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The role of histone chaperones in osteoblastic differentiation of C2C12 myoblasts

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ABSTRACT

Cellular differentiation is a process in which the cells gain a more specialized shape, metabolism, and function. These cellular changes are accompanied by dynamic changes in gene expression programs. In most cases, DNA methylation, histone modification, and variant histones drive the epigenetic transition that reprograms the gene expression. Histone chaperones, HIRA and Asf1a, have a role for cellular differentiation by deposition of one of variant histones, H3.3, during myogenesis of murine C2C12 cells. In this study, we accessed the roles of histone chaperones and histone H3.3 in osteoblastic conversion of C2C12 myoblasts and compared their roles with those for myogenic differentiation. The unbiased analysis of the expression pattern of histone chaperones and variant histones proposed their uncommon contribution to each pathway. HIRA and Asf1a decreased to ~50% and further diminished during differentiation into osteoblasts, while they were maintained during differentiation into myotubes. HIRA, Asf1a, and H3.3 were indispensable for expression of cell type-specific genes during conversion into osteoblasts or myotubes. RNA interference analysis indicated that histone chaperones and H3.3 were required for early steps of osteoblastic differentiation. Our results suggest that histone chaperones and variant histones might be differentially required for the distinct phases of differentiation pathway.

1. Introduction

Histones, which are key proteins that compact the structure of all eukaryotic DNA in the form of chromatin, have attracted much attention due to their impact on genome function [1]. They form the core of the repeated unit of chromatin, a nucleosome, in which 147 base pairs of DNA wrap around a histone octamer, comprising a tetramer of $(H3–H4)_2$ and two heterodimers of H2A–H2B. This organization contributes to the regulation of all cellular processes operating on DNA [2,3]. Eukaryotic cells contain variants of histones H2A and H3 that can be distinguished from the canonical histones by amino acid sequences. These variants are synthesized and assembled into nucleosomes independently of DNA replication, which strongly suggests that their incorporation into chromatin might have considerable impacts on epigenetic function of chromatin domains. Eukaryotic cells express several histone H3 variants. Among them, histone H3.3 has roles in transcription and in preventing heterochromatin spreading [4].

The correct incorporation of histones onto DNA requires the assistance of histone chaperones. Histone chaperones are thought to function in coordination with chromatin-remodeling factors to mediate the accurate positioning of nucleosomes on a DNA template. Several histone chaperones have been identified and characterized, both biochemically and genetically [5,6]. In particular, many chaperones are found to function in the deposition of specific histone variants [7]. The canonical H3 is associated mainly with complexes, which contains the histone chaperone chromatin assembly factor 1 (CAF1) and Asf1b, whereas HIRA and Asf1a mediate H3.3 deposition during transcription with unknown mechanism [8]. Nucleosomes containing either of these H3 forms are almost structurally identical, making it unlikely that the incorporation of H3.3 changes the overall structure of the nucleosome [9,10]. Nonetheless, it remains unclear why H3.3 is preferred to constitute nucleosomes for transcriptionally active regions.

Cell differentiation is a natural event and the process by which a less specialized cell undergoes an epigenetic programming to obtain a more specialized function [11]. These processes involve epigenetic factors, such as DNA methylation, histone modification, and variant histones. Recently, with regard to its role in transcription and cellular differentiation, H3.3 was reported to be associated with the epigenetic memory of a transcription state during muscle development [12,13]. Replication-independent (RI) deposition of H3.3 via histone chaperones HIRA/Asf1a play an important role in myogenic differentiation through the cell type-specific activation of *MyoD*, at the level of transcription [14,15]. In this study, we analyzed the roles of histone H3.3 and its chaperones, HIRA and Asf1a, during osteoblastic differentiation of C2C12 myoblasts. We

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depleted each factor during cell differentiation, and analyzed their effect on the expression of lineage-specific marker genes. Our data suggest that H3.3/HIRA/Asf1a plays an important role in early steps of osteogenic conversion, whereas it is persistently required for the initiation and maturation of C2C12 cells into myotubes.

2. Materials and methods

2.1. Cells and culture condition

The mouse myoblast cell line, C2C12, was obtained from American Type Culture Collection (ATCC). C2C12 was maintained in DMEM containing 15% fetal bovine serum (GM) at 37 °C with 5% CO_2. Cells were inoculated at 6×10^5 cells. To induce differentiation into myotube (Mt) and osteoblast (Ob), cells were incubated in DMEM containing 2% horse serum or 300 ng/ml of BMP-2 (recombinant human Bone Morphogenetic Protein-2, GenScript, Inc.). All media were supplemented with 1% penicillin and streptomycin. Generation of C2C12 stably expressing shRNA against HIRA (shHIRA C2C12) was described previously [14].

