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Wdr5, a WD-40 protein, regulates osteoblast differentiation during embryonic bone development

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Abstract

Wdr5 accelerates osteoblast and chondrocyte differentiation in vitro, and is developmentally expressed in osteoblasts as well as in proliferating and hypertrophic chondrocytes. To investigate the role of Wdr5 during endochondral bone development, transgenic mice overexpressing Wdr5 under the control of the 2.3-kb fragment of the mouse α(1) I collagen promoter were generated. The transgene was specifically expressed in the osteoblasts of transgene positive mice and was absent in the growth plate. Histological analyses at embryonic day 14.5 demonstrated that the humeri of transgene positive embryos were longer than those isolated from wild-type littermates largely due to an expansion of the hypertrophic chondrocyte layer. Acceleration of osteoblast differentiation was observed with greater and more extensive expression of type I collagen and more extensive mineral deposition in the bone collar of transgene positive embryos. Acceleration of vascular invasion was also observed in transgene positive mice. Postnatal analyses of transgenic mice confirmed persistent acceleration of osteoblast differentiation. Targeted expression of Wdr5 to osteoblasts resulted in earlier activation of the canonical Wnt signaling pathway in the bone collar as well as in primary calvarial osteoblast cultures. In addition, overexpression of Wdr5 increased the expression of OPG, a target of the canonical Wnt signaling pathway. Overall, our findings suggest that Wdr5 accelerates osteoblast differentiation in association with activation of the canonical Wnt pathway.

Keywords: Wdr5; BIG-3; Endochondral bone formation; Wnt signaling; Osteoblasts; Differentiation; Osteoprotegerin

Introduction

Skeletal development in vivo occurs via two major processes, intramembranous and endochondral ossification. Both intramembranous and endochondral ossifications begin with condensation of mesenchymal cells that form a template for the skeleton and end with formation of a mineralized skeleton. However, while intramembranous ossification occurs by direct differentiation of mesenchymal cells into osteoblasts, endochondral bone formation is a multistep process where chondrocytes form a mould, in which vascular invasion occurs followed by formation of an ossification center containing type I collagen-expressing osteoblasts. At the molecular level, several factors are known to cooperate in regulating the sequential steps of endochondral bone formation (Kronenberg, 2003), including the bone morphogenetic protein (BMP) (Canalis et al., 2003; Ducy and Karsenty, 2000; Hoffmann and Gross, 2001; Kronenberg, 2003), parathyroid hormone-related peptide (PTHrP), Indian Hedgehog (Ihh) (Chung et al., 1998, 2001; Karaplis et al., 1994; Karp et al., 2000; Kobayashi et al., 2002; Kronenberg, 2003; Lanske et al., 1999; Schipani et al., 1997; St-Jacques et al., 1999; Vortkamp et al., 1996), fibroblast growth factor (FGF) (Colvin et al., 1996; Kronenberg, 2003; Ornitz, 2005; Wang et al., 1999) and Wnt signaling pathways among others (Akiyama et al., 2004; Glass et al., 2005; Hartmann and Tabin, 2000; Hu et al., 2005; Kawakami et al., 1999, 2000; Westendorf et al., 2004; Yamaguchi et al., 1999).

We identified a novel BMP-2-induced gene named BIG-3 (*B*MP-2 *I*nduced *G*ene 3 kb) and recently renamed Wdr5, that dramatically accelerates the program of osteoblastic differentiation in MC3T3-E1 cells. Wdr5 encodes a protein belonging to the family of the WD-40 proteins. This family of proteins has been shown to play a role in numerous cellular functions including signal transduction, mRNA processing, gene regulation, vesicular trafficking and cell cycle regulation (Neer et al.,

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1994; Smith et al., 1999; Wysocka et al., 2003). Wdr5 is expressed in immortalized marrow stromal cells, osteoblasts, osteocytes and chondrocytes (Gori et al., 2001). Overexpression of Wdr5 dramatically accelerates the program of osteoblast and chondrocyte differentiation in cell culture models and is developmentally expressed in osteoblasts as well as in proliferative and hypertrophic chondrocytes during endochondral bone formation (Gori and Demay, 2004; Gori et al., 2001). To investigate whether Wdr5 has a functional role during endochondral bone formation in vivo, transgenic mice overexpressing Wdr5 under the control of the 2.3-kb fragment of the mouse α (1) I collagen promoter were generated. These studies demonstrate that overexpression of Wdr5 accelerates osteoblast differentiation and maturation by activating the canonical Wnt signaling pathway during skeletal development. An expansion of the hypertrophic chondrocyte layer is also observed, suggesting that Wdr5 expressed in the bone collar has paracrine actions on chondrocyte differentiation.

