

Gene Regulation Networks Related to Neural Differentiation of hESC

JIANG F. ZHONG,*†‡ YAHUI SONG,† JING DU,* CHRISTINE GAMACHE,*
KATHLEEN A. BURKE,†‡ BRETT T. LUND,* AND LESLIE P. WEINER*

*Department of Neurology, Keck School of Medicine, University of Southern California,
Los Angeles, CA 90033, USA

†Gene Therapy Laboratories, Keck School of Medicine, University of Southern California,
Los Angeles, CA 90033, USA

‡Department of Biochemistry and Molecular Biology, Keck School of Medicine,
University of Southern California, Los Angeles, CA 90033, USA

With the unique property of self-renewal and developmental pluripotency, human embryonic stem cells (hESC) provide an opportunity to study molecular aspects of developmental biology. Understanding gene regulation of hESC pluripotency is a critical step toward directing hESC differentiation for regenerative medicine. However, currently little is known about hESC gene regulation of hESC pluripotency. Applying network analysis to microarray gene expression profiling data, we compared gene expression profiles from pluripotent hESC to hESC-derived astrocytes and identified potential gene regulation networks. These gene regulation networks suggest that hESC has stringent control of cell cycle and apoptosis. Our data reveal several potential hESC differentiation biomarkers and suggest that *IGF2* and *A2M* could play a role in hESC pluripotency by altering the availability of cytokines at the local environment of hESC. These findings underscore the importance of network analysis among differentially expressed genes, and should facilitate future study for understanding the gene regulation of hESC pluripotency.

Key words: Pluripotent stem cells; Regulation of gene expression; Neuroglia; Cell differentiation; Microarray analysis

INTRODUCTION

Human embryonic stem cells (hESC) derived from inner cell mass of blastocysts can be maintained in vitro without losing their pluripotency and be differentiated into somatic cells (1,41,52). In addition to the promising clinical value for regenerative medicine, hESCs provide a unique in vitro system for studying molecular aspects of human developmental biology, including regulation of stem cell pluripotency. However, despite intensive efforts, the search of hESC pluripotency regulatory genes is far from being complete. One approach is to compare the gene expression profiles from mouse, rat, and human ESC with the assumption that the common orthologous

genes are the most relevant for pluripotency (21,60). Recent studies indicate that mouse embryonic stem cells (mESC) are very different from hESCs. Murine leukemia-inhibitory factor (LIF) plays a critical role in maintaining mESC undifferentiated growth in vitro, but has no such effect on hESC (10,14). In addition, bone morphogenetic protein 4 (BMP4) blocks neuroectoderm differentiation of mESC, but promotes the trophectoderm differentiation of hESC (66,68). These studies underscore the difficulty of using mESC as a model to study hESC, and the importance of identifying hESC specific pluripotency regulatory genes.

Microarray gene expression profiling has been rapidly transformed from a novel approach into a com-

Address correspondence to Jiang F. Zhong, Department of Neurology, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, USA. Tel: (323) 442-2371; Fax: (323) 442-2311; E-mail: jzhong@usc.edu

mon tool in study of gene regulation. However, interpreting the data generated from microarray gene expression profiling remains a challenging task. Differentially expressed genes from microarray studies can be organized into networks based on various relationships such as expression patterns, common promoter sequences, physical location on chromosomes, and the direct binding of gene products. We have utilized some of these gene relationships to analyze microarray gene expression data in a previous study of murine hematopoietic stem cells (72).

In this study we compare the gene expression profile of hESC (H1, WiCell Research Institute, Inc., Madison, WI) to that from astrocytes derived from this hESC line. By comparing cells between the pluripotent stage and the neuronal differentiated stage within the same hESC lines, genetic variation among hESC lines is eliminated from the analysis. A large number of relatively pure cells from each population was used to extract RNA for subsequent microarray gene expression profiling. Literature mining was performed to identify gene networks among differentially expressed genes. Instead of focusing on individual differentially expressed genes, we organized differentially expressed genes into networks based on the direct binding relationships of their encoded proteins. This approach can identify genes that play a critical role in pluripotency regulation, but are not in the differential expression gene list. We found that the expressions of multiple members of the p53 pathway are lower in hESC-derived astrocytes than in hESC. In contrast, the p53 mRNA level shows no significant difference between pluripotent hESC and the astrocytes differentiated from these hESC. p53, a tumor suppressor, has been shown to regulate mESC differentiation (34), but the role of p53 in regulation of pluripotency of human ESC remains unclear. Our data suggest that a posttranscriptional mechanism of p53 may be employed in the regulation of hESC differentiation. We also identified *IGF2* and alpha 2-macroglobulin (*A2M*) as potentially important factors for hESC pluripotency by this approach. Finally, we show that *A2M* induces cell death in pluripotent hESC without affecting differentiated hESC. These findings underscore the importance of network analysis among differentially expressed genes, and should facilitate future study for understanding the gene regulation of hESC pluripotency.