2.2. RNA interference

Transfection of siRNA was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The siRNAs were purchased from either Invitrogen or ST Pharm. The target sequences of siRNA and shRNA are as follows: mHIRA shRNA; 5′ CTC AAG CTG ATG ATC GAA GTT 3′, mHIRA siRNA-1; 5′ GAC CAA GTT TGC AAC TGG AGG ACA A 3′, mHIRA siRNA-2; 5′ CCG GAA AGC TGT GAC TGT TGT GAA A 3′, mHIRA siRNA-3; 5' CCU AUG GCA AGA GCC UGG CAA UAA U 3', mAsf1a siRNA; 5′ GGC ATA TGT TTG TGT TTC AGG CTG A 3′, mH3.3a siRNA-1; 5′ TGA AGA AAC CTC ATC GTT A 3′, mH3.3a siRNA-2; 5′ GAG AAA TTG CTC AGG ACT T 3′, mH3.3b siRNA-1; 5′ UUG GUG GCC AGC UGU UUG C 3′, mH3.3b siRNA-2; 5′ UUU CUG GUA ACG ACG GAU C 3′.

2.3. RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was purified using RNeasy plus mini kit (QIAGEN), according to the manufacturer's instructions. One microgram (1 μg) of purified RNA was used to synthesize cDNA using a reverse transcription reaction mixture, containing oligo dT or random hexamer and gene-specific primers (Fermentas). Real-time PCR was performed, using the CFX96 Real Time System (BioRad). PCR amplification was carried out, using KAPA SYBR FAST Master Mix (KAPA Biosystem) and relative quantification was performed with the 2^{-CT}(Livak) program method.

2.4. RNA-sequencing analysis

Total RNA was prepared from cells with RNeasy plus mini kit (QIAGEN), according to the manufacturer's protocol. RNA purity and integrity were evaluated by gel electrophoresis and OD 260/280 ratio (>1.8). Subsequent steps for cDNA synthesis, Illumina sequencing library preparation, sequencing, and analysis of RNA-seq reads were performed by DNALink, Korea. For analysis of RNA-seq expression data, the RPKM RNA-Seq pipeline was used. Gene ontology analysis of genes was performed using DAVID (http://david.abcc.ncifcrf.gov/home.jsp).

2.5. Immunoblot analysis

Lysates were prepared, using lysis buffer [Tris 25 mM (Ph 8.0), EDTA 1 mM, NaCl 150 mM, 0.5% NP40]. Equal amounts of protein extract were separated on SDS–polyacrylamide gels and transferred to nitrocellulose membranes. Blots were incubated with appropriate primary antibodies. After washing, membranes were incubated with HRP-conjugated secondary antibodies. Protein bands were visualized by ECL detection reagent (Abfrontier).

2.6. Alkaline phosphatase (ALP) staining

Cells were washed with PBS and fixed in fixation solution [Citrate, Acetone, 37% Formaldehyde]. Cells were stained and counterstained with alkaline-dye mixture/Neutral red solution (Sigma–Aldrich ALP staining kit). Cells stained were dried at room temperature for evaluation under a microscope.

3. Results

3.1. Differentiation of myogenic C2C12 cells into myotubes and osteoblasts

A subset of cells, such as osteoblasts, myocytes, chondrocytes, and adipocytes, can be derived from common progenitor cells. To study cellular differentiation *in vitro*, murine myoblastic C2C12 cells were used as a model system. C2C12 cells have myogenic origin and differentiate into either multinucleated myotubes upon serum withdrawal or osteoblasts by treatment with BMP-2. BMP-2 is one of the potent activator of osteoblast differentiation [16,17]. To investigate the role of histone H3.3 and cognate histone chaperones in two independent cellular differentiation pathways, we used C2C12 cells to induce differentiation into osteoblasts. Proliferating C2C12 cells were induced to differentiate into multinucleated myotubes, in parallel. The morphological changes were monitored by light microscopy (Fig. 1A, upper panel). C2C12 cells generated numerous multinucleated myotubes by day 3 in the myogenic differentiation condition (Mt), but these were not observed in osteoblastic differentiation condition (Ob) (Fig. 1A, upper panel). Instead, the expression of alkaline phosphatases (ALP), a bone marker, which is important for bone mineralization, greatly induced only in Ob as observed by cytochemical staining (Fig. 1A, lower panel). To understand the transition of cell states at the level of gene expression, we examined the global gene expression profiles in C2C12 cells, during myogenic or osteogenic differentiation using RNA-Seq. As shown in Fig. 1B, a total of 641 genes were either up- (328) or down- (313) regulated by more than 1.5-fold when proliferating C2C12 cells proceeded to myotubes. In the meanwhile, total 1175 genes were up- (502) or down- (673) regulated when cells proceeded toward osteoblasts. The heat map showed that genes were differentially regulated during myogenic or osteogenic transition, indicating that different set of genes are either activated or silenced in accordance to the differentiation condition. The level of genes that are well-characterized as differentiation markers was increased appropriately and grouped into different clusters (*ALP*, *Col1a2*, and *Runx-2* for osteogenic markers and *Musk*, *MCK*, and *MHC* for myogenic markers). To validate RNA-Seq data, we performed quantitative analysis of RT-PCR of cell type-specific genes for myotubes (MyoD and Myogenin) and osteoblasts (ALP and Osteocalcin) (Fig. 1C–F). BMP-2 efficiently stimulated osteoblastic conversion of C2C12 by day 3, while completely suppressing its myogenic differentiation. Taken all, cellular differentiation induced expression of marker genes with lack of cross-activation of the opposite pathway. Thus, our data indicate that C2C12 cells were well differentiated into each lineage with development of characteristic cellular phenotypes.