Materials and methods

Generation and identification of transgenic mice

Transgenic mice, overexpressing *Wdr5* under the control of the 2.3-kb fragment of the mouse α(1) I collagen promoter (Col I–Wdr5), were generated. The coding region of Wdr5 (1.9 Kb) was substituted for the lacZ gene in the plasmid pJ251 (a gift of Dr. Benoit de Crombrugghe, University of Texas, M.D. Anderson Cancer Center, Houston, TX, USA). This vector provides a 3′ intron and polyadenylation signal for the transgene, the latter of which was used as a probe for in situ hybridization analyses to distinguish the transgene from the endogenous transcript. An *Xho*I–*Hin*dIII fragment containing the 2.3-kb fragment of the mouse α(1) I collagen promoter, the coding region of Wdr5 and the 500 bp mouse protamine gene intron and polyadenylation signal (Fig. 1A) was isolated from the vector sequences and purified according to standard techniques. Pronuclear injections of fertilized mouse oocytes from FVB/N females were performed. Potential founders were screened by PCR analyses of genomic DNA isolated from tails using F1 and R1 primers (Fig. 1A) and subsequently by Southern analyses using *Pst*I digested genomic DNA probed with a ^{32}P-labeled 1100 bp *Apo*I fragment of Wdr5 (probe A in Fig. 1A), to identify those with a single transgene integration site. All studies were approved by the institutional animal care committee. Mice were maintained in a virus and parasite-free barrier facility and exposed to a 12 h light/dark cycle.

Northern analyses

Transgene expression was evaluated by Northern analysis of RNA isolated from calvariae of offspring from potential founders. Ten micrograms of total RNA was resolved on a 1% agarose/formaldehyde gel and transferred to a nylon membrane (Biotrans ICN, Aurora, OH) by capillary blotting. Probes were radiolabeled with [α-^{32}P] dATP (Dupont New England Nuclear, Boston, MA) to a specific activity of $\geq 10^8$ cpm/ng DNA (Megaprime™ DNA labeling systems, Amersham Piscataway, NJ).

Alcian Blue and Alizarin Red staining

Day 14.5 fetuses were fixed in 95% ethanol. After 5 days, specimens were transferred to acetone for an additional 7 days. The specimens were then stained with 0.3% Alcian Blue 8GS (Sigma, St. Louis, MO) in 70% ethanol and 1% Alizarin Red S (Sigma, St. Louis, MO) in 95% ethanol for 3 days at 37°C. Specimens were transferred to 1% KOH until the skeletons were clearly visible. Specimens were then incubated in 1% KOH containing increasing concentrations of glycerol (20%, 50% and 80% glycerol) over a period of 4 weeks.

Histological evaluation

Limbs from 14.5 dpc embryos, neonatal and 7- and 9-day-old mice were fixed in 10% neutral buffered formalin followed by dehydration and paraffin embedding. Tissue blocks were cut into 6 μm sections, deparaffinized and rehydrated. Forelimb sections were stained with hematoxylin and eosin (H&E) or von Kossa stained to permit phenotypic analyses. Measurements for the length of the humeri and the size of the flat and round proliferating chondrocytes layer and hypertrophic chondrocyte layer were performed using microscope software.

Immunohistochemistry

Immunohistochemical analyses were performed on forelimb sections isolated from 14.5 dpc embryos using anti-CD31(BD Biosciences, Franklin Lakes, NJ, USA) or a non-specific mouse IgG and on tibiae isolated from newborn mice using anti-Wdr5 (Gori and Demay, 2004) or a non-specific rabbit IgG (Sigma, St Louis, MO) and the TSA Biotin System Kit (PerkinElmer, Boston, MA). Immunoreactive proteins were visualized using Streptavidin HRP and Streptavidin Texas Red (PerkinElmer, Boston, MA). To examine activation of the Wnt signaling pathway during development, immunohistochemistry was performed using anti-β-catenin (BD Biosciences, Franklin Lakes, NJ, USA). Frozen sections isolated from 14.5 dpc forelmbs were incubated at room temperature with anti-β-catenin or a non-specific

