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Biochemical and Biophysical Research Communications 367 (2008) 97–102
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Hes1 stimulates transcriptional activity of Runx2 by increasing protein stabilization during osteoblast differentiation

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Received 3 December 2007
Available online 26 December 2007

Abstract

Runt-related transcription factor 2 (Runx2) is a key transcription factor for osteogenic gene expression and osteoblast differentiation. In order to maintain bone homeostasis, the transcriptional activity of Runx2 is tightly modulated by many intra- and extra-cellular factors. Here, we reveal the mechanism by which Hairy Enhancer of Split1 (Hes1) regulates the transcriptional activity of Runx2, and elucidate the potential role of Hes1 during osteoblast differentiation. Coexpression of Hes1 with Runx2 promoted an increase in Runx2 protein levels by increasing the half-life of Runx2; Hes1 thereby augmented the formation of a Runx2–DNA complex at Runx2 target sites. During osteoblast differentiation, the retroviral overexpression of Hes1 accelerated osteogenesis and stimulated the expression of osteogenic marker genes, including osteopontin and type 1 collagen. Taken together, these results suggest that Hes1 augments the protein level and transcriptional activity of Runx2, resulting in the stimulation of osteoblast differentiation.

Keywords: Runx2; Hes1; Osteoblast differentiation; Protein stability

Runt-related transcription factor 2 (Runx2; also known as Cbfa1, Pebp2αA, and AML3) is a master regulator of osteogenic gene expression and osteoblast differentiation [1]. Runx2 binds to the osteoblast-specific *cis*-acting element (OSE2) in the promoter region of several osteogenic genes, including osteocalcin, type 1 collagen, and osteopontin [1,2]. Runx2 knockout mice exhibited no bone tissues or osteoblasts, indicating that osteoblast differentiation is completely blocked in the absence of Runx2 [3]. Consistent with these observations, transgenic mice overexpressing the dominant negative form of Runx2 exhibited an osteopenic phenotype and reduced bone formation [4]. Recent studies have suggested that the expression and function of Runx2 are sensitively regulated by multiple factors, including post-translational modifications, hormones, and interactions with other proteins. For example, Runx2 activity is stimulated by MAPK signaling and negatively regulated by thrombin-like enzyme 2 (TLE2) [5,6]. Additionally, Runx2 protein can be controlled by treatment with cAMP, the major mediator of parathyroid hormone signaling, and cyclin D1-Cdk4 complex in osteoblasts [7,8]. Furthermore, the dissociation of Runx2 from histone deacetylases (HDACs) appears to accelerate the differentiation of osteoblasts by recruiting other transcriptional activators, such as p300 to Runx2 [9].

Hairy enhancer of split 1 (Hes1), a member of the basic helix-loop-helix (bHLH) transcription factor, is well known as a downstream negative regulator of Notch signaling [10]. Several *in vivo* and *in vitro* studies have indicated that Hes1 is essential for neurogenesis, myogenesis, hematopoiesis, and sex determination [10–13]. It has been demonstrated that Hes1 is also involved in several transcriptional activation processes. For instance, the binding of Hes1 to STAT-3 enhances gene expression in response to signals from epidermal growth factor [14]. In addition, the interaction between Hes1 and Runx2 enhances the

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0006-291X/$ - see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.bbrc.2007.12.100

transcriptional activity of Runx2 [15]. However, it is largely unknown how Hes1 regulates and/or enhances Runx2 activity during osteoblast differentiation.

In the present study, we reveal the regulatory mechanism by which Hes1 stimulates Runx2 activity in osteoblast differentiation. We observed that the stability of the Runx2 protein is specifically enhanced by Hes1, resulting in the augmented formation of a Runx2–DNA complex. Furthermore, overexpression of Hes1 in MC3T3-E1 cells promoted the expression of several Runx2 target genes, including type 1 collagen and osteopontin, and stimulated osteoblast differentiation, concomitantly increasing calcium nodule formation. Collectively, these results suggest a novel regulatory mechanism for Hes1-mediated Runx2 activation.

