Dynamic Changes in Histone H3 Phosphoacetylation during Early Embryonic Stem Cell Differentiation Are Directly Mediated by Mitogen- and Stress-activated Protein Kinase 1 via Activation of MAPK Pathways

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Embryonic stem (ES) cells are pluripotent cells capable of unlimited self-renewal and differentiation into the three embryonic germ layers under appropriate conditions. Mechanisms for control of the early period of differentiation, involving exit from the pluripotent state and lineage commitment, are not well understood. An emerging concept is that epigenetic histone modifications may play a role during this early period. We have found that upon differentiation of mouse ES cells by removal of the cytokine leukemia inhibitory factor, there is a global increase in coupled histone H3 phosphorylation (Ser-10)-acetylation (Lys-14) (H3 phosphoacetylation). We show that this occurs through activation of both the extracellular signal-regulated kinase (ERK) and p38 MAPK signaling pathways. Early ES cell differentiation is delayed using pharmacological inhibitors of the ERK and p38 pathways. One common point of convergence of these pathways is the activation of the mitogen- and stress-activated protein kinase 1 (MSK1). We show here that MSK1 is the critical mediator of differentiation-induced H3 phosphoacetylation using both the chemical inhibitor H89 and RNA interference. Interestingly, inhibition of H3 phosphoacetylation also alters gene expression during early differentiation. These results point to an important role for both epigenetic histone modifications and kinase pathways in modulating early ES differentiation.

Embryonic stem (ES) cells are pluripotent cells with the capacity for unlimited self-renewal or differentiation into the three germ layers: endoderm, ectoderm, and mesoderm. This ability to form different cell types under appropriate conditions makes them a powerful tool in the study of biological mechanisms and treatment of disease. Mouse ES cells are derived from the inner cell mass of the developing blastocyst (1). In vivo, the inner cell mass becomes organized into a pluripotent epithelial layer, the epiblast, from which embryonic tissues are derived (2). In vitro, mouse ES cells can be maintained in an undifferentiated state with the addition of the cytokine leukemia inhibitory factor (LIF) to culture media. LIF primarily acts via the JAK-STAT signaling pathway to maintain pluripotency (3). Self-renewal also is enhanced by inhibition of mitogen-activated protein kinase (MAPK) pathways (4). Withdrawal of LIF results in spontaneous differentiation of the ES cells into all three lineages, which is marked by changes in gene expression and cell morphology (see Fig. 1A).

Although much focus has been placed on factors involved in self-renewal, the mechanisms regulating exit from the pluripotent state followed by commitment to specific lineages remain largely unknown. An emerging concept is that alterations in epigenetic histone modifications may be important during this timeframe (5). The nucleosome, with DNA wrapped around an octamer of core histones (H2A, H2B, H3, and H4), forms the basic building blocks of chromatin. Histone tails are subject to numerous covalent modifications, including acetylation, phosphorylation, methylation, and ubiquitination (6). The various modifications of specific residues of the histone tail have led to the hypothesis of a histone code that functions to regulate the accessibility of chromatin to the transcriptional machinery. The different modifications may either permit or repress transcription by serving as recruitment marks for transcription complexes.

Epigenetic modifications usually associated with transcriptional competence include acetylation at lysines 9 and 14 on histone H3. These acetylated residues can occur in conjunction with phosphorylation of serine 10 on histone H3, producing H3 phosphoacetylation (7). Histone H3 is found to be rapidly and transiently phosphorylated upon stimulation with various growth factors or protein synthesis inhibitors. This effect has been termed the nucleosomal response (8). This response is frequently limited to localized nucleosomes and does not necessarily spread widely over an active gene, although only limited loci have been examined to date. A common thread among the
diverse stimulators of the nucleosomal response is the activation of the extracellular signal-regulated kinase (ERK) 1/2 or p38 branches of the MAPK pathway (9). A point of convergence between ERK1/2 and p38 is the activation of mitogen- and stress-activated protein kinase 1 and 2 (MSK1/2), which have been found to be the primary histone kinases for the nucleosomal response (10). This contrasts with genome-wide H3 phosphorylation at Ser-10 that occurs during mitosis and is carried out by the Aurora kinase family (11).