MATERIALS AND METHODS

hESC Culture and Differentiation

The hESC line (H1) was obtained from the WiCell Research Institute (Madison, WI) and maintained as

instructed (52). Undifferentiated hESC were cultured on an irradiated (5500 rads) layer of mouse embryonic fibroblast (MEF) feeder cells in six-well plates or Matrigel-coated plates with MEF conditioned medium. MEF were prepared from the embryos of 13–14-day pregnant CF-1 mice (Charles River Labs) and stocks were cryopreserved until required for culture of hESC. hESC were consistently observed as large clumps that appear on top of the MEF layer, consistent with the published observations of others. Under these conditions, the hESC remained continuously undifferentiated and periodic karyotyping has confirmed their human diploid chromosomal character. We have tested the hESC cultured in our laboratory for their potential to form all three embryonic germ layers. These cells produced teratomas after injection into severe combined immunodeficient (SCID) mice; the pathology included tissues of ectodermal, mesodermal, and endodermal origin. Immunostaining of the hESC for alkaline phosphatase and with antibodies specific for SSEA-4, TRA-1-60, TRA-1-81, or Oct-3/4 confirmed the stem cell phenotype of these cells. Prior to induction into astrocytes, hESC were transferred from MEF feeder layers onto six-well plates coated with Matrigel in the same culture medium. The medium was changed every other day. For differentiation, feeder-free hESCs were cultured in differentiation medium comprised of: DMEM/F12, N2 supplement, 0.1 mM nonessential amino acids, 1 mg/ml heparin, 10 ng/ml bFGF, 20 ng/ml EGF. The medium was changed every other day and cells were passaged as required by confluence. After 4 weeks in the differentiation medium, the majority of cells (>90%) differentiated into GFAP+ cells.

Immunofluorescent Staining

The hESC cells grown on cover slips were fixed for 20 min at room temperature in 4% paraformaldehyde and then washed three times in PBS. Cells were consequently permeabilized for 20 min in PBS containing 0.1% Triton X at room temperature. Cells were then washed three times with PBS and blocked for 1 h at room temperature in PBS containing 10% goat serum. Subsequently, cells were incubated with a primary antibody (Oct-3/4, 1:100; Santa Cruz) in PBS containing 1% goat serum at 37°C for 1 h. As a negative control, cells were also treated separately with a nonspecific anti-mouse IgG antibody (1:100; Chemicon, USA). After being washed three times in freshly made PBS containing 0.5% Tween 20, cells were then incubated with the secondary antibody (phycoerythrin-conjugated anti-mouse IgG, 1:200; Santa Cruz) in PBS containing 1% goat serum at 37°C for 45 min. The unbound secondary antibodies

were removed by washing three times in freshly made PBS containing 0.5% Tween 20. The cells were then washed briefly in PBS before mounting. The hESC-derived astrocytes were fixed with ethanol and labeled with Cy3-conjugated mouse anti-glial fibrillary acidic protein (GFAP) monoclonal antibody (Sigma, USA).

Microarray Gene Expression Profiling

Total RNA was extracted with Trizol (Invitrogen, USA) from pluripotent hESC and astrocytes differentiated from hESC. RNA quality was examined and hybridized to Applied Biosystems (ABI) 1700 chemiluminescent human microarray chips at ABI microarray facility (Foster City, CA). Triplicate samples were used from each group. A *t*-test was performed using Matlab (Natick, MA). Differentially expressed genes were selected with the criteria: 1) passed all ABI 1700 data control filters; and 2) at least a threefold difference with $p < 0.01$ between hESC and astrocytes. The fold changes for individual genes are calculated by dividing the average of normalized expression units from hESC and astrocytes. Literature mining and pathway analysis were performed with Pathwaystudio (Rockville, USA) and GenMAPP (Gladstone Institutes, UCSF, USA). To confirm microarray data, quantitative real-time RT-PCR was performed with Taqman primer sets purchased from ABI (Foster City, CA) on LightCycler (Roche, USA). The fold changes were calculated with the $\Delta\Delta C_t$ method.

RESULTS

hESC Culture and Differentiation

To identify genes that are related to neural differentiation, hESCs (H1 line) were differentiated into GFA-positive astrocytes using neural differentiation medium. As shown in Figure 1, before differentiation, hESC grew as colonies on Matrigel and only the undifferentiated pluripotent hESC in the center of the colony expressed Oct-3/4, a marker for pluripotent hESC. The spontaneous differentiated hESC cells at the edge of the colony did not express Oct3/4. After neural cell differentiation treatment, cells no longer grow as colonies. The dispersed neural differentiated cells expressed the astrocytic marker antigen, GFAP, and exhibited a flat morphology, which is typical for cultured astrocytes (Fig. 1B). Total RNAs were extracted from undifferentiated pluripotent hESC and differentiated astrocytes for microarray gene expression profiling. The gene expression profiles between hESC and hESC-derived astrocytes were compared.

Expression of Pluripotency-Related Genes

The expression of approximately 28,000 human genes (33,183 transcripts) was interrogated with Applied Biosystem's expression array system (Fig. 2). After statistical analysis, we identified 684 differentially expressed genes with at least a threefold difference ($p < 0.01$) between pluripotent hESC and hESC-derived astrocytes. As expected, many known stem cell genes such as *Oct3-4*, *Nodal*, and *Rex1*(45) were expressed higher in the hESC than in the astrocytes. The expression of *Oct-3/4*, *Nodal*, and *Rex1* in hESC was 66-fold, 15-fold, and 35-fold of that in astrocytes, respectively. Quantitative RT-PCR confirmed the perturbation of these representative genes. *BMP4* has been shown to sustain pluripotency of mESC (68), but induce differentiation of hESC (20,66). *BMP4* expression is threefold higher in hESC-derived astrocytes than that in hESC. The expression levels of *bFGF* (14-fold higher in hESC) and *BMP4* agree with a published report that *bFGF* represses *BMP4* in order to maintain hESC pluripotency (67). The high expression of *Oct-3/4* and *Nodal* in hESC agrees with the immunoassay data presented above (Fig. 1), and confirms reports from other investigators (53). Compared to undifferentiated hESC, there was 43-fold more *A2M* mRNA expressed in astrocytes than in pluripotent hESC. Quantitative RT-PCR with $\Delta\Delta C_t$ estimation confirmed the 43-fold upregulation of *A2M* in astrocytes (Table 1).