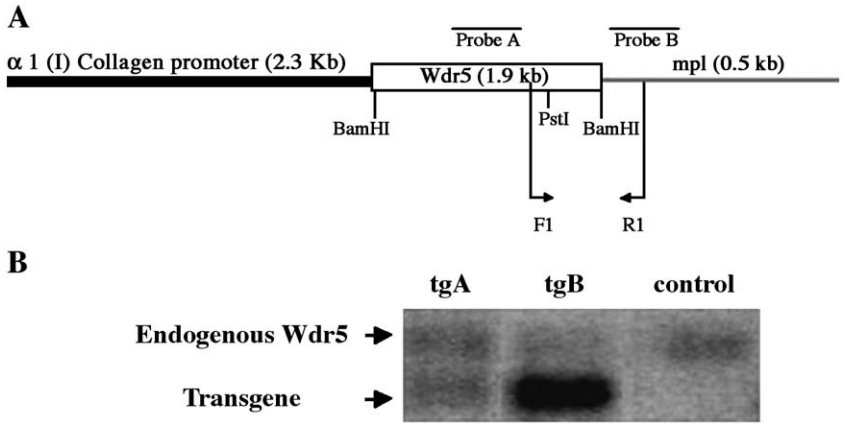

Fig. 1. Generation of transgenic mice. (A) Schematic representation of the transgene construct showing the position of the oligonucleotides F1 and R1 used for PCR of tail genomic DNA and Probe A and Probe B used for Southern and in situ hybridization analyses, respectively. Restriction sites for *Bam*HI and *Pst*I are also indicated. (B) Northern Analyses. Total RNA, isolated from calvaria of 3-day-old offspring from two founders (TgA and TgB) and a control wt (control) mouse.

mouse IgG (Sigma, St Louis, MO) in PBS containing 1% BSA. After 1 h, slides were washed in PBS and incubated with antimouse IgG-FITC (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h to visualize immunoreactive proteins.

In situ hybridization

Riboprobes were radiolabeled with ^{35}S-UTP to a specific activity of at least 10^8 cpm/μg of riboprobe template. Hybridization was performed at 55°C for 20 h in 50% formamide, 10 mM Tris–HCl pH 7.6, 200 μg/ml tRNA, 1× Denhardt's solution, 10% dextran sulfate, 600 mM NaCl, 0.25% SDS and 50 mM DTT. Sequential washes with graded stringency were performed, following which slides were dipped in photoemulsion (Autoradiography Emulsion, Type NTB, Kodak, Rochester NY) and exposed at 4°C for 6 to 14 days. The slides were developed and counter-stained with H&E to identify the cellular source of the signal.

β-gal staining

Embryonic day 14.5 offspring of TOPGAL (Jackson Institute, Bar Harbor, ME) mice and Col I–Wdr5 mice (Col I–Wdr5;TOPGAL), were fixed in 2% paraformaldeyde pH 7.1 and 0.02% glutaraldehyde pH 7.1. After 1 h, embryos were rinsed with PBS and stained with a solution of 20 mg/ml X-gal (American Bioanalytical Natick, MA), 0.25 M EGTA (Sigma, St Louis, MO), 0.02% IGEPAL (Sigma, St Louis, MO), 5 mM $K_3Fe(CN)_6$ (Sigma, St Louis, MO), 5 mM $K_4Fe(CN)_6$ $3H_2O_2$ (Sigma, St Louis, MO) in PBS at 30°C overnight. Embryos were then washed in PBS and postfixed in 4% paraformaldehyde for 45 min prior to dehydration and paraffin embedding (Hens et al., 2005). Tissue blocks were cut into 6 μm sections for evaluation of X-gal staining.

Calvarial osteoblast preparation and analysis

Calvariae were isolated from 3-day-old wild-type and Col I–Wdr5 mice according to a standard protocol (Divieti et al., 1998; Sooy et al., 2005). Alkaline phosphatase activity was evaluated from 4 to 25 days in culture. 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 M 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. Mineralized matrix formation was evaluated from 14 to 25 days in culture. Calcium accumulation in the matrix was quantitated by solubilizing the deposited calcium with 0.6 N HCl. The samples and a standard curve of calcium carbonate were reacted with methylthymol blue and measured spectrophotometrically at 620 nm.