Materials and methods

Cell culture. Mouse MC3T3-E1 osteoblasts were maintained and differentiated as described previously [9]. HEK293 cells were maintained in DMEM supplemented with 10% bovine calf serum at 37 °C in 5% CO_2.

Western blot analysis and immunoprecipitation. Western blot analysis and immunoprecipitation were performed as described previously [9]. The membranes were probed with primary antibodies against Hes1 (a gift from Tetsuo Sudo, Japan), Runx2 (Santa Cruz), β-tubulin (Sigma), GAPDH (LabFrontier) and Myc (Cell Signaling). The bound antibodies were visualized by incubation with mouse or rabbit horseradish peroxidase-conjugated secondary antibodies (Sigma) and enhanced chemiluminescence.

mRNA analysis. Total RNA was isolated from MC3T3-E1 cells with Trizol reagent (Invitrogen) according to the manufacturer's protocol. Real-time PCR were performed as described previously [9]. The relative values of each mRNA were normalized to GAPDH. The primer sequences for real-time PCR analyses are available upon request.

Chromatin immunoprecipitation (ChIP) assay. Chromatin was immunoprecipitated as described previously [9]. The PCR products were resolved on 6% polyacrylamide/1× Tris–borate/EDTA gels. The primer sequences for PCR analyses are available upon request.

Oligonucleotide binding assay. Biotinylated oligonucleotide interactions were measured as described previously [16]. Briefly, HEK293 cells were transfected and collected in lysis buffer (4 mM Tris pH 7.5, 20 mM Hepes, pH 7.5, 5% glycerol, 170 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM $MgCl_2$, and 0.1% Triton X-100). Double-stranded OSE2 probes (5′-CCCAGGCAGCTGCAATCACCAACCACAGCATCCTTTGGG TTTGAC-3′) modified by the addition of biotin to the 5′ end, were added to cell lysates and allowed to link to cell lysates at 4 °C for 1 h. Streptavidin magnetic beads (Promega) were then mixed with the OSE2-coupled cell lysates. Bound complexes were washed 3 times with wash buffer (16 mM Hepes, pH 7.6, 100 mM NaCl, 0.4 mM EDTA, 1 mM $MgCl_2$, and 1% glycerol) using a magnetic stand. The complexes were dissolved in 1× SDS sample buffer and analyzed by Western blotting.

Transient transfection and luciferase assay. HEK293 cells were cotransfected with a luciferase reporter containing 6 OSE2 probes (p6OSE2-luc) and Runx2 and Hes1 expression vectors using the calcium phosphate method [17]. The cells were lysed and assayed for luciferase activity.

Results

Hes1 specifically increases the levels of Runx2 protein

It has been reported that Hes1 increases the transcriptional activity of Runx2 by removal of the repressor protein TLE2, suggesting the passive role of the Hes1 by dissociating a transcription repressor [15]. In order to test the direct effect of Hes1 on Runx2 in the absence of TLE2, we coexpressed Runx2 with Hes1 and examined the Runx2 protein levels in HEK293 cells, which have low levels of endogenous TLE2 protein [18]. The amounts of Runx2 protein were significantly increased by Hes1 (Fig. 1A, lane 2 vs. 4) while Hes1 protein levels were not affected by Runx2 (Fig. 1A, lane 3 vs. 4). Consistent with previous report [15], we observed that Hes1 directly interacted with Runx2 and Hes1 increased Runx2 protein levels (Fig. 1B). Moreover, Hes1 significantly promoted the transcriptional activity of Runx2 in the absence of TLE2 (Fig. 1C), implying that Hes1 directly regulates Runx2 activity by enhancing Runx2 protein stability.

To determine whether the stability of Runx2 protein is specifically affected by Hes1, we examined the level of Runx2 protein in the absence or presence of other Runx2-interacting proteins such as HDAC1 or TLE2 [9,15]. As illustrated in Fig. 1D, neither HDAC1 nor TLE2 increased the levels of Runx2 protein. Next, to explore whether Hes1 also increases the levels of other proteins, we cotransfected cells with ADD1/SREBP1c and Hes1, followed by Western blot analysis. ADD1/SREBP1c belongs to the same bHLH transcription factor family as Hes1, and its activity is negatively regulated by the Hes1 [19]. Unlike Runx2, the coexpression of Hes1 with ADD1/SREBP1c had no discernible effect on the levels of ADD1/SREBP1c protein (Fig. 1E), indicating that Runx2 protein levels are specifically increased by Hes1.

