHAMM in maintenance of hESC pluripotency, viability, and cell cycle control. Interestingly, HAS2 knockdown results in suppression of hESC differentiation without affecting hESC pluripotency. This suggests an intrinsic role for HAS2 in hESC differentiation process. In accordance with this, addition of exogenous HA to the differentiation medium enhances hESC differentiation to mesodermal and cardiac lineages. Disclosure of potential conflicts of interest is found at the end of this article.

Cibelli, J. B., S. L. Stice, et al. (1998). "Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells." *Nat Biotechnol* **16**(7): 642-6.

We have developed a method, using nuclear transplantation, to produce transgenic embryonic stem (ES)-like cells from fetal bovine fibroblasts. These cells, when reintroduced into preimplantation embryos, differentiated into derivatives from the three

embryonic germ layers, ectoderm, mesoderm, and endoderm, in 5-month-old animals. Six out of seven (86%) calves born were found to be chimeric for at least one tissue. These experiments demonstrate that somatic cells can be genetically modified and then de-differentiated by nuclear transfer into ES-like cells, opening the possibility of using them in differentiation studies and human cell therapy.

Collodi, P., Y. Kamei, et al. (1992). "Fish embryo cell cultures for derivation of stem cells and transgenic chimeras." *Mol Mar Biol Biotechnol* **1**(4-5): 257-65.

It has been demonstrated in mammalian systems that techniques using embryonal stem cells provide advantages over conventional injection of DNA into embryos for generation of transgenic animals. We employed cell culture approaches in an attempt to develop this technology for fish transgenesis. Using a trout embryo-derived mitogenic preparation in a specialized culture medium, we initiated replication of zebrafish blastula-derived cell cultures and expressed marker genes introduced into the cells by plasmid transfection. Reintroduction of cells from the cultures into blastula-stage embryos indicated that the cultured cells survived and may contribute to the developing organism.

Conner, D. A. (2001). "Mouse embryonic stem (ES) cell culture." *Curr Protoc Mol Biol* **Chapter 23**: Unit 23 3.

Mouse embryonic stem (ES) cells are used to generate mouse mutants by gene targeting and blastocyst-mediated transgenesis. ES cells must be cultured under conditions that prevent differentiation to maintain their ability to transmit altered alleles by contributing to the germ line. This unit describes one of the most common methods for culturing ES cells using mouse embryo fibroblast (MEF) feeder layers and recombinant leukemia inhibitory factor (LIF) to prevent differentiation.

Constantinescu, S. N. (2000). "Stem cell generation and choice of fate: role of cytokines and cellular microenvironment." *J Cell Mol Med* **4**(4): 233-248.

Hematopoietic stem cells (HSC) have provided a model for the isolation, enrichment and transplantation of stem cells. Gene targeting studies in mice have shown that expression of the thrombopoietin receptor (TpoR) is linked to the accumulation of HSCs capable to generate long-term blood repopulation when injected into irradiated mice. The powerful increase in vivo in HSC numbers by retrovirally transduced HOX4B, a homeotic gene, along with the role of the TpoR, suggested that stem cell fate, renewal, differentiation and number can be controlled. The discovery of the precise region of the

mouse embryo where HSCs originate and the isolation of supporting stromal cell lines open the possibility of identifying the precise signals required for HSC choice of fate. The completion of human genome sequencing coupled with advances in gene expression profiling using DNA microarrays will enable the identification of key genes deciding the fate of stem cells. Downstream from HSCs, multipotent hematopoietic progenitor cells appear to co-express a multiplicity of genes characteristic of different blood lineages. Genomic approaches will permit the identification of the select group of genes consolidated by the commitment of these multipotent progenitors towards one or the other of the blood lineages. Studies with neural stem cells pointed to the unexpected plastic nature of these cells. Isolation of stem cells from multiple tissues may suggest that, providing the appropriate environment/ signal, tissues could be regenerated in the laboratory and used for transplantation. A spectacular example of influence of the environment on cell fate was revealed decades ago by using mouse embryonic stem cells (ES). Injected into blastocysts, ES cells contribute to the formation of all adult tissues. Injected into adult mice, ES cells become cancer cells. After multiple passages as ascites, when injected back into the blastocyst environment, ES- derived cancer cells behaved again as ES cells. More recently, the successful cloning of mammals and reprogramming of transferred nuclei by factors in the cytoplasm of oocytes turned back the clock by showing that differentiated nuclei can be "re-booted" to generate again the stem cells for different tissues.

Contramaestre, A. P., F. Sifontes, et al. (2008). "Secretion of stem cell factor and granulocyte-macrophage colony-stimulating factor by mouse embryos in culture: influence of group culture." *Zygote* **16**(4): 297-301.

Previous studies showed that the addition of a growth factor to the culture medium could modulate embryo development. The possible secretion of different factors to the culture medium by the embryo itself, however, has been poorly evaluated. The present study was designed to investigate: (1) the influence of single or group culture on the development of 2-cell mouse embryos (strain CD-1) to the blastocyst stage; (2) the release of granulocyte-macrophage colony-stimulating factor (GM-CSF) and stem cell factor (SCF) into the culture medium by the embryo; and (3) the levels of GM-CSF and SCF in the culture medium from both single and group embryos. Two-cell CD-1 mouse embryos were cultured for 96 h singly or in groups of five embryos per drop. GM-CSF and SCF were assayed by ELISA in the complete culture medium. It was found that embryos cultured in

groups gave a higher percentage of total blastocyst formation and hatched blastocyst when compared with single embryo culture. The mouse embryos secreted GM-CSF and SCF to the culture medium. The concentration of these cytokines is significantly higher in the group cultures than the level found in single cultures. In conclusion, mouse embryos in culture secrete GM-CSF and SCF to the culture medium and the concentration of these cytokines increases during communal culture. These factors may be operating in both autocrine and paracrine pathways to modulate embryo development during in vitro culture.

Cortes, J. L., L. Sanchez, et al. (2008). "Whole-blastocyst culture followed by laser drilling technology enhances the efficiency of inner cell mass isolation and embryonic stem cell derivation from good- and poor-quality mouse embryos: new insights for derivation of human embryonic stem cell lines." *Stem Cells Dev* **17**(2): 255-67.

The optimization of human embryonic stem (hES) cell line derivation methods is challenging because many worldwide laboratories have neither access to spare human embryos nor ethical approval for using supernumerary human embryos for hES cell derivation purposes. Additionally, studies performed directly on human embryos imply a waste of precious human biological material. In this study, we developed a new strategy based on the combination of whole-blastocyst culture followed by laser drilling destruction of the trophoectoderm for improving the efficiency of inner cell mass (ICM) isolation and ES cell derivation using murine embryos. Embryos were divided into good- and poor-quality embryos. We demonstrate that the efficiency of both ICM isolation and ES cell derivation using this strategy is significantly superior to whole-blastocyst culture or laser drilling technology itself. Regardless of the ICM isolation method, the ES cell establishment depends on a feeder cell growth surface. Importantly, this combined methodology can be successfully applied to poor-quality blastocysts that otherwise would not be suitable for laser drilling itself nor immunosurgery in an attempt to derive ES cell lines due to the inability to distinguish the ICM. The ES cell lines derived by this combined method were characterized and shown to maintain a typical morphology, undifferentiated phenotype, and in vitro and in vivo three germ layer differentiation potential. Finally, all ES cell lines established using either technology acquired an aneuploid karyotype after extended culture periods, suggesting that the method used for ES cell derivation does not seem to influence the karyotype of the ES cells after extended culture. This methodology may open up new avenues for further improvements for the

derivation of hES cells, the majority of which are derived from frozen, poor-quality human embryos.

Daley, G. Q. (2002). "Prospects for stem cell therapeutics: myths and medicines." *Curr Opin Genet Dev* **12**(5): 607-13.

With common scientific themes and experimental strategies, stem cell biology is evolving into a recognizable discipline. Its clinical arm, regenerative medicine, is also gaining momentum-invigorated by the potential of stem cells to provide treatments for a host of medical conditions that are poorly served by drug therapy. But are the expectations for stem cell therapies realistic or overstated? In the past year, neurons, insulin-producing cells, and hematopoietic stem cells have been generated from embryonic stem cells or cultivated from somatic tissues of the adult. These cells have yielded modest and preliminary hints of functional reconstitution in animal models. Although encouraging, significant hurdles remain before the promise of stem cells will be realized in the clinic.

Damjanov, I. (2004). "From stem cells to germ cell tumors and back." *Verh Dtsch Ges Pathol* **88**: 39-44.

Germ cell tumors originate from ovarian, testicular and extragonadal germ cells. Tumor stem cells can retain most of the features of germ cells and form seminomas or dysgerminomas or, transform into developmentally pluripotent embryonic stem cells and give rise to teratomas or teratocarcinomas. Similar tumors can be experimentally produced in mice from early mouse embryos transplanted to extrauterine sites. The malignant stem cells of teratocarcinomas, called in analogy with their human counterparts embryonal carcinoma (EC), can be isolated and grown in culture and or propagated indefinitely by isotransplantation in syngeneic inbred mice. When injected into the blastocyst, i.e., the embryonic environment from which they have been originally isolated, EC cells lose their malignancy and become benign, participating in the normal development of the injected blastocyst. Injection of EC cells into blastocysts has been used to generate transgenic mice. Developmentally pluripotent non-neoplastic embryonic stem (ES) cells can be produced from mouse blastocysts cultured in vitro. These cells are developmentally similar to EC cells. In contrast to EC cells, ES cells injected into adult mice do not produce teratocarcinomas but only teratomas. Similar ES cells were produced from human blastocysts cultured in vitro. Human ES injected into nude mice produce teratomas composed of various somatic tissues. Human ES cells resemble mouse ES cells, but differ from human EC cells. Like their mouse equivalents, human ES cells could be used for generating

experimental models of various diseases and, hopefully, for cell therapy in not so distant future.

Dhali, A., V. M. Anchamparathy, et al. (2009). "In vitro development of bovine embryos cultured with stem cell factor or insulin-like growth factor-I following IVF with semen of two bulls having different field fertility." *Anim Reprod Sci* **116**(3-4): 188-95.

The usefulness of IVF as a potential tool to evaluate the field fertility of bulls is equivocal and growth factor addition to culture media research is needed to delineate components needed for providing defined environments for embryos. The overall aim was to evaluate the in vitro development of embryos derived using a serum supplemented and serum-free production systems and semen from two bulls of different field fertility. The study was conducted to determine the combinatorial effect of stem cell factor (SCF) and/or insulin-like growth factor-I (IGF-I) in culture on subsequent embryo development in cattle. Oocytes were aspirated separately from ≥ 3 to < 3 mm follicles to test different follicle size populations and were matured in TCM-199 supplemented with LH, FSH, estradiol and BSA (Fraction V). Matured oocytes were fertilized in BSA supplemented synthetic oviductal fluid (SOF)-IVF medium. Presumptive zygotes were cultured for 8d (in humidified 5% CO₂) at 38.5 degrees C) in BSA supplemented SOF-in vitro culture (IVC) medium. SOF-IVC medium was supplemented with fetal bovine serum (4%), IGF-I (100ng/mL), SCF (50ng/mL) or IGF-I (100ng/mL)+SCF (50ng/mL). The development competence of embryos did not differ between the bulls and among the culture environments. Nevertheless, there was an effect of follicle size on cleavage rate ($P < 0.05$) and a greater cleavage rate resulted from oocytes aspirated from ≥ 3 mm follicles (71.0 \pm 1.5%) compared to those collected from < 3 mm follicles (64.8 \pm 1.6%). The overall cleavage rate (%); blastocyst formation (%); and expanded/hatched blastocyst formation (%) were 68.2 \pm 1.5 and 67.7 \pm 1.7; 29.4 \pm 1.4 and 28.6 \pm 1.5; and 18.6 \pm 1.2 and 18.5 \pm 1.1, respectively, for the bull of above and below average field fertility. The results indicate that follicle size for oocyte aspiration is effective for determining IVC success and that IVF may not discriminate among bulls of different field fertility.

Ding, H. and A. Guha (2001). "Mouse astrocytoma models: embryonic stem cell mediated transgenesis." *J Neurooncol* **53**(3): 289-96.

The development of rational and targeted therapies for human astrocytomas is heavily dependent on our knowledge of its molecular

pathogenesis, combined with the generation of appropriate pre-clinical mouse models. The ability to manipulate the mouse genome, which is nearing completion and is highly homologous to its human counterpart, has significantly accelerated our ability to create transgenic mouse models that replicate the pathological and molecular characteristics found in human astrocytomas. These models should serve to further our knowledge of the molecular pathogenesis of human astrocytomas, and serve as useful reagents to test conventional and novel therapeutics.

Doetschman, T., M. Shull, et al. (1993). "Embryonic stem cell model systems for vascular morphogenesis and cardiac disorders." *Hypertension* **22**(4): 618-29.

To better understand the formation of the cardiovascular system and its disease states, models amenable to manipulation must be developed. In this article we present two models. One is a small animal model for an inflammatory disorder that can lead to heart failure. Production of this model is based on the ability of blastocyst-derived embryonic stem cells, which can be genetically altered in vitro by a technique called gene targeting, to reconstitute an entire animal when reintroduced into a blastocyst and allowed to colonize the germ line of the resulting chimeric embryo. The other model is based on the capacity of embryonic stem cells to differentiate in culture into embryo-like structures called embryoid bodies. Embryoid bodies contain angioblasts, or prevascular endothelial cells, which can be induced to undergo aspects of vascular development by manipulation of culture conditions.

Doetschman, T. C., H. Eistetter, et al. (1985). "The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium." *J Embryol Exp Morphol* **87**: 27-45.

The in vitro developmental potential of mouse blastocyst-derived embryonic stem cell lines has been investigated. From 3 to 8 days of suspension culture the cells form complex embryoid bodies with endoderm, basal lamina, mesoderm and ectoderm. Many are morphologically similar to embryos of the 6- to 8-day egg-cylinder stage. From 8 to 10 days of culture about half of the embryoid bodies expand into large cystic structures containing alphafoetoprotein and transferrin, thus being analogous to the visceral yolk sac of the postimplantation embryo. Approximately one third of the cystic embryoid bodies develop myocardium and when cultured in the presence of human cord serum, 30% develop blood islands, thereby exhibiting a high level of organized development at a very high frequency. Furthermore, most embryonic stem cell lines observed exhibit

similar characteristics. The in vitro developmental potential of embryonic stem cell lines and the consistency with which the cells express this potential are presented as aspects which open up new approaches to the investigation of embryogenesis.

Donovan, P. J. (1998). "The germ cell--the mother of all stem cells." *Int J Dev Biol* **42**(7): 1043-50.

The germline, uniquely amongst the lineages of the embryo, carries the genome from generation to generation and is therefore the only lineage which retains true developmental totipotency. Paradoxically, when mouse primordial germ cells (PGCs) are introduced into a host blastocyst, they do not contribute to either the germline or the soma, suggesting that they are restricted in developmental potency. Conversely, in vivo PGCs give rise to embryonal carcinoma (EC) cells, the pluripotent stem cells of teratomas, benign tumors containing derivatives of the three primary germ layers. Similarly, PGCs can be converted in vitro into embryonic germ (EG) cells, pluripotent stem cells capable of giving rise to somatic and germline chimeras. The ability of PGCs to form EC cells in vivo and EG cells in vitro suggests that developmental potency of PGCs is regulateable. The molecular mechanisms controlling PGC growth and differentiation are gradually being elucidated through the characterization of sterile mutants and through the use of in vitro culture systems. Understanding how a PGC can give rise to a pluripotent stem cell could give significant insights into the regulation of developmental totipotency as well as having important implications for male fertility and the etiology of testicular cancer.

Draper, J. S. and A. Nagy (2007). "Improved embryonic stem cell technologies." *Handb Exp Pharmacol*(178): 107-28.

Murine embryonic stem (ES) cells have become an indispensable tool for investigating genetic function both in vitro and, importantly, in vivo. Recent advances, including tetraploid aggregation, new site-specific recombinases and RNAi, have enabled more sophisticated manipulation of the ES cell genome. For instance, it is now possible to control gene expression in both a temporally and spatially restricted manner. Such new technologies are answering complex questions surrounding the function and interaction of an increasing number of genes. This chapter will review both the history and recent technological progress that has been made in mouse ES cell derivation, genetic manipulation and the generation of ES cell-derived chimaeric animals.

Drukker, M. (2008). "Recent advancements towards the derivation of immune-compatible patient-specific human embryonic stem cell lines." *Semin Immunol* **20**(2): 123-9.

The derivation of human embryonic stem cell lines from blastocyst stage embryos, first achieved almost a decade ago, demonstrated the potential to prepare virtually unlimited numbers of therapeutically beneficial cells in vitro. Assuming that large-scale production of differentiated cells is attainable, it is imperative to develop strategies to prevent immune responses towards the grafted cells following transplantation. In this review, I will discuss recent advances in the production of pluripotent cell lines using three emerging techniques: somatic cell nuclear transfer into enucleated oocytes and zygotes, parthenogenetic activation of unfertilized oocytes and induction of pluripotency in somatic cells. Importantly, if these techniques can be harnessed for generating patient-specific pluripotent cell lines, then immunological processes are expected to be low or completely absent.

Eiges, R., M. Schuldiner, et al. (2001). "Establishment of human embryonic stem cell-transfected clones carrying a marker for undifferentiated cells." *Curr Biol* **11**(7): 514-8.

Human embryonic stem (ES) cells are pluripotent cell lines that have been derived from the inner cell mass (ICM) of blastocyst stage embryos [1--3]. They are characterized by their ability to be propagated indefinitely in culture as undifferentiated cells with a normal karyotype and can be induced to differentiate in vitro into various cell types [1, 2, 4--6]. Thus, human ES cells promise to serve as an unlimited cell source for transplantation. However, these unique cell lines tend to spontaneously differentiate in culture and therefore are difficult to maintain. Furthermore, colonies may contain several cell types and may be composed of cells other than pluripotent cells [1, 2, 6]. In order to overcome these difficulties and establish lines of cells with an undifferentiated phenotype, we have introduced a reporter gene that is regulated by a promoter of an ES cell-enriched gene into the cells. For the introduction of DNA into human ES cells, we have established a specific transfection protocol that is different from the one used for murine ES cells. Human ES cells were transfected with enhanced green fluorescence protein (EGFP), under the control of murine Rex1 promoter. The transfected cells show high levels of GFP expression when in an undifferentiated state. As the cells differentiate, this expression is dramatically reduced in monolayer cultures as well as in the primitive endoderm of early stage (simple) embryoid bodies (EBs) and in mature EBs. The undifferentiated

cells expressing GFP can be analyzed and sorted by using a Fluorescence Activated Cell Sorter (FACS). Thus, we have established lines of human ES cells in which only undifferentiated cells are fluorescent, and these cells can be followed and selected for in culture. We also propose that the pluripotent nature of the culture is made evident by the ability of the homogeneous cell population to form EBs. The ability to efficiently transfect human ES cells will provide the means to study and manipulate these cells for the purpose of basic and applied research.

Faast, R., S. J. Harrison, et al. (2006). "Use of adult mesenchymal stem cells isolated from bone marrow and blood for somatic cell nuclear transfer in pigs." *Cloning Stem Cells* **8**(3): 166-73.

Mesenchymal stem cells (MSCs) isolated from bone marrow were used to examine the hypothesis that a less differentiated cell type could increase adult somatic cell nuclear transfer (SCNT) efficiencies in the pig. SCNT embryos were produced using a fusion before activation protocol described previously and the rate at which these developed to the blastocyst stage compared with that using fibroblasts obtained from ear tissue from the same animal. The use of bone marrow MSCs did not increase cleavage rates compared with adult fibroblasts. However, the percentage of embryos that developed to the blastocyst stage was almost doubled, providing support for the hypothesis that a less differentiated cell can increase cloning efficiencies. As MSCs are relatively difficult to isolate from the bone marrow of live animals, a second experiment was undertaken to determine whether MSCs could be isolated from the peripheral circulation and used for SCNT. Blood MSCs were successfully isolated from four of the five pigs sampled. These cells had a similar differentiation capacity and marker profile to those isolated from bone marrow but did not result in increased rates of development. This is the first study to our knowledge, to report that MSCs can be derived from peripheral blood and used for SCNT for any species. These cells can be readily obtained under relatively sterile conditions compared with adult fibroblasts and as such, may provide an alternative cell type for cloning live animals.

Falco, G., S. L. Lee, et al. (2007). "Zscan4: a novel gene expressed exclusively in late 2-cell embryos and embryonic stem cells." *Dev Biol* **307**(2): 539-50.

The first wave of transcription, called zygotic genome activation (ZGA), begins during the 2-cell stage in mouse preimplantation development and marks a vital transition from the maternal genetic to the embryonic genetic program. Utilizing DNA microarray data, we looked for genes that are

expressed only during ZGA and found Zscan4, whose expression is restricted to late 2-cell stage embryos. Sequence analysis of genomic DNA and cDNA clones revealed nine paralogous genes tightly clustered in 0.85 Mb on mouse chromosome 7. Three genes are not transcribed and are thus considered pseudogenes. Among the six expressed genes named Zscan4a-Zscan4f, three - Zscan4c, Zscan4d, and Zscan4f - encode full-length ORFs with 506 amino acids. Zscan4d is a predominant transcript at the late 2-cell stage, whereas Zscan4c is a predominant transcript in embryonic stem (ES) cells. No transcripts of any Zscan4 genes are detected in any other cell types. Reduction of Zscan4 transcript levels by siRNAs delays the progression from the 2-cell to the 4-cell stage and produces blastocysts that fail to implant or proliferate in blastocyst outgrowth culture. Zscan4 thus seems to be essential for preimplantation development.

Fang, Z. F., H. Gai, et al. (2006). "Rabbit embryonic stem cell lines derived from fertilized, parthenogenetic or somatic cell nuclear transfer embryos." *Exp Cell Res* **312**(18): 3669-82.

Embryonic stem cells were isolated from rabbit blastocysts derived from fertilization (conventional rbES cells), parthenogenesis (pES cells) and nuclear transfer (ntES cells), and propagated in a serum-free culture system. Rabbit ES (rbES) cells proliferated for a prolonged time in an undifferentiated state and maintained a normal karyotype. These cells grew in a monolayer with a high nuclear/cytoplasm ratio and contained a high level of alkaline phosphatase activity. In addition, rbES cells expressed the pluripotent marker Oct-4, as well as EBAF2, FGF4, TDGF1, but not antigens recognized by antibodies against SSEA-1, SSEA-3, SSEA-4, TRA-1-10 and TRA-1-81. All 3 types of ES cells formed embryoid bodies and generated teratoma that contained tissue types of all three germ layers. rbES cells exhibited a high cloning efficiency, were genetically modified readily and were used as nuclear donors to generate a viable rabbit through somatic cell nuclear transfer. In combination with genetic engineering, the ES cell technology should facilitate the creation of new rabbit lines.

Fang, Z. F., F. Jin, et al. (2005). "Human embryonic stem cell lines derived from the Chinese population." *Cell Res* **15**(5): 394-400.

Six human embryonic stem cell lines were established from surplus blastocysts. The cell lines expressed alkaline phosphatase and molecules typical of primate embryonic stem cells, including Oct-4, Nanog, TDGF1, Sox2, EBAF, Thy-1, FGF4, Rex-1, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81. Five of

the six lines formed embryoid bodies that expressed markers of a variety of cell types; four of them formed teratomas with tissue types representative of all three embryonic germ layers. These human embryonic stem cells are capable of producing clones of undifferentiated morphology, and one of them was propagated to become a subline. Human embryonic stem cell lines from the Chinese population should facilitate stem cell research and may be valuable in studies of population genetics and ecology.

Fassler, R., M. Pfaff, et al. (1995). "Lack of beta 1 integrin gene in embryonic stem cells affects morphology, adhesion, and migration but not integration into the inner cell mass of blastocysts." *J Cell Biol* **128**(5): 979-88.

A gene trap-type targeting vector was designed to inactivate the beta 1 integrin gene in embryonic stem (ES) cells. Using this vector more than 50% of the ES cell clones acquired a disruption in the beta 1 integrin gene and a single clone was mutated in both alleles. The homozygous mutant did not produce beta 1 integrin mRNA or protein, while alpha 3, alpha 5, and alpha 6 integrin subunits were transcribed but not detectable on the cell surface. Heterozygous mutants showed reduced beta 1 expression and surface localization of alpha/beta 1 heterodimers. The alpha V subunit expression was not impaired on any of the mutants. Homozygous ES cell mutants lacked adhesiveness for laminin and fibronectin but not for vitronectin and showed a reduced association with a fibroblast feeder layer. Furthermore, they did not migrate towards chemoattractants in fibroblast medium. None of these functions were impaired in heterozygous mutants. Scanning electron microscopy revealed that homozygous cells showed fewer cell-cell junctions and had many microvilli not usually found on wild type and heterozygous cells. This profound change in cell shape is not associated with gross alterations in the expression and distribution of cytoskeletal components. Unexpectedly, microinjection into blastocysts demonstrated full integration of homozygous and heterozygous mutants into the inner cell mass. This will allow studies of the consequences of beta 1 integrin deficiency in several in vivo situations.

Findikli, N., N. Z. Candan, et al. (2006). "Human embryonic stem cell culture: current limitations and novel strategies." *Reprod Biomed Online* **13**(4): 581-90.

Embryonic stem cells (ESC) are multipotent cells isolated from blastocyst-stage preimplantation embryos. Since their first culture in 1998, human ESC have revolutionized reproductive and regenerative

medicine by allowing the establishment of detailed molecular and therapeutic models for certain metabolic pathways and life-threatening disorders. They also offer significant contributions to genetics and pharmacology in designing and analysing disease models that can be closer to in vivo than any other procedures available. However, the procedures by which they are obtained and manipulated also create intense ethical and social debates worldwide. This article discusses the current limitations and recent advances in isolation, culture and differentiation of human ESC from the laboratory perspective.

Findikli, N., S. Kahraman, et al. (2005). "Establishment and characterization of new human embryonic stem cell lines." *Reprod Biomed Online* **10**(5): 617-27.

Human embryonic stem cells (hESC), with their ability to differentiate into all cell types in the human body, are likely to play a very important therapeutic role in a variety of neurodegenerative and life-threatening disorders in the near future. Although more than 120 different human embryonic stem cell lines have been reported worldwide, only a handful are currently available for researchers, which limits the number of studies that can be performed. This study reports the isolation, establishment and characterization of new human embryonic stem cell lines, as well as their differentiation potential into variety of somatic cell types. Blastocyst-stage embryos donated for research after assisted reproductive techniques were used for embryonic stem cell isolation. A total of 31 blastocysts were processed either for immunosurgery or direct culture methods for inner cell mass isolation. A total of nine primary stem cell colonies were isolated and of these, seven cell lines were further expanded and passaged. Established lines were characterized by their cellular and colony morphology, karyotypes and immunocytochemical properties. They were also successfully cryopreserved/thawed and showed similar growth and cellular properties upon thawing. When induced to differentiate in vitro, these cells formed a variety of somatic cell lineages including cells of endoderm, ectoderm and mesoderm origin. There is now an exponentially growing interest in stem cell biology as well as its therapeutic applications for life-threatening human diseases. However, limited availability of stem cell lines as well as financial or ethical limitations restrict the number of research projects. The establishment of new hESC lines may create additional potential sources for further worldwide and nationwide research on stem cells.

Fletcher, J. M., P. M. Ferrier, et al. (2006). "Variations in humanized and defined culture conditions supporting derivation of new human embryonic stem cell lines." Cloning Stem Cells **8**(4): 319-34.

The evolution of "humanized" (i.e., free of animal sourced reagents) and ultimately chemically defined culture systems for human embryo stem cell (hESC) isolation and culture is of importance to improving their efficacy and safety in research and therapeutic applications. This can be achieved by integration of a multitude of individual approaches to replace or eliminate specific animal sourced reagents into a single comprehensive protocol. In the present study our objective was to integrate strategies obviating reliance on some of the most poorly defined and path-critical factors associated with hESC derivation, namely the use of animal immune compliment to isolate embryo inner cell mass, and animal sourced serum products and feeder cells to sustain hESC growth and attachment. As a result we report the derivation of six new hESC lines isolated by outgrowth from whole blastocysts on an extracellular matrix substrate of purified human laminin (Ln) with transitional reliance on mitotically inactivated human fibroblast (HDF) feeder cells. With this integrated system hESC lines were isolated using either HDF conditioned medium supplemented with a bovine-sourced serum replacement (bSRM), or a defined serum-free medium (SFM) containing only human sourced and recombinant protein. Further, outgrowth of embryonic cells from whole blastocysts in both media could be achieved for up to 1 week without reliance on feeder cells. All variant conditions sustained undifferentiated cell status, a stable karyotype and the potential to form cells representative of all three germinal lineages in vitro and in vivo, when transitioned off of feeders onto Laminin or Matrigel. Our study thus demonstrates the capacity to integrate derivation strategies eliminating a requirement for animal immune compliment and serum products, with a transitional requirement for human feeder cells. This represents another sequential step in the generation of therapeutic grade stem cells with reduced risk of zoonotic pathogen transmission.

Fong, C. Y., M. Richards, et al. (2006). "Unsuccessful derivation of human embryonic stem cell lines from pairs of human blastomeres." Reprod Biomed Online **13**(2): 295-300.

Human embryonic stem cells (hESC) that differentiate into all three primordial germ layers have been established. Differentiation of these cells into desirable lineages offers hope for future transplantation therapies. Currently, hESC lines are derived from the inner cell mass (ICM) of blastocysts,

leading to destruction of the embryo, and thus the process is ethically controversial. Successful attempts at deriving hESC lines from blastomeres without destruction of the ensuing embryo have not been reported. One or two blastomeres are routinely biopsied from 8-cell embryos for preimplantation genetic diagnosis. In this study it was therefore attempted to derive hESC lines from paired blastomeres. Of 66 pairs of 8-cell stage blastomeres, four pairs produced two morula and two blastocyst-like structures. When plated on mitomycin-C-treated mouse embryonic fibroblasts, one morula and one blastocyst-like structure separately produced small colonies containing hESC-like cells with prominent nucleoli and high nuclear-cytoplasmic ratios. When these colonies were detached and plated onto fresh feeders, there was no further colony formation or ensuing hESC lines. The results showed that it might not be possible to derive hESC lines directly from paired blastomeres. A minimum number of blastomeres in close contact with one another may be required to successfully generate an hESC line as blastomeres, like ICM and hESC cells, may be 'social' cells.

Forrester, L. M., A. Bernstein, et al. (1991). "Long-term reconstitution of the mouse hematopoietic system by embryonic stem cell-derived fetal liver." Proc Natl Acad Sci U S A **88**(17): 7514-7.

Murine embryonic stem (ES) cells are permanent blastocyst-derived cell lines capable of contributing to a wide variety of tissues, including the germ line, after injection into host blastocysts. Recently, we have shown that ES cells can produce all of the cells of the developing fetus after aggregation with developmentally compromised tetraploid embryos. Completely ES cell-derived embryos die perinatally, but the liver of these embryos is a source of entirely ES cell-derived hematopoietic progenitors. We have taken 14- to 15-day fetal liver cells from ES cell-tetraploid chimeras and reconstituted the hematopoietic system of lethally irradiated adult recipient mice. ES cell-derived hematopoietic stem cells were capable of long-term (greater than 6 months) repopulation of irradiated recipients, and all hematopoietic cell lineages analyzed (erythrocytes, T cells, mast cells, and macrophages) were derived exclusively from ES cells in such recipients. Thus, ES cells retain the capacity to differentiate into all hematopoietic cell types after prolonged passage in culture. This approach should provide a direct route to the production of mice whose hematopoietic cells carry genetic alterations that would be lethal if passed through the germ line.

Foshay, K. M. and G. I. Gallicano (2009). "miR-17 family miRNAs are expressed during early mammalian development and regulate stem cell differentiation." *Dev Biol* **326**(2): 431-43.

MicroRNAs are small non-coding RNAs that regulate protein expression by binding 3'UTRs of target mRNAs, thereby inhibiting translation. Similar to siRNAs, miRNAs are cleaved by Dicer. Mouse and ES cell Dicer mutants demonstrate that microRNAs are necessary for embryonic development and cellular differentiation. However, technical obstacles and the relative infancy of this field have resulted in few data on the functional significance of individual microRNAs. We present evidence that miR-17 family members, miR-17-5p, miR-20a, miR-93, and miR-106a, are differentially expressed in developing mouse embryos and function to control differentiation of stem cells. Specifically, miR-93 localizes to differentiating primitive endoderm and trophoctoderm of the blastocyst. We also observe high miR-93 and miR-17-5p expression within the mesoderm of gastrulating embryos. Using an ES cell model system, we demonstrate that modulation of these miRNAs delays or enhances differentiation into the germ layers. Additionally, we demonstrate that these miRNAs regulate STAT3 mRNA in vitro. We suggest that STAT3, a known ES cell regulator, is one target mRNA responsible for the effects of these miRNAs on cellular differentiation.

Frydman, N., O. Feraud, et al. (2009). "Characterization of human PGD blastocysts with unbalanced chromosomal translocations and human embryonic stem cell line derivation?" *Reprod Biomed Online* **19 Suppl 4**: 4199.

