

Stem Cell and Differentiation Literatures

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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 situation or become another type of cell with a more specialized function, such as a muscle cell, a red blood cell, a bone cell, a nerve cell, or a brain cell. Stem cell research is a typical and important topic of life science. This material collects some literatures on stem cell and differentiation.

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

Literatures

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.

Alzuherri, H. M. and R. J. White (1999). "Regulation of RNA polymerase I transcription in response to F9 embryonal carcinoma stem cell differentiation." *J Biol Chem* **274**(7): 4328-34.

Dramatic changes in the patterns of transcription are a common feature of early development. We have used F9 embryonal carcinoma cells as a model system to study gene regulation during an early stage of murine embryogenesis. We find that transcription by RNA polymerase I decreases when F9 cells differentiate into parietal endoderm. The reduced rate of transcription is associated with a down-regulation of several components of the class I transcription apparatus. The most substantial change involves the essential factor SL1, which is a multisubunit complex that contains the TATA-binding protein and three TATA-binding protein-associated factors (TAFs). The abundance of two of these TAFs, TAFI48 and TAFI95, decreases during F9 cell differentiation. Developmental regulation of a specific class of genes may therefore be achieved through changes in the availability of TAFs.

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 differentiation 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), trimethylation 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.

Attema, J. L., P. Papathanasiou, et al. (2007). "Epigenetic characterization of hematopoietic stem cell differentiation using miniChIP and bisulfite sequencing analysis." *Proc Natl Acad Sci U S A* **104**(30): 12371-6.

Hematopoietic stem cells (HSC) produce all blood cell lineages by virtue of their capacity to self-renew and differentiate into progenitors with decreasing cellular potential. Recent studies suggest that epigenetic mechanisms play an important role in controlling stem cell potency and cell fate decisions. To investigate this hypothesis in HSC, we have modified the conventional chromatin immunoprecipitation assay allowing for the analysis of 50,000 prospectively purified stem and progenitor cells. Together with bisulfite sequencing analysis, we found that methylated H3K4 and ACh3 and unmethylated CpG dinucleotides colocalize across defined regulatory regions of lineage-affiliated genes in HSC. These active epigenetic histone modifications

either accumulated or were replaced by increased DNA methylation and H3K27 trimethylation in committed progenitors consistent with gene expression. We also observed bivalent histone modifications at a lymphoid-affiliated gene in HSC and downstream transit-amplifying progenitors. Together, these data support a model in which epigenetic modifications serve as an important mechanism to control HSC multipotency.

Backdahl, L., M. Herberth, et al. (2009). "Gene body methylation of the dimethylarginine dimethylaminohydrolase 2 (Ddah2) gene is an epigenetic biomarker for neural stem cell differentiation." *Epigenetics* 4(4): 248-54.

DNA methylation is an important epigenetic mark that is involved in the regulation of many cellular processes such as gene expression, genomic imprinting and silencing of repetitive elements. Because of their ability to cause and capture phenotypic plasticity, epigenetic marks such as DNA methylation represent potential biomarkers to distinguish between different types of tissues and stages of differentiation. Here, we have identified differential DNA methylation in the gene body of the nitric oxide inhibitor Ddah2 that discriminates embryonic stem cells from neural stem cells and is positively correlated with differential gene expression.

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.

Balloni, S., E. M. Calvi, et al. (2009). "Effects of titanium surface roughness on mesenchymal stem cell commitment and differentiation signaling." *Int J Oral Maxillofac Implants* 24(4): 627-35.

PURPOSE: Human mesenchymal stem cells (hMSCs) are primary cells capable of differentiating to osteocytic lineage when stimulated under appropriate conditions. This study examined changes in hMSC morphology, proliferation, and gene expression after growth on machined or dual acid-etched (AE) titanium surfaces. **MATERIALS AND METHODS:** hMSCs, isolated from adult human bone marrow, were cultured on titanium surfaces. The two specimens of titanium surfaces in this study included machined and AE titanium disks. Cell morphology was evaluated by scanning electron microscopy, and cell proliferation and collagen synthesis were estimated by measuring the amount of 3H-thymidine incorporation into DNA and 3H-proline incorporation into collagen fibers. Alkaline phosphatase (ALP) activity was determined by measuring the release of p-nitrophenol from disodium p-nitrophenyl phosphate. Changes in gene expression for bone morphogenetic protein-2 (BMP-2), Runx2 type II, Osterix (Osx), osteopontin, type I collagen, ALP, osteocalcin, and bone sialoprotein were determined by reverse-transcriptase polymerase chain reaction after 22 days of in vitro culture in osteogenic medium. **RESULTS:** The two substrates had no significant effects on cell adhesion and proliferation. Morphologic characteristics were observed by scanning electron microscopy. hMSCs on the machined surface spread more and were flatter than cells cultured on the AE surface. Osteopontin mRNA expression was similar on all surfaces, and the other mRNA transcripts were increased in hMSC cultured on AE surface. In particular, BMP-2, Runx2, and Osx, three osteogenic factors that induce the progressive differentiation of multipotent mesenchymal cells into osteoblasts, were expressed more on AE titanium than on machined titanium. Collagen and ALP assays confirmed the highest level of mRNA transcripts correlated with increases in these proteins. **CONCLUSION:** These results showed that an AE titanium surface stimulated

the expression of markers of osteoblastic phenotype more than a machined titanium surface.

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. To assess their role in development, we studied the in vitro differentiation of embryonic stem (ES) cells that bear one or both disrupted Hmga1 alleles. Here, we report that Hmga1 null ES cells generate fewer T-cell precursors than do wild-type ES cells. Indeed, they preferentially differentiate to B cells, probably consequent to decreased interleukin 2 expression and increased interleukin 6 expression. Moreover, a lack of HMGA1 expression induces changes in hemopoietic differentiation, i.e., a reduced monocyte/macrophage population and an increase in megakaryocyte precursor numbers, erythropoiesis, and globin gene expression. Re-expression of the Hmga1 gene in Hmga1 null ES cells restores the wild-type phenotype. 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.

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.

Buzanska, L., N. Spassky, et al. (2001). "Human medulloblastoma cell line DEV is a potent tool to screen for factors influencing differentiation of neural stem cells." *J Neurosci Res* **65**(1): 17-23.

The aim of our study was to investigate whether a human neural cell line could be used as a reliable screening tool to examine the functional conservation, in humans, of transcription factors involved in neuronal or glial specification in other species. Gain-of-function experiments were performed on DEV cells, a cell line derived from a human medulloblastoma. Genes encoding nine different transcription factors were tested for their influence on the process of specification of human DEV cells towards a neuronal or glial fate. In a first series of experiments, DEV cells were transfected with murine genes encoding transcription factors known to be involved in the neuronal differentiation cascade. Neurogenins-1, -2, and -3; Mash-1; and NeuroD increased the differentiation of DEV cells towards a neuronal phenotype by a factor of 2-3.5. In a second series of experiments, we tested transcription factors involved in invertebrate glial specification. In the embryonic *Drosophila* CNS, the development of most glial cells depends on the master regulatory gene glial cell missing (*gcm*). Expression of *gcm* in DEV cells induced a twofold increase of astrocytic and a sixfold increase of oligodendroglial cell types. Interestingly, expression of *tramtrack69*, which is required in all *Drosophila* glial cells, resulted in a fourfold increase of only the oligodendrocyte phenotype. Expression of the related *tramtrack88* protein, which is not expressed in the fly glia, or the *C. elegans* *lin26* protein showed no effect. These results show that the *Drosophila* transcription factor genes tested can conserve their function upon transfection into the human DEV cells, qualifying this cell line as a screening tool to analyze the mechanisms of neuronal and glial specification.

Chen, S. and R. J. Lechleider (2004). "Transforming growth factor-beta-induced differentiation of smooth muscle from a neural crest stem cell line." *Circ Res* **94**(9): 1195-202.

During vascular development, nascent endothelial networks are invested with a layer of supporting cells called pericytes in capillaries or smooth muscle in larger vessels. The cellular lineage of smooth muscle precursors and factors responsible for regulating their differentiation remain uncertain. In vivo, cells derived from the multipotent neural crest can give rise to vascular smooth muscle in parts of the head and also the cardiac outflow tract. Although transforming growth factor-beta (TGF-beta) has previously been shown to induce some smooth muscle markers from primary cultures of neural crest stem cells, the extent of the differentiation induced was not clear. In this study, we demonstrate that TGF-beta can induce many of the markers and characteristics of vascular smooth muscle from a neural crest stem cell line, Monc-1. Within 3 days of in vitro treatment, TGF-beta induces multiple smooth muscle-specific markers, while downregulating epithelial markers present on the parent cells. Treatment with TGF-beta also induces a contractile phenotype that responds to the muscarinic agonist carbachol and is not immediately reversed on TGF-beta withdrawal. Examination of the signaling pathways involved revealed that TGF-beta activation of Smad2 and Smad3 appear to be essential for the observed differentiation. Taken together, this system provides a novel model of smooth muscle differentiation that reliably recapitulates the process observed in vivo and allows for dissection of the pathways and processes involved in this process.

Clement, C. A., L. A. Larsen, et al. (2009). "Using nucleofection of siRNA constructs for knockdown of primary cilia in P19.CL6 cancer stem cell differentiation into cardiomyocytes." Methods Cell Biol **94**: 181-97.