3.2. Histones and histone chaperones show different expression profiles during osteogenic and myogenic differentiation of C2C12 cells

To investigate the role of histone H3.3 and H3.3-specific histone chaperones, we first examined their shift of gene expression during

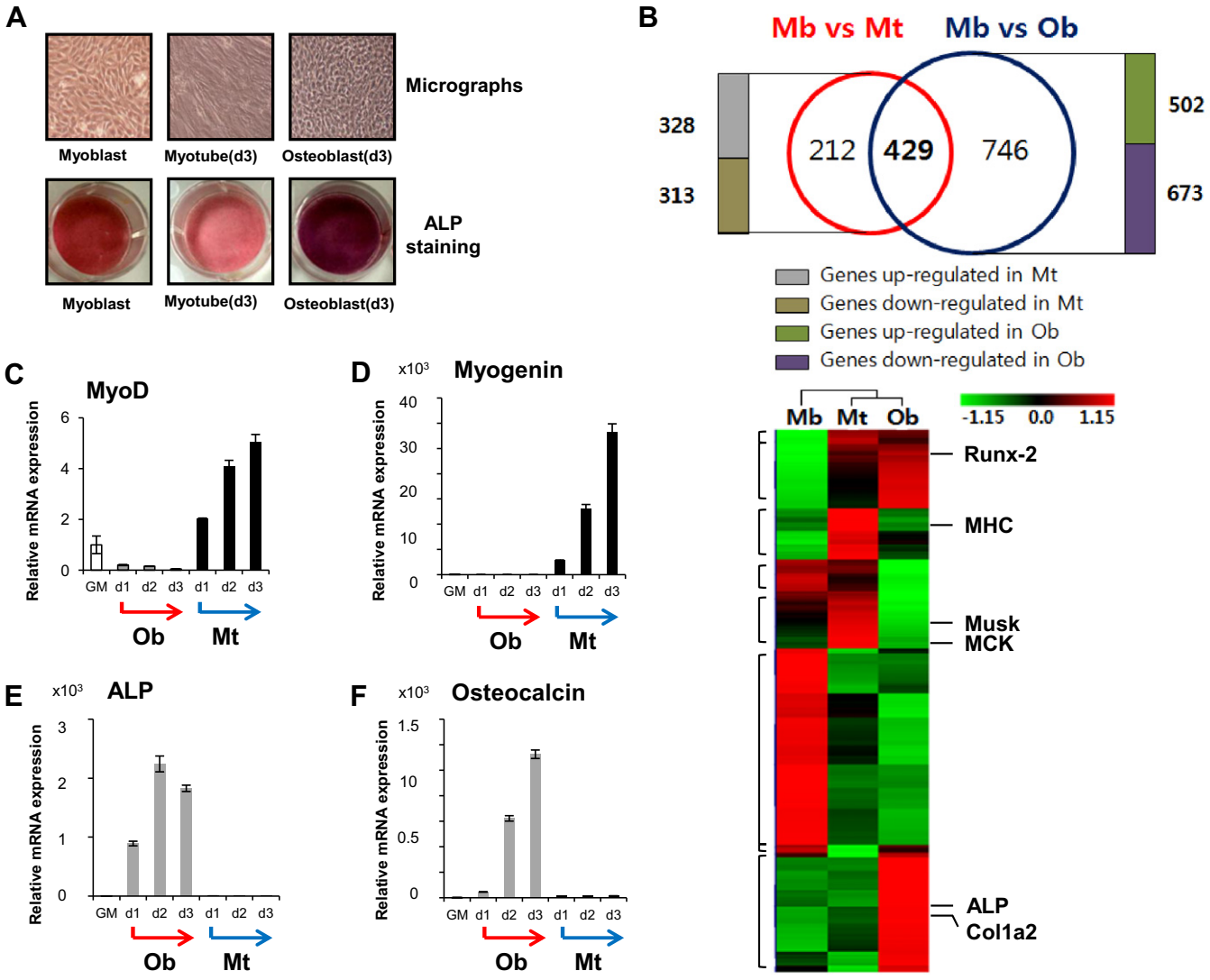

Fig. 1. Differentiation of C2C12 myoblasts into myogenic or osteogenic lineages. C2C12 cells were grown in proliferating growth medium (GM) and then induced for differentiation into myotubes (Mt) by incubation with 2% of horse serum or into osteoblasts (Ob) by addition of 300 ng/ml of BMP-2 for 3 days as described in Materials and Methods. (A) Myotube formation of the differentiated C2C12 was evaluated by light microscopy and the expression of alkaline phosphatases (ALP) was monitored histochemically at day 3. ALP-positive cells are stained purple. (B) The genome-wide gene expression profiles were analyzed by RNA-Seq as described in Materials and Methods. Venn-diagram shows protein coding genes differentially expressed in three types of cells. 328 and 502 genes were up-regulated by day 3 of differentiation into myotubes and osteoblasts, respectively, compared to genes of myoblasts in GM condition. 313 and 673 genes were down regulated in myotubes and osteoblasts, respectively. (C–F) The mRNA levels of cell type-specific genes were analyzed along the time course (d1–d3) by quantitative RT-PCR. C2C12 cells were differentiated into myotubes and osteoblasts for 3 days and cells were harvested at the indicated time points (day1, 2, and 3). RNAs were extracted and reversely transcribed with oligo-dT to synthesize cDNA. GM indicates growing states of cells before induction of differentiation. MyoD and Myogenin are myogenic genes. Alkaline phosphatase (ALP) and Osteocalcin are osteogenic genes.