Novel embryonic stem cell lines derived from embryos carrying structural chromosomal abnormalities obtained after preimplantation genetic diagnosis (PGD) are of interest to study in terms of the influence of abnormalities on further development. A total of 22 unbalanced blastocysts obtained after PGD were analysed for structural chromosomal defects. Morphological description and chromosomal status of these blastocysts was established and they were used to derive human embryonic stem cell (ESC) lines. An outgrowth of cells was observed for six blastocysts (6/22; 27%). For two blastocysts, the exact morphology was unknown since they were at early stage, and for four blastocysts, the inner cell mass was clearly visible. Fifteen blastocysts carried an unbalanced chromosomal defect linked to a reciprocal translocation, resulting in a positive outgrowth of cells for five blastocysts. One human ESC line was obtained from a blastocyst carrying a partial chromosome-21 monosomy and a partial chromosome-1 trisomy. Six blastocysts carried an

unbalanced chromosomal defect linked to a Robertsonian translocation, and one showed a positive outgrowth of cells. One blastocyst carried an unbalanced chromosomal defect linked to an insertion and no outgrowth was observed. The efficiency of deriving human ESC lines with constitutional chromosomal disorders was low and probably depends on the initial morphological aspect of the blastocysts and/or the type of the chromosomal disorders.

Furusawa, T., M. Ikeda, et al. (2006). "Gene expression profiling of mouse embryonic stem cell subpopulations." *Biol Reprod* **75**(4): 555-61.

We previously demonstrated that mouse embryonic stem (ES) cells show a wide variation in the expression of platelet endothelial cell adhesion molecule 1 (PECAM1) and that the level of expression is positively correlated with the pluripotency of ES cells. We also found that PECAM1-positive ES cells could be divided into two subpopulations according to the expression of stage-specific embryonic antigen (SSEA)-1. ES cells that showed both PECAM1 and SSEA-1 predominantly differentiated into epiblast after the blastocyst stage. In the present study, we performed pairwise oligo microarray analysis to characterize gene expression profiles in PECAM1-positive and -negative subpopulations of ES cells. The microarray analysis identified 2034 genes with a more than 2-fold difference in expression levels between the PECAM1-positive and -negative cells. Of these genes, 803 were more highly expressed in PECAM1-positive cells and 1231 were more highly expressed in PECAM1-negative cells. As expected, genes known to function in ES cells, such as Pou5f1(Oct3/4) and Nanog, were found to be upregulated in PECAM1-positive cells. We also isolated 23 previously uncharacterized genes. A comparison of gene expression profiles in PECAM1-positive cells that were either positive or negative for SSEA-1 expression identified only 53 genes that showed a more than 2-fold greater difference in expression levels between these subpopulations. However, many genes that are under epigenetic regulation, such as globins, Igf2, Igf2r, and H19, showed differential expression. Our results suggest that in addition to differences in gene expression profiles, epigenetic status was altered in the three cell subpopulations.

Furusawa, T., K. Ohkoshi, et al. (2004). "Embryonic stem cells expressing both platelet endothelial cell adhesion molecule-1 and stage-specific embryonic antigen-1 differentiate predominantly into epiblast cells in a chimeric embryo." *Biol Reprod* **70**(5): 1452-7.

We examined the expression of cell-surface markers on subpopulations of mouse embryonic stem (ES) cells to identify those that were associated with cells that had the highest pluripotency. Flow cytometry analysis revealed a wide variation in the expression of platelet endothelial cell adhesion molecule 1 (PECAM-1) and stage-specific embryonic antigen (SSEA)-1 in ES cells. Almost all SSEA-1+ cells expressed a high level of PECAM-1, and reversible repopulation was observed between PECAM-1+SSEA-1+ and PECAM-1-SSEA-1- cells. The ES cells carrying the lacZ gene were sorted into three subpopulations: PECAM-1-SSEA-1-, PECAM-1+SSEA-1-, and PECAM-1+SSEA-1+. Quantitative reverse transcription-polymerase chain reaction revealed a low level of Oct3/4 mRNA expression and an elevation in differentiation maker gene expression in PECAM-1- cells. To compare the pluripotency of these three subpopulations, a single cell from each was injected into eight-cell embryo and ES cells identified at later stages by X-gal staining. At the blastocyst stage, PECAM-1+ SSEA-1+/- cells were found to have differentiated into epiblast cells in high numbers. In contrast, PECAM-1- cell derivatives localized in the primitive endoderm or trophoderm. At 6.0-7.0 days post coitum, many PECAM-1+SSEA-1+ cells were found in the epiblast, but few beta-gal+ cells were detected in any regions of embryos that were injected with cells from the other two populations. These results showed that the expression levels of PECAM-1 and SSEA-1 in ES cells correlated closely with their pluripotency and/or their ability to incorporate into the epiblast of chimeric embryos.

Gan, Q., T. Yoshida, et al. (2007). "Concise review: epigenetic mechanisms contribute to pluripotency and cell lineage determination of embryonic stem cells." *Stem Cells* **25**(1): 2-9.

Epigenetic mechanisms, such as histone modifications and DNA methylation, have been shown to play a key role in the regulation of gene transcription. Results of recent studies indicate that a novel "bivalent" chromatin structure marks key developmental genes in embryonic stem cells (ESCs), wherein a number of untranscribed lineage-control genes, such as Sox1, Nkx2-2, Msx1, Irx3, and Pax3, are epigenetically modified with a unique combination of activating and repressive histone modifications that prime them for potential activation (or repression) upon cell lineage induction and differentiation. However, results of these studies also showed that a subset of lineage-control genes, such as Myf5 and Mash1, were not marked by these histone modifications, suggesting that distinct epigenetic mechanisms might exist for lineage-control genes in

ESCs. In this review article, we summarize evidence regarding possible mechanisms that control these unique histone modifications at lineage-control gene loci in ESCs and consider their possible contribution to ESC pluripotency. In addition, we propose a novel "histone modification pulsing" model wherein individual pluripotent stem cells within the inner cell mass of blastocysts undergo transient asynchronous histone modifications at these developmental gene loci, thereby conferring differential responsiveness to environmental cues and morphogenic gradients important for cell lineage determination. Finally, we consider how these rapid histone modification exchanges become progressively more stable as ESCs undergo differentiation and maturation into specialized cell lineages.

Gao, S., M. McGarry, et al. (2003). "Effect of cell confluence on production of cloned mice using an inbred embryonic stem cell line." *Biol Reprod* **68**(2): 595-603.

Mice have been successfully cloned from both somatic cells and hybrid embryonic stem (ES) cells. Heterozygosity of the donor ES cell genome has been suggested as a crucial factor for long-term survival of cloned mice. In the present study, an inbred ES cell line, HM-1 (129/Ola), and a well-tested ES cell line, R1 (129/Sv x 129/Sv-CP), were used as donor cells to evaluate the developmental potential of nuclear transfer embryos. We found that ES cell confluence dramatically affects the developmental potential of reconstructed embryos. With the ES cell line HM-1 and 80-90% confluence, 49% of reconstructed embryos developed to the morula/blastocyst stage, 9% of these embryos developed to live pups when transferred to the surrogate mothers, and 5 of 18 live pups survived to adulthood. By contrast, at 60-70% confluence, only 22% of embryos developed to the morula/blastocyst stage, and after transfer, only a single fetus reached term. Consistent with previous reports, the nuclei of R1 ES cells were also shown to direct development to term, but no live pups were derived from cells at later passages (>20). Our results show that the developmental potential of reconstructed embryos is determined by both cell confluence and cell passage. These results also demonstrate that the inbred ES cell line, HM-1, can be used to produce viable cloned mice, although less efficiently than most heterozygous ES cell lines.

Gautrey, H., J. McConnell, et al. (2008). "Staufen1 is expressed in preimplantation mouse embryos and is required for embryonic stem cell differentiation." *Biochim Biophys Acta* **1783**(10): 1935-42.

Pluripotent mouse embryonic stem (mES) cells derived from the blastocyst of the preimplantation embryo can be induced to differentiate in vitro along different cell lineages. However the molecular and cellular factors that signal and/or determine the expression of key genes, and the localisation of the encoded proteins, during the differentiation events are poorly understood. One common mechanism by which proteins can be targeted to specific regions of the cell is through the asymmetric localisation of mRNAs and Staufen, a double-stranded RNA binding protein, is known to play a direct role in mRNA transport and localisation. The aims of the present study were to describe the expression of Staufen in preimplantation embryos and mES cells and to use RNA interference (RNAi) to investigate the roles of Staufen1 in mES cell lineage differentiation. Western blotting and immunocytochemistry demonstrated that Staufen is present in the preimplantation mouse embryo, pluripotent mES cells and mES cells stimulated to differentiate into embryoid bodies, but the Staufen staining patterns did not support asymmetric distribution of the protein. Knockdown of Staufen1 gene expression in differentiating mES cells reduced the synthesis of lineage-specific markers including Brachyury, alpha-fetoprotein (AFP), PAX-6, and Vasa. There was however no significant change in either the gene expression of Nanog and Oct4, or in the synthesis of SSEA-1, all of which are key markers of pluripotency. These data indicate that inhibition of Staufen1 gene expression by RNAi affects an early step in mES cell differentiation and suggest a key role for Staufen in the cell lineage differentiation of mES cells.

Geens, M., I. Mateizel, et al. (2009). "Human embryonic stem cell lines derived from single blastomeres of two 4-cell stage embryos." Hum Reprod **24**(11): 2709-17.

BACKGROUND: Recently, we demonstrated that single blastomeres of a 4-cell stage human embryo are able to develop into blastocysts with inner cell mass and trophectoderm. To further investigate potency at the 4-cell stage, we aimed to derive pluripotent human embryonic stem cells (hESC) from single blastomeres. **METHODS:** Four 4-cell stage embryos were split on Day 2 of preimplantation development and the 16 blastomeres were individually cultured in sequential medium. On Day 3 or 4, the blastomere-derived embryos were plated on inactivated mouse embryonic fibroblasts (MEFs). **RESULTS:** Ten out of sixteen blastomere-derived morulae attached to the MEFs, and two produced an outgrowth. They were mechanically passaged onto fresh MEFs as described for blastocyst

ICM-derived hESC, and shown to express the typical stemness markers by immunocytochemistry and/or RT-PCR. In vivo pluripotency was confirmed by the presence of all three germ layers in the teratoma obtained after injection in immunodeficient mice. The first hESC line displays a mosaic normal/abnormal 46, XX, dup(7)(q33qter), del(18)(q23qter) karyotype. The second hESC line displays a normal 46, XY karyotype. **CONCLUSION:** We report the successful derivation and characterization of two hESC lines from single blastomeres of four split 4-cell stage human embryos. These two hESC lines were derived from distinct embryos, proving that at least one of the 4-cell stage blastomeres is pluripotent.

Gerecht-Nir, S. and J. Itskovitz-Eldor (2004). "Cell therapy using human embryonic stem cells." Transpl Immunol **12**(3-4): 203-9.

Cell therapy refers to the transplantation of healthy, functional and propagating cells to restore the viability or function of deficient tissues. Stem cells are characterized by self-renewal and the potential to form differentiated cells. In early mammalian embryos, at the blastocyst stage, the inner cell mass is pluripotent. Thus, it has been recognized that human embryonic stem cells (hESCs), which are derived from such cells of blastocysts, may serve as a source of numerous types of differentiated cells. The first part of this review summarizes different techniques for the derivation and maintenance of undifferentiated hESCs. In the second part, issues concerning the safety and bulk production, which may enable hESCs use in future clinical applications, are presented. The last part of this review details accumulated data regarding the in vitro differentiation potential of hESCs.

Gerlach, M., H. Braak, et al. (2002). "Current state of stem cell research for the treatment of Parkinson's disease." J Neurol **249** Suppl 3: III/33-5.

Current findings suggest that multipotent stem cells may be suitable for cell replacement therapies in the treatment of neurodegenerative disorders. Embryonic stem (ES) cells are pluripotent cells isolated from the inner cell mass of the preimplantation blastocyst, which give rise to all cells in the organism. Similarly, multipotent stem cells are also able to regenerate, but are believed to have a more restricted potential than ES cells, and are often defined by the organ from which they are derived. Neural stem cells have been categorized as multipotent stem cells derived from the nervous system with the capacity to regenerate and to give rise to cells belonging to all three cell lineages in the nervous system: neurons, oligodendrocytes, and astrocytes. It is hoped that research on stem cells may

reveal methods for producing an infinite supply of dopamine neurons for transplant into Parkinson's disease (PD) patients. The problem is controlling cell growth and differentiation. We will briefly review the current state of stem cell research and will critically discuss the potential of stem cells for the treatment of PD.

Gissel, C., C. Voolstra, et al. (2005). "An optimized embryonic stem cell model for consistent gene expression and developmental studies: a fundamental study." *Thromb Haemost* **94**(4): 719-27.

In vitro differentiation of embryonic stem (ES) cells results in generation of tissue-specific somatic cells and may represent a powerful tool for general understanding of cellular differentiation and development in vivo. Culturing of most ES cell lines requires murine embryonic fibroblasts (MEF), which may influence adventitiously the genetic differentiation program of ES cells. We compared the expression profile of key developmental genes in the MEF-independent CGR8 ES cell line and in the MEF-dependent D3 ES cell line. Using neomycin-resistant MEFs we demonstrated that MEFs are able to contaminate the D3 ES cells even after removing the MEFs. Subsequently, optimal differentiation conditions were established for the differentiation of CGR8 ES cells into various germ layer cells. Detailed gene expression studies in differentiating CGR8 cells were done by RT-PCR analysis and by microarray analysis demonstrating a general trend of the assessed genes to be expressed either in 3 days- or 10-days old embryoid bodies (EBs) when compared to undifferentiated ES cells. Subsets within the various functional gene classes were defined that are specifically up- or down-regulated in concert. Interestingly, the present results demonstrate that developmental processes toward germ layer formation are irreversible and mostly independent of the culture conditions. Notably, apoptotic and mitochondrial ribosomal genes were down- and up-regulated in 10-days old EBs, respectively, whereas compared to the 3-days old EBs whereas the activity of the extracellular signal-regulated kinase (ERK) 1/2 decreased with progressive development. This article defines a platform for ES cell differentiation and gene expression studies.

Goossens, E., V. Frederickx, et al. (2006). "Blastocyst development after assisted reproduction using spermatozoa obtained after testicular stem cell transplantation in mice." *Hum Reprod* **21**(7): 1759-64.

BACKGROUND: Since its introduction in 1994, testicular stem cell transplantation (TSCT) has been widely used for research. This technique may also become important for preserving fertility in pre-

pubertal cancer patients. Therefore, it is necessary to investigate the safety aspects of reproduction using spermatozoa obtained after TSCT. In this study, preimplantation development of mouse embryos, using spermatozoa obtained after TSCT, was examined. METHODS: TSCT-derived spermatozoa were used for IVF and ICSI. Embryos were cultured for five days until they reached blastocyst stage and were evaluated by differential staining. RESULTS: IVF revealed significantly lower fertilization and development rates after TSCT-IVF compared to control-IVF. Blastocysts derived from TSCT-IVF had significantly lower inner cell mass numbers (ICMs) and lower ICM/trophectoderm (TE) ratios compared to control-IVF blastocysts. No differences in fertilization and development rates were observed between TSCT-ICSI and control-ICSI, and blastocyst quality in the transplanted group was similar to that of the control blastocysts. CONCLUSION: Our study showed that after TSCT-IVF, fertilization and preimplantation development were disturbed and blastocysts showed reduced ICM and ICM/TE ratio. However, after TSCT-ICSI, both fertilization and preimplantation development were normal and blastocyst formation was comparable to control-ICSI.

Gossler, A., T. Doetschman, et al. (1986). "Transgenesis by means of blastocyst-derived embryonic stem cell lines." *Proc Natl Acad Sci U S A* **83**(23): 9065-9.

This study demonstrates that blastocyst-derived embryonic stem cells (ES cells) can be used as a vehicle for transgenesis. The method is nearly as efficient as other methods, and the introduced neomycin phosphotransferase (neo) gene is stably transmitted through several generations with no apparent loss in G418 resistance. An important factor contributing to the efficiency of this process is the rigorous selection, before blastocyst injection, of genetically transformed cells for in vitro developmental pluripotency. One of the advantages of the ES cell route to transgenesis is that it provides investigators with the opportunity to screen for the desired genetic alterations before reintroducing the ES cells into the animal.

Greco, B., H. P. Low, et al. (2004). "Differentiation prevents assessment of neural stem cell pluripotency after blastocyst injection." *Stem Cells* **22**(4): 600-8.

Earlier studies reported that neural stem (NS) cells injected into blastocysts appeared to be pluripotent, differentiating into cells of all three germ layers. In this study, we followed in vitro green fluorescent protein (GFP)-labeled NS and embryonic stem (ES) cells injected into blastocysts. Forty-eight hours after injection, significantly fewer blastocysts

contained GFP-NS cells than GFP-ES cells. By 96 hours, very few GFP-NS cells remained in blastocysts compared with ES cells. Moreover, 48 hours after injection, GFP-NS cells in blastocysts extended long cellular processes, ceased expressing the NS cell marker nestin, and instead expressed the astrocytic marker glial fibrillary acidic protein. GFP-ES cells in blastocysts remained morphologically undifferentiated, continuing to express the pluripotent marker stage-specific embryonic antigen-1. Selecting cells from the NS cell population that preferentially formed neurospheres for injection into blastocysts resulted in identical results. Consistent with this in vitro behavior, none of almost 80 mice resulting from NS cell-injected blastocysts replaced into recipient mothers were chimeric. These results strongly support the idea that NS cells cannot participate in chimera formation because of their rapid differentiation into glia-like cells. Thus, these results raise doubts concerning the pluripotency properties of NS cells.

Guest, D. J. and W. R. Allen (2007). "Expression of cell-surface antigens and embryonic stem cell pluripotency genes in equine blastocysts." Stem Cells Dev **16**(5): 789-96.

Embryonic stem-like (ES-like) cells have now been derived from the inner cell mass (ICM) of horse embryos at the blastocyst stage. Because they have been shown to express cell-surface antigens found in both human and mouse ES cells, the present study investigated gene expression patterns in day-7 horse blastocysts from which the horse ES-like cells had been derived originally. The genes studied included Oct-4, stage-specific embryonic antigen-1 (SSEA-1), SSEA-3, SSEA-4, tumor rejection antigen-1-60 (TRA-1-60), TRA-1-81, and alkaline phosphatase activity, and whereas all three of the SSEA antigens were expressed in both the ICM and the trophoblast on day 7, Oct-4, TRA-1-60, TRA-1-81, and alkaline phosphatase activity were localized mostly in the ICM. Upon in vitro differentiation of the horse ES-like cells, their expression of the stem cell markers was abolished. Therefore, the species-specific expression pattern of stem cell markers in horse ES-like cells reflects gene expression in the blastocysts from which they are derived.

Guo, Y., L. Einhorn, et al. (2004). "Redox regulation of the embryonic stem cell transcription factor oct-4 by thioredoxin." Stem Cells **22**(3): 259-64.

Oct-4 is a transcriptional regulator required to maintain the totipotentiality of embryonic stem (ES) cells. Downregulation of its activity is required for proper differentiation of the blastocyst during uterine implantation. Uterine implantation and subsequent vascularization increase oxygen exposure

of the developing embryo, thereby altering the intracellular reduction-oxidation status. We tested whether Oct-4 could be regulated by these changes in reduction-oxidation status. We found that Oct-4 DNA binding was exquisitely sensitive to abrogation by oxidation but that the DNA binding of another ES cell transcription factor, FoxD3, was much less sensitive to oxidation. The reducing enzyme Thioredoxin (but not Ape-1) could restore DNA-binding activity of Oct-4. Thioredoxin was less effective at restoring the DNA-binding ability of FoxD3. It was also found that Thioredoxin (but not Ape-1) could physically associate with cysteines in the POU domain of Oct-4. Finally, overexpressing normal Thioredoxin increased the transcriptional activity of Oct-4, while overexpressing a mutant Thioredoxin decreased the transcriptional activity of Oct-4. These data imply that ES cell transcription factors are differentially sensitive to oxidation and that Thioredoxin may differentially regulate ES cell transcription factors.

Hall, V. (2008). "Porcine embryonic stem cells: a possible source for cell replacement therapy." Stem Cell Rev **4**(4): 275-82.

The development of porcine embryonic stem cell lines (pESC) has received renewed interest given the advances being made in the production of immunocompatible transgenic pigs. However, difficulties are evident in the production of pESCs in vitro. This may largely be attributable to differences in porcine pre-implantation development compared to the mouse and human. Expression of oct4, nanog and sox2 differs in the zona-enclosed porcine blastocyst compared to its mouse and human counterparts, which may suggest that other factors may be responsible for maintaining porcine pluripotency in the early blastocyst. In addition, the epiblast forms considerably later, at days 7 to 8 when the porcine blastocyst begins to hatch and is maintained for 4 days before completely differentiating. This review covers an outline of the known molecular profile during porcine pre-implantation development and provides a history in the development of putative pESCs to date. Greater knowledge on the molecular mechanisms that underlie porcine pluripotency and pre-implantation development may aid in improving the development of pESCs.

Hanaoka, K., M. Hayasaka, et al. (1991). "The stem cells of a primordial germ cell-derived teratocarcinoma have the ability to form viable mouse chimeras." Differentiation **48**(2): 83-7.

A euploid testicular teratocarcinoma line, STT-3, has been established from a tumor spontaneously occurring in the testis of a 129/Sv-ter male. Developmental ability of the STT-3 stem cells

was tested by injecting these cells into mouse blastocysts. The frequency and the extent of chimerism were examined in mid-gestational fetuses and in live-born mice. STT-3 stem cells form viable chimeras at a high rate and differentiate into normal tissues. This is the first reported testicular teratocarcinoma-derived stem line with a proven capacity to form viable chimeric mice upon injection into the blastocysts.

Harrison, N. J., D. Baker, et al. (2007). "Culture adaptation of embryonic stem cells echoes germ cell malignancy." *Int J Androl* **30**(4): 275-81; discussion 281.

Teratocarcinomas are a subset of tumours that result from the neoplastic transformation of primordial germ cells. Such germ cell tumours (GCT) are histologically heterogeneous, reflecting a capacity for differentiation (pluripotency) of their embryonal carcinoma (EC) stem cells. However, malignant evolution of these tumours may ultimately correlate with a decrease in pluripotency, because this would tend to increase the propensity of EC cells for self-renewal. Human embryonic stem (ES) cells, derived from early blastocysts, closely resemble EC cells and, on prolonged culture in vitro, acquire progressive genetic changes that show striking similarity to those seen in GCT (e.g. gain of material from chromosome 12). In parallel, these abnormal ES cells show enhanced population growth rates and plating efficiencies, indicative of their adaptation to culture conditions. Understanding the mechanisms that drive such culture adaptation of ES cells may also provide insights into the development and progression of GCT.

Hearn, J. P. (2001). "Embryo implantation and embryonic stem cell development in primates." *Reprod Fertil Dev* **13**(7-8): 517-22.

The endocrine dialogue that results in implantation and the successful establishment of pregnancy in primates relies on embryonic secretion of chorionic gonadotrophin (CG). This hormone is a signal of embryo viability and capacity to support the corpus luteum. The expression of CG is apparently restricted to primates. Active or passive immunization of marmoset monkeys against the beta subunit of CG prevented implantation and early pregnancy, without disrupting the ovarian cycle. Studies of individual embryos cultured in vitro showed that CG is secreted at low levels by the blastocyst from before attachment, with secretion increasing exponentially after attachment. Gonadotrophin releasing hormone (GnRH) was also secreted, from mid-blastocyst stages, before the detection of CG. The secretion of GnRH by the embryo continued through the

attachment and outgrowth stages of embryonic differentiation in vitro. The hypothetical role of GnRH in regulating CG release during implantation was tested in recently completed experiments. Individual embryos cultured with GnRH, or with agonist or antagonist to GnRH, showed significant variations in their secretion of CG and in their survival in culture, suggesting a causal relationship between these hormones. Embryos cultured with natural GnRH showed enhanced growth and development. Embryonic stem cells, from the inner cell mass of marmoset and rhesus monkeys, were the first primate embryonic stem cells to be isolated and characterized, enabling the subsequent isolation of human embryonic stem cells.

Hedlund, E., J. Pruszek, et al. (2008). "Embryonic stem cell-derived Pitx3-enhanced green fluorescent protein midbrain dopamine neurons survive enrichment by fluorescence-activated cell sorting and function in an animal model of Parkinson's disease." *Stem Cells* **26**(6): 1526-36.

Both fetal ventral mesencephalic (VM) and embryonic stem (ES) cell-derived dopamine neurons have been used successfully to correct behavioral responses in animal models of Parkinson's disease. However, grafts derived from fetal VM cells or from ES cells contain multiple cell types, and the majority of these cells are not dopamine neurons. Isolation of ES cell-derived dopamine neurons and subsequent transplantation would both elucidate the capacity of these neurons to provide functional input and also further explore an efficient and safer use of ES cells for the treatment of Parkinson's disease. Toward this goal, we used a Pitx3-enhanced green fluorescent protein (Pitx3-eGFP) knock-in mouse blastocyst-derived embryonic stem (mES) cell line and fluorescence-activated cell sorting (FACS) to select and purify midbrain dopamine neurons. Initially, the dopaminergic marker profile of intact Pitx3-eGFP mES cultures was evaluated after differentiation in vitro. eGFP expression overlapped closely with that of Pitx3, Nurr1, Engrailed-1, Lmx1a, tyrosine hydroxylase (TH), l-aromatic amino acid decarboxylase (AADC), and vesicular monoamine transporter 2 (VMAT2), demonstrating that these cells were of a midbrain dopamine neuron character. Furthermore, postmitotic Pitx3-eGFP(+) dopamine neurons, which constituted 2%-5% of all live cells in the culture after dissociation, could be highly enriched to >90% purity by FACS, and these isolated neurons were viable, extended neurites, and maintained a dopaminergic profile in vitro. Transplantation to 6-hydroxydopamine-lesioned rats showed that an enriched dopaminergic population could survive and restore both amphetamine- and apomorphine-induced

functions, and the grafts contained large numbers of midbrain dopamine neurons, which innervated the host striatum. Disclosure of potential conflicts of interest is found at the end of this article.

Holm, S. (2008). "New embryos' - new challenges for the ethics of stem cell research." *Cells Tissues Organs* **187**(4): 257-62.

Among the many ethical issues raised by human embryonic stem cell research (in the following all references to 'stem cells' should be read as references to human embryonic stem cells), two have gained specific prominence: (1) whether stem cell research is ethically problematic because it entails the destruction of human embryos and (2) what kind of control embryo donors should have over the stem cell lines derived from their embryos. In the present paper, I will analyse how these two issues are engaged by various attempts to derive stem cells from anomalous embryos (e.g. embryos in cleavage arrest, embryos not implanted following pre-implantation genetic diagnosis or embryos created by altered nuclear transfer) or in ways that are claimed to be non-destructive for the embryo (e.g. blastocyst or blastomere biopsy).

Hong, Y., C. Winkler, et al. (1998). "Production of medakafish chimeras from a stable embryonic stem cell line." *Proc Natl Acad Sci U S A* **95**(7): 3679-84.

Embryonic stem (ES) cell lines provide a unique tool for introducing targeted or random genetic alterations through gene replacement, insertional mutagenesis, and gene addition because they offer the possibility for in vitro selection for the desired, but extremely rare, recombinant genotypes. So far only mouse blastocyst embryos are known to have the competence to give rise to such ES cell lines. We recently have established a stable cell line (Mes1) from blastulae of the medakafish (*Oryzias latipes*) that shows all characteristics of mouse ES cells in vitro. Here, we demonstrate that Mes1 cells also have the competence for chimera formation; 90% of host blastulae transplanted with Mes1 cells developed into chimeric fry. This high frequency was not compromised by cryostorage or DNA transfection of the donor cells. The Mes1 cells contributed to numerous organs derived from all three germ layers and differentiated into various types of functional cells, most readily observable in pigmented chimeras. These features suggest the possibility that Mes1 cells may be a fish equivalent of mouse ES cells and that medaka can be used as another system for the application of the ES cell technology.

Huang, J., K. Deng, et al. (2008). "Efficient production of mice from embryonic stem cells

injected into four- or eight-cell embryos by piezo micromanipulation." *Stem Cells* **26**(7): 1883-90.

The conventional method for producing embryonic stem (ES) cell-derived knockout or transgenic mice involves injection of ES cells into normal, diploid blastocysts followed by several rounds of breeding of resultant chimeras and thus is a time-consuming and inefficient procedure. F0 ES cell pups can also be derived directly from tetraploid embryo complementation, which requires fusion of two-cell embryos. Recently, F0 ES cell pups have been produced by injection of ES cells into eight-cell embryos using a laser-assisted micromanipulation system. We report a simple method for producing F0 ES cell germline-competent mice by piezo injection of ES cells into four- or eight-cell embryos. The efficiency of producing live, transgenic mice by this method is higher than that with the tetraploid blastocyst complementation method. This efficient and economical technique for directly producing F0 ES cell offspring can be applicable in many laboratories for creating genetically manipulated mice using ES cell technology and also for stringent testing of the developmental potency of new ES cell or other types of pluripotent stem cell lines.

Hussain, M. A. and N. D. Theise (2004). "Stem-cell therapy for diabetes mellitus." *Lancet* **364**(9429): 203-5.

CONTEXT: Curative therapy for diabetes mellitus mainly implies replacement of functional insulin-producing pancreatic beta cells, with pancreas or islet-cell transplants. However, shortage of donor organs spurs research into alternative means of generating beta cells from islet expansion, encapsulated islet xenografts, human islet cell-lines, and stem cells. Stem-cell therapy here implies the replacement of diseased or lost cells from progeny of pluripotent or multipotent cells. Both embryonic stem cells (derived from the inner cell mass of a blastocyst) and adult stem cells (found in the postnatal organism) have been used to generate surrogate beta cells or otherwise restore beta-cell functioning. STARTING POINT: Recently, Andreas Lechner and colleagues failed to see transdifferentiation into pancreatic beta cells after transplantation of bone-marrow cells into mice (*Diabetes* 2004; 53: 616-23). Last year, Jayaraj Rajagopal and colleagues failed to derive beta cells from embryonic stem cells (*Science* 2003; 299: 363). However, others have seen such effects. WHERE NEXT? As in every emerging field in biology, early reports seem confusing and conflicting. Embryonic and adult stem cells are potential sources for beta-cell replacement and merit further scientific investigation. Discrepancies between different results need to be reconciled. Fundamental processes in determining the

differentiation pathways of stem cells remain to be elucidated, so that rigorous and reliable differentiation protocols can be established. Encouraging studies in rodent models may ultimately set the stage for large-animal studies and translational investigation.

Hwang, W. S., Y. J. Ryu, et al. (2004). "Evidence of a pluripotent human embryonic stem cell line derived from a cloned blastocyst." *Science* **303**(5664): 1669-74.

Somatic cell nuclear transfer (SCNT) technology has recently been used to generate animals with a common genetic composition. In this study, we report the derivation of a pluripotent embryonic stem (ES) cell line (SCNT-hES-1) from a cloned human blastocyst. The SCNT-hES-1 cells displayed typical ES cell morphology and cell surface markers and were capable of differentiating into embryoid bodies in vitro and of forming teratomas in vivo containing cell derivatives from all three embryonic germ layers in severe combined immunodeficient mice. After continuous proliferation for more than 70 passages, SCNT-hES-1 cells maintained normal karyotypes and were genetically identical to the somatic nuclear donor cells. Although we cannot completely exclude the possibility that the cells had a parthenogenetic origin, imprinting analyses support a SCNT origin of the derived human ES cells.

Inoue, K., N. Ogonuki, et al. (2006). "Inefficient reprogramming of the hematopoietic stem cell genome following nuclear transfer." *J Cell Sci* **119**(Pt 10): 1985-91.

In general, cloning undifferentiated preimplantation embryos (blastomeres) or embryonic stem cells is more efficient than cloning differentiated somatic cells. Therefore, there has been an assumption that tissue-specific stem cells might serve as efficient donors for nuclear transfer because of the undifferentiated state of their genome. Here, we show that this is not the case with adult hematopoietic stem cells (HSCs). Although we have demonstrated for the first time that mouse HSCs can be cloned to generate offspring, the birth rates (0-0.7%) were lowest among the clones tested (cumulus, immature Sertoli and fibroblast cells). Only 6% of reconstructed embryos reached the morula or blastocyst stage in vitro (versus 46% for cumulus clones; $P < 5 \times 10^{-10}$). Transcription and gene expression analyses of HSC clone embryos revealed that they initiated zygotic gene activation (ZGA) at the appropriate timing, but failed to activate five out of six important embryonic genes examined, including Hdac1 (encoding histone deacetylase 1), a key regulator of subsequent ZGA. These results suggest that the HSC genome has less plasticity than we imagined, at least in terms of

reprogrammability in the ooplasm after nuclear transfer.

Iwasaki, S. and H. Kimura (2003). "Further studies on thermal treatment of two-cell stage embryos to produce complete embryonic stem-cell-derived mice by cell-aggregation methods." *Dev Growth Differ* **45**(5-6): 427-34.

Employing aggregation techniques with two embryonic sources, one from two-cell stage embryos treated by thermal stimulation and the other from mouse embryonic stem (ES) cells that had been obtained from a feeder layer, simple and most effective methods of producing a complete generation of mice from ES cells were explored. Although thermal treatment affected embryos at various developmental stages, the embryos at the two-cell stage of development were selected because of the remarkably reduced number of cells present in the inner cell mass (ICM) at blastocyst stage after thermal conditioning. Under these conditions, a combination of thermally treated host embryos and an aggregated ES cell-clump was found to produce a high rate of live newborns by natural delivery. That the newborns were completely derived from ES cells was checked by two criteria: microsatellite analysis and coat color analysis. Importantly, all of these mice were healthy and fertile. The aggregation techniques reported here might well be applied to other animal species whose ES cells form stable colonies on a feeder layer.

Jahagirdar, B. N. and C. M. Verfaillie (2005). "Multipotent adult progenitor cell and stem cell plasticity." *Stem Cell Rev* **1**(1): 53-9.