Gene Expression Networks Among Differentially Expressed Genes

Although differentially expressed genes may reveal valuable information, it is important to view them from a landscape or network point of view, rather than as individual entities. Cellular events and characteristics including pluripotency are rarely regulated by a single gene, but rather by networks of genes interacting with each other. While some genes are subjected to regulation at the protein or translational level, such regulations would cause mRNA level perturbation at downstream genes within the gene network. Even though gene expression profiling measures the mRNA level and cannot reveal gene regulation information at the translational regulation level (protein synthesis or miRNA regulation), valuable information can be inferred from differential expression genes with network analysis. In contrast to some gene expression profiling studies that only analyze differentially expressed genes at the individual gene level, in this study a data mining process was carried out on peer-reviewed literature with computational software. The network analysis revealed several networks among the differentially expressed

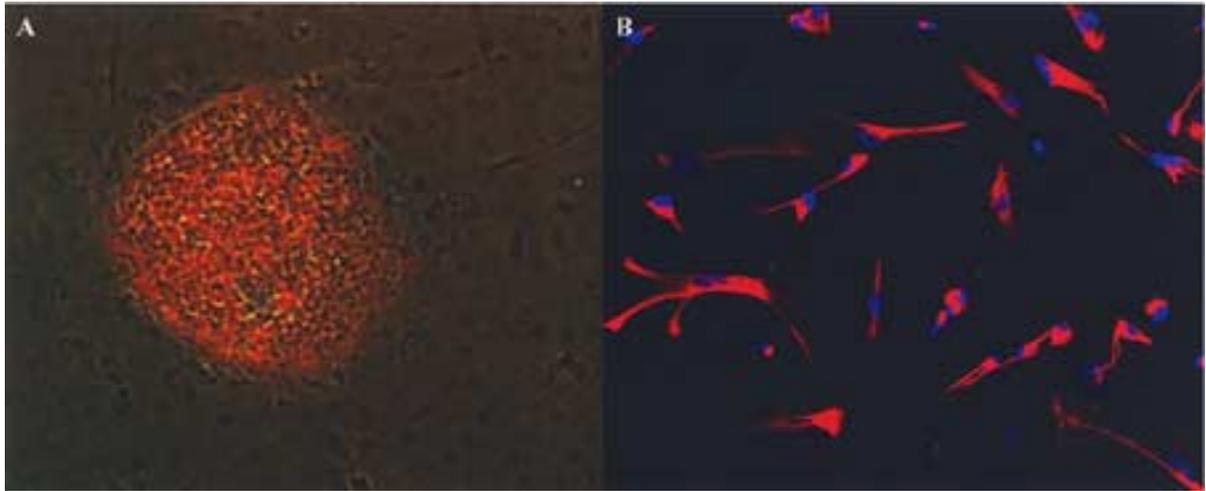


Figure 1. Pluripotent and neuronal differentiated hESC. (A) Merged image of an immunofluorescent-stained (Oct-3/4) and light microscope images from a pluripotent hESC colony. The hESC colony was labeled with mouse α human Oct-3/4 IgG and PE-conjugated rabbit anti-mouse IgG antibodies. Only cells in the center of the hESC colony expressed Oct-3/4. The spontaneous differentiated cells around the colony did not express Oct-3/4. (B) hESC-derived astrocytes were labeled with Cy3-conjugated mouse α human GFAP antibody as well as nuclei staining, DAPI (blue). Cells expressed GFAP (an astrocyte marker) and displayed astrocytes morphology.

