Wdr5 Is Essential for Osteoblast Differentiation

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Wdr5 is developmentally expressed in osteoblasts and accelerates osteoblast differentiation in vitro and in vivo. To address whether Wdr5 is essential for osteoblast differentiation, plasmid-based small interfering RNAs were used to stably suppress endogenous Wdr5 protein levels in MC3T3-E1 cells. Reduction of endogenous Wdr5 levels markedly inhibited osteoblast differentiation, evidenced by a significant decrease in alkaline phosphatase activity, Runx-2 and osteocalcin mRNAs, and absence of mineralized matrix formation. Wdr5 suppression also resulted in a reduction of histone H3 lysine 4 trimethylation, confirming its critical role in this modification. Because Wdr5 overexpression enhances canonical Wnt signaling in osteoblasts in vitro, the effects of Wdr5 silencing on this pathway were examined. The expression of the canonical Wnt target gene, c-myc, was decreased, whereas that of sfrp2, which is repressed by Wnt signaling, was increased with Wdr5 knockdown. Although only a minimal increase in apoptosis was observed, the antiapoptotic effect of Wnt signaling was also impaired with Wdr5 silencing. The expression of canonical Wnts was significantly decreased with Wdr5 knockdown, resulting in a decrease in nuclear β-catenin protein levels. Activation of the canonical Wnt signaling pathway did not overcome the effects of Wdr5 knockdown on the expression of Wnt target genes. Chromatin immunoprecipitation demonstrated that Wdr5 is present on the Wnt1 promoter and on canonical Wnt response elements of the c-myc and Runx-2 promoters. These studies demonstrate that Wdr5 suppression interferes with the canonical Wnt signaling pathway at multiple stages and that optimal Wdr5 levels are required for induction of the osteoblast phenotype.

Wdr5, a BMP-2-induced gene, is a WD repeat protein that is essential for histone H3 lysine 4 (H3K4) trimethylation, a marker of actively transcribed genes (1, 2). Targeting expression of Wdr5 to osteoblasts using the 2.3-kb fragment of the mouse α(1) 1 collagen promoter results in acceleration of endochondral bone formation during embryonic development (3).

Characterization of the molecular mechanisms by which Wdr5 overexpression exerts these effects demonstrated that Wdr5 enhances canonical Wnt signaling (3). Wnt proteins are secreted signaling factors that play a key role in development and in adult tissue homeostasis. These proteins bind to Frizzled G protein-coupled receptors and low density lipoprotein receptor-related protein (LRP) cell surface co-receptors. Signaling by the Wnt/β-catenin pathway, also referred to as the canonical Wnt signaling pathway, impairs degradation of cytoplasmic β-catenin, resulting in its nuclear translocation and regulation of target gene expression. The canonical Wnt signaling pathway has been shown to promote osteoblast differentiation during skeletal development and to regulate bone mass accrual postnatally (4–15). The findings that loss and gain of function mutations in the Wnt co-receptor LRP5 result in low and high bone mass, respectively, and that mice harboring gain or loss of function mutations in β-catenin in osteoblasts develop a high and low bone mass phenotype, respectively, (7, 15–17) underscore the important role of the canonical Wnt signaling pathway as a modulator of skeletal development and homeostasis.

Based on our previous investigations demonstrating that overexpression of Wdr5 enhances canonical Wnt signaling and osteoblast differentiation, studies were undertaken to determine whether Wdr5 was essential for osteoblast differentiation and optimal canonical Wnt signaling. Plasmid-based RNA-mediated interference was used to decrease endogenous Wdr5 protein levels in MC3T3-E1 cells. Although synthetic siRNAs have been successfully used to suppress gene expression, when this technique is used, the resultant reduction in gene expression is transient, allowing only short term analyses of the resultant phenotype (18–22). Thus, plasmid-based siRNA, which results in a more stable suppression of gene expression, was used for our analyses.