We study global histone modifications in early ES cell differentiation to understand how chromatin may be regulating differentiation. We report here that there is an increase in H3 phosphoacetylation during early ES cell differentiation induced by withdrawal of LIF for 3 days. We have defined the time period needed for exit from the undifferentiated state and commitment to a new differentiation status to occur as 24–36 h after LIF withdrawal.3 This differentiation-induced H3 phosphoacetylation gradually increases during this important period of pluripotent exit from the undifferentiated state and beginning lineage commitment. This increase in H3 phosphoacetylation also serves as a link to upstream signaling activity in the cells. The ERK and p38 MAPK pathways are activated during this early differentiation time period and are responsible for the increased H3 phosphoacetylation. We show that these pathways converge to activate MSK1, which serves as the critical mediator of differentiation-induced H3 phosphoacetylation. Inhibition of these processes alters ES cell differentiation as assayed by changes in the pattern of gene expression, showing that dynamic changes in epigenetic histone modifications and cell signaling pathways are an important regulatory mechanism in early ES cell differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—CCE mES cells were used for all studies and were obtained from Dr. John Gearhart (Johns Hopkins University) with permission from Dr. Gordon Keller (Mount Sinai School of Medicine). Plastic tissue culture dishes were pre-treated with 0.1% porcine gelatin Type A (Sigma) in water for 30 min at 37 °C. Undifferentiated mES cells were grown at 5% CO2 in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 3.7 g/liter sodium bicarbonate (pH to 7.4), 1 mM sodium pyruvate, 0.15 mM nonessential amino acids, 0.15 mM monothioglycerol (ICN Biomedicals Inc.), and 1800 units/ml LIF (Chemicon, Temecula, CA). Cells were passaged every 3 days at 1:100 and maintained at low cell density. Cells were induced to differentiate by removal of LIF from the media (all other components were unchanged). Addition of chemical inhibitors in the appropriate experiment took place when LIF was first withdrawn from media. H89 (suspended in 1:1 H2O:EtOH) was from Sigma. PD98059 (suspended in Me2SO) and SB203580 (suspended in H2O) were from Calbiochem. Previous work from our laboratory shows that removing LIF at 1:1000 concentrations do not affect ES cell morphology or gene expression.

**Immunoblotting**—Approximately 5–10 µg of acid extracted proteins was dissolved in 2× SDS sample buffer. For whole cell extract preparation, 2× SDS sample buffer was added to plated cells, and the cells were scraped off the plate. Extracts were then sheared with a 23-gauge needle. All samples were then subjected to 13% SDS-PAGE and transferred to a nitrocellulose membrane by standard methods. Membranes were blocked in 3% dry milk in 1× Tris-buffered saline with 0.1% Tween for 1 h at room temperature. Primary antibodies were then added. Phospho-S10-acetylK14 H3 (1:2000, 2 h at room temperature), phospho-HMG1 (1:1000, 4 °C, ON) antibodies were from Upstate Biotechnology, Inc., Charlottsville, VA. Phospho-ERK1/2 (1:1000, 4 °C, ON), phospho-p38 (1:1000, 4 °C, ON), phospho-MSK1 (1:1000, 4 °C, ON), phospho-CREB (1:1000, 4 °C, ON), and native histone H3 (1:5000, 2 h at room temperature) were from Cell Signaling Technologies, Beverly, MA. MSK1 (1:200, 2 h at room temperature), actin (1:1000, 2 h at room temperature), and total ERK1/2 (1:1000, 37 °C 2 h) were from Santa Cruz Biotechnology, Santa Cruz, CA. p38 (1:1000, 4 °C, ON), HMG1 (1:1000, 4 °C, ON) were from Abcam. MSK2 (1:250, 2 h at room temperature) was from Abgent, San Diego, CA. Membranes were washed and appropriate horseradish peroxidase (1:5000 donkey anti-rabbit or donkey anti-goat IgG, Santa Cruz Biotechnology)-conjugated secondary antibody was added for 45 min at room temperature. ECL developing reagent mix (Amersham Biosciences) was added, and blots were exposed to film. Image analysis was performed on a Macintosh computer using the public domain Image program (developed at the U.S. National Institutes of Health and available at rsb.info.nih.gov/nih-image/).

