Human Embryonic Stem Cell (hESC) Literatres

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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 situuition 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 tipical and important topic of life science. This material collects some literatures on human embryonic stem cell (hESC).
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Introduction

Scientific progress in human embryonic stem cell (hESC) research and increased funding make it imperative to look ahead to the ethical issues generated by the expected use of hESC for transplantation. Several issues should be addressed now, even though Phase I clinical trials of hESC transplantation are still in the future. To minimize the risk of hESC transplantation, donors of materials used to derive hESC lines will need to be recontacted to update their medical history and screening. Because of privacy concerns, such recontact needs to be discussed and agreed to at the time of donation, before new hESC lines are derived. Informed consent for Phase I clinical trials of hESC transplantation also raises ethical concerns. In previous Phase I trials of highly innovative interventions, allegations that trial participants had not really understood the risk and benefits caused delays in subsequent trials. Thus researchers should consider what information needs to be discussed during the consent process for hESC clinical trials and how to verify that participants have a realistic understanding of the study. Lack of attention to the special ethical concerns raised by clinical trials of hESC transplantation and their implications for the derivation of new hESC lines may undermine or delay progress towards stem cell therapies.

Increased funding and continued scientific progress have opened a new era in the ethics of human embryonic stem cell (hESC) research. These developments will reframe the ethical debate, which to date has focused on the moral status of the embryo and the acceptability of using embryos for research purposes. Although such philosophical questions have not been resolved, the issue is no longer *if* hESC research should proceed, but rather *how* it should proceed. The rapid pace of research makes it imperative to look ahead to the ethical issues generated by the expected use of hESC for transplantation. Some of these issues should be addressed now, even though Phase I clinical trials of hESC transplantation are still in the future. Crucial issues concerning safety of hESC transplantation and the need to recontact donors of materials used to derive new hESC lines are best resolved when these materials are donated. In addition, informed consent for hESC transplantation Phase I clinical trials will present particular challenges, which will require modification of the usual consent process for clinical trials. Failure to address these ethical issues may delay or preclude clinical trials that will test whether interventions based on hESC are safe and effective.

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.

Abeyta, M. J., A. T. Clark, et al. (2004). "Unique gene expression signatures of independently-derived human embryonic stem cell lines." Hum Mol Genet **13**(6): 601-8.

Human embryonic stem cells (hESCs) have the potential to differentiate to diverse cell types. This ability endows hESCs with promise for the development of novel therapeutics, as well as promise for the development of a rigorous genetic system to probe human gene function. However, in spite of the impending utility of hESCs for clinical and basic applications, little is known about their fundamental properties. Recent reports have documented transcriptional profiles of mouse embryonic stem cells (mESCs), adult stem cells and a single hESC line, H9. To date, however, the transcriptional profiles of independently-derived hESC lines have not been compared. In order to examine the similarities and differences in multiple hESC lines, we compared gene expression profiles of the HSF-1, HSF-6 and H9 lines. We found that the majority of genes examined were expressed in all three cell lines. However, we also observed that each line possessed a unique expression signature; the expression of many genes was limited to just one or two hESC lines. We suggest that the observed differences in gene expression between independently-derived hESC lines may reflect inherent differences in the initial culture of each line and/or the underlying genetics of the embryos from which the lines were derived.

Ahmad, S., R. Stewart, et al. (2007). "Differentiation of human embryonic stem cells into corneal epithelial-like cells by in vitro replication of the corneal epithelial stem cell niche." Stem Cells **25**(5): 1145-55.

Human embryonic stem cells (hESCs) are pluripotent cells capable of differentiating into any cell type of the body. It has long been known that the adult stem cell niche is vital for the maintenance of adult stem cells. The cornea at the front of the eye is covered by a stratified epithelium that is renewed by stem cells located at its periphery in a region known as the limbus. These so-called limbal stem cells are maintained by factors within the limbal microenvironment, including collagen IV in basement membrane and limbal fibroblasts in the stroma. Because this niche is very specific to the stem cells (rather than to the more differentiated cells) of the corneal epithelium, it was hypothesized that replication of these factors in vitro would result in hESC differentiation into corneal epithelial-like cells. Indeed, here we show that culturing of hESC on collagen IV using medium conditioned by the limbal fibroblasts results in the loss of pluripotency and differentiation into epithelial-like cells. Further differentiation results in the formation of terminally differentiated epithelial-like cells not only of the cornea but also of skin. Scanning electron microscopy shows that some differences exist between hESC-derived and adult limbal epithelial-like cells, necessitating further investigation using in vivo animal models of limbal stem cell deficiency. Such a model of hESC differentiation is useful for understanding the early events of epithelial lineage specification and to the eventual potential application of epithelium differentiated from hESC for clinical conditions of epithelial stem cell loss. Disclosure of potential conflicts of interest is found at the end of this article.

Aiba, K., T. Nedorezov, et al. (2009). "Defining developmental potency and cell lineage trajectories by expression profiling of differentiating mouse embryonic stem cells." DNA Res **16**(1): 73-80.

Biologists rely on morphology, function and specific markers to define the differentiation status of cells. Transcript profiling has expanded the repertoire of these markers by providing the snapshot of cellular status that reflects the activity of all genes. However, such data have been used only to assess relative similarities and differences of these cells. Here we show that principal component analysis of global gene expression profiles map cells in multidimensional transcript profile space and the positions of differentiating cells progress in a stepwise manner along trajectories starting from undifferentiated embryonic stem (ES) cells located in the apex. We present three 'cell lineage trajectories', which represent the differentiation of ES cells into the first three lineages in mammalian development: primitive endoderm, trophoblast and primitive ectoderm/neural ectoderm. The positions of the cells along these trajectories seem to reflect the developmental potency of cells and can be used as a scale for the potential of cells. Indeed, we show that embryonic germ cells and

induced pluripotent cells are mapped near the origin of the trajectories, whereas mouse embryo fibroblast and fibroblast cell lines are mapped near the far end of the trajectories. We suggest that this method can be used as the non-operational semi-quantitative definition of cell differentiation status and developmental potency. Furthermore, the global expression profiles of cell lineages provide a framework for the future study of in vitro and in vivo cell differentiation.

Anneren, C., C. A. Cowan, et al. (2004). "The Src family of tyrosine kinases is important for embryonic stem cell self-renewal." J Biol Chem **279**(30): 31590-8.

cYes, a member of the Src family of non-receptor tyrosine kinases, is highly expressed in mouse and human embryonic stem (ES) cells. We demonstrate that cYes kinase activity is regulated by leukemia inhibitory factor (LIF) and serum and is down-regulated when cells differentiate. Moreover, selective chemical inhibition of Src family kinases decreases growth and expression of stem cell genes that mark the undifferentiated state, including Oct3/4, alkaline phosphatase, fibroblast growth factor 4, and Nanog. A synergistic effect on differentiation is observed when ES cells are cultured with an Src family inhibitor and low levels of retinoic acid. Src family kinase inhibition does not interfere with LIF-induced JAK/STAT3 (Janus-associated tyrosine kinases/signal transducer and activator of transcription 3) or p42/p44 MAPK (mitogen-activated protein kinase) phosphorylation. Together the results suggest that the activation of the Src family is important for maintaining mouse and human ES in an undifferentiated state and may represent a third, independent pathway, downstream of LIF in mouse ES cells.

Anzai, H., M. Nagayoshi, et al. (1999). "Self-renewal and differentiation of a basic fibroblast growth factor-dependent multipotent hematopoietic cell line derived from embryonic stem cells." Dev Growth Differ **41**(1): 51-8.

Despite the accumulation of informat on on the origin of hematopoietic stem cells, it is still unclear how these cells are generated in ontogeny. Isolation of cell lines equivalent to early embryonic hematopoietic progenitor cells can be helpful. A multipotent hematopoietic progenitor cell line, A-6, was isolated from H-1 embryonic stem (ES) cells. The self-renewal of A-6 cells was supported by basic-fibroblast growth factor (b-FGF) and their differentiation into definitive erythroid cells, granulocytes and macrophages was induced after co-culture with ST-2 stromal cells. A-6 cells were positive for the surface markers of hematopoietic stem cell, c-kit, CD31, CD34, Flt3/Flk2, PgP-1, and HSA, but were negative for that of the differentiated cells. Reverse transcription-polymerase chain reaction analysis showed that A-6 cells produced mRNA from SCL/tal-1 and GATA-2 genes. Among various cytokines examined, on y stem cell factor (SCF) and Flt3/Flk2 ligand (FL) supported the proliferation of A-6 cells instead of b-FGF. The FL, as well as b-FGF, supported the self-renewal of A-6 cells, whereas SCF induced differentiation into myeloid cells. A-6 cells will be useful for the characterization of hematopoietic progenitor cells derived from ES cells and provide a model system to realize the control mechanisms between self-renewal and different ation of hematopoietic stem cells.

Aoto, T., N. Saitoh, et al. (2006). "Nuclear and chromatin reorganization in the MHC-Oct3/4 locus at developmental phases of embryonic stem cell differentiation." Dev Biol **298**(2): 354-67.

Epigenetic gene control is involved in mechanisms of development. Little is known about the cooperation of nuclear and chromatin events in programmed differentiation from mouse embryonic stem cells (ESC). To address this, Oct3/4-positive ESC and differentiated progenies, Sox1-positive neural precursor cells (NPC) and post-mitotic neurons (PMN), were isolated using a stage-selected culture system. We first investigated global nuclear organization at the each stage. Chromocenter preexists in ESC, disperses in NPC and becomes integrated into large heterochromatic foci in PMN, while the formation of PML bodies markedly decreases in neural differentiation. We next focused on the gene-dense MHC-Oct3/4 region. Oct3/4 gene is expressed preferentially adjacent to PML bodies in ESC and are repressed in the absence of chromocenter association in NPC and PMN. Histone deacetylation in NPC, demethylation of lysine 4 of histone H3 (H3K4), tri-methylation of H3K27, and CpG methylation in PMN are targeted for the Oct3/4 promoter within the region. Interestingly, di-methyl H3K4 mark is present in Oct3/4 promoter in NPC as well as ESC. These findings provide insights into the molecular basis of global nuclear reorganization and euchromatic gene silencing in differentiation through the spatiotemporal order of epigenetic controls.

Armstrong, L., M. Lako, et al. (2004). "A role for nucleoprotein Zap3 in the reduction of telomerase activity during embryonic stem cell differentiation." Mech Dev **121**(12): 1509-22.

Telomerase, the enzyme which maintains the ends of linear chromosomes in eukaryotic cells is found in murine embryonic stem cells; however, its

activity is downregulated during in vitro differentiation. Previous work has indicated that this is due to the transcriptional downregulation of murine reverse transcriptase unit (mTert) of telomerase. To investigate the factors that cause the transcriptional repression of mTert we defined a 300 bp region which is essential for its transcription and performed site directed mutagenesis and electrophoretic mobility shift assays. This analysis indicated that Sp1, Sp3 and c-Myc bind to the GC-boxes and E-boxes, respectively, within the promoter and help activate the transcription of mTert gene. We also identified a novel binding sequence, found repeated within the mTert core region, which when mutated caused increased mTert expression. Yeast one hybrid screening combined with electrophoretic mobility shift assays indicated that the nuclear protein Zap3 binds to this site and its overexpression leads to the downregulation of mTert during differentiation. This suggests that regulation of mTert transcription is a complex process which depends on a quantitative balance between transcription factors that cause activation or repression of this gene. Overexpression of Zap3 in murine embryonic stem cells results in reduction in telomerase activity and telomere length as well as reduced proliferative capacity and limited ability to contribute to the development of haematopoietic cells upon differentiation.

Arnaud, D., M. G. Mattei, et al. (1993). "A panel of deleted mouse X chromosome somatic cell hybrids derived from the embryonic stem cell line HD3 shows preferential breakage in the Hprt-DXHX254E region." Genomics **18**(3): 520-6.

A panel of 91 somatic cell hybrids containing deleted mouse X chromosomes and falling into seven nested intervals has been isolated and characterized from fusions involving the murine embryonic stem cell HD3. Many of the X chromosome breakpoints present in these hybrids fall within regions in which few or no other hybrids were previously available. The apparent enrichment for breakpoints lying within the Hprt-DXHX254E region is discussed in relation to both the nature of the embryonic stem cell fusions and the presence of the Fmr1 gene associated with FRAXA in man within this span.

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. 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.

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.

Baker, R. K., M. A. Haendel, et al. (1997). "In vitro preselection of gene-trapped embryonic stem cell clones for characterizing novel developmentally regulated genes in the mouse." Dev Biol **185**(2): 201-14.

We have developed an in vitro gene trap screen for novel murine genes that allows one to determine, prior to making chimeric or transgenic animals, if these genes are expressed in one or more specific embryonic tissues. Totipotent embryonic stem (ES) cells are infected with a retroviral gene trap construct encoding a selectable lacZ/neo fusion gene, which is expressed only if the gene trap inserts within an active transcription unit. Gene traps and their wild-type alleles are characterized in terms of copy number, alternate splicing of their transcripts, and the proportion of endogenous mRNA sequence that is replaced by lacZ/neo in the hybrid gene trap transcript. This approach, which we term "in vitro preselection," is more economical than standard in vivo gene trap screening because tissue-specific expression of probable knockout alleles is verified before transgenic animals are generated. These results also highlight the utility of ES cell differentiation in vitro as a method with which to study the molecular mechanisms regulating the specification and commitment of a variety of cell and tissue types.

Battista, S., F. Pentimalli, et al. (2003). "Loss of Hmga1 gene function affects embryonic stem cell lympho-hematopoietic differentiation." Faseb J **17**(11): 1496-8.

By interacting with transcription machinery, high-mobility group A 1 (HMGA1) proteins alter the chromatin structure and thereby regulate the transcriptional activity of several genes. The effect on megakaryocyte/erythrocyte lineages seems, at least in part, mediated by the GATA-1 transcription factor, a key regulator of red blood cell differentiation. In fact, we found that Hmga1-/- ES cells overexpress GATA-1 and that HMGA1 proteins directly control GATA-1 transcription. Taken together, these data indicate that HMGA1 proteins play a prime role in lymphohematopoietic differentiation.

Becker, K. A., P. N. Ghule, et al. (2006). "Self-renewal of human embryonic stem cells is supported by a shortened G1 cell cycle phase." J Cell Physiol **209**(3): 883-93.

Competency for self-renewal of human embryonic stem (ES) cells is linked to pluripotency. However, there is a critical paucity of fundamental parameters of human ES cell division. In this study we show that human ES cells (H1 and H9; NIH-designated WA01 and WA09) rapidly proliferate due to a very short overall cell cycle (15-16 h) compared to somatic cells (e.g., normal diploid IMR90 fibroblasts and NT-2 teratocarcinoma cells). The human ES cell cycle maintains the four canonical cell cycle stages G1, S, G2, and M, but the duration of G1 is dramatically shortened. Bromodeoxyuridine (BrdU) incorporation and FACS analysis demonstrated that 65% of asynchronously growing human ES cells are in S phase. Molecular analyses using quantitative RT-PCR demonstrate that human ES cells and somatic cells express similar cell cycle markers. However, among cyclins and cyclin-dependent kinases (CDKs), we observed high mRNA levels for the G1-related CDK4 and cyclin D2 genes. We conclude that human ES cells exhibit unique G1 cell cycle kinetics and use CDK4/cyclin D2 related mechanisms to attain competency for DNA replication.

Becker, K. A., J. L. Stein, et al. (2007). "Establishment of histone gene regulation and cell cycle checkpoint control in human embryonic stem cells." J Cell Physiol **210**(2): 517-26.

Rapid self-renewal of human embryonic stem (ES) cells (NIH designation WA01 and WA09) is accommodated by an abbreviated cell cycle due to a reduction in the G1 phase. Thus, molecular mechanisms operative in ES cells may expedite the cellular commitment to progress into S phase to initiate replication of DNA and biosynthesis of histone proteins to form new chromatin. Here we show that the selective cell cycle regulated expression of individual histone H4 gene copies, which is typical for somatic cell types, is already firmly established in human ES cells. This early establishment of H4 gene regulation, which is E2F independent, is consistent with co-expression of the cognate transcriptional regulators HiNF-P and p220(NPAT). Human ES cells differ from somatic cells in the expression of members of the E2F family and RB-related pocket proteins (p105(RB1), p107(RBL1), and p130(RBL2/RB2)) that control expression of genes encoding enzymes for nucleotide metabolism and DNA synthesis. Human ES cells rapidly and robustly (>200-fold) induce the cyclin dependent kinase (CDK) inhibitor p21(WAF1/CIP1) upon gamma-irradiation. This DNA

damage response promptly reduces histone gene expression as well as mRNA levels for HiNF-P and p220(NPAT) and causes accumulation of unprocessed histone H4 precursor RNAs. Furthermore, while E2F4, E2F5 and p130(RBL2/RB2) are the major E2F and pocket protein mRNAs in actively proliferating ES cells, expression levels of E2F5, E2F6, and p105(RB1) are most strongly elevated during cell cycle arrest in cells responding to DNA damage. Our data suggest that the brief G1 phase of ES cells is supported by a potent p21(WAF1/CIP1) related DNA damage response that functions through several mechanisms to rapidly inhibit cell cycle progression. This response may alter the E2F/pocket protein combinations that control E2F dependent genes and block H4 gene expression by inhibiting histone-specific transcription factors and processing of histone gene transcripts, as well as by destabilizing histone mRNAs.

Bhattacharya, B., S. Puri, et al. (2009). "A review of gene expression profiling of human embryonic stem cell lines and their differentiated progeny." Curr Stem Cell Res Ther **4**(2): 98-106.

One of the key characteristics of human embryonic stem cells (hESC) is their ability to proliferate for an indefinite period of time. Previous studies have shown that a unique network of transcription factors are involved in hESC self renewal. Since hESC lines have the potential to differentiate into cells of all three germ layers, cells derived from hESC may be useful for the treatment of a variety of inherited or acquired diseases. The molecular signal required to differentiate hESC into a particular cell type has not been defined. It is expected that global gene expression profiling of hESC may provide an insight into the critical genes involved in maintaining pluripotency of hESC and genes that are modulated when hESCs differentiate. Several groups have utilized a variety of high throughput techniques and performed gene expression profiling of undifferentiated hESCs and mouse ES cells (mESC) to identify a set of genes uniquely expressed in ES cells but not in mature cells and defined them as "stemness" genes. These molecular techniques include DNA microarray, EST-enumeration, MPSS profiling, and SAGE. Irrespective of the molecular technique used, highly expressed genes showed similar expression pattern in several ES cell lines supporting their importance. A set of approximately 100 genes were identified, which are highly expressed in ES cells and considered to be involved in maintaining pluripotency and self renewal of ES cells. Various studies have also reported on the gene expression profiling of differentiated embryoid bodies (EB) derived from hESCs and mESCs. When hESCs are differentiated, "stemness" genes are down-regulated and a set of genes are up-regulated. Together with down-modulation of "stemness" genes and up-regulation of new genes may provide a new insight into the molecular pathways of hESC differentiation and study of these genes may be useful in the characterization of differentiated cells.

Boheler, K. R. and K. V. Tarasov (2006). "SAGE analysis to identify embryonic stem cell-predominant transcripts." Methods Mol Biol **329**: 195-221.

The Human Genome Consortium has successfully sequenced the entire human genome (http://www.genome.gov/11006945), but an unfinished goal remains the identification of specific genes responsible for unique cellular processes. With respect to embryonic stem (ES) cells, this includes the identification of factors that govern self-renewal and pluripotentiality. One technique that facilitates this last goal is serial analysis of gene expression (SAGE), a functional genomics technique that identifies and quantifies mRNA transcripts. This technique relies on the preparation and sequencing of complementary DNA concatemers to rapidly generate a comprehensive profile of gene expression within a cell, and unlike microarrays, it does not require prior knowledge of the genes to be assayed. Because SAGE is a sequence-based technique, it can be used to search for ES-restricted genes (i.e., markers) by sequence comparisons among stem cells, differentiated cells, and tissues. These markers can then be genetically manipulated to understand the molecular basis for stem cell biology to help define how transcriptional mechanisms distinguish ES cells from other, less-pluripotent cell types. SAGE is, thus, a powerful technique that permits a comprehensive analysis of mRNA abundance that can define, at a molecular level, fundamental characteristics of ES cells. In this chapter, we illustrate the basic principles of SAGE, describe a complete protocol for the generation of SAGE libraries, and show how this technique can be employed to analyze embryonic stem cells.

Brenner, C. A., H. M. Kubisch, et al. (2004). "Role of the mitochondrial genome in assisted reproductive technologies and embryonic stem cell-based therapeutic cloning." Reprod Fertil Dev **16**(7): 743-51.

Mitochondria play a pivotal role in cellular metabolism and are important determinants of embryonic development. Mitochondrial function and biogenesis rely on an intricate coordination of regulation and expression of nuclear and mitochondrial genes. For example, several nucleus-derived transcription factors, such as mitochondrial transcription factor A, are required for mitochondrial

DNA replication. Mitochondrial inheritance is strictly maternal while paternally-derived mitochondria are selectively eliminated during early embryonic cell divisions. However, there are reports from animals as well as human patients that paternal mitochondria can occasionally escape elimination, which in some cases has led to severe pathologies. The resulting existence of different mitochondrial genomes within the same cell has been termed mitochondrial heteroplasmy. The increasing use of invasive techniques in assisted reproduction in humans has raised concerns that one of the outcomes of such techniques is an increase in the incidence of mitochondrial heteroplasmy. Indeed, there is evidence that heteroplasmy is a direct consequence of ooplasm transfer, a technique that was used to 'rescue' oocytes from older women by injecting ooplasm from young oocytes. Mitochondria from donor and recipient were found in varying proportions in resulting children. Heteroplasmy is also a byproduct of nuclear transfer, as has been shown in studies on cloned sheep, cattle and monkeys. As therapeutic cloning will depend on nuclear transfer into oocytes and the subsequent generation of embryonic stem cells from resulting blastocysts, the prospect of mitochondrial heteroplasmy and its potential problems necessitate further studies in this area.

Brimble, S. N., X. Zeng, et al. (2004). "Karyotypic stability, genotyping, differentiation, feeder-free maintenance, and gene expression sampling in three human embryonic stem cell lines derived prior to August 9, 2001." Stem Cells Dev **13**(6): 585-97.

The number of human embryonic stem cell (hESC) lines available to federally funded U.S. researchers is currently limited. Thus, determining their basic characteristics and disseminating these lines is important. In this report, we recovered and expanded the earliest available cryopreserved stocks of the BG01, BG02, and BG03 hESC lines. These cultures exhibited multiple definitive characteristics of undifferentiated cells, including long-term self-renewal, expression of markers of pluripotency, maintenance of a normal karyotype, and differentiation to mesoderm, endoderm, and ectoderm. Each cell line exhibited a unique genotype and human leukocyte antigen (HLA) isotype, confirming that they were isolated independently. BG01, BG02, and BG03 maintained in feederfree conditions demonstrated self-renewal, maintenance of normal karyotype, and gene expression indicative of undifferentiated pluripotent stem cells. A survey of gene expression in BG02 cells using massively parallel signature sequencing generated a digital read-out of transcript abundance and showed that this line was similar to other hESC lines. BG01, BG02, and BG03 hESCs are therefore independent, undifferentiated, and pluripotent lines that can be maintained without accumulation of karyotypic abnormalities.

Brown, J., M. F. Greaves, et al. (1991). "The gene encoding the stem cell antigen, CD34, is conserved in mouse and expressed in haemopoietic progenitor cell lines, brain, and embryonic fibroblasts." Int Immunol **3**(2): 175-84.

The human haemopoietic cell surface antigen, CD34, is a 105 - 120 kd cell surface glycoprotein whose stage-specific expression by stem cells and lineage-specific progenitor cells suggests a role in regulating early events in blood cell differentiation. A murine gene and cDNA encoding a closely homologous protein have been isolated. The gene is organized in eight exons in 22 kb of DNA. The first exon lies in a GC- and CpG-rich island. The sequence of the gene and the cDNA predict a 382 amino acid-long protein containing an N-terminal signal peptide and one transmembrane region 73 amino acids from the C-terminus. The extracellular part of the protein contains: a 140 amino acid-long-N-terminal region, 40% of whose residues are serine or threonine potential attachment sites for O-linked carbohydrate, as well as five potential attachment sites for N-linked carbohydrate. Proximal to the extracellular membrane there is a 79 amino acid-long cysteine-rich region. The homology with the human sequence is highest in the intracellular domain (90% amino acid identity) and lowest in the N-terminal region (43% amino acid identity). The protein is not homologous with any other proteins currently in the databases. The expression of the murine gene by a number of haemopoietic progenitor cell lines suggests that the CD34 function in haemopoiesis may be conserved between man and mouse. The high level of expression in a number of embryonic fibroblast cell lines and in brain imply a function outside of haemopoiesis.

Byrne, J. A., D. A. Pedersen, et al. (2007). "Producing primate embryonic stem cells by somatic cell nuclear transfer." Nature **450**(7169): 497-502.

Derivation of embryonic stem (ES) cells genetically identical to a patient by somatic cell nuclear transfer (SCNT) holds the potential to cure or alleviate the symptoms of many degenerative diseases while circumventing concerns regarding rejection by the host immune system. However, the concept has only been achieved in the mouse, whereas inefficient reprogramming and poor embryonic development characterizes the results obtained in primates. Here, we used a modified SCNT approach to produce rhesus macaque blastocysts from adult skin fibroblasts, and successfully isolated two ES cell lines from these

embryos. DNA analysis confirmed that nuclear DNA was identical to donor somatic cells and that mitochondrial DNA originated from oocytes. Both cell lines exhibited normal ES cell morphology, expressed key stem-cell markers, were transcriptionally similar to control ES cells and differentiated into multiple cell types in vitro and in vivo. Our results represent successful nuclear reprogramming of adult somatic cells into pluripotent ES cells and demonstrate proof-of-concept for therapeutic cloning in primates.

Cai, C., J. Thorne, et al. (2008). "Hedgehog serves as a mitogen and survival factor during embryonic stem cell neurogenesis." Stem Cells **26**(5): 1097-108.

Hedgehog (Hh) signaling is involved in a wide range of important biological activities. Within the vertebrate central nervous system, Sonic Hedgehog (Shh) can act as a morphogen or mitogen that regulates the patterning, proliferation, and survival of neural stem cells (NSCs). However, its role in embryonic stem cell (ESC) neurogenesis has not been explored in detail. We have previously shown that Hh signaling is required for ESC neurogenesis. In order to elucidate the underlying mechanism, we utilized the Sox1-GFP ESC line, which has a green fluorescent protein (GFP) reporter under the control of the Sox1 gene promoter, providing an easy means of detecting NSCs in live cell culture. We show here that ESC differentiation in adherent culture follows the ESC--> primitive ectoderm --> neurectoderm transitions observed in vivo. Selective death of the Sox1-GFP-negative cells contributes to the enrichment of Sox1-GFP-positive NSCs. Interestingly, Shh is expressed exclusively by the NSCs themselves and elicits distinct downstream gene expression in Sox1-GFP-positive and -negative cells. Suppression of Hh signaling by antagonist treatment leads to different responses from these two populations as well: increased apoptosis in Sox1-GFP-positive NSCs and decreased proliferation in Sox1-GFP-negative primitive ectoderm cells. Hedgehog agonist treatment, in contrast, inhibits apoptosis and promotes proliferation of Sox1-GFP-positive NSCs. These results suggest that Hh acts as a mitogen and survival factor during early ESC neurogenesis, and evidence is presented to support a novel autocrine mechanism for Hh-mediated effects on NSC survival and proliferation.

Cameron, C. M., F. Harding, et al. (2008). "Activation of hypoxic response in human embryonic stem cell-derived embryoid bodies." Exp Biol Med (Maywood) **233**(8): 1044-57.

Oxygen tension can provide an important determinant for differentiation and development of many cells and tissues. Genetic regulation of hemato-endothelial commitment is known to respond to oxygen deprivation via stimulation of hypoxia inducible factors (HIFs). Here, we use a closed bioreactor system to monitor and control the dissolved oxygen during differentiation of human embryonic stem cells (hESCs) via formation of embryoid bodies (hEBs). Exposing hESC-derived EBs to ambient oxygen at or below 5% results in stabilization of HIF-1alpha and increased transcription of hypoxic responsive genes. Interestingly, we find that rather than HIF-1alpha expression being stable over prolonged (7-16 days) culture in hypoxic conditions, HIF-1alpha expression peaks after approximately 48 hours of hypoxic exposure, and then declines to near undetectable levels, despite constant hypoxic exposure. This transient stabilization of HIF-1alpha during hESC-derived EB culture is demonstrated for four distinct stages of differentiation. Furthermore, we demonstrate hEB cell expansion is slowed by hypoxic exposure, with increased apoptosis. However, hEB cell proliferation returns to normal rates upon return to normoxic conditions. Therefore, although hypoxia effectively stimulates hypoxic responsive genes, this single variable was not sufficient to improve development of hemato-endothelial cells from hESCs.

Camper, S. A., T. L. Saunders, et al. (1995). "Implementing transgenic and embryonic stem cell technology to study gene expression, cell-cell interactions and gene function." Biol Reprod **52**(2): 246-57.

This review highlights the use of transgenic mice and gene targeting in the study of reproduction, pituitary gene expression, and cell lineage. Since 1980 numerous applications of transgenic animal technology have been reported. Altered phenotypes resulting from transgene expression demonstrated that introduced genes can exert profound effects on animal physiology. Transgenic mice have been important for the study of hormonal and developmental control of gene expression because gene expression in whole animals often requires more DNA sequence information than is necessary for expression in cell cultures. This point is illustrated by studies of pituitary glycoprotein hormone alpha- and beta-subunit gene expression (Kendall et al., Mol Endocrinol 1994; in press [1]. Transgenic mice have also been invaluable for producing animal models of cancer and other diseases and testing the efficacy of gene therapy. In addition, cell-cell interactions and cell lineage relationships have been explored by cell-specific expression of toxin genes in transgenic mice. Recent studies suggest that attenuated and inducible toxins hold promise for future transgene ablation experiments. Since 1987, embryonic stem (ES) cell

technology has been used to create numerous mouse strains with targeted gene alterations, contributing enormously to our understanding of the functional importance of individual genes. For example, the unexpected development of gonadal tumors in mice with a targeted disruption of the inhibin gene revealed a potential role for inhibin as a tumor suppressor (Matzuk et al., Nature 1992:360: 313-319 [2]. The transgenic and ES cell technologies will undoubtedly continue to expand our understanding and challenge our paradigms in reproductive biology.