Stem cells are defined by their biological function. A stem cell is an undifferentiated cell that self-renews to maintain the stem cell pool and at the single-cell level differentiates into more than one mature, functional cell. In addition, when transplanted, a stem cell should be capable of replacing a damaged organ or tissue for the lifetime of the recipient. Some would argue that stem cells should also be capable of functionally integrating into nondamaged tissues. Stem cells are critical to both embryogenesis and postnatal life.

Jiang, H., B. Sun, et al. (2007). "Activation of paternally expressed imprinted genes in newly derived germline-competent mouse parthenogenetic embryonic stem cell lines." *Cell Res* **17**(9): 792-803.

Parthenogenetic embryonic stem (pES) cells provide a valuable in vitro model system for studying the molecular mechanisms that underlie genomic imprinting. However, the pluripotency of pES cells and the expression profiles of paternally expressed imprinted genes have not been fully explored. In this

study, three mouse pES cell lines were established and the differentiation potential of these cells in extended culture was evaluated. The undifferentiated cells had a normal karyotype and homozygous genome, and expressed ES-cell-specific molecular markers. The cells remained undifferentiated after more than 50 passages and exhibited pluripotent differentiation capacity. All three lines of the established ES cells produced teratomas; two lines of ES cells produced chimeras and germline transmission. Furthermore, activation of the paternally expressed imprinted genes *Snrpn*, *U2af1-rs1*, *Peg3*, *Impact*, *Zfp127*, *Dlk1* and *Mest* in these cells was detected. Some paternally expressed imprinted genes were found to be expressed in the blastocyst stage of parthenogenetically activated embryos *in vitro* and their expression level increased with extended pES cell culture. Furthermore, our data show that the activation of these paternally expressed imprinted genes in pES cells was associated with a change in the methylation of the related differentially methylated regions. These findings provide direct evidence for the pluripotency of pES cells and demonstrate the association between the DNA methylation pattern and the activation of paternally expressed imprinted genes in pES cells. Thus, the established ES cell lines provide a valuable model for studying epigenetic regulation in mammalian development.

Jincho, Y., Y. Sotomaru, et al. (2008). "Identification of genes aberrantly expressed in mouse embryonic stem cell-cloned blastocysts." *Biol Reprod* **78**(4): 568-76.

During development, cloned embryos often undergo embryonic arrest at any stage of embryogenesis, leading to diverse morphological abnormalities. The long-term effects resulting from embryo cloning procedures would manifest after birth as early death, obesity, various functional disorders, and so forth. Despite extensive studies, the parameters affecting the developmental features of cloned embryos remain unclear. The present study carried out extensive gene expression analysis to screen a cluster of genes aberrantly expressed in embryonic stem cell-cloned blastocysts. Differential screening of cDNA subtraction libraries revealed 224 differentially expressed genes in the cloned blastocysts: eighty-five were identified by the BLAST search as known genes performing a wide range of functions. To confirm their differential expression, quantitative gene expression analyses were performed by real-time PCR using single blastocysts. The genes *Skp1a*, *Canx*, *Ctsd*, *Timd2*, and *Psmc6* were significantly up-regulated, whereas *Aqp3*, *Ak311*, *Rhot1*, *Sf3b3*, *Nid1*, *mt-Rnr2*, *mt-Nd1*, *mt-Cytb*, and *mt-Co2* were significantly down-regulated in the majority of

embryonic stem cell-cloned embryos. Our results suggest that an extraordinarily high frequency of multiple functional disorders caused by the aberrant expression of various genes in the blastocyst stage is involved in developmental arrest and various other disorders in cloned embryos.

Ju, J. Y., C. Y. Park, et al. (2008). "Establishment of stem cell lines from nuclear transferred and parthenogenetically activated mouse oocytes for therapeutic cloning." *Fertil Steril* **89**(5 Suppl): 1314-23.

OBJECTIVE: To establish embryonic stem cell lines from nuclear transfer of somatic cell nuclei isolated from the same oocyte donor and from parthenogenetic activation. The study also evaluated the effect of the micromanipulation procedure on the outcome of somatic cell nuclear transfer in mice. **DESIGN:** Randomized, prospective study. **SETTING:** Hospital-based assisted reproductive technology laboratory. **ANIMAL(S):** F(1) (C57BL/6 x 129P3/J) mice. **INTERVENTION(S):** Metaphase II-stage oocytes were either parthenogenetically activated or nuclear transferred with cumulus cell nuclei or parthenogenetically activated after a sham-manipulation procedure. **MAIN OUTCOME MEASURE(S):** Embryogenesis and embryonic stem cell establishment. **RESULT(S):** The development rate to morula/blastocyst of nuclear transferred oocytes (27.9% +/- 5.9%) was significantly lower than that of the sham-manipulated (84.1% +/- 5.6%) or parthenogenetic (98.6% +/- 1.4%) groups. A sharp decrease in cleavage potential was obvious in the two-to four-cell transition for the nuclear transferred embryos (79.0% +/- 4.6% and 43.3% +/- 5.0%), implying incomplete nuclear reprogramming in arrested oocytes. However, the cleavage, as well as the development rate, of parthenogenetic and sham-manipulated groups did not differ significantly. The embryonic stem cell line establishment rate was higher from parthenogenetically activated oocytes (15.7%) than nuclear transferred (4.3%) or sham-manipulated oocytes (12.5%). Cell colonies from all groups displayed typical morphology of mice embryonic stem cells and could be maintained successfully with undifferentiated morphology after continuous proliferation for more than 120 passages still maintaining normal karyotype. All these cells were positive for mice embryonic stem cell markers such as Oct-4 and SSEA-1 based on immunocytochemistry and reverse transcriptase-polymerase chain reaction. The clonal origin of the ntES cell line and the parthenogenetic embryonic stem cell lines were confirmed by polymerase chain reaction analysis of the polymorphic markers. Blastocyst injection experiments demonstrated that

these lines contributed to resulting chimeras and are germ-line competent. CONCLUSION(S): We report the establishment of ntES cell lines from somatic cells isolated from same individual. Our data also suggest that embryo micromanipulation procedure during the nuclear transfer procedure influences the developmental ability and embryonic stem cell establishment rate of nuclear transferred embryos.

Kanatsu-Shinohara, M., J. Lee, et al. (2008). "Pluripotency of a single spermatogonial stem cell in mice." *Biol Reprod* **78**(4): 681-7.

Although pluripotent stem cells were recently discovered in postnatal testis, attempts to analyze their developmental potential have led to conflicting claims that spermatogonial stem cells are pluripotent or that they lose spermatogenic potential after conversion into pluripotent stem cells. To examine this issue, we analyzed the developmental fate of a single spermatogonial stem cell that appeared during transfection experiments. After transfection of a neomycin-resistance gene into germline stem cells, we obtained an embryonic stem-like, multipotent germline stem cell line. Southern blot analysis revealed that the germline stem and multipotent germline stem clones have the same transgene integration pattern, demonstrating their identical origin. The two lines, however, have different DNA methylation patterns. The multipotent germline stem cells formed chimeras after blastocyst injection but did not produce sperm after germ cell transplantation, whereas the germline stem cells could produce only spermatozoa and did not differentiate into somatic cells. Interestingly, the germline stem cells expressed several transcription factors (Pou5f1, Sox2, Myc, and Klf4) required for reprogramming fibroblasts into a pluripotent state, suggesting that they are potentially pluripotent. Thus, our study provides evidence that a single spermatogonial stem cell can acquire pluripotentiality but that conversion into a pluripotent cell type is accompanied by loss of spermatogenic potential.

Kania, G., D. Corbeil, et al. (2005). "Somatic stem cell marker prominin-1/CD133 is expressed in embryonic stem cell-derived progenitors." *Stem Cells* **23**(6): 791-804.

Prominin-1/CD133 is a plasma membrane marker found in several types of somatic stem cells, including hematopoietic and neural stem cells. To study its role during development and with differentiation, we analyzed its temporal and spatial expression (mRNA and protein) in preimplantation embryos, undifferentiated mouse embryonic stem (ES) cells, and differentiated ES cell progeny. In early embryos, prominin-1 was expressed in trophoblast but

not in cells of the inner cell mass; however, prominin-1 transcripts were detected in undifferentiated ES cells. Both ES-derived cells committed to differentiation and early progenitor cells coexpressed prominin-1 with early lineage markers, including the cytoskeletal markers (nestin, cytokeratin 18, desmin), fibulin-1, and valosin-containing protein. After spontaneous differentiation at terminal stages, prominin-1 expression was downregulated and no coexpression with markers characteristic for neuroectodermal, mesodermal, and endodermal cells was found. Upon induction of neuronal differentiation, some prominin-1-positive cells, which coexpressed nestin and showed the typical morphology of neural progenitor cells, persisted until terminal stages of differentiation. However, no coexpression of prominin-1 with markers of differentiated neural cells was detected. In conclusion, we present the somatic stem cell marker prominin-1 as a new parameter to define ES-derived committed and early progenitor cells.

Kawase, E., Y. Yamazaki, et al. (2000). "Mouse embryonic stem (ES) cell lines established from neuronal cell-derived cloned blastocysts." *Genesis* **28**(3-4): 156-63.

We have established mouse embryonic stem (ES) cell lines from blastocysts derived by transfer of nuclei of fetal neuronal cells. These neuronal cell-derived embryonic cell lines had properties that characterize them as ES cells, including typical cell markers and alkaline phosphatase activity. Moreover, the cells had a normal karyotype and were pluripotent, as they were capable of differentiating into all three germ layers. Although they were derived from neuronal donor nuclei, the cells no longer expressed neuronal markers; however, they were capable of differentiating into cells with neuronal characteristics. These results suggest that the clone-derived cells have fully acquired an ES cell character. Thus, ES cells can be derived from embryos resulting from nuclear transfer, which results in reprogramming of the genetic information and acquisition of pluripotency. ES cells established from somatic cell-derived blastocysts could be useful not only as research tools for studying reprogramming but also as models for cell-based transplantation therapy.

Keskintepe, L., K. Norris, et al. (2007). "Derivation and comparison of C57BL/6 embryonic stem cells to a widely used 129 embryonic stem cell line." *Transgenic Res* **16**(6): 751-8.

Typically, embryonic stem (ES) cells derived from 129 mouse substrains are used to generate genetically altered mouse models. Resulting chimeric mice were then usually converted to a C57BL/6

background, which takes at least a year, even in the case of speed congenics. In recent years, embryonic stem cells have been derived from various mouse strains. However, 129 ES cells are still widely used partially due to poor germline transmission of ES cells derived from other strains. Availability of highly germline-competent C57BL/6 ES cells would enormously facilitate generation of genetically altered mice in a pure C57BL/6 genetic background by eliminating backcrossing time, and thus significantly reducing associated costs and efforts. Here, we describe establishment of a C57BL/6 ES cell line (LK1) and compare its efficacy to a widely used 129SvJ ES cell line (GSI-1) in generating germline chimeras. In contrast to earlier studies, our data shows that highly germline-competent C57BL/6 ES cell lines can be derived using a simple approach, and thus support broader use of C57BL/6 ES cell lines for genetically engineered mouse models.

Kidder, B. L., L. Oseth, et al. (2008). "Embryonic stem cells contribute to mouse chimeras in the absence of detectable cell fusion." *Cloning Stem Cells* **10**(2): 231-48.

Embryonic stem (ES) cells are capable of differentiating into all embryonic and adult cell types following mouse chimera production. Although injection of diploid ES cells into tetraploid blastocysts suggests that tetraploid cells have a selective disadvantage in the developing embryo, tetraploid hybrid cells, formed by cell fusion between ES cells and somatic cells, have been reported to contribute to mouse chimeras. In addition, other examples of apparent stem cell plasticity have recently been shown to be the result of cell fusion. Here we investigate whether ES cells contribute to mouse chimeras through a cell fusion mechanism. Fluorescence in situ hybridization (FISH) analysis for X and Y chromosomes was performed on dissociated tissues from embryonic, neonatal, and adult wild-type, and chimeric mice to follow the ploidy distributions of cells from various tissues. FISH analysis showed that the ploidy distributions in dissociated tissues, notably the tetraploid cell number, did not differ between chimeric and wild-type tissues. To address the possibility that early cell fusion events are hidden by subsequent reductive divisions or other changes in cell ploidy, we injected Z/EG (lacZ/EGFP) ES cells into ACTB-cre blastocysts. Recombination can only occur as the result of cell fusion, and the recombined allele should persist through any subsequent changes in cell ploidy. We did not detect evidence of fusion in embryonic chimeras either by direct fluorescence microscopy for GFP or by PCR amplification of the recombined Z/EG locus on genomic DNA from ACTB-cre::Z/EG chimeric embryos. Our results argue

strongly against cell fusion as a mechanism by which ES cells contribute to chimeras.

Kim, C., T. Amano, et al. (2009). "Improvement of embryonic stem cell line derivation efficiency with novel medium, glucose concentration, and epigenetic modifications." *Cloning Stem Cells* **11**(1): 89-100.

Although the first mouse embryonic stem (ES) cell lines were derived 2 decades ago, and standard protocols for ES cell derivation are widely used today, the technical difficulty of these protocols still pose a challenge for many investigators attempting to produce large numbers of ES cell lines, and are limited to only a few mouse strains. Recently, glucose concentration was shown to have a significant effect on the efficiency of ES cell derivation, but the mechanism(s) mediating this effect are still the subject of debate. In this report, we investigated the effect of glucose concentration on ES cell derivation efficiency from blastocysts in the context of a new medium, Minimum Essential Medium alpha (MEMalpha). Furthermore, we propose novel methods to improve mouse ES cell derivation efficiency using in vitro epigenetic modifications during early passages, combined with detection of Oct4-expressing cells. Based on the results reported here, modified MEMalpha containing high glucose improves the efficiency of ES cell derivation remarkably, compared with Knockout Dulbecco's-Modified Eagle Media (KDMEM). Epigenetic modifications are able to improve the efficiency even further.

Kim, I. W., S. P. Gong, et al. (2009). "Derivation of developmentally competent oocytes by the culture of preantral follicles retrieved from adult ovaries: maturation, blastocyst formation, and embryonic stem cell transformation." *Fertil Steril* **92**(5): 1716-24.

OBJECTIVE: To determine whether the preantral follicles in adult ovaries can generate developmentally competent oocytes after in vitro culture. DESIGN: Prospective, animal-model study. SETTING: Gamete and Stem Cell Biotechnology Laboratory, Seoul National University, Seoul, Korea. ANIMAL(S): B6CBAF1 mice. INTERVENTION(S): Preantral follicles collected from 8-week-old mice were cultured in vitro. MAIN OUTCOME MEASURE(S): Follicle development, embryogenesis, and embryonic stem cell characterization. RESULT(S): A mean of 50.3 preantral follicles were retrieved from one adult animal, which is significantly less than the number (88.7 follicles) retrieved from a prepubertal female. Extension of the culture period greatly improved oocyte maturation; increased follicular growth to the pseudo-antral (89%-91% vs. 32%) or mature oocyte stage (65%-77% vs. 13%) was observed after 12 or 13 days of culture compared with

9 days of culture. Blastocyst formation after parthenogenesis was detected in only one case; in comparison, the use of IVF yielded a large number of embryos that developed into blastocysts. A mean of 14.7 intrafollicular oocytes per animal were produced after 13 days of culture, and 41% of those developed into blastocysts after IVF. Embryonic stem cell-like colonies were established by subculturing the inner cell mass cells from the blastocysts. CONCLUSION(S): Developmentally competent oocytes can be generated by culturing adult preantral follicles. These results may help increase the feasibility of follicle culture systems.

Kim, M., A. Habiba, et al. (2009). "Regulation of mouse embryonic stem cell neural differentiation by retinoic acid." *Dev Biol* **328**(2): 456-71.

Pluripotent mouse embryonic stem cells (ESCs) derived from the early blastocyst can differentiate in vitro into a variety of somatic cell types including lineages from all three embryonic germ layers. Protocols for ES cell neural differentiation typically involve induction by retinoic acid (RA), or by exposure to growth factors or medium conditioned by other cell types. A serum-free differentiation (SFD) medium completely lacking exogenous retinoids was devised that allows for efficient conversion of aggregated mouse ESCs into neural precursors and immature neurons. Neural cells produced in this medium express neuronal ion channels, establish polarity, and form functional excitatory and inhibitory synapses. Brief exposure to RA during the period of cell aggregation speeds neuronal maturation and suppresses cell proliferation. Differentiation without RA yields neurons and neural progenitors with apparent telencephalic identity, whereas cells differentiated with exposure to RA express markers of hindbrain and spinal cord. Transcriptional profiling indicates a substantial representation of transit amplifying neuroblasts in SFD cultures not exposed to RA.

Kim, S. J., J. E. Lee, et al. (2005). "Efficient derivation of new human embryonic stem cell lines." *Mol Cells* **19**(1): 46-53.

Human embryonic stem (hES) cells, unlike most cells derived from adult or fetal human tissues, represent a potentially unlimited source of various cell types for basic clinical research. To meet the increased demand for characterized hES cell lines, we established and characterized nine new lines obtained from frozen-thawed pronucleus-stage embryos. In addition, we improved the derivation efficiency from inner cell masses (to 47.4%) and optimized culture conditions for undifferentiated hES cells. After these cell lines had been maintained for over a year in vitro,

they were characterized comprehensively for expression of markers of undifferentiated hES cells, karyotype, and in vitro/in vivo differentiation capacity. All of the cell lines were pluripotent, and one cell line was trisomic for chromosome 3. Improved culture techniques for hES cells should make them a good source for diverse applications in regenerative medicine, but further investigation is needed of their basic biology.

Kim, Y. H. and H. J. Han (2008). "High-glucose-induced prostaglandin E(2) and peroxisome proliferator-activated receptor delta promote mouse embryonic stem cell proliferation." *Stem Cells* **26**(3): 745-55.

Peroxisome proliferator-activated receptor is a nuclear receptor that has been implicated in blastocyst implantation, cell cycle, and pathogenesis of diabetes. However, the signal cascades underlying this effect are largely unknown in embryo stem cells. This study examined whether or not there is an association between the reactive oxygen species-mediated prostaglandin E(2) (PGE(2))/peroxisome proliferator-activated receptor (PPAR) delta and the growth response to high glucose levels in mouse ESCs. A high concentration of glucose (25 mM) significantly increased the level of [3H]thymidine incorporation, the level of 5-bromo-2'-deoxyuridine incorporation, and the number of cells. Moreover, 25 mM glucose increased the intracellular reactive oxygen species, phosphorylation of the cytosolic phospholipase A(2) (cPLA(2)), and the release of [3H]arachidonic acid ([3H]AA). In addition, 25 mM glucose also increased the level of cyclooxygenase-2 (COX-2) protein expression, which stimulated the synthesis of PGE(2). Subsequently, high glucose-induced PGE(2) stimulated PPARdelta expression directly or through Akt phosphorylation indirectly through the E type prostaglandin receptor receptors. The PPARdelta antagonist inhibited the 25 mM glucose-induced DNA synthesis. Moreover, transfection with a pool of PPARdelta-specific small interfering RNA inhibited the 25 mM glucose-induced DNA synthesis and G1/S phase progression. Twenty-five millimolar glucose also increased the level of the cell cycle regulatory proteins (cyclin E/cyclin-dependent kinase [CDK] 2 and cyclin D1/CDK 4) and decreased p21(WAF1/Cip1) and p27(Kip1), which were blocked by the inhibition of the cPLA(2), COX-2, or PPARdelta pathways. In conclusion, high glucose promotes mouse ESC growth in part through the cPLA(2)-mediated PGE(2) synthesis and in part through PPARdelta pathways.

Kruglova, A. A., E. A. Kizilova, et al. (2008). "Embryonic stem cell/fibroblast hybrid cells with

near-tetraploid karyotype provide high yield of chimeras." *Cell Tissue Res* **334**(3): 371-80.

Ten primary clones of hybrid cells were produced by the fusion of diploid embryonic stem (ES) cells, viz., line E14Tg2aSc4TP6.3 marked by green fluorescent protein (GFP), with diploid embryonic or adult fibroblasts derived from DD/c mice. All the hybrid clones had many characteristics similar to those of ES cells and were positive for GFP. Five hybrid clones having ploidy close to tetraploidy (over 80% of cells had 76-80 chromosomes) were chosen for the generation of chimeras via injection into C57BL blastocysts. These hybrid clones also contained microsatellites marking all ES cell and fibroblast chromosomes judging from microsatellite analysis. Twenty chimeric embryos at 11-13 days post-conception were obtained after injection of hybrid cells derived from two of three clones. Many embryos showed a high content of GFP-positive descendants of the tested hybrid cells. Twenty one adult chimeras were generated by the injection of hybrid cells derived from three clones. The contribution of GFP-labeled hybrid cells was significant and comparable with that of diploid E14Tg2aSc4TP6.3 cells. Cytogenetic and microsatellite analyses of cell cultures derived from chimeric embryos or adults indicated that the initial karyotype of the tested hybrid cells remained stable during the development of the chimeras, i.e., the hybrid cells were mainly responsible for the generation of the chimeras. Thus, ES cell/fibroblast hybrid cells with near-tetraploid karyotype are able to generate chimeras at a high rate, and many adult chimeras contain a high percentage of descendants of the hybrid cells.

Kuliev, A., S. Rechitsky, et al. (2005). "Preimplantation genetics: Improving access to stem cell therapy." *Ann N Y Acad Sci* **1054**: 223-7.

There has been progress in the application of stem cell transplantation for treatment of an increasing number of severe congenital and acquired bone marrow disorders, currently restricted by the availability of human leukocyte antigen (HLA)-matched related donors. Preimplantation HLA typing has recently been introduced to improve the access to stem cell therapy for inherited bone marrow failures. Preimplantation genetic diagnosis (PGD) provides an option not only for avoiding an affected pregnancy with thalassemia and other inherited disorders but also for preselection of the HLA-compatible donors for affected siblings. Multiple short tandem repeat markers throughout the HLA region are applied for this purpose, allowing 100% accuracy of HLA typing, through picking up possible recombination in the HLA region, as well as the copy number of

chromosome 6, which affect accuracy of preimplantation HLA typing. Present experience of preimplantation HLA typing includes preimplantation HLA typing in 180 cycles, 122 of which were done as part of PGD for Fanconi anemia, thalassemia, Wiscott-Aldrich syndrome, hyper-immunoglobulin M syndrome, hypohidrotic ectodermal dysplasia with immune deficiency, and X-linked adrenoleukodystrophy, and 58 for the sole purpose of HLA typing for leukemias and for aplastic and Diamond-Blackfan anemia. The applied method resulted in the accurate preselection and transfer of 100% HLA-matched embryos, yielding already three dozen clinical pregnancies and the birth of two dozen HLA-matched children to the siblings requiring stem cell transplantation. Successful therapy with HLA-matched stem cells, obtained from these PGD children, has been achieved already for Diamond-Blackfan anemia hypohidrotic ectodermal dysplasia with immune deficiency and thalassemia.

Labosky, P. A., D. P. Barlow, et al. (1994). "Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines." *Development* **120**(11): 3197-204.

Primordial germ cells of the mouse cultured on feeder layers with leukemia inhibitory factor, Steel factor and basic fibroblast growth factor give rise to cells that resemble undifferentiated blastocyst-derived embryonic stem cells. These primordial germ cell-derived embryonic germ cells can be induced to differentiate extensively in culture, form teratocarcinomas when injected into nude mice and contribute to chimeras when injected into host blastocysts. Here, we report the derivation of multiple embryonic germ cell lines from 8.5 days post coitum embryos of C57BL/6 inbred mice. Four independent embryonic germ cell lines with normal male karyotypes have formed chimeras when injected into BALB/c host blastocysts and two of these lines have transmitted coat color markers through the germline. We also show that pluripotent cell lines capable of forming teratocarcinomas and coat color chimeras can be established from primordial germ cells of 8.0 days p.c. embryos and 12.5 days p.c. genital ridges. We have examined the methylation status of the putative imprinting box of the insulin-like growth factor type 2 receptor gene (Igf2r) in these embryonic germ cell lines. No correlation was found between methylation pattern and germline competence. A significant difference was observed between embryonic stem cell and embryonic germ cell lines in their ability to maintain the methylation imprint of the Igf2r gene in

culture. This may illustrate a fundamental difference between these two cell types.

Lanzendorf, S. E., C. A. Boyd, et al. (2001). "Use of human gametes obtained from anonymous donors for the production of human embryonic stem cell lines." *Fertil Steril* **76**(1): 132-7.

OBJECTIVE: To investigate the use of donated gametes for the production of human embryonic stem cell lines. **DESIGN:** Basic research study. **SETTING:** Assisted Reproductive Technology (ART) program at an academic institution. **PATIENT(S):** Consenting oocyte and sperm donors. **INTERVENTION(S):** None. **MAIN OUTCOME MEASURE(S):** Oocytes were aspirated from oocyte donors (n = 12) and inseminated with frozen-thawed donor (n = 2) sperm followed by culture of embryos to day 5 or 6 in sequential media. The inner cell masses of expanded blastocysts were isolated using immunosurgery and cultured for 4-11 days on irradiated primary mouse embryonic fibroblasts (PMEFs). Viable cell colonies were passed every 7-10 days onto fresh PMEFs in the presence of leukemia inhibitory factor (0.1 microg/mL) and evaluated for appropriate cell surface markers. **RESULT(S):** Immunosurgery of 40 blastocysts resulted in the culture of 18 inner cell masses, which have produced three cell lines. One of these cell lines has been shown to stain positive for alkaline phosphatase and stage-specific embryonic antigen (SSEA)-4 and negative for SSEA-1, express telomerase activity, and produce hCG when allowed to differentiate. **CONCLUSION(S):** These findings demonstrate that the future production of human embryonic stem cell lines for therapeutic use is possible with the use of donated gametes. Many ethical issues were considered before the initiation of this study, and it was our goal to ensure that both oocyte and sperm donors understood the nature and purpose of the research before their participating in the study.

Latham, K. E. (2006). "The Primate Embryo Gene Expression Resource in embryology and stem cell biology." *Reprod Fertil Dev* **18**(8): 807-10.

The analysis of temporal patterns of gene expression in embryos is an essential component of any research program seeking to understand molecular mechanisms that control development. Little is known of early regulatory mechanisms that operate in primate oocytes and preimplantation-stage embryos. Such studies have been hindered by the cost of obtaining, and limited availability of, non-human primate oocytes and embryos, and by ethical and legal constraints on studies of human embryos. Over the past 4 years we have established the Primate Embryo Gene Expression Resource (PREGER) to circumvent

these limitations. A set of over 200 samples of rhesus monkey oocytes and embryos has been converted to cDNA libraries, which are, in turn, used for a variety of molecular analyses. Both the libraries and cDNA dot blots can be distributed free of charge to anyone wishing to study gene expression at these stages. This includes providing an inexpensive and rapid method for confirming and extending results of gene discovery approaches such as microarray analysis. PREGER includes an on-line resource with a database and other useful tools for embryologists. The resource is being expanded to incorporate samples from other species and from embryonic stem cells.

Ledermann, B. and K. Burki (1991). "Establishment of a germ-line competent C57BL/6 embryonic stem cell line." *Exp Cell Res* **197**(2): 254-8.

Embryonic stem (ES) cell lines have been derived from blastocysts of the inbred mouse strain C57BL/6. The highest frequencies of ES cell colonies were observed when blastocysts were explanted directly onto growth-arrested feeder layers of 5637 human bladder carcinoma cells in the presence of conditioned medium. One of the male ES cell lines tested (BL/6-III) was shown to be karyotypically stable and germ-line competent when introduced into BALB/c host blastocysts. These results demonstrate that ES cell lines from inbred mouse strains other than 129/Sv may be used as vectors to introduce selected mutations into the germ-line of mice.

Lee, J. B., J. E. Lee, et al. (2005). "Establishment and maintenance of human embryonic stem cell lines on human feeder cells derived from uterine endometrium under serum-free condition." *Biol Reprod* **72**(1): 42-9.

Human embryonic stem (hES) cells are usually established and maintained on mouse embryonic fibroblast (MEFs) feeder layers. However, it is desirable to develop human feeder cells because animal feeder cells are associated with risks such as viral infection and/or pathogen transmission. In this study, we attempted to establish new hES cell lines using human uterine endometrial cells (hUECs) to prevent the risks associated with animal feeder cells and for their eventual application in cell-replacement therapy. Inner cell masses (ICMs) of cultured blastocysts were isolated by immunosurgery and then cultured on mitotically inactivated hUEC feeder layers. Cultured ICMs formed colonies by continuous proliferation and were allowed to proliferate continuously for 40, 50, and 55 passages. The established hES cell lines (Miz-hES-14, -15, and -9, respectively) exhibited typical hES cells characteristics, including continuous growth, expression of specific markers, normal karyotypes, and differentiation capacity. The hUEC feeders have

the advantage that they can be used for many passages, whereas MEF feeder cells can only be used as feeder cells for a limited number of passages. The hUECs are available to establish and maintain hES cells, and the high expression of embryotrophic factors and extracellular matrices by hUECs may be important to the efficient growth of hES cells. Clinical applications require the establishment and expansion of hES cells under stable xeno-free culture systems.

Lengner, C. J., F. D. Camargo, et al. (2007). "Oct4 expression is not required for mouse somatic stem cell self-renewal." *Cell Stem Cell* **1**(4): 403-15.

The Pou domain containing transcription factor Oct4 is a well-established regulator of pluripotency in the inner cell mass of the mammalian blastocyst as well as in embryonic stem cells. While it has been shown that the Oct4 gene is inactivated through a series of epigenetic modifications following implantation, recent studies have detected Oct4 activity in a variety of somatic stem cells and tumor cells. Based on these observations it has been suggested that Oct4 may also function in maintaining self-renewal of somatic stem cells and, in addition, may promote tumor formation. We employed a genetic approach to determine whether Oct4 is important for maintaining pluripotency in the stem cell compartments of several somatic tissues including the intestinal epithelium, bone marrow (hematopoietic and mesenchymal lineages), hair follicle, brain, and liver. Oct4 gene ablation in these tissues revealed no abnormalities in homeostasis or regenerative capacity. We conclude that Oct4 is dispensable for both self-renewal and maintenance of somatic stem cells in the adult mammal.

Lerou, P. H., A. Yabuuchi, et al. (2008). "Human embryonic stem cell derivation from poor-quality embryos." *Nat Biotechnol* **26**(2): 212-4.

During in vitro fertilization, embryos deemed clinically useless based on poor morphology are typically discarded. Here we demonstrate a statistical correlation between the developmental stage of such poor-quality embryos and the yield of human embryonic stem (hES) cell lines. 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.

Lev, S., I. Kehat, et al. (2005). "Differentiation pathways in human embryonic stem cell-derived cardiomyocytes." *Ann N Y Acad Sci* **1047**: 50-65.

Human embryonic stem (hES) cells are pluripotent cell lines derived from the inner cell mass of the blastocyst-stage embryo. These unique cell lines can be propagated in the undifferentiated state in

culture, while retaining the capacity to differentiate into derivatives of all three germ layers, including cardiomyocytes. The derivation of the hES cell lines presents a powerful tool to explore the early events of cardiac progenitor cell specification and differentiation, and it also provides a novel cell source for the emerging field of cardiovascular regenerative medicine. A spontaneous differentiation system of these stem cells to cardiomyocytes was established and the generated myocytes displayed molecular, structural, and functional properties of early-stage heart cells. In order to follow the in vitro differentiation process, the temporal expression of signaling molecules and transcription factors governing early cardiac differentiation was examined throughout the process. A characteristic pattern was noted recapitulating the normal in vivo cardiac differentiation scheme observed in other model systems. This review discusses the known pathways involved in cardiac specification and the possible factors that may be used to enhance cardiac differentiation of hES cells, as well as the steps required to fully harness the enormous potential of these unique cells.

Li, C., Y. Yang, et al. (2009). "Derivation and transcriptional profiling analysis of pluripotent stem cell lines from rat blastocysts." *Cell Res* **19**(2): 173-86.

Embryonic stem (ES) cells are derived from blastocyst-stage embryos. Their unique properties of self-renewal and pluripotency make them an attractive tool for basic research and a potential cell resource for therapy. ES cells of mouse and human have been successfully generated and applied in a wide range of research. However, no genuine ES cell lines have been obtained from rat to date. In this study, we identified pluripotent cells in early rat embryos using specific antibodies against markers of pluripotent stem cells. Subsequently, by modifying the culture medium for rat blastocysts, we derived pluripotent rat ES-like cell lines, which expressed pluripotency markers and formed embryoid bodies (EBs) in vitro. Importantly, these rat ES-like cells were able to produce teratomas. Both EBs and teratomas contained tissues from all three embryonic germ layers. In addition, from the rat ES-like cells, we derived a rat primitive endoderm (PrE) cell line. Furthermore, we conducted transcriptional profiling of the rat ES-like cells and identified the unique molecular signature of the rat pluripotent stem cells. Our analysis demonstrates that multiple signaling pathways, including the BMP, Activin and mTOR pathways, may be involved in keeping the rat ES-like cells in an undifferentiated state. The cell lines and information obtained in this study will accelerate our understanding of the

molecular regulation underlying pluripotency and guide us in the appropriate manipulation of ES cells from a particular species.

Li, T., C. Q. Zhou, et al. (2005). "Establishment of human embryonic stem cell line from gamete donors." *Chin Med J (Engl)* **118**(2): 116-22.

BACKGROUND: Human embryonic stem (HES) cell derived from human blastocyst can be propagated indefinitely in the primitive undifferentiated state while remaining pluripotent. It has exciting potential in human developmental biology, drug discovery, and transplantation medicine. But there are insufficient HES cell lines for further study. **METHODS:** Three oocyte donors were studied, and 3 in vitro fertilization (IVF) cycles were carried out to get blastocysts for the establishment of HES cell line. Isolated from blastocysts immunosurgically, inner cell mass (ICM) was cultured and propagated on mouse embryonic fibroblasts (MEFs). Once established, morphology, cell surface markers, karyotype and differentiating ability of the cell line were thoroughly analyzed. **RESULTS:** Four ICMs from 7 blastocysts were cultured on MEFs. After culture, one cell line (cHES-1) was established and met the criteria for defining human pluripotent stem cells including a series of markers used to identify pluripotent stem cells, morphological similarity to primate embryonic stem cells and HES reported elsewhere. Normal and stable karyotype maintained over 60 passages, and demonstrated ability to differentiate into a wide variety of cell types. **CONCLUSIONS:** HES cell lines can be established from gamete donors at a relatively highly efficient rate. The establishment will exert a widespread impact on biomedical research.