myogenic and osteogenic change from RNA-Seq data. This analysis revealed that the expression of *HIRA* and *Asf1a* was fairly maintained during myogenic differentiation (Supplementary Fig. S1A), but their expression considerably decreased during osteogenic conversion (Supplementary Fig. S1B). The expression pattern of each gene was further examined in detail at mRNA and protein levels. As shown in Fig. 2A, the protein levels of HIRA and Asf1a significantly decreased in osteoblastic C2C12 cells by day 3, while their levels in myotubes were substantially maintained. The mRNA level of each gene was analyzed in the time course by quantitative RT-PCR. The expression of *HIRA* and *Asf1a* was significantly maintained during the myogenic differentiation, whereas their levels were gradually down-regulated to less than 20% of those of myoblasts during osteogenic conversion (Fig. 2B and C). The canonical H3-specific histone chaperone CAF1 (p150, the largest subunit) and Asf1b, the other Asf1 isotype associated with CAF1, were decreased as cells differentiated into both directions (Fig. 2D and E).

Mouse genome contains two H3.3 genes, H3.3a and H3.3b. Both were relatively unchanged for a while up to day 2, but decreased when further incubated during osteoblastic differentiation, while they were maintained or even increased during myotube formation (Fig. 2F and G), which is in part correlated with the expression fate of *HIRA* and *Asf1a* (Fig. 2A–C). The canonical H3s (*H3.1* and *H3.2*) and other forms of histone variant were largely decreased with both direction of differentiation (Fig. 2H–I and Supplementary Fig. S1). These results indicate that the variant histone H3.3 and the RI histone chaperones, HIRA and Asf1a, might play an uncommon role in transition into myotubes and osteoblasts.

3.3. HIRA, Asf1a, and variant histone H3.3 are essential for expression of cell type-specific genes during osteogenic conversion of C2C12 cells

HIRA interacts with Asf1a *in vivo* and both are essential factors for myogenic differentiation of C2C12 cells [14,18]. We asked