Davey, R. E. and P. W. Zandstra (2006). "Spatial organization of embryonic stem cell responsiveness to autocrine gp130 ligands reveals an autoregulatory stem cell niche." Stem Cells **24**(11): 2538-48.

Highly ordered aggregates of cells, or niches, regulate stem cell fate. Specific tissue location need not be an obligatory requirement for a stem cell niche, particularly during embryogenesis, where cells exist in a dynamic environment. We investigated autoregulatory fixed-location-independent processes controlling cell fate by analyzing the spatial organization of embryonic stem cells (ESCs) using quantitative single-cell immunocytochemistry and a computational approach involving Delaunay triangulation. ESC colonies demonstrated radial organization of phosphorylated signal transducer and activator of transcription 3, Nanog, and Oct4 (among others) in the presence and absence of exogenous leukemia inhibitory factor (LIF). Endogenous self-renewal signaling resulted from autocrine non-LIF gp130 ligands, which buffered cells against differentiation upon exogenous LIF deprivation. Together with a radial organization of differential responsiveness to gp130 ligands within colonies, autocrine signaling produced a radial organization of self-renewal, generating a fixed-location-independent autoregulatory niche. These findings reveal fundamental properties of niches and elucidate mechanisms colonies of cells use to transition between fates during morphogenesis.

David, R., H. Theiss, et al. (2008). "Connexin 40 promoter-based enrichment of embryonic stem cell-derived cardiovascular progenitor cells." Cells Tissues Organs **188**(1-2): 62-9.

BACKGROUND: Pluripotent embryonic stem (ES) cells that can differentiate into functional cardiomyocytes as well as vascular cells in cell culture may open the door to cardiovascular cell transplantation. However, the percentage of ES cells in embryoid bodies (EBs) which spontaneously undergo cardiovascular differentiation is low (<10%), making strategies for their specific labeling and purification indispensable. METHODS: The human connexin 40 (Cx40) promoter was isolated and cloned in the vector pEGFP. The specificity of the construct was initially assessed in Xenopus embryos injected with Cx40-EGFP plasmid DNA. Stable Cx40-EGFP ES cell clones were differentiated and fluorescent cells were enriched manually as well as via fluorescence-activated cell sorting. Characterization of these cells was performed with respect to spontaneous beating as well as via RT-PCRs and immunofluorescent stainings. RESULTS: Cx40-EGFP reporter plasmid injection led to EGFP fluorescence specifically in the abdominal aorta of frog tadpoles. After crude manual enrichment of highly Cx40-EGFP-positive EBs, the appearance of cardiac and vascular structures was increased approximately 3-fold. Immunofluorescent stainings showed EGFP expression exclusively in vascular-like structures simultaneously expressing von Willebrand factor and in formerly beating areas expressing alpha-actinin. Cx40-EGFP-expressing EBs revealed significantly higher numbers of beating cardiomyocytes and vascular-like structures. Semiquantitative RT-PCRs confirmed an enhanced cardiovascular differentiation as shown for the cardiac markers Nkx2.5 and MLC2v, as well as the endothelial marker vascular endothelial cadherin. CONCLUSIONS: Our work shows the feasibility of specific labeling and purification of cardiovascular progenitor cells from differentiating EBs based on the Cx40 promoter. We provide proof of principle that the deleted CD4 (DeltaCD4) surface marker-based method for magnetic cell sorting developed by our group will be ideally suitable for transference to this promoter.

de Waard, H., E. Sonneveld, et al. (2008). "Cell-type-specific consequences of nucleotide excision repair deficiencies: Embryonic stem cells versus fibroblasts." DNA Repair (Amst) **7**(10): 1659-69.

Pluripotent embryonic stem cells (ES cells) are the precursors of all different cell types comprising the organism. Since persistent DNA damage in this cell type might lead to mutations that cause huge malformations in the developing organism, genome caretaking is of prime importance. We first compared the sensitivity of wild type mouse embryonic fibroblasts (MEFs) and ES cells for various genotoxic agents and show that ES cells are more sensitive to treatment with UV-light, gamma-rays and mitomycin C than MEFs. We next investigated the contribution of the transcription-coupled (TC-NER) and global genome (GG-NER) sub-pathways of nucleotide excision repair (NER) in protection of ES cells, using cells from mouse models for the NER disorders xeroderma pigmentosum (XP) and Cockayne syndrome (CS). TC-NER-deficient Csb(-/-) and GG-NER/TC-NER-defective Xpa(-/-)

MEFs are hypersensitive to UV, whereas GG-NER-deficient Xpc(-/-) MEFs attribute intermediate UV sensitivity. The observed UV-hypersensitivity in Csb(-/-) and Xpa(-/-) MEFs correlates with increased apoptosis. In contrast, Xpa(-/-) and Xpc(-/-) ES cells are highly UV-sensitive, while a Csb deficiency only causes a mild increase in UV-sensitivity. Surprisingly, a UV-induced hyperapoptotic response is mainly observed in Xpa(-/-) ES cells, suggesting a different mechanism of apoptosis induction in ES cells, mainly triggered by damage in the global genome rather than in transcribed genes (as in MEFs). Moreover, we show a pronounced S-phase delay in Xpa(-/-) and Xpc(-/-) ES cells, which might well function as a safeguard mechanism for heavily damaged ES cells in case the apoptotic response fails. Although Xpa(-/-) and Xpc(-/-) ES cells are totally NER-defective or GG-NER-deficient respectively, mutation induction upon UV is similar compared to wild type ES cells indicating that the observed apoptotic and cell cycle responses are indeed sufficient to protect against proliferation of damaged cells. In conclusion, we show a double safeguard mechanism in ES cells against NER-type of damages, which mainly relies on damage detection in the global genome.

Deleu, S., E. Gonzalez-Merino, et al. (2009). "Human cystic fibrosis embryonic stem cell lines derived on placental mesenchymal stromal cells." Reprod Biomed Online **18**(5): 704-16.

This study describes the production of two new human embryonic stem cell (hESC) lines affected by cystic fibrosis. These cell lines are heterozygous compounds, each a carrier of the DF508 mutations associated either with E585X or with 3849+10 kb C-->T. The derivation process was performed on irradiated human placental mesenchymal stromal cells and designed to minimize contact with xeno-components. This new source of feeder cells is easy to obtain and devoid of ethical concerns. The cells have a great capacity to proliferate which reduces the need for continuous preparation of new feeder cell lines. In addition, three normal hESC lines were obtained in the same conditions. The five stem cell lines retained hESC-specific features, including an unlimited and undifferentiated proliferation capacity, marker expression and the maintenance of stable karyotype. They also demonstrated pluripotency in vitro, forming cell lineages of the three germ layers, as indicated by immunolocalization of beta-tubulin, alpha-fetoprotein and actin. These new genetic cell lines represent an important in-vitro tool to study the physiological processes underlying this genetic disease, drug screening, and tissue engineering.

Deng, T., Y. Kuang, et al. (2007). "Disruption of imprinting and aberrant embryo development in completely inbred embryonic stem cell-derived mice." Dev Growth Differ **49**(7): 603-10.

The completely embryonic stem (ES) cell-derived mice (ES mice) produced by tetraploid embryo complementation provide us with a rapid and powerful approach for functional genome analysis. However, inbred ES cell lines often fail to generate ES mice. The genome of mouse ES cells is extremely unstable during in vitro culture and passage, and the expression of the imprinted genes is most likely to be affected. Whether the ES mice retain or repair the abnormalities of the donor ES cells has still to be determined. Here we report that the inbred ES mice were efficiently produced with the inbred ES cell line (SCR012). The ES fetuses grew more slowly before day 17.5 after mating, but had an excessive growth from day 17.5 to birth. Five imprinted genes examined (H19, Igf2, Igf2r, Peg1, Peg3) were expressed abnormally in ES fetuses. Most remarkably, the expression of H19 was dramatically repressed in the ES fetuses through the embryo developmental stage, and this repression was associated with abnormal biallelic methylation of the H19 upstream region. The altered methylation pattern of H19 was further demonstrated to have arisen in the donor ES cells and persisted on in vivo differentiation to the fetal stage. These results indicate that the ES fetuses did retain the epigenetic alterations in imprinted genes from the donor ES cells.

Deshpande, A. M., Y. S. Dai, et al. (2009). "Cdk2ap1 is required for epigenetic silencing of Oct4 during murine embryonic stem cell differentiation." J Biol Chem **284**(10): 6043-7.

Oct4 is a known master regulator of stem cell renewal and differentiation. Expression of Oct4 during differentiation is regulated by promoter methylation by the nucleosome remodeling and histone deacetylation (NuRD) complex. Here, we show that Cdk2ap1, a negative regulator of Cdk2 function and cell cycle, promotes Oct4 promoter methylation during murine embryonic stem cell differentiation to down-regulate Oct4 expression. We further show that this repressor function of Cdk2ap1 is dependent on its physical interaction with the methyl DNA-binding protein, Mbd3. Our data support a potential molecular link between the known differentiation promoters, including bone morphogenetic proteins and transforming growth factor signaling, and embryonic stem cell differentiation.

Dighe, V., L. Clepper, et al. (2008). "Heterozygous embryonic stem cell lines derived from nonhuman primate parthenotes." Stem Cells **26**(3): 756-66.

Monoparental parthenotes represent a potential source of histocompatible stem cells that should be isogenic with the oocyte donor and therefore suitable for use in cell or tissue replacement therapy. We generated five rhesus monkey parthenogenetic embryonic stem cell (PESC) lines with stable, diploid female karyotypes that were morphologically indistinguishable from biparental controls, expressed key pluripotent markers, and generated cell derivatives representative of all three germ layers following in vivo and in vitro differentiation. Interestingly, high levels of heterozygosity were observed at the majority of loci that were polymorphic in the oocyte donors. Some PESC lines were also heterozygous in the major histocompatibility complex region, carrying haplotypes identical to those of the egg donor females. Expression analysis revealed transcripts from some imprinted genes that are normally expressed from only the paternal allele. These results indicate that limitations accompanying the potential use of PESC-derived phenotypes in regenerative medicine, including aberrant genomic imprinting and high levels of homozygosity, are cell line-dependent and not always present. PESC lines were derived in high enough yields to be practicable, and their derivatives are suitable for autologous transplantation into oocyte donors or could be used to establish a bank of histocompatible cell lines for a broad spectrum of patients.

Du, K. L., M. Chen, et al. (2004). "Megakaryoblastic leukemia factor-1 transduces cytoskeletal signals and induces smooth muscle cell differentiation from undifferentiated embryonic stem cells." J Biol Chem **279**(17): 17578-86.

The SAP domain transcription factor myocardin plays a critical role in the transcriptional program regulating smooth muscle cell differentiation. In this report, we describe the capacity of myocardin to physically associate with megakaryoblastic leukemia factor-1 (MKL1) and characterize the function of MKL1 in smooth muscle cells (SMCs). The MKL1 gene is expressed in most human tissues and myocardin and MKL are co-expressed in SMCs. MKL1 and myocardin physically associate via conserved leucine zipper domains. Overexpression of MKL1 transactivates serum response factor (SRF)-dependent SMC-restricted transcriptional regulatory elements including the SM22alpha promoter, smooth muscle myosin heavy chain promoter/enhancer, and SM-alpha-actin promoter/enhancer in non-SMCs. Moreover, forced expression of MKL1 and SRF in undifferentiated SRF(-/-) embryonic stem cells activates multiple endogenous SMC-restricted genes at levels equivalent to, or exceeding, myocardin. Forced expression of a dominant-negative MKL1 mutant reduces myocardin-induced activation of the SMC-specific SM22alpha promoter. In NIH3T3 fibroblasts MKL1 localizes to the cytoplasm and translocates to the nucleus in response to serum stimulation, actin treadmilling, and RhoA signaling. In contrast, in SMCs MKL1 is observed exclusively in the nucleus regardless of serum conditions or RhoA signaling. However, when actin polymerization is disrupted MKL1 translocates from the nucleus to the cytoplasm in SMCs. Together, these data were consistent with a model wherein MKL1 transduces signals from the cytoskeleton to the nucleus in SMCs and regulates SRF-dependent SMC differentiation autonomously or in concert with myocardin.

Duval, D., B. Reinhardt, et al. (2000). "Role of suppressors of cytokine signaling (Socs) in leukemia inhibitory factor (LIF) -dependent embryonic stem cell survival." Faseb J **14**(11): 1577-84.

Mouse embryonic stem (ES) cells remain pluripotent in vitro when grown in the presence of leukemia inhibitory factor (LIF). LIF withdrawal results in progressive ES cell differentiation. Here we show that during this differentiation process, part of the cells undergo apoptosis concomitant with an activation of the p38 MAP kinase. To gain insight into events mediated by LIF in ES cells, the expression of potential candidate genes was analyzed in the absence or presence of this cytokine by using a semiquantitative RT-PCR assay. We focused on early response genes and on a new type of cytokine repressors (the Socs proteins), some of which exhibit anti-apoptotic properties. We found that expression of c-Fos, c-Jun, and JunB was induced upon LIF treatment whereas that of JunD, the tyrosine phosphatase ESP, and the components of the LIF receptor remained unaffected. Expression of Socs-3, but not Socs-1 or Socs-2, was stimulated in the presence of LIF. Finally, uncontrolled overexpression of Socs-1 and Socs-3 led to repression of LIF-dependent transcription and severely reduced cell viability, suggesting that the disturbance of a well balanced Socs protein content has adverse effects on cell survival.

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.

Ezeh, U. I., P. J. Turek, et al. (2005). "Human embryonic stem cell genes OCT4, NANOG, STELLAR, and GDF3 are expressed in both seminoma and breast carcinoma." Cancer **104**(10): 2255-65.

BACKGROUND: The seminoma class of testicular germ cell tumor (TGCT) are characterized by a morphological resemblance to primordial germ cells (PGCs) or gonocytes, and chromosome duplications at 12p. Recently, it was determined that human embryonic stem cells (hESCs) express genes in common with PGCs, and that three of these genes, GDF3, STELLAR, and NANOG, are located on 12p. The current study was designed to identify whether expression of these 12p genes were elevated in seminoma relative to normal testis, and to determine whether elevated expression was unique to seminoma. METHODS: Real-time polymerase chain reaction (PCR) and immunohistochemistry were used to assess gene expression in seminoma samples relative to normal testis and endpoint PCR was used to identify the presence or absence of these genes in breast carcinoma. RESULTS: GDF3 expression was increased in eight of nine seminomas compared with normal testis, whereas NANOG, OCT4, or both were expressed at the highest levels in seminoma compared with all other markers analyzed. In addition, the NANOG protein was expressed in the majority of seminoma cells. The adult meiotic germ cell markers BOULE and TEKT1 were undetectable in seminoma, whereas the embryonic and adult germ cell markers DAZL and VASA were significantly reduced. Analysis of these markers in breast carcinoma and the MCF7 breast carcinoma cell line revealed that a core hESC-transcriptional profile could be identified consisting of OCT4, NANOG, STELLAR, and GDF3 and that NANOG protein could be detected in breast carcinoma. CONCLUSIONS: These observations suggest that seminoma and breast carcinoma express a common stem cell profile and that the expression of DAZL and VASA in seminoma mark the germ cell origin of seminoma that is absent in breast carcinoma. Our findings suggest that stem cell genes may either play a direct role in different types of carcinoma progression or serve as valuable markers of tumorigenesis.

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.

Faustino, R. S., A. Behfar, et al. (2008). "Genomic chart guiding embryonic stem cell cardiopoiesis." Genome Biol **9**(1): R6.

BACKGROUND: Embryonic stem cells possess a pluripotent transcriptional background with the developmental capacity for distinct cell fates. Simultaneous expression of genetic elements for multiple outcomes obscures cascades relevant to specific cell phenotypes. To map molecular patterns critical to cardiogenesis, we interrogated gene expression in stem cells undergoing guided differentiation, and defined a genomic paradigm responsible for confinement of pluripotency. RESULTS: Functional annotation analysis of the transcriptome of differentiating embryonic stem cells exposed downregulated components of DNA replication, recombination and repair machinery, cell cycling, cancer mechanisms, and RNA post-translational modifications. Concomitantly, cardiovascular development, cell-to-cell signaling, cell development and cell movement were upregulated. These simultaneous gene ontology rearrangements engaged a repertoire switch that specified lineage development. Bioinformatic integration of genomic and gene ontology data further unmasked canonical signaling cascades prioritized within discrete phases of cardiopoiesis. Examination of gene relationships revealed a non-stochastic network anchored by integrin, WNT/beta-catenin, transforming growth factor beta and vascular endothelial growth factor pathways, validated by manipulation of selected cascades that promoted or restrained cardiogenic yield. Moreover, candidate genes within anchor pathways acted as nodes that organized correlated expression profiles into functional clusters, which collectively orchestrated and secured an overall cardiogenic theme. CONCLUSION: The present systems biology approach reveals a dynamically integrated and tractable gene network fundamental to embryonic stem cell specification, and represents an initial step towards resolution of a genomic cardiopoietic atlas.

Filippi, M. D., F. Porteu, et al. (2000). "Embryonic stem cell differentiation to hematopoietic cells: A model to study the function of various regions of the intracytoplasmic domain of cytokine receptors in vitro." Exp Hematol **28**(12): 1363-72.

To examine whether the in vitro model of embryonic stem (ES) cell hematopoietic differentiation is suitable to study the function of intracytoplasmic regions of cytokine receptors, we used the thrombopoietin receptor Mpl as a typical cytokine receptor.ES cells deficient in c-mpl (mpl(-/)-) were transfected with genes encoding the full-length or two mutated forms of the intracytoplasmic domain of Mpl using the pEF-BOS expression vector. The mutated forms lack box1 or box2.pEF-BOS was able to maintain protein production during ES cell differentiation. Reintroduction of full-length-c-mpl into mpl(-/)- ES cells restored the response of megakaryocyte progenitors to a truncated form of human Mpl-ligand conjugated to polyethylene glycol (PEG-rhuMGDF) and the formation of platelets, for which mpl(-/)- ES cells are defective. In addition, enforced expression of Mpl resulted in the development of all myeloid progenitors and mature cells in the presence of PEG-rhuMGDF. Blast colony-forming cells, the in vitro equivalent of the hemangioblast, also generated blast cell colonies with a hematopoietic potential equivalent to that of the wild type in the presence of PEG-rhuMGDF, although its growth is normally dependent on vascular endothelial cell growth factor (VEGF). Thus, Mpl acts as a substitute for other cytokine receptors and for a tyrosine kinase receptor, Flk-1, indicating that Mpl has no instructive role in hematopoietic cell commitment and differentiation. The Mpl mutant forms lacking box1 or box2 prevented response of ES cell-derived blast colony-forming cells or progenitors to PEG-rhuMGDF. Therefore, these two regions, essential for signaling by cytokine receptors, are required for the responses of ES cell-derived hematopoietic cells to PEG-rhuMGDF.These results show that the in vitro hematopoietic differentiation of ES cells is suitable for studying the role of various intracytoplasmic regions of cytokine receptors.

Fu, J., S. S. Tay, et al. (2006). "High glucose alters the expression of genes involved in proliferation and cell-fate specification of embryonic neural stem cells." Diabetologia **49**(5): 1027-38.

AIMS/HYPOTHESIS: Maternal diabetes induces neural tube defects during embryogenesis. Since the neural tube is derived from neural stem cells (NSCs), it is hypothesised that in diabetic pregnancy neural tube defects result from altered expression of developmental control genes, leading to abnormal proliferation and cell-fate choice of NSCs. MATERIALS AND METHODS: Cell viability, proliferation index and apoptosis of NSCs and differentiated cells from mice exposed to physiological or high glucose concentration medium were examined by a tetrazolium salt assay, 5-bromo-2'-deoxyuridine incorporation, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling and immunocytochemistry. Expression of developmental genes, including sonic hedgehog (Shh), bone morphogenetic protein 4 (Bmp4),

neurogenin 1/2 (Neurog1/2), achaete-scute complex-like 1 (Ascl1), oligodendrocyte transcription factor 1 (Olig1), oligodendrocyte lineage transcription factor 2 (Olig2), hairy and enhancer of split 1/5 (Hes1/5) and delta-like 1 (Dll1), was analysed by real-time RT-PCR. Proliferation index and neuronal specification in the forebrain of embryos at embryonic day 11.5 were examined histologically. RESULTS: High glucose decreased the proliferation of NSCs and differentiated cells. The incidence of apoptosis was increased in NSCs treated with high glucose, but not in the differentiated cells. High glucose also accelerated neuronal and glial differentiation from NSCs. The decreased proliferation index and early differentiation of neurons were evident in the telencephalon of embryos derived from diabetic mice. Exposure to high glucose altered the mRNA expression levels of Shh, Bmp4, Neurog1/2, Ascl1, Hes1, Dll1 and Olig1 in NSCs and Shh, Dll1, Neurog1/2 and Hes5 in differentiated cells. CONCLUSIONS/INTERPRETATION: The changes in proliferation and differentiation of NSCs exposed to high glucose are associated with altered expression of genes that are involved in cell-cycle progression and cell-fate specification during neurulation. These changes may form the basis for the defective neural tube patterning observed in embryos of diabetic pregnancies.

Fujii-Yamamoto, H., J. M. Kim, et al. (2005). "Cell cycle and developmental regulations of replication factors in mouse embryonic stem cells." J Biol Chem **280**(13): 12976-87.

Embryonic stem (ES) cells can grow rapidly and permanently while maintaining their differentiation capacity. To gain insight into how the cell cycle progression of undifferentiated murine ES cells is regulated, we have examined the expression patterns of various replication and cell cycle regulators. Most factors including cyclins, Cdc6, and geminin are rather constitutively expressed during the cell cycle of ES cells. Furthermore, the transcript levels of almost all the cell cycle regulators we investigated except for p21 and p27 are higher in undifferentiated ES cells than in murine embryonic fibroblasts (MEFs), and the increased stability of mRNA in ES cells may be partially responsible for this at least with some of the factors. More strikingly, the transcriptional levels of these factors are strongly correlated with the acetylated state of histone H3 at their promoter regions. However, the methylation state of histone or CpG methylation of the promoter region is not generally correlated significantly with the expression pattern of these factors in both cell types. On the protein level, Cdc6, ASK, cyclin A2, and cyclin B1 are extremely abundant in ES cells compared with MEFs. Furthermore, they are rapidly down-regulated upon induction of differentiation of ES cells. The significance of these findings is discussed in relation to the unusual proliferative properties of ES cells in an undifferentiated state.

Fujimoto, T., M. Ogawa, et al. (2001). "Step-wise divergence of primitive and definitive haematopoietic and endothelial cell lineages during embryonic stem cell differentiation." Genes Cells **6**(12): 1113-27.

BACKGROUND: The developmental processes leading from the mesoderm to primitive and definitive haematopoietic and endothelial lineages, although of great importance, are still poorly defined. Recent studies have suggested a model in which common precursors give rise to endothelial progenitors and haematopoietic progenitors, the latter subsequently generating both primitive and definitive haematopoietic lineages. However, this model is contradicted by findings that suggest the emergence of haematopoietic cells from the endothelial lineage. RESULTS: We found sequential steps in the differentiation of FLK1+ mesoderm into haematopoietic and endothelial lineages in an in vitro differentiation system of embryonic stem (ES) cells: (i) the GATA-1+ subset of FLK1+ mesodermal cells loses the capacity to give rise to endothelial cells and is restricted to primitive erythroid, macrophage and definitive erythroid progenitors; (ii) the remaining GATA-1- cells give rise to VE-cadherin+ endothelial cells; and subsequently (iii) multiple definitive haematopoietic progenitors and endothelial cells branch off from a subset of VE-cadherin+ cells. CONCLUSIONS: These observations strongly suggest that the divergence of primitive and multilineage definitive haematopoietic/endothelial lineages occurs first, and then multilineage definitive haematopoietic progenitors arise from VE-cadherin+ endothelial cells in the development of haematopoietic and endothelial cells.

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, andH19, showed differential expression. Our results suggest that in addition to differences in gene expression profiles, epigenetic status was altered in the three cell subpopulations.

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, F., S. W. Kwon, et al. (2009). "PARP1 poly(ADP-ribosyl)ates Sox2 to control Sox2 protein levels and FGF4 expression during embryonic stem cell differentiation." J Biol Chem **284**(33): 22263-73.

Transcription factors Oct4 and Sox2 are key players in maintaining the pluripotent state of embryonic stem cells (ESCs). Small changes in their levels disrupt normal expression of their target genes. However, it remains elusive how protein levels of Oct4 and Sox2 and expression of their target genes are precisely controlled in ESCs. Here we identify PARP1, a DNA-binding protein with an NAD+-dependent enzymatic activity, as a cofactor of Oct4 and Sox2 to regulate expression of their target gene FGF4. We demonstrate for the first time that PARP1 binds the FGF4 enhancer to positively regulate FGF4 expression. Our data show that PARP1 interacts with and poly(ADP-ribosyl)ates Sox2 directly, which may be a step required for dissociation and degradation of inhibitory Sox2 proteins from the FGF4 enhancer. When PARP1 activity is inhibited or absent, poly(ADP-ribosyl)ation of Sox2 decreases and association of Sox2 with FGF4 enhancers increases, accompanied by an elevated level of Sox2 proteins and reduced expression of FGF4. Significantly, specific knockdown of Sox2 expression by RNA interference can considerably abrogate the inhibitory effect of the poly(ADP-ribose) polymerase inhibitor on FGF4 expression. Interestingly, PARP1 deficiency does not affect undifferentiated ESCs but compromises cell survival and/or growth when ESCs are induced into differentiation. Addition of FGF4 can partially rescue the phenotypes caused by PARP1 deficiency during ESC differentiation. Taken together, this study uncovers new mechanisms through which Sox2 protein levels and FGF4 expression are dynamically regulated during ESC differentiation and adds a new member to the family of proteins regulating the properties of ESCs.

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.

Garcia-Gonzalo, F. R. and J. C. Izpisua Belmonte (2008). "Albumin-associated lipids regulate human embryonic stem cell self-renewal." PLoS One **3**(1): e1384.

BACKGROUND: Although human embryonic stem cells (hESCs) hold great promise as a source of differentiated cells to treat several human diseases, many obstacles still need to be surmounted before this can become a reality. First among these, a robust chemically-defined system to expand hESCs in culture is still unavailable despite recent advances in the understanding of factors controlling hESC self-renewal. METHODOLOGY/PRINCIPAL FINDINGS: In this study, we attempted to find new molecules that stimulate long term hESC self-renewal. In order to do this, we started from the observation that a commercially available serum replacement product has a strong positive effect on the expansion of undifferentiated hESCs when added to a previously reported chemically-defined medium. Subsequent experiments demonstrated that the active ingredient within the serum replacement is lipid-rich albumin. Furthermore, we show that this activity is trypsin-resistant, strongly suggesting that lipids and not albumin are responsible for the effect. Consistent with this, lipid-poor albumin shows no detectable activity. Finally, we identified the major lipids bound to the lipid-rich albumin and tested several lipid candidates for the effect. CONCLUSIONS/SIGNIFICANCE: Our discovery of the role played by albumin-associated lipids in stimulating hESC self-renewal constitutes a significant advance in the knowledge of how hESC pluripotency is maintained by extracellular factors and has important applications in the development of increasingly chemically defined hESC culture systems.

Gasparrini, B., S. Gao, et al. (2003). "Cloned mice derived from embryonic stem cell karyoplasts and activated cytoplasts prepared by induced enucleation." Biol Reprod **68**(4): 1259-66.

Our objective was to induce enucleation (IE) of activated mouse oocytes to yield cytoplasts capable of supporting development following nuclear transfer. Fluorescence microscopy for microtubules, microfilaments, and DNA was used to evaluate meiotic resumption after ethanol activation and the effect of subsequent transient treatments with 0.4 micro g/ml of demecolcine. Using oocytes from B6D2F1 (C57BL/6 x DBA/2) donors, the success of IE of chromatin into polar bodies (PBs) was dependent on the duration of demecolcine treatment and the time that such treatment was initiated after activation. Similarly, variations in demecolcine treatment altered the proportions of oocytes exhibiting a reversible compartmentalization of chromatin into PBs. Treatment for 15 min begun immediately after activation yielded an optimized IE rate of 21% (n = 80) when oocytes were evaluated after overnight recovery in culture. With this protocol, 30-50% of oocytes were routinely scored as compartmentalized when assessed 90 min postactivation. No oocytes could be scored as such following overnight recovery, with 66% of treated oocytes cleaving to the 2-cell stage (n = 80). Activated cytoplasts were prepared by mechanical removal of PBs from oocytes whose chromatin had undergone IE or compartmentalization. These cytoplasts were compared with mechanically enucleated, metaphase (M) II cytoplasts whose activation was delayed in nuclear transfer experiments using HM-1 embryonic stem cells. Using oocytes from either B6D2F1 or B6CBAF1 (C57BL/6 x CBA) donors, the in vitro development of cloned embryos using activated cytoplasts was consistently inferior to that observed using MII cytoplasts. Live offspring were derived from both oocyte strains using the latter, whereas a single living mouse was cloned from activated B6CBAF1 cytoplasts.

Gerrard, L., D. Zhao, et al. (2005). "Stably transfected human embryonic stem cell clones express OCT4-specific green fluorescent protein and maintain self-renewal and pluripotency." Stem Cells **23**(1): 124-33.