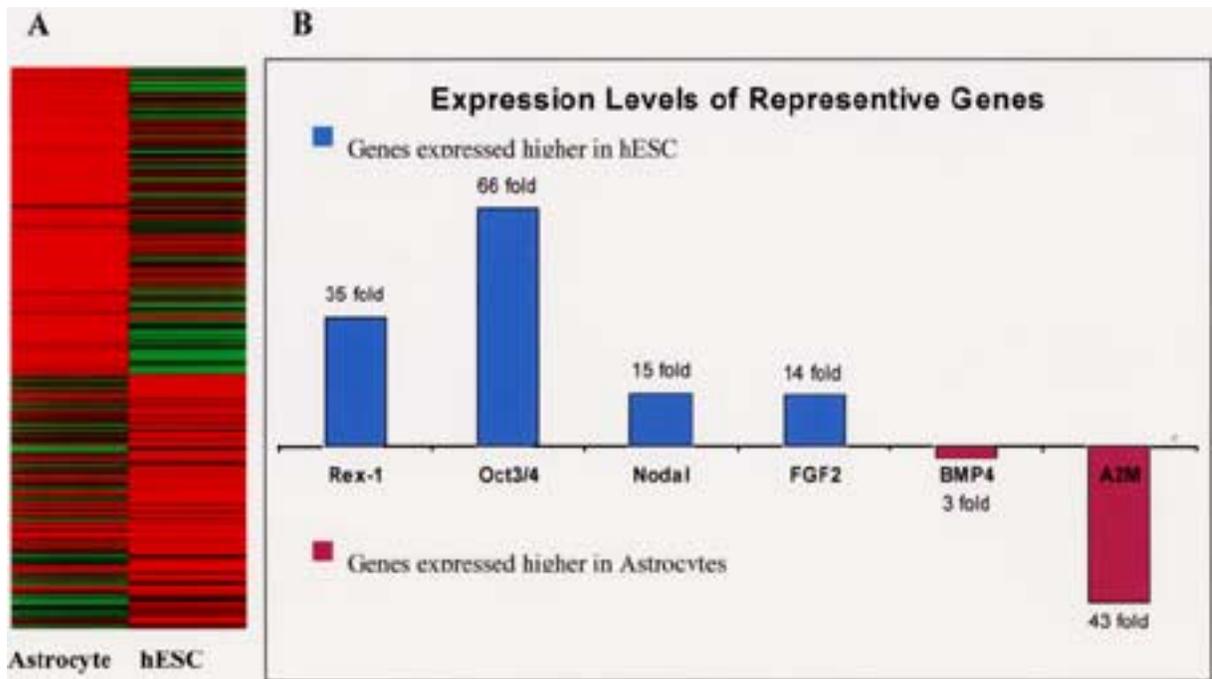


Figure 2. Differential gene expression between pluripotent hESC and hESC-derived astrocytes. (A) There are 684 differentially expressed genes between hESC and astrocytes derived from hESC. The gene expression levels are presented as an expression heatmap. Red: high; yellow: medium; green: low. (B) Expression levels of representative genes are shown with fold difference, which is calculated as described in Materials and Methods.

TABLE 1
QUANTITATIVE RT-PCR CONFIRMS THE UPREGULATION
OF A2M IN hESC-DERIVED ASTROCYTES

	ΔCt (hESC-Astrocyte)	$\Delta\Delta\text{Ct}$	Upregulation in Astrocytes
B2M (reference gene)	-2.8 ± 0.05	—	—
A2M	2.6 ± 0.03	5.4	42-fold

The fold change (estimated with the $\Delta\Delta\text{Ct}$ method) is very close to the microarray measurement.

genes based on direct-binding relationships of their gene products, the encoded proteins.

The regulatory relationships (e.g., induction, repression/degradation) of genes in published papers are not always consistent. On the other hand, the experimental facts of direct binding (e.g., immunoprecipitation, yeast two-hybrids assays) are less arguable. Therefore, we based our network analysis on reported direct-binding relationships of proteins. Among the 684 differentially expressed genes, 95 proteins formed 22 networks. Three representative networks with potential significance in astrocyte differentiation (based on high fold changes and significance of p -values) are presented in Figure 3.

While p53 is not among the differentially expressed genes (p53 is 2.2-fold lower at GFAP-positive cells with $p = 0.037$, and excluded from the differentially expressed gene list by our selection criteria), many members of the p53 pathway are downregulated in astrocytes (Fig. 3A). The downregulated p53-related genes are: *CDKN1A* (13-fold), *CDKN2A* (3-fold), *CDKN2B* (3.6-fold), *MDM2* (6-fold), and *BAX* (4-fold). *CDKN1A*, *CDKN2A*, and *CDKN2B* are three cyclin-dependent kinase inhibitors that regulate G_1/S progression. The promoter of *CDKN1A* (also called p21 or WAF1) has a p53 binding site and its expression is inducible by p53. Overexpression of p21 in tumor cell lines suppresses colony formation similar to that resulting from p53 overexpression, and this strongly suggests that p21 is a mediator of p53-dependent growth suppression (17). *CDKN2A* (also called p16) arrests normal diploid cells in late G_1 (35). An alternative beta transcript of *CDKN2A* encodes p14 (ARF), which binds to MDM2 and promotes the rapid degradation of MDM2 (71). MDM2 (downregulated sixfold in astrocytes) is a major regulator of the tumor suppressor p53 by targeting its destruction (25,37). *CDKN2B* (also called p15) is adjacent to *CDKN2A* (28), and a potential effector of TGF- β -induced cell cycle arrest in human keratinocytes (24). In addition to lower expression of cyclin-dependent kinase inhibitors, the *BAX* and its regulator *ETV4* (also called Pea3) (19) also expressed 4-fold

and 7.6-fold lower in hESC-derived astrocytes, respectively. *BAX* is a proapoptotic protein parent of *BCL2*, and p53 directly activated *BAX* in the absence of other proteins to permeabilize mitochondria and engage the apoptotic program (11). While cyclin-dependent kinase inhibitors and apoptotic genes are downregulated, the S-phase kinase-associated protein 2 (*SKP 2*, also called p45) is upregulated 3.3-fold in hESC-derived astrocytes. Microinjection of specific antibodies to *SKP 2* specifically blocked the cell cycle in G_1 , and suggest that *SKP 2* is essential for S-phase entry (69). Together, these data indicate a more constricted regulation of cell cycle and apoptosis in hESC compared to hESC-derived astrocytes. That agrees with our observation of the slow growth and apoptotic susceptibility of hESC in vitro.