Western analyses

Cells were lysed in 25 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM CaCl and 1% Triton after 4 and 7 days in culture. Five micrograms of protein was subjected to SDS-PAGE under reducing conditions. After transfer to Hybond ECL (Amersham, Piscataway, NJ), membranes were blocked in PBS containing 1% BSA and 5% non-fat dry milk for 1 h at room temperature. Membranes were incubated with anti-β-catenin (BD Biosciences, Franklin Lakes, NJ, USA) or anti-OPG (Santa Cruz Biotechnology Inc, Santa Cruz, CA) at 4°C overnight followed by washes and immunodetection with a peroxidase-conjugated goat antimouse IgG or goat antirabbit IgG, respectively (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Immunoreactive proteins were visualized using a chemiluminescence detection kit (NEN, Boston, MA) according to the manufacturer's instructions.

Statistical analysis

Student's paired *t* test was used to evaluate differences between calvarial osteoblast cultures isolated from wild-type and transgenic mice at each time point. A P value <0.05 was considered statistically significant.

Results

Wdr5 accelerates osteoblast differentiation in vitro and is developmentally expressed in osteoblasts during endochondral bone formation (Gori and Demay, 2004; Gori et al., 2001). To identify a role for Wdr5 in osteoblast differentiation, in vivo transgenic mice, overexpressing Wdr5 under the control of the 2.3-kb fragment of the mouse α(1) I collagen promoter (Col I–Wdr5), were generated (Fig. 1A). This fragment has been shown to target expression of transgenes specifically to osteoblasts and odontoblasts but not to other type I collagen-producing cells and is active at 13.5 dpc (Rossert et al., 1995). Five potential founders were identified by PCR analyses of genomic DNA, three of which had a single transgene integration site on Southern analysis (data not shown). Northern analyses of calvarial RNA isolated from transgene positive and transgene negative offspring of these three potential founders identified two true founders with varying degrees of transgene expression (Fig. 1B). Qualitatively similar results were obtained with both

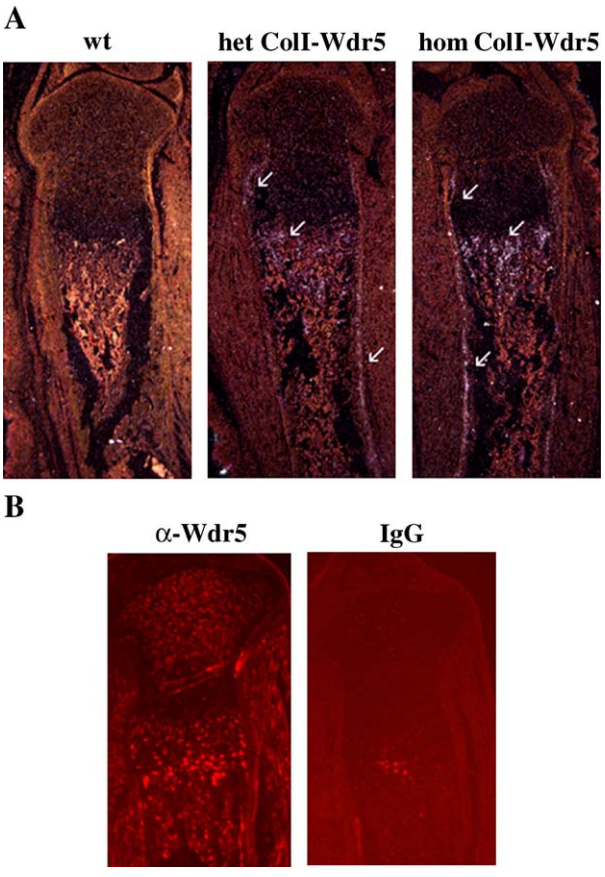

Fig. 2. Expression of the transgene. (A) Wdr5 antisense riboprobe was radiolabeled with ^{35}S-UTP to a specific activity of at least 10^8 cpm/μg of template. Dark-field photographs of proximal tibiae of wild-type (wt), het Col I–Wdr5 and hom Col I–Wdr5 newborns. Arrows indicate the expression of the transgene in the bone collar, periosteum and trabecular bone. There is no detectable expression of the transgene in the chondrocytes. (B) Immunohistochemistry on tibiae from newborn mice was performed using α-Wdr5. Endogenous Wdr5 is expressed in proliferating chondrocytes, hypertrophic chondrocytes, the periosteum and trabecular bone. Representative hindlimbs of three mice are shown.