Human embryonic stem cells (hESCs) are derived from the inner cell mass of preimplantation embryos; they can be cultured indefinitely and differentiated into many cell types in vitro. These cells therefore have the ability to provide insights into

human disease and provide a potential unlimited supply of cells for cell-based therapy. Little is known about the factors that are important for maintaining undifferentiated hESCs in vitro, however. As a tool to investigate these factors, transfected hES clonal cell lines were generated; these lines are able to express the enhanced green fluorescent protein (EGFP) reporter gene under control of the OCT4 promoter. OCT4 is an important marker of the undifferentiated state and a central regulator of pluripotency in ES cells. These OCT4-EGFP clonal cell lines exhibit features similar to parental hESCs, are pluripotent, and are able to produce all three embryonic germ layer cells. Expression of OCT4-EGFP is colocalized with endogenous OCT4, as well as the hESC surface antigens SSEA4 and Tra-1-60. In addition, the expression is retained in culture for an extensive period of time. Differentiation of these cells toward the neural lineage and targeted knockdown of endogenous OCT4 expression by RNA interference downregulated the EGFP expression in these cell lines, and this correlates closely with the reduction of endogenous OCT4 expression. Therefore, these cell lines provide an easy and noninvasive method to monitor expression of OCT4 in hESCs, and they will be invaluable for studying not only OCT4 function in hESC self-renewal and differentiation but also the factors required for maintenance of undifferentiated hESCs in culture.

Ghule, P. N., K. A. Becker, et al. (2007). "Cell cycle dependent phosphorylation and subnuclear organization of the histone gene regulator p220(NPAT) in human embryonic stem cells." J Cell Physiol **213**(1): 9-17.

Human embryonic stem (ES) cells have an expedited cell cycle (approximately 15 h) due to an abbreviated G1 phase (approximately 2.5 h) relative to somatic cells. One principal regulatory event during cell cycle progression is the G1/S phase induction of histone biosynthesis to package newly replicated DNA. In somatic cells, histone H4 gene expression is controlled by CDK2 phosphorylation of p220(NPAT) and localization of HiNF-P/p220(NPAT) complexes with histone genes at Cajal body related subnuclear foci. Here we show that this 'S point' pathway is operative in situ in human ES cells (H9 cells; NIH-designated WA09). Immunofluorescence microscopy shows an increase in p220(NPAT) foci in G1 reflecting the assembly of histone gene regulatory complexes in situ. In contrast to somatic cells where duplication of p220(NPAT) foci is evident in S phase, the increase in the number of p220(NPAT) foci in ES cells appears to precede the onset of DNA synthesis as measured by BrdU incorporation. Phosphorylation of p220(NPAT) at CDK dependent epitopes is most pronounced in S phase when cells exhibit elevated levels of cyclins E and A. Our data indicate that subnuclear organization of the HiNF-P/p220(NPAT) pathway is rapidly established as ES cells emerge from mitosis and that p220(NPAT) is subsequently phosphorylated in situ. Our findings establish that the HiNF-P/p220(NPAT) gene regulatory pathway operates in a cell cycle dependent microenvironment that supports expression of DNA replication-linked histone genes and chromatin assembly to accommodate human stem cell self-renewal.

Goulet, J. L., C. Y. Wang, et al. (1997). "Embryonic stem cell lines from MRL mice allow genetic modification in a murine model of autoimmune disease." J Immunol **159**(9): 4376-81.

The MRL/MpJ-Fas(lpr) (MRL-lpr/lpr) mouse spontaneously develops a generalized autoimmune disease with features similar to those of systemic lupus erythematosus. This mouse strain provides a valuable system for identifying and characterizing the multiple genetic factors that influence the pathogenesis of autoimmune diseases. One of the most powerful means of examining the role of a specific gene product in vivo is by inactivating the gene in mouse embryonic stem (ES) cells by homologous recombination and using these cells to derive mouse lines carrying the inactivated gene. The successful application of this approach, however, requires an ES cell line that will remain stable in culture during the processes of genetic manipulation and selection. To date, ES cell lines that meet this criterion have been derived from only a few mouse strains. Here we describe the production and characterization of stable ES cell lines from the MRL mouse strain. Approximately 7% of the blastocysts derived from the MRL/MpJ+ (MRL-+/+) strain gave rise to ES cell lines, and both of the male MRL-+/+ ES cell lines tested were shown to be germline competent. We show that the MRL-+/+ ES cell lines undergo gene targeting by homologous recombination at high frequency by inactivating the gene encoding the EP2 prostaglandin receptor. These Ep2-targeted MRL ES cell lines were used to generate MRL mouse lines heterozygous for the disrupted Ep2 gene, thus demonstrating the feasibility of using a genetic approach to dissect the pathobiology of the autoimmune disease in the MRL mouse.

Greber, B., H. Tandara, et al. (2005). "Comparison of PCR-based mutation detection methods and application for identification of mouse Sult1a1 mutant embryonic stem cell clones using pooled templates." Hum Mutat **25**(5): 483-90.

Reverse genetic approaches to generate mutants of model species are useful tools to assess

functions of unknown genes. Recent work has demonstrated the feasibility of such strategies in several organisms, exploiting the power of chemical mutagenesis to disrupt genes randomly throughout the genome. To increase the throughput of gene-driven mutant identification, efficient mutation screening protocols are needed. Given the availability of sequence information for large numbers of unknown genes in many species, mutation detection protocols are preferably based on PCR. Using a set of defined mutations in the Hprt1 gene of mouse embryonic stem (ES) cells, we have systematically compared several PCR-based point mutation and deletion detection methods available for their ability to identify lesions in pooled samples, which is a major criterion for an efficient large-scale mutation screening assay. Results indicate that point mutations are most effectively identified by heteroduplex cleavage using CEL I endonuclease. Small deletions can most effectively be detected employing the recently described "poison" primer PCR technique. Further, we employed the CEL I assay followed by conventional agarose gel electrophoresis analysis for screening a library of chemically mutagenized ES cell clones. This resulted in the isolation of several clones harboring mutations in the mouse Sult1a1 locus, demonstrating the high-throughput compatibility of this approach using simple and inexpensive laboratory equipment.

Green, J. J., B. Y. Zhou, et al. (2008). "Nanoparticles for gene transfer to human embryonic stem cell colonies." Nano Lett **8**(10): 3126-30.

We develop biodegradable polymeric nanoparticles to facilitate nonviral gene transfer to human embryonic stem cells (hESCs). Small (approximately 200 nm), positively charged (approximately 10 mV) particles are formed by the self assembly of cationic, hydrolytically degradable poly(beta-amino esters) and plasmid DNA. By varying the end group of the polymer, we can tune the biophysical properties of the resulting nanoparticles and their gene-delivery efficacy. We created an OCT4-driven GFP hES cell line to allow the rapid identification of nanoparticles that facilitate gene transfer while maintaining an hESC undifferentiated state. Using this cell system, we synthesized nanoparticles that have gene delivery efficacy that is up to 4 times higher than that of the leading commercially available transfection agent, Lipofectamine 2000. Importantly, these materials have minimal toxicity and do not adversely affect hESC colony morphology or cause nonspecific differentiation.

Grez, M., E. Akgun, et al. (1990). "Embryonic stem cell virus, a recombinant murine retrovirus with expression in embryonic stem cells." Proc Natl Acad Sci U S A **87**(23): 9202-6.

The expression of Moloney murine leukemia virus and vectors derived from it is restricted in undifferentiated mouse embryonal carcinoma and embryonal stem (ES) cells. We have developed a retroviral vector, the murine embryonic stem cell virus (MESV), that is active in embryonal carcinoma and ES cells. MESV was derived from a retroviral mutant [PCC4-cell-passaged myeloproliferative sarcoma virus (PCMV)] expressed in embryonal carcinoma cells but not in ES cells. The enhancer region of PCMV was shown to be functional in both cell types, but sequences within the 5' untranslated region of PCMV were found to restrict viral expression in ES cells. Replacement of this region by related sequences obtained from the dl-587rev retrovirus results in MESV, a modified PCMV virus that confers G418 resistance to fibroblasts and ES cells with similar efficiencies. Expression of MESV in ES cells is mediated by transcriptional regulatory elements within the 5' long terminal repeat of the viral genome.

Grinnell, K. L. and J. R. Bickenbach (2007). "Skin keratinocytes pre-treated with embryonic stem cell-conditioned medium or BMP4 can be directed to an alternative cell lineage." Cell Prolif **40**(5): 685-705.

OBJECTIVES: In this study, we have investigated whether secreted factors from embryonic stem cells (ESCs) could reprogramme keratinocytes and increase their potential to be directed into alternative cell lineages. MATERIALS AND METHODS: Contact and non-contact co-cultures of skin keratinocytes and murine ESCs were used initially to confirm any reprogramming ability of ESC-conditioned medium (CM). Immunofluoresence was used to assess nuclear expression of octamer-4 (Oct-4), as well as to confirm neuronal protein expression in neuroectodermally directed keratinocytes. Transcript expression changes were evaluated using semiquantitative reverse transcription-polymerase chain reaction. Western blotting, accompanied by densitometry analysis, was used to evaluate protein expression following morphology changes. RESULTS: We found that keratinocytes treated with ESC-CM changed their morphology and were stimulated to express the pluripotency regulator, Oct-4, and its target transcripts, Sox-2, Nanog, Utf1 and Rex-1. We demonstrate that at least one of the reprogramming factors is bone morphogenetic factor-4 (BMP4). Pre-treated keratinocytes could be specifically directed to differentiate into cells of the neuronal lineage. The majority of responsive keratinocytes were the epidermal stem cell population, with a small percentage of transit-amplifying cells also being affected. CONCLUSIONS: Our results

suggest that ESC-CM contains a number of factors, including BMP4, which are capable of reprogramming mouse skin keratinocytes to make them more developmentally potent, as evidenced by their ability to be re-differentiated into cells of the neuronal lineage. Our findings also imply a continuum of differentiation within the basal keratinocyte population. An increase in developmental potential combined with directed differentiation could increase the therapeutic relevancy of somatic cells.

Gu, P., D. LeMenuet, et al. (2005). "Orphan nuclear receptor GCNF is required for the repression of pluripotency genes during retinoic acid-induced embryonic stem cell differentiation." Mol Cell Biol **25**(19): 8507-19.

Embryonic stem (ES) cell pluripotency and differentiation are controlled by a network of transcription factors and signaling molecules. Transcription factors such as Oct4 and Nanog are required for self-renewal and maintain the undifferentiated state of ES cells. Decreases in the expression of these factors indicate the initiation of differentiation of ES cells. Inactivation of the gene encoding the orphan nuclear receptor GCNF showed that it plays an important role in the repression of Oct4 expression in somatic cells during early embryonic development. GCNF-/- ES cells were isolated to study the function of GCNF in the down-regulation of pluripotency genes during differentiation. Loss of repression of ES cell marker genes Oct4, Nanog, Sox2, FGF4, and Stella was observed upon treatment of GCNF-/- ES cells with retinoic acid. The loss of repression of pluripotency genes is either a direct or indirect consequence of loss of GCNF. Both the Oct4 and Nanog genes are direct targets of GCNF repression during ES cell differentiation and early mouse embryonic development. In contrast Sox2 and FGF4 are indirectly regulated by GCNF through Oct4. These findings establish a central role for GCNF in the repression of pluripotency gene expression during retinoic acid-induced ES cell differentiation.

Gunaratne, P. H. (2009). "Embryonic stem cell microRNAs: defining factors in induced pluripotent (iPS) and cancer (CSC) stem cells?" Curr Stem Cell Res Ther **4**(3): 168-77.

The discovery of microRNAs (miRNAs - small non-coding RNAs of approximately 22 nt) heralded a new and exciting era in biology. During this period miRNAs have gone from ignominy due to their origin mainly in 'junk DNA' to notoriety where they can be at once characterized as being all powerful (a single miRNA can target and potentially silence several hundred genes) and yet marginal (a given gene can be targeted by several miRNAs such that a given miRNA typically exerts a modest repression) [1-4]. The emerging paradox is exemplified by miRNAs that are prominently expressed in embryonic stem (ES) cells. The collective importance of miRNAs is firmly established by the fact that Dicer-/- mouse embryos die on day 7.5 due to defects in differentiation [5]. However, oppositely correlated expression that is expected of conventional repressors is increasingly being defied in multiple systems in relation to miRNA-mRNA target pairs. This is most evident in ES cells where miR-290-295 and 302 clusters the most abundant ES cell miRNAs are found to be driven by pluripotency genes Oct4, Nanog and Sox2 and also target these genes in 'incoherent feed-forward loops' [7]. Here the miRNAs are co-expressed and positively correlated with these targets that they repress suggesting that one of their primary roles is to fine tune gene expression rather than act as ON/OFF switches. On the other hand, let-7 family members that are notably low in ES cells and rapidly induced upon differentiation exhibit more conventional anti-correlated expression patterns with their targets [7-8]. In an intricately designed auto-regulatory loop, LIN28, a key 'keeper' of the pluripotent state binds and represses the processing of let-7 (a key 'keeper' of the differentiated state) [9-11]. One of the let-7 family members, let-7g targets and represses LIN28 through four 3'-UTR binding sites [12]. We propose that LIN28/let-7 pair has the potential to act as a 'toggle switch' that balances the decision to maintain pluripotency vs. differentiation. We also propose that the c-Myc/E2F driven miR17-92 cluster that together controls the G1 to S transition is fundamental for ES self-renewal and cell proliferation [13-18]. In that context it is no surprise that LIN28 and c-Myc (and therefore let-7 and miR-17-92 by association) and more recently Oct4/Sox2 regulated miR-302 has been shown to be among a handful of factors shown to be necessary and sufficient to convert differentiated cells to induced pluripotent stem (iPS) cells [19-29]. It is also no surprise that activation of miR-17-92 (OncomiRs) and down-regulation of let-7 (tumor suppressors) is a recurring theme in relation to cancers from multiple systems [30-48]. We speculate that the LIN28/let-7; c-MYC-E2F/miR-17-92 and Oct4/Sox2/miR-302-cyclin D1 networks are fundamental to properties of pluripotency and self-renewal associated with embryonic stem cells. We also speculate that ES cell miRNA-mRNA associations may also regulate tissue homeostasis and regeneration in the fully developed adult. Consequently, the appropriate regulation of LIN28/let-7; c-MYC-E2F/miR-17-92 and Oct4/Sox2/miR-302-cyclin D1 gene networks will be critical for the success of regenerative strategies that

involve iPS cells. Any perturbation in key ES cell miRNA-mRNA networks during any of the above processes maybe a hallmark of (CSCs).

Guo, Y., R. Costa, et al. (2002). "The embryonic stem cell transcription factors Oct-4 and FoxD3 interact to regulate endodermal-specific promoter expression." Proc Natl Acad Sci U S A **99**(6): 3663-7.

The POU homeodomain protein Oct-4 and the Forkhead Box protein FoxD3 (previously Genesis) are transcriptional regulators expressed in embryonic stem cells. Down-regulation of Oct-4 during gastrulation is essential for proper endoderm development. After gastrulation, FoxD3 is generally down-regulated during early endoderm formation, although it specifically remains expressed in the embryonic neural crest. In these studies, we have found that Oct-4 and FoxD3 can bind to identical regulatory DNA sequences. In addition, Oct-4 physically interacted with the FoxD3 DNA-binding domain. Cotransfection of Oct-4 and FoxD3 expression vectors activated the osteopontin enhancer, which is expressed in totipotent embryonic stem cells. FoxA1 and FoxA2 (previously HNF-3alpha and HNF-3beta) are Forkhead Box transcription factors that participate in liver and lung formation from foregut endoderm. Although FoxD3 activated the FoxA1 and FoxA2 promoters, Oct-4 inhibited FoxD3 activation of the FoxA1 and FoxA2 endodermal promoters. These data indicate that Oct-4 functions as a corepressor of FoxD3 to provide embryonic lineage-specific transcriptional regulatory activity to maintain appropriate developmental timing.

Hailesellasse Sene, K., C. J. Porter, et al. (2007). "Gene function in early mouse embryonic stem cell differentiation." BMC Genomics **8**: 85.

BACKGROUND: Little is known about the genes that drive embryonic stem cell differentiation. However, such knowledge is necessary if we are to exploit the therapeutic potential of stem cells. To uncover the genetic determinants of mouse embryonic stem cell (mESC) differentiation, we have generated and analyzed 11-point time-series of DNA microarray data for three biologically equivalent but genetically distinct mESC lines (R1, J1, and V6.5) undergoing undirected differentiation into embryoid bodies (EBs) over a period of two weeks. RESULTS: We identified the initial 12 hour period as reflecting the early stages of mESC differentiation and studied probe sets showing consistent changes of gene expression in that period. Gene function analysis indicated significant up-regulation of genes related to regulation of transcription and mRNA splicing, and down-regulation of genes related to intracellular signaling. Phylogenetic analysis indicated that the genes showing the largest expression changes were more likely to have originated in metazoans. The probe sets with the most consistent gene changes in the three cell lines represented 24 down-regulated and 12 up-regulated genes, all with closely related human homologues. Whereas some of these genes are known to be involved in embryonic developmental processes (e.g. Klf4, Otx2, Smn1, Socs3, Tagln, Tdgf1), our analysis points to others (such as transcription factor Phf21a, extracellular matrix related Lama1 and Cyr61, or endoplasmic reticulum related Sc4mol and Scd2) that have not been previously related to mESC function. The majority of identified functions were related to transcriptional regulation, intracellular signaling, and cytoskeleton. Genes involved in other cellular functions important in ESC differentiation such as chromatin remodeling and transmembrane receptors were not observed in this set. CONCLUSION: Our analysis profiles for the first time gene expression at a very early stage of mESC differentiation, and identifies a functional and phylogenetic signature for the genes involved. The data generated constitute a valuable resource for further studies. All DNA microarray data used in this study are available in the StemBase database of stem cell gene expression data 1 and in the NCBI's GEO database.

Hernandez, D., P. J. Mee, et al. (1999). "Transchromosomal mouse embryonic stem cell lines and chimeric mice that contain freely segregating segments of human chromosome 21." Hum Mol Genet **8**(5): 923-33.

At least 8% of all human conceptions have major chromosome abnormalities and the frequency of chromosomal syndromes in newborns is >0.5%. Despite these disorders making a large contribution to human morbidity and mortality, we have little understanding of their aetiology and little molecular data on the importance of gene dosage to mammalian cells. Trisomy 21, which results in Down syndrome (DS), is the most frequent aneuploidy in humans (1 in 600 live births, up to 1 in 150 pregnancies world-wide) and is the most common known genetic cause of mental retardation. To investigate the molecular genetics of DS, we report here the creation of mice that carry different human chromosome 21 (Hsa21) fragments as a freely segregating extra chromosome. To produce these 'transchromosomal' animals, we placed a selectable marker into Hsa21 and transferred the chromosome from a human somatic cell line into mouse embryonic stem (ES) cells using irradiation microcell-mediated chromosome transfer (XMMCT). 'Transchromosomal' ES cells containing different Hsa21 regions ranging in size from approximately 50 to approximately 0.2 Mb have been used to create

chimeric mice. These mice maintain Hsa21 sequences and express Hsa21 genes in multiple tissues. This novel use of the XMMCT protocol is applicable to investigations requiring the transfer of large chromosomal regions into ES or other cells and, in particular, the modelling of DS and other human aneuploidy syndromes.

Hiratani, I., T. Ryba, et al. (2008). "Global reorganization of replication domains during embryonic stem cell differentiation." PLoS Biol **6**(10): e245.

DNA replication in mammals is regulated via the coordinate firing of clusters of replicons that duplicate megabase-sized chromosome segments at specific times during S-phase. Cytogenetic studies show that these "replicon clusters" coalesce as subchromosomal units that persist through multiple cell generations, but the molecular boundaries of such units have remained elusive. Moreover, the extent to which changes in replication timing occur during differentiation and their relationship to transcription changes has not been rigorously investigated. We have constructed high-resolution replication-timing profiles in mouse embryonic stem cells (mESCs) before and after differentiation to neural precursor cells. We demonstrate that chromosomes can be segmented into multimegabase domains of coordinate replication, which we call "replication domains," separated by transition regions whose replication kinetics are consistent with large originless segments. The molecular boundaries of replication domains are remarkably well conserved between distantly related ESC lines and induced pluripotent stem cells. Unexpectedly, ESC differentiation was accompanied by the consolidation of smaller differentially replicating domains into larger coordinately replicated units whose replication time was more aligned to isochore GC content and the density of LINE-1 transposable elements, but not gene density. Replication-timing changes were coordinated with transcription changes for weak promoters more than strong promoters, and were accompanied by rearrangements in subnuclear position. We conclude that replication profiles are cell-type specific, and changes in these profiles reveal chromosome segments that undergo large changes in organization during differentiation. Moreover, smaller replication domains and a higher density of timing transition regions that interrupt isochore replication timing define a novel characteristic of the pluripotent state.

Hochedlinger, K., W. M. Rideout, et al. (2004). "Nuclear transplantation, embryonic stem cells and the potential for cell therapy." Hematol J **5 Suppl 3**: S114-7.

Nuclear transfer experiments in mammals have shown that the nucleus of an adult cell has the ability to direct the development of an entire organism, id est its genome is totipotent. However, these experiments did not conclusively demonstrate that the nuclei of terminally differentiated adult cells remain totipotent. It is possible that rare adult stem cells served as donors for the few surviving clones. To address this question, we have generated monoclonal mice from terminally differentiated lymphocytes that carry a single antigen receptor rearrangement in all tissues. Nuclear transfer technology may provide a powerful method for obtaining autologous cells for replacement therapy. We have demonstrated the feasibility of this concept by combining nuclear transfer with gene and cell therapy to treat the immune deficiency of Rag2 mutant mice, thus establishing a paradigm for 'therapeutic cloning'. Moreover, we will discuss the potential use of nuclear transfer to study the role of reversible genomic (epigenetic) modifications during tumorigenesis.

Honda, A., M. Hirose, et al. (2008). "Stable embryonic stem cell lines in rabbits: potential small animal models for human research." Reprod Biomed Online **17**(5): 706-15.

Although embryonic stem (ES) cell lines derived from mice and primates are used extensively, the development of such lines from other mammals is extremely difficult because of their rapid decline in proliferation potential and pluripotency after several passages. This study describes the establishment of rabbit ES cell lines with indefinite proliferation potential. It was found that the feeder cell density determines the fate of rabbit ES cells, and that maximum proliferation potential was obtained when they were cultured on a feeder cell density of one-sixth of the density at confluency. Higher and lower densities of feeder cells induced ES cell differentiation or division arrest. Under optimized conditions, rabbit ES cells were passaged 50 times, after which they still possessed high telomerase activity. This culture system enabled efficient gene transduction and clonal expansion from single cells. During culture, rabbit ES cells exhibited flattened monolayer cell colonies, as reported for monkey and human ES cells, and expressed pluripotency markers. Embryoid bodies and teratomas formed readily in vitro and in vivo respectively. These ES cell lines can be safely cryopreserved for later use. Thus, rabbit ES cells can be added to the list of stable mammalian ES cells, enabling the rabbit to be used as a small animal model for the study of human cell transplantation therapy.

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.

Huber, I., I. Itzhaki, et al. (2007). "Identification and selection of cardiomyocytes during human embryonic stem cell differentiation." Faseb J **21**(10): 2551-63.

Human embryonic stem cells (hESC) are pluripotent lines that can differentiate in vitro into cell derivatives of all three germ layers, including cardiomyocytes. Successful application of these unique cells in the areas of cardiovascular research and regenerative medicine has been hampered by difficulties in identifying and selecting specific cardiac progenitor cells from the mixed population of differentiating cells. We report the generation of stable transgenic hESC lines, using lentiviral vectors, and single-cell clones that express a reporter gene (eGFP) under the transcriptional control of a cardiac-specific promoter (the human myosin light chain-2V promoter). Our results demonstrate the appearance of eGFP-expressing cells during the differentiation of the hESC as embryoid bodies (EBs) that can be identified and sorted using FACS (purity>95%, viability>85%). The eGFP-expressing cells were stained positively for cardiac-specific proteins (>93%), expressed cardiac-specific genes, displayed cardiac-specific action-potentials, and could form stable myocardial cell grafts following in vivo cell transplantation. The generation of these transgenic hESC lines may be used to identify and study early cardiac precursors for developmental studies, to robustly quantify the extent of cardiomyocyte differentiation, to label the cells for in vivo grafting, and to allow derivation of purified cell populations of cardiomyocytes for future myocardial cell therapy strategies.

Imreh, M. P., S. Wolbank, et al. (2004). "Culture and expansion of the human embryonic stem cell line HS181, evaluated in a double-color system." Stem Cells Dev **13**(4): 337-43.

An approach of using RFP-transfected human foreskin fibroblasts (hFS-RFP) to support the growth of GFP expressing human embryonic stem cells (hES; HS181-GFP) is reported. The two-color system was applied to detect interactions between hFS and human embryonic stem cells (hES). After overnight culture, the hES cell colonies showed a behavior of "pushing away" the underlying feeder cells. This phenomenon occurred with both a low and high density of feeders. The density of the feeder cell layer, however, influenced the growth pattern of hES cell colonies. At a high feeder cell density, the hES colonies were more pointed and aligned with the direction of the fibroblasts, whereas less dense feeder layers allowed a more rounded and flat hES colony formation. Not surprisingly, a small fraction of mitotically inactivated feeder cells reattached after passage and remained viable in the cultures for up to four subsequent passages. The prospect of using the two-color system for detection of possible fusion events between hES cells and feeder cells was assessed by screening a large number of cell cultures for double RFP/EGFP expressing cells. The results indicate that fusion events are extremely rare (<10(-6)), or alternatively that after fusion the dual expression of both EGFP and RFP is not easily detected for other reasons. In summary, a two-color system allows analysis of colony formation and also helps to identify and follow the differentiation of cells.

Ioffe, E., Y. Liu, et al. (1995). "WW6: an embryonic stem cell line with an inert genetic marker that can be traced in chimeras." Proc Natl Acad Sci U S A **92**(16): 7357-61.

Mutant mice produced by gene targeting in embryonic stem (ES) cells often have a complex or embryonic lethal phenotype. In these cases, it would be helpful to identify tissues and cell types first affected in mutant embryos by following the contribution to chimeras of ES cells homozygous for the mutant allele. Although a number of strategies for following ES cell development in vivo have been reported, each has limitations that preclude its general application. In this paper, we describe ES cell lines that can be tracked to every nucleated cell type in chimeras at all developmental stages. These lines were

derived from blastocysts of mice that carry an 11-Mb beta-globin transgene on chromosome 3. The transgene is readily detected by DNA in situ hybridization, providing an inert, nuclear-localized marker whose presence is not affected by transcriptional or translational controls. The "WW" series of ES lines possess the essential features of previously described ES lines, including giving rise to a preponderance of male chimeras, all of which have to date exhibited germ-line transmission. In addition, clones selected for single or double targeting events form strong chimeras, demonstrating the feasibility of using WW6 cells to identify phenotypes associated with the creation of a null mutant.

Irioka, T., K. Watanabe, et al. (2005). "Distinct effects of caudalizing factors on regional specification of embryonic stem cell-derived neural precursors." Brain Res Dev Brain Res **154**(1): 63-70.

Recent embryological studies have implicated several "caudalizing factors" in the caudal specification of the central nervous system (CNS). In this study, we have examined the effects of three candidate caudalizing factors on neural precursors induced from embryonic stem (ES) cells by the stromal cell-derived inducing activity (SDIA) method. Among retinoic acid (RA), Wnt and FGF signals, RA causes the strongest level of caudalization: inducing suppression of forebrain differentiation and promotion of caudal CNS specification. Obvious suppression of the telencephalic marker Bf1 and that of the forebrain marker Otx2 occur at 2x10(-8) and 2x10(-7) M, respectively. Activation of the caudal marker genes such as Hoxb9 is observed in a dose-dependent manner over the range of 2x10(-9)-2x10(-6) M. Suppression of the forebrain genes has a narrow critical period of RA response during the early culture phase. In contrast, significant induction of the caudal genes is evoked by a 1-day exposure to RA at any time between days 3 and 8. RA treatment not only induces caudal specification but also inhibits differentiation of ventral CNS tissues, particularly of floor plate cells. FGF4 induces partial caudalization while Wnt-3A exhibits weak caudalizing activities only in the presence of RA. These findings provide useful information on the proper selection of combination of signaling molecules, doses and timing for steering ES cell differentiation by caudalizing factors into caudal neural fates.

Jaksch, M., J. Munera, et al. (2008). "Cell cycle-dependent variation of a CD133 epitope in human embryonic stem cell, colon cancer, and melanoma cell lines." Cancer Res **68**(19): 7882-6.

CD133 (Prominin1) is a pentaspan transmembrane glycoprotein expressed in several stem cell populations and cancers. Reactivity with an antibody (AC133) to a glycoslyated form of CD133 has been widely used for the enrichment of cells with tumor-initiating activity in xenograph transplantation assays. We have found by fluorescence-activated cell sorting that increased AC133 reactivity in human embryonic stem cells, colon cancer, and melanoma cells is correlated with increased DNA content and, reciprocally, that the least reactive cells are in the G(1)-G(0) portion of the cell cycle. Continued cultivation of cells sorted on the basis of high and low AC133 reactivity results in a normalization of the cell reactivity profiles, indicating that cells with low AC133 reactivity can generate highly reactive cells as they resume proliferation. The association of AC133 with actively cycling cells may contribute to the basis for enrichment for tumor-initiating activity.

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, Ak3l1, 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.

Josephson, R., G. Sykes, et al. (2006). "A molecular scheme for improved characterization of human embryonic stem cell lines." BMC Biol **4**: 28.

BACKGROUND: Human embryonic stem cells (hESC) offer a renewable source of a wide range of cell types for use in research and cell-based therapies to treat disease. Inspection of protein markers provides important information about the current state of the cells and data for subsequent manipulations. However, hESC must be routinely analyzed at the genomic level to guard against deleterious changes during extensive propagation, expansion, and manipulation in vitro. RESULTS: We found that short tandem repeat (STR) analysis, human leukocyte antigen (HLA) typing, single nucleotide polymorphism (SNP) genomic analysis, mitochondrial DNA sequencing, and gene expression analysis by microarray can be used to fully describe any hESC culture in terms of its identity, stability, and undifferentiated state. CONCLUSION: Here we describe, using molecular biology alone, a comprehensive characterization of 17 different hESC lines. The use of amplified nucleic acids means that for the first time full characterization of hESC lines can be performed with little time investment and a minimum of material. The information thus gained will facilitate comparison of lines and replication of results between laboratories.