Li, X., S. G. Zhou, et al. (2006). "Horse embryonic stem cell lines from the proliferation of inner cell mass cells." *Stem Cells Dev* **15**(4): 523-31.

Inner cell mass (ICM) cells were isolated immunosurgically from day 7-8 horse blastocysts and, after proliferation in vitro for 15-28 passages, three lines of cells were confirmed to be embryonic stem (ES) cells by their continued expression of alkaline phosphatase activity and their ability to bind antisera specific for the recognized stem cell markers, SSEA-1, TRA-1-60, TRA-1-81, and the key embryonic gene Oct-4. When maintained under feeder cell-free conditions in vitro, the three lines of cells differentiated into cells of ectodermal, endodermal, and mesodermal lineages. However, they did not form teratomata when injected into the testes of severe combined immunodeficiency (SCID)/beige immunoincompetent mice, thereby indicating a

significant difference in phenotype between ES cells of the horse and those of the mouse and human.

Liang, L. and J. R. Bickenbach (2002). "Somatic epidermal stem cells can produce multiple cell lineages during development." *Stem Cells* **20**(1): 21-31.

It has been demonstrated that several types of somatic stem cells have the remarkable capacity to differentiate into other types of tissues. We demonstrate here that stem cells from the skin, the largest organ of the body, have the capacity to form multiple cell lineages during development. Using our recently developed sorting technique, we isolated viable homogeneous populations of somatic epidermal stem and transient amplifying cells from the skin of 3-day old transgenic mice, who carried the enhanced green fluorescent protein transgene, and injected stem, TA, or unsorted basal epidermal cells into 3.5-day C57BL/6 blastocysts. Only the stem-injected blastocysts produced mice with GFP(+) cells in their tissues. We found GFP(+) cells in ectodermal, mesenchymal, and neural-crest-derived tissues in E13.5 embryos, 13-day-old neonates, and 60-day-old adult mice, proving that epidermal stem cells survived the blastocyst injection and multiplied during development. Furthermore, the injected stem cells altered their epidermal phenotype and expressed the appropriate proteins for the tissues into which they developed, demonstrating that somatic epidermal stem cells have the ability to produce cells of different lineages during development. These data suggest that somatic epidermal stem cells may show a generalized plasticity expected only of embryonic stem cells and that environmental (extrinsic) factors may influence the lineage pathway for somatic stem cells. Thus, the skin could be a source of easily accessible stem cells that are able to be reprogrammed to form multiple cell lineages.

Liao, S. M. (2005). "Rescuing human embryonic stem cell research: the Blastocyst Transfer Method." *Am J Bioeth* **5**(6): 8-16.

Despite the therapeutic potential of human embryonic stem (HES) cells, many people believe that HES cell research should be banned. The reason is that the present method of extracting HES cells involves the destruction of the embryo, which for many is the beginning of a person. This paper examines a number of compromise solutions such as parthenogenesis, the use of defective embryos, genetically creating a "pseudo embryo" that can never form a placenta, and determining embryo death, and argues that none of these proposals are likely to satisfy embryoists, that is, those who regard the embryo as a person. This paper then proposes a

method of extracting HES cells, what might be called the Blastocyst Transfer Method, that meets the ethical requirements of embryoists, and it considers some possible concerns regarding this method. It concludes by encouraging future HES cell research to investigate this method.

Lim, C. Y., W. L. Tam, et al. (2008). "Sall4 regulates distinct transcription circuitries in different blastocyst-derived stem cell lineages." *Cell Stem Cell* **3**(5): 543-54.

Stem cells self-renew or differentiate under the governance of a stem-cell-specific transcriptional program, with each transcription factor orchestrating the activities of a particular set of genes. Here we demonstrate that a single transcription factor is able to regulate distinct core circuitries in two different blastocyst-derived stem cell lines, embryonic stem cells (ESCs) and extraembryonic endoderm (XEN) cells. The transcription factor Sall4 is required for early embryonic development and for ESC pluripotency. Sall4 is also expressed in XEN cells, and depletion of Sall4 disrupts self-renewal and induces differentiation. Genome-wide analysis reveals that Sall4 is regulating different gene sets in ESCs and XEN cells, and depletion of Sall4 targets in the respective cell types induces differentiation. With Oct4, Sox2, and Nanog, Sall4 forms a crucial interconnected autoregulatory network in ESCs. In XEN cells, Sall4 regulates the key XEN lineage-associated genes Gata4, Gata6, Sox7, and Sox17. Our findings demonstrate how Sall4 functions as an essential stemness factor for two different stem cell lines.

Lin, T. C., J. M. Yen, et al. (2003). "IGF-1/IGFBP-1 increases blastocyst formation and total blastocyst cell number in mouse embryo culture and facilitates the establishment of a stem-cell line." *BMC Cell Biol* **4**: 14.

BACKGROUND: Apoptosis occurs frequently for blastocysts cultured in vitro, where conditions are suboptimal to those found in the natural environment. Insulin-like growth factor-1 (IGF-1) plays an important role in preventing apoptosis in the early development of the embryo, as well as in the progressive regulation of organ development. We hypothesize that IGF-1 and its dephosphorylated binding protein (IGFBP-1) may be able to improve embryo culture with an associated reduced cell death, and that the resultant increase in the total cell number of the embryo could increase the chances of establishing an embryonic stem-cell line. **RESULTS:** In vivo fertilized zygotes were cultured in medium containing supplementary IGF-1, or IGFBP-1/IGF-1. The stages of the resultant embryos were evaluated at

noon on day five post-hCG injection. The extent of apoptosis and necrosis was evaluated using Annexin V and propidium iodine staining under fluorescent microscopy. The establishment of embryonic stem-cell lines was performed using the hatching blastocysts that were cultured in the presence of IGF-1 or IGFBP-1/IGF-1. The results show that the rate of blastocyst formation in a tissue-culture system in the presence of IGF-1 was 88.7% and IGFBP-1/IGF-1 it was 94.6%, respectively, and that it was significantly greater than the figure for the control group (81.9%). IGFBP-1/IGF-1 also resulted in a higher hatching rate than was the case for the control group (68.8% vs. 48.6% respectively). IGF-1 also increased the number of Annexin V-free and propidium iodine-free blastocysts in culture (86.8% vs. 75.9% respectively). Total cell number of blastocyst in culture was increased by 18.9% for those examples cultured with dephosphorylated IGFBP-1/IGF-1. For subsequent stem-cell culture, the chances of the successful establishment of a stem-cell line was increased for the IGF-1 and IGFBP-1/IGF-1 groups (IGF-1 vs. IGFBP-1/IGF-1 vs. control: 45.8% vs. 59.6% vs. 27.3% respectively). **CONCLUSION:** IGF-1 or dephosphorylated IGFBP-1/IGF-1 supplement does result in an anti-apoptotic effect for early embryo development in culture, with a subsequent increased total cell number resulting from cell culture. The effect is beneficial for the later establishment of a stem-cell line.

Liu, W., Y. Yin, et al. (2009). "Derivation and characterization of human embryonic stem cell lines from poor quality embryos." *J Genet Genomics* **36**(4): 229-39.

Poor quality embryos discarded from in vitro fertilization (IVF) laboratories are good sources for deriving human embryonic stem cell (hESC) lines. In this study, 166 poor quality embryos donated from IVF centers on day 3 were cultured in a blastocyst medium for 2 days, and 32 early blastocysts were further cultured in a blastocyst optimum culture medium for additional 2 days so that the inner cell masses (ICMs) could be identified and isolated easily. The ICMs of 17 blastocysts were isolated by a mechanical method, while those of the other 15 blastocysts were isolated by immunosurgery. All isolated ICMs were inoculated onto a feeder layer for subcultivation. The rates of ICM attachment, primary ICM colony formation and the efficiency of hESC derivation were similar between the ICMs isolated by the two methods ($P > 0.05$). As a result, four new hESC lines were established. Three cell lines had normal karyotypes and one had an unbalanced Robertsonian translocation. All cell lines showed normal hESC characteristics and had the differentiation ability. In

conclusion, we established a stable and effective method for hESC isolation and culture, and it was confirmed that the mechanical isolation was an effective method to isolate ICMs from poor embryos. These results further indicate that hESC lines can be derived from poor quality embryos discarded by IVF laboratories.

Lodge, P., J. McWhir, et al. (2005). "Increased gp130 signaling in combination with inhibition of the MEK/ERK pathway facilitates embryonic stem cell isolation from normally refractory murine CBA blastocysts." *Cloning Stem Cells* 7(1): 2-7.

The standard method for isolation of ES cells from strain 129 mice does not give rise to ES lines of CBA origin. We investigated the effect of inhibition of MEK/ERK signaling in combination with increased stimulation of gp130 signaling on derivation of ES cells from CBA blastocysts. Inhibition of MEK1 and MEK2 using the drug U0126 and stimulation of gp130 signaling by elevating the level of LIF present gave rise to ES-like lines in 22.6% of explants. No lines arose when MEK was inhibited in the absence of additional LIF stimulation, nor when additional LIF stimulation occurred in the absence of MEK inhibition. Typically for ES cell lines, CBA-derived cells contributed to chimeric mice and differentiated broadly in vitro. Increased levels of gp130 signaling led to similar levels of STAT3 activation in strain 129 and CBA ES cells. We conclude that CBA ES cells have a requirement for additional STAT3 activation.

Longo, L., A. Bygrave, et al. (1997). "The chromosome make-up of mouse embryonic stem cells is predictive of somatic and germ cell chimaerism." *Transgenic Res* 6(5): 321-8.

Mouse pluripotent embryonic stem (ES) cells, once reintroduced into a mouse blastocyst, can contribute to the formation of all tissues, including the germline, of an organism referred to as a chimaeric. However, the reasons why this contribution often appears erratic are poorly understood. We have tested the notion that the chromosome make-up may be important in contributing both to somatic cell chimaerism and to germ line transmission. We found that the percentage of chimaerism of ES cell-embryo chimaeras, the absolute number of chimaeras and the ratio of chimaeras to total pups born all correlate closely with the percentage of euploid metaphases in the ES cell clones injected into the murine blastocyst. The majority of the ES cell clones that we tested, which were obtained from different gene targeting knockout experiments and harboured 50 to 100% euploid metaphases, did transmit to the germline; in contrast, none of the ES cell clones with more than 50% of chromosomally abnormal metaphases

transmitted to the germline. Euploid ES cell clones cultured in vitro for more than 20 passages rapidly became severely aneuploid, and again this correlated closely with the percentage of chimaerism and with the number of ES cell-embryo chimaeras obtained per number of blastocysts injected. At the same time, the ability of these clones to contribute to the germline was lost when the proportion of euploid cells dropped below 50%. This study suggests that aneuploidy, rather than 'loss of totipotency', in ES cells, is the major cause of failure in obtaining contributions to all tissues of the adult chimaera, including the germline. Because euploidy is predictive of germline transmission, karyotype analysis is crucial and time/cost saving in any gene-targeting experiment.

Lorthongpanich, C., S. H. Yang, et al. (2008). "Chemical enhancement in embryo development and stem cell derivation from single blastomeres." *Cloning Stem Cells* 10(4): 503-12.

Several chemicals targeting the mitogen-activated protein (MAP) kinase signaling pathway, which play an important role in regulating cell growth and differentiation, have shown enhancing effects on the development of the inner cell mass (ICM) and the derivation of ES cells. However, investigation of such chemicals on early embryonic development and the establishment of ES cell lines has not been elucidated. This study was aimed to determine if ACTH, MAP2K1 inhibitor [MAP2K1 (I)], and MAPK14 inhibitor [MAPK14 (I)] could enhance the development of the ICM in preimplantation mouse embryos and blastocyst outgrowths, and the establishment of ES cell lines from blastomeres of early embryos. We have demonstrated that both MAP2K1 (I) and MAPK14 (I) delay early embryo development and inhibit the development of embryos from early blastomeres. On the other hand, ACTH had a positive effect on embryos derived from early blastomeres. As a result, 17 ES cell lines were established. Among these ES cell lines, nine and five ES cell lines were established from single blastomeres of two-cell embryos with and without the supplement of ACTH, respectively. In addition to two-cell isolated blastomeres, three ES cell lines were established from blastomeres of four-cell embryos only with the supplement of ACTH. Our results suggest that ACTH can enhance the derivation of ES cells from single blastomere-derived embryos.

Low, H. P., B. Greco, et al. (2009). "Embryonic stem cell rescue of tremor and ataxia in myelin-deficient shiverer mice." *J Neurol Sci* 276(1-2): 133-7.

Transplantation of neural precursor cells has been proposed as a possible approach for replacing missing or damaged central nervous system myelin.

Neonatal and adult myelin-deficient shiverer (shi) mice, bearing a mutation of the myelin basic protein (MBP) gene, have been used extensively as hosts for testing cell engraftment, migration, and myelination, but relatively little progress has been made in reversing shi motor deficits. Here we describe a prenatal cell replacement strategy, showing that embryonic stem cells injected into shi blastocyst embryos can generate chimeric mice with strong and widespread immunoreactive MBP expression throughout the brain and a behavioral (motor) phenotype that appears essentially rescued.

Mai, Q., Y. Yu, et al. (2007). "Derivation of human embryonic stem cell lines from parthenogenetic blastocysts." *Cell Res* **17**(12): 1008-19.

Parthenogenesis is one of the main, and most useful, methods to derive embryonic stem cells (ESCs), which may be an important source of histocompatible cells and tissues for cell therapy. Here we describe the derivation and characterization of two ESC lines (hPES-1 and hPES-2) from in vitro developed blastocysts following parthenogenetic activation of human oocytes. Typical ESC morphology was seen, and the expression of ESC markers was as expected for alkaline phosphatase, octamer-binding transcription factor 4, stage-specific embryonic antigen 3, stage-specific embryonic antigen 4, TRA-1-60, and TRA-1-81, and there was absence of expression of negative markers such as stage-specific embryonic antigen 1. Expression of genes specific for different embryonic germ layers was detected from the embryoid bodies (EBs) of both hESC lines, suggesting their differentiation potential in vitro. However, in vivo, only hPES-1 formed teratoma consisting of all three embryonic germ layers (hPES-2 did not). Interestingly, after continuous proliferation for more than 100 passages, hPES-1 cells still maintained a normal 46 XX karyotype; hPES-2 displayed abnormalities such as chromosome translocation after long term passages. Short Tandem Repeat (STR) results demonstrated that the hPES lines were genetic matches with the egg donors, and gene imprinting data confirmed the parthenogenetic origin of these ES cells. Genome-wide SNP analysis showed a pattern typical of parthenogenesis. All of these results demonstrated the feasibility to isolate and establish human parthenogenetic ESC lines, which provides an important tool for studying epigenetic effects in ESCs as well as for future therapeutic interventions in a clinical setting.

Mann, J. R. (2001). "Deriving and propagating mouse embryonic stem cell lines for studying genomic imprinting." *Methods Mol Biol* **181**: 21-39.

Embryonic stem (ES) cells are a cell culture derivative of the blastocyst inner cell mass (ICM), the latter giving rise to the embryo, the amnion, the yolk sac, and the chorioallantoic portion of the placenta. Blastocyst injection chimera experiments show that ES cells are similar to early-stage ICM cells in that they contribute to the primitive ectoderm and endoderm derivatives (1). However, it is probably not possible to equate these two cell types, as ES cells appear to be produced by the cell culture environment and have no exact counterpart in the blastocyst. Instead, ES cells could be thought of as being ICM cells that, instead of undergoing rapid differentiation as they would in vivo, are abnormally locked into continuing cycles of division in the undifferentiated state by virtue of the action of exogenous factors. Leukemia inhibitory factor, LIF, is one such factor (2,3) and is indispensable for the propagation of mouse ES cells at least when primary embryo fibroblasts (PEFs) are used as feeder layers (4).

Markoulaki, S., A. Meissner, et al. (2008). "Somatic cell nuclear transfer and derivation of embryonic stem cells in the mouse." *Methods* **45**(2): 101-14.

Addressing the fundamental questions of nuclear equivalence in somatic cells has fascinated scientists for decades and has resulted in the development of somatic cell nuclear transfer (SCNT) or animal cloning. SCNT involves the transfer of the nucleus of a somatic cell into the cytoplasm of an egg whose own chromosomes have been removed. In the mouse, SCNT has not only been successfully used to address the issue of nuclear equivalence, but has been used as a model system to test the hypothesis that embryonic stem cells (ESCs) derived from NT blastocysts have the potential to correct--through genetic manipulations--degenerative diseases. This paper aims to provide a comprehensive description of SCNT in the mouse and the derivation of ESCs from blastocysts generated by this technique. SCNT is a very challenging and inefficient procedure because it is technically complex, it bypasses the normal events of gamete interactions and egg activation, and it depends on adequate reprogramming of the somatic cell nucleus in vivo. Improvements in any or all those aspects may enhance the efficiency and applicability of SCNT. ESC derivation from SCNT blastocysts, on the other hand, requires the survival of only a few successfully reprogrammed cells, which have the capacity to proliferate indefinitely in vitro, maintain correct genetic and epigenetic status, and differentiate into any cell type in the body--characteristics that are essential for transplantation therapy or any other in vivo application.

Markoullis, K., D. Bulian, et al. (2009). "Mycoplasma contamination of murine embryonic stem cells affects cell parameters, germline transmission and chimeric progeny." *Transgenic Res* **18**(1): 71-87.

Murine embryonic stem cells (mESCs) inoculated at passage P13 with the mycoplasma species *M. hominis*, *M. fermentans* and *M. orale* and cultured over 20 passages showed reduced growth rate and viability ($P < 0.0001$) compared to control mESCs. Spectral karyotypic analysis of mycoplasma-infected mESCs showed a number of non-clonal chromosomal aberrations which increased with the duration of infection. The differentiation status of the infected mESCs was most affected at passage P13+6 where the infection was strongest and 46.3% of the mESCs expressed both POU5F1 and SSEA-1 markers whereas 84.8% of control mESCs expressed both markers. The percentage of germline chimeras from mycoplasma-infected mESCs was examined after blastocyst injection and embryo transfer to suitable recipients at different passages and, compared to the respective control group, was most affected at passage P13+5 (50% vs. 90%; $P < 0.07$). Further reductions were obtained at the same passage in the percentage of litters born (50% vs. 100%; $P < 0.07$) and in the percentage of pups born (22% vs. 45%; $P < 0.001$). Thirty three chimeras (39.8%) obtained from blastocyst injection with mycoplasma-infected mESCs showed reduced body weight ($P < 0.0001$), nasal discharge, osteoarthropathia, and cachexia. Flow cytometric analysis of plasma from chimeras produced with mycoplasma-infected mESCs revealed statistically significant differences in the proportions of T-cells and increased levels of IgG1 ($P < 0.001$), IgG2a ($P < 0.05$) and IgM ($P < 0.05$), anti-DNA antibodies ($P < 0.05$) and rheumatoid factor ($P < 0.01$). The present data indicate that mycoplasma contamination of mESCs affects various cell parameters, germline transmission, and postnatal development of the resulting chimeras.

Maslov, A. Y., K. J. Bailey, et al. (2007). "Stem/progenitor cell-specific enhanced green fluorescent protein expression driven by the endogenous *Mcm2* promoter." *Stem Cells* **25**(1): 132-8.

Previous studies have demonstrated expression of the minichromosome maintenance protein *Mcm2* in cells that remain competent to divide, including stem/progenitor cells of the subventricular zone (SVZ) within the brain. Here, a transgenic mouse line in which the *Mcm2* gene drives expression of enhanced green fluorescent protein (EGFP) was constructed by insertion of an internal ribosomal entry site (IRES)-EGFP cassette into the last exon of the gene, 3' to the stop codon. In these

mice, expression of EGFP is observed in the SVZ and several other tissues with high proliferative activity, including the spleen, intestine, hair follicles, and bone marrow. These observations suggest that EGFP fluorescence in this mouse line provides an index of the proliferative capacity of different tissues. Immunohistological analysis demonstrates a direct concordance between expression of EGFP and *Mcm2*, consistent with a transcriptional level downregulation of *Mcm2* expression in postmitotic cells. To test the utility of EGFP expression for recovery of live cells retaining the capacity to divide, EGFP-expressing and -nonexpressing cells from bone marrow and brain were isolated from an adult *Mcm2*(IRES-EGFP) mouse by fluorescence-activated cell sorting and assayed for clonal growth. The EGFP-positive fraction contained the entire clonogenic population of the bone marrow and greater than 90% of neurosphere-forming cells from the brain. Brain-derived clonogenic cells were shown to remain competent to differentiate towards all three neural lineages. These studies demonstrate that the *Mcm2*(IRES-EGFP) transgenic line constructed here can be used for recovery of proliferation competent cells from different tissue types.

Metzger, J. M., W. I. Lin, et al. (1995). "Myosin heavy chain expression in contracting myocytes isolated during embryonic stem cell cardiogenesis." *Circ Res* **76**(5): 710-9.

Mouse embryonic stem (ES) cells are totipotent cells derived from the inner cell mass of the preimplantation blastocyst and are capable of differentiating in vitro into cardiac myocytes. Attached cultures of differentiating ES cells were established to document the timing of contractile development by microscopic observation and to permit the microdissection of cardiac myocytes from culture. The onset of spontaneous contraction varied markedly in differentiation culture, with contraction being maintained on average for 9 days (range, 1 to 75 days). Indirect immunofluorescence in microscopy showed that myosin expression was localized to the contracting cardiac myocytes in culture. Myosin heavy chain (MHC) isoform expression in microdissected ES cell-derived cardiac myocytes was determined by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The distribution of MHC isoform expression in isolated ES cell cardiac myocytes was as follows: 27% expressed the beta-MHC isoform, 33% expressed both the alpha- and beta-MHC isoforms, and 40% expressed the alpha-MHC isoform. MHC phenotype was correlated to the duration of continuous contractile activity of the myocytes. Myocytes that had just initiated spontaneous contractile activity predominantly

expressed the beta-MHC (average days of contraction before isolation, 2.5 +/- 0.7). The alpha-MHC isoform was detected after mouse prolonged contractile activity in vitro (1 to 5 weeks). A strong correlation was obtained between MHC phenotype and days of contraction of the cardiac myocyte preparations isolated from ES cell cultures ($r = .93$). The apparent transition in MHC isoform expression during ES cell differentiation parallels the beta- to alpha-MHC isoform transition characteristic of murine cardiac development in vivo. These findings are evidence that ES cell cardiac myocyte differentiation follows the normal developmental program of murine cardiogenesis.

Metzger, J. M., L. C. Samuelson, et al. (1997). "Embryonic stem cell cardiogenesis applications for cardiovascular research." *Trends Cardiovasc Med* 7(2): 63-8.

Mouse embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of the preimplantation blastocyst. These cells can be maintained in culture in an undifferentiated state, or they can be induced to differentiate in vitro into multiple cell types, including spontaneously beating cardiac myocytes. The ability to engineer these ES cells genetically, together with their noted rapid differentiation into cardiac myocytes in vitro, makes this a useful tool for the study of cardiac gene expression and function. This in vitro cardiogenesis system may be particularly advantageous for pharmacological studies focusing on discovery of cardioactive drugs and for specifying the functional alterations associated with ablated or mutated cardiac genes that result in a lethal phenotype in vivo. (*Trends Cardiovasc Med* 1997;7:63-68). (c) 1997, Elsevier Science Inc.

Mitsunari, M., T. Harada, et al. (1999). "The potential role of stem cell factor and its receptor c-kit in the mouse blastocyst implantation." *Mol Hum Reprod* 5(9): 874-9.

Embryo implantation is a complex process that requires the interaction of embryo and endometrium. Several growth factors and cytokines appear to be involved in this process. Stem cell factor (SCF) and its receptor c-kit regulate the proliferation and survival of germ cells and play an important role in follicular development. However, little information is available on the role of SCF and c-kit in the process of blastocyst implantation. In the present study, we examined the expression of SCF and c-kit mRNA in mouse embryos and in the stromal and epithelial cells of the uterine endometrium by reverse transcription-polymerase chain reaction (RT-PCR). SCF mRNA was expressed in the spreading blastocysts and

endometrial cells, with especially strong expression occurring in the stromal cells. Expression of c-kit mRNA was detected in the blastocysts and spreading blastocysts, as well as in the endometrial cells. By immunocytochemical studies, staining for c-kit protein was observed in the in-vitro spreading trophoblasts. We found that 50-100 ng/ml SCF significantly promoted the expansion of the surface area of the spreading blastocysts ($P < 0.01$). These results are consistent with the hypothesis that SCF derived from endometrial cells and the implanting embryo exerts paracrine and/or autocrine action on the process of implantation by stimulating trophoblast outgrowth through its receptor c-kit.

Muller, T., G. Fleischmann, et al. (2009). "A novel embryonic stem cell line derived from the common marmoset monkey (*Callithrix jacchus*) exhibiting germ cell-like characteristics." *Hum Reprod* 24(6): 1359-72.

BACKGROUND: Embryonic stem cells (ESC) hold great promise for the treatment of degenerative diseases. However, before clinical application of ESC in cell replacement therapy can be achieved, the safety and feasibility must be extensively tested in animal models. The common marmoset monkey (*Callithrix jacchus*) is a useful preclinical non-human primate model due to its physiological similarities to human. Yet, few marmoset ESC lines exist and differences in their developmental potential remain unclear. **METHODS:** Blastocysts were collected and immunosurgery was performed. cjes001 cells were tested for euploidy by karyotyping. The presence of markers for pluripotency was confirmed by immunofluorescence staining and RT-PCR. Histology of teratoma, in vitro differentiation and embryoid body formation revealed the differentiation potential. **RESULTS:** cjes001 cells displayed a normal 46,XX karyotype. Alkaline phosphatase activity, expression of telomerase and the transcription factors OCT4, NANOG and SOX2 as well as the presence of stage-specific embryonic antigen (SSEA)-3, SSEA-4, tumor rejection antigens (TRA)-1-60, and TRA-1-81 indicated pluripotency. Teratoma formation assay displayed derivatives of all three embryonic germ layers. Upon non-directed differentiation, the cells expressed the germ cell markers VASA, BOULE, germ cell nuclear factor and synaptonemal complex protein 3 and showed colocalization of VASA protein within individual cells with the germ line stem cell markers CD9, CD49f, SSEA-4 and protein gene product 9.5, respectively. **CONCLUSIONS:** The cjes001 cells represent a new pluripotent ESC line with evidence for enhanced spontaneous differentiation potential into germ cells.

This cjes001 line will be very valuable for comparative studies on primate ESC biology.

Mummery, C. (2004). "Stem cell research: immortality or a healthy old age?" Eur J Endocrinol **151 Suppl 3**: U7-12.

Stem cell research holds the promise of treatments for many disorders resulting from disease or trauma where one or at most a few cell types have been lost or do not function. In combination with tissue engineering, stem cells may represent the greatest contribution to contemporary medicine of the present century. Progress is however being hampered by the debate on the origin of stem cells, which can be derived from human embryos and some adult tissues. Politics, religious beliefs and the media have determined society's current perception of their relative value while the ethical antipathy towards embryonic stem cells, which require destruction of a human embryo for their derivation, has in many countries biased research towards adult stem cells. Many scientists believe this bias may be premature and basic research on both cell types is still required. The media has created confusion about the purpose of stem cell research: treating chronic ailments or striving for immortality. Here, the scientific state of the art on adult and embryonic stem cells is reviewed as a basis for a debate on whether research on embryonic stem cells is ethically acceptable.

Munne, S., E. Velilla, et al. (2005). "Self-correction of chromosomally abnormal embryos in culture and implications for stem cell production." Fertil Steril **84**(5): 1328-34.

OBJECTIVE: To ascertain whether embryos classified by preimplantation genetic diagnosis (PGD) for infertility as abnormal and then plated to obtain stem cells would self-correct partially or totally in culture, producing disomic stem cells. **DESIGN:** Prospective study to determine the chromosome status of embryos on day 3 and 6, as well as cultured cells derived from inner cell masses from the same embryos when cultured up to day 12. **SETTING:** Research laboratory. **PATIENT(S):** Patients undergoing PGD of aneuploidy. **INTERVENTION(S):** Of 142 embryos classified by PGD for aneuploidy as abnormal, 50 were cultured to the blastocyst stage. At that stage a fraction of the embryos underwent trophoctoderm biopsy to reconfirm the PGD diagnosis. After further co-culture with feeders up to day 12, 34 embryos attached to the feeder cells. Of those, 24 were analyzed by fluorescence in situ hybridization (FISH) and the rest for the expression of Oct-4, SSEA-3, SSEA-4, TRA1-60, and TRA1-80. **MAIN OUTCOME MEASURE(S):** Disomic cells obtained from trisomic

embryos. **RESULT(S):** Analysis by FISH of day-12 cultures showed that 7 were totally normal, 6 were mostly abnormal, and 11 had experienced some chromosome normalization, having between 21% and 88% normal cells. Day-12 culture was positive for Oct-4 expression by reverse transcriptase polymerase chain reaction analysis and for SSEA-3, SSEA-4, TRA1-60, and TRA1-80 by immunocytochemistry. **CONCLUSION(S):** Chromosome self-normalization occurs in a significant proportion of chromosomally abnormal embryos, possibly because of the loss of a chromosome in trisomic cells after blastocyst stage. Thus chromosomally abnormal embryos are a potential source of disomic stem cells. Not all chromosomally abnormal embryos self-corrected. Abnormal stem cells that might be derived could be used as models to study the effect of chromosomal abnormalities on human development.

Munsie, M. J., A. E. Michalska, et al. (2000). "Isolation of pluripotent embryonic stem cells from reprogrammed adult mouse somatic cell nuclei." Curr Biol **10**(16): 989-92.

Pluripotent human stem cells isolated from early embryos represent a potentially unlimited source of many different cell types for cell-based gene and tissue therapies [1-3]. Nevertheless, if the full potential of cell lines derived from donor embryos is to be realised, the problem of donor-recipient tissue matching needs to be overcome. One approach, which avoids the problem of transplant rejection, would be to establish stem cell lines from the patient's own cells through therapeutic cloning [3,4]. Recent studies have shown that it is possible to transfer the nucleus from an adult somatic cell to an unfertilised oocyte that is devoid of maternal chromosomes, and achieve embryonic development under the control of the transferred nucleus [5-7]. Stem cells isolated from such a cloned embryo would be genetically identical to the patient and pose no risk of immune rejection. Here, we report the isolation of pluripotent murine stem cells from reprogrammed adult somatic cell nuclei. Embryos were generated by direct injection of mechanically isolated cumulus cell nuclei into mature oocytes. Embryonic stem (ES) cells isolated from cumulus-cell-derived blastocysts displayed the characteristic morphology and marker expression of conventional ES cells and underwent extensive differentiation into all three embryonic germ layers (endoderm, mesoderm and ectoderm) in tumours and in chimaeric fetuses and pups. The ES cells were also shown to differentiate readily into neurons and muscle in culture. This study shows that pluripotent stem cells can be derived from nuclei of terminally differentiated adult somatic cells and offers a model

system for the development of therapies that rely on autologous, human pluripotent stem cells.

Nagy, A., J. Rossant, et al. (1993). "Derivation of completely cell culture-derived mice from early-passage embryonic stem cells." *Proc Natl Acad Sci U S A* **90**(18): 8424-8.

Several newly generated mouse embryonic stem (ES) cell lines were tested for their ability to produce completely ES cell-derived mice at early passage numbers by ES cell \longleftrightarrow tetraploid embryo aggregation. One line, designated R1, produced live offspring which were completely ES cell-derived as judged by isoenzyme analysis and coat color. These cell culture-derived animals were normal, viable, and fertile. However, prolonged in vitro culture negatively affected this initial totipotency of R1, and after passage 14, ES cell-derived newborns died at birth. However, one of the five subclones (R1-S3) derived from single cells at passage 12 retained the original totipotency and gave rise to viable, completely ES cell-derived animals. The total in vitro culture time of the sublines at the time of testing was equivalent to passage 24 of the original line. Fully potent early passage R1 cells and the R1-S3 subclone should be very useful not only for ES cell-based genetic manipulations but also in defining optimal in vitro culture conditions for retaining the initial totipotency of ES cells.

Newman-Smith, E. D. and Z. Werb (1995). "Stem cell defects in parthenogenetic peri-implantation embryos." *Development* **121**(7): 2069-77.

Mouse embryos containing only maternal chromosomes (parthenotes) develop abnormally in vivo, usually failing at the peri-implantation stage. We have analyzed the development of parthenote embryos by using an inner cell mass (ICM) outgrowth assay that mimics peri-implantation development. ICMs from normal embryos maintained undifferentiated stem cells positive for stage-specific embryonic antigen-1 and Rex-1 while differentiating into a variety of cell types, including visceral endoderm-like cells and parietal endoderm cells. In contrast, ICMs from parthenotes failed to maintain undifferentiated stem cells and differentiated almost exclusively into parietal endoderm. This suggests that parthenote ICMs have a defect that leads to differentiation, rather than maintenance, of the stem cells, and a defect that leads to a parietal endoderm fate for the stem cells. To test the hypothesis that the ICM population is not maintained owing to a lack of proliferation of the stem cells, we investigated whether mitogenic agents were able to maintain the ICM population in parthenotes. When parthenote blastocysts were supplied with the insulin-like growth factor-1 receptor (Igf-1r) and

insulin-like growth factor-2 (Igf-2), two genes not detectable in parthenote blastocysts by in situ hybridization, the ICM population was maintained. Similarly, culture of parthenote blastocysts in medium conditioned by embryonic fibroblasts and supplemented with the maternal factor leukemia inhibitory factor maintained the ICM population. However, once this growth factor-rich medium was removed, the parthenote ICM cells still differentiated predominantly into parietal endoderm. (ABSTRACT TRUNCATED AT 250 WORDS)

Oda, M., S. Tanaka, et al. (2009). "Establishment of trophoblast stem cell lines from somatic cell nuclear-transferred embryos." *Proc Natl Acad Sci U S A* **106**(38): 16293-7.