As expected, the p21 (*CDKN1A*)-activated kinase 1 (*PAK1*) and the gene of its binding protein *TC10L* (55) are downregulated with *CDKN1A* in hESC-derived astrocytes. VEGF-related genes are upregulated in astrocytes compared to hESC. These genes include: *VEGFR1* (11-fold), *VEGFR2* (7.6-fold), *ETK* (6-fold), *HOXB5* (5.9-fold), and *HOXB6* (6-fold). *ETK* directly associated with *PAK1* and *VEGFR2*. Wild-type *ETK* in noninvasive human breast cancer MCF-7 cells increased proliferation and anchorage-independent growth (3,70), and *VEGFR2* (Flk-1) is the earliest marker of cells fated to endothelial and hematopoietic lineages (38). Studies in the murine model showed that *HOXB5* is a potent transcriptional regulator of *VEGFR2* (65). The relative low expression of *HOXB5*, *HOXB6*, *VEGFR1*, and *VEGFR2* in hESC compared to hESC-derived astrocytes suggests they may be involved in the initiation of hESC differentiation.

Another interesting network centers on a growth factor: insulin-like growth factors II (*IGF2*) (Fig. 3B). *IGF2* and its major carrying protein *IGFBP3* (18) are upregulated 183-fold and 7-fold, respectively in hESC-derived astrocytes. The latent TGF- β binding protein (*LTBP-1*), which targets latent complex of TGF- β to the extracellular matrix where the latent complex is activated, is also upregulated 3.8-fold. *LTBP-1* also binds *IGFBP3* and is a potential connector of *IGFBP3* to the TGF family proteins (23). Three extracellular matrix protein genes, Fibrilin 2 (*FBN2*), matrilin 2 (*MATN2*), and vitronectin (*VTN*), are also upregulated in hESC-derived astrocytes. It has been reported that *IGF2* binds to *VTN* with an affinity similar to that for the IGF receptor, and the *IGF2/VTN* complex enhances migration of breast cancer cells (40). This network suggests that *IGF2/IGFBP3/LTBP1* complex may target *IGF2* to the extracellular *FBN2/MATN2/VTN* complex in hESC-derived astrocytes. Alpha-fetoprotein (AFP), a major plasma pro-