**Flow Cytometric Analysis**—Flow cytometry protocols were adapted from Hasegawa et al. (13) and Cell Signaling Technologies. All work until primary antibody addition was performed at 4 °C. Cells were washed in wash solution (containing 1× PBS with 50 mM NaF and 100 mM okadaic acid). Cells were trypsinized and centrifuged at 100 × g. Cells were resuspended in 2 ml of PBS, and 2 ml of 2% paraformaldehyde was slowly added dropdown. Cells were placed on ice for 15 min, centrifuged at 500 × g for 10 min, then resuspended in 1× PBS, and methanol was added to 90% final concentration dropwise at 4 °C while gently vortexing. Cells were stored on ice for 15 min.

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and centrifuged at 1500 × g for 10 min. One milliliter of incubation buffer (containing 0.5% bovine serum albumin in 1× PBS) was added, and cells were incubated on ice for 10 min. 1:100 pS10-aK14 H3 antibody was added, and cells were kept at room temperature for 60 min. Cells were centrifuged for 10 min at 500 × g and washed three times in incubation buffer. Alexa 488 fluorescein isothiocyanate chicken anti-rabbit IgG (Molecular Probes, Eugene, OR) antibody was diluted 1:100 in incubation buffer and added to samples for 30 min at room temperature. Cells were centrifuged for 10 min at 500 × g and washed three times in incubation buffer. Cells were resuspended in 1 ml of PBS with 33 µg/ml propidium iodide (Molecular Probes) and 20 µg/ml RNase (molecular biology grade, Sigma) and stored at 4 °C overnight. Samples were analyzed on a FACSCalibur (BD Biosciences) cytometer. Data were analyzed using CellQuest (BD Biosciences) and Modfit (Verity Software House) software. Technical assistance was provided by Kathy Schell and the staff at the University of Wisconsin-Madison Comprehensive Cancer Center flow cytometry facility.

Immunofluorescence—Cells were grown on coverslips (Fisher Scientific, Pittsburgh, PA) and washed with 1× PBS. Cells were fixed for 20 min with 2% paraformaldehyde dissolved in H2O at room temperature for 20 min. Cells were washed with 1× PBS then twice with wash solution I (1× PBS with 0.1% Triton X-100) for 4 min each. Incubation solution (3% goat serum in 1× PBS with 0.1% Triton X-100) was added for 30 min at room temperature. Primary antibody (1:100) in incubation solution was added for 120 min at room temperature. Cells were washed three times with wash solution I for 3 min each. 1:1000 Alexa 488 fluorescein isothiocyanate chicken anti-rabbit IgG secondary antibody in incubation solution was added for 60 min at room temperature. Cells were washed twice in 1× PBS and stained with 1:50,000 DAPI for 3 min. Coverslips were mounted (Fluoromount G SouthernBiotech, Birmingham, AL) on a glass slide and examined under a microscope with a 400× objective.

Cell Staining—Cells were stained with HEMA 3 hematological staining solution (Fisher Scientific). The product protocol was followed, and cells were examined under a microscope with a 400× objective.

RNA Preparation—Lysis buffer (Qiagen, Valencia, CA) was added to cells and sheared using Qiagen QiaShredder columns. Samples were then purified using a Qiagen RNAeasy mini-kit, following the manufacturer’s instructions, including a DNase digestion step. RNA was dissolved in RNase free water and stored at −80 °C.