Placental abnormalities occur frequently in cloned animals. Here, we attempted to isolate trophoblast stem (TS) cells from mouse blastocysts produced by somatic cell nuclear transfer (NT) at the blastocyst stage (NT blastocysts). Despite the predicted deficiency of the trophoblast cell lineage, we succeeded in isolating cell colonies with typical morphology of TS cells and cell lines from the NT blastocysts (ntTS cell lines) with efficiency as high as that from native blastocysts. The established 10 ntTS cell lines could be maintained in the undifferentiated state and induced to differentiate into several trophoblast subtypes in vitro. A comprehensive analysis of the transcriptional and epigenetic traits demonstrated that ntTS cells were indistinguishable from control TS cells. In addition, ntTS cells contributed exclusively to the placenta and survived until term in chimeras, indicating that ntTS cells have developmental potential as stem cells. Taken together, our data show that NT blastocysts contain cells that can produce TS cells in culture, suggesting that proper commitment to the trophoblast cell lineage in NT embryos occurs by the blastocyst stage.

Odorico, J. S., D. S. Kaufman, et al. (2001). "Multilineage differentiation from human embryonic stem cell lines." *Stem Cells* **19**(3): 193-204.

Stem cells are unique cell populations with the ability to undergo both self-renewal and differentiation. A wide variety of adult mammalian tissues harbors stem cells, yet "adult" stem cells may be capable of developing into only a limited number of cell types. In contrast, embryonic stem (ES) cells, derived from blastocyst-stage early mammalian embryos, have the ability to form any fully differentiated cell of the body. Human ES cells have a normal karyotype, maintain high telomerase activity, and exhibit remarkable long-term proliferative potential, providing the possibility for unlimited expansion in culture. Furthermore, they can

differentiate into derivatives of all three embryonic germ layers when transferred to an in vivo environment. Data are now emerging that demonstrate human ES cells can initiate lineage-specific differentiation programs of many tissue and cell types in vitro. Based on this property, it is likely that human ES cells will provide a useful differentiation culture system to study the mechanisms underlying many facets of human development. Because they have the dual ability to proliferate indefinitely and differentiate into multiple tissue types, human ES cells could potentially provide an unlimited supply of tissue for human transplantation. Though human ES cell-based transplantation therapy holds great promise to successfully treat a variety of diseases (e.g., Parkinson's disease, diabetes, and heart failure) many barriers remain in the way of successful clinical trials.

Ohta, H., Y. Ohinata, et al. (2009). "Male germline and embryonic stem cell lines from NOD mice: efficient derivation of GS cells from a nonpermissive strain for ES cell derivation." *Biol Reprod* **81**(6): 1147-53.

The nonobese diabetic (NOD) mouse is a valuable model for human type 1 diabetes and the development of humanized mice. Although the importance of this mouse strain is widely recognized, its usefulness is constrained by the absence of NOD embryonic stem (ES) lines with adequate germline transmission competence. In the present study, we established two germline transmission-competent types of cell lines from NOD mice; these cell lines, male germline stem (GS) cells and ES cells, were derived from NOD spermatogonia and blastocysts, respectively. NOD-GS cells proliferated in vitro and differentiated into mature sperm after transplantation into testis. NOD-ES cell lines were effectively established from NOD blastocysts using culture medium containing inhibitors for fibroblast growth receptor, MEK, and GSK3. Both the NOD-GS and NOD-ES cell lines transmitted their haplotypes to progeny, revealing a novel strategy for gene modification in a pure NOD genetic background. Our results also suggest that the establishment of GS cells is an effective procedure in nonpermissive mouse strains or other species for ES cell derivation.

Ostenfeld, T. and C. N. Svendsen (2003). "Recent advances in stem cell neurobiology." *Adv Tech Stand Neurosurg* **28**: 3-89.

1. Neural stem cells can be cultured from the CNS of different mammalian species at many stages of development. They have an extensive capacity for self-renewal and will proliferate ex vivo in response to mitogenic growth factors or following genetic modification with immortalising oncogenes. Neural

stem cells are multipotent since their differentiating progeny will give rise to the principal cellular phenotypes comprising the mature CNS: neurons, astrocytes and oligodendrocytes. 2. Neural stem cells can also be derived from more primitive embryonic stem (ES) cells cultured from the blastocyst. ES cells are considered to be pluripotent since they can give rise to the full cellular spectrum and will, therefore, contribute to all three of the embryonic germ layers: endoderm, mesoderm and ectoderm. However, pluripotent cells have also been derived from germ cells and teratocarcinomas (embryonal carcinomas) and their progeny may also give rise to the multiple cellular phenotypes contributing to the CNS. In a recent development, ES cells have also been isolated and grown from human blastocysts, thus raising the possibility of growing autologous stem cells when combined with nuclear transfer technology. 3. There is now an emerging recognition that the adult mammalian brain, including that of primates and humans, harbours stem cell populations suggesting the existence of a previously unrecognised neural plasticity to the mature CNS, and thereby raising the possibility of promoting endogenous neural reconstruction. 4. Such reports have fuelled expectations for the clinical exploitation of neural stem cells in cell replacement or recruitment strategies for the treatment of a variety of human neurological conditions including Parkinson's disease (PD), Huntington's disease, multiple sclerosis and ischaemic brain injury. Owing to their migratory capacity within the CNS, neural stem cells may also find potential clinical application as cellular vectors for widespread gene delivery and the expression of therapeutic proteins. In this regard, they may be eminently suitable for the correction of genetically-determined CNS disorders and in the management of certain tumors responsive to cytokines. Since large numbers of stem cells can be generated efficiently in culture, they may obviate some of the technical and ethical limitations associated with the use of fresh (primary) embryonic neural tissue in current transplantation strategies. 5. While considerable recent progress has been made in terms of developing new techniques allowing for the long-term culture of human stem cells, the successful clinical application of these cells is presently limited by our understanding of both (i) the intrinsic and extrinsic regulators of stem cell proliferation and (ii) those factors controlling cell lineage determination and differentiation. Although such cells may also provide accessible model systems for studying neural development, progress in the field has been further limited by the lack of suitable markers needed for the identification and selection of cells within proliferating heterogeneous populations of precursor cells. There is a further need to

distinguish between the committed fate (defined during normal development) and the potential specification (implying flexibility of fate through manipulation of its environment) of stem cells undergoing differentiation. 6. With these challenges lying ahead, it is the opinion of the authors that stem-cell therapy is likely to remain within the experimental arena for the foreseeable future. In this regard, few (if any) of the in vivo studies employing neural stem cell grafts have shown convincingly that behavioural recovery can be achieved in the various model paradigms. Moreover, issues relating to the quality control of cultured cells and their safety following transplantation have only begun to be addressed. 7. While on the one hand cell biotechnologists have been quick to realise the potential commercial value, human stem cell research and its clinical applications has been the subject of intense ethical and legislative considerations. The present chapter aims to review some recent aspects of stem cell research applicable to developmental neurobiology and the potential applications in clinical neuroscience.

Park, S. P., Y. J. Lee, et al. (2004). "Establishment of human embryonic stem cell lines from frozen-thawed blastocysts using STO cell feeder layers." *Hum Reprod* **19**(3): 676-84.

BACKGROUND: Recently, human embryonic stem (hES) cells have become very important resources for basic research on cell replacement therapy and other medical applications. The purpose of this study was to test whether pluripotent hES cell lines could be successfully derived from frozen-thawed embryos that were destined to be discarded after 5 years in a routine human IVF-embryo transfer programme and whether an STO cell feeder layer can be used for the culture of hES cells. **METHODS:** Donated frozen embryos (blastocysts or pronuclear) were thawed, and recovered or in vitro developed blastocysts were immunosurgically treated. All inner cell masses were cultured continuously on an STO cell feeder layer and then presumed hES cell colonies were characterized. **RESULTS:** Seven and two cell lines were established from frozen-thawed blastocysts (7/20, 35.0%) and pronuclear stage embryos (2/20, 10.0%), respectively. The doubling time of hES cells on the immortal STO cell feeder layer was approximately 36 h, similar to that of cells grown using fresh mouse embryonic fibroblast (MEF) feeder conditions. Subcultured hES cell colonies showed strong positive immunostaining for alkaline phosphatase, stage-specific embryonic antigen-4 (SSEA-4) and tumour rejection antigen 1-60 (TRA1-60) cell surface markers. Also, the hES colonies retained normal karyotypes and Oct-4

expression in prolonged subculture. When in vitro differentiation of hES cells was induced by retinoic acid, three embryonic germ layer cells were identified by RT-PCR or indirect immunocytochemistry. **CONCLUSIONS:** This study indicates that establishment of hES cells from frozen-thawed blastocysts minimizes the ethical problem associated with the use of human embryos in research and that the STO cell feeder layer can be used for the culture of hES cells.

Pawar, S. S., D. Malakar, et al. (2009). "Stem cell-like outgrowths from in vitro fertilized goat blastocysts." *Indian J Exp Biol* **47**(8): 635-42.

With an aim to isolate, culture and characterize goat embryonic stem cell-like cells derived from in vitro fertilized goat blastocysts, slaughterhouse derived goat oocytes were in vitro matured in maturation medium in 5% CO₂ air at 38.5 degrees C. Matured oocytes were fertilized in vitro with fresh capacitated spermatozoa. Total 636 (36.5%) cleaved embryos were obtained which were further co-cultured with goat oviductal epithelial cells (GOEC) for 7-10 days. GOEC culture system was better for formation of morula (150; 44.3%) and hatched blastocyst (13; 3.8%) than embryo development medium culture system, [morula (69; 23.1%) and hatched blastocyst (5; 1.6%)]. Out of total blastocysts (48) the primary colonies were formed in 23.3% (7/30) blastocysts, and 66.6% (12/18) of hatched blastocysts. The cells of the inner cell mass (ICM) derived primary colonies were small, aggregated and tightly packed in nature forming embryoid bodies on further subculture. The colonies were stained to see the expression of alkaline phosphatase and positive result was obtained. Goat embryonic stem cell like outgrowths were also characterized for Oct-4 expression and positive result was found. It could be concluded that ICM cells were isolated from in vitro fertilized goat blastocysts and cultured for embryonic stem cell-like cells and expression of alkaline phosphatase and Oct-4 in these cells were positive.

Pereira, L., F. Yi, et al. (2006). "Repression of Nanog gene transcription by Tcf3 limits embryonic stem cell self-renewal." *Mol Cell Biol* **26**(20): 7479-91.

The dual function of stem cells requires them not only to form new stem cells through self-renewal but also to form lineage-committed cells through differentiation. Embryonic stem cells (ESC), which are derived from the blastocyst inner cell mass, retain properties of self-renewal and the potential for lineage commitment. To balance self-renewal and differentiation, ESC must carefully control the levels of several transcription factors, including Nanog,

Sox2, and Oct4. While molecular mechanisms promoting transcription of these genes have been described, mechanisms preventing excessive levels in self-renewing ESC remain unknown. By examining the function of the TCF family of transcription factors in ESC, we have found that Tcf3 is necessary to limit the steady-state levels of Nanog mRNA, protein, and promoter activity in self-renewing ESC. Chromatin immunoprecipitation and promoter reporter assays showed that Tcf3 bound to a promoter regulatory region of the Nanog gene and repressed its transcriptional activity in ESC through a Groucho interaction domain-dependent process. The absence of Tcf3 caused delayed differentiation of ESC in vitro as elevated Nanog levels persisted through 5 days of embryoid body formation. These new data support a model wherein Tcf3-mediated control of Nanog levels allows stem cells to balance the creation of lineage-committed and undifferentiated cells.

Peura, T., A. Bosman, et al. (2008). "Karyotypically normal and abnormal human embryonic stem cell lines derived from PGD-analyzed embryos." Cloning Stem Cells **10**(2): 203-16.

Although a normal karyotype is generally a requirement for stem cell lines, new applications are likely to emerge for stem cells with defined chromosomal aneuploidies. We therefore investigated the use of embryos found to be aneuploid on biopsy followed by preimplantation genetic diagnosis (PGD) with fluorescent in situ hybridization (FISH), and developmentally arrested embryos for stem cell derivation. Eleven stem cell lines were obtained from 41 embryos in 36 cultures, with higher success rate achieved from PGD-analyzed, developmentally advanced embryos (45%) than from clinically unsuitable non-PGD embryos (13%). The resulting stem cell lines were karyotyped, and surprisingly, six of the nine lines from aneuploid embryos as well as both lines from non-PGD embryos were karyotypically normal. Three lines from PGD embryos were aneuploid exhibiting trisomy 5, trisomy 16, and an isochromosome 13, respectively. None of the aneuploid lines presented the same anomaly as the original PGD analysis. Our study has three important implications. First, we confirm the ability to produce stem cell lines from PGD-tested embryos as well as developmentally abnormal embryos, offering specialty stem cell lines for research into the clinically important aneuploidies. Second, we observe that stem cell derivation from apparently aneuploid embryos is often thwarted by underlying mosaicism and emerging dominance of the stem cell line by karyotypically normal cells. The corollary, however, is that regular production of normal stem cell lines from developmentally abnormal embryos ordinarly

discarded opens a new source of embryos for stem cells, whether for research or for eventual therapeutic use within the donating families.

Pickering, S. J., P. R. Braude, et al. (2003). "Preimplantation genetic diagnosis as a novel source of embryos for stem cell research." Reprod Biomed Online **7**(3): 353-64.

The generation of human embryonic stem (hES) cells has captured the public and professional imagination, largely due their potential as a means of overcoming many debilitating and degenerative diseases by cell replacement therapy. Despite this potential, few well-characterized hES cell lines have been derived. Indeed, in the UK, despite several centres having been active in this area for more than 2 years, there are as yet no published reports of human embryonic stem cells having been generated. Part of the reason for this lack of progress may relate to the quality of embryos available for research. Embryos surplus to therapeutic requirements following routine assisted reproduction treatment are often of poor quality and a large proportion may be aneuploid. This study reports a new approach to hES cell derivation. Embryos surplus to therapeutic requirements following preimplantation genetic diagnosis were used. Although unsuitable for embryo transfer due to the high risk of genetic disease, these embryos are from fertile couples and thus may be of better quality than fresh embryos surplus to assisted reproduction treatment cycles. Embryos donated after cryopreservation were also used, and putative hES lines were derived from both sources of embryos. The cell lines described here are thought to be the first reported hES cell lines to have been derived in the UK.

Ponsaerts, P., V. F. van Tendeloo, et al. (2004). "Current challenges in human embryonic stem cell research: directed differentiation and transplantation tolerance." J Biol Regul Homeost Agents **18**(3-4): 347-51.

Research towards potential curative transplantation of human embryonic stem (hES) cell-derived grafts in a variety of diseases has become an important topic since the successful derivation and propagation of hES cells from the inner cell mass of a blastocyst. However, clinical applicability can only be established after intensive laboratory studies that should elaborate on two major topics: A) the development of efficient, controlled and stable hES cell differentiation protocols for any specific cell type, and B) the induction of immunological tolerance against transplanted allogeneic hES cell-derived cell types. This review will briefly discuss: A) current possibilities in hES cell differentiation, followed by

the development of viral, DNA and mRNA-based gene transfer strategies for hES cells, and B) possible immune modulation strategies for inducing immune tolerance against allogeneic hES cell transplants.

Pralong, D., M. L. Lim, et al. (2005). "Tetraploid embryonic stem cells contribute to the inner cell mass of mouse blastocysts." *Cloning Stem Cells* 7(4): 272-8.

The demonstration that mouse somatic cells can be reprogrammed following fusion with embryonic stem (ES) cells may provide an alternative to somatic cell nuclear transfer (therapeutic cloning) to generate autologous stem cells. In an attempt to produce cells with an increased pool of reprogramming factors, tetraploid ES cells were produced by polyethylene glycol mediated fusion of two ES cell lines transfected with plasmids carrying puromycin or neomycin resistance cassettes, respectively, followed by double antibiotic selection. Tetraploid ES cells retain properties characteristic of diploid ES cells, including the expression of pluripotent gene markers Oct4 and Rex1. On injection into the testis capsule of severe combined immunodeficient (SCID) mice, tetraploid ES cells are able to form teratomas containing cells representative of all three germ layers. Further, these cells demonstrated the ability to integrate into the inner cell mass of blastocysts. This study indicates that tetraploid ES cells are promising candidates as cytoplasm donors for reprogramming studies.

Prelle, K., N. Zink, et al. (2002). "Pluripotent stem cells--model of embryonic development, tool for gene targeting, and basis of cell therapy." *Anat Histol Embryol* 31(3): 169-86.

Embryonic stem (ES) cells are pluripotent cell lines with the capacity of self-renewal and a broad differentiation plasticity. They are derived from pre-implantation embryos and can be propagated as a homogeneous, uncommitted cell population for an almost unlimited period of time without losing their pluripotency and their stable karyotype. Murine ES cells are able to reintegrate fully into embryogenesis when returned into an early embryo, even after extensive genetic manipulation. In the resulting chimeric offspring produced by blastocyst injection or morula aggregation, ES cell descendants are represented among all cell types, including functional gametes. Therefore, mouse ES cells represent an important tool for genetic engineering, in particular via homologous recombination, to introduce gene knock-outs and other precise genomic modifications into the mouse germ line. Because of these properties ES cell technology is of high interest for other model organisms and for livestock species like cattle and

pigs. However, in spite of tremendous research activities, no proven ES cells colonizing the germ line have yet been established for vertebrate species other than the mouse (Evans and Kaufman, 1981; Martin, 1981) and chicken (Pain et al., 1996). The in vitro differentiation capacity of ES cells provides unique opportunities for experimental analysis of gene regulation and function during cell commitment and differentiation in early embryogenesis. Recently, pluripotent stem cells were established from human embryos (Thomson et al., 1998) and early fetuses (Shablott et al., 1998), opening new scenarios both for research in human developmental biology and for medical applications, i.e. cell replacement strategies. At about the same time, research activities focused on characteristics and differentiation potential of somatic stem cells, unravelling an unexpected plasticity of these cell types. Somatic stem cells are found in differentiated tissues and can renew themselves in addition to generating the specialized cell types of the tissue from which they originate. Additional to discoveries of somatic stem cells in tissues that were previously not thought to contain these kinds of cells, they also appear to be capable of developing into cell types of other tissues, but have a reduced differentiation potential as compared to embryo-derived stem cells. Therefore, somatic stem cells are referred to as multipotent rather than pluripotent. This review summarizes characteristics of pluripotent stem cells in the mouse and in selected livestock species, explains their use for genetic engineering and basic research on embryonic development, and evaluates their potential for cell therapy as compared to somatic stem cells.

Pruksananonda, K., R. Rungsiwiwut, et al. (2009). "Development of human embryonic stem cell derivation." *J Med Assoc Thai* 92(4): 443-50.

OBJECTIVE: To establish human embryonic stem (hES) cells from human embryos. **DESIGN:** Experimental study. **SETTING:** Reproductive Medicine Unit, Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn University. **MATERIAL AND METHOD:** Abnormal and normal fertilization embryos were cultured in vitro until reaching blastocyst stage. Four different methods for isolation of ICMs were used. Immunosurgery, mechanical isolation, laser assists, and whole blastocyst culture were performed. The feeder layers used in the present study were fibroblasts, isolated from either mouse or human. Mechanical splitting of ICM outgrowths or hES-like cells was performed for propagation of cells. Characterization of hES-like cells was conducted by morphology, detection of immunostaining of Oct-4, and enzymatic activity of alkaline phosphatase (AP).

HES-like cells were spontaneously differentiated through suspension culture of embryoid body (EB). Subsequent differentiation was done on gelatin-coated dishes. **MAIN OUTCOME MEASURE:** Establishment of hES cells. **RESULTS:** By using abnormal fertilization embryos, 80.0% (8/10) of blastocysts were able to attach on the feeder layers, 50% (4/8) formed ICM outgrowths, but no hES-like cells were established. By using normal fertilization embryos, 84.6% (22/26) of blastocysts were able to attach on feeder layers, 18.2% (4/22) formed ICM outgrowths. One hES-like cell line was successfully established by using mechanical isolation of ICMs and human adult skin fibroblasts as feeder layers. This hES-like cells exhibited typical morphology of hES cells, positive staining for Oct-4 and AP. hES-like cells were able to form EB and differentiated into neural-like cells. **CONCLUSION:** This is the first report in Thailand that hES-like cells can be isolated from normal development human embryos at blastocysts-stage using mechanical isolation of ICM and culture with human adult skin fibroblast as feeder layers.

Ralston, A. and J. Rossant (2005). "Genetic regulation of stem cell origins in the mouse embryo." *Clin Genet* **68**(2): 106-12.

'Stem cell' has practically become a household term, but what is a stem cell and where does it come from? Insight into these questions has come from the early mouse embryo, or blastocyst, from which three kinds of stem cells have been derived: embryonic stem (ES) cells, trophoblast stem (TS) cells, and extraembryonic endoderm (XEN) cells. These stem cells appear to derive from three distinct tissue lineages within the blastocyst: the epiblast, the trophectoderm, and the extraembryonic endoderm. Understanding how these lineages arise during development will illuminate efforts to understand the establishment and maintenance of the stem cell state and the mechanisms that restrict stem cell potency. Genetic analysis has enabled the identification of several genes important for lineage decisions in the mouse blastocyst. Among these, Oct4, Nanog, Cdx2, and Gata6 encode transcription factors required for the three lineages of the blastocyst and for the maintenance their respective stem cell types. Interestingly, genetic manipulation of several of these factors can cause lineage switching among these stem cells, suggesting that knowledge of key lineage-determining genes could help control differentiation of stem cells more generally. Pluripotent stem cells have also been isolated from the human blastocyst, but the relationship between these cells and stem cells of the mouse blastocyst remains to be explored. This review describes the genetic regulation of lineage

allocation during blastocyst formation and discusses similarities and differences between mouse and human ES cells.

Ramirez, M. A., R. Fernandez-Gonzalez, et al. (2009). "Effect of stem cell activation, culture media of manipulated embryos, and site of embryo transfer in the production of F0 embryonic stem cell mice." *Biol Reprod* **80**(6): 1216-22.

Recently, F0 embryonic stem (ES) cell mice have been produced by injection of ES cells into eight-cell embryos using either laser- or piezo-assisted injection systems. To simplify the injection procedure, we have optimized the conventional blastocyst injection method, free of laser- or piezo-assisted micromanipulation systems, to produce F0 ES cell pups. To increase the efficiency of producing mice from ES cell injection into eight-cell and blastocyst stage embryos, we have tested: 1) the effect of activating ES cell before injection, 2) the effect of in vitro culture in medium optimized for the survival of both ES cells and embryos, and 3) the effect of transferring the micromanipulated embryos into the oviduct versus into the uterus of CD1 foster mice. Two B6D2 hybrid ES cell lines were used for injection in a multifactorial analysis to evaluate the efficiency of producing live chimeric and F0 ES cell mice. Our results demonstrate that the activation of ES cells and the appropriate culture conditions are crucial parameters influencing the generation of F0 ES cell offspring. Transfer of blastocysts injected with ES cells into the oviduct of 0.5-day postcoitum pseudopregnant females increased the number of live animals with higher chimera proportion. Under these conditions, injections into eight-cell embryos produce a high number of F0 ES mice, and the conventional blastocyst injection method produces a lower number of F0 ES cell pups; however, the efficiency of production of chimeric mice with germline transmission was high. We have developed an economical and efficient technique for producing fully ES cell-derived F0 mice with full germline transmission that can be applied in many laboratories without the use of piezo or laser instruments.

Ratajczak, M. Z., E. K. Zuba-Surma, et al. (2008). "Hunt for pluripotent stem cell -- regenerative medicine search for almighty cell." *J Autoimmun* **30**(3): 151-62.

Regenerative medicine and tissue engineering are searching for a novel stem cell based therapeutic strategy that will allow for efficient treatment or even potential replacement of damaged organs. The pluripotent stem cell (PSC), which gives rise to cells from all three germ lineages, seems to be the most ideal candidate for such therapies. PSC could

be extracted from developing embryos. However, since this source of stem cells for potential therapeutic purposes remains controversial, stem cell researchers look for PSC that could be isolated from the adult tissues or generated from already differentiated cells. True PSC should possess both potential for multilineage differentiation in vitro and, more importantly, also be able to complement in vivo blastocyst development. This review will summarize current approaches and limitations to isolate PSC from adult tissues or, alternatively, to generate it by nuclear reprogramming from already differentiated somatic cells.

Raya, A., I. Rodriguez-Piza, et al. (2008). "Generation of cardiomyocytes from new human embryonic stem cell lines derived from poor-quality blastocysts." *Cold Spring Harb Symp Quant Biol* **73**: 127-35.

Human embryonic stem (hES) cells represent a potential source for cell replacement therapy of many degenerative diseases. Most frequently, hES cell lines are derived from surplus embryos from assisted reproduction cycles, independent of their quality or morphology. Here, we show that hES cell lines can be obtained from poor-quality blastocysts with the same efficiency as that obtained from good- or intermediate-quality blastocysts. Furthermore, we show that the self-renewal, pluripotency, and differentiation ability of hES cell lines derived from either source are comparable. Finally, we present a simple and reproducible embryoid body-based protocol for the differentiation of hES cells into functional cardiomyocytes. The five new hES cell lines derived here should widen the spectrum of available resources for investigating the biology of hES cells and advancing toward efficient strategies of regenerative medicine.

Rebel, V. I., A. L. Kung, et al. (2002). "Distinct roles for CREB-binding protein and p300 in hematopoietic stem cell self-renewal." *Proc Natl Acad Sci U S A* **99**(23): 14789-94.

Hematopoietic stem cells (HSC) are tightly regulated through, as yet, undefined mechanisms that balance self-renewal and differentiation. We have identified a role for the transcriptional coactivators CREB-binding protein (CBP) and p300 in such HSC fate decisions. A full dose of CBP, but not p300, is crucial for HSC self-renewal. Conversely, p300, but not CBP, is essential for proper hematopoietic differentiation. Furthermore, in chimeric mice, hematologic malignancies emerged from both CBP(-/-) and p300(-/-) cell populations. Thus, CBP and p300 play essential but distinct roles in maintaining normal hematopoiesis, and, in mice, both are required for preventing hematologic tumorigenesis.

Reijo Pera, R. A., C. DeJonge, et al. (2009). "Gene expression profiles of human inner cell mass cells and embryonic stem cells." *Differentiation* **78**(1): 18-23.

Human embryonic stem cell (hESC) lines are derived from the inner cell mass (ICM) of preimplantation human blastocysts obtained on days 5-6 following fertilization. Based on their derivation, they were once thought to be the equivalent of the ICM. Recently, however, studies in mice reported the derivation of mouse embryonic stem cell lines from the epiblast; these epiblast lines bear significant resemblance to human embryonic stem cell lines in terms of culture, differentiation potential and gene expression. In this study, we compared gene expression in human ICM cells isolated from the blastocyst and embryonic stem cells. We demonstrate that expression profiles of ICM clusters from single embryos and hESC populations were highly reproducible. Moreover, comparison of global gene expression between individual ICM clusters and human embryonic stem cells indicated that these two cell types are significantly different in regards to gene expression, with fewer than one half of all genes expressed in both cell types. Genes of the isolated human inner cell mass that are upregulated and downregulated are involved in numerous cellular pathways and processes; a subset of these genes may impart unique characteristics to hESCs such as proliferative and self-renewal properties.

Reubinoff, B. E., M. F. Pera, et al. (2000). "Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro." *Nat Biotechnol* **18**(4): 399-404.

We describe the derivation of pluripotent embryonic stem (ES) cells from human blastocysts. Two diploid ES cell lines have been cultivated in vitro for extended periods while maintaining expression of markers characteristic of pluripotent primate cells. Human ES cells express the transcription factor Oct-4, essential for development of pluripotential cells in the mouse. When grafted into SCID mice, both lines give rise to teratomas containing derivatives of all three embryonic germ layers. Both cell lines differentiate in vitro into extraembryonic and somatic cell lineages. Neural progenitor cells may be isolated from differentiating ES cell cultures and induced to form mature neurons. Embryonic stem cells provide a model to study early human embryology, an investigational tool for discovery of novel growth factors and medicines, and a potential source of cells for use in transplantation therapy.

Revazova, E. S., N. A. Turovets, et al. (2008). "HLA homozygous stem cell lines derived from human

parthenogenetic blastocysts." Cloning Stem Cells **10**(1): 11-24.

Individual HLA homozygous parthenogenetic human stem cell (hpSC-Hhom) lines have the potential for cell-based therapy in a significant number of individuals, provided the HLA haplotype is prevalent. We report the successful derivation of four stable hpSC-Hhom lines from both HLA homozygous and HLA heterozygous donors. Of these, the hpSC-Hhom-4 line carries the HLA haplotype found most commonly within the U.S. population, and is shared by different racial groups. These hpSC-Hhom lines demonstrate typical human embryonic stem cell morphology, expressing appropriate stem cell markers and possessing high levels of alkaline phosphatase and telomerase activity. Additionally, injection of these cell lines into immunodeficient animals leads to teratoma formation. G-banded karyotyping demonstrates a normal 46,XX karyotype in lines hpSC-Hhom-1 and hpSC-Hhom-4, and chromosomal anomalies in lines hpSC-Hhom-2 and hpSC-Hhom-3, both derived from the same donor. HLA genotyping of all four hpSC-Hhom lines demonstrates that they are HLA homozygous. Furthermore, in the case of HLA heterozygous donors, the hpSC-Hhom lines inherit the haplotype from only one of the donor's parents. Single-nucleotide polymorphism (SNP) data analysis suggests that hpSC-Hhom lines derived from HLA heterozygous oocyte donors are homozygous throughout the genome as assessed by SNP analysis. The protocol used for deriving these HLA homozygous stem cell lines minimizes the use of animal-derived components, which makes them more appealing for potential clinical application.

Revazova, E. S., N. A. Turovets, et al. (2007). "Patient-specific stem cell lines derived from human parthenogenetic blastocysts." Cloning Stem Cells **9**(3): 432-49.

Parthenogenetic activation of human oocytes may be one way to produce histocompatible cells for cell-based therapy. We report the successful derivation of six pluripotent human embryonic stem cell (hESC) lines from blastocysts of parthenogenetic origin. The parthenogenetic human embryonic stem cells (phESC) demonstrate typical hESC morphology, express appropriate markers, and possess high levels of alkaline phosphatase and telomerase activity. The phESC lines have a normal 46, XX karyotype, except one cell line, and have been cultured from between 21 to 35 passages. The phESC lines form embryoid bodies in suspension culture and teratomas after injection to immunodeficient animals and give differentiated derivatives of all three embryonic germ layers. DNA profiling of all six phESC lines

demonstrates that they are MHC matched with the oocyte donors. The study of imprinted genes demonstrated further evidence of the parthenogenetic origin of the phESC lines. Our research has resulted in a protocol for the production of human parthenogenetic embryos and the derivation of stem cell lines from them, which minimizes the presence of animal-derived components, making the derived phESC lines more suitable for potential clinical use.

Risau, W., H. Sariola, et al. (1988). "Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies." Development **102**(3): 471-8.

Embryonic stem cells (ESC) have been established previously from the inner cell mass cells of mouse blastocysts. In suspension culture, they spontaneously differentiate to blood-island-containing cystic embryoid bodies (CEB). The development of blood vessels from in situ differentiating endothelial cells of blood islands, a process which we call vasculogenesis, was induced by injecting ESC into the peritoneal cavity of syngeneic mice. In the peritoneum, fusion of blood islands and formation of an in vivo-like primary capillary plexus occurred. Transplantation of ESC and ESC-derived complex and cystic embryoid bodies (ESC-CEB) onto the quail chorioallantoic membrane (CAM) induced an angiogenic response, which was directed by nonyolk sac endoderm structures. Neither yolk sac endoderm from ESC-CEB nor normal mouse yolk sac tissue induced angiogenesis on the quail CAM. Extracts from ESC-CEB stimulated the proliferation of capillary endothelial cells in vitro. Mitogenic activity increase during in vitro culture and differentiation of ESC. Almost all growth factor activity was associated with the cells. The ESC-CEB derived endothelial cell growth factor bound to heparin-sepharose. The identification of acidic fibroblast growth factor (FGF) in heparin-sepharose-purified material was accomplished by immunoblot experiments involving antibodies against acidic and basic FGF. We conclude that vasculogenesis, the development of blood vessels from in situ differentiating endothelial cells, and angiogenesis, the sprouting of capillaries from preexisting vessels are very early events during embryogenesis which can be studied using ESC differentiating in vitro. Our results suggest that vasculogenesis and angiogenesis are differently regulated.

Roach, M. L., J. L. Stock, et al. (1995). "A new embryonic stem cell line from DBA/1lacJ mice allows genetic modification in a murine model of human inflammation." Exp Cell Res **221**(2): 520-5.

The development of embryonic stem (ES) cells and their capacity to generate mice with

mutations at specific loci has provided a powerful resource for functional analysis of genes in pathological processes. However, the ability to combine this technology with the large number of existing murine models of human genetic disease has been complicated by the inability to routinely generate ES cell lines from strains other than 129. Here, we report the production of a novel ES cell line derived from an inbred mouse, DBA/1lacJ. This new ES cell line undergoes homologous recombination and efficient colonization of the germline of male chimeric offspring with ES cell microinjection into C57B1/6 embryos. The DBA/1lacJ mouse is a murine model of human inflammation, therefore genetic modifications in the DBA ES cells will allow evaluation of the target gene's role in the inflammatory process.