EXPERIMENTAL PROCEDURES

Cell Culture—MC3T3-E1 cells were cultured in αMEM, 10% fetal bovine serum, and 1% penicillin/streptomycin for periods ranging from 4 to 29 days. In experiments performed to evaluate mineralized matrix formation, medium was supplemented with β-glycerolphosphate (10 mM) and ascorbic acid (50 μg/ml) (Sigma). Cells, stably transfected either with Wnt-3a (L-Wnt-3a) or with an empty vector (L) as source for production of Wnt-3a conditioned medium (CM) or control CM, were purchased from ATCC (Manassas, VA). Conditioned media were prepared following the ATCC protocol. To evaluate the activation of the Wnt signaling pathway, confluent cells were treated for 5 h with control CM or Wnt-3a CM prior to RNA extraction. To evaluate the effect of BMP-2, confluent cells were treated for 2, 4, or 24 h with BMP-2 (100 ng/ml) prior to RNA extraction.
siRNA Design—Using the “design tool” provided by Ambion (Ambion, Austin, TX), four siRNAs (siRNAs 1, 2, 3, and 4) targeting the mouse Wdr5 cDNA sequences were designed as was one scrambled sequence (siRNA-S). BLAST (NCBI) analyses were performed to exclude homology with other genes in the eukaryotic genome. The selected siRNAs were then subcloned into the pSilencer 3.1-H1 neo siRNA expression vector (Ambion), downstream from the H1 RNA polymerase III promoter. DNA sequence analysis was performed to confirm insertion of the selected siRNAs.

Transfections—MC3T3-E1 cells were plated at a density of 3 × 10^5 cells/cm². After 24 h, they were stably transfected with each of the vectors using calcium phosphate precipitation. The following day and every 48 h thereafter for 14 days, medium was replaced with fresh medium containing 300 μg/ml G418. This dose of G418 has been shown to kill 100% of untransfected cells in 10 days. G418-resistant colonies were then isolated to evaluate Wdr5 protein levels.

Western Analysis—Cells were plated at a density of 5 × 10^5 cells/cm² and cultured for 4 and 7 days. Cells were lysed in 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton. Ten μg of protein was subjected to SDS-PAGE under reducing conditions of the selected siRNAs. Blots were probed with antibodies specific to Wdr5 (BD Biosciences), downstream from the H1 RNA polymerase III promoter, with GAPDH (Cell Signaling Technologies) as a loading control. Immunoreactive bands were visualized using a chemiluminescence detection kit (PerkinElmer Life Sciences) according to the manufacturer’s instructions.

Alkaline Phosphatase Activity—Cells were plated at a density of 5 × 10^5 cells/cm² and cultured for periods ranging from 4 to 29 days. At each time point, the alkaline phosphatase activity in cell lysates was assessed in assay buffer (50 mM Tris-HCl, pH 7.6, and 0.1% Triton X-100) containing 1.5 μM 2-amino-2-methyl-1-propanol for 1 h at 37°C using p-nitrophenylphosphate as a substrate. The release of p-nitrophenol was monitored by measuring absorbance at 405 nm.

Real-time PCR—Cells were plated at a density of 5 × 10^5 cells/cm² and cultured for periods ranging from 4 to 29 days. Total RNA was extracted using TRI reagent (Sigma). Twenty μg of total RNA was DNase-digested (MessageClean kit, Genhunter Corp., Brookline, MA) following which cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using the Opticon DNA engine system (MJ Research, Waltham, MA). The efficacy of DNase digestion was confirmed by performing analyses in the presence and absence of reverse transcriptase. mRNA levels encoding each gene of interest were normalized for actin mRNA in the same sample using the formula of Livak and Schmittgen (24), in a method identical to that used to normalize levels of RNA of interest to those of housekeeping genes (actin or GAPDH). Fold enrichment reflects the ratio of promoter/control sequences in the immunoprecipitate versus the input samples. Normal rabbit IgG was used as a control.

Statistical Analyses—All values are expressed as mean ± S.E. Student’s paired t test was used to identify significant differences between the scrambled and specific siRNA-transfected cells at each time point. A p value <0.05 was considered statistically significant.

RESULTS

Overexpression of Wdr5 dramatically accelerates the program of osteoblast differentiation in vitro and in vivo. Studies were, therefore, undertaken to determine whether Wdr5 is essential for osteoblastic differentiation. The murine osteoblast cell line MC3T3-E1 was chosen for these studies since these cells have features of preosteoblasts and recapitulate the program of osteoblast differentiation when cultured for up to 4 weeks (28). In addition, MC3T3-E1 cells have been used by other investigators to study the canonical Wnt signaling pathway (29–31). MC3T3-E1 cells were stably transfected with vectors containing siRNAs 1, 2, 3, and 4, which specifically target the Wdr5 coding region, or with a scrambled siRNA (siRNA-S).
Endogenous Wdr5 protein levels were assessed by Western analyses after 4 and 7 days in culture. At 4 days, clones expressing siRNA-2 and siRNA-4 demonstrated a significant decrease in endogenous Wdr5 protein levels relative to that of the siRNA-S clones (Fig. 1, B and C). This decrease persisted at 7 days in culture, when Wdr5 protein levels are maximally expressed (23) (Fig. 1, B and C). Wdr5 protein levels were not affected by either siRNA-1 or siRNA-3 (data not shown); therefore, studies were performed with clones expressing siRNA-2, siRNA-4, and siRNA-S.