transgenic lines; gene dosage effects were observed with the most profound phenotype being present in homozygous transgene positive mice from line B. Mice from line B were used for these studies. To ensure that the transgene was specifically targeted to cells of the osteoblastic lineage, transgene expression was analyzed by in situ hybridization using a probe complementary to the bGH polyadenylation sequence, which does not recognize the endogenous Wdr5 transcript. As shown in Fig. 2A, the transgene was specifically expressed in the bone collar, primary spongiosa and cortical bone of heterozygous (het) Col I–Wdr5 and homozygous (hom) Col I–Wdr5 mice. No signal was observed with Wdr5 sense riboprobe (data not shown). Unlike the endogenous Wdr5 gene (Fig. 2B), the transgene was not expressed in the growth plate. To assess whether targeted expression of Wdr5 to osteoblasts results in acceleration of skeletal development, whole-mount skeletons from het Col I–Wdr5, hom Col I–Wdr5 and wild-type fetuses at embryonic day 14.5 were analyzed. As shown in Fig. 3A, the expression of the transgene resulted in an overall bigger skeleton in a gene dosage-dependent fashion [wt < het Col I–Wdr5 < hom Col I–Wdr5]. To further characterize the phenotype of the transgenic mice, histological analyses of humeri isolated from 14.5 dpc embryos were performed. As shown in Fig. 3B, the humeri of Col I–Wdr5 embryos were longer than those isolated from wild-type littermates at 14.5 dpc. While no difference was observed in the length of the zone of flat and round proliferating chondrocytes, the hypertrophic layer was expanded in Col I–Wdr5 embryos compared to wild-type littermates (Fig. 3B and Table 1) with a more profound phenotype being observed in humeri isolated from hom Col I–Wdr5 embryos. This expansion of the hypertrophic chondrocyte layer was not observed at 13.5 dpc (data not shown), prior to the appearance of osteoblasts in the bone collar, suggesting that the chondrocyte phenotype is a paracrine consequence of Wdr5 overexpression in the osteoblasts of the bone collar. As shown by immunostaining for CD31, blood vessels were present in the humeri isolated from both transgene positive embryos and wild-type embryos at 14.5 dpc. However, there was evidence of vascular invasion into the humeri isolated from transgene positive embryos, whereas in the humeri isolated from wild-type embryos blood vessels were present only in the bone collar, suggesting that overexpression of Wdr5 resulted in accelerated