Jung, C. G., H. Hida, et al. (2004). "Pleiotrophin mRNA is highly expressed in neural stem (progenitor) cells of mouse ventral mesencephalon and the product promotes production of dopaminergic neurons from embryonic stem cell-derived nestin-positive cells." Faseb J **18**(11): 1237-9.

Neural stem cells are promising candidates for donor cells in neural transplantation. However, the mechanism by which neural stem cells differentiate into neurons is not well understood. In the present study, a serial analysis of gene expression (SAGE) was carried out to generate a gene file of neural stem (progenitor) cells from the mouse ventral mesencephalon. Among the 15,815 tags investigated, the mRNA of the housekeeping genes (elongation factor 1-alpha, ATPase subunit 6, GAPDH, actin), laminin receptor 1, HSP 70, pleiotrophin, and nestin were highly expressed. Because pleiotrophin (PTN) exhibits mitogenic and trophic effects on neural development and exhibits trophic effects on survival of dopaminergic (DAergic) neurons, we investigated the role of PTN in neurogenesis, especially to DAergic neurons. Here, we show that PTN increased the production of tyrosine hydroxylase (TH)-positive neurons from embryonic stem (ES) cell-derived nestin-positive cells. The expression of Nurr1 mRNA was enhanced by PTN. L-dopa in the culture medium was increased by PTN. This effect was as strong as with sonic hedgehog. Data suggest that PTN mRNA is highly expressed in neural stem (progenitor) cells of mouse ventral mesencephalon, and PTN promotes the production of DAergic neurons from ES cell-derived nestin-positive cells.

Kang, H. B., J. S. Kim, et al. (2005). "Basic fibroblast growth factor activates ERK and induces c-fos in human embryonic stem cell line MizhES1." Stem Cells Dev **14**(4): 395-401.

Human embryonic stem (hES) cells can be maintained in a proliferative undifferentiated state in vitro by growing them on feeder layers of mouse embryonic fibroblast (MEF) cells along with basic fibroblast growth factor (bFGF/FGF-2). To understand the molecular mechanisms involved in the requirement of bFGF in human ES cells, we investigated expression of FGF receptors and intracellular signaling events in response to bFGF in human ES cell line MizhES1. On the basis of the

results of RT-PCR, clear expression of FGF receptors FGFR1, FGR2, and FGFR3 was noticed. Because MAPK, PI3K, and PKC pathways are well-known pathways triggered by bFGF in other cells, these pathways were investigated after stimulation with bFGF. bFGF did not induce activation of PI3K or PKC, but induced activation of ERK (extracellular signal-regulated kinase). To monitor the consequences of ERK activation, we examined expression of the immediate early gene c-fos, one downstream target of the MEK1/ERK pathway. mRNA and protein levels of the c-fos gene were increased by bFGF. Induction of c-Fos was dependent on MEKl. Therefore, it is likely that bFGF contributes to maintenance of human ES cells, at least in part, through the MEK1/ERK pathway.

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.

Kawai, T., T. Takahashi, et al. (2004). "Efficient cardiomyogenic differentiation of embryonic stem cell by fibroblast growth factor 2 and bone morphogenetic protein 2." Circ J **68**(7): 691-702.

BACKGROUND: Despite the pluripotency of embryonic stem (ES) cells, the specific control of their cardiomyogenic differentiation remains difficult. The aim of the present study was to investigate whether growth factors may efficiently enhance the in vitro cardiac differentiation of ES cells. METHODS AND RESULTS: Recombinant growth factors at various concentrations or their inhibitors were added according to various schedules during the cardiomyogenic differentiation of ES cells. Cardiomyogenic differentiation was assessed by mRNA and protein expressions of several cardiomyocyte-specific genes. Basic fibroblast growth factor-2 (FGF-2) and/or bone morphogenetic protein-2 (BMP-2) efficiently enhanced the cardiomyogenic differentiation, but only when they were added at the optimal concentration (1.0 ng/ml in FGF-2 and 0.2 ng/ml in BMP-2; relatively lower than expected in both cases) for the first 3 days. Inhibition of FGF-2 and/or BMP-2 drastically suppressed the cardiomyogenic differentiation. CONCLUSION: FGF-2 and BMP-2 play a crucial role in early cardiomyogenesis. The achievement of efficient cardiac differentiation using both growth factors may facilitate ES cell-derived cell therapy for heart diseases as well as contribute to developmental studies of the heart.

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, D. W., S. Chung, et al. (2006). "Stromal cell-derived inducing activity, Nurr1, and signaling molecules synergistically induce dopaminergic neurons from mouse embryonic stem cells." Stem Cells **24**(3): 557-67.

To induce differentiation of embryonic stem cells (ESCs) into specialized cell types for therapeutic purposes, it may be desirable to combine genetic manipulation and appropriate differentiation signals. We studied the induction of dopaminergic (DA) neurons from mouse ESCs by overexpressing the transcription factor Nurr1 and coculturing with PA6 stromal cells. Nurr1-expressing ESCs (N2 and N5) differentiated into a higher number of neurons (approximately twofold) than the naive ESCs (D3). In addition, N2/N5-derived cells contained a significantly higher proportion (>50%) of tyrosine hydroxylase (TH)+ neurons than D3 (<30%) and an even greater proportion of TH+ neurons (approximately 90%) when treated with the signaling molecules sonic hedgehog, fibroblast growth factor 8, and ascorbic acid. N2/N5-derived cells express much higher levels of DA markers (e.g., TH, dopamine transporter, aromatic amino acid decarboxylase, and G protein-regulated inwardly rectifying K+ channel 2) and produce and release a higher level of dopamine, compared with D3-derived cells. Furthermore, the majority of generated neurons exhibited electrophysiological properties characteristic of midbrain DA neurons. Finally, transplantation experiments showed efficient in vivo integration/generation of TH+ neurons after implantation into mouse striatum. Taken together, our results show that the combination of genetic manipulation(s) and in vitro cell differentiation conditions offers a reliable and effective induction of DA neurons from ESCs and may pave the way for future cell transplantation therapy in Parkinson's disease.

Kim, K. P., A. Thurston, et al. (2007). "Gene-specific vulnerability to imprinting variability in human embryonic stem cell lines." Genome Res **17**(12): 1731-42.

Disregulation of imprinted genes can be associated with tumorigenesis and altered cell differentiation capacity and so could provide adverse outcomes for stem cell applications. Although the maintenance of mouse and primate embryonic stem cells in a pluripotent state has been reported to disrupt the monoallelic expression of several imprinted genes, available data have suggested relatively higher imprint stability in the human equivalents. Identification of 202 heterozygous loci allowed us to examine the allelic expression of 22 imprinted genes in 22 human embryonic stem cell lines. Half of the genes examined (IPW, H19, MEG3, MEST isoforms 1 and 2, PEG10, MESTIT1, NESP55, ATP10A, PHLDA2, IGF2) showed variable allelic expression between lines, indicating vulnerability to disrupted imprinting. However, seven genes showed consistent monoallelic expression (NDN, MAGEL2, SNRPN, PEG3, KCNQ1, KCNQ1OT1, CDKN1C). Furthermore, four genes known to be monoallelic or to exhibit polymorphic imprinting in later-developing human tissues (TP73, IGF2R, WT1, SLC22A18) were always biallelic in hESCs. MEST isoform 1, PEG10, and NESP55 showed an association between the variability observed in interline allelic expression status and the DNA methylation of previously identified regulatory regions. Our results demonstrate gene-specific differences in the stability of imprinted loci in human embryonic stem cells and identify disrupted DNA methylation as one potential mechanism. We conclude the prudence of including comprehensive imprinting analysis in the continued characterization of human embryonic stem cell lines.

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.

Korhonen, L., K. Brannvall, et al. (2003). "Tumor suppressor gene BRCA-1 is expressed by embryonic and adult neural stem cells and involved in cell proliferation." J Neurosci Res **71**(6): 769-76.

BRCA-1 is a tumor suppressor gene that plays a role in DNA repair and cellular growth control. Here we show that BRCA-1 mRNA is expressed by embryonic rat brain and is localized to the neuroepithelium containing neuronal precursor cells. The expression of BRCA-1 decreases during rat brain development, but BRCA-1 is expressed postnatally by proliferating neuronal precursor cells in the developing cerebellum. Neural stem cells (NSC) prepared from embryonic rat brain and cultured in the presence of epidermal growth factor were positive for BRCA-1. Induction of NSC differentiation resulted in down-regulation of BRCA-1 expression as shown by RNA and protein analyses. In addition to embryonic cells, BRCA-1 is also present in NSC prepared from adult rat brain. In adult rats, BRCA1 was expressed by cells in the walls of brain ventricles and in choroid plexus. The results show that BRCA-1 is present in embryonic and adult rat NSC and that the expression is linked to NSC proliferation.

Kramer, J., C. Hegert, et al. (2000). "Embryonic stem cell-derived chondrogenic differentiation in vitro: activation by BMP-2 and BMP-4." Mech Dev **92**(2): 193-205.

Differentiation of mouse embryonic stem (ES) cells via embryoid bodies was established as a suitable model to study development in vitro. Here, we show that differentiation of ES cells in vitro into chondrocytes can be modulated by members of the transforming growth factor-beta family (TGF-beta(1), BMP-2 and -4). ES cell differentiation into chondrocytes was characterized by the appearance of Alcian blue-stained areas and the expression of cartilage-associated genes and proteins. Different stages of cartilage differentiation could be distinguished according to the expression pattern of the transcription factor scleraxis, and the cartilage matrix protein collagen II. The number of Alcian-blue-stained areas decreased slightly after application of TGF-beta(1), whereas BMP-2 or -4 induced chondrogenic differentiation. The inducing effect of BMP-2 was found to be dependent on the time of application, consistent with its role to recruit precursor cells to the chondrogenic fate.

Krumenacker, J. S., S. Katsuki, et al. (2006). "Differential expression of genes involved in cGMP-dependent nitric oxide signaling in murine embryonic stem (ES) cells and ES cell-derived cardiomyocytes." Nitric Oxide **14**(1): 1-11.

Nitric oxide (NO) performs multiple physiological roles as a biological signaling molecule. The role of NO and cGMP signaling in embryonic stem (ES) cell-derived cardiomyocytes (CM) has been investigated but many questions remain. In this study, we examined the expression of the NO signaling pathway components nitric oxide synthase (NOS-1, 2, 3), soluble guanylyl cyclase (sGCalpha(1) and beta(1)) and protein kinase G (PKG) genes and sGC activity in murine ES cells subjected to differentiation by embryoid body (EB) formation. We found that in undifferentiated ES cells, NOS-1, NOS-3, and sGCbeta(1) were detected while NOS-2, sGCalpha(1), and PKG were very low or undetectable. When ES cells were subjected to differentiation, NOS-1 abruptly decreased within one day, NOS-2 mRNA became detectable after several days, and NOS-3 increased after 7-10 days. Levels of sGCalpha(1), sGCbeta(1), and PKG all increased gradually over a several day time course of differentiation in EB outgrowths. Analysis of sGC activity in cell lysates derived from undifferentiated ES cells revealed that NO could not stimulate cGMP. However, lysates from differentiated EB outgrowths produced abundant cGMP levels after NO stimulation. Purification of ES-cell derived CM revealed that mRNA expression of all the NOS isoforms was very low to absent while sGCalpha(1) and beta(1) subunit mRNAs were abundant and sGC-mediated cGMP production was apparent in this population of cells. These data suggest that cGMP-mediated NO signaling may play a minor role, if any, in undifferentiated ES cells but could be involved in the early differentiation events or physiological processes of ES cells or ES cell-derived lineages.

Kumar, R. A., K. L. Chan, et al. (2004). "Unexpected embryonic stem (ES) cell mutations represent a concern in gene targeting: lessons from "fierce" mice." Genesis **38**(2): 51-7.

The exceptional value of gene targeting technology to generate mouse models of human disease exists under the shadow of potential genetic errors. We previously observed an unexpected brain-behavior phenotype that resulted from a gene-targeting experiment designed to delete the Zfa gene. Given that the transcription of Zfa is restricted to the germ cell lineage of adult testis, it was both a surprise and a concern when the resulting mice had a phenotype present in both sexes that included abnormal brains and violent behavior. We hypothesized that an unrelated mutation may have been responsible for the unexpected phenotype. Here we show that the single gene mutation, Nr2e1(frc) (fierce), which was responsible for the brain-behavior phenotype, existed in the embryonic stem (ES) cell even before the derivation of the Zfa knockout mice. Our work thus highlights a concern in gene targeting, namely, that ES cells can harbor unexpected mutations, which can lead to genotype-phenotype misattribution. Based on our findings, we caution the gene-targeting community to use low-passage ES cells, to characterize mice derived from more than one independently targeted ES cell clone, and to backcross mice to allow for segregation of distant but linked mutations.

Kunarso, G., K. Y. Wong, et al. (2008). "Detailed characterization of the mouse embryonic stem cell transcriptome reveals novel genes and intergenic splicing associated with pluripotency." BMC Genomics **9**: 155.

BACKGROUND: Transcriptional control of embryonic stem (ES) cell pluripotency has been a subject of intense study. Transcriptional regulators including Oct4 (Oct3/4 index), Sox2 and Nanog are fundamental for maintaining the undifferentiated state. However, the ES cell transcriptome is not limited to their targets, and exhibits considerable complexity when assayed with microarray, MPSS, cDNA/EST sequencing, and SAGE technologies. To identify novel genes associated with pluripotency, we globally searched for ES transcripts not corresponding to known genes, validated their sequences, determined their expression profiles, and employed RNAi to test their function. RESULTS: Gene Identification Signature (GIS) analysis, a SAGE derivative distinguished by paired 5' and 3' transcript end tags, identified 153 candidate novel transcriptional units (TUs) distinct from known genes in a mouse E14 ES mRNA library. We focused on 16 TUs free of artefacts and mapping discrepancies, five of which were validated by RTPCR product sequencing. Two of the TUs were revealed by annotation to represent novel protein-coding genes: a PRY-domain cluster member and a KRAB-domain zinc finger. The other three TUs represented intergenic splicing events involving adjacent, functionally unrelated protein-coding genes transcribed in the same orientation, with one event potentially encoding a fusion protein containing domains from both component genes (Clk2 and Scamp3). Expression profiling using embryonic samples and adult tissue panels confirmed that three of the TUs were unique to or most highly expressed in ES cells. Expression levels of all five TUs dropped dramatically during three distinct chemically induced differentiation treatments of ES cells in culture. However, siRNA knockdowns of the TUs did not alter mRNA levels of pluripotency or differentiation markers, and did not affect cell morphology. CONCLUSION: Transcriptome libraries retain considerable potential for novel gene discovery despite massive recent cDNA and EST sequencing efforts; cDNA and EST evidence for these ES cell TUs had been limited or absent. RTPCR and full-length sequencing remain essential in resolving the bottleneck between numerous candidate novel transcripts inferred from high-throughput sequencing and the small fraction that can be validated. RNAi results indicate that, despite their strong association with pluripotency, these five transcriptomic novelties may not be required for maintaining it.

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.

Lagarkova, M. A., P. Y. Volchkov, et al. (2006). "Diverse epigenetic profile of novel human embryonic stem cell lines." Cell Cycle **5**(4): 416-20.

Human embryonic stem cells (hESCs) are a promising model for studying mechanisms of regulation of early development and differentiation. OCT4, NANOG, OCT4-related genes and some others were recently described to be important in pluripotency maintenance. Lesser is known about molecular mechanisms involved in their regulation. Apart from genetic regulation of gene expression epigenetic events, particularly methylation, play an important role in early development. Using RT-PCR we studied the expression of pluripotency-related genes OCT4, NANOG, DPPA3 and DPPA5 during hESCs differentiation to embryoid bodies. Analysis of methylation profiles of promoter or putative regulatory regions of the indicated genes demonstrated that expression of the pluripotency-maintaining genes correlated with their methylation status, whereas methylation of DPPA3 and DPPA5 varied between cell lines. We propose that DNA methylation underlies the developmental stage-specific mechanisms of pluripotency-related genes expression and reactivation and may have an impact on differentiation potential of hESC lines.

Laurent, L. C., J. Chen, et al. (2008). "Comprehensive microRNA profiling reveals a unique human embryonic stem cell signature dominated by a single seed sequence." Stem Cells **26**(6): 1506-16.

Embryonic stem cells are unique among cultured cells in their ability to self-renew and differentiate into a wide diversity of cell types, suggesting that a specific molecular control network underlies these features. Human embryonic stem cells (hESCs) are known to have distinct mRNA expression, global DNA methylation, and chromatin profiles, but the involvement of high-level regulators, such as microRNAs (miRNA), in the hESC-specific molecular network is poorly understood. We report that global miRNA expression profiling of hESCs and a variety of stem cell and differentiated cell types using a novel microarray platform revealed a unique set of miRNAs differentially regulated in hESCs, including numerous miRNAs not previously linked to hESCs. These hESC-associated miRNAs were more likely to be located in large genomic clusters, and less likely to be located in introns of coding genes. hESCs had higher expression of oncogenic miRNAs and lower expression of tumor suppressor miRNAs than the other cell types. Many miRNAs upregulated in hESCs share a common consensus seed sequence, suggesting that there is cooperative regulation of a critical set of target miRNAs. We propose that miRNAs are coordinately controlled in hESCs, and are key regulators of pluripotence and differentiation. Disclosure of potential conflicts of interest is found at the end of this article.

Lee, J. H., S. R. Hart, et al. (2004). "Histone deacetylase activity is required for embryonic stem cell differentiation." Genesis **38**(1): 32-8.

Mammalian development requires commitment of cells to restricted lineages, which requires epigenetic regulation of chromatin structure. Epigenetic modifications were examined during in vitro differentiation of murine embryonic stem (ES) cells. Global histone acetylation, a euchromatin marker, declines dramatically within 1 day of differentiation induction and partially rebounds by day 2. Histone H3-Lys9 methylation, a heterochromatin marker, increases during in vitro differentiation. Conversely, the euchromatin marker H3-Lys4 methylation transiently decreases, then increases to undifferentiated levels by day 4, and decreases by day 6. Global cytosine methylation, another heterochromatin marker, increases slightly during ES cell differentiation. Chromatin structure of the Oct4 and Brachyury gene promoters is modulated in concert with their pattern of expression during ES cell differentiation. Importantly, prevention of global histone deacetylation by treatment with trichostatin A prevents ES cell differentiation. Hence, ES cells undergo functionally important global and gene-specific remodeling of chromatin structure during in vitro differentiation. genesis 38:32-38, 2004.

Lee, S. H., J. S. Heo, et al. (2007). "Effect of hypoxia on 2-deoxyglucose uptake and cell cycle regulatory protein expression of mouse embryonic stem cells: involvement of Ca2+ /PKC, MAPKs and HIF-1alpha." Cell Physiol Biochem **19**(5-6): 269-82.

This study investigated the signal molecules linking the alteration in 2-dexoyglucose (2-DG) uptake and DNA synthesis in mouse embryonic stem (ES) cells under hypoxia. Hypoxia increased the 2-DG uptake and GLUT-1 protein expression level while the undifferentiated state of ES cells and cell viability

were not affected by the hypoxia (1 - 48h). Subsequently, [(3)H] thymidine incorporation was significantly increased at 12 hours of hypoxic exposure. Hypoxia increased the Ca(2+) uptake and PKC beta (I), epsilon, and zeta translocation from the cytosol to the membrane fraction. Moreover, hypoxia increased the level of p44/42 mitogen-activated protein kinases (MAPKs) phosphorylation and hypoxia inducible factor-1alpha (HIF-1alpha) in a time-dependent manner. On the other hand, inhibition of these pathways blocked the hypoxia-induced increase in the 2-DG uptake and GLUT-1 protein expression level. Under hypoxia, cell cycle regulatory protein expression [cyclin D1, cyclin E, cyclin-dependent kinase (CDK) 2, and CDK 4] were increased in a time-dependent manner, which were blocked by PD 98059. pRB protein was also increased in a time-dependent manner. In conclusion, under hypoxia, there might be a parallel relationship between the expression of GLUT1 and DNA synthesis, which is mediated by the Ca(2+) /PKC, MAPK, and the HIF-1alpha signal pathways in mouse ES cells.

Li, L., E. Arman, et al. (2004). "Distinct GATA6- and laminin-dependent mechanisms regulate endodermal and ectodermal embryonic stem cell fates." Development **131**(21): 5277-86.

This study investigates the establishment of alternative cell fates during embryoid body differentiation when ES cells diverge into two epithelia simulating the pre-gastrulation endoderm and ectoderm. We report that endoderm differentiation and endoderm-specific gene expression, such as expression of laminin 1 subunits, is controlled by GATA6 induced by FGF. Subsequently, differentiation of the non-polar primitive ectoderm into columnar epithelium of the epiblast is induced by laminin 1. Using GATA6 transformed Lamc1-null endoderm-like cells, we demonstrate that laminin 1 exhibited by the basement membrane induces epiblast differentiation and cavitation by cell-to-matrix/matrix-to-cell interactions that are similar to the in vivo crosstalk in the early embryo. Pharmacological and dominant-negative inhibitors reveal that the cell shape change of epiblast differentiation requires ROCK, the Rho kinase. We also show that pluripotent ES cells display laminin receptors; hence, these stem cells may serve as target for columnar ectoderm differentiation. Laminin is not bound by endoderm derivatives; therefore, the sub-endodermal basement membrane is anchored selectively to the ectoderm, conveying polarity to its assembly and to the differentiation induced by it. Unique to these interactions is their flow through two cell layers connected by laminin 1 and their involvement in the differentiation of two epithelia from the same stem cell pool: one into endoderm controlled by FGF and GATA6; and the other into epiblast regulated by laminin 1 and Rho kinase.

Li, Y., J. McClintick, et al. (2005). "Murine embryonic stem cell differentiation is promoted by SOCS-3 and inhibited by the zinc finger transcription factor Klf4." Blood **105**(2): 635-7.

Embryonic stem (ES) cells homozygous for a Shp-2 mutation (Shp-2(Delta46-110)) demonstrate leukemia inhibitory factor (LIF) hypersensitivity and increased LIF-stimulated phosphorylation of signal transducer and activator of transcription (STAT3). We hypothesized that LIF-responsive genes in Shp-2(Delta46-110) cells would represent potential candidates for molecules vital for ES cell self-renewal. Using microarray analysis, we detected 41 genes whose expression was modified by LIF in Shp-2(Delta46-110) ES cells. Induction of 2 significantly up-regulated genes, suppressor of cytokine signaling-3 (SOCS-3) and Kruppel-like factor 4 (Klf4), was verified using Northern blotting. ES cells overexpressing SOCS-3 had an increased capacity to differentiate to hematopoietic progenitors, rather than to self-renew. In contrast, ES cells overexpressing Klf4 had a greater capacity to self-renew based on secondary embryoid body (EB) formation. Klf4-transduced d6 EBs expressed higher levels of Oct-4, consistent with the notion that Klf4 promotes ES cell self-renewal. These findings verify the negative role of SOCS-3 on LIF signaling and provide a novel role for Klf4 in ES cell function.

Lien, L. L., Y. Lee, et al. (1997). "Regulation of the myeloid-cell-expressed human gp91-phox gene as studied by transfer of yeast artificial chromosome clones into embryonic stem cells: suppression of a variegated cellular pattern of expression requires a full complement of distant cis elements." Mol Cell Biol **17**(4): 2279-90.

Identifying the full repertoire of cis elements required for gene expression in mammalian cells (or animals) is challenging, given the moderate sizes of many loci. To study how the human gp91-phox gene is expressed specifically in myeloid hematopoietic cells, we introduced yeast artificial chromosome (YAC) clones and derivatives generated in yeast into mouse embryonic stem cells competent to differentiate to myeloid cells in vitro or into mouse chimeras. Fully appropriate regulation was recapitulated with a 130-kb YAC containing 60 and 30 kb of 5' and 3' flanking sequences, respectively. Immunodetection of human gp91-phox protein revealed uniform expression in individual myeloid cells. The removal of upstream sequences led to

decreased overall expression which reflected largely a variegated pattern of expression, such that cells were either "on" or "off," rather than pancellular loss of expression. The proportion of clones displaying marked variegation increased with progressive deletion. DNase I mapping of chromatin identified two hypersensitive clusters, consistent with the presence of multiple regulatory elements. Our findings point to cooperative interactions of complex regulatory elements and suggest that the presence of an incomplete set of elements reduces the probability that an open chromatin domain (or active transcriptional complex) may form or be maintained in the face of repressive influences of neighboring chromatin.

Lin, G., Q. OuYang, et al. (2007). "A highly homozygous and parthenogenetic human embryonic stem cell line derived from a one-pronuclear oocyte following in vitro fertilization procedure." Cell Res **17**(12): 999-1007.

Homozygous human embryonic stem cells (hESCs) are thought to be better cell sources for hESC banking because their human leukocyte antigen (HLA) haplotype would strongly increase the degree of matching for certain populations with relatively smaller cohorts of cell lines. Homozygous hESCs can be generated from parthenogenetic embryos, but only heterozygous hESCs have been established using the current strategy to artificially activate the oocyte without second polar body extrusion. Here we report the first successful derivation of a human homozygous ESC line (chHES-32) from a one-pronuclear oocyte following routine in vitro fertilization treatment. chHES-32 cells express common markers and genes with normal hESCs. They have been propagated in an undifferentiated state for more than a year (>P50) and have maintained a stable karyotype of 46, XX. When differentiated in vivo and in vitro, chHES-32 cells can form derivatives from all three embryonic germ layers. The almost undetectable expression of five paternally expressed imprinted genes and their HLA genotype identical to the oocyte donor indicated their parthenogenetic origin. Using genome-wide single-nucleotide polymorphism analysis and DNA fingerprinting, the homozygosity of chHES-32 cells was further confirmed. The results indicated that 'unwanted' one-pronuclear oocytes might be a potential source for human homozygous and parthenogenetic ESCs, and suggested an alternative strategy for obtaining homozygous hESC lines from parthenogenetic haploid oocytes.

Lin, H. T., C. L. Kao, et al. (2007). "Enhancement of insulin-producing cell differentiation from embryonic stem cells using pax4-nucleofection method." World J Gastroenterol **13**(11): 1672-9.

AIM: To enhance the differentiation of insulin producing cell (IPC) ability from embryonic stem (ES) cells in vitro. METHODS: Four-day embryoid body (EB)-formatted ES cells were dissociated as single cells for the followed plasmid DNA delivery. The use of Nucleofector electroporator (Amaxa biosystems, Germany) in combination with medium-contained G418 provided a high efficiency of gene delivery for advanced selection. Neucleofected cells were plated on the top of fibronectin-coated Petri dishes. Addition of Ly294002 and raised the glucose in medium at 24 h before examination. The differentiation status of these cells was monitored by semi-quantitative PCR (SQ-PCR) detection of the expression of relative genes, such as oct-4, sox-17, foxa2, mixl1, pdx-1, insulin 1, glucagons and somatostatin. The percentage of IPC population on d 18 of the experiment was investigated by immunohistochemistry (IHC), and the content/secretion of insulin was estimated by ELISA assay. The mice with severe combined immunodeficiency disease (SCID) pretreated with streptozotocin (STZ) were used to eliminate plasma glucose restoration after pax4+ ES implantation. RESULTS: A high efficiency of gene delivery was demonstrated when neucleofection was used in the present study; approximately 70% cells showed DsRed expression 2 d after neucleofection. By selection of medium-contained G418, the percentage of DsRed expressing cells kept high till the end of study. The pancreatic differentiation seemed to be accelerated by pax4 nucleofection. When compared to the group of cells with mock control, foxa2, mixl1, pdx1, higher insulin and somatostatin levels were detected by SQ-PCR 4 d after nucleofection in the group of pax4 expressing plasmid delivery. Approximately 55% of neucleofected cells showed insulin expression 18 d after neucleofection, and only 18% of cells showed insulin expression in mock control. The disturbance was shown by nucleofected pax4 RNAi vector; only 8% of cells expressed insulin 18 d after nucleofection. A higher IPC population was also detected in the insulin content by ELISA assay, and the glucose dependency was demonstrated in insulin secretion level. In the animal model, improvement of average plasma glucose concentration was observed in the group of pax-4 expressed ES of SCID mice pretreated with STZ, but no significant difference was observed in the group of STZ-pretreated SCID mice who were transplanted ES with mock plasmid. CONCLUSION: Enhancement of IPC differentiation from EB-dissociated ES cells can be revealed by simply using pax4 expressing plasmid delivery. Not only more IPCs but also pancreatic

differentiation-related genes can be detected by SQ-PCR. Expression of relative genes, such as foxa 2, mixl 1, pdx-1, insulin 1 and somatostatin after nucleofection, suggests that pax4 accelerates the whole differentiation progress. The higher insulin production with glucose dependent modulation suggests that pax4 expression can drive more mature IPCs. Although further determination of the entire mechanism is required, the potential of pax-4-nucleofected cells in medical treatment is promising.

Love, P. E., M. L. Tremblay, et al. (1992). "Targeting of the T-cell receptor zeta-chain gene in embryonic stem cells: strategies for generating multiple mutations in a single gene." Proc Natl Acad Sci U S A **89**(20): 9929-33.

The T-cell receptor zeta chain is a member of a family of related proteins that play a critical role in coupling cell-surface receptors to intracellular signaling pathways. To study the role of zeta chain in T-cell ontogeny, we generated targeted mutations of the zeta-chain gene in murine embryonic stem cells. The mutant alleles are predicted to result either in a null phenotype or in the synthesis of a truncated protein capable of supporting T-cell-receptor surface expression but deficient in transmembrane signaling. Both of these targeting events were recovered in a single electroporation experiment with either coelectroporation or a combination deletion/truncation construct. Our results suggest that similar approaches could be used to generate multiple single mutations, modifications of more than one site within a gene, or subtle alterations that rely upon coconversion with the selectable marker gene.