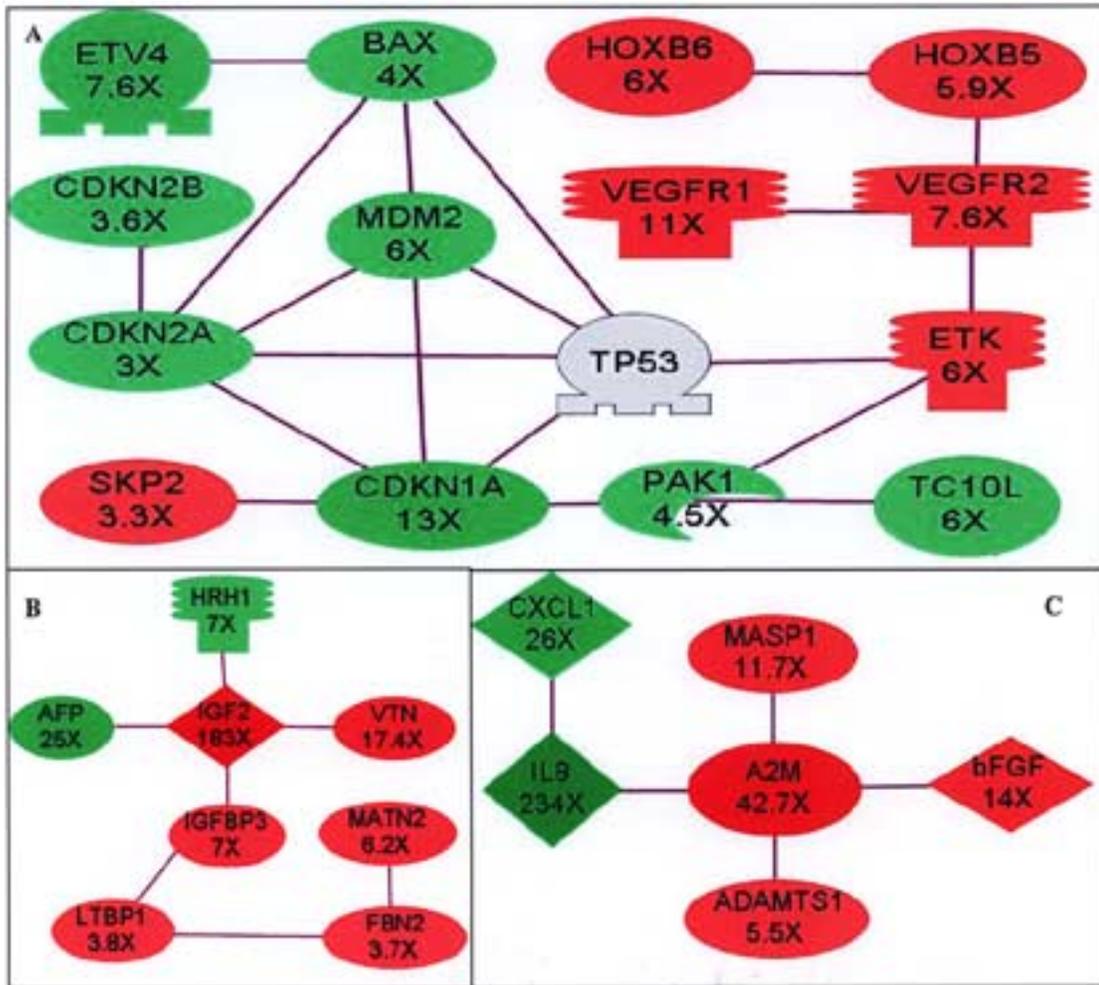


Figure 3. Gene expression networks among differentially expressed genes. Networks are defined by the relationship of protein–protein direct binding among differentially expressed genes. Lines connecting genes represent the protein–protein binding relationships from published peer-reviewed articles. Red: genes are upregulated in hESC-derived astrocytes; green: genes are downregulated in hESC-derived astrocytes. The fold changes between hESC and hESC-derived astrocytes are indicated under each corresponding gene name. (A) p53-related network. The TP53 is not differentially expressed, and is included to show its relationship with other genes in the network. (B) IGF2-related network. (C) A2M-related network. A2M: alpha-2-macroglobulin; ADAMTS1: a disintegrin-like and metalloprotease with thrombospondin type 1 motif 1; AFP: alpha-fetoprotein; BAX: Bcl2-associated X protein; CDKN1A: cyclin-dependent kinase inhibitor 1A (p21); CDKN2A: cyclin-dependent kinase inhibitor 2A (p16); CDKN2B: cyclin-dependent kinase inhibitor 2B (p15); ETK: ephrin receptor A3; ETV4: ets variant gene 4 (E1AF); FBN2: fibrillin 2; HOXB5: homeo box B5; HOXB6: homeo box B6; HRH1: histamine receptor H1; IGF2: insulin-like growth factor 2; IGF1BP3: insulin-like growth factor binding protein 3; IL8: interleukin 8 (CXCL8); LTBP1: latent transforming growth factor beta binding protein 1; MASP1: mannan-binding lectin serine protease 1; MATN2: matrilin 2; MDM2: mouse double minute 2 homolog; PAK1: p21 (CDKN1A)-activated kinase 1; SKP2: S-phase kinase-associated protein 2 (p45); TC10L: TC10-like protein (ras homolog gene family Y, member J); VEGFR1: vascular endothelial growth factor receptor 1(FLT1); VEGFR2: vascular endothelial growth factor receptor 2(KDR); VTN: vitronectin.

tein produced by the yolk sac and the liver during fetal life, is downregulated in astrocytes. AFP is the fetal counterpart of adult serum albumin, and its lower expression in hESC-derived astrocytes is interesting but not unexpected. Histamine receptor H1 (*HRH1*), which can mediate the internalization and degradation of IGF2 (22), is downregulated also. The downregulation of *HRH1* and the upregulation of *IGF2* as well as IGF2 extra cellular binding proteins in hESC-derived astrocytes compared to hESC sug-

gest an important role of IGF2 in the hESC differentiation. We are currently investigating the function of IGF2 protein in hESC pluripotency.

The most interesting network centers on A2M (Fig. 3C). The expression of *ADAMTS1* and *MASP1* is upregulated 5.5- and 11.7-fold, respectively, with A2M (upregulated 42.7-fold) in hESC-derived astrocytes. *ADAMTS1* is a secreted protein that has an N-terminal signal peptide, a zinc metalloprotease domain containing a zinc-binding site, and a cysteine-rich re-

gion containing two putative disintegrin loops (54). It is associated with the inflammation response (29). MASP1 is an extracellular serine protease that activates complement components C3 and C2 (51). Because A2M binds to both ADAMTS1 (an active metalloprotease that is associated with the extracellular matrix) (30) and MASP1 (50), it is possible that A2M (a protease inhibitor and cytokine binding protein) is expressed to suppress these two proteases. However, A2M is upregulated to a much higher magnitude than the two proteases. This suggests that it may also play a role in cytokine binding. Interestingly, the *IL8* and *CXCL1* are downregulated in hESC-derived astrocytes 234-fold and 36-fold, respectively. Structurally, CXCL1 is related to a superfamily of inflammatory proteins that includes IL8 and platelet factor-4 (PF4) (26). Both IL8 and CXCL1 bind to the class II IL-8 receptor (CXCR2) with high affinity during inflammation (33). IL8 and CXCR2 are responsible for the resistance of Fas-induced cell death in astrocytes (46). The upregulation of A2M (which binds IL8) as well as the downregulation of IL8 and CXCL1 suggests that Fas-induced cell death may be a part of normal astrocyte maturation from stem cells.

Effects of A2M on hESC Culture

In the A2M network, A2M is upregulated approximately 43-fold, while IL8 is downregulated 234-fold. A2M has been recognized as a binding protein for various growth factors, particularly those of the TGF- β family (7,12,32). The expression of *bFGF*, a member of the TGF- β family and key player of hESC pluripotency, is significantly downregulated in hESC-derived astrocytes (Fig. 2), while A2M expression is significantly upregulated in hESC-derived astrocytes. To investigate the role of A2M in hESC pluripotency, we added human A2M (final concentration of 77 $\mu\text{g}/\text{ml}$) into undifferentiated hESC culture. This concentration of A2M is significantly lower than the A2M concentration in human plasma, which is 2–5 μM (1440–3590 $\mu\text{g}/\text{ml}$) (36). Preliminary experiments on 293T cells also showed no effects of adding A2M at this concentration (data not shown). Interestingly, A2M induces hESC colony detachments and cell death on Oct-3/4-positive pluripotent hESC, but has no such effects on Oct-3/4-negative hESC cells (Fig. 4).

DISCUSSION

Human embryonic stem cells have the ability to self-renew and differentiate into all tissue types in the human body. Various animal cloning experiments (4,43,47,56) and stem cell fusion experiments (13,48)

indicate that somatic cells can be reprogrammed to pluripotent stem cells. Therefore, identifying the genes that control hESC pluripotency and genes that initiate specific lineage differentiation is critically important for clinical and research stem cell applications. Once the pluripotency-controlling genes and their interaction networks are known, somatic cells could be reprogrammed to pluripotent stem cells by gene manipulation techniques such as siRNA, micro-RNA, and gene transfer. Reprogramming somatic cells to pluripotent stem cells with gene manipulation rather than with nuclear transfer technique may overcome many difficulties in hESC study such as the ethical issues of using human embryos, and low efficiency of nuclear transfer.