Primary cilia assemble as solitary organelles in most mammalian cells during growth arrest and are thought to coordinate a series of signal transduction pathways required for cell cycle control, cell migration, and cell differentiation during development and in tissue homeostasis. Recently, primary cilia were suggested to control pluripotency, proliferation, and/or differentiation of stem cells, which may comprise an important source in regenerative biology. We here provide a method using a P19.CL6 embryonic carcinoma (EC) stem cell line to study the function of the primary cilium in early cardiogenesis. By knocking down the formation of the primary cilium by nucleofection of plasmid DNA with siRNA sequences against genes essential in ciliogenesis (IFT88 and IFT20) we block hedgehog (Hh) signaling in P19.CL6 cells as well as the differentiation of the cells into beating cardiomyocytes (Clement et al., 2009). Immunofluorescence microscopy, western

blotting, and quantitative PCR analysis were employed to delineate the molecular and cellular events in cilia-dependent cardiogenesis. We optimized the nucleofection procedure to generate strong reduction in the frequency of ciliated cells in the P19.CL6 culture.

Dansranjav, T., S. Krehl, et al. (2009). "The role of promoter CpG methylation in the epigenetic control of stem cell related genes during differentiation." Cell Cycle **8**(6): 916-24.

Differentiation and malignant transformation of stem cells are regulated by epigenetic mechanisms. We analyzed promoter methylation and expression of the stem cell determining genes Brachyury, DPPA5, FGF4, FOXD3, LIN28, NESTIN and ZFP42 depending on the differentiation state in human mesenchymal stem cells (MSC), human embryonal carcinoma cells (ECC) and somatic tumor cells. Differentiation of MSC into osteoblasts and adipocytes was accompanied with a loss of expression of the Brachyury gene and downregulation of LIN28. Inactivation of Brachyury was associated with progressive methylation of its CpG island promoter. In ECC promoter methylation of stem cell markers was more frequent in the differentiated subgroup (71%) compared to undifferentiated ECC (29%) and this was associated with downregulation of Brachyury, DPPA5, FGF4, FOXD3, LIN28 and ZFP42. DPPA5 was methylated and NESTIN was unmethylated in most tumor cells. In somatic tumor cells, methylation of stem cell markers (Brachyury, DPPA5, FGF4, FOXD3, LIN28 and ZFP42) was frequently observed (85%). Treatment of cell lines with an inhibitor of DNA methyltransferase reactivated the expression of DPPA5, FGF4, FOXD3, LIN28 and ZFP42, indicating that aberrant promoter methylation is a crucial event that results in their silencing. Our results suggest that epigenetic inactivation of stem cell associated genes is mediated by promoter methylation and that this may represent a fundamental mechanism during normal differentiation processes.

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.

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.

Eistetter, H. R. (1988). "A mouse pluripotent embryonic stem cell line stage-specifically regulates expression of homeo-box containing DNA sequences during differentiation in vitro." *Eur J Cell Biol* **45**(2): 315-21.

Mouse embryonic stem (ES) cells have been shown to provide a new model system suitable for the analysis of different aspects of murine development. This report gives evidence that ES cell lines are also most useful for the study of developmentally regulated gene expression in vitro. Homeo-box containing genes which are suggested to play a key role in the regulation of differentiation steps occurring during embryogenesis are stage-specifically transcribed in differentiating murine ES cells: (i) A mouse embryonic stem cell line (ES-12957) was isolated and characterized with respect to its differentiation potential. When injected subcutaneously into syngeneic mice, ES-12957 cells formed fully differentiated teratomas representing derivatives of all three germ layers. When allowed to grow in suspension cultures in vitro, the cells followed a reproducible developmental pathway forming complex organized 'embryoid bodies' which resembled mouse early postimplantation embryos. (ii) A mouse DNA sequence with homeo-box homology (MH-121) was isolated and structurally analyzed. Transcription of a 1.7 kb RNA species from this DNA sequence was demonstrated in ES-12957 cells which were differentiated in vitro. A second, previously described homeo-box gene (Mo-10) was also shown to be expressed in ES-12957 cells in a stage-specific manner. A 4-kb transcript could be identified exclusively in RNA of cells which were allowed to differentiate for 9 days. These findings support the suggestion that the homeo-box genes of mammals, like those of *Drosophila*, may have important functions during embryonic development.

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.

Frostesjo, L., I. Holm, et al. (1997). "Interference with DNA methyltransferase activity and genome methylation during F9 teratocarcinoma stem cell differentiation induced by polyamine depletion." *J Biol Chem* **272**(7): 4359-66.

When ornithine decarboxylase, the initial and highly regulated enzyme in polyamine biosynthesis, is irreversibly inactivated by alpha-difluoromethylornithine, F9 teratocarcinoma stem cells are depleted of putrescine and spermidine and as a result differentiate into a cell type which phenotypically resembles the parietal endoderm cells of the early mouse embryo. Simultaneously the level of decarboxylated S-adenosylmethionine (dcAdoMet), the aminopropyl group donor in spermidine and spermine synthesis, increases dramatically, as the aminopropyl group acceptor molecules (putrescine and spermidine) become limiting. When this excessive accumulation of dcAdoMet is prevented by specific inhibition of the AdoMet decarboxylase activity, the differentiative effect is counteracted, despite the fact that the extent of polyamine depletion remains almost identical. Therefore, it may be concluded that dcAdoMet plays an important role in the induction of differentiation. Moreover, this key metabolite acts as a competitive inhibitor of DNA methyltransferase and is therefore capable of interfering with the maintenance methylation of newly replicated DNA. During the course of F9 cell differentiation, the highly methylated genome is gradually demethylated, and its pattern of gene expression is changed. Our present findings, that the DNA remains highly methylated and that the

differentiative process is counteracted when the build-up of dcAdoMet is prevented, provide strong evidence for a causative relation between the level of dcAdoMet and the state of DNA methylation as well as cell differentiation.

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.

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-ribosylation) 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.

Gordeeva, O., R. Zinovieva, et al. (2005). "Differentiation of embryonic stem cells after transplantation into peritoneal cavity of irradiated mice and expression of specific germ cell genes in pluripotent cells." *Transplant Proc* 37(1): 295-8.

Permanent embryonic stem cell lines (ES cells) are considered as one of the most promising cellular sources for regenerative medicine. ES cells have a high proliferative potency and ability to differentiate into all kinds of somatic and germ cells. However, transplantation of undifferentiated ES cells into adult recipient tissue results in the formation of teratomas. To understand the mechanisms underlying self-renewal and determination of pluripotent cells, we investigated differentiation potencies of undifferentiated ES cells and differentiating embryoid bodies (EB). ES cells and EBs growing on acetate-cellulose membranes were transplanted into the peritoneal cavity of irradiated mice. Behavior and differentiation of transplanted cells were studied within 1, 2, 3, and 6 weeks after transplantation. No differences in the cell composition were found in the teratomas formed by ES cells and differentiating EBs. The pattern of expression of the genes specific for pluripotent and germ cells was studied in all types of experimental teratomas. The expression of oct4, stella, fragilis was detected in the teratomas, but nanog was not expressed. We conclude that pluripotent cells are retained in the experimental teratomas formed after transplantation of ES cells and EBs but the pattern of expression of the studied genes underwent changes.

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.

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*, *Snm1*, *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.

Hamada, T., S. Murasawa, et al. (2009). "Changing modified regions in the genome in hematopoietic stem cell differentiation." *Biochem Biophys Res Commun* **381**(2): 135-8.

In the process of hematopoietic stem cell (CD133+ cell) differentiation, a drastic change in gene expression occurs which must be regulated by epigenetic mechanisms. One strategy for CD133+ cell differentiation analysis is to identify genomic DNA regions that have been modified in the process of differentiation. However, it is difficult to obtain large amounts of genomic DNA from uniform CD133+ cells. Based on this situation, we screened genomic DNA regions where modifications change during the process of differentiation in human CD133+ cells using differential methylation site scanning (DMSS), which is a method of identifying differentially methylated regions of the genome from a small number of cells. As a result, we cloned three DNA fragments which corresponded to centrosomal protein 68kDA (*Cep68*), TRIO and F-actin binding protein (TRIOBP), and AMP-activated protein kinase beta (AMPKb).

Hatakeyama, J., Y. Bessho, et al. (2004). "Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation." *Development* **131**(22): 5539-50.

Radial glial cells derive from neuroepithelial cells, and both cell types are identified as neural stem cells. Neural stem cells are known to change their competency over time during development: they initially undergo self-renewal only and then give rise to neurons first and glial cells later. Maintenance of neural stem cells until late stages is thus believed to be essential for generation of cells in correct numbers and diverse types, but little is known about how the timing of cell differentiation is regulated and how its deregulation influences brain organogenesis. Here, we report that inactivation of *Hes1* and *Hes5*, known Notch effectors, and additional inactivation of *Hes3* extensively accelerate cell differentiation and cause a wide range of defects in brain formation. In *Hes*-deficient embryos, initially formed neuroepithelial cells are not properly maintained, and radial glial cells are prematurely differentiated into neurons and depleted without generation of late-born cells. Furthermore, loss of radial glia disrupts the inner and outer barriers of the neural tube, disorganizing the histogenesis. In addition, the forebrain lacks the optic vesicles and the ganglionic eminences. Thus, *Hes* genes are essential for generation of brain structures of appropriate size, shape and cell arrangement by controlling the timing of cell differentiation. Our data also indicate that embryonic neural stem cells change their characters over time in the following order: *Hes*-independent neuroepithelial cells, transitory *Hes*-dependent neuroepithelial cells and *Hes*-dependent radial glial cells.

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.