Lu, M., C. H. Glover, et al. (2007). "Involvement of tyrosine kinase signaling in maintaining murine embryonic stem cell functionality." Exp Hematol **35**(8): 1293-302.

OBJECTIVE: We previously demonstrated that c-kit expression decreases during murine embryonic stem cell (ESC) differentiation induced by leukemia inhibitory factor removal. In this study, we addressed the possibility that c-kit is a marker of undifferentiated murine ESC and, moreover, that it plays a role in maintaining the undifferentiated state of these cells. MATERIALS AND METHODS: c-kit expression was analyzed under various differentiation conditions by flow cytometry and quantitative reverse transcription polymerase chain reaction. ESC were then sorted on the basis of c-kit expression and functionality was investigated using embryoid body and colony-forming cell assays. Imatinib (Gleevec) and ACK2 were used to block, and stem cell factor was used to stimulate, c-kit activity. RESULTS: c-kit expression decreased in two murine ESC lines under various differentiation conditions. Sorting of ESC populations on the basis of c-kit expression revealed significant differences in the functional capacities and gene expression profiles of the sorted populations. The inhibition studies revealed an important role for tyrosine kinase activity in maintaining ESC viability and differentiation capacity, at least in part by preventing apoptosis and enhancing cell cycle progression. However, activation of c-kit alone is not sufficient for maintaining undifferentiated ESC. CONCLUSION: The results suggest that c-kit may represent a useful marker for monitoring ESC functionality. Moreover, tyrosine kinase signaling plays an important role in maintaining undifferentiated ESC. This work provides valuable insights into the complex signaling pathways that synergize to maintain the undifferentiated state of murine ESC.

Lu, S. J., J. A. Hipp, et al. (2007). "GeneChip analysis of human embryonic stem cell differentiation into hemangioblasts: an in silico dissection of mixed phenotypes." Genome Biol **8**(11): R240.

BACKGROUND: Microarrays are being used to understand human embryonic stem cell (hESC) differentiation. Most differentiation protocols use a multi-stage approach that induces commitment along a particular lineage. Therefore, each stage represents a more mature and less heterogeneous phenotype. Thus, characterizing the heterogeneous progenitor populations upon differentiation are of increasing importance. Here we describe a novel method of data analysis using a recently developed differentiation protocol involving the formation of functional hemangioblasts from hESCs. Blast cells are multipotent and can differentiate into multiple lineages of hematopoeitic cells (erythroid, granulocyte and macrophage), endothelial and smooth muscle cells. RESULTS: Large-scale transcriptional analysis was performed at distinct time points of hESC differentiation (undifferentiated hESCs, embryoid bodies, and blast cells, the last of which generates both hematopoietic and endothelial progenies). Identifying genes enriched in blast cells relative to hESCs revealed a genetic signature indicative of erythroblasts, suggesting that erythroblasts are the predominant cell type in the blast cell population. Because of the heterogeneity of blast cells, numerous comparisons were made to publicly available data sets in silico, some of which blast cells are capable of differentiating into, to assess and characterize the blast cell population. Biologically relevant comparisons masked particular genetic signatures within the heterogeneous population and identified genetic signatures indicating the presence of endothelia, cardiomyocytes, and hematopoietic lineages in the

blast cell population. CONCLUSION: The significance of this microarray study is in its ability to assess and identify cellular populations within a heterogeneous population through biologically relevant in silico comparisons of publicly available data sets. In conclusion, multiple in silico comparisons were necessary to characterize tissue-specific genetic signatures within a heterogeneous hemangioblast population.

Luo, G., M. S. Yao, et al. (1999). "Disruption of mRad50 causes embryonic stem cell lethality, abnormal embryonic development, and sensitivity to ionizing radiation." Proc Natl Acad Sci U S A **96**(13): 7376-81.

The Mre11/Rad50 protein complex functions in diverse aspects of the cellular response to double-strand breaks (DSBs), including the detection of DNA damage, the activation of cell cycle checkpoints, and DSB repair. Whereas genetic analyses in Saccharomyces cerevisiae have provided insight regarding DSB repair functions of this highly conserved complex, the implication of the human complex in Nijmegen breakage syndrome reveals its role in cell cycle checkpoint functions. We established mRad50 mutant mice to examine the role of the mammalian Mre11/Rad50 protein complex in the DNA damage response. Early embryonic cells deficient in mRad50 are hypersensitive to ionizing radiation, consistent with a role for this complex in the repair of ionizing radiation-induced DSBs. However, the null mrad50 mutation is lethal in cultured embryonic stem cells and in early developing embryos, indicating that the mammalian Mre11/Rad50 protein complex mediates functions in normally growing cells that are essential for viability.

Luo, Y., C. Schwartz, et al. (2006). "A focused microarray to assess dopaminergic and glial cell differentiation from fetal tissue or embryonic stem cells." Stem Cells **24**(4): 865-75.

We designed oligonucleotide gene-specific probes to develop a focused array that can be used to discriminate between neural phenotypes, identify biomarkers, and provide an overview of the process of dopaminergic neuron and glial differentiation. We have arrayed approximately 100 genes expressed in dopaminergic neurons, oligodendrocytes, and astrocytes, an additional 200 known cytokines, chemokines, and their respective receptors, as well as markers for pluripotent and progenitor cells. The gene-specific 60-mer 3' biased oligonucleotides for these 281 genes were arrayed in a 25 x 12 format based on function. Using human adult brain substantia nigra, human embryonic stem cells (ESCs), and the differentiated progeny of pluripotent cells, we showed that this array was capable of distinguishing dopaminergic neurons, glial cells, and pluripotent cells by their gene expression profiles in a concentration-dependent manner. Using linear correlation coefficients of input RNA with output intensity, we identified a list of genes that can serve as reporting genes for detecting dopaminergic neurons, glial cells, and contaminating ESCs and progenitors. Finally, we monitored NTera2 differentiation toward dopaminergic neurons and have shown the ability of this array to distinguish stages of differentiation and provide important clues to factors regulating differentiation, the degree of contaminating populations, and stage of cell maturity. We suggest that this focused array will serve as a useful complement to other large-scale arrays in routine assessment of cell properties prior to their therapeutic use.

Ma, D. K., C. H. Chiang, et al. (2008). "G9a and Jhdm2a regulate embryonic stem cell fusion-induced reprogramming of adult neural stem cells." Stem Cells **26**(8): 2131-41.

Somatic nuclei can be reprogrammed to pluripotency through fusion with embryonic stem cells (ESCs). The underlying mechanism is largely unknown, primarily because of a lack of effective approaches to monitor and quantitatively analyze transient, early reprogramming events. The transcription factor Oct4 is expressed specifically in pluripotent stem cells, and its reactivation from somatic cell genome constitutes a hallmark for effective reprogramming. Here we developed a double fluorescent reporter system using engineered ESCs and adult neural stem cells/progenitors (NSCs) to simultaneously and independently monitor cell fusion and reprogramming-induced reactivation of transgenic Oct4-enhanced green fluorescent protein (EGFP) expression. We demonstrate that knockdown of a histone methyltransferase, G9a, or overexpression of a histone demethylase, Jhdm2a, promotes ESC fusion-induced Oct4-EGFP reactivation from adult NSCs. In addition, coexpression of Nanog and Jhdm2a further enhances the ESC-induced Oct4-EGFP reactivation. Interestingly, knockdown of G9a alone in adult NSCs leads to demethylation of the Oct4 promoter and partial reactivation of the endogenous Oct4 expression from adult NSCs. Our results suggest that ESC-induced reprogramming of somatic cells occurs with coordinated actions between erasure of somatic epigenome and transcriptional resetting to restore pluripotency. These mechanistic findings may guide more efficient reprogramming for future therapeutic applications of stem cells. Disclosure of potential conflicts of interest is found at the end of this article.

Manceur, A., A. Wu, et al. (2007). "Flow cytometric screening of cell-penetrating peptides for their uptake into embryonic and adult stem cells." Anal Biochem **364**(1): 51-9.

There is an increasing appreciation of the potential of cell-penetrating peptides (CPPs) as vectors to deliver peptides, proteins, and DNA into cells. However, the absolute and relative efficacy of various CPPs for applications targeting stem cells and primary cells is unclear. In this study, we have developed a two-step loading method and a flow cytometric assay to systematically compare the cellular uptake of five CPPs into embryonic stem cells, neurospheres (NSs), primary bone marrow hematopoietic progenitor (Sca-1(+)Lin(-)) cells, and hematopoietic cell lines (TF-1, K562, and FDCP Mix). The series of CPPs tested included three arginine-rich peptides; one was derived from HIV transactivator of transcription (TAT), one was derived from Antennapedia (Antp), and the third was a synthetic peptide known as protein transduction domain 4 (PTD4). Two hydrophobic peptides were also tested; one was derived from Kaposi fibroblast growth factor (K-FGF), and one was derived from PreS2 surface antigen of hepatitis B virus (PreS2-TLM). Our results indicate, for the first time, that arginine-rich CPPs can internalize into primary NSs and bone marrow Sca-1(+)Lin(-) cells. In addition, in all cell types examined, the uptake of arginine-rich CPPs is significantly greater than that of hydrophobic peptides.

Martin, G. R., L. M. Silver, et al. (1987). "Establishment of embryonic stem cell lines from preimplantation mouse embryos homozygous for lethal mutations in the t-complex." Dev Biol **121**(1): 20-8.

We have determined the frequency at which embryonic stem cell (ESC) lines can be established from inner cell masses (ICMs) isolated from blastocysts homozygous for lethal mutations in the mouse t-complex. Approximately one-third of the expected number, 3/29, of the ESC lines established from embryos obtained by inter-se mating of +/tw18 mice are homozygous for the tw18 haplotype. These tw18/tw18 ESC lines form a variety of cell types in vitro and in vivo, including mesodermal derivatives such as cartilage and muscle. On the basis of these and data from other studies, we suggest that the normal function of the gene represented by the tw18 lethal allele is required for multiplication/survival of mesodermal precursors in the embryo rather than the specification of the mesodermal lineage, and that the lethal effects of this mutation are expressed in only the highly structured environment of the early postimplantation embryo. In studies of the lethal tw5 haplotype, we found that 2/2 ESC lines obtained are mutant homozygotes. Analysis of these data, in conjunction with the results of our earlier study (Magnuson, T., Epstein, C. J., Silver, L. M., and Martin, G. R. (1982), Nature (London) 298, 750-753), suggests that homozygosity for the genes found in the tw5 haplotype does not reduce cell viability. By contrast, 0/16 ESC lines isolated from embryos obtained from matings of +/t0 mice are mutant homozygotes. Analysis of the genotypes of ICM-derived primary stem cell colonies suggests that t0 homozygous ICM cells are unable to undergo sufficient proliferation in vitro to give rise to ESC lines.

Martinat, C., J. J. Bacci, et al. (2006). "Cooperative transcription activation by Nurr1 and Pitx3 induces embryonic stem cell maturation to the midbrain dopamine neuron phenotype." Proc Natl Acad Sci U S A **103**(8): 2874-9.

Midbrain dopamine (DA) neurons play a central role in the regulation of voluntary movement, and their degeneration is associated with Parkinson's disease. Cell replacement therapies, and in particular embryonic stem (ES) cell-derived DA neurons, offer a potential therapeutic venue for Parkinson's disease. We sought to identify genes that can potentiate maturation of ES cell cultures to the midbrain DA neuron phenotype. A number of transcription factors have been implicated in the development of midbrain DA neurons by expression analyses and loss-of-function knockout mouse studies, including Nurr1, Pitx3, Lmx1b, Engrailed-1, and Engrailed-2. However, none of these factors appear sufficient alone to induce the mature midbrain DA neuron phenotype in ES cell cultures in vitro, suggesting a more complex regulatory network. Here we show that Nurr1 and Pitx3 cooperatively promote terminal maturation to the midbrain DA neuron phenotype in murine and human ES cell cultures.

Masui, S., D. Shimosato, et al. (2005). "An efficient system to establish multiple embryonic stem cell lines carrying an inducible expression unit." Nucleic Acids Res **33**(4): e43.

The growing use of mouse embryonic stem (ES) cells in research emphasizes their importance in studies of molecular mechanisms that maintain pluripotency and direct cellular differentiation. Although systems for regulatable transgene expression are essential for fine analysis of cellular processes at the molecular level, a strategy for the establishment of multiple ES cell lines carrying any of these systems has not yet been described. Here, we report our development of the ROSA-TET system, an effective system for the establishment of multiple ES

cell lines carrying a tetracycline (Tc)-regulatable transgene at the Gt (ROSA)26asSor (ROSA26) locus. This system contains a knock-in step of a construct carrying both loxP and its mutant sequences into the ROSA26 locus, followed by a subsequent exchange step that introduces a cDNA to be Tc-regulated to the locus using the recombinase-mediated cassette exchange reaction. Both steps are demonstrated to give desired clones with high efficiency, suggesting that this system can be introduced readily into any ES cell lines, leading to the simultaneous establishment of multiple cell lines carrying different Tc-regulated cDNAs. We believe that use of this system will strongly accelerate molecular biological research using ES cells.

Mathur, D., T. W. Danford, et al. (2008). "Analysis of the mouse embryonic stem cell regulatory networks obtained by ChIP-chip and ChIP-PET." Genome Biol **9**(8): R126.

BACKGROUND: Genome-wide approaches have begun to reveal the transcriptional networks responsible for pluripotency in embryonic stem (ES) cells. Chromatin Immunoprecipitation (ChIP) followed either by hybridization to a microarray platform (ChIP-chip) or by DNA sequencing (ChIP-PET), has identified binding targets of the ES cell transcription factors OCT4 and NANOG in humans and mice, respectively. These studies have provided an outline of the transcriptional framework involved in maintaining pluripotency. Recent evidence with comparing multiple technologies suggests that expanding these datasets using different platforms would be a useful resource for examining the mechanisms underlying pluripotency regulation. RESULTS: We have now identified OCT4 and NANOG genomic targets in mouse ES cells by ChIP-chip and provided the means to compare these data with previously reported ChIP-PET results in mouse ES cells. We have mapped the sequences of OCT4 and NANOG binding events from each dataset to genomic coordinates, providing a valuable resource to facilitate a better understanding of the ES cell regulatory circuitry. Interestingly, although considerable differences are observed in OCT4 and NANOG occupancy as identified by each method, a substantial number of targets in both datasets are enriched for genes that have known roles in cell-fate specification and that are differentially expressed upon Oct4 or Nanog knockdown. CONCLUSION: This study suggests that each dataset is a partial representation of the overall ES cell regulatory circuitry, and through integrating binding data obtained by ChIP-chip and ChIP-PET, the methods presented here provide a useful means for integrating datasets obtained by different techniques in the future.

Miller-Hance, W. C., M. LaCorbiere, et al. (1993). "In vitro chamber specification during embryonic stem cell cardiogenesis. Expression of the ventricular myosin light chain-2 gene is independent of heart tube formation." J Biol Chem **268**(33): 25244-52.

The molecular cues that control patterning of the heart tube during early cardiogenesis are largely unknown. The present study has explored the embryonic stem (ES) cell differentiation system to determine if this in vitro model could be useful in studying the process of regional specification of cardiac muscle cells at the earliest possible stages. As assessed by polymerase chain reaction, ribonuclease protection, in situ hybridization, and immunohistochemical analyses, ES cell differentiation into embryoid bodies is characterized by the transcriptional and translational activation of the ventricular regulatory (phosphorylatable) myosin light chain gene, demonstrating that ventricular specification occurs during ES cell cardiogenesis. The finding of a ventricular-specific marker in an in vitro system in the absence of an intact heart tube provides evidence for cardiac regional specification independent of positional cues or physiologic stimuli. The temporal expression of the myogenic regulatory factors, myogenin and MyoD, suggests activation of the skeletal muscle program following cardiac myogenesis in vitro, indicating temporal fidelity to the progression of in vivo myogenesis. These data establish the mouse embryonic stem cell system as a model for cardiac chamber specification and suggest a promising approach in the study of regional specification in genetically engineered cardiac muscle cells.

Mombaerts, P., A. R. Clarke, et al. (1991). "Creation of a large genomic deletion at the T-cell antigen receptor beta-subunit locus in mouse embryonic stem cells by gene targeting." Proc Natl Acad Sci U S A **88**(8): 3084-7.

Recently it has become possible to introduce predesigned mutations into a given gene in the mouse germ line by homologous recombination in embryonic stem cells. The mutations are usually introduced by inserting the neomycin phosphotransferase gene into an exon of a particular gene. Here we describe an extension of this method that can result in at least a 15-kilobase-long deletion. The deletion created in the present work encompasses one of the two diversity gene segments of the mouse T-cell receptor beta-subunit locus, 10 out of the 12 joining gene segments, and both constant gene segments. This strategy is a valuable alternative to sequential targeting of multiple genes forming a gene cluster, could simplify the construction of plasmids to be used for targeting, and

could be the solution for inactivating small genes that have eluded conventional targeting approaches.

Mukhopadhyay, A., D. Das, et al. (2003). "Embryonic stem cell and tissue-specific expression of a novel conserved gene, asrij." Dev Dyn **227**(4): 578-86.

We have identified a novel gene expressed in murine embryonic stem (ES) cells and in a restricted, tissue-specific pattern during mouse development. The gene is also expressed in blood vessels; hence, we have named it asrij (Sanskrit; asRij= blood). The gene encodes a novel conserved, predicted transmembrane protein of 247 amino acids, which is localized to lysosomes and endosomes. During ES cell-derived blood vessel formation in vitro, Asrij expression precedes and partially overlaps with the vascular markers Flk-1 and PECAM. During development, Asrij is expressed predominantly in mouse embryonic blood vessels. The asrij transcript is alternatively spliced, and its expression is regulated in a tissue-specific manner. An asrij splice variant that is enriched in the adult mouse brain encodes a protein of 196 amino acids. Asrij can serve as an early stem cell marker that is down-regulated in nonvascular tissues. Our data indicate that Asrij belongs to a novel class of conserved proteins with a complex developmental profile and suggests multiple functions for the gene.

Naujok, O., F. Francini, et al. (2008). "An efficient experimental strategy for mouse embryonic stem cell differentiation and separation of a cytokeratin-19-positive population of insulin-producing cells." Cell Prolif **41**(4): 607-24.

OBJECTIVES: Embryonic stem cells are a potential source for insulin-producing cells, but existing differentiation protocols are of limited efficiency. Here, the aim has been to develop a new one, which drives development of embryonic stem cells towards insulin-producing cells rather than to neuronal cell types, and to combine this with a strategy for their separation from insulin-negative cells. MATERIALS AND METHODS: The cytokeratin-19 (CK19) promoter was used to control the expression of enhanced yellow fluorescence protein in mouse embryonic stem cells during their differentiation towards insulin-producing cells, using a new optimized four-stage protocol. Two cell populations, CK19(+) and CK19(-) cells, were successfully fluorescence sorted and analysed. RESULTS: The new method reduced neuronal progeny and suppressed differentiation into glucagon- and somatostatin-producing cells. Concomitantly, beta-cell like characteristics of insulin-producing cells were strengthened, as documented by high gene expression of the Glut2 glucose transporter and the transcription factor Pdx1. This novel protocol was combined with a cell-sorting technique. Through the combined procedure, a fraction of glucose-responsive insulin-secreting CK19(+) cells was obtained with 40-fold higher insulin gene expression and 50-fold higher insulin content than CK19(-) cells. CK19(+) cells were immunoreactive for C-peptide and had ultrastructural characteristics of an insulin-secretory cell. CONCLUSION: Differentiated CK19(+) cells reflect an endocrine precursor cell type of ductal origin, potentially suitable for insulin replacement therapy in diabetes.

Nikolova, T., J. Czyz, et al. (2005). "Electromagnetic fields affect transcript levels of apoptosis-related genes in embryonic stem cell-derived neural progenitor cells." Faseb J **19**(12): 1686-8.

Mouse embryonic stem (ES) cells were used as an experimental model to study the effects of electromagnetic fields (EMF). ES-derived nestin-positive neural progenitor cells were exposed to extremely low frequency EMF simulating power line magnetic fields at 50 Hz (ELF-EMF) and to radiofrequency EMF simulating the Global System for Mobile Communication (GSM) signals at 1.71 GHz (RF-EMF). Following EMF exposure, cells were analyzed for transcript levels of cell cycle regulatory, apoptosis-related, and neural-specific genes and proteins; changes in proliferation; apoptosis; and cytogenetic effects. Quantitative RT-PCR analysis revealed that ELF-EMF exposure to ES-derived neural cells significantly affected transcript levels of the apoptosis-related bcl-2, bax, and cell cycle regulatory "growth arrest DNA damage inducible" GADD45 genes, whereas mRNA levels of neural-specific genes were not affected. RF-EMF exposure of neural progenitor cells resulted in down-regulation of neural-specific Nurr1 and in up-regulation of bax and GADD45 mRNA levels. Short-term RF-EMF exposure for 6 h, but not for 48 h, resulted in a low and transient increase of DNA double-strand breaks. No effects of ELF- and RF-EMF on mitochondrial function, nuclear apoptosis, cell proliferation, and chromosomal alterations were observed. We may conclude that EMF exposure of ES-derived neural progenitor cells transiently affects the transcript level of genes related to apoptosis and cell cycle control. However, these responses are not associated with detectable changes of cell physiology, suggesting compensatory mechanisms at the translational and posttranslational level.

Nishimoto, M., A. Fukushima, et al. (1999). "The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2." Mol Cell Biol **19**(8): 5453-65.

UTF1 is a transcriptional coactivator which has recently been isolated and found to be expressed mainly in pluripotent embryonic stem (ES) cells (A. Okuda, A. Fukushima, M. Nishimoto, et al., EMBO J. 17:2019-2032, 1998). To gain insight into the regulatory network of gene expression in ES cells, we have characterized the regulatory elements governing UTF1 gene expression. The results indicate that the UTF1 gene is one of the target genes of an embryonic octamer binding transcription factor, Oct-3/4. UTF1 expression is, like the FGF-4 gene, regulated by the synergistic action of Oct-3/4 and another embryonic factor, Sox-2, implying that the requirement for Sox-2 by Oct-3/4 is not limited to the FGF-4 enhancer but is rather a general mechanism of activation for Oct-3/4. Our biochemical analyses, however, also reveal one distinct difference between these two regulatory elements: unlike the FGF-4 enhancer, the UTF1 regulatory element can, by its one-base difference from the canonical octamer-binding sequence, selectively recruit the complex comprising Oct-3/4 and Sox-2 and preclude the binding of the transcriptionally inactive complex containing Oct-1 or Oct-6. Furthermore, our analyses reveal that these properties are dictated by the unique ability of the Oct-3/4 POU-homeodomain that recognizes a variant of the Octamer motif in the UTF1 regulatory element.

Nishimoto, M., S. Miyagi, et al. (2005). "Oct-3/4 maintains the proliferative embryonic stem cell state via specific binding to a variant octamer sequence in the regulatory region of the UTF1 locus." Mol Cell Biol **25**(12): 5084-94.

The POU transcription factor Oct-3/4 has been shown to be critical for maintaining embryonic stem (ES) cell character. However, the molecular mechanisms underlying its function remain elusive. We have previously shown that among the POU transcription factor family of proteins, Oct-3/4 alone is able to bind to the regulatory region of the UTF1 gene bearing a variant octamer sequence together with Sox-2. Here, we demonstrate using Oct-3/4-Oct-6 chimeras that there is a precise correlation between the ability of proteins to form a complex on the UTF1 enhancer with Sox-2 and the ability to maintain the stem cell state in ES cells. Different chimeric proteins show differential abilities to form a Sox-2-containing complex on the UTF1 regulatory region, with a decrease in efficiency of the complex formation accompanied by a decrease in the level of UTF1 expression and the rate of cell proliferation. Overexpression of UTF1 in these slow-growing cells was able to restore their proliferation rate to wild-type levels. Moreover, UTF1 was also observed to have an effect on teratoma formation. These results suggest a molecular pathway by which Oct-3/4 induces rapid proliferation and tumorigenic properties of ES cells through activation of the UTF1 gene.

Nogueira, M. M., M. T. Mitjavila-Garcia, et al. (2000). "Regulation of Id gene expression during embryonic stem cell-derived hematopoietic differentiation." Biochem Biophys Res Commun **276**(2): 803-12.

To elucidate the role of helix-loop-helix (HLH) Id proteins in hematopoietic differentiation, we used a model of embryonic stem (ES) cell differentiation in vitro which gives access not only to hematopoietic myeloid progenitor cells but also to the more primitive blast colony-forming cell (BL-CFC), the in vitro equivalent of the hemangioblast that gives rise to blast cell colonies in the presence of VEGF. We first demonstrated that ES cell-derived blast cell colonies could be used as a model to study hematopoietic differentiation and maturation. We next established the expression profile of Id genes in this model. Transcripts of the four Id genes were present in ES cells. Id1, Id3 and Id4 gene expression was down-regulated during the development of blast cell colonies while that of Id2 was maintained. Thus, Id1, Id3, and Id4 proteins are candidates for being negative regulators of hematopoiesis in the model of hematopoietic ES cell differentiation in vitro.

Novosadova, E. V., E. S. Manuilova, et al. (2005). "Different effects of enhanced and reduced expression of pub gene on the formation of embryoid bodies by cultured embryonic mouse stem cell." Bull Exp Biol Med **140**(1): 153-8.

The effects of pub gene on proliferation and initial stages of differentiation of embryonic mouse stem cells were studied in vitro. To this end we used enhanced expression of human pub gene (hpub) and suppression of expression of mouse endogenous pub gene with RNA-interference in embryonic stem cells. Proliferative activity of genetically modified polyclonal lines of the embryonic stem cells transfected with plasmids carrying expressing hpub gene or plasmids generating small interference RNA to this gene did not differ from that of the control cells. Inhibition of expression of endogenous pub gene in embryonic stem cells using small interference RNA 2-fold decreased the formation of embryoid bodies, at the same time additional expression of exogenous hpub gene almost 2-fold increased their number in comparison with the control. It was hypothesized that pub gene participates in early stages of differentiation of embryonic stem cells leading to the formation of embryoid bodies.

Ohba, S., T. Ikeda, et al. (2007). "Identification of a potent combination of osteogenic genes for bone

regeneration using embryonic stem (ES) cell-based sensor." Faseb J **21**(8): 1777-87.

To identify potent bioactive factors for in vivo tissue regeneration by comprehensive screening remains a challenge for regenerative medicine. Here we report the development of an ES cell-based monitoring system for osteogenic differentiation, the identification of a potent combination of osteogenic genes using such a system, and an evaluation of its therapeutic potentials. ES cells were isolated from mice carrying a transgene expressing GFP driven by the 2.3 kb fragment of rat type I collagen alpha1 promoter. Using these cells engineered to fluoresce on osteogenic differentiation, we screened cDNA libraries and combinations of major osteogenesis-related genes. Among them, the combination of constitutively active activin receptor-like kinase 6 (caALK6) and runt-related transcription factor 2 (Runx2) was the minimal unit that induced fluorescence. The combination efficiently induced osteogenic differentiation in various cell types, including terminally differentiated nonosteogenic cells. The cooperative action of the combination occurred through protein stabilization of core binding factor beta (Cbfb), induction of Runx2-Cbfb complex formation, and its DNA binding. Furthermore, transplantation of a monolayer sheet of fibroblasts transduced with the combination achieved bone regeneration within 4 wk in mouse calvarial bone defects. Thus, we successfully identified the potent combination of genes for bone regeneration, which helped broaden cell sources.

Parekkadan, B., Y. Berdichevsky, et al. (2008). "Cell-cell interaction modulates neuroectodermal specification of embryonic stem cells." Neurosci Lett **438**(2): 190-5.

The controlled differentiation of embryonic stem (ES) cells is of utmost interest to their clinical, biotechnological, and basic science use. Many investigators have combinatorially assessed the role of specific soluble factors and extracellular matrices in guiding ES cell fate, yet the interaction between neighboring cells in these heterogeneous cultures has been poorly defined due to a lack of conventional tools to specifically uncouple these variables. Herein, we explored the role of cell-cell interactions during neuroectodermal specification of ES cells using a microfabricated cell pair array. We tracked differentiation events in situ, using an ES cell line expressing green fluorescent protein (GFP) under the regulation of the Sox1 gene promoter, an early marker of neuroectodermal germ cell commitment in the adult forebrain. We observed that a previously specified Sox1-GFP+ cell could induce the specification of an undifferentiated ES cell. This induction was modulated by the two cells being in contact and was dependent on the age of previously specified cell prior to coculture. A screen of candidate cell adhesion molecules revealed that the expression of connexin (Cx)-43 correlated with the age-dependent effect of cell contact in cell pair experiments. ES cells deficient in Cx-43 showed aberrant neuroectodermal specification and lineage commitment, highlighting the importance of gap junctional signaling in the development of this germ layer. Moreover, this study demonstrates the integration of microscale culture techniques to explore the biology of ES cells and gain insight into relevant developmental processes otherwise undefined due to bulk culture methods.

Park, C. H., Y. K. Minn, et al. (2005). "In vitro and in vivo analyses of human embryonic stem cell-derived dopamine neurons." J Neurochem **92**(5): 1265-76.