Saburi, S., S. Azuma, et al. (1997). "Developmental fate of single embryonic stem cells microinjected into 8-cell-stage mouse embryos." *Differentiation* **62**(1): 1-11.

Embryonic stem (ES) cells are pluripotent and capable of differentiating into somatic as well as germ cell lineages when conjoined with blastomeres of early mouse embryos. However, the developmental potential of single ES cells has not been fully investigated. We injected single murine ES cells (A3-1 cell line) of 129 origin into 8-cell mouse embryos (B6xBDF1) and examined the patterns of distribution of ES-cell-derived cells in the blastocysts as well as in the fully grown chimeric mice. The ES cells underwent 1-2 cycles of mitosis between the 8-cell and the blastocyst stage when they were introduced as single cells, whereas those introduced as groups of 2-5 cells did not proliferate during the same period of development. The ES cells and their daughter cells were predominantly incorporated into the ICM. From the 63 8-cell embryos which received single ES cells microinjected into the perivitelline space, 24 newborns were obtained, and 4 (2 fertile males, 1 sterile female and 1 hermaphrodite) of them (16.6%) were chimeric. The test breeding studies revealed that all the progeny of the two chimeric males were derived from spermatozoa of 129 genotype. The relative contribution of the host-derived and the ES-cell-derived cells in different tissues of the chimeric mice was assessed by PCR analyses of the microsatellite polymorphism of genomic DNA extracted from the tissues. In two male germ line chimeras, the testes, the kidneys and the dorsal skeletal muscles exhibited exceptionally high 129 contents. Our results demonstrated that single ES cells which maintain totipotency or pluripotency of high degree are present in a colony of ES cells, and that single ES cells conjoined with the blastomeres of 8-

cell-stage embryos may colonize, if the circumstances allow, almost exclusively the germ cells and concomitantly the urogenital cell lineages. Possible correlation between the allocation of the germ line and the urogenital lineages is discussed.

Sakaki-Yumoto, M., C. Kobayashi, et al. (2006). "The murine homolog of SALL4, a causative gene in Okihiro syndrome, is essential for embryonic stem cell proliferation, and cooperates with Sall1 in anorectal, heart, brain and kidney development." *Development* **133**(15): 3005-13.

Mutations in SALL4, the human homolog of the *Drosophila* homeotic gene spalt (*sal*), cause the autosomal dominant disorder known as Okihiro syndrome. In this study, we show that a targeted null mutation in the mouse *Sall4* gene leads to lethality during peri-implantation. Growth of the inner cell mass from the knockout blastocysts was reduced, and *Sall4*-null embryonic stem (ES) cells proliferated poorly with no aberrant differentiation. Furthermore, we demonstrated that anorectal and heart anomalies in Okihiro syndrome are caused by *Sall4* haploinsufficiency and that *Sall4/Sall1* heterozygotes exhibited an increased incidence of anorectal and heart anomalies, exencephaly and kidney agenesis. *Sall4* and *Sall1* formed heterodimers, and a truncated *Sall1* caused mislocalization of *Sall4* in the heterochromatin; thus, some symptoms of Townes-Brocks syndrome caused by SALL1 truncations could result from SALL4 inhibition.

Sathanathan, H., S. Gunasheela, et al. (2003). "Critical evaluation of human blastocysts for assisted reproduction techniques and embryonic stem cell biotechnology." *Reprod Biomed Online* **7**(2): 219-27.

Critical examination of 30 blastocysts by transmission electron microscopy (TEM) reveals cellular features not usually evident, including abnormalities of cell structure and aberrations such as multinucleation, internal fragmentation, phagocytic or degenerating cells. Invariably, such blastocysts are inactive and delay or fail to expand and hatch in vitro. Hatching seems to be a major problem in ageing blastocysts due to inactivity of the surface epithelium of trophoblast cells that do not stretch and expand. These lack surface microvilli and contractile tonofilaments that anchor on to specialized cell junctions such as desmosomes. Trophoblast expansion and consequent thinning of the zona is a prerequisite to proper hatching aided by the hydrostatic pressure in the blastocoele and by specialized cells at hatching points. Proper assessment of the inner cell mass is required if a healthy population of cells is to be harvested for embryonic stem cell culture. An inactive blastocyst is obviously not good material and could

have a defective inner cell mass (ICM). Normally approximately 3-5% of cells are mitotic in blastocysts and arrested cell division is also an indicator of inactivity. An attempt has been made to evaluate blastocyst internal structure for both assisted reproduction techniques and embryonic stem cell biotechnology.

Schoonjans, L., V. Kreemers, et al. (2003). "Improved generation of germline-competent embryonic stem cell lines from inbred mouse strains." *Stem Cells* **21**(1): 90-7.

Genetically altered mice may exhibit highly variable phenotypes due to the variation in genetic background, which can only be circumvented by generation of inbred, isogenic gene-targeted and control mice. Here we report that an embryonic stem (ES) cell culture medium conditioned by a rabbit fibroblast cell line transduced with genomic rabbit leukemia inhibitory factor allows efficient derivation and maintenance of ES cell lines from all of 10 inbred mouse strains tested, including some that were presumed to be nonpermissive for ES cell derivation (129/SvEv, 129/SvJ, C57BL/6N, C57BL/6JOLA, CBA/CaOla, DBA/2N, DBA/1Ola, C3H/HeN, BALB/c, and FVB/N). Germline transmission was established by blastocyst injection of established ES cell lines after 10 or more passages from all of seven strains tested (129/SvJ, C57BL/6N, C57BL/6JOLA, DBA/2N, DBA/1Ola, BALB/c, and FVB/N), by diploid aggregation of ES cell lines from all of four strains tested (129/SvEv, C57BL/6N, CBA/ CaOla, and FVB/N), or by tetraploid aggregation of ES cell lines from all of three strains tested (129/SvEv, C57BL/6N, and CBA/CaOla). Thus, these inbred ES cell lines may constitute useful tools to derive gene-targeted mice and isogenic controls in selected genetic backgrounds.

Schwenk, F., B. Zevnik, et al. (2003). "Hybrid embryonic stem cell-derived tetraploid mice show apparently normal morphological, physiological, and neurological characteristics." *Mol Cell Biol* **23**(11): 3982-9.

ES cell-tetraploid (ES) mice are completely derived from embryonic stem cells and can be obtained at high efficiency upon injection of hybrid ES cells into tetraploid blastocysts. This method allows the immediate generation of targeted mouse mutants from genetically modified ES cell clones, in contrast to the standard protocol, which involves the production of chimeras and several breeding steps. To provide a baseline for the analysis of ES mouse mutants, we performed a phenotypic characterization of wild-type B6129S6F(1) ES mice in relation to controls of the same age, sex, and genotype raised

from normal matings. The comparison of 90 morphological, physiological, and behavioral parameters revealed elevated body weight and hematocrit as the only major difference of ES mice, which exhibited an otherwise normal phenotype. We further demonstrate that ES mouse mutants can be produced from mutant hybrid ES cells and analyzed within a period of only 4 months. Thus, ES mouse technology is a valid research tool for rapidly elucidating gene function in vivo.

Sharp, J. and H. S. Keirstead (2009). "Stem cell-based cell replacement strategies for the central nervous system." *Neurosci Lett* **456**(3): 107-11.

During human development, cells of the blastocyst inner cell mass proliferate and give rise to each cell in the human body. It is that potential which focuses intense interest on these stem cells as a substrate for cell-based regenerative medicine. An increased understanding of the interrelation of processes that govern the formation of various cell types will allow for the directed differentiation of stem cells into specified cells or tissues that can ameliorate the effects of disease or damage. Perhaps the most difficult cells and tissues to derive for use in cell replacement strategies are the diverse neurons, glia and complex networks of the central nervous system (CNS). Here we present emerging perspectives on the development of neuronal and glial cells from stem cells for clinical application to CNS diseases and injury.

Shibayama, M., S. Ohno, et al. (2009). "Polypyrimidine tract-binding protein is essential for early mouse development and embryonic stem cell proliferation." *Febs J* **276**(22): 6658-68.

Polypyrimidine tract-binding protein (PTB) is a widely expressed RNA-binding protein with multiple roles in RNA processing, including the splicing of alternative exons, mRNA stability, mRNA localization, and internal ribosome entry site-dependent translation. Although it has been reported that increased expression of PTB is correlated with cancer cell growth, the role of PTB in mammalian development is still unclear. Here, we report that a homozygous mutation in the mouse *Ptb* gene causes embryonic lethality shortly after implantation. We also established *Ptb*(-/-) embryonic stem (ES) cell lines and found that these mutant cells exhibited severe defects in cell proliferation without aberrant differentiation in vitro or in vivo. Furthermore, cell cycle analysis and a cell synchronization assay revealed that *Ptb*(-/-) ES cells have a prolonged G(2)/M phase. Thus, our data indicate that PTB is essential for early mouse development and ES cell proliferation.

Shimizu, R., A. Sakata, et al. (2005). "Establishment of a new embryonic stem cell line derived from C57BL/6 mouse expressing EGFP ubiquitously." *Genesis* **42**(1): 47-52.

Transgenic mice ubiquitously expressing enhanced green fluorescent protein (EGFP) are useful as marker lines in chimera experiments. We established a new embryonic stem (ES) cell line (named B6G-2) from a C57BL/6 blastocyst showing ubiquitous EGFP expression. Undifferentiated B6G-2 cells showed strong green fluorescence and mRNAs of pluripotent marker genes. B6G-2 cells were transferred into a C57BL/6 blastocyst to generate a germline chimera, the progeny of which inherited ubiquitous EGFP expression. Mice derived completely from B6G-2 cells were also developed from the ES cells; these were tetraploid chimeras. The established B6G-2 cells were shown to be pluripotent and to be capable of differentiating into cells of all lineages. Thus, the new ES cell line expressing EGFP ubiquitously is useful for basic research in the field of regenerative medicine. The B6G-2 cell line is freely available from the BioResource Center, RIKEN Tsukuba Institute (<http://www.brc.riken.jp/lab/cell/english/>).

Siu, C. W., J. C. Moore, et al. (2007). "Human embryonic stem cell-derived cardiomyocytes for heart therapies." *Cardiovasc Hematol Disord Drug Targets* **7**(2): 145-52.

Cardiovascular diseases remain the leading cause of mortality and morbidity worldwide. Despite substantial improvements in acute management, survivors of myocardial infarction often progress to heart failure. Since adult cardiomyocytes (CMs) do not regenerate, their loss permanently compromises myocardial contractile function. Heart transplantation is currently the last resort for end-stage heart failure, but is hampered by a severe shortage of donor organs and rejection. Cell-based therapies are a promising alternative: Various cell types such as human fetal CMs, skeletal muscle myoblasts and smooth muscle cells have been tested but these approaches are also limited by cell availability or side effects (e.g. due to their non-cardiac identity). In recent years, clinical studies exploiting adult bone marrow mesenchymal stem cells for transplantation in patients with coronary artery disease have reported favorable outcomes but their cardiomyogenic ability is limited. By contrast, human embryonic stem cells (hESCs), derived from the inner cell mass of blastocyst-stage embryos, are pluripotent and can self-renew and differentiate into all cell types including CMs. Furthermore, hESC-derived CMs (hESC-CMs) are viable human heart cells that can functionally integrate with the recipient

organ after transplantation. This article reviews the current state and hurdles of hESC-CM research, as well as their therapeutic potentials and limitations.

Suemori, H., T. Tada, et al. (2001). "Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI." *Dev Dyn* **222**(2): 273-9.

Human embryonic stem (ES) cells are predicted to be a valuable source for producing ES-derived therapeutic spare tissues to treat diseases by controlling their growth and differentiation. To understand the regulative mechanisms of their differentiation in vivo and in vitro, ES cells derived from nonhuman primates could be a powerful tool. We established four ES cell lines from cynomolgus monkey (*Macaca fascicularis*) blastocysts produced by in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). The ES cells were characterized by the expression of specific markers such as alkaline phosphatase and stage-specific embryonic antigen-4. They were successfully maintained in an undifferentiated state and with a normal karyotype even after more than 6 months of culture. Pluripotential competence was confirmed by the formation of teratomas containing ectoderm-, mesoderm-, and endoderm- derivatives after subcutaneous injection into SCID mice. Differentiation to a variety of tissues was identified by immunohistochemical analyses using tissue-specific antibodies. Therefore, we established pluripotent ES cell lines derived from monkeys that are widely used as experimental animals. These lines could be a useful resource for preclinical stem cell research, including allogenic transplantation into monkey models of disease.

Sukoyan, M. A., A. N. Golubitsa, et al. (1992). "Isolation and cultivation of blastocyst-derived stem cell lines from American mink (*Mustela vison*)." *Mol Reprod Dev* **33**(4): 418-31.

Ten embryonic stem (ES) cell lines from mink blastocysts were isolated and characterized. All the lines had a normal diploid karyotype; of the ten lines studied, five had the XX and five had the XY constitution. Testing of the pluripotency of the ES-like cells demonstrated that 1) among four lines of genotype XX, and X was late-replicating in three; both Xs were active in about one-third of cells of line MES8, and analysis of glucose-6-phosphate dehydrogenase revealed no dosage compensation for the X-linked gene; 2) when cultured in suspension, the majority of lines were capable of forming "simple" embryoid bodies (EB), and two only showed the capacity for forming "cystic" multilayer EBs. However, formation of ectoderm or foci of yolk sac

hematopoiesis, a feature of mouse ES cells, was not observed in the "cystic" EB; 3) when cultured as a monolayer without feeder, the ES cells differentiated into either vimentin-positive fibroblast-like cells or cytokeratin-positive epithelial-like cells (less frequently); neural cells appeared in two lines; 4) when injected into athymic mice, only one of the four tested lines gave rise to tumors. These were fibrosarcomas composed of fibroblast-like cells, with an admixture of smooth muscular elements and stray islets of epithelial tissue; (5) when the ES cells of line MES1 were injected into 102 blastocyst cavities and subsequently transplanted into foster mothers, we obtained 30 offspring. Analysis of the biochemical markers and coat color did not demonstrate the presence of chimaeras among offspring. Thus the cell lines derived from mink blastocysts are true ES cells. However, their pluripotential capacities are restricted.

Sukoyan, M. A., S. Y. Vatolin, et al. (1993). "Embryonic stem cells derived from morulae, inner cell mass, and blastocysts of mink: comparisons of their pluripotencies." *Mol Reprod Dev* **36**(2): 148-58.

A characterization of cell lines that we derived from morulae (three lines), blastocysts (two lines), and the inner cell mass (ICM) is given. The karyotype of all the lines was normal; the genotype of four lines was XX, and four lines were genotypically XY. The pluripotencies and commitment status of the derived lines were estimated. First, there were not less than two-thirds of cells in the populations of the lines derived from morulae and the ICM with both Xs active; 70-100% of cells of the blastocyst-derived lines had one of the Xs in an inactive state. The activity of glucose-6-phosphate dehydrogenase (G6PD) in the lines (genotype XX) derived from morulae and ICM was found to be twofold higher than in lines with genotype XY, and G6PD activity was the same in the blastocyst-derived XX lines and XY lines. Second, when injected intraperitoneally into athymic mice, morulae- and ICM-derived cells gave rise to simple and complex embryoid bodies (EB) resembling to typical "cystic" mouse EBs. Third, when injected subcutaneously to athymic mice, the ICM- or morula-derived cells gave rise to typical teratomas containing derivatives of the three germ layers and components of organogenesis. Comparisons of cell lines of different derivations demonstrated that the pluripotencies of the ES cells derived from morulae or the ICM are higher than those of blastocyst derivation.

Sumida, T., T. Maeda, et al. (1990). "Expression of IgH promoter/enhancer Ly-1 transgene in hematopoietic chimeric mice generated by embryonic stem cell line." *Int Arch Allergy Appl Immunol* **93**(2-3): 155-64.

We have produced hematopoietic chimeric mice from an embryonic stem (ES) cell line carrying Ly-1 cDNA under the control of IgH promoter and enhancer. Various amounts of serum IgM (5-86% of total IgM) in chimeric mice were of ES origin and 30-60% of IgM-positive B cells from the chimeric mice analyzed were reconstituted from ES cells. Using these chimeric mice, the expression of the Ly-1 transgene on lymphoid tissues was examined by polymerase chain reaction assay with primers specific for the transgene, and by cell sorter analysis. Transcription of the Ly-1 transgene was detected in spleen cells, thymocytes and lymph node cells; however, the expression of the Ly-1 molecule was observed only on lipopolysaccharide (LPS)-stimulated splenic IgM-positive B cells but not on resting splenic B cells. There was no significantly increased expression of Ly-1 on splenic T cells and thymocytes. Thus, our findings demonstrate that conventional splenic B cells could express the Ly-1 transgene on their surface *in vivo* after LPS stimulation. Also discussed is the ES-derived chimeric hematopoietic system.

Suss-Toby, E., S. Gerecht-Nir, et al. (2004). "Derivation of a diploid human embryonic stem cell line from a mononuclear zygote." *Hum Reprod* **19**(3): 670-5.

BACKGROUND: IVF occasionally produces aneuploid zygotes with one or three pronuclei (PN). Routinely, these zygotes are discarded. The aim of this work was to establish human embryonic stem cell (hESC) lines from blastocysts resulting from abnormal fertilization. **METHODS:** Abnormally fertilized zygotes were cultured to the blastocyst stage and, following zona pellucida digestion, zona-free blastocysts were placed on a mouse feeder layer. Culture of hESCs was carried out as described earlier. **RESULTS:** Six out of the nine developing blastocysts attached to the feeder layer. One hESC line, originating from a mononuclear zygote following ICSI, was successfully derived. This line displayed typical phenotype and embryonic surface markers, and exhibited the potential to develop into all three embryonic germ layers both *in vitro* (by embryoid body formation) and *in vivo* (teratoma generation). Genetic examination revealed normal diploid karyotype and heterozygotic appearance for metachromatic leukodystrophy (MLD). **CONCLUSION:** This method, which requires neither immuno nor mechanical removal of the trophectoderm, may facilitate the derivation of hESC lines in general, and those from abnormal embryos in particular. Furthermore, it is shown that aneuploid zygotes can be used as a source for normal hESC

derivation and hold the potential to generate aneuploid hESC lines for research purposes.

Talbot, N. C. and W. M. Garrett (2001). "Ultrastructure of the embryonic stem cells of the 8-day pig blastocyst before and after in vitro manipulation: development of junctional apparatus and the lethal effects of PBS mediated cell-cell dissociation." *Anat Rec* **264**(1): 101-13.

Ultrastructural examination of 8-day hatched pig blastocysts (large and small), their cultured inner cell mass (ICM), and cultured epiblast tissue (embryonic stem cells) was undertaken to assess the development of epiblast cell junctions and cytoskeletal elements. In small blastocysts, epiblast cells had no desmosomes or tight junction (TJ) connections and few organized microfilament bundles, whereas in large blastocysts the epiblast cells were connected by TJ and desmosomes with associated microfilaments. ICM isolation by immunodissection damaged the endoderm cells beneath the trophoderm cells but did not appear to damage the epiblast cells or their associated endoderm cells. Epiblast cells in cultured ICMs were similar in character to those in the intact large blastocyst except that perinuclear microfilaments were observed. Isolated pig epiblasts, cultured for approximately 36 hr on STO feeder layers, formed a monolayer whose cells were connected by TJ, adherens junctions and desmosomes with prominent microfilament bundles running parallel to the apical cytoplasmic membranes. Perinuclear microfilaments were a consistent feature in the approximately 36 hr cultured epiblast cells. A feature characteristic of differentiation into notochordal cells, i.e., a solitary cilium, was also observed in the cultured epiblast. Exposure of the cultured epiblast cells to Ca(++)-Mg(++)-free phosphate buffered saline (PBS) for 5-10 min resulted in extensive cell blebbing and lysis. The results may indicate that pig epiblast cells could be more easily dissociated from early blastocysts (approximately 400 microm in diameter) if immunodissection damage to the ICM can be avoided. It may be difficult, however, to establish them as embryonic stem cell lines because the cultured pig epiblast cells were easily lysed by standard cell-cell dissociation methods.

Tam, W. L., C. Y. Lim, et al. (2008). "T-cell factor 3 regulates embryonic stem cell pluripotency and self-renewal by the transcriptional control of multiple lineage pathways." *Stem Cells* **26**(8): 2019-31.

The Wnt signaling pathway is necessary both for maintaining undifferentiated stem cells and for directing their differentiation. In mouse embryonic stem cells (ESCs), Wnt signaling preferentially maintains "stemness" under certain permissive

conditions. T-cell factor 3 (Tcf3) is a component of the Wnt signaling and a dominant downstream effector in ESCs. Despite the wealth of knowledge regarding the importance of Wnt signaling underlying stem cells functions, the precise mechanistic explanation by which the effects are mediated is unknown. In this study, we identified new regulatory targets of Tcf3 using a whole-genome approach and found that Tcf3 transcriptionally represses many genes important for maintaining pluripotency and self-renewal, as well as those involved in lineage commitment and stem cell differentiation. This effect is in part mediated by the corepressors transducin-like enhancer of split 2 and C-terminal Binding Protein (CtBP). Notably, Tcf3 binds to and represses the Oct4 promoter, and this repressive effect requires both the Groucho and CtBP interacting domains of Tcf3. Interestingly, we find that in mouse preimplantation development embryos, Tcf3 expression is coregulated with Oct4 and Nanog and becomes localized to the inner cell mass of the blastocyst. These data demonstrate an important role for Tcf3 in modulating the appropriate level of gene transcription in ESCs and during embryonic development. Disclosure of potential conflicts of interest is found at the end of this article.

Tanaka, N., T. Takeuchi, et al. (2006). "Laser-assisted blastocyst dissection and subsequent cultivation of embryonic stem cells in a serum/cell free culture system: applications and preliminary results in a murine model." *J Transl Med* **4**: 20.

BACKGROUND: To evaluate embryonic stem cell (ESC) harvesting methods with an emphasis on derivation of ESC lines without feeder cells or sera. Using a murine model, laser-assisted blastocyst dissection was performed and compared to conventional immunosurgery to assess a novel laser application for inner cell mass (ICM) isolation. **METHODS:** Intact blastocysts or isolated ICMs generated in a standard mouse strain were plated in medium with or without serum to compare ESC harvesting efficiency. ESC derivation was also undertaken in a feeder cell-free culture system. **RESULTS:** Although ICM growth and dissociation was comparable irrespective of the media components, an enhanced ESC harvest was observed in our serum-free medium ($p < 0.01$). ESC harvest rate was not affected by ICM isolation technique but was attenuated in the feeder cell-free group. **CONCLUSION:** Achieving successful techniques for human ESC research is fundamentally dependent on preliminary work using experimental animals. In this study, all experimentally developed ESC lines manifested similar features to ESCs obtained from intact blastocysts in standard culture. Cell/sera free

murine ESC harvest and propagation are feasible procedures for an embryology laboratory and await refinements for translation to human medical research.

Thomson, J. A., J. Itskovitz-Eldor, et al. (1998). "Embryonic stem cell lines derived from human blastocysts." *Science* **282**(5391): 1145-7.

Human blastocyst-derived, pluripotent cell lines are described that have normal karyotypes, express high levels of telomerase activity, and express cell surface markers that characterize primate embryonic stem cells but do not characterize other early lineages. After undifferentiated proliferation in vitro for 4 to 5 months, these cells still maintained the developmental potential to form trophoblast and derivatives of all 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). These cell lines should be useful in human developmental biology, drug discovery, and transplantation medicine.

Thomson, J. A., J. Kalishman, et al. (1995). "Isolation of a primate embryonic stem cell line." *Proc Natl Acad Sci U S A* **92**(17): 7844-8.

Embryonic stem cells have the ability to remain undifferentiated and proliferate indefinitely in vitro while maintaining the potential to differentiate into derivatives of all three embryonic germ layers. Here we report the derivation of a cloned cell line (R278.5) from a rhesus monkey blastocyst that remains undifferentiated in continuous passage for > 1 year, maintains a normal XY karyotype, and expresses the cell surface markers (alkaline phosphatase, stage-specific embryonic antigen 3, stage-specific embryonic antigen 4, TRA-1-60, and TRA-1-81) that are characteristic of human embryonal carcinoma cells. R278.5 cells remain undifferentiated when grown on mouse embryonic fibroblast feeder layers but differentiate or die in the absence of fibroblasts, despite the presence of recombinant human leukemia inhibitory factor. R278.5 cells allowed to differentiate in vitro secrete bioactive chorionic gonadotropin into the medium, express chorionic gonadotropin alpha- and beta-subunit mRNAs, and express alpha-fetoprotein mRNA, indicating trophoblast and endoderm differentiation. When injected into severe combined immunodeficient mice, R278.5 cells consistently differentiate into derivatives of all three embryonic germ layers. These results define R278.5 cells as an embryonic stem cell line, to our knowledge, the first to be derived from any primate species.

Tielens, S., B. Verhasselt, et al. (2006). "Generation of embryonic stem cell lines from mouse blastocysts developed in vivo and in vitro: relation to Oct-4 expression." *Reproduction* **132**(1): 59-66.

Embryonic stem (ES) cells are the source of all embryonic germ layer tissues. Oct-4 is essential for their pluripotency. Since in vitro culture may influence Oct-4 expression, we investigated to what extent blastocysts cultured in vitro from the zygote stage are capable of expressing Oct-4 and generating ES cell lines. We compared in vivo with in vitro derived blastocysts from B6D2 mice with regard to Oct-4 expression in inner cell mass (ICM) outgrowths and blastocysts. ES cells were characterized by immunostaining for alkaline phosphatase (ALP), stage-specific embryonic antigen-1 (SSEA-1) and Oct-4. Embryoid bodies were made to evaluate the ES cells' differentiation potential. ICM outgrowths were immunostained for Oct-4 after 6 days in culture. A quantitative real-time PCR assay was performed on individual blastocysts. Of the in vitro derived blastocysts, 17% gave rise to ES cells vs 38% of the in vivo blastocysts. Six-day old outgrowths from in vivo developed blastocysts expressed Oct-4 in 55% of the cases vs 31% of the in vitro derived blastocysts. The amount of Oct-4 mRNA was significantly higher for freshly collected in vivo blastocysts compared to in vitro cultured blastocysts. In vitro cultured mouse blastocysts retain the capacity to express Oct-4 and to generate ES cells, be it to a lower level than in vivo blastocysts.

Tojo, H., M. Nishida, et al. (1995). "Establishment of a novel embryonic stem cell line by a modified procedure." *Cytotechnology* **19**(2): 161-5.

To generate mutant mice, embryonic stem (ES) cells are used as a vehicle for introducing mutations. The establishment of ES cells is difficult because it requires specific skills and it is time-consuming. We established a novel ES cell line derived from hybrid mice between C57BL/6 and DBA/2 using a modified method. To collect a large number of preimplantational embryos, we collected embryos at the 8-cell stage and cultured them to blastocysts, whereas the usual procedure of preparing the delayed blastocysts demands technical skills. To eliminate unnecessary female cells at an initial stage of inner cell mass culture, male clones were selected by polymerase chain reaction to detect the mouse Sry gene. The established ES cell line efficiently contributed to the germ-line when injected into 8-cell embryos of ICR mice. This potency was maintained after manipulation throughout gene targeting.

Tokuzawa, Y., E. Kaiho, et al. (2003). "Fbx15 is a novel target of Oct3/4 but is dispensable for

embryonic stem cell self-renewal and mouse development." *Mol Cell Biol* **23**(8): 2699-708.

Embryonic stem (ES) cells are immortal and pluripotent cells derived from early mammalian embryos. Transcription factor Oct3/4 is essential for self-renewal of ES cells and early mouse development. However, only a few Oct3/4 target genes have been identified. In this study, we found that F-box-containing protein Fbx15 was expressed predominantly in mouse undifferentiated ES cells. Inactivation of Oct3/4 in ES cells led to rapid extinction of Fbx15 expression. Reporter gene analyses demonstrated that this ES cell-specific expression required an 18-bp enhancer element located approximately 500 nucleotides upstream from the transcription initiation site. The enhancer contained an octamer-like motif and an adjacent Sox-binding motif. Deletion or point mutation of either motif abolished the enhancer activity. The 18-bp fragment became active in NIH 3T3 cells when Oct3/4 and Sox2 were coexpressed. A gel mobility shift assay demonstrated cooperative binding of Oct3/4 and Sox2 to the enhancer sequence. In mice having a beta-galactosidase gene knocked into the Fbx15 locus, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside staining was detected in ES cells, early embryos (two-cell to blastocyst stages), and testis tissue. Despite such specific expression of Fbx15, homozygous mutant mice showed no gross developmental defects and were fertile. Fbx15-null ES cells were normal in morphology, proliferation, and differentiation. These data demonstrate that Fbx15 is a novel target of Oct3/4 but is dispensable for ES cell self-renewal, development, and fertility.

Trivedi, H. L., V. V. Mishra, et al. (2006). "Embryonic stem cell derived and adult hematopoietic stem cell transplantation for tolerance induction in a renal allograft recipient:--a case report." *Transplant Proc* **38**(9): 3103-8.

We generated an human embryonic stem cell (hESC) line to augment chimerism-associated tolerance. A 40-year-old African with chronic glomerulonephritis-chronic renal failure with 100% G6PD enzyme deficiency presented for renal transplantation with a 27-year-old, 6/6 HLA-matched sister as a willing donor. METHOD: We generated an hESC line from the donor's oocytes using long ovarian stimulation protocol simultaneously with tolerance induction protocol. A nuclear transfer (NT)-hESC line was derived by transferring a donor cumulus cell into an enucleated oocyte, subjected to electrical fusion, and cultured for 5 days. ESCs hatched from the blastocyst on day 6 were cocultured with her unmodified bone marrow for 2 days and suspended in Ringer's lactate. Five milliliters of suspension were

collected for cell counting, viability, pluripotency, flow cytometry, and karyotyping. The remaining suspension was infused into the periphery of the recipient. Transplantation was performed 1 week later following a negative lymphocytotoxicity cross-match test using no immunosuppression. Peripheral blood chimerism (PBC) was studied using fluorescent in situ hybridization technique. Allograft biopsy was performed on day 7. RESULTS: NT-hESC CD34+ count was 7.6%, viability 100%, karyotyping normal, pluripotency markers: SSEA-1, SSEA-4, OCT-3/4, TRA-1/60:positive; 12% PBC was noted at 1 week after transplantation. Serum creatinine was 1.2 mg%, graft biopsy was unremarkable, and G6PD enzyme deficiency was corrected to 0% at 100 days posttransplant. Liver function tests and hematology profile were unremarkable for graft-versus-host disease. CONCLUSION: This is the first report of tolerance induction using NT-hESC-induced hematopoietic chimerism with synergistic use of adult bone marrow. It was safe and effective.

Trounson, A. (2002). "Human embryonic stem cells: mother of all cell and tissue types." *Reprod Biomed Online* **4 Suppl 1**: 58-63.

Pluripotential embryonic stem (ES) cells have been derived very efficiently from spare human embryos produced by IVF and grown in culture to the nascent blastocyst stage. The inner cell mass (ICM) is isolated by immunosurgery and grown on selected embryonic fibroblast monolayer cultures. ICM cells lose their memory for axis during formation of ES cell colonies and are then unable to integrate tissue formation with a body plan. ES cells form teratomas in vivo with cells and tissues representative of the three major embryonic lineages (ectoderm, mesoderm, endoderm). The ES cells are continuously renewable and can be directed to differentiate into early progenitors of neural stem cells (Noggin cells) and from there into mature neurons and glia (astrocytes and oligodendrocytes). The neural stem cells formed from human ES cells repopulate the brains of newborn mice when injected into the lateral cerebral ventricles, forming astrocytes dominantly in the parenchyma. The human neural cells can be observed migrating from the subventricular areas along the rostral migratory stream. Human neurons can be found in the olfactory bulb. Human ES cells can also be directed into cardiomyocytes when co-cultured with visceral endoderm-like cells (END-2). These observations provide further scope to explore stem cell therapies, gene therapies and drug discovery. For compatible transplantation, ES may need to be derived with a range of HLA types or by nuclear transplantation or stem cell fusion.

Trounson, A. (2005). "Human embryonic stem cell derivation and directed differentiation." Ernst Schering Res Found Workshop(54): 27-44.

Human embryonic stem cells (hESCs) are produced from normal, chromosomally aneuploid and mutant human embryos, which are available from in vitro fertilisation (IVF) for infertility or preimplantation diagnosis. These hESC lines are an important resource for functional genomics, drug screening and eventually cell and gene therapy. The methods for deriving hESCs are well established and repeatable, and are relatively successful, with a ratio of 1:10 to 1:2 hESC lines established to embryos used. hESCs can be formed from morula and blastocyst-stage embryos and from isolated inner cell mass cell (ICM) clusters. The hESCs can be formed and maintained on mouse or human somatic cells in serum-free conditions, and for several passages in cell-free cultures. The hESCs can be transfected with DNA constructs. Their gene expression profiles are being described and immunological characteristics determined. They may be grown indefinitely in culture while maintaining their original karyotype but this must be confirmed from time to time. hESCs spontaneously differentiate in the absence of the appropriate cell feeder layer, when overgrown in culture and when isolated from the ESC colony. All three major embryonic lineages are produced in differentiating attachment cultures and in unattached embryoid bodies. Cell progenitors of interest can be identified by markers, expression of reporter genes and characteristic morphology, and the culture thereafter enriched for further culture to more mature cell types. The most advanced directed differentiation pathways have been developed for neural cells and cardiac muscle cells, but many other cell types including haematopoietic progenitors, endothelial cells, lung alveoli, keratinocytes, pigmented retinal epithelium, neural crest cells and motor neurones, hepatic progenitors and cells that have some markers of gut tissue and pancreatic cells have been produced. The prospects for regenerative medicine are significant and there is much optimism for their contribution to human medicine.