Hjertson, M., C. Sundstrom, et al. (1996). "Stem cell factor, but not flt3 ligand, induces differentiation and activation of human mast cells." *Exp Hematol* **24**(6): 748-54.

We have examined the effect of human ligand for the flt3/flk2 tyrosine kinase receptor on the differentiation of human mast cells in suspension cultures. We also explored the effect of flt3 ligand (FL) on the human mast cell line HMC-1 and mRNA expression of flt3/flk2 on in vitro developed human mast cells and HMC-1. The growth of cord blood mononuclear cells in suspension cultures was increased when cells were cultured in the presence of FL compared with cells cultured in the presence of stem cell factor (SCF). When SCF and FL were combined, the total cell growth was increased further. Our data show that FL by itself neither induced differentiation of mast cells nor acted in the SCF-dependent differentiation of human cord blood-derived mast cells (CBMC). Furthermore, no effects of FL were found on the proliferation of HMC-1 cells or the induction of early-immediate response genes in HMC-1 cells. In addition, neither HMC-1 cells nor CBMC expressed mRNA for flt3. As has been shown before, SCF and FL have little biological effect on their own but synergize well with a number of other hematopoietic growth factors. This study shows that a major difference between FL and SCF is that only SCF affects the differentiation and activation of mast cells.

Hole, N., G. J. Graham, et al. (1996). "A limited temporal window for the derivation of multilineage repopulating hematopoietic progenitors during embryonic stem cell differentiation in vitro." *Blood* **88**(4): 1266-76.

Embryonic stem cells have been shown to differentiate in vitro into all hematopoietic lineages. This has been used successfully as one approach to the study of genetic events occurring during haematopoiesis. However, studies on the commitment of mesodermal precursors to the hematopoietic lineage have been limited due to the inability to define a system in which embryonic stem (ES) cells will give rise to primitive hematopoietic stem cells in vitro. Using a colony forming assay (CFU-A), we determined that the earliest time point at which primitive multilineage hematopoietic precursors can be detected during ES cell differentiation in vitro in the absence of exogenous conditioned medium or stromal cell culture is 4 days. Lethally irradiated adult recipient mice that received differentiated ES cells from this time point survived for more than 3 weeks; and in two out of three experiments, peripheral blood from these animals contained ES-derived progeny. Fluorescence activated cell sorting (FACS) found ES-derived CD45+ hematopoietic cells in both lymphoid and myeloid compartments at 12 weeks posttransplantation, suggesting that the population of day 4 differentiated ES cells contains primitive hematopoietic precursors. A preliminary RT-PCR analysis of gene expression around this time point suggests that there are very few hematopoietic cells present. This approach should prove useful in studies of genetic control of commitment to and maintenance of hematopoietic lineages in vitro and in vivo.

Horn, L. C., C. Hanel, et al. (2008). "Mixed serous carcinoma of the endometrium with trophoblastic differentiation: analysis of the p53 tumor suppressor gene suggests stem cell origin." *Ann Diagn Pathol* **12**(1): 1-3.

The pathogenesis of mixed endometrial adenocarcinoma with trophoblastic differentiation is quite unclear at times. The present study examines a serous carcinoma with choriocarcinomatous differentiation. p53 staining was seen in the serous component and the cytotrophoblastic cells of the choriocarcinomatous component, but not in the syncytiotrophoblastic cells. p53 mutational analysis showed a heterozygotic mutation at exon 8 for the choriocarcinomatous component and a homozygote deletion at exon 7 for the serous component. These alterations suggest that the multidirectional tumor differentiation might occur from a common stem cell in these malignancies.

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.

Jacob, A., S. Budhiraja, et al. (1994). "Retinoic acid-mediated activation of HNF-3 alpha during EC stem cell differentiation." *Nucleic Acids Res* **22**(11): 2126-33.

We present evidence demonstrating that the liver-enriched transcription factor HNF-3 alpha is activated upon retinoic acid-induced differentiation of mouse F9 embryonal carcinoma cells. We have detected increases in the DNA binding activity and mRNA level of HNF-3 alpha. Both are reflections of the actual activation mechanism at the level of transcriptional initiation, which we showed with the help of HNF-3 alpha promoter constructs. Time course studies clearly show that HNF-3 alpha activation is a transient event. Employing Northern blots, HNF-3 alpha mRNA can be detected between 16 and 24 hours post-differentiation, reaches its zenith at approximately 1 day, and then declines to virtually undetectable levels. F9 cells can give rise to three distinct differentiated cell types; visceral endoderm, parietal endoderm, and primitive endoderm. We have clearly shown that HNF-3 alpha stimulation occurs upon primitive endoderm formation. In addition, the transcription factor is also activated during the induction of cell lineages that give rise to parietal and visceral endoderm. HNF-3 alpha stimulation upon visceral endoderm differentiation is accompanied by the activation of HNF-3 target genes such as

transthyretin, suggesting that HNF-3 alpha is involved in the developmental activation of this gene. In contrast, HNF-3 alpha target genes in parietal and primitive endoderm have yet to be identified. However, the stimulation of HNF-3 alpha during primitive endoderm formation, which is an extremely early event during murine embryogenesis, points towards a role for the factor in crucial determination processes that occur early during development.

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.

Kim, D. H., K. H. Yoo, et al. (2005). "Gene expression profile of cytokine and growth factor during differentiation of bone marrow-derived mesenchymal stem cell." *Cytokine* **31**(2): 119-26.

Mesenchymal stem cells (MSCs), which are adherent stromal cells of a nonhematopoietic origin, have the ability to give rise to various differentiated cell types. MSCs regulate localization, self-renewal and differentiation of hematopoietic stem cells (HSCs) due to MSCs' secretion of cytokines and growth factors, the cell-to-cell interactions and the influence of the extracellular matrix proteins. Using RT-PCR analysis, we examined the expression levels of cytokines and growth factors from MSCs and their

differentiated cell types, including osteoblasts, adipocytes and endothelial cells. Cytokine and growth factor genes, including IL-6, IL-8, IL-11, IL-12, IL-14, IL-15, LIF, G-CSF, GM-CSF, M-SCF, FL and SCF, were found to be expressed in the MSCs. In contrast, there was no IL-1alpha, IL-1beta, or IL-7 expression observed. The IL-12, IL-14, G-CSF, and GM-CSF mRNA expression levels either disappeared or decreased after the MSCs differentiated into osteoblasts, adipocytes, and endothelial cells. Among the differentiated cells derived from MSCs, osteoblasts, adipocytes, and endothelial cells expressed the osteopontin, aP2, and the VEGFR-2 gene, respectively. These profiles could help determine future clinical applications of MSCs and their derivatives for cell therapy.

Koutna, I., M. Klabusay, et al. (2006). "Evaluation of CD34+ - and Lin- -selected cells from peripheral blood stem cell grafts of patients with lymphoma during differentiation in culture ex vivo using a cDNA microarray technique." *Exp Hematol* **34**(7): 832-40.

OBJECTIVE: Hematopoietic stem cells (enriched in fraction of CD34+ cells) have the ability to regenerate hematopoiesis in all of its lineages, and this potential is clinically used in transplanting bone marrow or peripheral blood stem cells. Our objective was to assemble a suitable method for evaluating gene expression in enriched populations of hematopoietic stem cells. We compared biologic properties of cells cultured ex vivo obtained using two different ways of immunomagnetic separation (positive selection of CD34+ cells and negative selection of Lin- cells) by means of a cDNA microarray technique. **METHODS:** CD34+ and Lin- cells were enriched from peripheral blood stem cell (PBSCs) grafts of patients with non-Hodgkin's lymphoma. Isolated cells were in the presence of cytokine PBSCs, Flt-3 ligand, interleukin-3, interleukin-6, and granulocyte colony-stimulating factor. At days 0, 4, 6, 8, 10, 12, and 14 cells were harvested and analyzed by cDNA microarrays. Total cell expansion, CD34+, colony-forming unit for granulocyte-macrophage and megakaryocytes expansion, vitality, and phenotype of cells were also analyzed. **RESULTS:** cDNA microarray analysis of cultured hematopoietic cells proved equivalence of the two enrichment methods for PBSC samples and helped us characterize differentiating cells cultured ex vivo. **CONCLUSION:** Our methodologic approach is helpful in characterizing cultured hematopoietic cells cultured ex vivo, but it is also suitable for more general purposes. Equivalence of CD34+ and Lin-selection methods from PBSC samples proved by cDNA microarray may have an implication for graft manipulation in an experimental setting of hematopoietic transplantation. Total cell expansion

and colony formation and phenotype from CD34+ selected and from Lin- samples were comparable.

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.

Kristeva, M. A. (2008). "Stem cells in light of a new concept for cell differentiation." *Stem Cells Dev* **17**(5): 849-52.

My concept of cell differentiation involves genetic information from DNA being transcribed into mRNA proteins-morphogenes (mRNAs+ homeodomain proteins)-and stored in the ovoplasm as maternal inheritance, or cytoplasmic genetic memory. Feedback mechanism(s) allow these morphogenes to selectively unlock new genes, regulating the development of the embryo. The blastomeres and the embryonic pluripotent cells of the inner cell mass of early (5 day) blastocysts are loaded with morphogenes which hamper the production of cell lines and are responsible for the formation of embryoid bodies in vitro and teratomas in vivo. There are therefore legitimate concerns as to proposals to use embryonic pluripotent cells for cell therapy and regenerative medicine. An alternative cell therapy would involve the production of tailored growth-related genes-morphogenes-and hence selective in vitro differentiation of adult de-differentiated cells.