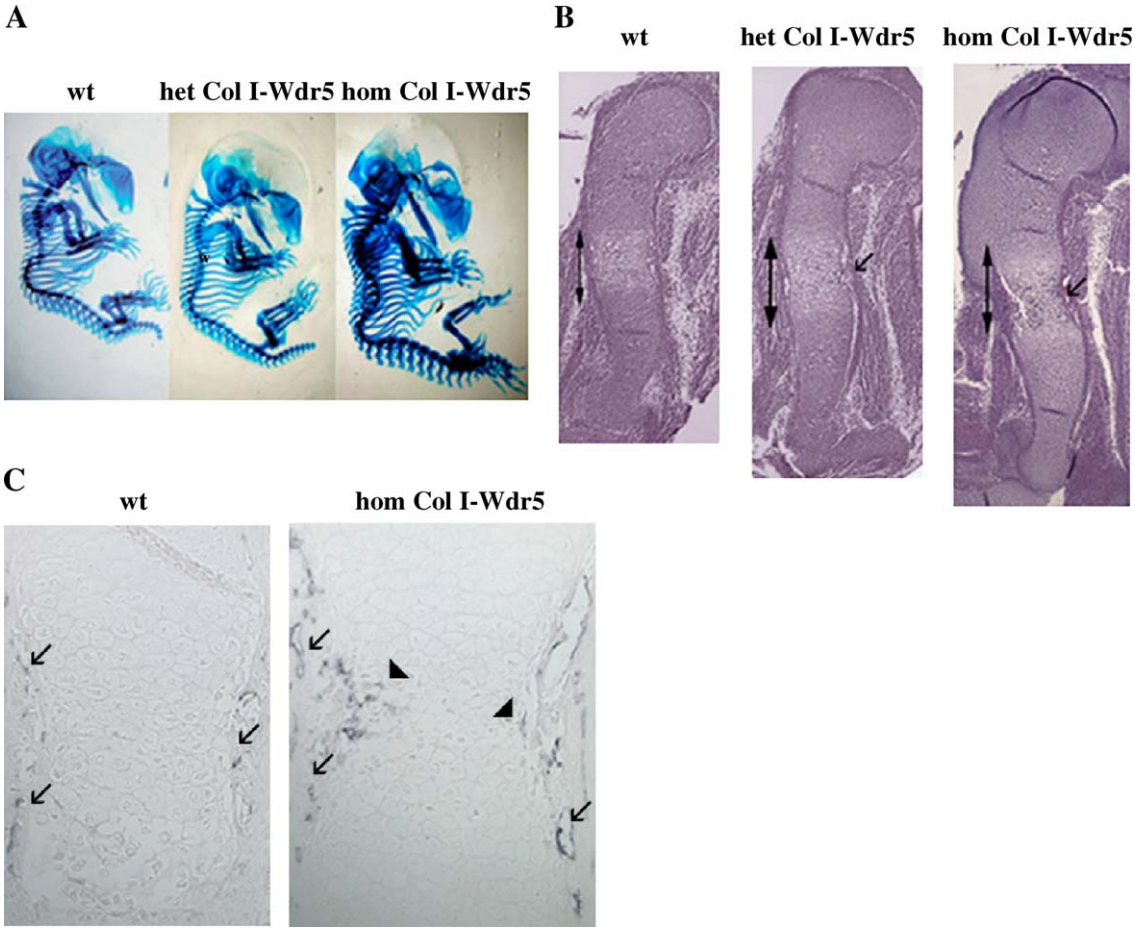

Fig. 3. The expression of the transgene leads to a larger skeleton and accelerated vascular invasion. Alcian Blue and Alizarin Red staining was performed on skeletons (A) of 14.5 dpc wt, het Col I–Wdr5 and hom Col I–Wdr5 embryos. (B) Hematoxylin and eosin. H&E staining was performed on humeri isolated from wt, het Col I–Wdr5 and hom Col I–Wdr5 embryos at 14.5 dpc. Double arrowheaded lines indicate the hypertrophic chondrocyte layer and arrows point to the bone collar. Representative sections of forelimbs from at least four wt, het Col I–Wdr5 and hom Col I–Wdr5 embryos at 14.5 dpc are shown. (C) Immunohistochemistry was performed on humeri isolated from 14.5 dpc embryos using an anti-CD31 antibody. Arrows indicate blood vessels in the bone collar and arrowheads indicate blood vessels invading the hypertrophic chondrocyte layer.

Table 1
Length of humeri, round and flat proliferating chondrocytes and hypertrophic chondrocytes layers

Genotype	Round and flat proliferating chondrocytes	Hypertrophic chondrocytes	Length of the humeri
Wt	0.862 ± 0.02 (n = 8)	0.416 ± 0.02 (n = 8)	1.84 ± 0.03 (n = 5)
het Col I–Wdr5	0.898 ± 0.03 (n = 7)	0.492 ± 0.01 (n = 7)*	2.085 ± 0.03 (n = 6)**
hom Col I–Wdr5	0.815 ± 0.03 (n = 4)	0.530 ± 0 (n = 4)**	2.165 ± 0.07 (n = 4)**

Measurements are expressed in mm ± SEM. *P < 0.001 and **P < 0.005 by Student's t test.

vascular invasion (Fig. 3C). Histological analyses of tibiae isolated from 7- (Figs. 4A and B) and 9- (Figs. 4C and D) day-old mice demonstrated accelerated formation of the secondary ossification center, confirming persistent acceleration of endochondral bone development in transgene positive mice postnatally (arrows in Fig. 4).

To confirm that targeted expression of Wdr5 to osteoblasts results in accelerated osteoblast differentiation, in situ hybridization analyses were performed using type I collagen (Col I), a specific marker for preosteoblasts and osteoblasts (Stein et al., 2004), and osteopontin (OP), a marker for mature osteoblasts and for terminally differentiated hypertrophic chondrocytes (Gerstenfeld and Shapiro, 1996; Stein et al., 2004). As shown in Fig. 5, Col I was expressed more extensively and at higher levels in the bone collar of humeri isolated from 14.5 dpc het Col I–Wdr5 embryos compared to those of their wild-type littermates, demonstrating accelerated maturation of the bone collar. The expression domain of OP was more extensive in the bone collar and interestingly was more expanded in the hypertrophic chondrocyte layer of the humeri isolated from het Col I–Wdr5 embryos compared to that of their wild-type littermates at 14.5 dpc (Fig. 5). Because the expression of type X collagen, a marker for hypertrophic chondrocytes (Schmid and Linsenmayer, 1985), overlapped that of osteopontin in the wild-type and het Col I–Wdr5 embryos (data not shown), the expansion of the domain of osteopontin-expressing cells was due to an expansion of the terminally differentiated hypertrophic chondrocyte layer. The acceleration in osteoblast differentiation was also reflected by more extensive mineral deposition in the bone collar of the het Col I–Wdr5 embryos at 14.5 dpc (Fig. 5). This acceleration of osteoblast differentiation persists in newborn mice, as shown by higher levels of osteocalcin (OC) expression in the primary spongiosa and cortical bone of tibiae isolated from transgene positive mice compared to their wild-type littermates (Fig. 5).