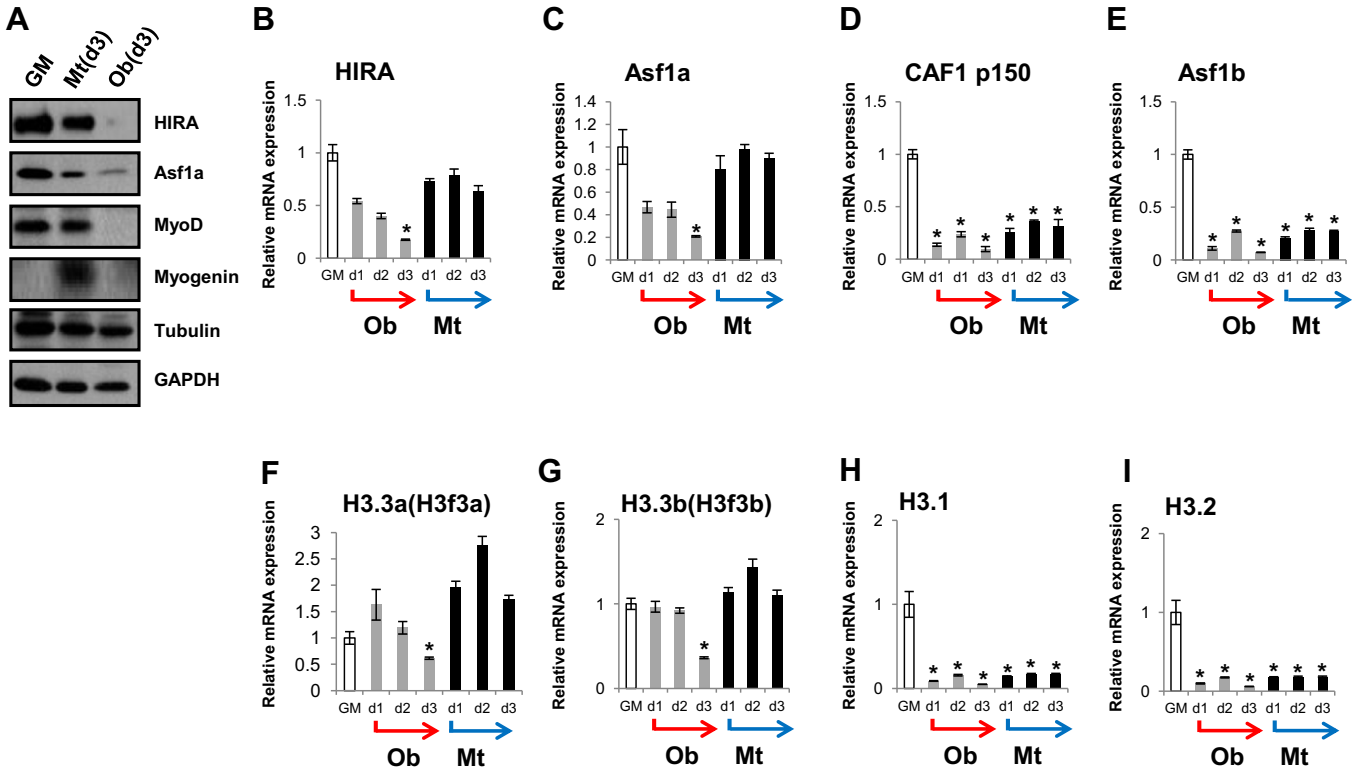

Fig. 2. The expression of RI-pathway histone chaperones and histone H3.3 are maintained during skeletal myogenesis but gradually decreased during osteogenic conversion. Either whole cell extract (A) or RNA (B–I) was prepared from the C2C12 cells to detect protein or mRNA levels by immunoblotting and qRT-PCR, respectively. (A) MyoD and Myogenin are myogenic genes. GAPDH and Tubulin were used as loading controls. mRNA levels of histone chaperone HIRA (B), Asf1a (C), Asf1b (E), the largest subunit of RC histone chaperone CAF1 (D), variant histone H3.3 (F and G), and canonical histone H3.1 (H)/H3.2 (I), were analyzed. The mRNA levels were normalized to the level of *GAPDH* and shown as relative values. Error bars represent standard deviation (SD) of three independent experiments ($n = 3$). The significance of difference was evaluated (*P value < 0.05).

whether HIRA and Asf1a also have any role during osteogenic differentiation of C2C12 cells. To directly ascertain the role of histone chaperones, we analyzed the effect of RNA interference-mediated knock-down of HIRA in C2C12 cells stably expressing shRNA against HIRA. We also attempted to deplete HIRA or Asf1a when cells were subjected to differentiation by adding siRNAs at day 0 (Fig. 3D). The knock-down efficiency was confirmed to effectively deplete HIRA by immunoblot analysis and quantitative RT-PCR, compared to their controls (Fig. 3A and B). In this condition, the cell type-specific genes representing each differentiation were analyzed in the control shRNA or shHIRA C2C12 cells. Interestingly, the shHIRA C2C12 showed diminished levels of not only myogenic (*MyoD*, *Myogenin*) but osteogenic (*ALP*, *Osteocalcin*, and *Runx-2*) genes when cells allowed differentiation into myotubes and osteoblasts, respectively (Fig. 3C). To confirm this again, C2C12 cells were treated with three independent siRNAs that target the different regions of HIRA mRNA (Supplementary Fig. S2A). This trial also showed that both myogenic and osteogenic genes were affected by depletion of HIRA (Supplementary Fig. S2B), which indicated that H3.3-specific histone chaperone HIRA is important for the cellular differentiation into both directions. We also examined the knock-down effect of Asf1a, using siRNAs (Fig. 3E). C2C12 cells treated with siRNA targeting Asf1a reduced the expression of each gene (*MyoD*, *Myogenin*, *ALP*, *Osteocalcin*, and *Runx-2*) at the level of mRNA (Fig. 3F). These results demonstrated that both HIRA and Asf1a were essential factors for myogenic and osteogenic differentiation of C2C12 cells, even though their expression levels, along the respective direction, display different profiles.