La Thangue, N. B., B. Thimmappaya, et al. (1990). "The embryonal carcinoma stem cell Ela-like activity involves a differentiation-regulated transcription factor." *Nucleic Acids Res* **18**(10): 2929-38.

Murine F9 embryonal carcinoma (EC) stem cells have an Ela-like transcription activity that is undetectable in F9 cells differentiated to parietal endoderm-like cells (F9-PE). The Ela-inducible adenovirus E2A promoter has been used to further define this activity and we show that in vitro the transcription of this promoter in F9 EC and F9-PE cell extracts reflects the regulation in vivo. In EC cell extracts several trans-acting protein factors bind to E2A promoter sequences. A distal domain containing a CRE binds proteins present in F9 EC, F9-PE and HeLa cell extracts. Sequences between -71 and -50 define a multiplicity of binding activities, termed DRTF1, all of which are down regulated as EC stem cells differentiate. DRTF2, a low abundance, regulated binding activity requires DNA sequences that overlap those required by DRTF1. The CRE and the DRTF1 binding site compete for transcription in vitro, indicating that in EC cell extracts the respective proteins function as positively acting, binding site dependent transcription factors. Comparison of DRTF1 with the previously defined HeLa cell factor E2F, induced during adenovirus infection, indicates that although both factors recognise the same region of the promoter there are clear differences between them. These data indicate that multiple factors are necessary for efficient transcription of the E2A promoter in F9 EC cell extracts and suggest that DRTF1 is responsible, at least in part, for the developmental regulation of the cellular Ela-like activity.

Lang, D., M. M. Lu, et al. (2005). "Pax3 functions at a nodal point in melanocyte stem cell differentiation." *Nature* **433**(7028): 884-7.

Most stem cells are not totipotent. Instead, they are partially committed but remain undifferentiated. Upon appropriate stimulation they are capable of regenerating mature cell types. Little is known about the genetic programmes that maintain the undifferentiated phenotype of lineage-restricted stem cells. Here we describe the molecular details of a nodal point in adult melanocyte stem cell differentiation in which Pax3 simultaneously functions to initiate a melanogenic cascade while acting downstream to prevent terminal differentiation. Pax3 activates expression of Mitf, a transcription factor critical for melanogenesis, while at the same time it competes with Mitf for occupancy of an enhancer required for expression of dopachrome tautomerase, an enzyme that functions in melanin synthesis. Pax3-expressing melanoblasts are thus committed but undifferentiated until Pax3-mediated repression is relieved by activated beta-catenin. Thus, a stem cell transcription factor can both determine cell fate and simultaneously maintain an undifferentiated

state, leaving a cell poised to differentiate in response to external stimuli.

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.

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.

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 nucleofection was used in the present study; approximately 70% cells showed DsRed expression 2 d after nucleofection. 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 nucleofected cells showed insulin expression 18 d after nucleofection, 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.

Lin, P., L. Chen, et al. (2009). "Evaluation of stem cell differentiation in diabetic rats transplanted with bone marrow mesenchymal stem cells." Transplant Proc **41**(5): 1891-3.

OBJECTIVE: Bone marrow mesenchymal stem cell (BM-MSC) transplantation has generated a great deal of excitement as a promising therapeutic strategy for diabetes mellitus. However, the exact mechanisms of reversing hyperglycemia remain elusive. Our objective was to investigate whether stem cell differentiation determined therapeutic efficacy. **MATERIALS AND METHODS:** Wistar rats were rendered diabetic by an intraperitoneal injection of streptozotocin. BM-MSCs isolated from diabetic Wistar rats were analyzed for phenotype characteristics. Subsequently, BM-MSCs were transplanted into diabetic rats, followed by intravenous injection of recombinant lentiviruses encoding 2 different small hairpin RNAs (shRNAs) for specific interference with neurogenin 3 (Ngn3). We measured blood glucose levels and insulin and performed histological analysis of the pancreas. **RESULTS:** BM-MSCs lowered blood glucose by increasing beta-cell mass compared with sham-operated controls, but this effect was inhibited by interference with the Ngn3 gene. **CONCLUSION:** Differentiation of stem cells, including BM-MSCs and endogenous pancreatic stem cells, plays a major role in the process of reversing hyperglycemia.

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 hematopoietic 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, 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.

Maines, J. Z., J. K. Park, et al. (2007). "Stonewalling Drosophila stem cell differentiation by epigenetic controls." *Development* **134**(8): 1471-9.

During Drosophila oogenesis, germline stem cell (GSC) identity is maintained largely by preventing the expression of factors that promote differentiation. This is accomplished via the activity of several genes acting either in the GSC or in its niche. The translational repressors Nanos and Pumilio act in GSCs to prevent differentiation, probably by inhibiting the translation of early differentiation factors, whereas niche signals prevent differentiation by silencing transcription of the differentiation factor Bam. We have found that the DNA-associated protein Stonewall (Stwl) is also required for GSC maintenance. stwl is required cell-autonomously; clones of stwl(-) germ cells were lost by differentiation, and ectopic Stwl caused an expansion of GSCs. stwl mutants acted as Suppressors of variegation, indicating that stwl normally acts in chromatin-dependent gene repression. In contrast to several previously described GSC maintenance factors, Stwl probably functions epigenetically to prevent GSC differentiation. Stwl-dependent transcriptional repression does not target bam, but rather Stwl represses the expression of many genes, including those that may be targeted by Nanos and Pumilio translational inhibition.

Margariti, A., Q. Xiao, et al. (2009). "Splicing of HDAC7 modulates the SRF-myocardin complex during stem-cell differentiation towards smooth muscle cells." *J Cell Sci* **122**(Pt 4): 460-70.

Histone deacetylases (HDACs) have a central role in the regulation of gene expression. Here we investigated whether HDAC7 has an impact on

embryonic stem (ES) cell differentiation into smooth muscle cells (SMCs). ES cells were seeded on collagen-IV-coated flasks and cultured in the absence of leukemia inhibitory factor in differentiation medium to induce SMC differentiation. Western blots and double-immunofluorescence staining demonstrated that HDAC7 has a parallel expression pattern with SMC marker genes. In ex vivo culture of embryonic cells from SM22-LacZ transgenic mice, overexpression of HDAC7 significantly increased beta-galactosidase-positive cell numbers and enzyme activity, indicating its crucial role in SMC differentiation during embryonic development. We found that HDAC7 undergoes alternative splicing during ES cell differentiation. Platelet-derived growth factor enhanced ES cell differentiation into SMCs through upregulation of HDAC7 splicing. Further experiments revealed that HDAC7 splicing induced SMC differentiation through modulation of the SRF-myocardin complex. These findings suggest that HDAC7 splicing is important for SMC differentiation and vessel formation in embryonic development.

McCulloch, E. A., M. D. Minden, et al. (1988). "Stem cell renewal and differentiation in acute myeloblastic leukaemia." *J Cell Sci Suppl* **10**: 267-81.

The defining properties of stem cells are capacities for self-renewal and, after determination, a limited number of terminal divisions. The blast cells of acute myeloblastic leukaemia (AML) are maintained by stem cells with these two properties. Since renewal and differentiation can be assessed separately in cultures of AML blasts, these cancer cells provide a useful model for examining stem regulation; such studies have practical importance for future developments in the treatment of AML. This paper considers three aspects of blast cell biology. First, evidence is presented that self-renewal and differentiation are regulated by specific genes; further, the DNA encoding these genes has structural features that affect the chemosensitivity of self-renewal. This sensitivity varies from patient-to-patient and is an important attribute contributing to variation in treatment efficacy. Second, the effects of myelopoietic growth factors on blast stem cells are presented and discussed, as these bear on the regulation of the balance between renewal and differentiation. Finally, models of leukaemic haemopoiesis are considered in light of the experimental findings. The suggestion is advanced that leukaemia can be explained better by abnormalities of gene expression than by blocked differentiation.

Meshorer, E. (2007). "Chromatin in embryonic stem cell neuronal differentiation." *Histol Histopathol* **22**(3): 311-9.

Chromatin, the basic regulatory unit of the eukaryotic genetic material, is controlled by epigenetic mechanisms including histone modifications, histone variants, DNA methylation and chromatin remodeling. Cellular differentiation involves large changes in gene expression concomitant with alterations in genome organization and chromatin structure. Such changes are particularly evident in self-renewing pluripotent embryonic stem cells, which begin, in terms of cell fate, as a tabula rasa, and through the process of differentiation, acquire distinct identities. Here I describe the changes in chromatin that accompany neuronal differentiation, particularly of embryonic stem cells, and discuss how chromatin serves as the master regulator of cellular destiny.

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-secreting cell. **CONCLUSION:** Differentiated CK19(+) cells reflect an endocrine precursor cell type of ductal origin, potentially suitable for insulin replacement therapy in diabetes.

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.

Ogawa, K., M. Tashima, et al. (1994). "Inhibition of erythroid differentiation by stem cell factor in K562 cells expressing the c-kit gene." Exp Hematol **22**(1): 45-51.