Turetsky, T., E. Aizenman, et al. (2008). "Laser-assisted derivation of human embryonic stem cell lines from IVF embryos after preimplantation genetic diagnosis." Hum Reprod **23**(1): 46-53.

BACKGROUND: Human embryonic stem cells (hESCs) suitable for future transplantation therapy should preferably be developed in an animal-free system. Our objective was to develop a laser-based system for the isolation of the inner cell mass (ICM) that can develop into hESC lines, thereby circumventing immunosurgery that utilizes animal

products. **METHODS:** Hatching was assisted by micromanipulation techniques through a laser-drilled orifice in the zona pellucida of 13 abnormal preimplantation genetic diagnosed blastocysts. ICMs were dissected from the trophectoderm by a laser beam and plated on feeders to derive hESC lines. **RESULTS:** eight ICMs were isolated from nine hatched blastocysts and gave rise to three hESC lines affected by myotonic dystrophy type 1, hemophilia A and a carrier of cystic fibrosis 405 + 1G > A mutation. Five blastocysts that collapsed during assisted hatching or ICM dissection were plated whole, giving rise to an additional line affected by fragile X. All cell lines expressed markers of pluripotent stem cells and differentiated in vitro and in vivo into the three germ layers. **CONCLUSIONS:** These hESC lines can serve as an important model of the genetic disorders that they carry. Laser-assisted isolation of the ICMs may be applied for the derivation of new hESC lines in a xeno-free system for future clinical applications.

Uy, G. D., K. M. Downs, et al. (2002). "Inhibition of trophoblast stem cell potential in chorionic ectoderm coincides with occlusion of the ectoplacental cavity in the mouse." Development **129**(16): 3913-24.

At the blastocyst stage of pre-implantation mouse development, close contact of polar trophectoderm with the inner cell mass (ICM) promotes proliferation of undifferentiated diploid trophoblast. However, ICM/polar trophectoderm intimacy is not maintained during post-implantation development, raising the question of how growth of undifferentiated trophoblast is controlled during this time. The search for the cellular basis of trophoblast proliferation in post-implantation development was addressed with an in vitro spatial and temporal analysis of fibroblast growth factor 4-dependent trophoblast stem cell potential. Two post-implantation derivatives of the polar trophectoderm - early-streak extra-embryonic ectoderm and late-streak chorionic ectoderm - were microdissected into fractions along their proximodistal axis and thoroughly dissociated for trophoblast stem cell culture. Results indicated that cells with trophoblast stem cell potential were distributed throughout the extra-embryonic/chorionic ectoderm, an observation that is probably attributable to non-coherent growth patterns exhibited by single extra-embryonic ectoderm cells at the onset of gastrulation. Furthermore, the frequency of cells with trophoblast stem cell potential increased steadily in extra-embryonic/chorionic ectoderm until the first somite pairs formed, decreasing thereafter in a manner independent of proximity to the allantois. Coincident with occlusion of the ectoplacental cavity via union between chorionic ectoderm and the ectoplacental cone, a decline in the frequency of mitotic chorionic

ectoderm cells in vivo, and of trophoblast stem cell potential in vitro, was observed. These findings suggest that the ectoplacental cavity may participate in maintaining proliferation throughout the developing chorionic ectoderm and, thus, in supporting its stem cell potential. Together with previous observations, we discuss the possibility that fluid-filled cavities may play a general role in the development of tissues that border them.

Vanikar, A. V., V. V. Mishra, et al. (2007). "Successful generation of donor specific hematopoietic stem cell lines from co-cultured bone marrow with human embryonic stem cell line: a new methodology." Transplant Proc **39**(3): 658-61.

We report the generation of 30 healthy human embryonic stem cell (h-ESC) lines from 33 voluntary oocyte donors using a donor somatic cell nuclear transfer (SCNT) technique on 190 oocytes. Our aim was to coculture them with their own bone marrow (BM) to generate hematopoietic progenitor cells for therapeutic purposes. Pluripotency and undifferentiated stage were confirmed using molecular cell surface markers. Normal karyotype of these cell lines was confirmed. Here we demonstrate that SCNT-h-ESCs differentiate to hematopoietic precursors when cocultured with unmodified, nonirradiated donor BM. We did not use any xenogeneic material for this hematopoietic differentiation. Hematopoietic precursors derived from them expressed cell surface antigens CD45/34. When further cultured with hematopoietic growth factors these hematopoietic precursors formed characteristic myeloid, erythroid, and megakaryocyte lineages. Phenotypic CD34⁺ cells derived from NT-h-ESCs were functionally similar to their counterparts in primary hematopoietic tissues like BM, umbilical cord, and blood. More terminally differentiated hematopoietic cells derived from h-ESCs under these culture conditions also expressed normal surface antigens like glycophorin A on erythroid cells, CD15 on myeloid cells, and CD41 on megakaryocytes. We report generation of hematopoietic progenitor cells from h-ESC lines by a SCNT technique, with differentiation into further lineages with structural and functional similarities to their adult counterparts in vivo. This novel alternative source of CD34⁺ stem cells from h-ESC lines generated without any xenogeneic material might be used to create transplantation tolerance, to implement regenerative medicine, and to treat autoimmune disorders.

Verlinsky, Y., N. Strelchenko, et al. (2005). "Human embryonic stem cell lines with genetic disorders." Reprod Biomed Online **10**(1): 105-10.

A previous study described the establishment of human embryonic stem cell (ESC) lines from different sources of embryonic material, including morula, whole blastocyst and isolated inner cell mass. Using these methods, a repository of ESC lines has been established with different genetic abnormalities, which provides an unlimited source of disease cells in culture for undertaking research on the primary disturbances of the cellular processes in the genetically abnormal cells. ESC lines with genetic disorders were derived from the mutant embryos detected and avoided from transfer in the ongoing practice of preimplantation genetic diagnosis (PGD). The current repository contains 18 ESC lines with genetic disorders, including adrenoleukodystrophy, Duchenne and Becker muscular dystrophy, Fanconi anaemia, complementation group A, fragile-X syndrome, Huntington disease (three lines), Marfan syndrome, myotonic dystrophy (two lines), neurofibromatosis type I (five lines) and thalassaemia (two lines). These ESC lines are presently used for research purposes and may be available on request.

Verlinsky, Y., N. Strelchenko, et al. (2009). "Impact of meiotic and mitotic non-disjunction on generation of human embryonic stem cell lines." Reprod Biomed Online **18**(1): 120-6.

At least 50-60% of oocytes derived from IVF procedures are chromosomally abnormal due to meiotic I or II errors. Through the use of polar body and blastomere diagnosis, euploid embryos suitable for transfer can be identified. Those embryos that are aneuploid are usually discarded, or otherwise can be used to generate chromosomally abnormal human embryonic stem cell (hESC) lines. The authors' centre has one of the largest repositories of hESC lines with genetic and chromosomal disorders generated from preimplantation genetic diagnosis (PGD) abnormal embryos. The results, studying hESC lines derived from PGD abnormal zygotes, imply that aneuploidies resulting from meiotic non-disjunction have a greater impact on viability of cells of the human embryos than those originating from post-zygotic mitotic non-disjunction.

Verma, V., S. K. Gautam, et al. (2007). "Isolation and characterization of embryonic stem cell-like cells from in vitro-produced buffalo (*Bubalus bubalis*) embryos." Mol Reprod Dev **74**(4): 520-9.

This study was carried out to isolate and characterize buffalo embryonic stem (ES) cell-like cells from in vitro-produced embryos. Inner cell mass (ICM) cells were isolated either mechanically or by enzymatic digestion from 120 blastocysts whereas 28 morulae were used for the isolation of blastomeres mechanically. The ICM cells/ blastomeres were

cultured on mitomycin-C-treated feeder layer. Primary cell colony formation was higher ($P < 0.05$) for hatched blastocysts (73.1%, 30/41) than that for early/expanded blastocysts (25.3%, 20/79). However, no primary cell colonies were formed when blastomeres obtained from morulae were cultured. Primary colonies were formed in 14.1% (12/85) of intact blastocyst culture, which was significantly lower ($P < 0.05$) than that of 41.6% for ICM culture. These colonies were separated by enzymatic or mechanical disaggregation. Using mechanical disaggregation method, the cells remained undifferentiated and two buffalo ES cell-like cell lines (bES1, bES2) continued to grow in culture up to eight passages. However, disassociation through enzymatic method resulted in differentiation. Undifferentiated cells exhibited stem cell morphological features, normal chromosomal morphology, and expressed specific markers such as alkaline phosphatase (AP) and Oct-4. Cells formed embryoid bodies (EBs) in suspension culture; extended culture of EBs resulted in formation of cystic EBs. Following prolonged in vitro culture, these cells differentiated into several types of cells including neuron-like and epithelium-like cells. Furthermore, the vitrified-thawed ES cell-like cells also exhibited typical stem cell characteristics. In conclusion, buffalo ES cell-like cells could be isolated from in vitro-produced blastocysts and maintained in vitro for prolonged periods of time.

Wakayama, S., H. Ohta, et al. (2005). "Establishment of male and female nuclear transfer embryonic stem cell lines from different mouse strains and tissues." *Biol Reprod* **72**(4): 932-6.

Nuclear transfer can be used to generate embryonic stem cell lines from somatic cells, and these have great potential in regenerative medicine. However, it is still unclear whether any individual or cell type can be used to generate such lines. Here, we tested seven different male and female mouse genotypes and three cell types as sources of nuclei to determine the efficiency of establishing nuclear transfer embryonic stem cell lines. Lines were successfully established from all sources. Cumulus cell nuclei from F(1) mouse genotypes showed a significantly higher cumulative establishment rate from reconstructed oocytes than from other cells; however, there were no genotype differences in success rates from cloned blastocysts. Thus, the overall success depends on preimplantation development, and, once embryos have reached the blastocyst stage, the genotype differences disappear. All mouse genotypes that were tested demonstrated at least one cell line that subsequently contributed to germline transmission in chimeric mice, so these cell

lines clearly possess the same potential as embryonic stem cells derived from fertilized embryos. Thus, nuclear transfer embryonic stem cells can be generated relatively easily from a variety of inbred mouse genotypes and cell types of both sexes, even though it may be more difficult to generate clones directly.

Wakayama, T., V. Tabar, et al. (2001). "Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer." *Science* **292**(5517): 740-3.

Embryonic stem (ES) cells are fully pluripotent in that they can differentiate into all cell types, including gametes. We have derived 35 ES cell lines via nuclear transfer (ntES cell lines) from adult mouse somatic cells of inbred, hybrid, and mutant strains. ntES cells contributed to an extensive variety of cell types, including dopaminergic and serotonergic neurons in vitro and germ cells in vivo. Cloning by transfer of ntES cell nuclei could result in normal development of fertile adults. These studies demonstrate the full pluripotency of ntES cells.

Wang, F., S. Thirumangalathu, et al. (2006). "Establishment of new mouse embryonic stem cell lines is improved by physiological glucose and oxygen." *Cloning Stem Cells* **8**(2): 108-16.

Embryonic stem cell lines are routinely selected and cultured in glucose and oxygen concentrations that are well above those of the intrauterine environment. Supraphysiological glucose and hyperoxia each increase oxidative stress, which could be detrimental to survival in vitro by inhibiting proliferation and/or inducing cell death. The aim of this study was to test whether isolation of new embryonic stem cell lines from murine blastocysts is improved by culture in physiological (5%) oxygen instead of approximately 20%, the concentration of oxygen in room air, or in media containing physiological (100 mg/dL) instead of 450 mg/dL glucose. We found that culturing in either physiological oxygen or physiological glucose improved the success of establishing new murine embryonic stem cell lines, and that culture when concentrations of both oxygen and glucose were physiological improved the success of establishing new lines more than culture in either alone. Physiological oxygen and glucose reduce oxidative stress, as determined by 2',7'-dichlorodihydrofluorescein fluorescence. BrdU incorporation suggests that physiological oxygen and glucose increase the pool of proliferating cells. Cells isolated in physiological oxygen and glucose are capable of self-renewal and differentiation into all three germ layers in vitro. However, none of the culture

conditions prevents cytogenetic instability with prolonged passage. These results suggest that culture of cells derived from murine blastocysts in physiological oxygen and glucose reduces oxidant stress, which increases the success of establishing new embryonic stem cell lines.

Wang, Z. Q., F. Kiefer, et al. (1997). "Generation of completely embryonic stem cell-derived mutant mice using tetraploid blastocyst injection." *Mech Dev* **62**(2): 137-45.

Embryonic stem (ES) cells provide a unique tool for producing specifically designed mutations in mice. Here, we describe an alternative approach toward the generation of mice which are derived completely from ES cells (ES mice), as judged by glucose phosphate isomerase (GPI) analysis, without prior passage through the germline. By injecting wild-type and mutant ES cells into tetraploid blastocysts, viable and fertile ES mice were generated, suggesting that totipotency of ES cells was not affected by long-term culture and experimental manipulation in vitro. When ES cell clones harboring a lacZ reporter gene introduced by either targeted insertion or a gene-trap approach were used, the expression pattern of the lacZ gene in ES fetuses was identical to that of fetuses that were derived from breeding of chimeric mice. Thus, this technique can be considered as a useful and rapid approach to produce fetuses and mice directly from ES cells carrying predetermined genetic changes and offers many applications for studies in molecular genetics and developmental biology.

Watt, S. M. and M. Contreras (2005). "Stem cell medicine: umbilical cord blood and its stem cell potential." *Semin Fetal Neonatal Med* **10**(3): 209-20.

The ultimate aim of stem cell research is to improve patient outcomes and quality of life, and/or to effect a cure for a variety of inherited or acquired diseases. Improved treatments rely on developments in stem cell therapies and the discovery of new therapeutic drugs that regulate stem cell functions. These complement each other for the repair, regeneration and replacement of damaged or defective tissues. Stem cells may be sourced or derived from blood and tissues postnatally ('adult' stem cells), from the fetus (fetal stem cells) or from the blastocyst in the developing embryo prior to implantation (embryonic stem cells), each forming a unique component of the revolution in stem cell research and therapies. This review will concentrate on recent developments in the use of haemopoietic stem cells from umbilical cord blood for the transplantation of patients with haematological disorders. It will conclude with a summary of the potential of other umbilical cord

blood-derived stem cells for tissue repair or regeneration.

Wernig, M. and O. Brustle (2002). "Fifty ways to make a neuron: shifts in stem cell hierarchy and their implications for neuropathology and CNS repair." *J Neuropathol Exp Neurol* **61**(2): 101-10.

During embryogenesis, the developmental potential of individual cells is continuously restricted. While embryonic stem (ES) cells derived from the inner cell mass of the blastocyst can give rise to all tissues and cell types, their progeny segregates into a multitude of tissue-specific stem and progenitor cells. Following organogenesis, a pool of resident "adult" stem cells is maintained in many tissues. In this hierarchical concept, transition through defined intermediate stages of decreasing potentiality is regarded as prerequisite for the generation of a somatic cell type. Several recent findings have challenged this view. First, adult stem cells have been shown to adopt properties of pluripotent cells and contribute cells to a variety of tissues. Second, a direct transition from a pluripotent ES cell to a defined somatic phenotype has been postulated for the neural lineage. Finally, nuclear transplantation has revealed that the transcriptional machinery associated with a distinct somatic cell fate can be reprogrammed to totipotency. The possibility to bypass developmental hierarchies in stem cell differentiation opens new avenues for the study of nervous system development, disease, and repair.

Wianny, F., A. Bernat, et al. (2008). "Derivation and cloning of a novel rhesus embryonic stem cell line stably expressing tau-green fluorescent protein." *Stem Cells* **26**(6): 1444-53.

Embryonic stem cells (ESC) have the ability of indefinite self-renewal and multilineage differentiation, and they carry great potential in cell-based therapies. The rhesus macaque is the most relevant preclinical model for assessing the benefit, safety, and efficacy of ESC-based transplantations in the treatment of neurodegenerative diseases. In the case of neural cell grafting, tracing both the neurons and their axonal projections in vivo is essential for studying the integration of the grafted cells in the host brain. Tau-Green fluorescent protein (tau-GFP) is a powerful viable lineage tracer, allowing visualization of cell bodies, dendrites, and axons in exquisite detail. Here, we report the first rhesus monkey ESC line that ubiquitously and stably expresses tau-GFP. First, we derived a new line of rhesus monkey ESC (LYON-ES1) that show marker expression and cell cycle characteristics typical of primate ESCs. LYON-ES1 cells are pluripotent, giving rise to derivatives of the three germ layers in vitro and in vivo through

teratoma formation. They retain all their undifferentiated characteristics and a normal karyotype after prolonged culture. Using lentiviral infection, we then generated a monkey ESC line stably expressing tau-GFP that retains all the characteristics of the parental wild-type line and is clonogenic. We show that neural precursors derived from the tau-GFP ESC line are multipotent and that their fate can be precisely mapped in vivo after grafting in the adult rat brain. Disclosure of potential conflicts of interest is found at the end of this article.

Wilcox, J. T., E. Semple, et al. (2009). "Characterization of canine embryonic stem cell lines derived from different niche microenvironments." *Stem Cells Dev* **18**(8): 1167-78.

Embryo-derived stem cells hold enormous potential for producing cell-based transplantation therapies, allowing high-throughput drug screening and delineating early embryonic development. However, potential clinical applications must first be tested for safety and efficacy in preclinical animal models. Due to physiological and genetic parity to humans, the domestic dog is widely used as a clinically relevant animal model for cardiovascular, neurodegenerative, orthopedic, and oncologic diseases. Therefore, we established numerous putative canine embryonic stem cell (cESC) lines by immunodissection of the inner cell mass (ICM), which we termed OVC.ID.1-23, and by explant outgrowths from whole canine blastocysts, named OVC.EX.1-16. All characterized lines were immunopositive for OCT4, SOX2, NANOG, SSEA-3, and SSEA-4; displayed high telomerase and alkaline phosphatase (ALP) activities; and were maintained in this state up to 37 passages (approximately 160 days). Colonies from OVC.EX lines showed classic domed hESC-like morphology surrounded by a ring of fibroblast-like cells, whereas all OVC.ID lines exhibited a mixed cell colony of tightly packed cESCs surrounded by a GATA6+/CDX2- hypoblast-derived support layer. Spontaneous serum-only differentiation without feeder layers demonstrated a strong lineage selection associated with the colony niche type, and not the isolation method. Upon differentiation, cESC lines formed embryoid bodies (EB) comprised of cells representative of all germinal layers, and differentiated into cell types of each layer. Canine ESC lines such as these have the potential to identify differences between embryonic stem cell line derivations, and to develop or to test cell-based transplantation therapies in the dog before attempting human clinical trials.

Xiao, Y., Y. Ye, et al. (2008). "The lymphovascular embolus of inflammatory breast cancer expresses a

stem cell-like phenotype." *Am J Pathol* **173**(2): 561-74.

Inflammatory breast carcinoma (IBC) is a particularly lethal form of breast cancer characterized by exaggerated lymphovascular invasion, which is a phenotype recapitulated in our human xenograft MARY-X. MARY-X generated spheroids in vitro that resemble the embryonal blastocyst. Because of the resemblance of the spheroids to the embryonal blastocyst and their resistance to traditional chemotherapy/radiotherapy, we hypothesized that the spheroids expressed a stem cell-like phenotype. MARY-X spheroids expressed embryonal stem cell markers including *stella*, *rex-1*, *nestin*, *H19*, and potent transcriptional factors, *oct-4*, *nanog*, and *sox-2*, which are associated with stem cell self-renewal and developmental potential. Most importantly, MARY-X spheroids expressed a cancer stem cell profile characterized by CD44(+)/CD24(-/low), ALDH1, and most uniquely, CD133. A significant percentage of single cells of MARY-X exhibited distinct proliferative and morphogenic potencies in vitro. As few as 100 cells derived from single-cell clonogenic expansion were tumorigenic with recapitulation of the IBC phenotype. Prototype stem cell signaling pathways such as *notch3* were active in MARY-X. The stem cell phenotype exhibited by MARY-X also was exhibited by the lymphovascular emboli of human IBC cases independent of their molecular subtype. This stem cell-like phenotype may contribute to the aggressive nature of IBC but also may lend itself to selective targeting.

Yamada, S., T. J. Nelson, et al. (2009). "Stem cell transplant into preimplantation embryo yields myocardial infarction-resistant adult phenotype." *Stem Cells* **27**(7): 1697-705.

Stem cells are an emerging strategy for treatment of myocardial infarction, limited however to postinjury intervention. Preventive stem cell-based therapy to augment stress tolerance has yet to be considered for lifelong protection. Here, pluripotent stem cells were microsurgically introduced at the blastocyst stage of murine embryo development to ensure stochastic integration and sustained organ contribution. Engineered chimera displayed excess in body weight due to increased fat deposits, but were otherwise devoid of obesity-related morbidity. Remarkably, and in sharp contrast to susceptible nonchimeric offspring, chimera was resistant to myocardial infarction induced by permanent coronary occlusion. Infarcted nonchimeric adult hearts demonstrated progressive deterioration in ejection fraction, while age-matched 12-14-months-old chimera recovered from equivalent ischemic insult to regain within one-month preocclusion contractile

performance. Electrical remodeling and ventricular enlargement with fibrosis, prominent in failing nonchimera, were averted in the chimeric cohort characterized by an increased stem cell load in adipose tissue and upregulated markers of biogenesis Ki67, c-Kit, and stem cell antigen-1 in the myocardium. Favorable outcome in infarcted chimera translated into an overall benefit in workload capacity and survival. Thus, prenatal stem cell transplant yields a cardioprotective phenotype in adulthood, expanding cell-based indications beyond traditional postinjury applications to include pre-emptive therapy.

Yang, Z. and J. N. Petite (1994). "Use of avian cytokines in mammalian embryonic stem cell culture." *Poult Sci* **73**(7): 965-74.

Mouse blastocyst-derived embryonic stem (ES) cells are multipotent cells that can be used in vitro as models of differentiation and in vivo can contribute to all embryonic tissues including the germ line. The culture of ES cells requires a source of leukemia inhibitory factor (LIF), often provided by culture with a mouse fibroblast (STO) feeder layer, buffalo rat liver cell-conditioned media (BRL-CM), or the addition of recombinant LIF. To date, all of the ES cell culture systems use mammalian sources of LIF. We found that mouse ES cells can be maintained for over 10 passages in an undifferentiated state with media conditioned by a chicken liver cell line (LMH-CM) or on a feeder layer made with primary chicken embryonic fibroblasts (CEF). These ES cells can undergo both spontaneous and induced differentiation, which is associated with the disappearance or reduction of the expression of alkaline phosphatase and SSEA-1, similar to that observed for ES cells cultured with BRL-CM or STO feeder layers. The ES cells cultured in LMH-CM did not express cytokeratin Endo-A antigen recognized by TROMA-1, but their differentiated progeny did express this antigen. In contrast to LMH-CM, Endo-A was expressed in ES cells cultured on CEF feeder layers and in differentiated progeny. These results indicate that avian cells can produce a LIF-like cytokine that is active in inhibiting the differentiation of mouse ES cells. This could provide a biological end point for the isolation and characterization of avian LIF.

Zech, N. H., S. Koestenbauer, et al. (2005). "Paraffin-embedded manipulated blastocysts: a tool to demonstrate stem cell plasticity?" *Reprod Biomed Online* **10**(3): 406-14.

One of the big question marks in current stem cell research is whether there is true plasticity of adult progenitor cells (APC) or if cell fusion is the principle source of the supposed plasticity. The generation of chimeras by injecting adult progenitor

cells into blastocysts is not new. This paper describes an efficient embedding technique for murine blastocysts injected with human APC. This method could help in establishing a novel tool to analyse the process of plasticity, if it truly exists. If this is the case, this technology could be of great help to characterize surface markers of stem cells in great detail. On the other hand, fusion of cells could also be investigated. A system of embedding blastocysts was set up using paraffin for further analysis by means of light microscopy and immunohistochemistry. The embedding of the chimeras consists of fixing them first with paraformaldehyde in phosphate-buffered saline (PFA/PBS), embedding them in gelatine, fixing the gelatine block with PFA/PBS and finally fixing the gelatine block in a Petri dish by embedding it in paraffin. Using this protocol, the morphology of the blastocysts is well preserved.

Zhao, C., R. Yao, et al. (2007). "Establishment of customized mouse stem cell lines by sequential nuclear transfer." *Cell Res* **17**(1): 80-7.

Therapeutic cloning, whereby embryonic stem cells (ESCs) are derived from nuclear transfer (NT) embryos, may play a major role in the new era of regenerative medicine. In this study we established forty nuclear transfer-ESC (NT-ESC) lines that were derived from NT embryos of different donor cell types or passages. We found that NT-ESCs were capable of forming embryoid bodies. In addition, NT-ESCs expressed pluripotency stem cell markers in vitro and could differentiate into embryonic tissues in vivo. NT embryos from early passage R1 donor cells were able to form full term developed pups, whereas those from late passage R1 ES donor cells lost the potential for reprogramming that is essential for live birth. We subsequently established sequential NT-R1-ESC lines that were developed from NT blastocyst of late passage R1 ESC donors. However, these NT-R1-ESC lines, when used as nuclear transfer donors at their early passages, failed to result in live pups. This indicates that the therapeutic cloning process using sequential NT-ESCs may not rescue the developmental deficiencies that resided in previous donor generations.

Zhou, Q., A. Jouneau, et al. (2001). "Developmental potential of mouse embryos reconstructed from metaphase embryonic stem cell nuclei." *Biol Reprod* **65**(2): 412-9.

Mice have recently been successfully cloned from embryonic stem (ES) cells. However, these fast dividing cells provide a heterogeneous population of donor nuclei, in terms of cell cycle stage. Here we used metaphases as a source of donor nuclei because they offer the advantage of being both unambiguously

recognizable and synchronous with the recipient metaphase II oocyte. We showed that metaphases from ES cells can provide a significantly higher development rate to the morula or blastocyst stage (56--70%) than interphasic nuclei (up to 28%) following injection into a recipient oocyte. Selective detachment of mitotic cells after a demecolcin treatment greatly facilitates and accelerates the reconstruction of embryos by providing a nearly pure population of cells in metaphase and did not markedly affect the developmental rate. Most of the blastocysts obtained by this procedure were normal in terms of both morphology and ratio of inner cell mass and total cell number. After transfer into pseudopregnant recipients at the one- or two-cell stage, the ability of metaphase to be fully reprogrammed was demonstrated by the birth of two pups (1.5% of activated oocytes). Although the implantation rate was quite high (up to 32.9% of activated oocytes), the postimplantation development was characterized by a high and rapid mortality. Our data provide a clear situation to explore the long-lasting effects that can be induced by early reprogramming events.

References

- Abdelalim, E. M. and I. Tooyama (2009). "BNP signaling is crucial for embryonic stem cell proliferation." *PLoS One* **4**(4): e5341.
- Adamson, E. D., S. Strickland, et al. (1985). "A teratocarcinoma-derived endoderm stem cell line (1H5) that can differentiate into extra-embryonic endoderm cell types." *Differentiation* **29**(1): 68-76.
- Aejaz, H. M., A. K. Aleem, et al. (2007). "Stem cell therapy-present status." *Transplant Proc* **39**(3): 694-9.
- Ahuja, Y. R., V. Vijayalakshmi, et al. (2007). "Stem cell test: a practical tool in toxicogenomics." *Toxicology* **231**(1): 1-10.
- Aiba, K., M. G. Carter, et al. (2006). "Genomic approaches to early embryogenesis and stem cell biology." *Semin Reprod Med* **24**(5): 330-9.
- Amano, T., Y. Kato, et al. (2001). "Full-term development of enucleated mouse oocytes fused with embryonic stem cells from different cell lines." *Reproduction* **121**(5): 729-33.
- Amit, M., M. K. Carpenter, et al. (2000). "Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture." *Dev Biol* **227**(2): 271-8.
- Andang, M., J. Hjerling-Leffler, et al. (2008). "Histone H2AX-dependent GABA(A) receptor regulation of stem cell proliferation." *Nature* **451**(7177): 460-4.
- Araki, K., N. Takeda, et al. (2009). "Establishment of germline-competent embryonic stem cell lines from the MSM/Ms strain." *Mamm Genome* **20**(1): 14-20.
- Auerbach, W., J. H. Dunmore, et al. (2000). "Establishment and chimera analysis of 129/SvEv- and C57BL/6-derived mouse embryonic stem cell lines." *Biotechniques* **29**(5): 1024-8, 1030, 1032.
- Baharvand, H. and K. I. Matthaei (2004). "Culture condition difference for establishment of new embryonic stem cell lines from the C57BL/6 and BALB/c mouse strains." *In Vitro Cell Dev Biol Anim* **40**(3-4): 76-81.
- Baharvand, H., S. K. Ashtiani, et al. (2004). "Establishment and in vitro differentiation of a new embryonic stem cell line from human blastocyst." *Differentiation* **72**(5): 224-9.
- Bendall, S. C., M. H. Stewart, et al. (2008). "Human embryonic stem cells: lessons from stem cell niches in vivo." *Regen Med* **3**(3): 365-76.
- Ben-Yosef, D., M. Malcov, et al. (2008). "PGD-derived human embryonic stem cell lines as a powerful tool for the study of human genetic disorders." *Mol Cell Endocrinol* **282**(1-2): 153-8.
- Berthier, R., M. H. Prandini, et al. (1997). "The MS-5 murine stromal cell line and hematopoietic growth factors synergize to support the megakaryocytic differentiation of embryonic stem cells." *Exp Hematol* **25**(6): 481-90.
- Beyhan, Z., A. E. Iager, et al. (2007). "Interspecies nuclear transfer: implications for embryonic stem cell biology." *Cell Stem Cell* **1**(5): 502-12.
- Boiani, M., L. Gentile, et al. (2005). "Variable reprogramming of the pluripotent stem cell marker Oct4 in mouse clones: distinct developmental potentials in different culture environments." *Stem Cells* **23**(8): 1089-104.
- Bongso, A. and M. Richards (2004). "History and perspective of stem cell research." *Best Pract Res Clin Obstet Gynaecol* **18**(6): 827-42.
- Bongso, A. and S. Tan (2005). "Human blastocyst culture and derivation of embryonic stem cell lines." *Stem Cell Rev* **1**(2): 87-98.
- Boyd, A. S., Y. Higashi, et al. (2005). "Transplanting stem cells: potential targets for immune attack. Modulating the immune response against embryonic stem cell transplantation." *Adv Drug Deliv Rev* **57**(13): 1944-69.
- Brachvogel, B., H. Moch, et al. (2005). "Perivascular cells expressing annexin A5 define a novel mesenchymal stem cell-like population with the capacity to differentiate into multiple mesenchymal lineages." *Development* **132**(11): 2657-68.
- Brehany, J. (2005). "Nontraditional sources of pluripotent stem cells: a new chapter in the debate about embryonic stem cell research." *Health Care Ethics USA* **13**(2): E3.
- Chang, J. C., L. Ye, et al. (2006). "Correction of the sickle cell mutation in embryonic stem cells." *Proc Natl Acad Sci U S A* **103**(4): 1036-40.
- Chen, H., K. Qian, et al. (2005). "The derivation of two additional human embryonic stem cell lines from day 3 embryos with low morphological scores." *Hum Reprod* **20**(8): 2201-6.
- Chen, U., M. Kosco, et al. (1992). "Establishment and characterization of lymphoid and myeloid mixed-cell populations from mouse late embryoid bodies, "embryonic-stem-cell fetuses"." *Proc Natl Acad Sci U S A* **89**(7): 2541-5.