Quantitative RT-PCR—cDNA was synthesized using 1.25 µg of RNA with avian myeloblastosis virus-reverse transcriptase (AMV-RT) enzyme and random hexamers (Promega). An iCycler system (Bio-Rad) was used for quantitative analysis. Real-time PCR was performed using a SYBR Green supermix kit (Bio-Rad). The level of each gene transcript was normalized to β-actin expression levels. All primers showed 90–100% efficiency. Expression was calculated as [2ΔCt(gene of interest control treatment − gene of interest experimental treatment)] / [2ΔCt(β-actin control treatment − β-actin experimental treatment)]. Primers used included (from 5’ to 3’): nestin F: GCAGGGTCTACAGAGTCAG, R: GCAAGCGAGAGTTTCTCAG; oct4 F: CCACCATC-TGTGCTTTCG, R: GCTTCTCCACCACCTTCTC; hoxb1 F: CTATGGAGCGGTGGAGTC, R: CAGAGAGGTGTCGTA-GG; progesterone receptor F: CTACCCGCAATCTTTAAC, R: CGCCATAGTGACGCCAGATG; brachyury F: CACCC-ACTGAGCAACAG, R: ACCAGGTATGAGGAGGTTC; FGF5 F: AACAAGAGGGGAGCCCAAGAG, R: GAAACTGTCG-TAGGGAAGGAAG; BMP4 F: GTGTGTGATGCGGCT-GAG, R: GTATGTGTAGGTGGTATG; and β-actin F: GCCAACCGTAAAGAGATGACC, R: GGTGAGGAGAGCATAGC.

Small Interfering RNAs and Transfection—MSK1 small interfering (si)RNAs (SMARTpool) were obtained from Dharmacon Research, Inc Lafayette, CO. The SMARTpool was prepared as recommended by the manufacturer at 20 µM. Transfection complexes were prepared in Opti-Mem (Invitrogen). ES cells grown in 6-well plates were transfected with 100 nM of the siRNA mix with Lipofectamine 2000 at a ratio of 2:1 in a protocol adapted from Hay et al. (15). Cells were plated in +LIF media 24 h prior to transfection. The first transfection was for 16 h in −LIF media. Fresh −LIF media was then added. The second transfection was for 12 h at time 64 h of culture. Cells were harvested at 96 h of culture time (total 72 h in −LIF media). Mock treatments contained either BLOCK-iT fluorescent oligonucleotide (Invitrogen) or no siRNA with no differences noted between the two mock treatments.

RESULTS

H3 Phosphoacetylation Increases during Early ES Cell Differentiation—We analyzed global changes in H3 phosphoacetylation during early ES cell differentiation to determine if this epigenetic mark may be playing a role during this early period. This early differentiation period was assessed with plated ES cells. ES cells are maintained in a pluripotent state by including LIF in the culture media. Withdrawal of LIF produces characteristic changes in gene expression and morphology (Fig. 1A) (16). Gene expression of the differentiation markers hoxb1 (patternning), FGF5 (primitive ectoderm), and progesterone receptor increases early during this time period (16–18). Lineage-specific markers such as brachyury (mesoderm) and nestin (neuroectoderm) begin to be expressed at about 3 days following LIF withdrawal (19, 20). The pluripotent marker oct4 and the differentiation inducer BMP4 show decreased expression levels during this time, although oct4 levels drop very slowly during the first 3 days following LIF withdrawal (21, 22). Cells in the pluripotent state form tight spherical clusters, whereas differentiating cells begin to spread out and have distinct cell borders. Analysis of H3 phosphoacetylation was performed by immunoblotting analysis of acid-extracted histones with an antibody against histone H3 dually phosphorylated at serine 10 and acetylated at lysine 14 (pS10-aK14) (7). An antibody to total histone H3 was used as a loading control. This shows that global H3 phosphoacetylation gradually increased during this early differentiation period peaking with a 3-fold increase after 3 days of LIF withdrawal (Fig. 1B). This increase in H3 phosphoacetylation occurs over a period of days, and we have termed this “differentiation-induced H3 phosphoacetylation.” A possible mechanism for this increase in H3 phosphoacetylation is through activation of MSK1. MSK1 phospho-
rylates chromatin proteins histone H3 and HMGN1, as well as transcription factors such as CREB (9). Immunoblot analysis using antibodies specific for the active phosphorylated forms of these proteins showed that HMGN1 and CREB also are gradually phosphorylated during early ES cell differentiation (Fig. 1C). Non-phospho-HMGN1 and actin were used as loading controls for p-HMGN1 and p-CREB, respectively. Phosphorylation of these separate targets points to activation of MSK1 during early ES cell differentiation.