We found a K562 subclone (K562YO) that highly expressed the c-kit gene. K562YO had a higher capability of erythroid differentiation by hemin and cytosine arabinoside (Ara-C) than its parent K562 (KIT-). We obtained the transfectant expressing c-kit by introducing c-kit cDNA into K562 (KIT-). The differentiation of the transfectant was similar to that of the parent cell. Thus the difference described above was not due to the expression of c-kit. Next, we investigated the effects of stem cell factor (SCF) on the differentiation of the K562 cell expressing c-kit. SCF did not enhance the cell growth of K562YO. On the other hand, SCF suppressed induction of benzidine-positive cells when c-kit-positive cells were treated with hemin and Ara-C, especially at a low concentration. Furthermore, c-kit mRNA and protein were down-regulated during erythroid differentiation. SCF also downregulated the c-kit proteins. Our results suggest that the SCF/c-kit signals could act negatively for erythroid differentiation of the K562 cells expressing c-kit. K562YO is also useful for studying the mechanism that controls the expression of the c-kit gene because there is a K562 counterpart cell line that does not express this gene.

Pacary, E., H. Legros, et al. (2006). "Synergistic effects of CoCl₂ and ROCK inhibition on mesenchymal stem cell differentiation into neuron-like cells." J Cell Sci **119**(Pt 13): 2667-78.

Bone-marrow-derived mesenchymal stem cells (MSCs) constitute an interesting cellular source to promote brain regeneration after neurodegenerative diseases. Recently, several studies suggested that oxygen-dependent gene expression is of crucial importance in governing the essential steps of neurogenesis such as cell proliferation, survival and differentiation. In this context, we analysed the effect of the HIF-1 (hypoxia inducible factor-1) activation-mimicking agent CoCl₂ on MSCs. CoCl₂ treatment increased the expression of the anti-proliferative gene BTG2/PC3 and decreased cyclin D1 expression. Expression of HIF-1 α and its target genes EPO, VEGF and p21 was also upregulated. These changes were followed by inhibition of cell proliferation and morphological changes resulting in neuron-like cells, which had increased neuronal marker expression and responded to neurotransmitters. Echinomycin, a molecule inhibiting HIF-1 DNA-binding activity, blocked the CoCl₂ effect on MSCs. Additionally, by using Y-27632, we demonstrated that Rho kinase (ROCK) inhibition potentiated CoCl₂-induced MSC differentiation in particular into dopaminergic neuron-like cells as attested by its effect on tyrosine hydroxylase expression. Altogether, these results support the ability of MSCs to differentiate into neuron-like cells in response to CoCl₂, an effect that might act, in part, through HIF-1 activation and cell-cycle arrest, and which is potentiated by inhibition of ROCK.

Pasini, D., A. P. Bracken, et al. (2008). "Regulation of stem cell differentiation by histone methyltransferases and demethylases." Cold Spring Harb Symp Quant Biol **73**: 253-63.

The generation of different cell types from stem cells containing identical genetic information and their organization into tissues and organs during development is a highly complex process that requires defined transcriptional programs. Maintenance of such programs is epigenetically regulated and the factors involved in these processes are often essential for development. The activities required for cell-fate decisions are frequently deregulated in human tumors, and the elucidation of the molecular mechanisms that regulate these processes is therefore important for understanding both developmental processes and tumorigenesis.

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.

Pek, J. W., A. K. Lim, et al. (2009). "Drosophila maelstrom ensures proper germline stem cell lineage differentiation by repressing microRNA-7." *Dev Cell* 17(3): 417-24.

Nuage is a germline-unique perinuclear structure conserved throughout the animal kingdom. Maelstrom (Mael) is an unusual nuage component, as it is also found in the nucleus. Mael contains a High Mobility Group box, known to mediate DNA binding. We show that Mael nuclear function is required for proper differentiation in the Drosophila germline stem cell (GSC) lineage. In mael mutant testes, transit-amplifying cysts fail to differentiate into primary spermatocytes, instead breaking down into ectopic GSCs and smaller cysts, due to a depletion of Bag-of-marbles (Bam) protein. Mael regulates Bam via repression of miR-7. Mael binds the miR-7 promoter and is required for the local accumulation of HP1 and H3K9me3. miR-7 targets bam directly at its 3'UTR, and a reduction in miR-7 expression can rescue germline differentiation defects found in mael mutants by alleviating Bam repression. We propose that Mael ensures proper differentiation in the GSC lineage by repressing miR-7.

Pekkinen, M., M. E. Ahlstrom, et al. (2008). "Effects of phosphodiesterase 7 inhibition by RNA interference on the gene expression and differentiation of human mesenchymal stem cell-derived osteoblasts." *Bone* 43(1): 84-91.

The second messenger molecule cyclic adenosine monophosphate (cAMP) plays an important role in the hormonal regulation of bone metabolism. cAMP is inactivated by the cyclic nucleotide phosphodiesterases (PDEs), a superfamily of enzymes divided into 11 known families designated PDE 1-11. The aim of this study was to investigate the effect of PDE7 and PDE8 inhibition on the gene expression and differentiation of human osteoblasts. Osteoblasts differentiated from human mesenchymal stem cells (hMSC) were cultured and treated with short interfering RNAs (siRNAs) generated from PDE7 and PDE8 PCR products. Total RNA was isolated from the cells, and gene expression was assayed with cDNA microarray and quantitative real-time PCR. bALP measurements were assayed during differentiation, and mineralization was determined by quantitative Alizarin red S staining. PDE7 and PDE8 inhibition by RNA interference decreased the gene expression of PDE7A by 60-70%, PDE7B by 40-50%, and PDE8A by 30%. PDE7 silencing increased the expression of beta-catenin, osteocalcin, caspase-8, and cAMP-responsive element-binding protein 5 (CREB-5) genes and decreased the expression of the 1, 25-dihydroxyvitamin D3 receptor gene. PDE8A silencing increased the expression of anti-apoptotic genes, but decreased the expression of osteoglycin (osteoinductive factor) and bone morphogenetic protein 1 (BMP-1). PDE7 silencing increased bALP and mineralization up to three-fold compared to controls. Treatment with the PDE7-selective PDE inhibitor BRL-50481 had similar effects on mineralization as the gene silencing. The PDE7 silencing also increased forskolin stimulated cAMP response, but had no effect on the proliferation rate. Furthermore, osteocalcin expression was increased by PDE7 silencing by a mechanism dependent on protein kinase A. Our results show that specific gene silencing with the RNAi method is a useful tool for inhibiting the gene expression of specific PDEs and that PDE7 silencing upregulates several osteogenic genes and increases mineralization. PDE7 may play an important role in the regulation of osteoblastic differentiation.

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.

Poirier, F., C. T. Chan, et al. (1991). "The murine H19 gene is activated during embryonic stem cell differentiation in vitro and at the time of implantation in the developing embryo." *Development* **113**(4): 1105-14.

The differentiation in vitro of murine embryonic stem cells to embryoid bodies mimics events that occur in vivo shortly before and after embryonic implantation. We have used this system, together with differential cDNA cloning, to identify genes the expression of which is regulated during early embryogenesis. Here we describe the isolation of several such cDNA clones, one of which corresponds to the gene H19. This gene is activated in extraembryonic cell types at the time of implantation, suggesting that it may play a role at this stage of development, and is subsequently expressed in all of the cells of the mid-gestation embryo with the striking exception of most of those of the developing central and peripheral nervous systems. After birth, expression of this gene ceases or is dramatically reduced in all tissues.

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

Research towards potential curative transplantation of human embryonic stem (hES) cell-derived grafts in a variety of diseases has become an

important topic since the successful derivation and propagation of hES cells from the inner cell mass of a blastocyst. However, clinical applicability can only be established after intensive laboratory studies that should elaborate on two major topics: A) the development of efficient, controlled and stable hES cell differentiation protocols for any specific cell type, and B) the induction of immunological tolerance against transplanted allogeneic hES cell-derived cell types. This review will briefly discuss: A) current possibilities in hES cell differentiation, followed by the development of viral, DNA and mRNA-based gene transfer strategies for hES cells, and B) possible immune modulation strategies for inducing immune tolerance against allogeneic hES cell transplants.

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 (angiogenin, alpha_v 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.

Qihao, Z., C. Xigu, et al. (2007). "Spheroid formation and differentiation into hepatocyte-like cells of rat mesenchymal stem cell induced by co-culture with liver cells." *DNA Cell Biol* **26**(7): 497-503.

Mesenchymal stem cells (MSCs) derived from bone marrow have been shown to differentiate into hepatocytes, which would be an ideal resource for transplantation or artificial liver devices. Here we investigated the efficiency of co-culture system consisting of rat MSCs and adult liver cells to induce differentiation of MSCs into hepatocyte-like cells. Marked MSCs were either co-cultured with freshly isolated liver cells or treated with hepatocyte growth factor (HGF) for 21 days. In co-culture systems, MSCs formed spheroids of round-shaped cells while keeping normal proliferation and viability, strongly expressed albumin, alpha-fetoprotein, and cytokeratin-18 in mRNA and protein level from day 3 to 21. As a control, MSCs treated with HGF showed weak gene expressions in day 14 and had a few cells of protein staining in day 21. These results indicate that the co-culture microenvironment plays a decisive role for the hepatic differentiation of MSCs, and it is more efficient than HGF treatment. Insights gained from this study will be helpful to design optimal culture systems for the hepatic differentiation of human MSCs and the hepatic function maintenance of hepatocytes in vitro.

Rao, M. S., M. Yukawa, et al. (1996). "Expression of transcription factors and stem cell factor precedes hepatocyte differentiation in rat pancreas." *Gene Expr* **6**(1): 15-22.