To define the molecular basis for this accelerated osteoblast differentiation, the Wnt signaling pathway, known to play a crucial role in osteoblast differentiation, was investigated. The expression of β-catenin, a key mediator of the canonical Wnt signaling pathway, was examined in humeri isolated from wild-

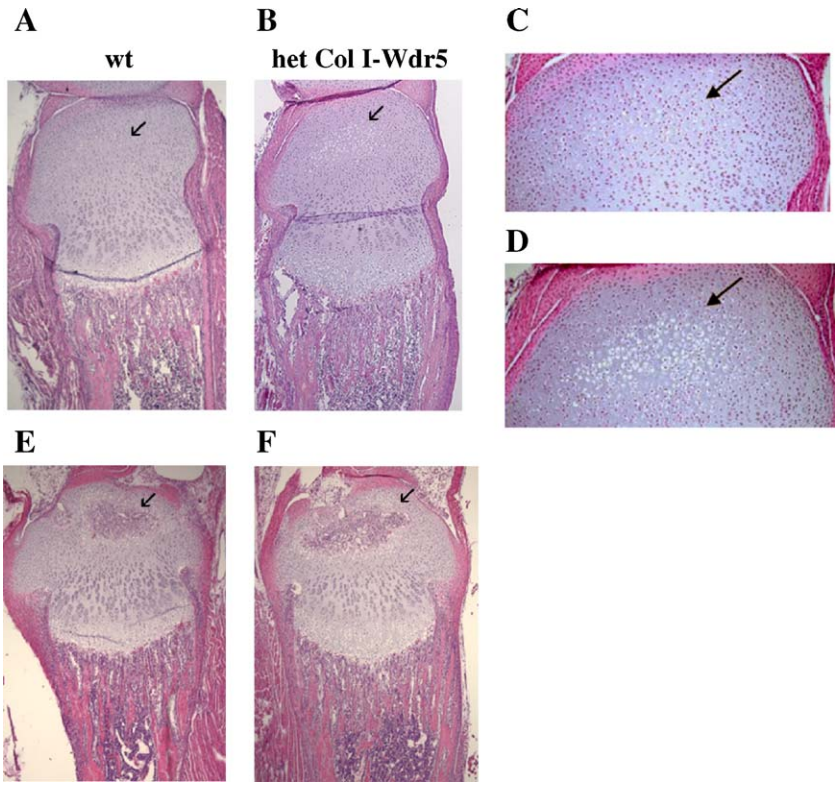

Fig. 4. Expression of the transgene leads to accelerated formation of the secondary ossification center. H&E staining was performed on tibiae isolated from 7- (A and B) and 9- (E and F) day-old wt and het Col I–Wdr5 mice. (C and D) Magnification of the secondary ossification center shown in panels A and B, respectively. Arrows indicate the secondary ossification center. Representative sections of hindlimbs from three wt and het Col I–Wdr5 mice are shown.