Hes1 increases the steady state level of Runx2 protein and Runx2–DNA complex formation

To test whether Hes1 could affect the half-life of Runx2 protein, cells were cotransfected with Runx2 and Hes1, and then treated with cycloheximide to inhibit the cellular protein synthesis. The abundance of the Runx2 protein was subsequently examined at several time points. As shown in Fig. 2A and B, the half-life of Runx2 protein was approximately 2 h 30 min; however, coexpression of Runx2 with Hes1 produced a substantial increase in the half-life of Runx2 (to approximately 5 h), implying that Hes1 enhances Runx2 protein stability by increasing the half-life of the Runx2 protein rather than by elevating Runx2 protein synthesis.

The Hes1-dependent increase in Runx2 protein stability prompted us to test the possibility that the Hes1-mediated increase in Runx2 protein levels might increase Runx2 binding to its target sequence such as OSE2 [20]. In order to assess whether Hes1 affects the target DNA binding activity of Runx2 to the OSE2 sequence, we conducted an *in vitro* biotinylated oligonucleotides pull-down assay, and this allowed us to analyze the protein composition in protein–DNA complexes (Fig. 2C). Consistent with the increase in Runx2 protein mediated by Hes1, Runx2–DNA complex formation at its target sequence was also augmented by Hes1 (Fig. 2D, lane 6 vs. 8). Additionally, we observed that Hes1 alone did not bind to the OSE2