Multiple foci of morphologically and functionally differentiated hepatocytes are induced in the pancreas of adult rats subjected to a copper depletion-repletion regimen. Differentiation of hepatocytes in pancreas is preceded by irreversible depletion of over 90% of pancreatic acinar cells. Progressive acinar cell loss during 4-6 weeks of copper deficiency results in the proliferation of oval cells, some of which may serve as the hepatocyte precursor or stem cells. Albumin mRNA is detected in oval cells at 5 and 6 weeks by in situ hybridization at which time no morphologically identifiable hepatocytes are evident in the pancreas. Immunocytochemical analysis demonstrated the presence of stem cell factor (SCF) in proliferating oval cells during 6 weeks of copper depletion, and Northern blot analysis revealed the expression of liver-enriched transcription factors in the rat pancreas during this 4-6-week period of copper deficiency. CCAAT/enhancer binding protein alpha (C/EBP alpha) mRNA was detected first at 4 weeks of copper deficiency. By 5 and 6 weeks of copper deficiency, the expression of mRNAs of C/EBP alpha, beta, and

delta, and hepatocyte nuclear factor-3 factor (HNF-3 beta) was markedly enhanced. This enhanced expression of liver-enriched transcription factors and the SCF during oval cell proliferation in the pancreas preceding the expression of albumin mRNA and subsequent differentiation of hepatocyte phenotype further supports the identity of these oval cells as hepatocyte precursors or stem cells.

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.

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.

Rogler, C. E., L. Levoci, et al. (2009). "MicroRNA-23b cluster microRNAs regulate transforming growth factor-beta/bone morphogenetic protein signaling and liver stem cell differentiation by targeting Smads." *Hepatology* **50**(2): 575-84.

Transforming growth factor-beta / bone morphogenetic protein (TGFbeta/BMP) signaling has a gradient of effects on cell fate choice in the fetal mouse liver. The molecular mechanism to understand why adjacent cells develop into bile ducts or grow actively as hepatocytes in the ubiquitous presence of both TGFbeta ligands and receptors has been unknown. We hypothesized that microRNAs (miRNAs) might play a role in cell fate decisions in the liver. miRNA profiling during late fetal development in the mouse identified miR-23b cluster miRNAs comprising miR-23b, miR-27b, and miR-24-1 and miR-10a, miR-26a, and miR-30a as up-regulated. *In situ* hybridization of fetal liver at embryonic day 17.5 of gestation revealed miR-23b cluster expression only in fetal hepatocytes. A complementary (c)DNA microarray approach was used to identify genes with a reciprocal expression pattern to that of miR-23b cluster miRNAs. This approach identified Smads (mothers against decapentaplegic homolog), the key TGFbeta signaling molecules, as putative miR-23b cluster targets. Bioinformatic analysis identified multiple candidate target sites in the 3' UTRs (untranslated regions) of Smads 3, 4, and 5. Dual luciferase reporter assays confirmed down-regulation of constructs containing Smad 3, 4, or 5, 3' UTRs by a mixture of miR-23b cluster mimics. Knockdown of miR-23b miRNAs during hepatocytic differentiation of a fetal liver stem cell line, HBC-3, promoted expression of bile duct genes, in addition to Smads, in these cells. In contrast, ectopic expression of miR-23b mimics during bile duct differentiation of HBC-3 cells blocked the process. **CONCLUSION:** Our data provide a model in which miR-23b miRNAs repress bile duct gene expression in fetal hepatocytes while promoting their growth by down-regulating Smads and consequently TGFbeta signaling. Concomitantly, low levels of the

miR-23b miRNAs are needed in cholangiocytes to allow TGFbeta signaling and bile duct formation.

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 cholinergic receptors 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.

Sakai, D., J. Mochida, et al. (2005). "Differentiation of mesenchymal stem cells transplanted to a rabbit degenerative disc model: potential and limitations for stem cell therapy in disc regeneration." *Spine (Phila Pa 1976)* **30**(21): 2379-87.

STUDY DESIGN: An in vivo study to assess the differentiation status of mesenchymal stem cells (MSCs) transplanted to the nucleus pulposus of degenerative discs in a rabbit model. **OBJECTIVES:** To evaluate the fate of MSCs transplanted to the nucleus pulposus of degenerative discs in a rabbit and to determine whether they are a suitable alternative for cell transplantation therapy for disc degeneration. **SUMMARY OF BACKGROUND DATA:** Although MSCs have been proposed as candidate donor cells for transplantation to treat intervertebral disc degeneration, their differentiation after transplantation has not been adequately investigated. **METHODS:** Autologous MSCs, labeled with green fluorescent protein, were transplanted into mature rabbits. Consecutive counts of transplanted MSCs in the nucleus area were performed for 48 weeks after transplantation. Differentiation of transplanted cells was determined by immunohistochemical analysis. The proteoglycan content of discs was measured quantitatively using a dimethylmethylene blue assay, and mRNA expression of Type I and II collagen, aggrecan and versican was measured semi-quantitatively using reverse transcription polymerase chain reaction. **RESULTS:** Many cells that were positive for green fluorescent protein were observed in the nucleus pulposus of cell-transplanted rabbit discs 2 weeks after transplantation. Their number increased significantly by 48 weeks. Some GFP-positive cells were positive for cell-associated matrix molecules, such as Type II collagen, keratan sulfate, chondroitin sulfate, aggrecan, and the nucleus pulposus phenotypic markers, hypoxia inducible factor 1 alpha, glutamine transporter 1, and matrix metalloproteinase 2. MSCs did not show significant expression of these molecules before transplantation. Biochemical and gene expression analyses showed significant restoration of total proteoglycan content and matrix-related genes compared with nontransplanted discs. **CONCLUSIONS:** MSCs transplanted to degenerative discs in rabbits proliferated and differentiated into cells expressing some of the major phenotypic characteristics of nucleus pulposus cells, suggesting that these MSCs may have undergone site-dependent differentiation. Further studies are needed to evaluate their functional role.

Sandberg, M. L., S. E. Sutton, et al. (2005). "c-Myb and p300 regulate hematopoietic stem cell

proliferation and differentiation." *Dev Cell* **8**(2): 153-66.

Precise control of hematopoietic stem cell (HSC) proliferation and differentiation is needed to maintain a lifetime supply of blood cells. Using genome-wide ENU mutagenesis and phenotypic screening, we have identified a mouse line that harbors a point mutation in the transactivation (TA) domain of the transcription factor c-Myb (M303V), which reduces c-Myb-dependent TA by disrupting its interaction with the transcriptional coactivator p300. The biological consequences of the c-Myb(M303V/M303V) mutation include thrombocytosis, megakaryocytosis, anemia, lymphopenia, and the absence of eosinophils. Detailed analysis of hematopoiesis in c-Myb(M303V/M303V) mice reveals distinct blocks in T cell, B cell, and red blood cell development, as well as a remarkable 10-fold increase in the number of HSCs. Cell cycle analyses show that twice as many HSCs from c-Myb(M303V/M303V) animals are actively cycling. Thus c-Myb, through interaction with p300, controls the proliferation and differentiation of hematopoietic stem and progenitor cells.

Schugar, R. C., P. D. Robbins, et al. (2008). "Small molecules in stem cell self-renewal and differentiation." *Gene Ther* **15**(2): 126-35.

The use of stem cells in regenerative medicine is a promising approach to the treatment of disease and injury. Natural and synthetic small molecules have been shown to be useful chemical tools for controlling and manipulating the fates of cells. Small molecules can target signaling transduction pathways (for example, tyrosine kinase receptors) and affect DNA replication, cell differentiation, tumor metastasis and apoptosis. Stem cells share many properties with cancer cells and these similarities can provide insights to control and direct cell behavior; small molecules are already standard chemotherapeutics in the treatment of cancer. Libraries of small molecules have been examined for anticancer behavior (especially apoptosis), and, more recently, for stem cell self-renewal and differentiation capabilities in potential approaches to regenerative medicine. Differentiation therapy for cancer is based on the idea that cancer cells are undifferentiated embryonic-like cells and proposes to promote the differentiation and hence block cell proliferation. For example, retinoids have a role in stem cell differentiation to several lineages and have also been used to promote differentiation of acute promyelocytic leukemic cells. Small molecules are also important tools for understanding mechanistic and developmental processes. Strategies for generating functional small molecule libraries have been outlined

previously. In this review, we will look at several small molecules that have been described in the recent literature as effectors of stem cell self-renewal or differentiation as associated with the Wnt, Hedgehog or NF-kappaB pathways.

Schuringa, J. J., K. Y. Chung, et al. (2004). "Constitutive activation of STAT5A promotes human hematopoietic stem cell self-renewal and erythroid differentiation." *J Exp Med* **200**(5): 623-35.

Activation of the transcription factor signal transducer and activator of transcription (STAT)5 is involved in various aspects of hematopoiesis, affecting cell proliferation, differentiation, and cell survival. Constitutive activation of STAT5 has also been associated with leukemic transformation. We overexpressed the constitutively active mutant STAT5A(1*6) in human cord blood CD34+ cells and evaluated the effects on the hematopoietic potential of stem cells in a variety of in vitro and in vivo systems. The observed phenotypic changes were correlated with differential gene expression patterns induced by STAT5A(1*6). Our data indicate that a persistent activation of STAT5A in human hematopoietic stem and progenitor cells results in their enhanced self-renewal and diverts differentiation to the erythroid lineage.

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.

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.

Shimamoto, T., K. Ohyashiki, et al. (1995). "The expression pattern of erythrocyte/megakaryocyte-related transcription factors GATA-1 and the stem cell leukemia gene correlates with hematopoietic differentiation and is associated with outcome of acute myeloid leukemia." *Blood* **86**(8): 3173-80.