HIRA, together with Asf1a, has a role in the *MyoD* activation and this ability relates to the replication-independent H3.3 deposition activity [14]. To investigate whether the requirement of HIRA and Asf1a for osteogenic conversion of C2C12 cells rely on their histone substrate, thereby on histone chaperoning activity, we treated the cells with two independent sets of siRNA mixture that targets H3.3a and H3.3b simultaneously, without impeding the level of canonical histone H3.1 or H3.2. The siRNAs were added at day 0 upon shifting C2C12 cells to each differentiation condition. First, we show that H3.3a and H3.3b were down-regulated to a different extent by treatment of cells with siRNAs (Fig. 3G and Supplementary Fig. S2C). Although the knockdown efficiency was not as good as that of HIRA or Asf1a, the expression of *MyoD* and *myogenin* was reduced significantly upon partial depletion of histone H3.3, as reported (Fig. 3H and Supplementary Fig. S2D). In this condition, the expression of *ALP*, *Osteocalcin*, and *Runx-2* were also diminished, which indicated that the differentiation process of C2C12 cells towards osteoblasts depends on H3.3, as well as HIRA and Asf1a. Our data suggest that the deposition of H3.3 via HIRA/Asf1a-mediated replication-independent pathway might play an important role not only in myogenesis, but also in osteogenic conversion of C2C12 cells.

3.4. HIRA, Asf1a, and histone H3.3 participate in the initial commitment of osteogenic differentiation of C2C12 cells

The expression of HIRA/Asf1a/H3.3 decreases gradually when C2C12 cells differentiate into osteoblasts (Fig. 2). In detail, the levels of HIRA/Asf1a were kept temporally as high as ~50% of myoblastic level for up to 2 days, before further decreased by day 3. In addition, the level of H3.3 started to decrease on day 3. Such profile raises the possibility that HIRA/Asf1a/histone