Because Wdr5 is essential for H3K4 trimethylation (1, 2), a marker of actively transcribed genes, the effect of Wdr5 knockdown on H3K4 trimethylation was investigated. Suppression of Wdr5 expression resulted in a significant decrease in H3K4 trimethylation (Fig. 1 D).

Acquisition of the osteoblast phenotype is characterized by the ability of cells to synthesize alkaline phosphatase; an increase in the specific activity of this enzyme is directly correlated with a more differentiated state. Therefore, the time course of onset and magnitude of alkaline phosphatase activity was evaluated. In contrast to cells expressing siRNA-S, siRNA-2 and siRNA-4 cells failed to show a significant increase in alkaline phosphatase activity from 4 to 29 days in culture (Fig. 2 A).

To determine whether the expression of other markers of osteoblast differentiation was altered when Wdr5 expression was suppressed, the level of the mRNAs encoding Runx2, a transcription factor involved in osteoblast commitment and differentiation, and of osteocalcin, a marker of late osteoblast differentiation, was examined by quantitative real-time PCR. At 4 days in culture, Runx2 mRNA expression was significantly decreased by 5.2 ± 0.18 S.E. and 3-fold ±0.57 S.E. in siRNA-2 and siRNA-4 cells, respectively, relative to that of cells expressing siRNA-S. This decrease in Runx2 mRNA persisted until 29 days in both the siRNA-2 and the siRNA-4 cells (Fig. 2 B). As shown in Fig. 2 B, cells expressing siRNA-2 and siRNA-4 failed to demonstrate the differentiation-dependent increase in osteocalcin mRNA expression observed in the siRNA-S cells.

The final stage of osteoblast differentiation is the formation of mineralized matrix. Cells expressing siRNA-2 and siRNA-4 failed to deposit mineralized matrix (Fig. 2 C), demonstrating that Wdr5 knockdown dramatically impairs osteoblast differentiation. This impairment in differentiation was not associated with an alteration in cell doubling time (data not shown).

Based on our previous studies demonstrating that overexpression of Wdr5 enhances canonical Wnt signaling in osteoblasts in vivo, investigations were performed to determine
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FIGURE 2. Wdr5 is essential for osteoblast differentiation. Osteoblast differentiation was evaluated in MC3T3-E1 cells stably expressing siRNA-2, siRNA-4, or siRNA-S, cultured from 4 to 29 days (hatched bars = 4 days; light gray bars = 7 days; open bars = 14 days; gray bars = 21 days; black bars = 29 days). A, alkaline phosphatase activity. Data shown are based on four independent experiments. The results are the mean ± S.E., p < 0.005 by Student’s t test for siRNA-2 and siRNA-4 versus siRNA-S at all time points. PNPP, p-nitrophenylphosphate. B, quantitative RT-PCR. Expression of Runx-2 and osteocalcin mRNA was normalized to that of actin in the same sample. Values are expressed as relative expression of the normalized mRNAs levels of the siRNA-2 and siRNA-4 cells relative to those of siRNA-S cells. Data shown are based on three independent mRNA isolations ± S.E. Runx-2, p < 0.005 by Student’s t test for siRNA-4 and siRNA-2 versus siRNA-S at all time points, and osteocalcin, p < 0.005 by Student’s t test for siRNA-4 and siRNA-2 versus siRNA-S at all time points. C, calcium content. The results are the mean ± S.E. of that obtained in six independent samples. p < 0.005 by Student’s t test for siRNA-2 and siRNA-4 versus siRNA-S at all time points.