To understand the clinical implications of transcription factors and their biologic roles during cellular differentiation in the hematopoietic system, we examined the expression of GATA-1, GATA-2, and stem cell leukemia (SCL) gene in human leukemia cell lines and various leukemia patients using the reverse transcriptase-polymerase chain reaction. Cell lines exhibiting megakaryocytic or erythrocytic phenotypes had GATA-1, GATA-2, and SCL gene transcripts, while monocytic cell lines had no detectable GATA-1, GATA-2, or SCL gene mRNA. In some myeloid cell lines, GATA-1 expression, but not SCL gene expression, was detected; GATA-1 expression in HL-60 cells was downregulated during the process of monocytic differentiation. We next examined GATA-1, GATA-2, and SCL gene expression in 110 leukemia samples obtained from 76 patients with acute myeloid leukemia (AML), 19 with acute lymphoblastic leukemia (ALL), and 15 with chronic myeloid leukemia in blast crisis (CML-BC). SCL gene expression was usually accompanied by GATA-1 expression and was preferentially detected in patients with leukemia exhibiting megakaryocytic or erythrocytic phenotypes, while patients with monocytic leukemia were clustered in the group with no detectable GATA-1 expression. None of the patients with ALL or CML-lymphoid-BC expressed SCL. De novo AML patients with SCL gene expression had a lower complete remission (CR) rate and had a significantly poorer prognosis. Among the patients with AML not expressing SCL, a high

percentage of patients with CD7+ AML and CD19+ AML had detectable GATA-1, while patients with GATA-1-negative AML had the best CR rate (87.5%). Our results suggest that the expression pattern of transcription factors reflects the lineage potential of leukemia cells, and GATA-1 and SCL gene expression may have prognostic value for the outcome of patients with AML.

Shimizu, E., Y. Takahashi, et al. (1996). "Stem-cell factor regulates the expression of cyclin A and retinoblastoma gene product in the growth and differentiation pathway of human megakaryocytic cells." *J Cancer Res Clin Oncol* **122**(8): 445-52.

The biological effects of c-kit ligand (stem-cell factor: SCF) on an immortalized human megakaryocytic cell line (CMK) was evaluated using methods including the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, surface marker analysis, DNA cell-cycle analysis and immunoblotting. SCF stimulated the growth of CMK cells. Incubation with SCF resulted in increased expression of IIb/IIIa platelet-related glycoprotein (gpIIb, IIIa), indicating enhanced differentiation of CMK cells. Treatment of CMK cells with SCF resulted in a decrease in the subpopulation in the G1 phase, with a reciprocal increase in those in the S phase and the G2 + M phase. Moreover, SCF significantly increased cellular expression of cyclin A, a regulatory subunit of cyclin-dependent protein kinase (CDK), and the ratio of phosphorylated/dephosphorylated retinoblastoma gene product (RB protein). These results suggest that SCF stimulates the growth and differentiation of megakaryocytic cells possibly through mechanisms related to the activation of cell-cycle-dependent serine/threonine kinase and inactivation of the nuclear tumor-suppressor gene product.

Snykers, S., T. Henkens, et al. (2009). "Role of epigenetics in liver-specific gene transcription, hepatocyte differentiation and stem cell reprogramming." *J Hepatol* **51**(1): 187-211.

Controlling both growth and differentiation of stem cells and their differentiated somatic progeny is a challenge in numerous fields, from preclinical drug development to clinical therapy. Recently, new insights into the underlying molecular mechanisms have unveiled key regulatory roles of epigenetic marks driving cellular pluripotency, differentiation and self-renewal/proliferation. Indeed, the transcription of genes, governing cell-fate decisions during development and maintenance of a cell's differentiated status in adult life, critically depends on the chromatin accessibility of transcription factors to genomic regulatory and coding regions. In this

review, we discuss the epigenetic control of (liver-specific) gene-transcription and the intricate interplay between chromatin modulation, including histone (de)acetylation and DNA (de)methylation, and liver-enriched transcription factors. Special attention is paid to their role in directing hepatic differentiation of primary hepatocytes and stem cells in vitro.

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-rTA 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.

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.

Taga, T. and S. Fukuda (2005). "Role of IL-6 in the neural stem cell differentiation." *Clin Rev Allergy Immunol* **28**(3): 249-56.

In the developing brain neurons, astrocytes, and oligodendrocytes are differentiated from common multipotent progenitors called neural stem cells. We have examined the effect of cytokines on mouse neuroepithelial cells that are known to contain neural stem cells. Cytokines belonging to the interleukin (IL)-6 family and those classified into the bone morphogenetic protein (BMP) family act in synergy on neuroepithelial cells to induce astrocyte differentiation. Cooperation of these two types of cytokines is explained by the formulation of a complex between their respective downstream transcription factors (signal transducer and activator of transcription [STAT]-3 and Smad1), bridged by a transcriptional coactivator (p300). Whereas BMPs family cytokines are involved in astrocyte differentiation, they inhibit neuronal differentiation. This appears to change the fate of neural progenitor cells from neurogenesis to astrocytogenesis. Interestingly, during brain development, neuronal differentiation starts at a very early stage and continues throughout development, whereas astrocytes appear just before term. We have shown that astrocyte differentiation is largely dependent on IL-6 family cytokine-mediated STAT3 activation and that there exists a STAT3 binding element in the promoter region of the gene for the astrocyte marker glial fibrillary acidic protein. A cytosine residue in

this element is highly methylated in neuroepithelial cells in a mid-gestational stage but becomes demethylated in accordance with brain development. Because this methylation inhibits STAT3 binding, we suggest that DNA methylation is a critical determinant in the developmental stage-dependent regulation of astrocytogenesis. In conclusion, fate of neural stem cells during development is regulated by cell-intrinsic programs, such as epigenetic modification (including DNA methylation), and signaling crosstalk of cell-external mediators (including IL-6 family cytokines).

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.

Testa, U., G. F. Torelli, et al. (2002). "Human acute stem cell leukemia with multilineage differentiation potential via cascade activation of growth factor receptors." *Blood* **99**(12): 4634-7.

The morphologic, immunophenotypic, genotypic, genomic, and functional features of an undifferentiated acute leukemia with stem cell features are reported. At light and electron

microscopy, the leukemic population was represented by primitive progenitor cells with no evidence of differentiation. The blasts were CD34(+), AC133(+), CD71(-), HLA-DR(-), CD38(-/dim+), CD90(+), CD117(dim+), flt3(+); did not express B, T, or myeloid-associated antigens; and showed a germline configuration of the immunoglobulin and T-cell receptor. Genomic profiling documented the expression of early stem cell and myeloid-associated genes. Receptors for early-acting hemopoietic growth factors (HGFs) were detected, while receptors for unilineage HGF were not expressed. Incubation with the flt3 or Kit ligand induced the expression of unilineage HGF receptors, allowing these cells to respond to their respective ligands. Growth without differentiation was sustained only in the presence of early-acting HGF, namely flt3 ligand, while early and unilineage HGF gave rise to all types of hemopoietic colonies.

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.

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.

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, Z. and H. Lin (2007). "Sex-lethal is a target of Bruno-mediated translational repression in promoting the differentiation of stem cell progeny during Drosophila oogenesis." *Dev Biol* **302**(1): 160-8.

The RNA Recognition Motif (RRM) type RNA binding protein Bruno is required for the differentiation of cystoblasts, the committed daughters of germline stem cells in the Drosophila ovary. To understand how Bruno controls cystoblast differentiation, we used a bioinformatics approach to identify potential mRNA targets of Bruno. One such target is the Sex-lethal (Sxl) transcript MS11, which contains four Bruno Response Elements (BREs) in its 3' untranslated region. Electrophoresis mobility shift

assays demonstrated that Bruno specifically binds to the BREs of Sxl MS11 mRNA. Tagged transgenic Sxl MS11 cDNA constructs were used to show that Bruno represses the translation of the Sxl MS11 mRNA via the BRE-containing region in the 3'UTR. The lack of either Bruno or the BRE-containing region leads to overexpression of SXL, which in turn causes defects in cystoblast differentiation similar to the Bruno mutant phenotype. Therefore, Sxl MS11 represents a novel target of Bruno-mediated translational repression required for cystoblast differentiation.

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 F1p 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.

White, J., E. Stead, et al. (2005). "Developmental activation of the Rb-E2F pathway and establishment of cell cycle-regulated cyclin-dependent kinase activity during embryonic stem cell differentiation." *Mol Biol Cell* **16**(4): 2018-27.

To understand cell cycle control mechanisms in early development and how they change during differentiation, we used embryonic stem cells to model embryonic events. Our results demonstrate that as pluripotent cells differentiate, the length of G(1) phase increases substantially. At the molecular level, this is associated with a significant change in the size of active cyclin-dependent kinase (Cdk) complexes,

the establishment of cell cycle-regulated Cdk2 activity and the activation of a functional Rb-E2F pathway. The switch from constitutive to cell cycle-dependent Cdk2 activity coincides with temporal changes in cyclin A2 and E1 protein levels during the cell cycle. Transcriptional mechanisms underpin the down-regulation of cyclin levels and the establishment of their periodicity during differentiation. As pluripotent cells differentiate and pRb/p107 kinase activities become cell cycle dependent, the E2F-pRb pathway is activated and imposes cell cycle-regulated transcriptional control on E2F target genes, such as cyclin E1. These results suggest the existence of a feedback loop where Cdk2 controls its own activity through regulation of cyclin E1 transcription. Changes in rates of cell division, cell cycle structure and the establishment of cell cycle-regulated Cdk2 activity can therefore be explained by activation of the E2F-pRb pathway.