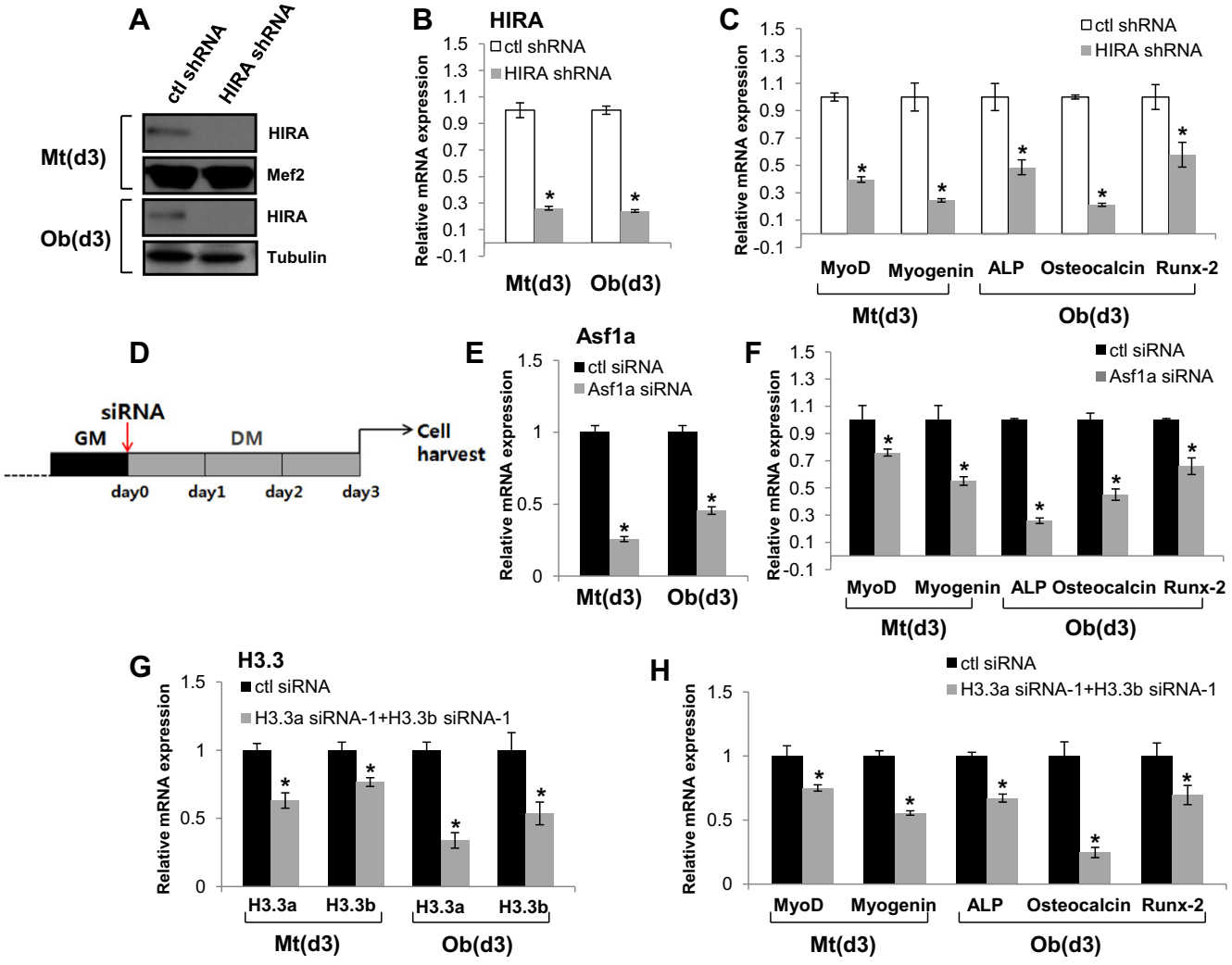

Fig. 3. HIRA, Asf1a and H3.3 are required for efficient expression of osteogenic and myogenic genes. (A and B) HIRA was reduced in C2C12 cells stably expressing shRNA against HIRA (HIRA shRNA) compared to that of cells expressing control shRNA (ctl shRNA) with shuffled RNA sequences. Mef2 and Tubulin were used as loading controls. (D) C2C12 cells were treated with siRNAs at day 0 and allowed to differentiate into myotubes or osteoblasts for 3 days. (B-H) The expression level of each gene was analyzed by the quantitative RT-PCR analysis at day 3. C2C12 cells were treated with siRNAs for knocking down Asf1a (E) or H3.3 (G). MyoD and Myogenin are myogenic genes. ALP, Osteocalcin, and Runx-2 are osteogenic genes. Error bars represent SD, $n = 3$.*, P value < 0.05.

H3.3 might be transiently required at early stages of the differentiation process. We therefore promptly examined whether they were engaged more importantly in the initial steps of osteoblastic conversion. To do this, we tried to deplete HIRA using siRNAs at different time window (Fig. 4A). This time, we treated the cells with control or each specific siRNAs 24 h after the cells had been induced for differentiation. Cells were allowed to differentiate for 2 more days and the expression of cell type-specific genes was analyzed by qRT-PCR. Interestingly, the expression of myogenic genes (*MyoD* and *Myogenin*) was affected even when HIRA was depleted once cells had entered myogenic differentiation pathway (Fig. 4B and C). However, the expression of osteogenic genes (*ALP*, *Osteocalcin*, and *Runx-2*) was barely affected by HIRA depletion, if the cells had undergone differentiation to the osteogenic direction (Fig. 4B and C). In addition, the effect of depletion of H3.3 or Asf1a was examined a day after the cells were induced for differentiation. Expression of osteoblastic marker genes was not significantly affected by knock-down of H3.3 or Asf1a, while that of myogenic genes decreased in this condition (Fig. 4D, E, and Supplementary Fig. S3). Our data showed that HIRA/Asf1a/H3.3 play important roles for the early steps of osteoblastic conversion of C2C12 cells.

4. Discussion

This study demonstrates the role of the histone chaperones, HIRA and Asf1a and variant histone H3.3, during myogenic or osteogenic differentiation of C2C12 cells.

C2C12 cells undergo differentiation into the multinucleated myotubes when fetal bovine serum in the culture medium is withdrawn by replacing it with low levels (2%) of horse serum. C2C12 cells also have a potential to differentiate into osteoblasts by BMP-2, which is known to completely inhibit the formation of the myotubes by repressing *MyoD* and *Myogenin* and to induce the expression of the osteoblastic markers [16,19,20], therefore establishing C2C12 cells as a model system to study cell fates, depending on culture condition. In this study, we sought to understand how replication-independent histone chaperones and variant histone H3.3 take a role to mediate two different lineages of differentiation. We previously reported that HIRA/Asf1a is required for efficient differentiation of C2C12 myoblasts into myotubes [14]. The similar strategy was applied to examine the contribution of H3.3-specific histone chaperones to osteogenesis of C2C12. In the direction of osteoblastic differentiation, expression of HIRA and Asf1a were maintained transiently around 50 percent, but