Differentiation-induced H3 Phosphoacetylation Is Not Associated with Cell-Cycle Changes—To distinguish between mitosis-induced phosphorylation of Ser-10 on histone H3 and differentiation-induced H3 phosphoacetylation, we have used fluorescence-activated cell staining analysis. Studies of stimulus-induced H3 phosphoacetylation, mediated by MSK1/2, normally occur in cells that have been serum-starved to enter the quiescent G0 phase of the cell cycle. This allows separation from mitotic H3 phosphorylation, usually mediated by the Aurora kinases. However, ES cells lack the normal cell-cycle checkpoint control of other cells and do not enter G0 (23). Attempts at serum starvation also perturb the delicate balance of factors needed to maintain pluripotency. The ES cells used in this study are rapidly dividing, doubling roughly every 12 h. Previous studies (24) have indicated that withdrawal of LIF does not alter the cell cycle or cell proliferation. To confirm this in our model system we used a propidium iodide DNA stain and analyzed cell-cycle distribution. This shows that there is no difference in the cell cycle between +LIF ES cells and ES cells withdrawn from LIF for 3 days (Fig. 2A). Around 60% of the
cells grown in either + or − LIF media are in the S phase of the cell cycle. To further examine distribution of H3 phosphoacetylation during the cell cycle, we stained cells with both the anti-pS10-aK14 antibody and propidium iodide. This allows examination of H3 phosphoacetylation by phase of the cell cycle, by gating different H3 phosphoacetylation populations. Consistent gates were drawn around G1/S and G2/M phase populations. There is a strong increase in H3 phosphoacetylation in G1/S phase (Fig. 2B) indicating that the increase in H3 phosphoacetylation is a stimulus-induced response. Smaller increases are also observed in the G2/M phase of the cell cycle. Furthermore, LIF-withdrawn cells (− LIF 3 days) also exhibit an increase in a punctate H3 phosphoacetylation nuclear staining pattern using immunofluorescent staining with the anti-pS10-aK14 antibody and the nuclear stain DAPI (Fig. 2C, bottom panel) compared with +LIF cells (Fig. 2C, top panel). This pattern is consistent with localized changes in H3 phosphoacetylation during differentiation and is in contrast to extensive H3 phosphoacetylation observed in mitotic figures approximately in equal numbers for + and − LIF-treated cells (data not shown). These results indicate that localized H3 phosphoacetylation increases in both G1/S and G2/M phase ES cells during early differentiation but with a much larger increase seen in the G1/S phase. 

MAPK Pathways Are Activated during Early ES Cell Differentiation—We next analyzed the activation of upstream cell signaling pathways to determine how differentiation-induced H3 phosphoacetylation occurs. We focused on activation of the ERK and p38 branches of the MAPK pathways. Each pathway has been shown to be responsible for mitogen-induced H3 phosphoacetylation, depending on the stimulus. Activation of these pathways was assayed by immunoblotting using phospho-specific antibodies to the active form of either ERK1/2 or p38. Antibodies against total ERK1/2 or p38 antibodies were used as loading controls for total kinase present (Fig. 3A). This analysis showed that active ERK1/2 is gradually increased during early ES cell differentiation, peaking at 3 days of LIF withdrawal in a pattern similar to H3 phosphoacetylation. Activated p38 also increased during early differentiation, but showed a sudden increase at 3 days of LIF withdrawal. Activation of these kinases was also observed in immunofluorescent staining of +/− LIF-treated ES cells with phospho-specific antibodies (Fig. 3B). Nuclear staining was performed using DAPI. This showed that there was an increase in phospho-ERK nuclear distribution in LIF withdrawal-induced differentiation (Fig. 3B, lower left panels). Phospho-p38 also increased following LIF withdrawal, but had a more diffuse increase, including both nuclear and cytoplasmic distributions (Fig. 3B, lower right panels). These results show activation of upstream MAPK signaling pathways during early ES cell differentiation.