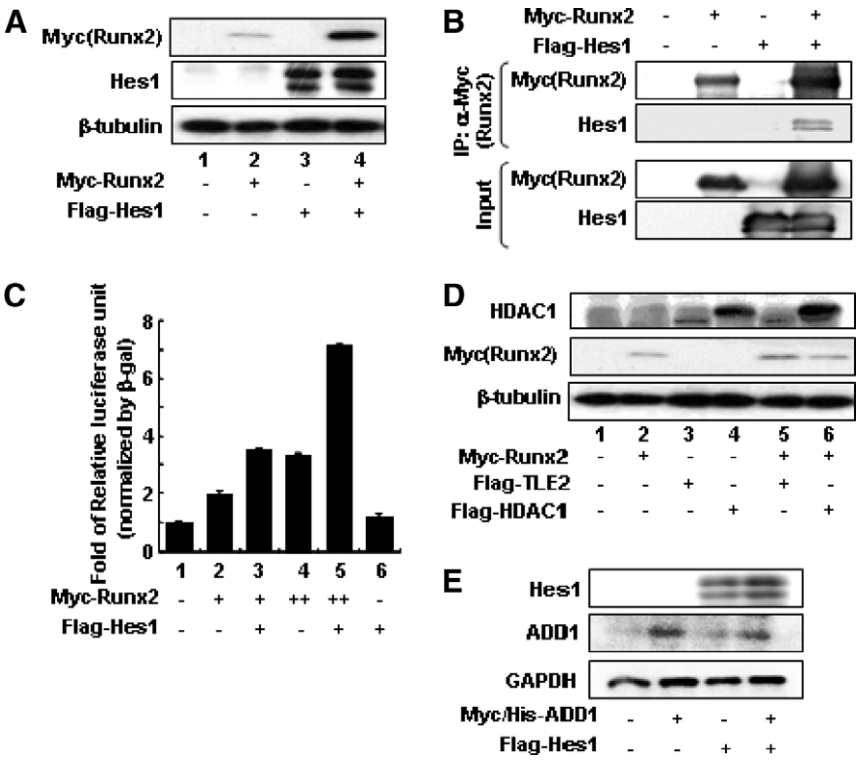

Fig. 1. Increase in Runx2 protein expression mediated by Hes1. (A and B) HEK293 cells were transfected with Myc-tagged Runx2 expression plasmid vector with or without Flag-tagged Hes1 expression vector. (A) Total cell lysates were subjected to Western blot analyses using antibodies against Myc and Hes1. (B) Association of Hes1 in the Runx2 immunocomplex. Total cell lysates were immunoprecipitated with anti-Myc antibody, followed by Western blot analyses with anti-Myc and anti-Hes1 antibodies. (C) OSE2-luciferase reporter construct was cotransfected with Myc-tagged Runx2 expression vector with or without Flag-tagged Hes1 into HEK293 cells. The luciferase activities were normalized using β-galactosidase assays. Error bars indicate standard deviations ($n = 2$). (D) HEK293 cells were transfected in combination with Flag-HDAC1, Flag-TLE2, or Myc-Runx2 expression vectors. Total cell lysates were subjected to Western blot analyses with antibodies against Myc and Hes1. (E) HEK293 cells were transfected in combination with Flag-Hes1 and Myc/His-nADD1/SREBP1c. Western blot analyses were performed using Hes1 and ADD1/SREBP1c antibodies. The levels of β-tubulin and GAPDH were used as an internal control. +, present; −, absent.

sequence (Fig. 2D, lane 7), and that Hes1 was barely detectable in the Runx2–DNA complex (Fig. 2D, lane 8). This implies that the Runx2–Hes1 protein complex would not preferentially bind to Runx2 target DNA elements. These results suggest that the increase in Runx2 protein stability mediated by Hes1 might lead to an increase in Runx2–DNA complex formation.

Expression of Hes1 during osteoblast differentiation

Osteoblast differentiation is accompanied by the induction of osteoblast-specific transcription factors, such as Runx2 and osterix [1,21]. Since, Hes1 enhanced the transcriptional activity of Runx2 (Fig. 1C), we decided to examine the expression levels of Hes1 during osteoblast differentiation. In mouse calvaria osteoblast MC3T3-E1 cells, substantial amounts of Hes1 protein were expressed and induced at a very early stage of osteoblast differentiation (Fig. 3A). Consistent with this observation, increased Hes1 mRNA was also detected during early osteogenesis (at differentiation day 1), similar to the levels of other osteogenic genes such as osteopontin and Runx2 (Fig. 3B). During osteogenesis, similar expression pattern of Hes1 were observed in other osteogenic cell line ROS 17/2.8 (data not shown). Therefore, it is likely that the early induction of Hes1 might be involved in osteoblast differentiation, probably by potentiating Runx2 activity.

Hes1 overexpression accelerates osteoblast differentiation

The observation that Hes1 expression was induced at the early stage of osteoblast differentiation led us to ascertain the effect of Hes1 overexpression on osteoblast differentiation. Retroviral overexpression of Hes1 in MC3T3-E1 cells resulted in a 1.75-fold increase in its mRNA compared to the mRNA in mock-infected cells (Fig. 4A). Compared with mock virus infected cells, Hes1 overexpression promoted the expression of osteogenic genes, including osteopontin and type 1 collagen (Fig. 4B). Moreover, the recruitment of Runx2 protein to the osteopontin promoter was substantially enhanced by Hes1 overexpression (Fig. 4C). However, the weak binding of Hes1 to the osteopontin promoter was not affected by Hes1 overexpression. Consistent with the gene expression profiles, the Hes1-overexpressing MC3T3-E1 cells stimulated the development of calcified nodules (Fig. 4D). These results clearly suggest that Hes1 overexpression stimulates osteogenesis, presumably by increasing the activity of Runx2.