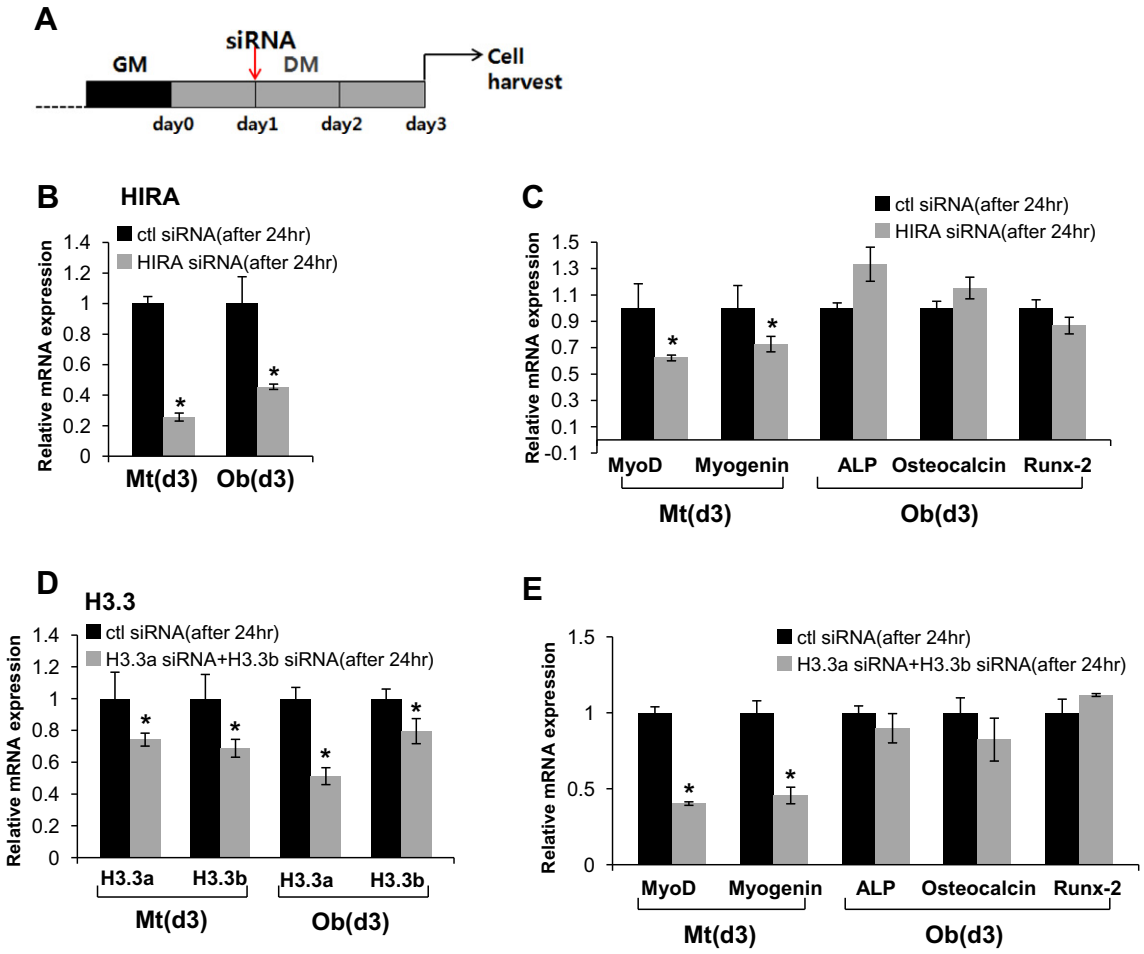

Fig. 4. Depletion of HIRA and H3.3 24-h-post differentiation shows different effect on gene expression. (A) Experimental scheme is depicted. C2C12 cells were treated with control siRNA or siRNAs for HIRA (B) or H3.3 (D) 24 h after cells were placed in the myogenic or osteoblastic differentiation condition. Cells were further incubated in each differentiation medium for 2 days. The expression level of each cell type-specific gene was evaluated by the quantitative RT-PCR analysis (C and E). Myogenic genes: MyoD, Myogenin; osteogenic genes: ALP, Osteocalcin, and Runx-2. Error bars represent SD, $n = 3$.*, P value < 0.05.

decreased further by day 3, whereas their expression was unchanged during myogenesis for days examined. In consistent with their cellular levels, differentiation of cells monitored by osteoblastic markers was affected by knock-down of HIRA, Asf1a, or H3.3. However, it was hardly affected when this treatment was applied to the cells that were under progress of osteoblastic differentiation. Current study suggests that HIRA, Asf1a, and H3.3 might be involved in not only myogenesis, but also osteogenesis of C2C12 cells. But their role is very likely distinguished in two differentiation pathways by a sustained requirement of H3.3-specific histone chaperones during myogenesis vs. transient requirement at a certain window of osteogenesis. Present study will add a clue on distinct management of epigenetic landscape for cell specification during differentiation.

It is interesting that histone H3.3 and histone chaperones are not equally required in every type of cells, but may function depending on the type and the state of cells, which can be explained with the concept of the H3 barcode hypothesis [21]. It speculates that H3 variants are deposited to form different epigenetic patterns depending on cell types and these patterns are important to ensure proper gene expression profiles. H3 variants are likely to index the genome in a way that the actively transcribed domain is enriched with H3.3, while transcriptionally silenced region is predominated by canonical histones, H3.1/H3.2. It is proposed that this barcoding of genomic regions with appropriate H3 variants ensures a long term cellular memory of the transcriptional status of genes and can be inherited through cell generations [12]. In this view, it is very likely that histone chaperones that are responsible for the deposition of each histone variants must play an active role in the remodeling of the chromatin domains, according to cell-type specific gene expression programs. H3.3 and HIRA/Asf1a might be needed in distinctive ways for myogenic and osteogenic programs. H3 barcoding and accompanied post translational modification of histones could enable cells to turn specific genes on or off after the cells receive appropriate directions of differentiation. Although the selective employment of histone H3 variants is supposed to play a critical role in cellular functions, further study is required to delineate which part of epigenome is actively remodeled to give the cell type-specific signatures. Future study will determine how the variant histone H3.3 is genome-wide distributed and which H3.3 loci are critical for cellular identity during muscle and bone differentiation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.026.

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