26. Chen, Y. M., Z. W. Du, et al. (2005). "Molecular cloning and functional analysis of ESGP, an embryonic stem cell and germ cell specific protein." *Acta Biochim Biophys Sin (Shanghai)* **37**(12): 789-96.
27. Cho, M., M. Jang, et al. (2006). "An alternative method of deriving embryonic stem cell-like clones by aggregation of diploid cells with tetraploid embryos." *Fertil Steril* **85 Suppl 1**: 1103-10.
28. Choudhary, M., X. Zhang, et al. (2007). "Putative role of hyaluronan and its related genes, HAS2 and RHAMM, in human early preimplantation embryogenesis and embryonic stem cell characterization." *Stem Cells* **25**(12): 3045-57.
29. Cibelli, J. B., S. L. Stice, et al. (1998). "Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells." *Nat Biotechnol* **16**(7): 642-6.
30. Collodi, P., Y. Kamei, et al. (1992). "Fish embryo cell cultures for derivation of stem cells and transgenic chimeras." *Mol Mar Biol Biotechnol* **1**(4-5): 257-65.
31. Conner, D. A. (2001). "Mouse embryonic stem (ES) cell culture." *Curr Protoc Mol Biol* **Chapter 23**: Unit 23 3.
32. Constantinescu, S. N. (2000). "Stem cell generation and choice of fate: role of cytokines and cellular microenvironment." *J Cell Mol Med* **4**(4): 233-248.
33. Contraestre, A. P., F. Sifontes, et al. (2008). "Secretion of stem cell factor and granulocyte-macrophage colony-stimulating factor by mouse embryos in culture: influence of group culture." *Zygote* **16**(4): 297-301.
34. Cortes, J. L., L. Sanchez, et al. (2008). "Whole-blastocyst culture followed by laser drilling technology enhances the efficiency of inner cell mass isolation and embryonic stem cell derivation from good- and poor-quality mouse embryos: new insights for derivation of human embryonic stem cell lines." *Stem Cells Dev* **17**(2): 255-67.
35. Daley, G. Q. (2002). "Prospects for stem cell therapeutics: myths and medicines." *Curr Opin Genet Dev* **12**(5): 607-13.
36. Damjanov, I. (2004). "From stem cells to germ cell tumors and back." *Verh Dtsch Ges Pathol* **88**: 39-44.
37. Dhali, A., V. M. Anchamparathy, et al. (2009). "In vitro development of bovine embryos cultured with stem cell factor or insulin-like growth factor-I following IVF with semen of two bulls having different field fertility." *Anim Reprod Sci* **116**(3-4): 188-95.
38. Ding, H. and A. Guha (2001). "Mouse astrocytoma models: embryonic stem cell mediated transgenesis." *J Neurooncol* **53**(3): 289-96.
39. Doetschman, T. C., H. Eistetter, et al. (1985). "The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium." *J Embryol Exp Morphol* **87**: 27-45.
40. Doetschman, T., M. Shull, et al. (1993). "Embryonic stem cell model systems for vascular morphogenesis and cardiac disorders." *Hypertension* **22**(4): 618-29.
41. Donovan, P. J. (1998). "The germ cell--the mother of all stem cells." *Int J Dev Biol* **42**(7): 1043-50.
42. Draper, J. S. and A. Nagy (2007). "Improved embryonic stem cell technologies." *Handb Exp Pharmacol*(178): 107-28.
43. Drukker, M. (2008). "Recent advancements towards the derivation of immune-compatible patient-specific human embryonic stem cell lines." *Semin Immunol* **20**(2): 123-9.
44. Eiges, R., M. Schuldiner, et al. (2001). "Establishment of human embryonic stem cell-transfected clones carrying a marker for undifferentiated cells." *Curr Biol* **11**(7): 514-8.
45. Faast, R., S. J. Harrison, et al. (2006). "Use of adult mesenchymal stem cells isolated from bone marrow and blood for somatic cell nuclear transfer in pigs." *Cloning Stem Cells* **8**(3): 166-73.
46. Falco, G., S. L. Lee, et al. (2007). "Zscan4: a novel gene expressed exclusively in late 2-cell embryos and embryonic stem cells." *Dev Biol* **307**(2): 539-50.
47. Fang, Z. F., F. Jin, et al. (2005). "Human embryonic stem cell lines derived from the Chinese population." *Cell Res* **15**(5): 394-400.
48. Fang, Z. F., H. Gai, et al. (2006). "Rabbit embryonic stem cell lines derived from fertilized, parthenogenetic or somatic cell nuclear transfer embryos." *Exp Cell Res* **312**(18): 3669-82.
49. Fassler, R., M. Pfaff, et al. (1995). "Lack of beta 1 integrin gene in embryonic stem cells affects morphology, adhesion, and migration but not integration into the inner cell mass of blastocysts." *J Cell Biol* **128**(5): 979-88.
50. Findikli, N., N. Z. Candan, et al. (2006). "Human embryonic stem cell culture: current limitations and novel strategies." *Reprod Biomed Online* **13**(4): 581-90.
51. Findikli, N., S. Kahraman, et al. (2005). "Establishment and characterization of new human embryonic stem cell lines." *Reprod Biomed Online* **10**(5): 617-27.
52. Fletcher, J. M., P. M. Ferrier, et al. (2006). "Variations in humanized and defined culture conditions supporting derivation of new human embryonic stem cell lines." *Cloning Stem Cells* **8**(4): 319-34.
53. Fong, C. Y., M. Richards, et al. (2006). "Unsuccessful derivation of human embryonic stem cell lines from pairs of human blastomeres." *Reprod Biomed Online* **13**(2): 295-300.
54. Forrester, L. M., A. Bernstein, et al. (1991). "Long-term reconstitution of the mouse hematopoietic system by embryonic stem cell-derived fetal liver." *Proc Natl Acad Sci U S A* **88**(17): 7514-7.
55. Foshay, K. M. and G. I. Gallicano (2009). "miR-17 family miRNAs are expressed during early mammalian development and regulate stem cell differentiation." *Dev Biol* **326**(2): 431-43.
56. Frydman, N., O. Feraud, et al. (2009). "Characterization of human PGD blastocysts with unbalanced chromosomal translocations and human embryonic stem cell line derivation?" *Reprod Biomed Online* **19 Suppl 4**: 4199.
57. Furusawa, T., K. Ohkoshi, et al. (2004). "Embryonic stem cells expressing both platelet endothelial cell adhesion molecule-1 and stage-specific embryonic

- antigen-1 differentiate predominantly into epiblast cells in a chimeric embryo." *Biol Reprod* **70**(5): 1452-7.
58. Furusawa, T., M. Ikeda, et al. (2006). "Gene expression profiling of mouse embryonic stem cell subpopulations." *Biol Reprod* **75**(4): 555-61.
 59. Gan, Q., T. Yoshida, et al. (2007). "Concise review: epigenetic mechanisms contribute to pluripotency and cell lineage determination of embryonic stem cells." *Stem Cells* **25**(1): 2-9.
 60. Gao, S., M. McGarry, et al. (2003). "Effect of cell confluence on production of cloned mice using an inbred embryonic stem cell line." *Biol Reprod* **68**(2): 595-603.
 61. Gautrey, H., J. McConnell, et al. (2008). "Staufen1 is expressed in preimplantation mouse embryos and is required for embryonic stem cell differentiation." *Biochim Biophys Acta* **1783**(10): 1935-42.
 62. Geens, M., I. Mateizel, et al. (2009). "Human embryonic stem cell lines derived from single blastomeres of two 4-cell stage embryos." *Hum Reprod* **24**(11): 2709-17.
 63. Gerecht-Nir, S. and J. Itskovitz-Eldor (2004). "Cell therapy using human embryonic stem cells." *Transpl Immunol* **12**(3-4): 203-9.
 64. Gerlach, M., H. Braak, et al. (2002). "Current state of stem cell research for the treatment of Parkinson's disease." *J Neurol* **249** Suppl 3: III/33-5.
 65. Gissel, C., C. Voolstra, et al. (2005). "An optimized embryonic stem cell model for consistent gene expression and developmental studies: a fundamental study." *Thromb Haemost* **94**(4): 719-27.
 66. Goossens, E., V. Frederickx, et al. (2006). "Blastocyst development after assisted reproduction using spermatozoa obtained after testicular stem cell transplantation in mice." *Hum Reprod* **21**(7): 1759-64.
 67. Gossler, A., T. Doetschman, et al. (1986). "Transgenesis by means of blastocyst-derived embryonic stem cell lines." *Proc Natl Acad Sci U S A* **83**(23): 9065-9.
 68. Greco, B., H. P. Low, et al. (2004). "Differentiation prevents assessment of neural stem cell pluripotency after blastocyst injection." *Stem Cells* **22**(4): 600-8.
 69. Guest, D. J. and W. R. Allen (2007). "Expression of cell-surface antigens and embryonic stem cell pluripotency genes in equine blastocysts." *Stem Cells Dev* **16**(5): 789-96.
 70. Guo, Y., L. Einhorn, et al. (2004). "Redox regulation of the embryonic stem cell transcription factor oct-4 by thioredoxin." *Stem Cells* **22**(3): 259-64.
 71. Hall, V. (2008). "Porcine embryonic stem cells: a possible source for cell replacement therapy." *Stem Cell Rev* **4**(4): 275-82.
 72. Hanaoka, K., M. Hayasaka, et al. (1991). "The stem cells of a primordial germ cell-derived teratocarcinoma have the ability to form viable mouse chimeras." *Differentiation* **48**(2): 83-7.
 73. Harrison, N. J., D. Baker, et al. (2007). "Culture adaptation of embryonic stem cells echoes germ cell malignancy." *Int J Androl* **30**(4): 275-81; discussion 281.
 74. Hearn, J. P. (2001). "Embryo implantation and embryonic stem cell development in primates." *Reprod Fertil Dev* **13**(7-8): 517-22.
 75. Hedlund, E., J. Pruszk, et al. (2008). "Embryonic stem cell-derived Pitx3-enhanced green fluorescent protein midbrain dopamine neurons survive enrichment by fluorescence-activated cell sorting and function in an animal model of Parkinson's disease." *Stem Cells* **26**(6): 1526-36.
 76. Holm, S. (2008). "New embryos' - new challenges for the ethics of stem cell research." *Cells Tissues Organs* **187**(4): 257-62.
 77. Hong, Y., C. Winkler, et al. (1998). "Production of medakafish chimeras from a stable embryonic stem cell line." *Proc Natl Acad Sci U S A* **95**(7): 3679-84.
 78. Huang, J., K. Deng, et al. (2008). "Efficient production of mice from embryonic stem cells injected into four- or eight-cell embryos by piezo micromanipulation." *Stem Cells* **26**(7): 1883-90.
 79. Hussain, M. A. and N. D. Theise (2004). "Stem-cell therapy for diabetes mellitus." *Lancet* **364**(9429): 203-5.
 80. Hwang, W. S., Y. J. Ryu, et al. (2004). "Evidence of a pluripotent human embryonic stem cell line derived from a cloned blastocyst." *Science* **303**(5664): 1669-74.
 81. Inoue, K., N. Ogonuki, et al. (2006). "Inefficient reprogramming of the hematopoietic stem cell genome following nuclear transfer." *J Cell Sci* **119**(Pt 10): 1985-91.
 82. Iwasaki, S. and H. Kimura (2003). "Further studies on thermal treatment of two-cell stage embryos to produce complete embryonic stem-cell-derived mice by cell-aggregation methods." *Dev Growth Differ* **45**(5-6): 427-34.
 83. Jahagirdar, B. N. and C. M. Verfaillie (2005). "Multipotent adult progenitor cell and stem cell plasticity." *Stem Cell Rev* **1**(1): 53-9.
 84. Jiang, H., B. Sun, et al. (2007). "Activation of paternally expressed imprinted genes in newly derived germline-competent mouse parthenogenetic embryonic stem cell lines." *Cell Res* **17**(9): 792-803.
 85. Jincho, Y., Y. Sotomaru, et al. (2008). "Identification of genes aberrantly expressed in mouse embryonic stem cell-cloned blastocysts." *Biol Reprod* **78**(4): 568-76.
 86. Ju, J. Y., C. Y. Park, et al. (2008). "Establishment of stem cell lines from nuclear transferred and parthenogenetically activated mouse oocytes for therapeutic cloning." *Fertil Steril* **89**(5 Suppl): 1314-23.
 87. Kanatsu-Shinohara, M., J. Lee, et al. (2008). "Pluripotency of a single spermatogonial stem cell in mice." *Biol Reprod* **78**(4): 681-7.
 88. Kania, G., D. Corbeil, et al. (2005). "Somatic stem cell marker prominin-1/CD133 is expressed in embryonic stem cell-derived progenitors." *Stem Cells* **23**(6): 791-804.
 89. Kawase, E., Y. Yamazaki, et al. (2000). "Mouse embryonic stem (ES) cell lines established from neuronal cell-derived cloned blastocysts." *Genesis* **28**(3-4): 156-63.

90. Keskinetepe, L., K. Norris, et al. (2007). "Derivation and comparison of C57BL/6 embryonic stem cells to a widely used 129 embryonic stem cell line." Transgenic Res **16**(6): 751-8.
91. Kidder, B. L., L. Oseth, et al. (2008). "Embryonic stem cells contribute to mouse chimeras in the absence of detectable cell fusion." Cloning Stem Cells **10**(2): 231-48.
92. Kim, C., T. Amano, et al. (2009). "Improvement of embryonic stem cell line derivation efficiency with novel medium, glucose concentration, and epigenetic modifications." Cloning Stem Cells **11**(1): 89-100.
93. Kim, I. W., S. P. Gong, et al. (2009). "Derivation of developmentally competent oocytes by the culture of preantral follicles retrieved from adult ovaries: maturation, blastocyst formation, and embryonic stem cell transformation." Fertil Steril **92**(5): 1716-24.
94. Kim, M., A. Habiba, et al. (2009). "Regulation of mouse embryonic stem cell neural differentiation by retinoic acid." Dev Biol **328**(2): 456-71.
95. Kim, S. J., J. E. Lee, et al. (2005). "Efficient derivation of new human embryonic stem cell lines." Mol Cells **19**(1): 46-53.
96. Kim, Y. H. and H. J. Han (2008). "High-glucose-induced prostaglandin E(2) and peroxisome proliferator-activated receptor delta promote mouse embryonic stem cell proliferation." Stem Cells **26**(3): 745-55.
97. Kruglova, A. A., E. A. Kizilova, et al. (2008). "Embryonic stem cell/fibroblast hybrid cells with near-tetraploid karyotype provide high yield of chimeras." Cell Tissue Res **334**(3): 371-80.
98. Kuliev, A., S. Rechitsky, et al. (2005). "Preimplantation genetics: Improving access to stem cell therapy." Ann N Y Acad Sci **1054**: 223-7.
99. Labosky, P. A., D. P. Barlow, et al. (1994). "Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines." Development **120**(11): 3197-204.
100. Lanzendorf, S. E., C. A. Boyd, et al. (2001). "Use of human gametes obtained from anonymous donors for the production of human embryonic stem cell lines." Fertil Steril **76**(1): 132-7.
101. Latham, K. E. (2006). "The Primate Embryo Gene Expression Resource in embryology and stem cell biology." Reprod Fertil Dev **18**(8): 807-10.
102. Ledermann, B. and K. Burki (1991). "Establishment of a germ-line competent C57BL/6 embryonic stem cell line." Exp Cell Res **197**(2): 254-8.
103. Lee, J. B., J. E. Lee, et al. (2005). "Establishment and maintenance of human embryonic stem cell lines on human feeder cells derived from uterine endometrium under serum-free condition." Biol Reprod **72**(1): 42-9.
104. Lengner, C. J., F. D. Camargo, et al. (2007). "Oct4 expression is not required for mouse somatic stem cell self-renewal." Cell Stem Cell **1**(4): 403-15.
105. Lerou, P. H., A. Yabuuchi, et al. (2008). "Human embryonic stem cell derivation from poor-quality embryos." Nat Biotechnol **26**(2): 212-4.
106. Lev, S., I. Kehat, et al. (2005). "Differentiation pathways in human embryonic stem cell-derived cardiomyocytes." Ann N Y Acad Sci **1047**: 50-65.
107. Li, C., Y. Yang, et al. (2009). "Derivation and transcriptional profiling analysis of pluripotent stem cell lines from rat blastocysts." Cell Res **19**(2): 173-86.
108. Li, T., C. Q. Zhou, et al. (2005). "Establishment of human embryonic stem cell line from gamete donors." Chin Med J (Engl) **118**(2): 116-22.
109. Li, X., S. G. Zhou, et al. (2006). "Horse embryonic stem cell lines from the proliferation of inner cell mass cells." Stem Cells Dev **15**(4): 523-31.
110. Liang, L. and J. R. Bickenbach (2002). "Somatic epidermal stem cells can produce multiple cell lineages during development." Stem Cells **20**(1): 21-31.
111. Liao, S. M. (2005). "Rescuing human embryonic stem cell research: the Blastocyst Transfer Method." Am J Bioeth **5**(6): 8-16.
112. Lim, C. Y., W. L. Tam, et al. (2008). "Sall4 regulates distinct transcription circuitries in different blastocyst-derived stem cell lineages." Cell Stem Cell **3**(5): 543-54.
113. Lin, T. C., J. M. Yen, et al. (2003). "IGF-1/IGFBP-1 increases blastocyst formation and total blastocyst cell number in mouse embryo culture and facilitates the establishment of a stem-cell line." BMC Cell Biol **4**: 14.
114. Liu, W., Y. Yin, et al. (2009). "Derivation and characterization of human embryonic stem cell lines from poor quality embryos." J Genet Genomics **36**(4): 229-39.
115. Lodge, P., J. McWhir, et al. (2005). "Increased gp130 signaling in combination with inhibition of the MEK/ERK pathway facilitates embryonic stem cell isolation from normally refractory murine CBA blastocysts." Cloning Stem Cells **7**(1): 2-7.
116. Longo, L., A. Bygrave, et al. (1997). "The chromosome make-up of mouse embryonic stem cells is predictive of somatic and germ cell chimaerism." Transgenic Res **6**(5): 321-8.
117. Lorthongpanich, C., S. H. Yang, et al. (2008). "Chemical enhancement in embryo development and stem cell derivation from single blastomeres." Cloning Stem Cells **10**(4): 503-12.
118. Low, H. P., B. Greco, et al. (2009). "Embryonic stem cell rescue of tremor and ataxia in myelin-deficient shiverer mice." J Neurol Sci **276**(1-2): 133-7.
119. Mai, Q., Y. Yu, et al. (2007). "Derivation of human embryonic stem cell lines from parthenogenetic blastocysts." Cell Res **17**(12): 1008-19.
120. Mann, J. R. (2001). "Deriving and propagating mouse embryonic stem cell lines for studying genomic imprinting." Methods Mol Biol **181**: 21-39.
121. Markoulaki, S., A. Meissner, et al. (2008). "Somatic cell nuclear transfer and derivation of embryonic stem cells in the mouse." Methods **45**(2): 101-14.
122. Markoullis, K., D. Bulian, et al. (2009). "Mycoplasma contamination of murine embryonic stem cells affects cell parameters, germline transmission and chimeric progeny." Transgenic Res **18**(1): 71-87.

123. Maslov, A. Y., K. J. Bailey, et al. (2007). "Stem/progenitor cell-specific enhanced green fluorescent protein expression driven by the endogenous Mcm2 promoter." Stem Cells **25**(1): 132-8.
124. Metzger, J. M., L. C. Samuelson, et al. (1997). "Embryonic stem cell cardiogenesis applications for cardiovascular research." Trends Cardiovasc Med **7**(2): 63-8.
125. Metzger, J. M., W. I. Lin, et al. (1995). "Myosin heavy chain expression in contracting myocytes isolated during embryonic stem cell cardiogenesis." Circ Res **76**(5): 710-9.
126. Muller, T., G. Fleischmann, et al. (2009). "A novel embryonic stem cell line derived from the common marmoset monkey (*Callithrix jacchus*) exhibiting germ cell-like characteristics." Hum Reprod **24**(6): 1359-72.
127. Mummery, C. (2004). "Stem cell research: immortality or a healthy old age?" Eur J Endocrinol **151 Suppl 3**: U7-12.
128. Munne, S., E. Velilla, et al. (2005). "Self-correction of chromosomally abnormal embryos in culture and implications for stem cell production." Fertil Steril **84**(5): 1328-34.
129. Munsie, M. J., A. E. Michalska, et al. (2000). "Isolation of pluripotent embryonic stem cells from reprogrammed adult mouse somatic cell nuclei." Curr Biol **10**(16): 989-92.
130. Nagy, A., J. Rossant, et al. (1993). "Derivation of completely cell culture-derived mice from early-passage embryonic stem cells." Proc Natl Acad Sci U S A **90**(18): 8424-8.
131. Newman-Smith, E. D. and Z. Werb (1995). "Stem cell defects in parthenogenetic peri-implantation embryos." Development **121**(7): 2069-77.
132. Oda, M., S. Tanaka, et al. (2009). "Establishment of trophoblast stem cell lines from somatic cell nuclear-transferred embryos." Proc Natl Acad Sci U S A **106**(38): 16293-7.
133. Odorico, J. S., D. S. Kaufman, et al. (2001). "Multilineage differentiation from human embryonic stem cell lines." Stem Cells **19**(3): 193-204.
134. Ohta, H., Y. Ohinata, et al. (2009). "Male germline and embryonic stem cell lines from NOD mice: efficient derivation of GS cells from a nonpermissive strain for ES cell derivation." Biol Reprod **81**(6): 1147-53.
135. Ostenfeld, T. and C. N. Svendsen (2003). "Recent advances in stem cell neurobiology." Adv Tech Stand Neurosurg **28**: 3-89.
136. Park, S. P., Y. J. Lee, et al. (2004). "Establishment of human embryonic stem cell lines from frozen-thawed blastocysts using STO cell feeder layers." Hum Reprod **19**(3): 676-84.
137. Pawar, S. S., D. Malakar, et al. (2009). "Stem cell-like outgrowths from in vitro fertilized goat blastocysts." Indian J Exp Biol **47**(8): 635-42.
138. Pereira, L., F. Yi, et al. (2006). "Repression of Nanog gene transcription by Tcf3 limits embryonic stem cell self-renewal." Mol Cell Biol **26**(20): 7479-91.
139. Peura, T., A. Bosman, et al. (2008). "Karyotypically normal and abnormal human embryonic stem cell lines derived from PGD-analyzed embryos." Cloning Stem Cells **10**(2): 203-16.
140. Pickering, S. J., P. R. Braude, et al. (2003). "Preimplantation genetic diagnosis as a novel source of embryos for stem cell research." Reprod Biomed Online **7**(3): 353-64.
141. Ponsaerts, P., V. F. van Tendeloo, et al. (2004). "Current challenges in human embryonic stem cell research: directed differentiation and transplantation tolerance." J Biol Regul Homeost Agents **18**(3-4): 347-51.
142. Pralong, D., M. L. Lim, et al. (2005). "Tetraploid embryonic stem cells contribute to the inner cell mass of mouse blastocysts." Cloning Stem Cells **7**(4): 272-8.
143. Prelle, K., N. Zink, et al. (2002). "Pluripotent stem cells--model of embryonic development, tool for gene targeting, and basis of cell therapy." Anat Histol Embryol **31**(3): 169-86.
144. Pruksananonda, K., R. Rungsiwirut, et al. (2009). "Development of human embryonic stem cell derivation." J Med Assoc Thai **92**(4): 443-50.
145. Ralston, A. and J. Rossant (2005). "Genetic regulation of stem cell origins in the mouse embryo." Clin Genet **68**(2): 106-12.
146. Ramirez, M. A., R. Fernandez-Gonzalez, et al. (2009). "Effect of stem cell activation, culture media of manipulated embryos, and site of embryo transfer in the production of F0 embryonic stem cell mice." Biol Reprod **80**(6): 1216-22.
147. Ratajczak, M. Z., E. K. Zuba-Surma, et al. (2008). "Hunt for pluripotent stem cell -- regenerative medicine search for almighty cell." J Autoimmun **30**(3): 151-62.
148. Raya, A., I. Rodriguez-Piza, et al. (2008). "Generation of cardiomyocytes from new human embryonic stem cell lines derived from poor-quality blastocysts." Cold Spring Harb Symp Quant Biol **73**: 127-35.
149. Rebel, V. I., A. L. Kung, et al. (2002). "Distinct roles for CREB-binding protein and p300 in hematopoietic stem cell self-renewal." Proc Natl Acad Sci U S A **99**(23): 14789-94.
150. Reijo Pera, R. A., C. DeJonge, et al. (2009). "Gene expression profiles of human inner cell mass cells and embryonic stem cells." Differentiation **78**(1): 18-23.
151. Reubinoff, B. E., M. F. Pera, et al. (2000). "Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro." Nat Biotechnol **18**(4): 399-404.
152. Revazova, E. S., N. A. Turovets, et al. (2007). "Patient-specific stem cell lines derived from human parthenogenetic blastocysts." Cloning Stem Cells **9**(3): 432-49.
153. Revazova, E. S., N. A. Turovets, et al. (2008). "HLA homozygous stem cell lines derived from human parthenogenetic blastocysts." Cloning Stem Cells **10**(1): 11-24.
154. Risau, W., H. Sariola, et al. (1988). "Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies." Development **102**(3): 471-8.

155. Roach, M. L., J. L. Stock, et al. (1995). "A new embryonic stem cell line from DBA/1lacJ mice allows genetic modification in a murine model of human inflammation." *Exp Cell Res* **221**(2): 520-5.
156. Saburi, S., S. Azuma, et al. (1997). "Developmental fate of single embryonic stem cells microinjected into 8-cell-stage mouse embryos." *Differentiation* **62**(1): 1-11.
157. Sakaki-Yumoto, M., C. Kobayashi, et al. (2006). "The murine homolog of SALL4, a causative gene in Okihiro syndrome, is essential for embryonic stem cell proliferation, and cooperates with Sall1 in anorectal, heart, brain and kidney development." *Development* **133**(15): 3005-13.
158. Sathananthan, H., S. Gunasheela, et al. (2003). "Critical evaluation of human blastocysts for assisted reproduction techniques and embryonic stem cell biotechnology." *Reprod Biomed Online* **7**(2): 219-27.
159. Schoonjans, L., V. Kreemers, et al. (2003). "Improved generation of germline-competent embryonic stem cell lines from inbred mouse strains." *Stem Cells* **21**(1): 90-7.
160. Schwenk, F., B. Zevnik, et al. (2003). "Hybrid embryonic stem cell-derived tetraploid mice show apparently normal morphological, physiological, and neurological characteristics." *Mol Cell Biol* **23**(11): 3982-9.
161. Sharp, J. and H. S. Keirstead (2009). "Stem cell-based cell replacement strategies for the central nervous system." *Neurosci Lett* **456**(3): 107-11.
162. Shibayama, M., S. Ohno, et al. (2009). "Polypyrimidine tract-binding protein is essential for early mouse development and embryonic stem cell proliferation." *Febs J* **276**(22): 6658-68.
163. Shimizukawa, R., A. Sakata, et al. (2005). "Establishment of a new embryonic stem cell line derived from C57BL/6 mouse expressing EGFP ubiquitously." *Genesis* **42**(1): 47-52.
164. Siu, C. W., J. C. Moore, et al. (2007). "Human embryonic stem cell-derived cardiomyocytes for heart therapies." *Cardiovasc Hematol Disord Drug Targets* **7**(2): 145-52.
165. Suemori, H., T. Tada, et al. (2001). "Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI." *Dev Dyn* **222**(2): 273-9.
166. Sukoyan, M. A., A. N. Golubitsa, et al. (1992). "Isolation and cultivation of blastocyst-derived stem cell lines from American mink (*Mustela vison*)." *Mol Reprod Dev* **33**(4): 418-31.
167. Sukoyan, M. A., S. Y. Vatolin, et al. (1993). "Embryonic stem cells derived from morulae, inner cell mass, and blastocysts of mink: comparisons of their pluripotencies." *Mol Reprod Dev* **36**(2): 148-58.
168. Sumida, T., T. Maeda, et al. (1990). "Expression of IgH promoter/enhancer Ly-1 transgene in hematopoietic chimeric mice generated by embryonic stem cell line." *Int Arch Allergy Appl Immunol* **93**(2-3): 155-64.
169. Suss-Toby, E., S. Gerecht-Nir, et al. (2004). "Derivation of a diploid human embryonic stem cell line from a mononuclear zygote." *Hum Reprod* **19**(3): 670-5.
170. Talbot, N. C. and W. M. Garrett (2001). "Ultrastructure of the embryonic stem cells of the 8-day pig blastocyst before and after in vitro manipulation: development of junctional apparatus and the lethal effects of PBS mediated cell-cell dissociation." *Anat Rec* **264**(1): 101-13.
171. Tam, W. L., C. Y. Lim, et al. (2008). "T-cell factor 3 regulates embryonic stem cell pluripotency and self-renewal by the transcriptional control of multiple lineage pathways." *Stem Cells* **26**(8): 2019-31.
172. Tanaka, N., T. Takeuchi, et al. (2006). "Laser-assisted blastocyst dissection and subsequent cultivation of embryonic stem cells in a serum/cell free culture system: applications and preliminary results in a murine model." *J Transl Med* **4**: 20.
173. Thomson, J. A., J. Itskovitz-Eldor, et al. (1998). "Embryonic stem cell lines derived from human blastocysts." *Science* **282**(5391): 1145-7.
174. Thomson, J. A., J. Kalishman, et al. (1995). "Isolation of a primate embryonic stem cell line." *Proc Natl Acad Sci U S A* **92**(17): 7844-8.
175. Tielens, S., B. Verhasselt, et al. (2006). "Generation of embryonic stem cell lines from mouse blastocysts developed in vivo and in vitro: relation to Oct-4 expression." *Reproduction* **132**(1): 59-66.
176. Tojo, H., M. Nishida, et al. (1995). "Establishment of a novel embryonic stem cell line by a modified procedure." *Cytotechnology* **19**(2): 161-5.
177. Tokuzawa, Y., E. Kaiho, et al. (2003). "Fbx15 is a novel target of Oct3/4 but is dispensable for embryonic stem cell self-renewal and mouse development." *Mol Cell Biol* **23**(8): 2699-708.
178. Trivedi, H. L., V. V. Mishra, et al. (2006). "Embryonic stem cell derived and adult hematopoietic stem cell transplantation for tolerance induction in a renal allograft recipient--a case report." *Transplant Proc* **38**(9): 3103-8.
179. Trounson, A. (2002). "Human embryonic stem cells: mother of all cell and tissue types." *Reprod Biomed Online* **4 Suppl 1**: 58-63.
180. Trounson, A. (2005). "Human embryonic stem cell derivation and directed differentiation." *Ernst Schering Res Found Workshop* (54): 27-44.
181. Turetsky, T., E. Aizenman, et al. (2008). "Laser-assisted derivation of human embryonic stem cell lines from IVF embryos after preimplantation genetic diagnosis." *Hum Reprod* **23**(1): 46-53.
182. Uy, G. D., K. M. Downs, et al. (2002). "Inhibition of trophoblast stem cell potential in chorionic ectoderm coincides with occlusion of the ectoplacental cavity in the mouse." *Development* **129**(16): 3913-24.
183. Vanikar, A. V., V. V. Mishra, et al. (2007). "Successful generation of donor specific hematopoietic stem cell lines from co-cultured bone marrow with human embryonic stem cell line: a new methodology." *Transplant Proc* **39**(3): 658-61.
184. Verlinsky, Y., N. Strelchenko, et al. (2005). "Human embryonic stem cell lines with genetic disorders." *Reprod Biomed Online* **10**(1): 105-10.

185. Verlinsky, Y., N. Strelchenko, et al. (2009). "Impact of meiotic and mitotic non-disjunction on generation of human embryonic stem cell lines." Reprod Biomed Online **18**(1): 120-6.
186. Verma, V., S. K. Gautam, et al. (2007). "Isolation and characterization of embryonic stem cell-like cells from in vitro-produced buffalo (*Bubalus bubalis*) embryos." Mol Reprod Dev **74**(4): 520-9.
187. Wakayama, S., H. Ohta, et al. (2005). "Establishment of male and female nuclear transfer embryonic stem cell lines from different mouse strains and tissues." Biol Reprod **72**(4): 932-6.
188. Wakayama, T., V. Tabar, et al. (2001). "Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer." Science **292**(5517): 740-3.
189. Wang, F., S. Thirumangalathu, et al. (2006). "Establishment of new mouse embryonic stem cell lines is improved by physiological glucose and oxygen." Cloning Stem Cells **8**(2): 108-16.
190. Wang, Z. Q., F. Kiefer, et al. (1997). "Generation of completely embryonic stem cell-derived mutant mice using tetraploid blastocyst injection." Mech Dev **62**(2): 137-45.
191. Watt, S. M. and M. Contreras (2005). "Stem cell medicine: umbilical cord blood and its stem cell potential." Semin Fetal Neonatal Med **10**(3): 209-20.
192. Wernig, M. and O. Brustle (2002). "Fifty ways to make a neuron: shifts in stem cell hierarchy and their implications for neuropathology and CNS repair." J Neuropathol Exp Neurol **61**(2): 101-10.
193. Wianny, F., A. Bernat, et al. (2008). "Derivation and cloning of a novel rhesus embryonic stem cell line stably expressing tau-green fluorescent protein." Stem Cells **26**(6): 1444-53.
194. Wilcox, J. T., E. Semple, et al. (2009). "Characterization of canine embryonic stem cell lines derived from different niche microenvironments." Stem Cells Dev **18**(8): 1167-78.
195. Xiao, Y., Y. Ye, et al. (2008). "The lymphovascular embolus of inflammatory breast cancer expresses a stem cell-like phenotype." Am J Pathol **173**(2): 561-74.
196. Yamada, S., T. J. Nelson, et al. (2009). "Stem cell transplant into preimplantation embryo yields myocardial infarction-resistant adult phenotype." Stem Cells **27**(7): 1697-705.
197. Yang, Z. and J. N. Petite (1994). "Use of avian cytokines in mammalian embryonic stem cell culture." Poult Sci **73**(7): 965-74.
198. Zech, N. H., S. Koestenbauer, et al. (2005). "Paraffin-embedded manipulated blastocysts: a tool to demonstrate stem cell plasticity?" Reprod Biomed Online **10**(3): 406-14.
199. Zhao, C., R. Yao, et al. (2007). "Establishment of customized mouse stem cell lines by sequential nuclear transfer." Cell Res **17**(1): 80-7.
200. Zhou, Q., A. Jouneau, et al. (2001). "Developmental potential of mouse embryos reconstructed from metaphase embryonic stem cell nuclei." Biol Reprod **65**(2): 412-9.
201. Ma H, Chen G. Stem Cell. J Am. Sci 2005;1(2):90-92. <http://www.sciencepub.net/american/0102/14-mahongbao.pdf>.
202. Ma H, Cheng S. Eternal Life and Stem Cell. Nat Sci 2007;5(1):81-96. <http://www.sciencepub.net/nature/0501/10-0247-mahongbao-eternal-ns.pdf>.
203. Ma H, Cheng S. Review of Stem Cell Studies. Nat Sci 2007;5(2):45-65. <http://www.sciencepub.net/nature/0502/09-0247-mahongbao-stem-ns.pdf>.
204. Yang Y, Ma H. Germ Stem Cell. Stem Cell 2010;1(2):38-60]. http://www.sciencepub.net/stem/stem0102/07_1348stem0102_38_60.pdf.
205. Pubmed. Stem Cell. <http://www.ncbi.nlm.nih.gov/pubmed/?term=stem+cell>.
206. Wikipedia. Stem Cell. http://en.wikipedia.org/wiki/Stem_cell.

10/9/2010