Because canonical Wnt signaling has been shown to inhibit osteoblast and osteocyte apoptosis in vitro and in vivo, investigations were performed to address whether enhanced apoptosis was observed with Wdr5 knockdown. After 4 days in culture, no significant differences were observed in activated (cleaved) caspase-9, a key effector of the mitochondrial apoptotic pathway in siRNA-2 and siRNA-4 cells relative to siRNA-S cells (Fig. 4). However, a significant increase in total and cleaved caspase-9 was observed at 7 days in culture in both siRNA-2 and siRNA-4 cells relative to that of cells expressing siRNA-S. The increase in caspase-9 was accompanied by decreased expression of the antiapoptotic protein Bcl-2 at both 4 and 7 days in culture in siRNA-2 and siRNA-4 cells when compared with siRNA-S cells (Fig. 4). To determine whether increased apoptosis could be responsible for the impaired osteoblast differentiation observed when Wdr5 protein levels are suppressed, apoptosis was evaluated by flow cytometry using double staining with annexin V-phycocerythin to detect phosphatidylserine on the external membrane of early apoptotic cells and the cell-impermeant dye, Nexin 7-AAD, as an indicator of membrane structural integrity. Quantitation of early and late apoptotic cells at 4 and 10 days in culture revealed that suppression of Wdr5 resulted in a significant increase in apoptotic cells in the siRNA-2- and siRNA-4-4 cells relative to that of cells expressing siRNA-S. (Fig. 4C) However, the absolute increase in apoptosis remained less than 4% in the siRNA-2 and siRNA-4 cells (Fig. 4C), suggesting that the observed impairment in osteoblast differentiation is not a reflection of a profound increase in cell apoptosis.

Investigations were then performed to address whether changes in the expression of canonical Wnts could account for impaired canonical Wnt signaling pathway. As shown in Fig. 5A, the expression of Wnt1 and Wnt3a was markedly decreased in the siRNA-2- and siRNA-4 cells at both 4 and 7 days in culture when compared with siRNA-S cells. To address the functional consequences of the decreased expression of canonical Wnts, nuclear β-catenin levels were examined. A decrease in nuclear β-catenin levels was observed in the siRNA-2- and
siRNA-4 cells relative to the siRNA-S cells (Fig. 5, B and C), whereas expression of cytosolic β-catenin was not affected at either time point (data not shown).

Since BMP-2 has been shown to activate canonical Wnt signaling, we addressed whether our observations could reflect impaired responsiveness to endogenous BMPs produced by MC3T3-E1 cells. Thus, the expression of Runx2 and Wnt1 was examined after treatment of cells with 100 ng/ml BMP-2. As shown in Fig. 6A, Runx-2 and Wnt1 mRNA expression was significantly induced by 2 h of BMP-2 treatment in siRNA-S cells and was markedly impaired by Wdr5 knockdown (Fig. 6A). These findings demonstrate that BMP signaling is impaired when Wdr5 levels are suppressed. However, induction of a transiently expressed BRE-Luc fusion gene was not impaired in the siRNA-2 and siRNA-4 cells (Fig. 6B), suggesting that optimal Wdr5 levels are required for induction of gene expression only when chromatin modifications are involved.

To address whether activation of the canonical Wnt signaling pathway could overcome the effects of Wdr5 knockdown, induction of gene expression in response to Wnt3a was examined. As shown in Fig. 6C, whereas treatment with Wnt3a conditioned medium resulted in a significant increase in Runx-2 and c-myc mRNA expression in siRNA-S cells, this increase was significantly attenuated in siRNA-2 and siRNA-4 cells.

Chromatin immunoprecipitation analyses were then performed to assess whether Wdr5 interacts with the promoters of these genes. As shown in Fig. 7, Wdr5 was specifically recruited to the Wnt1 promoter and to Wnt response elements of c-myc (−1447/−1144) and Runx-2 (−123/+123), the latter of which overlaps its promoter (27). These findings demonstrate that when endogenous Wdr5 levels are suppressed, BMP and canonical Wnt signaling is impaired, establishing that Wdr5 is critical for the effects of these pathways on induction of the osteoblast phenotype.

**DISCUSSION**

A number of transcription factors, cytokines, growth factors, and hormones have been shown to play an important role in osteoblast differentiation. However, investigations in cells or animal models in which expression of these factors is suppressed or ablated do not uniformly identify an essential role for these factors in osteoblast differentiation. In contrast, our investigations into the role of Wdr5 in osteoblast differentiation demonstrate that this WD repeat protein, which activates gene transcription via chromatin remodeling, is not only capable of
accelerating osteoblast differentiation when it is overexpressed (3, 23) but is actually essential for osteoblast differentiation. Although cells with siRNA-mediated Wdr5 knockdown exhibited low levels of Runx2 early in culture, they were incapable of acquiring markers of early or late osteoblasts, including expression of alkaline phosphatase activity, expression of osteocalcin mRNA, or mineralized matrix formation. The finding that trimethylation of H3K4 was impaired when Wdr5 is suppressed is consistent with previous findings that Wdr5 plays a crucial role in histone modification (1, 2).