Human embryonic stem (hES) cells, due to their capacity of multipotency and self-renewal, may serve as a valuable experimental tool for human developmental biology and may provide an unlimited cell source for cell replacement therapy. The purpose of this study was to assess the developmental potential of hES cells to replace the selectively lost midbrain dopamine (DA) neurons in Parkinson's disease. Here, we report the development of an in vitro differentiation protocol to derive an enriched population of midbrain DA neurons from hES cells. Neural induction of hES cells co-cultured with stromal cells, followed by expansion of the resulting neural precursor cells, efficiently generated DA neurons with concomitant expression of transcriptional factors related to midbrain DA development, such as Pax2, En1 (Engrailed-1), Nurr1, and Lmx1b. Using our procedure, the majority of differentiated hES cells (> 95%) contained neuronal or neural precursor markers and a high percentage (> 40%) of TuJ1+ neurons was tyrosine hydroxylase (TH)+, while none of them expressed the undifferentiated ES cell marker, Oct 3/4. Furthermore, hES cell-derived DA neurons demonstrated functionality in vitro, releasing DA in response to KCl-induced depolarization and reuptake of DA. Finally, transplantation of hES-derived DA neurons into the striatum of hemi-parkinsonian rats failed to result in improvement of their behavioral deficits as determined by amphetamine-induced rotation and step-adjustment. Immunohistochemical analyses of grafted brains revealed that abundant hES-derived cells (human nuclei+ cells) survived in the grafts, but none of them were TH+. Therefore, unlike those from mouse ES cells, hES cell-derived DA neurons either do not survive or their DA phenotype is unstable when grafted into rodent brains.

Pasini, D., A. P. Bracken, et al. (2007). "The polycomb group protein Suz12 is required for embryonic stem cell differentiation." Mol Cell Biol **27**(10): 3769-79.

Polycomb group (PcG) proteins form multiprotein complexes, called Polycomb repressive complexes (PRCs). PRC2 contains the PcG proteins EZH2, SUZ12, and EED and represses transcription through methylation of lysine (K) 27 of histone H3 (H3). Suz12 is essential for PRC2 activity and its inactivation results in early lethality of mouse embryos. Here, we demonstrate that Suz12(-/-) mouse embryonic stem (ES) cells can be established and expanded in tissue culture. The Suz12(-/-) ES cells are characterized by global loss of H3K27 trimethylation (H3K27me3) and higher expression levels of differentiation-specific genes. Moreover, Suz12(-/-) ES cells are impaired in proper differentiation, resulting in a lack of repression of ES cell markers as well as activation of differentiation-specific genes. Finally, we demonstrate that the PcGs are actively recruited to several genes during ES cell differentiation, which despite an increase in H3K27me3 levels is not always sufficient to prevent transcriptional activation. In summary, we demonstrate that Suz12 is required for the establishment of specific expression programs required for ES cell differentiation. Furthermore, we provide evidence that PcGs have different mechanisms to regulate transcription during cellular differentiation.

Pasumarthi, K. B., S. C. Tsai, et al. (2001). "Coexpression of mutant p53 and p193 renders embryonic stem cell-derived cardiomyocytes responsive to the growth-promoting activities of adenoviral E1A." Circ Res **88**(10): 1004-11.

Expression of adenoviral E1A in cardiomyocytes results in the activation of DNA synthesis followed by apoptosis. In contrast, expression of simian virus 40 large T antigen induces sustained cardiomyocyte proliferation. Previous studies have shown that T antigen binds to 2 proapoptotic proteins in cardiomyocytes, namely the p53 tumor suppressor and p193 (a new member of the BH3-only proapoptosis subfamily). Structure-function analyses identified a p193 C-terminal truncation mutant that encodes prosurvival activity. This mutant was used to test the role of p193 in E1A-induced cardiomyocyte apoptosis. E1A induced apoptosis in cardiomyocytes derived from differentiating embryonic stem cells. Expression of the prosurvival p193 mutant alone or a mutant p53 alone did not block E1A-induced apoptosis. In contrast, combinatorial expression of mutant p193 and mutant p53 blocked E1A-induced apoptosis, resulting in a proliferative response indistinguishable from that seen with T antigen. These results confirm the hypothesis that there are 2 proapoptotic pathways, encoded by p53 and p193, respectively, which restrict cardiomyocyte cell cycle activity in differentiating embryonic stem cell cultures. Furthermore, these results explain in molecular terms the phenotypic differences of E1A versus T-antigen gene transfer in cardiomyocytes.

Paulis, M., M. Bensi, et al. (2007). "Transfer of a human chromosomal vector from a hamster cell line to a mouse embryonic stem cell line." Stem Cells **25**(10): 2543-50.

Two transchromosomic mouse embryonic stem (ES) sublines (ESMClox1.5 and ESMClox2.1) containing a human minichromosome (MC) were established from a sample of hybrid colonies isolated in fusion experiments between a normal diploid mouse ES line and a Chinese hamster ovary line carrying the MC. DNA cytometric and chromosome analyses of ESMClox1.5 and ESMClox2.1 indicated a mouse chromosome complement with a heteroploid constitution in a subtetraploid range; the karyotypes showed various degrees of polysomy for different chromosomes. A single copy of the MC was found in the majority of cells in all the isolated hybrid colonies and was stably maintained in the established sublines for more than 100 cell generations either with or without the selective agent. No significant differences from the ES parental cells were observed in growth characteristics of the transchromosomic ES sublines. ESMClox1.5 cells were unable to grow in soft agar; when cultured in hanging drops, they formed embryoid bodies, and when inoculated in nude mice, they produced teratomas. They were able to express the early development markers Oct4 and Nanog, as demonstrated by reverse transcription-polymerase chain reaction assay. All these features are in common with the ES parental line. Further research using the transchromosomic ES sublines described here may allow gene expression studies on transferred human minichromosomes and could shed light on the relationships among ploidy, pluripotency, cell transformation, and tumorigenesis. Disclosure of potential conflicts of interest is found at the end of this article.

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.

Peterson, L. F., M. C. Lo, et al. (2007). "Inability of RUNX1/AML1 to breach AML1-ETO block of embryonic stem cell definitive hematopoiesis." Blood Cells Mol Dis **39**(3): 321-8.

The t(8;21)(q22:q22) translocation associated with acute myeloid leukemia fuses the AML1/RUNX1 N-terminal portion located on chromosome 21 to most of the ETO/MTG8 gene on chromosome 8. Various investigators have shown that the fusion product AML1-ETO on its own is unable to promote leukemia. Early studies using transgenic mouse models demonstrated that the direct knock-in of the fusion protein expression is embryonic lethal, similar to the AML1 knockout, suggesting that AML1-ETO has a dominant negative role over AML1. Using the embryonic stem cells generated for such studies, we show here that the presence of the fusion product AML1-ETO blocks definitive hematopoiesis in vitro as well, in both one and two step methylcellulose methods of embryonic stem cell hematopoietic differentiation. However, there is a very low occurrence of macrophage colonies, similar to the knock-in mice that display macrophages in cell cultures of yolk sac derived cells. In addition, we show that exogenous expression of AML1 is unable to bypass this AML1-ETO induced definitive hematopoietic block in these cells. This inability is not linked to an inability to reverse gene expression inhibition by AML1-ETO of the PU.1 gene associated with stem cell maintenance and myeloid differentiation. Our results suggest that AML1-ETO functions in a complex competitive manner with AML1 involving transcriptional regulation, protein-protein interactions and post-transcriptional mechanism(s) affecting early embryonic hematopoiesis and possibly leukemogenesis.

Phipps, S. M., W. K. Love, et al. (2009). "Differential expression of epigenetic modulators during human embryonic stem cell differentiation." Mol Biotechnol **41**(3): 201-7.

Although the progression of aging and the diseases associated with it are extensively studied, little is known about the initiation of the aging process. Telomerase is down-regulated early in embryonic differentiation, thereby contributing to telomeric attrition and aging. The mechanisms underlying this inhibition remain elusive, but epigenetic studies in differentiating human embryonic stem (hES) cells could give clues about how and when DNA methylation and histone deacetylation work together to contribute to the inactivation of hTERT, the catalytic subunit of telomerase, at the onset of the aging process. We have confirmed the differentiation status of cultured hES colonies with morphological assessment and immunohistochemical stainings for pluripotent stem cells. In hES cells with varying degrees of differentiation, we have shown a stronger association between hES differentiation and expression of the epigenetic regulators DNMT3A and DNMT3B than between genetic modulators of differentiation such as c-MYC. We also propose a new model system for analyses of stem cell regions, which are differentially down-regulating the expression of hTERT and the actions of epigenetic modulators such as the DNMTs and histone methyltransferases.

Plaia, T. W., R. Josephson, et al. (2006). "Characterization of a new NIH-registered variant human embryonic stem cell line, BG01V: a tool for human embryonic stem cell research." Stem Cells **24**(3): 531-46.

Human embryonic stem cells (hESCs) offer a renewable source of a wide range of cell types for use in research and cell-based therapies. Characterizing these cells provides important information about their current state and affords relevant details for subsequent manipulations. For example, identifying genes expressed during culture, as well as their temporal expression order after passaging and conditions influencing the formation of all three germ layers may be helpful for the production of functional beta islet cells used in treating type I diabetes. Although several hESC lines have demonstrated karyotypic instability during extended time in culture, select variant lines exhibit characteristics similar to their normal parental lines. Such variant lines may be

excellent tools and abundant sources of cells for pilot studies and in vitro differentiation research in which chromosome number is not a concern, similar to the role currently played by embryonal carcinoma cell lines. It is crucial that the cells be surveyed at a genetic and proteomic level during extensive propagation, expansion, and manipulation in vitro. Here we describe a comprehensive characterization of the variant hESC line BG01V, which was derived from the karyotypically normal, parental hESC line BG01. Our characterization process employs cytogenetic analysis, short tandem repeat and HLA typing, mitochondrial DNA sequencing, gene expression analysis using quantitative reverse transcription-polymerase chain reaction and microarray, assessment of telomerase activity, methylation analysis, and immunophenotyping and teratoma formation, in addition to screening for bacterial, fungal, mycoplasma, and human pathogen contamination.

Player, A., Y. Wang, et al. (2006). "Comparisons between transcriptional regulation and RNA expression in human embryonic stem cell lines." Stem Cells Dev **15**(3): 315-23.

Recent studies have focused on transcriptional regulation and gene expression profiling of human embryonic stem cells (hESCs). However, little information is available regarding the relationship between RNA expression and transcriptional regulation, which is critical in the complete understanding of pluripotency and differentiation of hESCs. In the current study, we determined RNA expression of three different hESC lines compared to Human universal reference RNA expression (HuU-RNA) using a full genome expression microarray, and compared our results to target genes previously identified using ChIP-on-chip analysis. The objective was to identify genes common between the two methods, and generate a more reliable list of embryonic signature genes. Even though hESCs were obtained from different sources and maintained under different conditions, a considerable number of genes could be identified as common between RNA expression and transcriptional regulation analyses. As an example, results from ChIP-on-chip studies show that OCT4, SOX2, and NANOG co-occupy SOX2, OCT4, TDGF1, GJA1, SET, and DPPA4 genes. The results are consistent with RNA expression analyses that demonstrate these genes as differently expressed in our hESC lines, further substantiating their role across cell types and confirming their importance as embryonic signatures. In addition, we report the differential expression of growth arrest-specific (GAS) family of genes in hESC. GAS2L1 and GAS3 members of this family appear to be transcriptionally regulated by OCT4, SOX2, or NANOG, whereas GAS5 and GAS6 are not; all of the genes are differentially expressed, as determined by microarray and validated via quantitative (Q)- PCR. Collectively, these data provide insight into the relationship between gene expression and transcriptional regulation, resulting in a reliable list of genes associated with hESCs.

Prandini, M. H., A. Desroches-Castan, et al. (2007). "No evidence for vasculogenesis regulation by angiostatin during mouse embryonic stem cell differentiation." J Cell Physiol **213**(1): 27-35.

During embryogenesis, the formation of blood vessels proceeds by both vasculogenesis and angiogenesis. Both processes appear to be finely regulated. To date, factors and genes involved in the negative regulation of embryonic vasculogenesis remain largely unknown. Angiostatin is a proteolytic fragment of plasminogen that acts as an inhibitor of angiogenesis. In this study, we analyzed the potential role of angiostatin during early stages of embryonic stem (ES) cell endothelial in vitro differentiation, as a model of vasculogenesis. We found an early expression of the known angiostatin binding sites (angiomotin, alphav integrin and c-met oncogene) during ES cell differentiation. Nevertheless, we did not detect any significant effect of angiostatin on mesoderm induction and on differentiation commitment into cells of the endothelial lineage. In both control and angiostatin-treated conditions, the temporal and extent of formation of the Flk1 positive and Flk-1/CD31 (PECAM-1) positive cell populations were not significantly different. Quantitative RT-PCR experiments of endothelial gene expression (Flk-1, PECAM-1 and tie-2) confirm a lack of interference with early steps of endothelial differentiation in embryoid bodies. No evidence for an angiostatin effect on endothelial cord-like formation could be detected at later differentiation stages. On the other hand, angiostatin inhibits vascular endothelial growth factor-induced endothelial sprouting from embryoid bodies cultured in three dimensional type I collagen gels. Taken together, these findings support a selective inhibitory effect on the sprouting angiogenesis response for angiostatin during embryonic vascular development.

Prost, S., C. O. Bellamy, et al. (1998). "p53-independent DNA repair and cell cycle arrest in embryonic stem cells." FEBS Lett **425**(3): 499-504.

The role of p53 in DNA repair and cell cycle checkpoint after ultraviolet irradiation was investigated in an embryonic stem cell line homozygous for a targeted deletion of p53. Results indicate that loss of p53 does not alter the capacity of

ES cells to respond to DNA damage. Wild-type and p53-deficient cells showed similar cessation of DNA synthesis after UV damage and similar ultimate capacity to repair a transiently transfected reporter plasmid. Interestingly, in the absence of DNA damaging treatment, the transit of p53-deficient cells through S phase was slower than wild-type cells. We suggest that this may result from the absence of a p53-dependent response to endogenous DNA damage: without p53 sensing endogenous damage leading to immediate repair, such damage may persist and thus delay DNA synthesis.

Rasmussen, T. P. (2003). "Embryonic stem cell differentiation: a chromatin perspective." Reprod Biol Endocrinol **1**: 100.

Embryonic stem (ES) cells hold immense promise for the treatment of human degenerative disease. Because ES cells are pluripotent, they can be directed to differentiate into a number of alternative cell-types with potential therapeutic value. Such attempts at "rationally-directed ES cell differentiation" constitute attempts to recapitulate aspects of normal development in vitro. All differentiated cells retain identical DNA content, yet gene expression varies widely from cell-type to cell-type. Therefore, a potent epigenetic system has evolved to coordinate and maintain tissue-specific patterns of gene expression. Recent advances show that mechanisms that govern epigenetic regulation of gene expression are rooted in the details of chromatin dynamics. As embryonic cells differentiate, certain genes are activated while others are silenced. These activation and silencing events are exquisitely coordinated with the allocation of cell lineages. Remodeling of the chromatin of developmentally-regulated genes occurs in conjunction with lineage commitment. Oocytes, early embryos, and ES cells contain potent chromatin-remodeling activities, an observation that suggests that chromatin dynamics may be especially important for early lineage decisions. Chromatin dynamics are also involved in the differentiation of adult stem cells, where the assembly of specialized chromatin upon tissue-specific genes has been studied in fine detail. The next few years will likely yield striking advances in the understanding of stem cell differentiation and developmental biology from the perspective of chromatin dynamics.

Raz, R., C. K. Lee, et al. (1999). "Essential role of STAT3 for embryonic stem cell pluripotency." Proc Natl Acad Sci U S A **96**(6): 2846-51.

Propagation of mouse embryonic stem (ES) cells in vitro requires exogenous leukemia inhibitory factor (LIF) or related cytokines. Potential downstream effectors of the LIF signal in ES cells include kinases of the Src, Jak, and mitogen-activated protein families and the signal transducer and transcriptional activator STAT3. Activation of nuclear STAT3 and the ability of ES cells to grow as undifferentiated clones were monitored during LIF withdrawal. A correlation was found between levels of STAT3 activity and maintenance of an undifferentiated phenotype at clonal density. In contrast, variation in STAT3 activity did not affect cell proliferation. The requirement for STAT3 was analyzed by targeted mutagenesis in ES cell lines exhibiting different degrees of LIF dependency. An insertional mutation was devised that abrogated Stat3 gene expression but could be reversed by Cre recombination-mediated excision. ES cells heterozygous for the Stat3 mutation could be isolated only from E14 cells, the line least dependent on LIF for self-renewal. Targeted clones isolated from other ES cell lines were invariably trisomic for chromosome 11, which carries the Stat3 locus, and retained normal levels of activated STAT3. Cre-regulated reduction of Stat3 gene copy number in targeted, euploid E14 clones resulted in dose-dependent losses of STAT3 activity and the efficiency of self-renewal without commensurate changes in cell cycle progression. These results demonstrate an essential role for a critical amount of STAT3 in the maintenance of an undifferentiated ES cell phenotype.

Ren, C. P., M. Zhao, et al. (2005). "Establishment of human embryonic stem cell line stably expressing Epstein-Barr virus-encoded nuclear antigen 1." Acta Biochim Biophys Sin (Shanghai) **37**(1): 68-73.

Human embryonic stem (hES) cells have the capability of unlimited undifferentiated proliferation, yet maintain the potential to form perhaps any cell type in the body. Based on the high efficiency of the Epstein-Barr virus-based episomal vector in introducing exogenous genes of interest into mammalian cells, we applied this system to hES cells, expecting that this would resolve the problem of poor transfection efficiency existing in current hES cell research. Therefore, the first step was to establish EBNA1-positive hES cells. Using the Fugene 6 transfection reagent, we transfected hES cells with the EBNA1 expression vector and subsequently generated hES cell clones that stably expressed EBNA1 under drug selection. These clones were confirmed to express EBNA1 mRNA by RT-PCR and to express EBNA1 protein by Western blotting. Furthermore, luciferase reporter gene analysis was performed on the EBNA1 clones and revealed that the expressed EBNA1 protein was functional. When the EBNA1-positive cells were injected into severe combined immunodeficient (SCID) mice, they formed teratoma tissues containing all three embryonic germ layers and

EBNA1 protein was detected in these teratoma tissues by Western blotting. All the results show that we have successfully created stable EBNA1-hES cells, thus laying a good foundation for further research.

Rieske, P., B. Krynska, et al. (2005). "Human fibroblast-derived cell lines have characteristics of embryonic stem cells and cells of neuro-ectodermal origin." Differentiation **73**(9-10): 474-83.

Fibroblasts are the most ubiquitous cells in complex organisms. They are the main cells of stromal tissue and play an important role in repair and healing of damaged organs. Here we report new data-initially serendipitous findings-that fibroblast-derived cell line (human fetal lung derived cells, MRC-5) have the morphology, growth rate and gene expression pattern characteristic of embryonic stem cells and cells of neuro-ectodermal origin. We have developed a serum-free culture system to maintain these cells in proliferative state. We discovered that, at proliferative state, these cells express transcription factors of pluripotent cells, OCT-3/4 and REX-1, and embryonic cell surface antigens SSEA-1, SSEA-3, and SSEA-4, as well as TRA-1-60 and TRA-1-81. In addition to embryonic cell markers, the fibroblasts expressed neuroectodermal genes: Musashi-1, nestin, medium neurofilament, and beta-III tubulin. RT-PCR data revealed that mesencephalic transcription factors, Nurr-1 and PTX-3, were also expressed in MRC-5 cells, and that these cells could be induced to express tyrosine hydroxylase (TH). Expression of TH followed down-regulation of genes associated with cell proliferation, OCT-3/4, REX-1, and beta-catenin. These data indicate that the cells commonly known as fibroblasts have some of the characteristics of stem cells, and can be induced to become neuroectodermal cells and perhaps even mature neurons.

Rodda, S. J., S. J. Kavanagh, et al. (2002). "Embryonic stem cell differentiation and the analysis of mammalian development." Int J Dev Biol **46**(4): 449-58.

Molecular and cellular analysis of early mammalian development is compromised by the experimental inaccessibility of the embryo. Pluripotent embryonic stem (ES) cells are derived from and retain many properties of the pluripotent founder population of the embryo, the inner cell mass. Experimental manipulation of these cells and their environment in vitro provides an opportunity for the development of differentiation systems which can be used for analysis of the molecular and cellular basis of embryogenesis. In this review we discuss strengths and weaknesses of the available ES cell differentiation methodologies and their relationship to events in vivo. Exploitation of these systems is providing novel insight into embryonic processes as diverse as cell lineage establishment, cell progression during differentiation, patterning, morphogenesis and the molecular basis for cell properties in the early mammalian embryo.

Rohwedel, J., V. Maltsev, et al. (1994). "Muscle cell differentiation of embryonic stem cells reflects myogenesis in vivo: developmentally regulated expression of myogenic determination genes and functional expression of ionic currents." Dev Biol **164**(1): 87-101.

The mouse blastocyst-derived embryonic stem cell (ES cell) line BLC6 efficiently differentiates into myosin heavy chain-, desmin- and myogenin-positive skeletal muscle cells when cultivated in embryo-like aggregates (embryoid bodies). Here, we show that the muscle-specific determination genes myf5, myogenin, myoD, and myf6 are expressed in these embryoid bodies in a characteristic temporal pattern which precisely reflects the sequence observed during mouse development in vivo. Myf5 is the first gene to be expressed followed by myogenin, myoD, and myf6, in this order. In situ hybridization demonstrates transcripts for myogenin and myoD accumulating in mono- and multinucleated myogenic cells, while myf5 mRNA is already found in mononucleated myoblasts. The myocytes also express functional nicotinic cholinoceptors and exhibit T-type Ca2+ currents and later L-type Ca2+ currents, demonstrating physiological properties of skeletal muscle cells. During myocyte differentiation the density of L-type Ca2+ channels significantly increases while the density of T-type Ca2+ channels decreases. The effect of external signals on myogenic differentiation of BLC6 cells was demonstrated by cocultivation with visceral endodermal END-2 cells and the activin A-secreting WEHI-3 cells. END-2 cells essentially prevent skeletal muscle differentiation, whereas basic fibroblast growth factor, transforming growth factor-beta, and WEHI-3 cells have no or an attenuating effect, respectively. Our results suggest that ES cells recapitulate closely the early steps of muscle development in vivo and may serve as an excellent in vitro system to study this process.

Rolletschek, A., H. Chang, et al. (2001). "Differentiation of embryonic stem cell-derived dopaminergic neurons is enhanced by survival-promoting factors." Mech Dev **105**(1-2): 93-104.

Here, we describe the generation of viable and dopamine-producing neurons derived from pluripotent mouse embryonic stem cells. Neurotrophic factors in combination with survival-promoting factors, such as interleukin-1beta, glial cell line-

derived neurotrophic factor, neurturin, transforming growth factor-beta(3) and dibutyryl-cyclic AMP, significantly enhanced Nurr1 and tyrosine hydroxylase (TH) mRNA levels, whereas En-1, mash-1 and dopamine-2-receptor mRNA levels were not upregulated. In parallel, mRNA levels of the anti-apoptotic gene bcl-2 were found to be upregulated at terminal stages. Double immunofluorescence analysis revealed increased numbers of TH- and dopamine transporter-, but not gamma-aminobutyric acid- and serotonin-positive neurons in relation to synaptophysin-labeled cells by survival-promoting factors. Moreover, high-performance liquid chromatography analysis showed detectable levels of intracellular dopamine. We conclude that survival-promoting factors enhance differentiation, survival and maintenance of dopaminergic neurons derived from embryonic stem cells.

Roth, T. M., P. Ramamurthy, et al. (2007). "A mouse embryonic stem cell model of Schwann cell differentiation for studies of the role of neurofibromatosis type 1 in Schwann cell development and tumor formation." Glia **55**(11): 1123-33.

The neurofibromatosis Type 1 (NF1) gene functions as a tumor suppressor gene. One known function of neurofibromin, the NF1 protein product, is to accelerate the slow intrinsic GTPase activity of Ras to increase the production of inactive rasGDP, with wide-ranging effects on p21ras pathways. Loss of neurofibromin in the autosomal dominant disorder NF1 is associated with tumors of the peripheral nervous system, particularly neurofibromas, benign lesions in which the major affected cell type is the Schwann cell (SC). NF1 is the most common cancer predisposition syndrome affecting the nervous system. We have developed an in vitro system for differentiating mouse embryonic stem cells (mESC) that are NF1 wild type (+/+), heterozygous (+/-), or null (-/-) into SC-like cells to study the role of NF1 in SC development and tumor formation. These mES-generated SC-like cells, regardless of their NF1 status, express SC markers correlated with their stage of maturation, including myelin proteins. They also support and preferentially direct neurite outgrowth from primary neurons. NF1 null and heterozygous SC-like cells proliferate at an accelerated rate compared to NF1 wild type; this growth advantage can be reverted to wild type levels using an inhibitor of MAP kinase kinase (Mek). The mESC of all NF1 types can also be differentiated into neuron-like cells. This novel model system provides an ideal paradigm for studies of the role of NF1 in cell growth and differentiation of the different cell types affected by NF1 in cells with differing levels of neurofibromin that are neither transformed nor malignant.

Roth, T. M., P. Ramamurthy, et al. (2008). "Influence of hormones and hormone metabolites on the growth of Schwann cells derived from embryonic stem cells and on tumor cell lines expressing variable levels of neurofibromin." Dev Dyn **237**(2): 513-24.

Loss of neurofibromin, the protein product of the tumor suppressor gene neurofibromatosis type 1 (NF1), is associated with neurofibromas, composed largely of Schwann cells. The number and size of neurofibromas in NF1 patients have been shown to increase during pregnancy. A mouse embryonic stem cell (mESC) model was used, in which mESCs with varying levels of neurofibromin were differentiated into Schwann-like cells. NF1 cell lines derived from a malignant and a benign human tumor were used to study proliferation in response to hormones. Estrogen and androgen receptors were not expressed or expressed at very low levels in the NF1+/+ cells, at low levels in NF1+/-cells, and robust levels in NF1-/-cells. A 17beta-estradiol (E2) metabolite, 2-methoxy estradiol (2ME2) is cytotoxic to the NF1-/- malignant tumor cell line, and inhibits proliferation in the other cell lines. 2ME2 or its derivatives could provide new treatment avenues for NF1 hormone-sensitive tumors at times of greatest hormonal influence.

Sakaguchi, T., M. Nishimoto, et al. (2006). "Putative "stemness" gene jam-B is not required for maintenance of stem cell state in embryonic, neural, or hematopoietic stem cells." Mol Cell Biol **26**(17): 6557-70.

Many genes have been identified that are specifically expressed in multiple types of stem cells in their undifferentiated state. It is generally assumed that at least some of these putative "stemness" genes are involved in maintaining properties that are common to all stem cells. We compared gene expression profiles between undifferentiated and differentiated embryonic stem cells (ESCs) using DNA microarrays. We identified several genes with much greater signal in undifferentiated ESCs than in their differentiated derivatives, among them the putative stemness gene encoding junctional adhesion molecule B (Jam-B gene). However, in spite of the specific expression in undifferentiated ESCs, Jam-B mutant ESCs had normal morphology and pluripotency. Furthermore, Jam-B homozygous mutant mice are fertile and have no overt developmental defects. Moreover, we found that neural and hematopoietic stem cells recovered from Jam-B mutant mice are not impaired in their ability to self-renew and differentiate. These results demonstrate that Jam-B is dispensable for normal

mouse development and stem cell identity in embryonic, neural, and hematopoietic stem cells.

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.

Scotland, K. B., S. Chen, et al. (2009). "Analysis of Rex1 (zfp42) function in embryonic stem cell differentiation." Dev Dyn **238**(8): 1863-77.

Rex1 (zfp42) is a zinc finger protein expressed primarily in undifferentiated stem cells, both in the embryo and the adult. Upon all-trans retinoic acid induced differentiation of murine embryonic stem (ES) cells, Rex1 mRNA levels decrease several fold. To characterize the function(s) of Rex1 more extensively, we generated Rex1 double knockout ES cell lines. The disruption of the Rex1 gene enhanced the expression of ectoderm, mesoderm, and endoderm markers as compared to wild-type (Wt) cells. We propose that Rex1 acts to reduce retinoic acid induced differentiation in ES cells. We performed microarray analyses on Wt and Rex1-/- cells cultured in the presence or absence of LIF to identify potential Rex1 targets. We also evaluated gene expression in a Wt line that overexpresses Rex1 and in a Rex1-/- line in which Rex1 expression was restored. These data, taken together, suggest that Rex1 influences differentiation, cell cycle regulation, and cancer progression.

Seigel, G. M., A. S. Hackam, et al. (2007). "Human embryonic and neuronal stem cell markers in retinoblastoma." Mol Vis **13**: 823-32.

PURPOSE: Retinoblastoma (RB) is the most common intraocular tumor of early childhood. The early onset of RB, coupled with our previous findings of cancer stem cell characteristics in RB, led us to hypothesize that subpopulations of RB tumors harbor markers and behaviors characteristic of embryonic and neuronal origin. METHODS: Our RB sources included: human pathological tissues, and the human RB cell lines Y79 and WERI-RB27. Microarray screening, single and dual-label immunocytochemistry and RT-PCR were performed to detect embryonic and neuronal stem cell markers, such as Oct3/4, Nanog, CD133, and Musashi-1. To test for functional evidence of stem cell behavior, we examined RB cells for their ability to form neurospheres and retain BrdU label as indicators of self-renewal and slow cell cycling, respectively. RESULTS: Microarray comparisons of human RB tumors with normal retinal tissue detected upregulation of a number of genes involved in embryonic development that were also present in Y79 cells, including Oct3/4, Nanog, Musashi-1 and Musashi-2, prominin-1 (CD133), Jagged-2, Reelin, Thy-1, nestin, Meis-1,NCAM, Patched, and Notch4. Expression of Musashi-1, Oct3/4 and Nanog was confirmed by immunostaining and RT-PCR analyses of RB tumors and RB cell lines. CD133 expression was confirmed by PCR analysis. Y79 and WERI-RB27 contained populations of Hoechst-dim/ABCG2-positive cells that co-localized with embryonic stem cell markers Oct3/4-ABCG2 and Nanog-ABCG2. Subpopulations of Y79 and WERI-RB27 cells were label-retaining (as seen by BrdU incorporation) and were able to generate neurospheres, both hallmarks of a stem cell phenotype. CONCLUSIONS: Small subpopulation(s) of RB cells express human embryonic and neuronal stem cell markers. There are also subpopulations that demonstrate functional behavior (label retention and self-renewal) consistent with cancer stem cells. These findings support the hypothesis that RB is a heterogeneous tumor comprised of subpopulation(s) with stem cell-like properties.