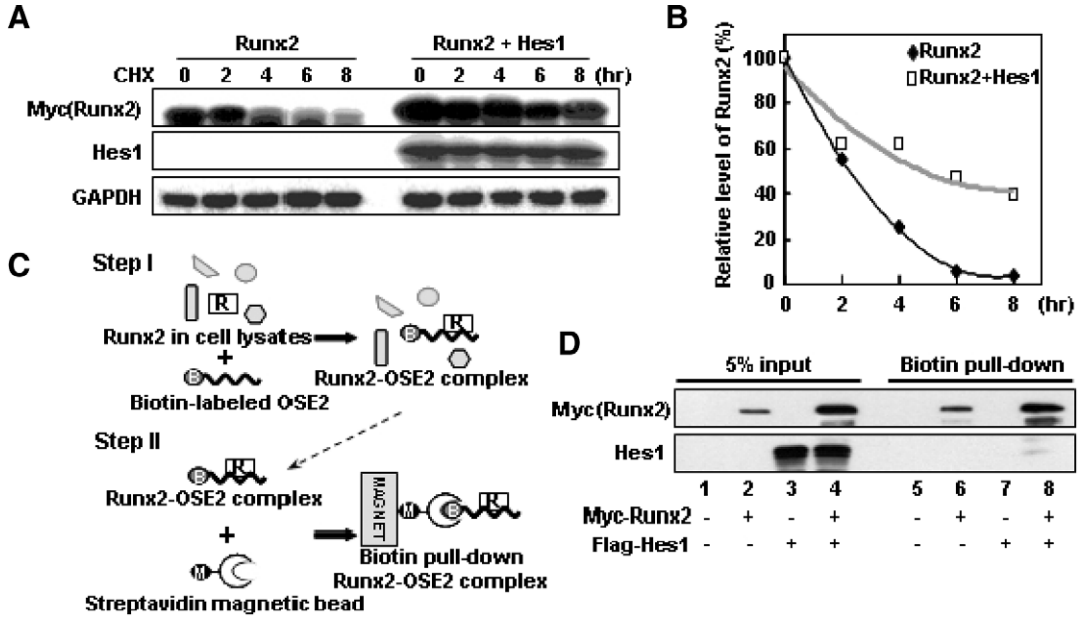

Fig. 2. Hes1 stabilized Runx2 protein and enhanced Runx2 binding to DNA. (A) HEK293 cells were transfected with Myc-tagged Runx2 plasmid with or without Hes1 expression vector. Transfected cells were treated with cycloheximide (CHX, 40 μg/ml) and harvested at the indicated time points. The levels of Runx2 and Hes1 proteins were analyzed by Western blotting using anti-Myc and anti-Hes1, respectively. The levels of GAPDH were used as the internal control. (B) Band intensities of Runx2 protein analyzed in (A) were quantified and plotted against time intervals to determine the half-life of Runx2 protein with or without Hes1 expression. (C) A diagram depicting the *in vitro* oligonucleotide pull-down binding assay. Nuclear extracts are mixed with biotin-labeled oligonucleotides. Runx2–OSE2 complexes are incubated with streptavidin magnetic beads and pulled down with a magnet. (D) Oligonucleotide binding assay were performed with cell lysates from HEK293 cells transfected with combinations of Myc-Runx2 and Flag-Hes1. +, present; −, absent.

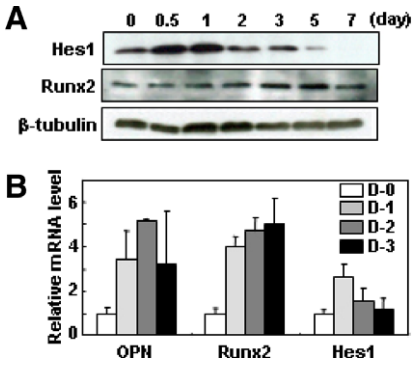

Fig. 3. Expression of Hes1 protein and mRNA during osteoblast differentiation. (A) During the differentiation of osteoblastic MC3T3-E1 cells, total cell lysates were prepared at the indicated time points. Immunoblottings were performed with anti-Runx2 and anti-Hes1 antibodies. The levels of β-tubulin were used as an internal control. (B) Total RNA was isolated during the differentiation of MC3T3-E1 cells at days (d) 0, 1, 2, and 3. The levels of Runx2, Hes1, osteopontin, and GAPDH mRNAs were determined using real-time PCR. Results are represented as the mean standard deviation of two independent experiments performed in triplicate.