Chemical Inhibition of MAPK Pathways Blocks H3 Phosphoacetylation and Inhibits Early ES Cell Differentiation during LIF Withdrawal—We used MAPK chemical inhibitors to block ERK1/2 and p38 pathways and determine if these signaling pathways are responsible for differentiation-induced H3 phosphoacetylation. H3 phosphoacetylation was again observed using immunoblotting of acid-extracted histones with the pS10-aK14 antibody. Our results indicated differentiation-induced H3 phosphoacetylation was significantly decreased in the presence of the ERK inhibitor PD98059 (25 μM) in ES cells withdrawn from LIF for 3 days (Fig. 4A, +PD) (25). Any remaining H3 phosphoacetylation was completely abolished when the p38 inhibitor SB203580 (10 μM) was added in conjunction with PD98059 in LIF withdrawn cells (Fig. 4A, +PD+SB) (26). SB203580 alone causes only a small decrease in phosphoacetylation (Fig. 4A, +SB).

These kinase inhibitors also had effects on cell morphology during differentiation (Fig. 4B). Using PD98059 alone or in combination with SB203580 produced ball-like clusters of cells even after 3 days of LIF withdrawal (Fig. 4B, −LIF 3d +PD+SB) and LIF 3d +PD+SB), consistent with the morphology of the undifferentiated state (Fig. 4B, +LIF). This appearance contrasted sharply with the branched cell pattern normally observed in ES cells after 3 days of LIF withdrawal alone (Fig. 4B, −LIF 3d). Treatment with SB alone during 3 days of LIF withdrawal (Fig. 4B, −LIF 3d +SB203580) did not alter morphology significantly compared with LIF withdrawal alone. Neither compound had an effect on morphology when treat-
ment occurred in the presence of LIF for 3 days (Fig. 4B, top panels). We used quantitative RT-PCR to analyze expression of differentiation markers in LIF withdrawn cells with and without kinase inhibitors present. Expression of the early differentiation marker *hoxb1* was reduced when the ERK or p38 pathways were inhibited (Fig. 4C). Expression was further reduced when both pathways were inhibited in LIF withdrawn cells. FGF5 expression was also reduced when both pathways were inhibited. Induction of the mesoderm marker *brachyury* was markedly inhibited when the ERK, p38, or both pathways were inhibited. However, the pluripotent marker *oct4* showed increased expression compared with undifferentiated cells (Fig. 4C, +LIF expression set equal to 1.0) when both the ERK and p38 pathways were inhibited (Fig. 4C, −LIF/+PD+SB). These results indicate that the ERK and p38 pathways are responsible for differentiation-induced H3 phosphoacetylation and that activation of these pathways is necessary for morphology and gene expression changes indicative of early ES cell differentiation by LIF withdrawal.

**Kinase Inhibitor H89 Blocks Differentiation-induced H3 Phosphoacetylation and Delays Molecular Differentiation**—MSK1 phosphorylates histone H3 in the nucleosomal response and lies downstream of the ERK and p38 pathways. We analyzed MSK1 activation during early ES cell differentiation using a phospho-specific anti-MSK1 antibody with immunoblotting. A non-phospho anti-MSK1 antibody was used to measure total protein, and an anti-actin antibody was used as a loading control (Fig. 5A). This analysis showed an increase in active MSK1 in ES cells following 1, 2, or 3 days of LIF withdrawal. We next used the small molecule MSK1 inhibitor H89 to determine the role of MSK1 in differentiation-induced H3 phosphoacetylation. Treatment of LIF withdrawn ES cells with H89 (20 μM) caused a marked inhibition of differentiation-induced H3 phosphoacetylation (Fig. 5B). Treatment with H89 also altered cell morphology in both the presence and absence of LIF (Fig. 5C). To determine if this altered morphology correlated with changes in gene expression, we employed quantitative RT-PCR on the early ES cell differentiation marker *hoxb1*, the lineage-specific markers *brachyury* (mesoderm) and nestin (neuroectoderm), and the
pluripotent marker Oct4. This showed that H89 in LIF-withdrawn cells caused a delay in molecular differentiation as measured by marker gene expression (Fig. 5D). Oct4 expression was not altered from the undifferentiated state. These data suggest that the inhibitor H89 may be modulating ES cell differentiation by blocking MSK1 and H3 phosphoacetylation.