Selfridge, J., A. M. Pow, et al. (1992). "Gene targeting using a mouse HPRT minigene/HPRT-deficient embryonic stem cell system: inactivation of the mouse ERCC-1 gene." Somat Cell Mol Genet **18**(4): 325-36.

A convenient system for gene targeting that uses hypoxanthine phosphoribosyltransferase (HPRT) minigenes as the selectable marker in HPRT-deficient mouse embryonic stem (ES) cells is described. Improvements to the expression of HPRT minigenes in ES cells were achieved by promoter substitution and the provision of a strong translational initiation signal. The use of minigenes in the positive-negative

selection strategy for gene targeting was evaluated and the smaller minigenes were found to be as effective as a more conventional marker--the herpes simplex virus thymidine kinase gene. Minigenes were used to target the DNA repair gene ERCC-1 in ES cells. A new HPRT-deficient ES cell line was developed that contributes with high frequency to the germ line of chimeric animals. The ability to select for and against HPRT minigene expression in the new HPRT-deficient ES cell line will make this system useful for a range of gene-targeting applications.

Sharpe, N. G., D. G. Williams, et al. (1990). "Regulated expression of the small nuclear ribonucleoprotein particle protein SmN in embryonic stem cell differentiation." Mol Cell Biol **10**(12): 6817-20.

The SmN protein is a component of small nuclear ribonucleoprotein particles and is closely related to the ubiquitous SmB and B' splicing proteins. It is expressed in a limited range of tissues and cell types, including several undifferentiated embryonal carcinoma cell lines and undifferentiated embryonic stem cells. The protein declines to undetectable levels when embryonal carcinoma or embryonic stem cells are induced to differentiate, producing primitive endoderm or parietal endoderm or yielding embryonal bodies. This decline is due to a corresponding decrease in the level of the SmN mRNA. The potential role of SmN in the regulation of alternative splicing in embryonic cell lines and early embryos is discussed.

Shimozaki, K., M. Namihira, et al. (2005). "Stage- and site-specific DNA demethylation during neural cell development from embryonic stem cells." J Neurochem **93**(2): 432-9.

Activation of the transcription factor STAT3 is important for astrocyte differentiation during neural development. Demethylation of the methyl-CpG dinucleotide in the STAT3 binding site in the promoter of the glial fibrillary acidic protein (GFAP) gene, a marker for astrocytes, was previously shown to be a crucial cue for neural progenitors to express this gene in response to astrogenic signals during brain development. In this study, we analyzed the methylation status of the STAT3 binding site in the GFAP gene promoter during neural cell development from mouse embryonic stem (ES) cells in vitro. The CpG dinucleotide in the STAT3 binding site in the GFAP gene promoter exhibited a high incidence of cytidine-methylation in undifferentiated pluripotent ES cells. The high incidence of methylation of this particular cytidine was maintained in ES cell-derived neuroectoderm-like cells, but it underwent demethylation when the neural lineage cells became competent to express GFAP in response to a STAT3 activation signal. In contrast, hypermethylation of the CpG site was maintained in non-neural cells generated from the same ES cells. Progressive demethylation of the STAT3 binding site in the GFAP gene promoter was also observed in primary embryonic neuroepithelial cells during in vitro culture, whereas non-neural cells maintained hypermethylation of this site even after culture. Taken together, these results demonstrate that the astrocyte gene-specific cytidine-demethylation is programmed when neural progenitors from pluripotent cells are committed to a neural lineage that is capable of producing astrocytes.

Sonntag, K. C., R. Simantov, et al. (2004). "Temporally induced Nurr1 can induce a non-neuronal dopaminergic cell type in embryonic stem cell differentiation." Eur J Neurosci **19**(5): 1141-52.

The nuclear transcription factor Nurr1 is involved in the development and maintenance of the midbrain dopaminergic (DA) neuronal phenotype. We analysed the cellular and biological effects of Nurr1 during embryonic stem (ES) cell differentiation using the ROSA26-engineered Tet-inducible ES cell line J1-rtTA that does not express transgenes in mature neurons. Induction of Nurr1 at nestin-positive precursor and later stages of ES cell differentiation produced a non-neuronal DA cell type including functional DA transporters. In these cells, we found a clear correlation between Nurr1 and TH gene expression and specific midbrain DA cellular markers such as AADC, AHD2 and calbindin. Nurr1 did not alter gene expression of non-DA neuronal phenotypes and did not influence other midbrain developmental transcription factors, such as Otx1, Otx2, En-1, GBX2, Pitx3 and lmx1b. In addition, Nurr1 expression was required for maintenance of the DA phenotype and mediated up-regulation of the tyrosine kinase Ret and associated trophic factor GDNF-family receptors alpha 1, 2, and 4. This demonstrates that Nurr1 is sufficient to induce and maintain a midbrain-like DA biochemical and functional cellular phenotype independent of neurogenesis.

Sorrentino, E., V. Nazzicone, et al. (2007). "Comparative transcript profiles of cell cycle-related genes in mouse primordial germ cells, embryonic stem cells and embryonic germ cells." Gene Expr Patterns **7**(6): 714-21.

We used cDNA array to compare the relative transcript levels of 96 cell cycle-related genes in mouse primordial germ cells (PGCs), embryonic germ (EG) cells and embryonic stem (ES) cells. Among 38 genes of the G1 phase analysed, Ccnd3 (CyclinD3), Cdkn1c (p57(kip2)), Rb1, and Tceb1l (Skip1-like) were expressed at significantly higher levels in PGCs

than in EG and ES cells; Ccnd1 (CyclinD1) was more abundant in EG cells than in PGCs. Except for higher mRNA levels of Ccng (CyclinG1) in EG and ES cells in comparison to PGCs, no difference among 20 genes of the S and 12 genes of G2/M phases was found. Less than half of the 26 genes regarded as DNA damage checkpoint/Trp53/Atm pathway genes showed significant transcript levels in all three cell populations. Among these, the transcript levels of Ube1x and Atm were significantly higher in PGCs than in EG and ES cells while that of Ube3a was higher in these latter. In addition, relatively high mRNA levels of Timp3 characterizes EG cells while transcripts of this gene were very low in PGCs and barely detectable in ES cells. With the exception of Tceb1l, differential transcript levels found in the cDNA array assay were confirmed by real time RT-PCR. Using this method, we also analysed the transcripts of two genes not present in the cDNA array: c-myc, known to be critical for the control of cell cycle in many cell types, and Eras, specifically expressed in ES cells and involved in the control of ES cell proliferation and their tumorigenic properties. While c-myc transcripts were present at similar levels in all three cell types examined, Eras was expressed at high levels in ES cells (10-fold) and even more so in EG cells (almost 40-fold) in comparison to PGCs. Taken together, these results indicate that despite similarities between PGCs and ES or EG cells, their cell cycles are differently regulated. In particular, it appears that PGCs, like most mitotic cells, possess a more regulatable control of G1 phase than EG and ES cells. Moreover, our data provide useful clues for further studies aimed at identifying cell cycle genes critical for PGC growth and their transformation in tumorigenic cells.

Sperger, J. M., X. Chen, et al. (2003). "Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors." Proc Natl Acad Sci U S A **100**(23): 13350-5.

Remarkably little is known about the transcriptional profiles of human embryonic stem (ES) cells or the molecular mechanisms that underlie their pluripotency. To identify commonalties among the transcriptional profiles of different human pluripotent cells and to search for clues into the genesis of human germ cell tumors, we compared the expression profiles of human ES cell lines, human germ cell tumor cell lines and tumor samples, somatic cell lines, and testicular tissue samples by using cDNA microarray analysis. Hierarchical cluster analysis of gene expression profiles showed that the five independent human ES cell lines clustered tightly together, reflecting highly similar expression profiles. The gene expression patterns of human ES cell lines showed many similarities with the human embryonal carcinoma cell samples and more distantly with the seminoma samples. We identified 895 genes that were expressed at significantly greater levels in human ES and embryonal carcinoma cell lines than in control samples. These genes are candidates for involvement in the maintenance of a pluripotent, undifferentiated phenotype.

Steele, W., C. Allegrucci, et al. (2005). "Human embryonic stem cell methyl cycle enzyme expression: modelling epigenetic programming in assisted reproduction?" Reprod Biomed Online **10**(6): 755-66.

To investigate a possible mechanism for inducing epigenetic defects in the preimplantation embryo, a human embryonic stem cell model was developed, and gene expression of the key methyl cycle enzymes, MAT2A, MAT2B, GNMT, SAHH, CBS, CGL, MTR, MTRR, BHMT, BHMT2, mSHMT, cSHMT and MTHFR was demonstrated, while MAT1 was barely detectable. Several potential acceptors of cycle-generated methyl groups, the DNA methyltransferases (DNMT1, DNMT3A, DNMT3B and DNMT3L), glycine methyltransferase and the polyamine biosynthetic enzymes, SAM decarboxylase and ornithine decarboxylase, were also expressed. Expression of folate receptor alpha suggests a propensity for folate metabolism. Methotrexate-induced depletion of folate resulted in elevated intracellular homocysteine concentration after 7 days in culture and a concomitant increase in cysteine and glutathione, indicating clearance of homocysteine through the transulphuration pathway. These studies indicate that altered methyl group metabolism provides a potential mechanism for inducing epigenetic changes in the preimplantation embryo.

Sullivan, S., S. Pells, et al. (2006). "Nuclear reprogramming of somatic cells by embryonic stem cells is affected by cell cycle stage." Cloning Stem Cells **8**(3): 174-88.

Hybrid embryonic stem (ES)-like clones were generated by fusion of murine ES cells with somatic cells that carried a neo resistance gene under the transcriptional control of the Oct-4 promoter. The Oct-4 promoter was reactivated in hybrid ES cells formed by fusion with fetal fibroblasts, and all hybrid colonies were of ES rather than fibroblast phenotype, suggesting efficient reprogramming of fibroblast chromosomes. Like normal diploid murine ES cells, hybrid lines expressed alkaline phosphatase activity and formed differentiated cells derived from the three embryonic germ layers both in vitro and in vivo. Treatments thought to affect nuclear transfer efficiency (ES cell confluence and serum starvation of primary embryonic fibroblasts) were investigated to

determine whether they had an effect on reprogramming in cell hybrids. Serum starvation of primary embryonic fibroblasts increased hybrid colony number 50-fold. ES cells were most effective at reprogramming when they contained a high proportion of cells in the S and G2/M phases of the cell cycle. These data suggest that nuclear reprogramming requires an initial round of somatic DNA replication of quiescent chromatin in the presence of ES-derived factors produced during S and G2/M phases.

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 carring 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.

Sun, B. W., A. C. Yang, et al. (2006). "Temporal and parental-specific expression of imprinted genes in a newly derived Chinese human embryonic stem cell line and embryoid bodies." Hum Mol Genet **15**(1): 65-75.

Although the study of imprinted genes in human development is very important, little is known about their expression and regulation in the early differentiation of human tissues due to lack of an appropriate model. In this study, a Chinese human embryonic stem (hES) cell line, SHhES1, was derived and fully characterized. Expression profiles of human imprinted genes were determined by Affymetrix Oligo micro-array in undifferentiated SHhES1 cells and SHhES1-derived embryoid bodies (EBs) at day 3, 8, 13 and 18. Thirty-two known human imprinted genes were detected in undifferentiated ES cells. Significantly, differential expression was found in nine genes at different stages of EB formation. Expression profile changes were confirmed by quantitative real-time reverse transcriptase-polymerase chain reaction in SHhES1 cells as well as in another independently derived hES cell line, HUES-7. In addition, the monoallelic expressions of four imprinted genes were examined in three different passages of undifferentiated ES cells and EBs of both hES cell lines. The monoallelic expressions of imprinted genes, H19, PEG10, NDNL1 and KCNQ1 were maintained in both undifferentiated hES cells and derived EBs. More importantly, with the availability of maternal peripheral blood lymphocyte sample, we demonstrated that the maternal expression of KCNQ1 and the paternal expression of NDNL1 and PEG10 were maintained in SHhES1 cells. These data provide the first demonstration that the parental-specific expression of imprinted genes is stable in EBs after extensive differentiation, also indicating that in vitro fertilization protocol does not disrupt the parental monoallelic expression of the imprinted genes examined.

Sun, C., Y. Nakatake, et al. (2008). "Stem cell-specific expression of Dax1 is conferred by STAT3 and Oct3/4 in embryonic stem cells." Biochem Biophys Res Commun **372**(1): 91-6.

Embryonic stem (ES) cells are pluripotent cells derived from inner cell mass of blastocysts. An orphan nuclear receptor, Dax1, is specifically expressed in undifferentiated ES cells and plays an important role in their self-renewal. The regulatory mechanism of Dax1 expression in ES cells, however, remains unknown. In this study, we found that STAT3 and Oct3/4, essential transcription factors for ES cell self-renewal, are involved in the regulation of Dax1 expression. Suppression of either STAT3 or Oct3/4 resulted in down-regulation of Dax1. Reporter assay identified putative binding sites for these factors in the promoter/enhancer region of the Dax1 gene. Chromatin immunoprecipitation analysis suggested the in vivo association of STAT3 and Oct3/4 with the putative sites. Furthermore, gel shift assay indicated that these transcription factors directly bind to their putative binding sites. These results suggest that STAT3 and Oct3/4 control the expression of Dax1 to maintain the self-renewal of ES cells.

Sun, X., X. Long, et al. (2008). "Similar biological characteristics of human embryonic stem cell lines with normal and abnormal karyotypes." Hum Reprod **23**(10): 2185-93.

BACKGROUND: Human embryonic stem cell (hESC) lines derived from poor quality embryos usually have either normal or abnormal karyotypes. However, it is still unclear whether their biological characteristics are similar. METHODS: Seven new hESC lines were established using discarded embryos. Five cell lines had normal karyotype, one was with an unbalanced Robertsonian translocation and one had a triploid karyotype. Their biological characteristics, short tandem repeat loci, HLA typing, differentiation capability and imprinted gene, DNA methylation and X chromosome inactivation status were compared between different cell lines. RESULTS: All seven hESC lines had similar biological characteristics regardless of karyotype (five normal and two abnormal), such as expression of stage-specific embryonic antigen (SSEA)-4, tumor-rejection antigen (TRA)-1-81 and TRA-1-60 proteins, transcription factor octamer binding protein 4 mRNA, no detectable expression of SSEA-1 protein and high levels of alkaline phosphatase activity. All cell lines were able to undergo differentiation. Imprinted gene expression and DNA methylation were also similar among these cell lines. Non-random X chromosome inactivation patterns were found in XX cell lines. CONCLUSIONS: The present results suggest that hESC lines with abnormal karyotype are also useful experimental materials for cell therapy, developmental biology and genetic research.

Suzuki, A., A. Raya, et al. (2006). "Maintenance of embryonic stem cell pluripotency by Nanog-mediated reversal of mesoderm specification." Nat Clin Pract Cardiovasc Med **3 Suppl 1**: S114-22.

Embryonic stem cells (ESCs) can be propagated indefinitely in culture, while retaining the ability to differentiate into any cell type in the organism. The molecular and cellular mechanisms underlying ESC pluripotency are, however, poorly understood. We characterize a population of early mesoderm-specified (EM) progenitors that is generated from mouse ESCs by bone morphogenetic protein stimulation. We further show that pluripotent ESCs are actively regenerated from EM progenitors by the action of the divergent homeodomain-containing protein Nanog, which, in turn, is upregulated in EM progenitors by the combined action of leukemia inhibitory factor and the early mesoderm transcription factor T/Brachyury. These findings uncover specific roles of leukemia inhibitory factor, Nanog, and bone morphogenetic protein in the self-renewal of ESCs and provide novel insights into the cellular bases of ESC pluripotency.

Swindle, C. S., H. G. Kim, et al. (2004). "Mutation of CpGs in the murine stem cell virus retroviral vector long terminal repeat represses silencing in embryonic stem cells." J Biol Chem **279**(1): 34-41.

Although DNA methylation and transcriptional repression are generally associated, a causal role for DNA methylation in silencing of retroviral vectors has not been established. The newer generation murine stem cell virus retroviral vector (MSCV) lacks many of the repressive cis-acting DNA sequences identified in Moloney murine leukemia virus but remains sensitive to transcriptional silencing in various cell types. To determine the contribution of cytosine methylation to MSCV silencing, we mutated CpG dinucleotides located in the MSCV long terminal repeat (LTR) that are clustered in the U3 region and directly spanning the transcription start site in the R region. Effects of the CpG mutations on MSCV silencing were assessed in murine embryonic stem cells. An analysis of numerous clonal proviral integrants showed that mutation of CpGs in both clusters eliminated proviral integrants that were completely silenced. Variegated expression was shown to represent a substantial component of intraclonal silencing and was independent of the presence of CpGs in the LTR. Treatment of transduced cells with 5-azadeoxycytidine delayed establishment of the silenced state but had only a modest effect on expression of some proviral integrants at late times post-transduction. These results are direct evidence for a causal contribution of DNA methylation in the LTR to MSCV silencing and define the promoter region CpGs as a repressive element in embryonic stem cells. Furthermore, distinct mechanisms are suggested for establishment and maintenance of the silenced proviral state.

Szabo, P. and J. R. Mann (1994). "Expression and methylation of imprinted genes during in vitro differentiation of mouse parthenogenetic and androgenetic embryonic stem cell lines." Development **120**(6): 1651-60.

Messenger RNA and methylation levels of four imprinted genes, H19, Igf2r, Igf-2 and Snrpn were examined by northern and Southern blotting in mouse parthenogenetic, androgenetic and normal or wild-type embryonic stem cell lines during their differentiation in vitro as embryoid bodies. In most instances, mRNA levels in parthenogenetic and androgenetic embryoid bodies differed from wild type as expected from previously determined patterns of monoallelic expression in midgestation embryos and at later stages of development. These findings implicate aberrant mRNA levels of these genes in the abnormal development of parthenogenetic and androgenetic embryos and chimeras. Whereas complete silence of one of the parental alleles has previously been observed in vivo, we detected some

mRNA in the corresponding embryonic stem cell line. This 'leakage' phenomenon could be explained by partial erasure, bypass or override of imprints, or could represent the actual activity status at very early stages of development. The mRNA levels of H19, Igf2r and Igf-2 and the degree of methylation at specific associated sequences were correlated according to previous studies in embryos, and thereby are consistent with suggestions that the methylation might play a role in controlling transcription of these genes. Paternal-specific methylation of the H19 promoter region is absent in sperm, yet we observed its presence in undifferentiated androgenetic embryonic stem cells, or before the potential expression phase of this gene in embryoid bodies. As such methylation is likely to invoke a repressive effect, this finding raises the possibility that it is part of the imprinting mechanism of H19, taking the form of a secondary imprint or postfertilization epigenetic modification necessary for repression of the paternal allele.

Szutorisz, H., C. Canzonetta, et al. (2005). "Formation of an active tissue-specific chromatin domain initiated by epigenetic marking at the embryonic stem cell stage." Mol Cell Biol **25**(5): 1804-20.

The differentiation potential of stem cells is determined by the ability of these cells to establish and maintain developmentally regulated gene expression programs that are specific to different lineages. Although transcriptionally potentiated epigenetic states of genes have been described for haematopoietic progenitors, the developmental stage at which the formation of lineage-specific gene expression domains is initiated remains unclear. In this study, we show that an intergenic cis-acting element in the mouse lambda5-VpreB1 locus is marked by histone H3 acetylation and histone H3 lysine 4 methylation at a discrete site in embryonic stem (ES) cells. The epigenetic modifications spread from this site toward the VpreB1 and lambda5 genes at later stages of B-cell development, and a large, active chromatin domain is established in pre-B cells when the genes are fully expressed. In early B-cell progenitors, the binding of haematopoietic factor PU.1 coincides with the expansion of the marked region, and the region becomes a center for the recruitment of general transcription factors and RNA polymerase II. In pre-B cells, E2A also binds to the locus, and general transcription factors are distributed across the active domain, including the gene promoters and the intergenic region. These results suggest that localized epigenetic marking is important for establishing the transcriptional competence of the lambda5 and VpreB1 genes as early as the pluripotent ES cell stage.

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.

Tay, Y., J. Zhang, et al. (2008). "MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation." Nature **455**(7216): 1124-8.

MicroRNAs (miRNAs) are short RNAs that direct messenger RNA degradation or disrupt mRNA translation in a sequence-dependent manner. For more than a decade, attempts to study the interaction of miRNAs with their targets were confined to the 3' untranslated regions of mRNAs, fuelling an underlying assumption that these regions are the principal recipients of miRNA activity. Here we focus on the mouse Nanog, Oct4 (also known as Pou5f1) and Sox2 genes and demonstrate the existence of many naturally occurring miRNA targets in their amino acid coding sequence (CDS). Some of the mouse targets analysed do not contain the miRNA seed, whereas others span exon-exon junctions or are not conserved in the human and rhesus genomes. miR-134, miR-296 and miR-470, upregulated on

retinoic-acid-induced differentiation of mouse embryonic stem cells, target the CDS of each transcription factor in various combinations, leading to transcriptional and morphological changes characteristic of differentiating mouse embryonic stem cells, and resulting in a new phenotype. Silent mutations at the predicted targets abolish miRNA activity, prevent the downregulation of the corresponding genes and delay the induced phenotype. Our findings demonstrate the abundance of CDS-located miRNA targets, some of which can be species-specific, and support an augmented model whereby animal miRNAs exercise their control on mRNAs through targets that can reside beyond the 3' untranslated region.

Thorsteinsdottir, S., B. A. Roelen, et al. (1999). "Expression of the alpha 6A integrin splice variant in developing mouse embryonic stem cell aggregates and correlation with cardiac muscle differentiation." Differentiation **64**(3): 173-84.

Mouse embryonic stem (ES) cells grown in aggregates give rise to several different cell types, including cardiac muscle. Given the lack of cardiac muscle cell lines, ES cells can be a useful tool in the study of cardiac muscle differentiation. The laminin-binding integrin alpha 6 beta 1 exists in two different splice variant forms of the alpha chain (alpha 6A and alpha 6B), the alpha 6A form having been implicated as possibly playing a role in cardiac muscle development, based on its distribution pattern [4, 53]. In this study we characterise the ES cell model system in terms of the expression of the two different alpha 6 splice variants. We correlate their expression with that of muscle markers and the transcription factor GATA-4, using the reverse transcription-polymerase chain reaction (RT-PCR). We confirm that alpha 6B is constitutively expressed by ES cells. In contrast, alpha 6A expression appears later and overlaps in time with a period when the muscle marker myosin light chain-2V (MLC-2V) is expressed, but no MyoD is present, which indicates the presence of cardiac muscle cells in the aggregates. We further show that GATA-4 is present at the same time. Culturing the aggregates under conditions that stimulate (transforming growth factor beta 1 supplement) or inhibit (TGF beta 1 plus 10(-9) M retinoic acid supplement) cardiac muscle differentiation does not lead to any qualitative differences in the timing of expression of these genes, but quantitative changes cannot be excluded. The TGF beta 1 supplement does, however, lead to a relatively greater expression of alpha 6A compared to alpha 6B than the TGF beta 1 plus 10(-9) M RA supplement after 6 days in culture, suggesting that alpha 6A expression is favoured under conditions that stimulate cardiac muscle differentiation. The switch towards alpha 6A expression in ES cell aggregates is paralleled by expression of the binding receptor for TGF beta (T beta RII). Stable expression of a mutated (dominant negative) T beta RII in ES cells, however, still resulted in (TGF beta-independent) upregulation of alpha 6A, demonstrating that these events were not causally related and that parallel or alternative regulatory pathways exist. The initial characterisation of differentiating ES cell aggregates in terms of alpha 6A integrin subunit expression suggests that this model system could be a valuable tool in the study of the role of the alpha 6A beta 1 integrin in cardiac muscle differentiation.

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.

Tribioli, C. and T. Lufkin (2006). "Long-term room temperature storage of high-quality embryonic stem cell genomic DNA extracted with a simple and rapid procedure." J Biomol Tech **17**(4): 249-51.

A very simple procedure for the isolation of high-quality, high-molecular-weight genomic DNA from embryonic stem cells is described. The DNA is very stable once dried and can be stored for long periods of time without refrigeration. Living cells are lysed in a sodium dodecyl sulfate and EDTA buffer containing proteinase K and then air-dried. Samples can be processed in bulk, and an individual can easily process thousands of samples for extraction and shipment on a daily basis using only common laboratory materials such as plastic ware and a multichannel pipetteman.

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.

Tsuda, H., C. E. Maynard-Currie, et al. (1997). "Inactivation of the mouse HPRT locus by a 203-bp retroposon insertion and a 55-kb gene-targeted deletion: establishment of new HPRT-deficient mouse embryonic stem cell lines." Genomics **42**(3): 413-21.

To obtain useful hypoxanthine phosphoribosyl-transferase (HPRT)-deficient mouse ES cell lines, two different methods were employed: (i) selection of spontaneous 6-TG-resistant mutants and (ii) gene targeting of the HPRT locus. The first approach resulted in the establishment of E14.1TG3B1, a spontaneous HPRT-deficient cell line with an insertional mutation of 203 bp in the third exon of the HPRT gene. The insert is highly homologous to the B2 mouse repetitive element and has all the expected retroposon characteristics, thus providing an example of gene inactivation by retroposon insertion. This clone exhibited stable 6-TG resistance and high germ-line transmission frequency. Thus E14.1TG3B1 is a useful ES cell line for modifying the mouse genome using the HPRT gene as a selection marker and for transmission at a high frequency into the mouse germ line. The second approach resulted in a 55-kb deletion of the mouse HPRT locus, demonstrating the feasibility of replacement-targeting vectors to generate large genomic DNA deletions.

Tsuzuki, T. and D. E. Rancourt (1998). "Embryonic stem cell gene targeting using bacteriophage lambda vectors generated by phage-plasmid recombination." Nucleic Acids Res **26**(4): 988-93.

Targeted mutagenesis is an extremely useful experimental approach in molecular medicine, allowing the generation of specialized animals that are mutant for any gene of interest. Currently the rate determining step in any gene targeting experiment is construction of the targeting vector (TV). The resulting lambdaTV DNA can then be cleaved with restriction endonucleases to release the bacteriophage arms and can subsequently be electroporated directly into ES cells to yield gene targets. We demonstrate that in vivo phage-plasmid recombination can be used to introduce neo and lacZ - neo mutations into precise positions within a lambda2TK subclone via double crossover recombination. We describe two methods for eliminating single crossover recombinants, spi selection and size restriction, both of which result in phage TVs bearing double crossover insertions. Thus TVs can be easily and quickly generated in bacteriophage without plasmid subcloning and with little genomic sequence or restriction site information.

Udy, G. B. and M. J. Evans (1994). "Microplate DNA preparation, PCR screening and cell freezing for gene targeting in embryonic stem cells." Biotechniques **17**(5): 887-94.

We have developed a new method for genomic DNA microextraction using acetone/N,N-dimethylformamide. Combining this with 96-well plate PCR allows rapid analysis of large numbers of genomic DNA samples. One application is the analysis of embryonic stem cells for rare gene targeting events. The efficacy of the combined microextraction and amplification procedure has been demonstrated by the identification of gene targeting events at the mOR gene locus. In addition, we have also detailed an improved 96-well plate freezing protocol for embryonic stem cells that allows long-term storage at -70 degrees C. The simple nature of the combined procedures described lend themselves to future automation.

Valbuena, D., A. Galan, et al. (2006). "Derivation and characterization of three new Spanish human embryonic stem cell lines (VAL -3 -4 -5) on human feeder and in serum-free conditions." Reprod Biomed Online **13**(6): 875-86.

A total of 184 human embryos, frozen for >5 years, were donated; informed consent was obtained according to Spanish law 45/2003. Survival rate was 40% and three out of 24 blastocysts (12.5%) developed into putative hESC lines, named VAL-3, VAL-4, and VAL-5. The derivation process was performed on microbiologically tested and irradiated human foreskin fibroblasts and designed to minimize contact with xeno-components in knockout DMEM supplemented with knockout serum replacement, and basic fibroblast growth factor. Fingerprinting and HLA typing of the cell lines allowed their identification and traceability. Karyotype was normal for VAL-3 (46XY), VAL-4 (46XX) and VAL-5 (46XX). All three hESC lines expressed specific markers for non-differentiation (Nanog, stage-specific embryonic antigen-4 [SSEA-4], tumour-related antigen [TRA]-1-60, and TRA-1-81) and were negative for SSEA-1. RT-PCR further demonstrated the expression of Oct-4, Sox2, Rex-1, Nanog, Cripto, Thy-1, and Lefty-A. Furthermore, they were found to be negative for classical differentiation markers such as neurofilament heavy chain (ectoderm), renin (mesoderm), and amylase (endoderm). All three cell lines displayed high levels of telomerase activity, and were shown to successfully overcome cryopreservation and thawing. Finally, these three new hESC lines have demonstrated the potential to differentiate in vitro and in vivo (teratoma formation) into cell types originating from all three germ layers.

van Eekelen, J. A., C. K. Bradley, et al. (2003). "Expression pattern of the stem cell leukaemia gene in the CNS of the embryonic and adult mouse." Neuroscience **122**(2): 421-36.

The basic helix-loop-helix (bHLH) transcription factor stem cell leukaemia (SCL) is a 'master regulator' of haematopoiesis, where SCL is pivotal in cell fate determination and differentiation. SCL has also been detected in CNS, where other members of the bHLH-family have been shown to be indispensable for neuronal development; however, no detailed expression pattern of SCL has so far been described. We have generated a map of SCL expression in the embryonic and adult mouse brain based on histochemical analysis of LacZ reporter gene expression in sequential sections of brain tissue derived from SCL-LacZ knockin mice. The expression of LacZ was confirmed to reflect SCL expression by in situ hybridisation. LacZ expression was found in a range of different diencephalic, mesencephalic and metencephalic brain nuclei in adult CNS. Co-localisation of LacZ with the neuronal marker NeuN indicated expression in post-mitotic neurons in adulthood. LacZ expression by neurons was confirmed in tissue culture analysis. The nature of the pretectal, midbrain and hindbrain regions expressing LacZ suggest that SCL in adult CNS is potentially involved in processing of visual, auditory and pain related information. During embryogenesis, LacZ expression was similarly confined to thalamus, midbrain and hindbrain. LacZ staining was also evident in parts of the intermediate and marginal zone of the aqueduct and ventricular zone of the fourth ventricle at E12.5 and E14. These cells may represent progenitor stages of differentiating neural cells. Given

the presence of SCL in both the developing brain and in post-mitotic neurons, it seems likely that the function of SCL in neuronal differentiation may differ from its function in maintaining the differentiated state of the mature neuron.