Overexpression of Wdr5 in osteoblasts accelerates endochondral bone formation by interacting with and enhancing the activity of the canonical Wnt signaling pathway (3). This pathway has several roles in osteoblastogenesis; it is required for osteoblast differentiation during skeletal development and participates in the regulation of skeletal homeostasis during postnatal life (6–14). Consistent with our previous findings demonstrating enhanced canonical Wnt signaling when Wdr5 was overexpressed in vivo, suppression of Wdr5 protein levels results in decreased expression of Wnt1 and Wnt3a. As a result, nuclear β-catenin is decreased, and impaired regulation of target genes of the canonical Wnt signaling pathway occurs when Wdr5 levels are suppressed. Chromatin immunoprecipitation experiments also show that Wdr5 is found in association with the β-catenin/TCF response elements of the Runx2 promoter (27), with the Left/TCF binding elements of c-myc promoter as well as with the Wnt1 promoter. Thus, Wdr5 is required for optimal expression of canonical Wnts and for their downstream actions. Similar to studies demonstrating that β-catenin recruits the MLL2 complex (which contains Wdr5) to the c-myc gene (26), Wdr5 may be required to promote chromatin modifications at the promoters of genes involved in osteoblast differentiation.

Although suppression of Wdr5 protein levels leads to impaired expression of mRNAs encoding markers of osteoblast differentiation and of c-myc, which is up-regulated in response to canonical Wnt signaling, this does not reflect a global suppression of gene expression. The expression of the housekeeping gene, actin, was not altered by Wdr5 silencing, and the expression of Sfrp2, which is suppressed by canonical Wnt signaling, was enhanced in response to Wdr5 silencing. Furthermore, BMP-2 induction of a transiently expressed fusion gene was not impaired in cells with Wdr5 knockdown.

BMP-2 has been shown to enhance Wnt1 and Wnt3a expression and to activate β-catenin signaling (30, 32). Our findings that Wdr5 is required for effects of BMP signaling on canonical Wnt signaling suggest that Wdr5 may be required for the expression of genes that promote osteoblast differentiation in response to these two signaling pathways.

The observation that activation of the canonical Wnt signaling pathway does not overcome the effects of Wdr5 suppres-
sion on Wnt target genes confirms that the impairment in osteoblast differentiation observed in cells stably expressing siRNAs targeting Wdr5 is due, at least in part, to a decrease in canonical Wnt signaling. These data are consistent with in vivo findings that loss or gain of function of several key effectors of the canonical Wnt signaling pathway is associated with either a decrease or an increase in osteoblast differentiation and function. However, our investigations cannot exclude the possibility that Wdr5 also interacts with other signaling pathways that play a role in osteoblast differentiation.

Activation of the canonical Wnt signaling pathway prevents osteoblast apoptosis (4, 29, 33–35). Wnt1 inhibits apoptosis by blocking caspase-9 activation, and Wnt3a induces expression of Bcl-2 in MC3T3-E1 cells, thereby prolonging their survival (29, 36). Thus, the increased cleavage of caspase-9 and decreased expression of the antiapoptotic protein, Bcl-2, observed in cells stably expressing Wdr5 siRNAs are most likely a consequence of impaired canonical Wnt signaling. However, our results cannot rule out the possibility that Wdr5 plays a role in cell survival by activating or inactivating additional signaling pathways involved in cell survival. Notable in this respect is the observation that, despite the marked effects of Wdr5 silencing on expression of Bcl2 and activation of caspase-9, there is only a minimal increase in apoptosis in these cells. Thus, these data suggest that other factors are called into play in the cells with Wdr5 knockdown that modulate the downstream consequences of enhanced activation of the mitochondrial apoptotic pathway. Furthermore, this modest increase in cell death observed cannot account for the dramatic effect of Wdr5 silencing on the program of osteoblast differentiation. Additional investigations will be required to determine the mechanisms by which the effects of Wdr5 on chromatin lead to enhanced osteoblast survival and differentiation.

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