**MSK1 Directly Mediates Differentiation-induced H3 Phosphoacetylation**—A difficulty in using H89 is its lack of specificity for MSK1 (27). To determine the specific role of MSK1 in differentiation-induced histone H3 phosphoacetylation, we utilized RNA interference technology (28). An anti-MSK1 antibody was used for immunoblotting to determine MSK1 knockdown using small inhibitory RNA (siRNA) targeted to MSK1 (Fig. 6A). An anti-actin antibody was used as a loading control. This shows that MSK1 protein expression is successfully inhibited following MSK1 siRNA treatment during 3 days of LIF withdrawal. Samples were compared with mock transfection during 3 days of LIF withdrawal. The specificity of the siRNA was demonstrated by the lack of knockdown of MSK2 protein (Fig. 6B). The anti-pS10-aK14 antibody was used for immunoblotting to determine H3 phosphoacetylation in MSK1 knockdown cells. This analysis showed that H3 phosphoacetylation was almost completely inhibited in MSK1 knockdown cells. This indicated that MSK1 is indeed the histone kinase responsible for differentiation-induced H3 phosphoacetylation.

There were no obvious morphological differences between mock and MSK1 siRNA-treated cells (data not shown). Analysis of gene expression in MSK1 knockdown during 3 days of LIF withdrawal showed a modulation of differentiation marker gene expression but not a definitive delay in differentiation as was seen using H89. Gene expression was compared relative to mock transfection during 3 days of LIF withdrawal.
Expression of the early differentiation marker *hoxb1* was decreased as was the mesoderm marker *brachyury*. The differentiation inducer BMP4 also showed a 2-fold decrease in MSK1 knockdown cells. However, nestin, FGF5, and *oct4* remained the same or slightly increased in MSK1 knockdown cells. These data indicate that MSK1 mediates differentiation-induced H3 phosphoacetylation and this phosphoacetylation plays at least a partial role in modulating gene expression levels from a limited set of genes critical in the process of early ES cell differentiation.

**DISCUSSION**

The importance of early epigenetic modifications is of great interest in understanding programs of stem cell differentiation. Changes in histone acetylation and methylation during differentiation and reprogramming have been studied (29, 30). However, the exact roles these modifications play in ES cell fate and lineage selection are still unclear. In this study, we have found that H3 phosphoacetylation acts as a dynamic epigenetic mark during early ES cell differentiation. This histone modification provides a link to upstream cell signaling pathways in differentiating cells. Both the ERK and p38 pathways are activated during early ES cell differentiation and are responsible for differentiation-induced H3 phosphoacetylation. These pathways converge on MSK1, which is the critical mediator of differentiation-induced H3 phosphoacetylation. Our results show that these dynamic epigenetic and cell signaling cascades are involved in modulating gene expression patterns during early ES cell differentiation. The effects on long term lineage selection during *in vitro* differentiation of mES cells remain to be determined.