Vasilkova, A. A., H. A. Kizilova, et al. (2007). "Dominant manifestation of pluripotency in embryonic stem cell hybrids with various numbers of somatic chromosomes." Mol Reprod Dev **74**(8): 941-51.

Developmental potential was assessed in 8 intra-specific and 20 inter-specific hybrid clones obtained by fusion of embryonic stem (ES) cells with either splenocytes or fetal fibroblasts. Number of chromosomes derived from ES cells in these hybrid clones was stable while contribution of somatic partner varied from single chromosomes to complete complement. This allowed us to compare pluripotency of the hybrid cells with various numbers of somatic chromosomes. Three criteria were used for the assessment: (i) expression of Oct-4 and Nanog genes; (ii) analyses of teratomas generated by subcutaneous injections of the tested cells into immunodeficient mice; (iii) contribution of the hybrid cells in chimeras generated by injection of the tested cells into C57BL blastocysts. All tested hybrid clones showed expression of Oct-4 and Nanog at level comparable to ES cells. Histological and immunofluorescent analyses demonstrated that most teratomas formed from the hybrid cells with different number of somatic chromosomes contained derivatives of three embryonic layers. Tested hybrid clones make similar contribution in various tissues of chimeras in spite of significant differences in the number of somatic chromosomes they contained. The data indicate that pluripotency is manifested as a dominant trait in the ES hybrid cells and does not depend substantially on the number of somatic chromosomes. The latter suggests that the developmental potential derived from ES cells is maintained in ES-somatic cell hybrids by cis-manner and is rather resistant to trans-acting factors emitted from the somatic one.

Vassilieva, S., K. Guan, et al. (2000). "Establishment of SSEA-1- and Oct-4-expressing rat embryonic stem-like cell lines and effects of cytokines of the IL-6 family on clonal growth." Exp Cell Res **258**(2): 361-73.

Here, we demonstrate long-term cultivation of alkaline phosphatase-positive rat embryonic stem-like (RES) cell lines. RES cells were characterized by their typical growth in highly compacted cell clusters, which were found to be sensitive against enzymatic dissociation. RES cells expressed stage-specific embryonic antigen-1 (SSEA-1) and transcription factor Oct-4, but Oct-4 mRNA was detected at lower levels compared to mouse ES cells. Once established to tissue culture, RES cells were able to grow in the absence of feeder cells under clonal conditions. Cytokines of the interleukin-6 family known to maintain the undifferentiated state of mouse ES cells were comparatively analyzed for their capacity to maintain the undifferentiated growth of two cell lines, RES-1 and RES-15, in a clonal assay. Rat ciliary neurotrophic factor (rCNTF), human oncostatin M (hOSM), and interleukin-6 and soluble interleukin-6 receptor (IL-6/sIL-6R) were found to support clonal growth of RES cells, but the cytokines did not reach the efficiency of the colony forming ability of leukemia inhibitory factor (LIF). When RES-1 and RES-15 cells were cultivated without feeder cells, SSEA-1 expression was maintained after clonal growth in the presence of LIF and LIF + rCNTF, respectively. Oct-4 mRNA was significantly detected in RES-15 cells when cultivated in the absence of feeder cells in media substituted by LIF and/or IL-6/sIL-6R, as well as without cytokines. In summary, rat embryonic stem-like cell lines could be established from rat blastocysts and were able to proliferate as undifferentiated alkaline phosphatase-positive cells. Embryonal stem cell properties, such as SSEA-1 and Oct-4 expression, were maintained by members of the IL-6 family of cytokines, but most significantly by LIF.

Vincent, R., N. Treff, et al. (2006). "Generation and characterization of novel tetracycline-inducible pancreatic transcription factor-expressing murine embryonic stem cell lines." Stem Cells Dev **15**(6): 953-62.

Pancreatic development in mammals is controlled in part by the expression and function of numerous genes encoding transcription factors. Yet, how these regulate each other and their target genes is incompletely understood. Embryonic stem (ES) cells have recently been shown to be capable of differentiating into pancreatic progenitor cells and insulin-producing cells, representing a useful in vitro model system for studying pancreatic and islet development. To generate tools to study the relationships of transcription factors in pancreatic development we have established seven unique mouse ES cell lines with tetracycline-inducible expression of either Hnf4alpha, Hnf6, Nkx2.2, Nkx6.1, Pax4, Pdx1, and Ptf1a cDNAs. Each of the cell lines was characterized for induction of transgene expression after exposure to doxycycline (DOX) by quantitative real-time PCR and immunofluorescence microscopy. Transgene expression in the presence of DOX was at least 97-fold that seen in untreated cells. Immunofluorescent staining of DOX-treated cultures

showed efficient (>95% of cells) transgene protein expression while showing <5% positive staining in uninduced cells. Each of the ES cell lines maintained their pluripotency as measured by teratoma formation. Furthermore, transgene expression can be efficiently achieved in vivo through DOX administration to mice. The establishment of ES cell lines with temporally controllable induction of critical pancreatic transcription factor genes provides a new set of tools that could be used to interrogate gene regulatory networks in pancreatic development and potentially generate greater numbers of beta cells from ES cells.

Vodyanik, M. A., J. A. Bork, et al. (2005). "Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential." Blood **105**(2): 617-26.

Embryonic stem (ES) cells have the potential to serve as an alternative source of hematopoietic precursors for transplantation and for the study of hematopoietic cell development. Using coculture of human ES (hES) cells with OP9 bone marrow stromal cells, we were able to obtain up to 20% of CD34+ cells and isolate up to 10(7) CD34+ cells with more than 95% purity from a similar number of initially plated hES cells after 8 to 9 days of culture. The hES cell-derived CD34+ cells were highly enriched in colony-forming cells, cells expressing hematopoiesis-associated genes GATA-1, GATA-2, SCL/TAL1, and Flk-1, and retained clonogenic potential after in vitro expansion. CD34+ cells displayed the phenotype of primitive hematopoietic progenitors as defined by co-expression of CD90, CD117, and CD164, along with a lack of CD38 expression and contained aldehyde dehydrogenase-positive cells as well as cells with verapamil-sensitive ability to efflux rhodamine 123. When cultured on MS-5 stromal cells in the presence of stem cell factor, Flt3-L, interleukin 7 (IL-7), and IL-3, isolated CD34+ cells differentiated into lymphoid (B and natural killer cells) as well as myeloid (macrophages and granulocytes) lineages. These data indicate that CD34+ cells generated through hES/OP9 coculture display several features of definitive hematopoietic stem cells.

Wamstad, J. A., C. M. Corcoran, et al. (2008). "Role of the transcriptional corepressor Bcor in embryonic stem cell differentiation and early embryonic development." PLoS One **3**(7): e2814.

Bcor (BCL6 corepressor) is a widely expressed gene that is mutated in patients with X-linked Oculofaciocardiodental (OFCD) syndrome. BCOR regulates gene expression in association with a complex of proteins capable of epigenetic modification of chromatin. These include Polycomb group (PcG) proteins, Skp-Cullin-F-box (SCF) ubiquitin ligase components and a Jumonji C (Jmjc) domain containing histone demethylase. To model OFCD in mice and dissect the role of Bcor in development we have characterized two loss of function Bcor alleles. We find that Bcor loss of function results in a strong parent-of-origin effect, most likely indicating a requirement for Bcor in extraembryonic development. Using Bcor loss of function embryonic stem (ES) cells and in vitro differentiation assays, we demonstrate that Bcor plays a role in the regulation of gene expression very early in the differentiation of ES cells into ectoderm, mesoderm and downstream hematopoietic lineages. Normal expression of affected genes (Oct3/4, Nanog, Fgf5, Bmp4, Brachyury and Flk1) is restored upon re-expression of Bcor. Consistent with these ES cell results, chimeric animals generated with the same loss of function Bcor alleles show a low contribution to B and T cells and erythrocytes and have kinked and shortened tails, consistent with reduced Brachyury expression. Together these results suggest that Bcor plays a role in differentiation of multiple tissue lineages during early embryonic development.

Wang, Y., F. Yates, et al. (2005). "Embryonic stem cell-derived hematopoietic stem cells." Proc Natl Acad Sci U S A **102**(52): 19081-6.

Despite two decades of studies documenting the in vitro blood-forming potential of murine embryonic stem cells (ESCs), achieving stable long-term blood engraftment of ESC-derived hematopoietic stem cells in irradiated mice has proven difficult. We have exploited the Cdx-Hox pathway, a genetic program important for blood development, to enhance the differentiation of ESCs along the hematopoietic lineage. Using an embryonic stem cell line engineered with tetracycline-inducible Cdx4, we demonstrate that ectopic Cdx4 expression promotes hematopoietic mesoderm specification, increases hematopoietic progenitor formation, and, together with HoxB4, enhances multilineage hematopoietic engraftment of lethally irradiated adult mice. Clonal analysis of retroviral integration sites confirms a common stem cell origin of lymphoid and myeloid populations in engrafted primary and secondary mice. These data document the cardinal stem cell features of self-renewal and multilineage differentiation of ESC-derived hematopoietic stem cells.

Wang, Z. Q., A. E. Grigoriadis, et al. (1991). "A novel target cell for c-fos-induced oncogenesis: development of chondrogenic tumours in embryonic stem cell chimeras." Embo J **10**(9): 2437-50.

Embryonic stem (ES) cells were used to investigate the target cell specificity and consequences

of c-fos when expressed ectopically during embryonic development. Chimeric mice generated with different ES cell clones selected for high exogenous c-fos expression were not affected during embryonic development; however, a high frequency of cartilage tumours developed as early as 3-4 weeks of age apparently independent of the extent of chimerism. The tumours originated from cartilagenous tissues and contained many chondrocytes. Expression of exogenous c-fos RNA and Fos protein was observed during development but was highest in tumour tissues, predominantly in differentiating chondrocytes. A number of primary and clonal tumour-derived cell lines were established which expressed high levels of c-fos, c-jun as well as the cartilage-specific gene type II collagen and which gave rise to cartilage tumours in vivo, some of which also contained bone. Interestingly, the levels of c-Fos and c-Jun appeared to be coordinately regulated in the cell lines as well as in chimeric tissues. Thus, we demonstrate that chondrogenic cells and earlier progenitors are specially transformed by Fos/Jun and therefore represent a novel mesenchymal target cell for c-fos overexpression.

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.

Wegmuller, D., I. Raineri, et al. (2007). "A cassette system to study embryonic stem cell differentiation by inducible RNA interference." Stem Cells **25**(5): 1178-85.

Although differentiation of pluripotent embryonic stem cells is restricted by a hierarchy of transcription factors, little is known about whether post-transcriptional mechanisms similarly regulate early embryoid differentiation. We developed a system where small hairpin (sh)RNAs can be induced in embryonic stem (ES) cells from a defined locus following integration by Flp recombinase-mediated DNA recombination. To verify the system, the key transcription factor Stat3, which maintains pluripotency, was downregulated by shRNA, and the expected morphological and biochemical markers of differentiation were observed. Induction of shRNA specific for the post-transcriptional regulator Brf1 (Zfp36L1) amplified the cardiac markers with strong stimulation of cardiomyocyte formation within embryoid bodies. These findings identify Brf1 as a novel potential regulator of cardiomyocyte formation and suggest that post-transcriptional mechanisms are of importance to early development and, possibly, to regenerative medicine. The inducible RNA interference system presented here should also allow assignment of function for candidate genes with suspected roles in ES cell development. Disclosure of potential conflicts of interest is found at the end of this article.

Wilson, P. G., J. J. Cherry, et al. (2007). "An SMA project report: neural cell-based assays derived from human embryonic stem cells." Stem Cells Dev **16**(6): 1027-41.

Human embryonic stem (ES) cells are promising resources for developing new treatments for neurodegenerative diseases. Spinal muscular atrophy (SMA) is one of the leading causes of childhood paralysis and infant mortality. SMA is caused by inactivation of the survival motor neuron-1 (SMN1) gene. The nearly identical SMN2 gene contains a silent polymorphism that disrupts splicing and as a result cannot compensate for loss of SMN1. The SMA Project was established by the National Institute of Neurological Disorders and Stroke (NINDS) as a pilot effort to establish a fully transparent coalition between academics, industry, and government to create a centralized network of shared resources and information to identify and test new SMA therapeutics. As one of the funded projects, the work described here tested the feasibility of generating a SMA cell-based assay using neural lineages derived from human ES cells approved for National Institutes of Health (NIH)-funded research. Minigene cassettes were constructed, employing firefly luciferase or green fluorescent protein (GFP) as reporters for splicing efficiency of SMN1 and/or SMN2 under the control of the SMN1, SMN2, or cytomegalovirus (CMV) promoters. Transient

transfection of proliferating neuroprogenitors in a 96-well format with plasmid DNA or adenoviral vectors showed differential levels that correlated with the splicing minigene and the promoter used; luciferase activities with SMN1 splicing minigenes were higher than SMN2, and the CMV promoter generated higher levels of activity than the SMN1 and SMN2 promoters. Our results indicate that human ES cell-derived neuroprogenitors provide a promising new primary cell source for assays of new therapeutics for neurodegenerative diseases.

Woll, N. L., J. D. Heaney, et al. (2006). "Osteogenic nodule formation from single embryonic stem cell-derived progenitors." Stem Cells Dev **15**(6): 865-79.

The process of bone formation can be approximated in vitro in the form of a mineralized nodule. Osteoprogenitors and mesenchymal stem cells (MSCs), the immediate precursors of the osteoprogenitor, proliferate and differentiate into osteoblasts when placed into culture. These osteoblasts secrete and mineralize a matrix during a period of 3-4 weeks. The differentiation potential of embryonic stem (ES) cells suggests that ES cells should also have the ability to form osteogenic nodules in vitro. ES cells were allowed to form embryoid bodies (EBs) and were cultured in suspension for 2 days; EBs were disrupted and plated as single cells at concentrations as low as 25 cells/cm(2). We provide five lines of evidence for osteogenesis in these ES cell-derived cultures: (1) cell and colony morphology as revealed by phase-contrast microscopy, (2) mineralization of extracellular matrix as revealed by von Kossa staining, (3) quantitative real-time PCR (QRT-PCR) analysis of cDNA from entire plates and individual colonies revealing expression of genes characteristic of, and specific for, osteoblasts, (4) confocal microscopy of nodules from osteocalcin-green fluorescent protein (GFP) ES cell lines demonstrating the appropriate stage and position of osteoblasts expressing the reporter, and (5) immunostaining of nodules with a type I collagen antibody. Our method of initiating osteogenesis from ES cell-derived cultures is the only described method that allows for the observation and manipulation of the commitment stage of mesengenesis from single embryonic progenitors.

Woltjen, K., G. Bain, et al. (2000). "Retro-recombination screening of a mouse embryonic stem cell genomic library." Nucleic Acids Res **28**(9): E41.

Targeted gene disruption is an important tool in molecular medicine, allowing for the generation of animal models of human disease. Conventional methods of targeting vector (TV) construction are difficult and represent a rate limiting step in any targeting experiment. We previously demonstrated that bacteriophage are capable of acting as TVs directly, obviating the requirement for 'rolling out' plasmids from primary phage clones and thus eliminating an additional, time consuming step. We have also developed methods which facilitate the construction of TVs using recombination. In this approach, modification cassettes and point mutations are shuttled to specific sites in phage TVs using phage-plasmid recombination. Here, we report a further improvement in TV generation using a recombination screening-based approach deemed 'retro-recombination screening' (RRS). We demonstrate that phage vectors containing specific genomic clones can be genetically isolated from a lambdaTK embryonic stem cell genomic library using a cycle of integrative recombination and condensation. By introducing the gam gene of bacteriophage lambda into the probe plasmid it is possible to select for positive clones which have excised the plasmid, thus returning to their native conformation following purification from the library. Rapid clone isolation using the RRS protocol provides another method by which the time required for TV construction may be further reduced.

Xu, C., E. Rosler, et al. (2005). "Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium." Stem Cells **23**(3): 315-23.

Previous studies have shown that prolonged propagation of undifferentiated human embryonic stem cells (hESCs) requires conditioned medium from mouse embryonic feeders (MEF-CM) as well as matrix components. Because hESCs express growth factor receptors, including those for basic fibroblast growth factor (bFGF), stem cell factor (SCF), and fetal liver tyrosine kinase-3 ligand (Flt3L), we evaluated these and other growth factors for their ability to maintain undifferentiated hESCs in the absence of conditioned medium. We found cultures maintained in bFGF alone or in combination with other factors showed characteristics similar to MEF-CM control cultures, including morphology, surface marker and transcription factor expression, telomerase activity, differentiation, and karyotypic stability. In contrast, cells in media containing Flt-3L, thrombopoietin, and SCF, individually or in combination, showed almost complete differentiation after 6 weeks in culture. These data demonstrate that hESCs can be maintained in nonconditioned medium using growth factors.

Xu, W., H. Qian, et al. (2004). "A novel tumor cell line cloned from mutated human embryonic bone

marrow mesenchymal stem cells." Oncol Rep **12**(3): 501-8.

A novel tumor cell line, denominated F6, was established from mutated human embryonic bone marrow mesenchymal stem cells (MSCs) which were induced by the GM-CSF and IL-4 in vitro. The characteristics of the F6 cell line, such as surface antigens, cell cycle, growth curve, gene expression, morphology, cytogenetics and tumor model were analyzed. The F6 cells were round and grew suspended in a plastic dish. The cell line has a strong self-renewal capability, was positive for CD13, CD29, CD44, but negative for CD1alpha, CD3, CD10, CD14, CD23, CD33, CD34, CD38, CD41, CD45, CD54 and HLA-DR. The surface antigens were lower than those of human embryonic MSCs. The karyotype of F6 cells was abnormal. The cell cycle included: G0/G1 phase, 52.24%; G2/M phase, 8.00%; S phase, 41.76%. After the cells had been passaged serially for more than 17 months (62 passages), their characteristics were still retained. The F6 cells resulted in tumors in SCID nude mice in vivo (8/8) and caused metastasis (3/8). The pathologic examination revealed that the tumor cells extensively invaded surrounding normal tissues such as dermis, muscular tissue, nerve tissue, adipose tissue and lymphoid tissue. F6 cell line, tumor tissues derived from F6 cells and the MSCs expressed different levels of the nucleostemin gene. These findings suggested that F6 may be a novel tumor cell line. It may provide evidence for the theory that cancer originates from stem cells, and may be useful for the investigation on safety of human MSCs in the clinical application.

Yamauchi, K., K. Hasegawa, et al. (2009). "In vitro germ cell differentiation from cynomolgus monkey embryonic stem cells." PLoS One **4**(4): e5338.

BACKGROUND: Mouse embryonic stem (ES) cells can differentiate into female and male germ cells in vitro. Primate ES cells can also differentiate into immature germ cells in vitro. However, little is known about the differentiation markers and culture conditions for in vitro germ cell differentiation from ES cells in primates. Monkey ES cells are thus considered to be a useful model to study primate gametogenesis in vitro. Therefore, in order to obtain further information on germ cell differentiation from primate ES cells, this study examined the ability of cynomolgus monkey ES cells to differentiate into germ cells in vitro. METHODS AND FINDINGS: To explore the differentiation markers for detecting germ cells differentiated from ES cells, the expression of various germ cell marker genes was examined in tissues and ES cells of the cynomolgus monkey (Macaca fascicularis). VASA is a valuable gene for the detection of germ cells differentiated from ES cells. An increase of VASA expression was observed when differentiation was induced in ES cells via embryoid body (EB) formation. In addition, the expression of other germ cell markers, such as NANOS and PIWIL1 genes, was also up-regulated as the EB differentiation progressed. Immunocytochemistry identified the cells expressing stage-specific embryonic antigen (SSEA) 1, OCT-4, and VASA proteins in the EBs. These cells were detected in the peripheral region of the EBs as specific cell populations, such as SSEA1-positive, OCT-4-positive cells, OCT-4-positive, VASA-positive cells, and OCT-4-negative, VASA-positive cells. Thereafter, the effect of mouse gonadal cell-conditioned medium and growth factors on germ cell differentiation from monkey ES cells was examined, and this revealed that the addition of BMP4 to differentiating ES cells increased the expression of SCP1, a meiotic marker gene. CONCLUSION: VASA is a valuable gene for the detection of germ cells differentiated from ES cells in monkeys, and the identification and characterization of germ cells derived from ES cells are possible by using reported germ cell markers in vivo, including SSEA1, OCT-4, and VASA, in vitro as well as in vivo. These findings are thus considered to help elucidate the germ cell developmental process in primates.

Yang, C., S. Przyborski, et al. (2008). "A key role for telomerase reverse transcriptase unit in modulating human embryonic stem cell proliferation, cell cycle dynamics, and in vitro differentiation." Stem Cells **26**(4): 850-63.

Embryonic stem cells (ESC) are a unique cell population with the ability to self-renew and differentiate into all three germ layers. Human ESC express the telomerase reverse transcriptase (TERT) gene and the telomerase RNA (TR) and show telomerase activity, but TERT, TR, and telomerase are all downregulated during the differentiation process. To examine the role of telomerase in human ESC self-renewal and differentiation, we modulated the expression of TERT. Upregulation of TERT and increased telomerase activity enhanced the proliferation and colony-forming ability of human ESC, as well as increasing the S phase of the cell cycle at the expense of a reduced G1 phase. Upregulation of TERT expression was associated with increases in CYCLIN D1 and CDC6 expression, as well as hyperphosphorylation of RB. The differentiated progeny of control ESC showed shortening of telomeric DNA as a result of loss of telomerase activity. In contrast, the differentiated cells from TERT-overexpressing ESC maintained high telomerase activity and accumulated lower concentrations of peroxides than wild-type cells,

implying greater resistance to oxidative stress. Although the TERT-overexpressing human ESC are able to form teratoma composed of three germ layers in vivo, their in vitro differentiation to all primitive and embryonic lineages was suppressed. In contrast, downregulation of TERT resulted in reduced ESC proliferation, increased G1, and reduced S phase. Most importantly, downregulation of TERT caused loss of pluripotency and human ESC differentiation to extraembryonic and embryonic lineages. Our results indicate for the first time an important role for TERT in the maintenance of human ESC pluripotency, cell cycle regulation, and in vitro differentiation capacity.

Yeo, S., S. Jeong, et al. (2007). "Characterization of DNA methylation change in stem cell marker genes during differentiation of human embryonic stem cells." Biochem Biophys Res Commun **359**(3): 536-42.

Pluripotent human embryonic stem cells (hESCs) have the distinguishing feature of innate capacity to allow indefinite self-renewal. This attribute continues until specific constraints or restrictions, such as DNA methylation, are imposed on the genome, usually accompanied by differentiation. With the aim of utilizing DNA methylation as a sign of early differentiation, we probed the genomic regions of hESCs, particularly focusing on stem cell marker (SCM) genes to identify regulatory sequences that display differentiation-sensitive alterations in DNA methylation. We show that the promoter regions of OCT4 and NANOG, but not SOX2, REX1 and FOXD3, undergo significant methylation during hESCs differentiation in which SCM genes are substantially repressed. Thus, following exposure to differentiation stimuli, OCT4 and NANOG gene loci are modified relatively rapidly by DNA methylation. Accordingly, we propose that the DNA methylation states of OCT4 and NANOG sequences may be utilized as barometers to determine the extent of hESC differentiation.

Yi, F., L. Pereira, et al. (2008). "Tcf3 functions as a steady-state limiter of transcriptional programs of mouse embryonic stem cell self-renewal." Stem Cells **26**(8): 1951-60.

Elucidating the underlying transcriptional control of pluripotent cells is necessary for the development of new methods of inducing and maintaining pluripotent cells in vitro. Three transcription factors, Nanog, Oct4, and Sox2, have been reported to form a feedforward circuit promoting pluripotent cell self-renewal in embryonic stem cells (ESC). Previously, we found that a transcriptional repressor activity of Tcf3, a DNA-binding effector of Wnt signaling, reduced Nanog promoter activity and Nanog levels in mouse embryonic stem cells (mESC). The objective of this study was to determine the scope of Tcf3 effects on gene expression and self-renewal beyond the regulation of Nanog levels. We show that Tcf3 acts broadly on a genome-wide scale to reduce the levels of several promoters of self-renewal (Nanog, Tcl1, Tbx3, Esrrb) while not affecting other ESC genes (Oct4, Sox2, Fgf4). Comparing effects of Tcf3 ablation with Oct4 or Nanog knockdown revealed that Tcf3 counteracted effects of both Nanog and Oct4. Interestingly, the effects of Tcf3 were more strongly correlated with Oct4 than with Nanog, despite the normal levels of Oct4 in TCF3-/- mESC. The deranged gene expression allowed TCF3-/- mESC self-renewal even in the absence of leukemia inhibitory factor and delayed differentiation in embryoid bodies. These findings identify Tcf3 as a cell-intrinsic inhibitor of pluripotent cell self-renewal that functions by limiting steady-state levels of self-renewal factors. Disclosure of potential conflicts of interest is found at the end of this article.

Ying, Q. L., J. Nichols, et al. (2003). "BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3." Cell **115**(3): 281-92.

The cytokine leukemia inhibitory factor (LIF) drives self-renewal of mouse embryonic stem (ES) cells by activating the transcription factor STAT3. In serum-free cultures, however, LIF is insufficient to block neural differentiation and maintain pluripotency. Here, we report that bone morphogenetic proteins (BMPs) act in combination with LIF to sustain self-renewal and preserve multilineage differentiation, chimera colonization, and germline transmission properties. ES cells can be propagated from single cells and derived de novo without serum or feeders using LIF plus BMP. The critical contribution of BMP is to induce expression of Id genes via the Smad pathway. Forced expression of Id liberates ES cells from BMP or serum dependence and allows self-renewal in LIF alone. Upon LIF withdrawal, Id-expressing ES cells differentiate but do not give rise to neural lineages. We conclude that blockade of lineage-specific transcription factors by Id proteins enables the self-renewal response to LIF/STAT3.

Zampetaki, A., Q. Xiao, et al. (2006). "TLR4 expression in mouse embryonic stem cells and in stem cell-derived vascular cells is regulated by epigenetic modifications." Biochem Biophys Res Commun **347**(1): 89-99.

Embryonic stem (ES) cells and ES cell-derived differentiated cells can be used in tissue regeneration approaches. However, inflammation may

pose a major hurdle. To define the inflammatory response of ES and ES cell-derived vascular cells, we exposed these cells to LPS. With the exception of MIF no significant cytokine mRNA levels were observed either at baseline or after stimulation. Further experiments revealed that these cells do not express TLR4. Analysis of the DNA methylation status of the TLR4 upstream region showed increased methylation. Moreover, in vitro methylation suppressed TLR4 promoter activity in reporter gene assays. ChIP assays showed that in this region histones H3 and H4 are hypoacetylated in ES cells. Interestingly, 5-aza-dC or TSA partially relieves this gene repression. Finally, the increased levels of TLR4 observed in ES cells after treatment with 5-aza-dC or TSA confer responsiveness to LPS, as induction of IL-6 and TNFalpha mRNA was detected in endotoxin stimulated ES cells.

Zardo, G., G. Cimino, et al. (2008). "Epigenetic plasticity of chromatin in embryonic and hematopoietic stem/progenitor cells: therapeutic potential of cell reprogramming." Leukemia **22**(8): 1503-18.

During embryonic development and adult life, the plasticity and reversibility of modifications that affect the chromatin structure is important in the expression of genes involved in cell fate decisions and the maintenance of cell-differentiated state. Epigenetic changes in DNA and chromatin, which must occur to allow the accessibility of transcriptional factors at specific DNA-binding sites, are regarded as emerging major players for embryonic and hematopoietic stem cell (HSC) development and lineage differentiation. Epigenetic deregulation of gene expression, whether it be in conjunction with chromosomal alterations and gene mutations or not, is a newly recognized mechanism that leads to several diseases, including leukemia. The reversibility of epigenetic modifications makes DNA and chromatin changes attractive targets for therapeutic intervention. Here we review some of the epigenetic mechanisms that regulate gene expression in pluripotent embryonic and multipotent HSCs but may be deregulated in leukemia, and the clinical approaches designed to target the chromatin structure in leukemic cells.

Zhang, J., W. L. Tam, et al. (2006). "Sall4 modulates embryonic stem cell pluripotency and early embryonic development by the transcriptional regulation of Pou5f1." Nat Cell Biol **8**(10): 1114-23.

Embryonic stem (ES) cells are pluripotent cells that can self-renew or differentiate into many cell types. A unique network of transcription factors and signalling molecules are essential for maintaining this capability. Here, we report that a spalt family member, Sall4, is required for the pluripotency of ES cells. Similarly to Oct4, a reduction in Sall4 levels in mouse ES cells results in respecification, under the appropriate culture conditions, of ES cells to the trophoblast lineage. Sall4 regulates transcription of Pou5f1 which encodes Oct4. Sall4 binds to the highly conserved regulatory region of the Pou5f1 distal enhancer and activates Pou5f1 expression in vivo and in vitro. Microinjection of Sall4 small interfering (si) RNA into mouse zygotes resulted in reduction of Sall4 and Oct4 mRNAs in preimplantation embryos and significant expansion of Cdx2 expression into the inner cell mass. These results demonstrate that Sall4 is a transcriptional activator of Pou5f1 and has a critical role in the maintenance of ES cell pluripotency by modulating Oct4 expression. The data also indicates that Sall4 is important for early embryonic cell-fate decisions.

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