Discussion

In this study, we investigated the molecular mechanism whereby Hes1 promotes the transcriptional activity of Runx2, knowledge of which is also important for a detailed understanding of osteoblast differentiation. We demonstrated that Hes1 positively regulates Runx2 activity by increasing its protein levels, and that Hes1 specifically enhances the level of Runx2 protein (Fig. 1). These results raised the possibility that Hes1 might promote the expression of Runx2 mRNA. However, Hes1 did not influence the mRNA levels of Runx2 (data not shown), suggesting that Hes1 modulates the activity and function of Runx2 at post-transcriptional levels. Apparently, the half-life of Runx2 when coexpressed with Hes1 was two-fold longer than that of Runx2 expressed alone (Fig. 2). Thus, we assumed that the increase in Runx2 protein expression accelerates the function of Runx2 in osteogenic gene expression. Clearly, Hes1 alone was able to augment the transcriptional activity of Runx2 (Fig. 1), which is consistent with previous observation [15]. Even though Hes1 is well known as a transcriptional repressor in neural differentiation [11], several recent studies have suggested that Hes1 is also actively involved in transcriptional activation processes. For instance, the interaction between Hes1 and Runx2 accelerates $1,25(OH)_2D_3$-induced osteopontin expression by dissociating HDAC1 in the promoter regions [22]. Also, the interaction between STAT3 and Hes1 augments JAK-STAT3 signaling by promoting the phosphorylation of STAT3 [14]. To the best of our knowledge, this is the first study to provide evidence that Hes1 positively regulates transcriptional activity by elevating the stability of another protein. Here, we demonstrated that Hes1 evidently increases Runx2 protein levels and augments Runx2–DNA complex formation, implicating the role of Hes1 in potentiating Runx2 transcriptional activity.

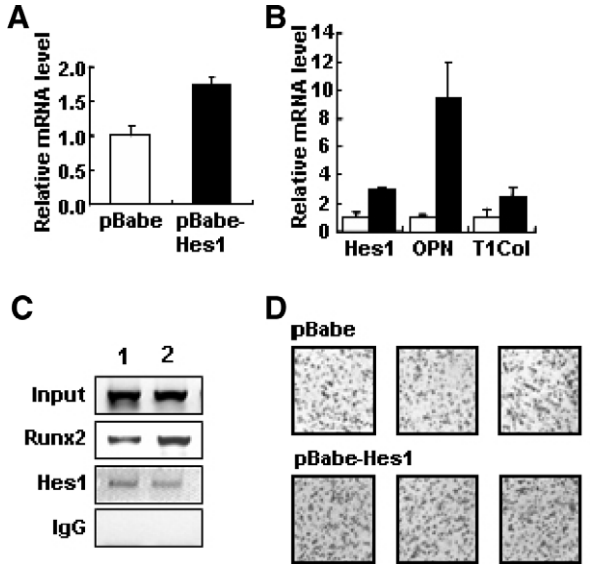

Fig. 4. Retroviral overexpression of Hes1 stimulated expression of osteoblast differentiation target genes. (A) Relative mRNA levels of Hes1. Total RNA from each cell type was isolated from pBabe or pBabe-Hes1-overexpressing MC3T3-E1 cells before inducing differentiation. The relative amount of Hes1 mRNA was analyzed by real-time PCR. (B) Retroviral mock or Hes1-overexpressing MC3T3-E1 cells were differentiated into mature osteoblast. Total RNAs from differentiated pBabe or pBabe-Hes1-overexpressing MC3T3-E1 cells (d 8) were isolated and analyzed by quantitative real-time PCR. OPN, osteopontin; T1Col, type 1 collagen. (C) Association of Runx2 and Hes1 in the promoter region of osteopontin genes. ChIP assays were performed with differentiated MC3T3-E1 osteoblasts (d 8), which were retrovirally overexpressed with mock or Hes1 constructs. Anti-Runx2, anti-Hes1, and rabbit preimmune serum were used for ChIP assays. (D) Confluent pBabe or pBabe-Hes1-overexpressing MC3T3-E1 cells were differentiated for 8d and monitored by photomicroscopy using Alizarin Red staining to measure the calcium deposition.