Various attempts have been made to understand stem cell gene regulation. A recent proteomic study conducted by Wang et al. explored the direct binding relationship of proteins in murine stem cells (57). This study demonstrated the network nature of stem cell pluripotency regulation. Comparisons of mouse and human ESC indicate that they are very different (10,14,66,68). It is now known that gene regulation is orchestrated by multiple genes at multiple levels, such as protein modification, micro-RNA interaction, mRNA alternative splicing, and stability. One of the major concerns of microarray gene expression experiment is mRNA expression levels do not always correlate with protein levels. The conventional microarray data analysis approaches such as fold changes, statistical analysis, and gene annotation often overlook valuable information. Rather than focusing on individual differentially expressed genes, in this study we analyzed differentially expressed genes from a network point of view to obtain information beyond conventional approaches. By performing network analysis with differentially expressed genes between hESC and hESC-derived astrocytes, we identified three networks among differentially expressed genes based on the binding relationships of their encoded proteins. These networks support previous published studies of hESC and reveal new information of hESC pluripotency.

In one network, p53-related cyclin-dependent kinase inhibitors and proapoptotic genes were expressed at a lower level in hESC-derived astrocytes compared to that in hESC. Interestingly, the expression level of p53 itself did not change significantly in our study and was not on the differentially expressed gene list. It has been reported that p53 expression is higher in mESC than that in hESC (21). Study of mESC showed that p53 induces differentiation of mESC and leads to apoptosis by suppressing *Nanog* expression (34). But the role of p53 in hESC differentiation is not clear. Our data show that many members of the