Changes in H3 phosphoacetylation have not been reported in early ES cell differentiation. However, cardiovascular lineage formation (a mesoderm derivative) by treatment of ES cells with laminar shear stress results in increased H3 phosphoacetylation (31). This occurs through activation of the p38 MAPK pathway. The p38 pathway has been shown to be active in cardiovascular formation from ES cells and plays a role in apoptotic decisions in early ES cell differentiation (32, 33). The ERK/MAPK pathway has also been shown to be important in development, with ERK2 knock-out mice lacking mesoderm formation (34). The ERK and p38 pathways are inhibited in the pluripotent state by BMP4, which was found in our study to be modulated by inhibition of MSK1 (35). BMP4 is a critical regulator of mesoderm and neural ectoderm differentiation. High levels of BMP4 favor mesoderm differentiation.
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and inhibit neural ectoderm differentiation (22, 36, 37). In addition, retinoic acid dramatically decreases BMP4 levels, represses mesoderm differentiation, and initiates neural differentiation (38, 39). In our results the effects of direct MSK1 knockdown on gene expression seemed to be limited to a small set of genes, including a reduction in the levels of BMP4 and brachyury. Taken together these data suggest that phosphoacetylation during early ES cell differentiation induced by LIF withdrawal aids in regulating the levels of genes involved in mesoderm differentiation. Our data indicate that the epigenetic change (phosphoacetylation) alone does not act as an “on-off” switch for these genes but, rather, appears to act more as a rheostat capable of modulating the expression levels of genes involved in mesoderm selection. Our results are also in agreement with previous studies that show the ERK and p38 pathways are vital for proper gene expression and morphology changes during ES cell differentiation.

The ERK and p38 pathways converge to activate MSK1/2, which can phosphorylate histone H3. Previous studies have created MSK1/2 knock-out mice, which are viable (10). These mice show marked reduction of Ser-10 phosphorylation and dampened responses to stress but continue to show changes in gene expression as well as increases in K14 acetylation. Our results and the findings with the knock-out mice for MSK1 support a role for phosphorylation of Ser-10 on H3 in modulating gene expression levels, but this is not the on/off switch for transcription. It is also possible that there are redundant mechanisms that compensate for MSK1/2 activity in knock-out mice (the authors (10) do report low levels of Ser-10 phosphorylation), and these mechanisms are not utilized in our mES cell model of acute, in vitro siRNA knockdown. We have found that MSK1 is activated during early ES cell differentiation induced by LIF withdrawal. The small molecule MSK1 inhibitor H89 blocks differentiation-induced H3 phosphoacetylation and markedly delays molecular differentiation. Cell morphology is also altered in the pluripotent and differentiating state by H89 treatment. However, H89 also inhibits other kinases such as protein kinase A and ROCK-II (27). We tested whether the protein kinase A peptide inhibitor PKI-tide could alter cell morphology or gene expression and saw no effect (data not shown). We have also tested the ROCK-II-specific inhibitor, Y27632, which alters cell morphology similarly in both undifferentiated mES cells and LIF-withdrawn cells. Y27632 treatment during 3 days of LIF withdrawal does not delay differentiation as H89 does. However, we observed slightly reduced levels of both hoxb1 and brachyury gene expression (data not shown). These reductions are similar to the levels following MSK1 siRNA knockdown (Fig. 6C). Therefore, we do not believe the significant effects of H89 on delaying ES cell differentiation are mediated through protein kinase A or ROCK-II. Further study is necessary to sort out whether there may be synergistic effects between MSK1 and ROCK-II, because ROCK-II has been found to play a role in epiblast differentiation (40). Our data suggest that blockade of the upstream kinase pathways has more dramatic effects on mES cell differentiation after LIF withdrawal.

Specific inhibition of MSK1 was achieved using RNA interference. This is the first reported use of this technology to inhibit MSK1, as H89 and dominant-negative MSK1 constructs were previously used to study this signaling pathway (41). We show that specific knockdown of MSK1 results in inhibition of differentiation-induced H3 phosphoacetylation. This shows that MSK1 is responsible for this dynamic epigenetic change during ES cell differentiation. In other model systems, inhibition of H3 phosphoacetylation alters gene expression efficiency (42). In agreement with this previous understanding of H3 phosphoacetylation, our data show that MSK1 inhibition during early ES cell differentiation modulates expression levels of genes important in the differentiation process, such as hoxb1, brachyury, and BMP4. In other model systems H3 phosphoacetylation occurring through activation of the nucleosomal response is usually rapid, transient, and localized within a restricted region of a gene. In our studies the global phosphoacetylation induced by LIF withdrawal slowly increased over 3 days and appeared to be maintained during this time.
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