Further, when the levels of input proteins (Fig. 2D, lanes 2 and 4) were compared with those of DNA-bound proteins (Fig. 2D, lanes 6 and 8), the folds of increased Runx2 protein were similar in the two groups. This observation suggests that the increase in levels of DNA-binding complex mediated by Hes1 is mainly due to the stabilization of the Runx2 protein and not due to a change in the target DNA binding affinity of Runx2. In connection with this increase in DNA-binding complex, another interesting question is whether Hes1 forms a protein complex with Runx2 when Runx2 binds to its target promoters. Although Hes1 was able to form a protein complex with Runx2 (Fig. 1B), only a very small portion of Hes1 protein was detected in the Runx2–DNA complex (Fig. 2D, lane 8). Similarly, as demonstrated by the ChIP assays, the amounts of amplified DNA fragment from the osteopontin promoter did not differ between Hes1-overexpressing cells and control cells (Fig. 4C), implying that overexpressed Hes1 protein does not bind to the osteopontin promoter as a Runx2–Hes1 complex. Based upon these results, it is possible to propose that Hes1 modulates Runx2 activity via Runx2 protein stabilization and not by forming the Runx2–Hes1–DNA complex.

As described above, we observed that Hes1 could specifically increase the protein levels and elevate the half-life of Runx2. Thus, we postulated that Hes1 might also affect the degradation of Runx2, particularly via Runx2 ubiquitination. Although treatment with the 26S proteasomal inhibitor MG132 increased the protein levels and transcriptional activity of Runx2, treatment with MG132 did not increase the protein levels of Runx2 when Runx2 was coexpressed with Hes1 (Supplementary Fig. 1 and data not shown). Moreover, we observed that Hes1 did not decrease the ubiquitination of Runx2, which is somewhat contrary to expectations (Supplementary Fig. 1). There are several possible explanations for these conflicting results. For example, Runx2 interacts with a ubiquitin E3 ligase, Smad ubiquitin regulatory factor 1 (Smurf1), which inhibits osteoblast differentiation and bone formation [23]. Although Hes1 did not decrease the ubiquitination of Runx2 in the absence of Smurf1, it is possible that Hes1 might play a role in protecting Runx2 from degradation in the presence of Smurf1. Another possible explanation for this observation is that Hes1 might inhibit the degradation of Runx2 independent of the ubiquitination process. Clearly, further studies are required in order to completely decipher the detailed mechanism by which Hes1 specifically increases the levels of Runx2 protein.

We observed that Hes1 expression increased at an early stage of osteoblast differentiation in MC3T3-E1 cells (Fig. 3). This change in Hes1 expression suggests a role for Hes1 in regulating osteoblast differentiation, possibly by controlling Runx2 activity during osteogenesis. The expression of Hes1 might lead to an increase in Runx2 protein, thereby elevating the expression of Runx2 target genes. Consistent with this idea, exogenous Hes1 overexpression led to an increase in osteoblast differentiation, presumably resulting from enhanced Runx2 activity elevating Runx2 target gene expression (Fig. 4).

In conclusion, we have revealed a novel pathway by which Hes1 regulates the transcriptional activity of Runx2 at the protein level. It is likely that stabilization of the Runx2 protein mediated by Hes1 increases the expression of Runx2 target genes, thereby causing enhanced osteoblast differentiation. Thus, the regulation of Runx2 by Hes1 offers a new approach to the regulation of osteoblast differentiation.

Acknowledgments

We thank the following investigators for generously supplying materials and reagents: Dr. T. Sudo for the anti-Hes1 Antibody; Dr. S.-C. Bae and Dr. H.M. Ryu for the Ose2-luciferase vector and the Myc-Runx2 expression vector; and Dr. R. Kageyama for the pMSCV-Hes1 vector.

This study was supported in part by grants from the Stem Cell Research Center of the 21st Century Frontier

Research Program and the National Research Laboratory Program of the Korea Science and Engineering Foundation. J.H.S, H.W.L. J.-W.L., and J.B.K. were supported by a BK21 Research Fellowship from the Ministry of Education and Human Resources Development.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.12.100.

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