White, R. J., D. Stott, et al. (1989). "Regulation of RNA polymerase III transcription in response to F9 embryonal carcinoma stem cell differentiation." *Cell* **59**(6): 1081-92.

B2 genes are rodent-specific middle repetitive elements transcribed by RNA polymerase III. They are expressed in the ectoderm and mesoderm but not in the embryonic or extraembryonic endoderm of early mouse embryos. This tissue specificity is mimicked in vitro by embryonal carcinoma and embryonic stem cell lines. Nuclear run-on experiments show that the down-regulation of B2 genes during F9 embryonal carcinoma cell differentiation into endoderm occurs at the transcriptional level and that other class III genes, including those encoding tRNA, show a similar response. We have used cell-free extracts to investigate the molecular mechanisms responsible. The specific down-regulation of transcription by RNA polymerase III during F9 cell differentiation is due to a reduction in the activity of the general class III transcription factor TFIIB.

Wu, J. X. and E. D. Adamson (1996). "Kinase-negative mutant epidermal growth factor receptor (EGFR) expression during embryonal stem cell differentiation favours EGFR-independent lineages." *Development* **122**(10): 3331-42.

EGF receptors are expressed on most fetal and adult cells but their precise roles are not well known. We previously reported that, in P19 embryonal carcinoma cells, the expression of kinase-negative EGFR inhibits retinoic acid (RA)-induced differentiation to nervous tissue, suggesting that EGFR plays a role in differentiation (J.-X. Wu and E. D. Adamson (1993) *Dev. Biol.* 159, 208-222).

Embryo stem (ES) cells differentiate into a wide range of tissue types after the removal of the cytokine LIF from the culture medium. We demonstrate here that the induction of some early markers of differentiation, tissue-type plasminogen activator (tPA), AFP and keratins 8 and 19 is inhibited, whilst brachyury and myosin are increased, in clones containing kinase-negative mutant EGFR. After an extended period of differentiation, the cell types present in mutant and control cultures differed. Mutant clones produced frequent cardiac and skeletal muscle as the predominant differentiated cell types *in vitro*; other cell types were sparse or absent. Teratocarcinomas formed by EGFR-deltakinase-expressing ES cells contained frequent skeletal and cardiac muscle as well as apoptotic nuclei, while normal ES cells produced no detectable muscle and less apoptoses. Since mutant differentiated cultures had slower growth rates and increased levels of cell death, we concluded that: (1) inactive EGFR does not allow some cell types to survive and/or proliferate; (2) tissues that do not require EGFR for their survival, development or function predominate in long-term mutant cultures; (3) EGFR activity is not necessary for cardiac and skeletal muscle or endoderm formation and (4) Impaired survival of EGF-dependent lineages leads to preferential selection of muscle in differentiating ES cells.

Xu, J., X. Liu, et al. (2008). "MAPK/ERK signalling mediates VEGF-induced bone marrow stem cell differentiation into endothelial cell." *J Cell Mol Med* 12(6A): 2395-406.

Multi-potent adult progenitor cells (MAPCs) differentiate into endothelial cells (ECs) in the presence of vascular endothelial growth factor (VEGF). The mechanism(s) of VEGF-induced differentiation of MAPCs to ECs are not yet known. We, therefore, examined the role of mitogen-activated protein kinase/extracellular signal-regulated kinase (p42/44-MAPK/ERK1/2) signalling in endothelial differentiation from bone marrow stem cells. We observed that VEGF stimulation of MAPCs for 14 days results in a significant expression of endothelial-specific gene and/or proteins including von Willebrand factor (vWF), vascular endothelial-cadherin (VE-cadherin), VEGF receptor-2 (VEGFR2), and CD31. Up-regulation of EC-specific markers was accompanied by a cobblestone morphology, expression of endothelial nitric oxide synthase (eNOS), and Dil-Ac-LDL uptake, typical for EC morphology and function. VEGF induced a sustained activation of p42 MAPK/ERK, but not that of p44 MAPK/ERK during the course of MAPCs differentiation in a time-dependent manner up to 14 days. VEGF-induced activation of p42 MAPK/ERK

also led to the nuclear translocation of MAPK/ERK1/2. Incubation of MAPCs with MAPK/ERK1/2 phosphorylation inhibitor PD98059 blocked the sustained VEGF-induced MAPK/ERK1/2 phosphorylation as well as its nuclear translocation in the differentiating MAPCs. Inhibition of MAPK/ERK1/2 phosphorylation by PD98059 also blocked the expression of EC-specific genes in these cells and their differentiation to ECs. These data suggest that VEGF induces MAPC differentiation into EC via a MAPK/ERK1/2 signalling pathway-mediated mechanism *in vitro*.

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.

Yan, J., L. Xu, et al. (2007). "Extensive neuronal differentiation of human neural stem cell grafts in adult rat spinal cord." *PLoS Med* 4(2): e39.

BACKGROUND: Effective treatments for degenerative and traumatic diseases of the nervous system are not currently available. The support or replacement of injured neurons with neural grafts, already an established approach in experimental therapeutics, has been recently invigorated with the addition of neural and embryonic stem-derived precursors as inexhaustible, self-propagating alternatives to fetal tissues. The adult spinal cord, *i.e.*, the site of common devastating injuries and motor neuron disease, has been an especially challenging target for stem cell therapies. In most cases, neural stem cell (NSC) transplants have shown either poor differentiation or a preferential choice of glial lineages. **METHODS AND FINDINGS:** In the present investigation, we grafted NSCs from human fetal spinal cord grown in monolayer into the lumbar cord of normal or injured adult nude rats and observed large-scale differentiation of these cells into neurons that formed axons and synapses and established extensive contacts with host motor neurons. Spinal cord microenvironment appeared to influence fate choice, with centrally located cells taking on a predominant neuronal path, and cells located under the pia membrane persisting as NSCs or presenting with astrocytic phenotypes. Slightly fewer than one-tenth of grafted neurons differentiated into oligodendrocytes. The presence of lesions increased the frequency of astrocytic phenotypes in the white matter. **CONCLUSIONS:** NSC grafts can show substantial neuronal differentiation in the normal and injured adult spinal cord with good potential of integration into host neural circuits. In view of recent similar findings from other laboratories, the extent of neuronal differentiation observed here disputes the notion of a spinal cord that is constitutively unfavorable to neuronal repair. Restoration of spinal cord circuitry in traumatic and degenerative diseases may be more realistic than previously thought, although major challenges remain, especially with respect to the establishment of neuromuscular connections.

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.

Yang, Z., Y. Sui, et al. (2007). "Switched alternative splicing of oncogene CoAA during embryonal carcinoma stem cell differentiation." *Nucleic Acids Res* 35(6): 1919-32.

Alternative splicing produces functionally distinct proteins participating in cellular processes including differentiation and development. CoAA is a coactivator that regulates transcription-coupled splicing and its own pre-mRNA transcript is alternatively spliced. We show here that the CoAA gene is embryonically expressed and alternatively spliced in multiple tissues to three splice variants, CoAA, CoAM and CoAR. During retinoic-acid-induced P19 stem cell differentiation, the expression of CoAA undergoes a rapid switch to its dominant negative splice variant CoAM in the cavity of the embryoid body. CoAM functionally inhibits CoAA,

and their switched expression up-regulates differentiation marker Sox6. Using a CoAA minigene cassette, we find that the switched alternative splicing of CoAA and CoAM is regulated by the cis-regulating sequence upstream of the CoAA basal promoter. Consistent to this, we show that p54(nrb) and PSF induce CoAM splice variant through the cis-regulating sequence. We have previously shown that the CoAA gene is amplified in human cancers with a recurrent loss of this cis-regulating sequence. These results together suggest that the upstream regulatory sequence contributes to alternative splicing of the CoAA gene during stem cell differentiation, and its selective loss in human cancers potentially deregulates CoAA alternative splicing and alters stem cell differentiation.

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.

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.

Yoshida, T., I. Hazan, et al. (2008). "The role of the chromatin remodeler Mi-2beta in hematopoietic stem cell self-renewal and multilineage differentiation." *Genes Dev* **22**(9): 1174-89.

The ability of somatic stem cells to self-renew and differentiate into downstream lineages is dependent on specialized chromatin environments that keep stem cell-specific genes active and key differentiation factors repressed but poised for activation. The epigenetic factors that provide this type of regulation remain ill-defined. Here we provide the first evidence that the SNF2-like ATPase Mi-2beta of the Nucleosome Remodeling Deacetylase (NuRD) complex is required for maintenance of and multilineage differentiation in the early hematopoietic hierarchy. Shortly after conditional inactivation of Mi-2beta, there is an increase in cycling and a decrease in quiescence in an HSC (hematopoietic stem cell)-enriched bone marrow population. These cycling mutant cells readily differentiate into the erythroid lineage but not into the myeloid and lymphoid lineages. Together, these effects result in an initial expansion of mutant HSC and erythroid progenitors that are later depleted as more differentiated proerythroblasts accumulate at hematopoietic sites exhibiting features of erythroid leukemia. Examination of gene expression in the mutant HSC reveals changes in the expression of genes associated with self-renewal and lineage priming and a pivotal role of Mi-2beta in their regulation. Thus, Mi-2beta provides the hematopoietic system with immune cell capabilities as well as with an extensive regenerative capacity.

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