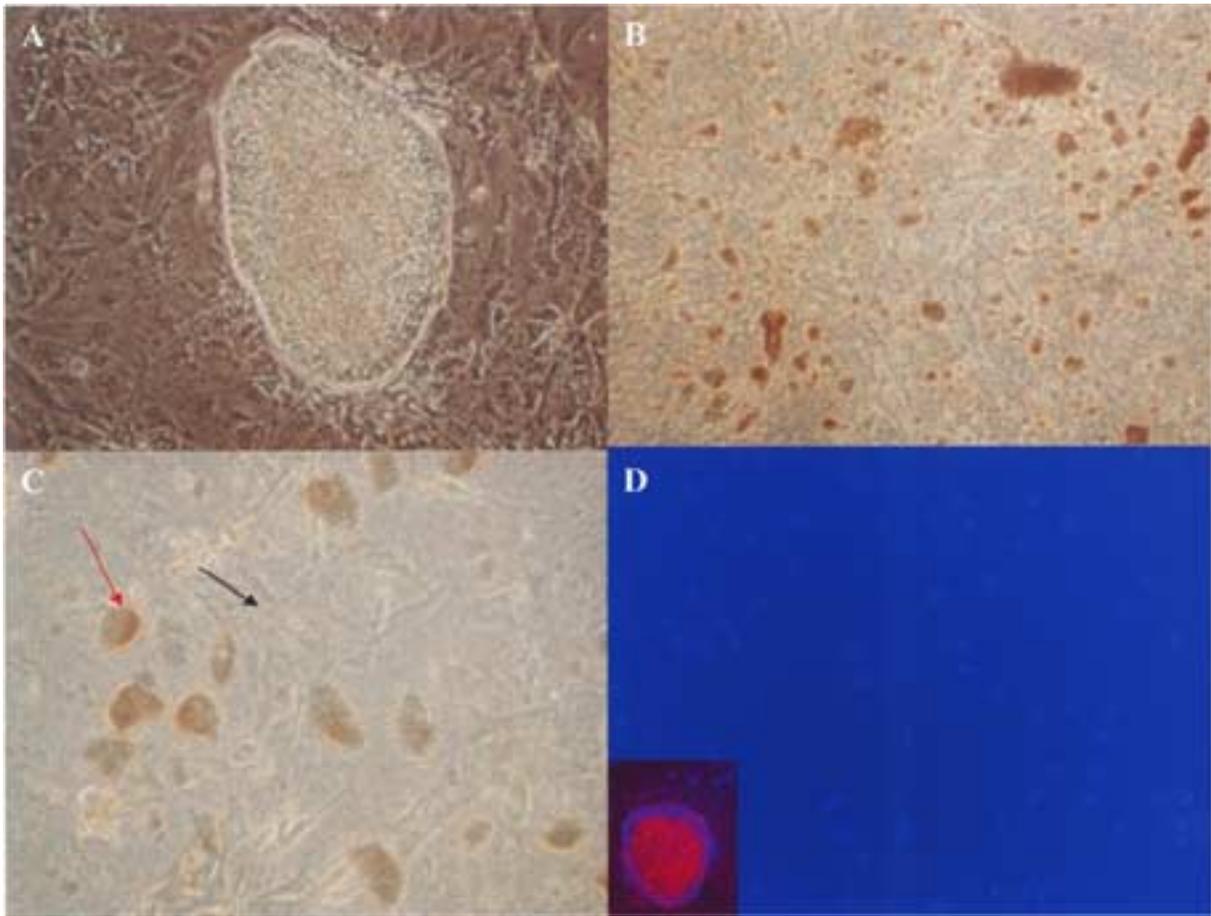


Figure 4. A2M induces detachment and cell death in pluripotent hESC. (A) A2M was added to hESC growing on a Matrigel-coated six-well plate at a final concentration of 77 $\mu\text{g/ml}$. hESC cell detachment and cell death were observed 72 h after adding A2M. In wells without adding A2M, a hESC colony is surrounded by differentiated hESC. (B) In wells with A2M treatment, hESC colonies disappeared and small clumps of dead cells were observed. (C) Differentiated cells still attached to Matrigel have healthy morphology (black arrow), while detached and fragmented hESC clumps appeared (red arrow). (D) Immunostaining after A2M treatment showed that Oct-3/4-positive cells disappeared, and the remaining cells are Oct-3/4 negative. The areas occupied by hESC colonies have no remaining cells. Immunostaining of wells without A2M treatment showed pluripotency marker Oct-3/4 expressed by cells inside the hESC colony, but not by the differentiated cells around it (inset). Red: Oct-3/4; blue: DAPI.

p53 pathway are expressed significantly lower in hESC-derived astrocytes compared to pluripotent hESC. This supports the idea that p53, a guardian of the genome, prevents cells entering the cell cycle, and could play a role in hESC pluripotency, but with a mechanism different from that in mESC. The downregulation of both *MDM2* and genes of the p53 pathway at first glance is a paradox. However, Schreiber's group recently demonstrated that p53 accumulated at apoptotic neurons despite the presence of high levels of MDM2 and p53–MDM2 complex (49). They further showed that ubiquitin regulates p53 stability at neurons. Together with the data from Schreiber's group, the downregulation of *MDM2* in our data suggests the co-regulation of p53 and *MDM2* at neural lineages. It is generally believed that the principle mechanisms governing p53 activity occur at the pro-

tein level, such as posttranslational modifications as well as regulation of the stability and subcellular location of p53 (63). Our data suggest that in hESC, the p53 pathway needs to be downregulated to allow differentiated cells to enter cell cycle; but rather than downregulating p53 mRNA level directly, this is to be accomplished by downregulating expression of other members of the p53 pathway.

In addition to the p53 pathway, we also found that IGF2 may play a role in neural differentiation of hESC toward astrocytes. The IGF2 and its related proteins are expressed significantly higher in astrocytes. Increasing expression of IGF2 at both mRNA and protein levels in tumor tissues has been reported (8,58). The most interesting network is the A2M network. A2M is a 720-kDa homotetrameric serum glycoprotein, and a major plasma and inflammatory

fluid proteinase scavenger. Besides binding proteinase, A2M binds to cytokines and growth factors such as CXCL8 (IL8) (31), human tumor necrosis factor- α (TNF- α) (61), platelet-derived growth factor (PDGF) (5,6,44), transforming growth factor (15,59), bFGF (16), and activin (39,42). Because bFGF irreversibly binds to A2M and the resulting complex has diminished ability to recognize either the high- or low-affinity bFGF binding site in cells (2,16,36,64), A2M appears to act as a limiter of bFGF. Activin can bind to both the native and active form of A2M with a higher affinity for the active form (42). Because Activin and bFGF are needed to maintain the pluripotency of hESC (53), A2M could affect hESC pluripotency by removing critical cytokines and growth factors that maintain hESC pluripotency, such as bFGF and activin.

Our gene expression data indicate that A2M is upregulated 43-fold in astrocytes differentiated from hESC and our in vitro study confirms that adding A2M to an hESC culture results in cell death of pluripotent hESC while not having such effects on differentiated hESC. These results agree with our observation that hESC differentiation is difficult to be reversed after differentiation is initiated. A2M may be responsible for removing critical growth factors such as bFGF and activin from the medium once differentiation is initiated. It is worth pointing out that A2M is an extracellular secretory protein; therefore, a high A2M concentration could be built up around the cells producing it. Also, not all cytokines that bind to A2M will lose their function. Some cytokines are protected by A2M from degradation (6,27). Therefore, A2M could create an environment for particular cells to grow while eliminate other cell types such as the pluripotent hESC. This may be the reason why daily medium change is necessary for maintaining pluripotent hESC cultures. As an acute-phase protein in brain, A2M is involved in a variety of neuropathological conditions (9). Examination of postmortem

multiple sclerosis (MS) tissues has shown that a large number of mature oligodendrocytes survived after the loss of myelin sheaths throughout the center of the recent MS lesions, but differentiation of oligodendrocyte precursor cells (OPC) is difficult to demonstrate (62). Our in vitro data showed that undifferentiated hESC are susceptible to A2M-induced cell death. If this mechanism occurs in OPC differentiation, A2M could be a potential factor to limit the replenishment of the oligodendrocyte precursor cells from stem cells.

In this study, with an approach of network analysis on differentially expressed genes, we identify three potentially significant gene regulation networks related to hESC pluripotency and neural differentiation. These gene regulation networks should provide useful information for better understanding of the initiation of hESC differentiation. However, it is important to know the limitation of such network analysis. Current knowledge of protein binding relationships is far from complete. Therefore, the networks we identified are not complete and need to be studied further. It is expected that these gene regulation networks will expand with increasing proteomic knowledge of genes. Although protein-protein binding is currently the most reliable evidence for gene regulation networks, the binding of two proteins does not equal to regulatory relationship. Network analysis is a powerful tool to identify potential gene regulation networks, but these networks must be confirmed with functional experiments such as gene manipulation. We are in the process to further investigate these identified networks with functional study.

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