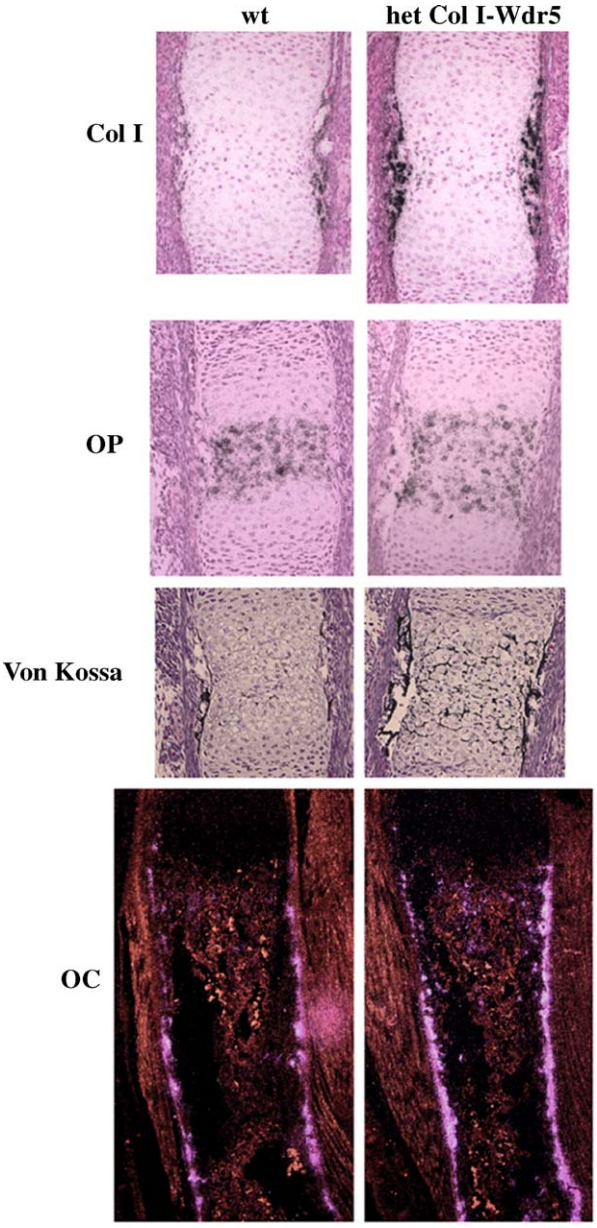

Fig. 5. Molecular analyses of osteoblast differentiation. Sections of humeri of wt and het Col I–Wdr5 embryos are shown. Type I collagen (Col I), ostepontin (OP) and osteocalcin (OC) antisense riboprobes were radiolabeled with ^{35}S-UTP to a specific activity of at least 10^8 cpm/μg of template. von Kossa staining. Deposited mineral is stained black by von Kossa staining. Representative forelimbs from at least four wt and het Col I–Wdr5 littermates are shown.

type and transgene positive embryos at 14.5 dpc. Higher levels of nuclear β-catenin protein were found in the bone collar of humeri isolated from het Col I–Wdr5 embryos compared to humeri isolated from wild-type embryos (Figs. 6A–B). To further confirm the activation of canonical Wnt signaling pathway, het Col I–Wdr5 mice were crossed with TOPGAL mice to obtain mice expressing both the Wdr5 and the *lacZ* transgenes (Col I–Wdr5;TOPGAL). TOPGAL transgenic mice express the *lacZ* gene under the control of a regulatory sequence consisting of three consensus LEF/TCF-binding motifs upstream of a minimal c-*fos* promoter. Since when β-catenin is activated, it translocates to the nucleus, where in concert with TCF, it modulates gene expression, β-gal activity in these mice reflects activation of the canonical Wnt signaling pathway. As expected, no difference in β-gal activity was observed in chondrocytes since the Wdr5 transgene was specifically expressed in osteoblasts (Figs. 6C–D). However, more extensive β-gal staining was observed in the bone collar of 14.5 dpc Col I–Wdr5;TOPGAL embryos compared to wt;TOPGAL embryos (Figs. 6E–F), demonstrating that acceleration of osteoblast differentiation by Wdr5 is associated with earlier activation of the canonical Wnt signaling pathway in osteoblasts.

To demonstrate that the effects of Wdr5 on osteoblast differentiation were cell-autonomous, primary calvarial osteoblasts were isolated from 3-day old het Col I–Wdr5, hom Col I–Wdr5 and wild-type mice. Analyses investigating the time of onset and level of expression of markers of osteoblast differentiation were performed. It is generally accepted that the acquisition of the mature osteoblast phenotype is characterized by the ability of these cells to synthesize Alkaline Phosphatase (AP), and that an increase in the specific activity of this enzyme is strongly correlated with a shift to a more differentiated state (Lian and Stein, 1993). As shown in Fig. 7A, by 4 days in culture, osteoblasts isolated from the het Col I–Wdr5 and hom Col I–Wdr5 show a 2.3-fold increase in AP activity compared to that of the cells isolated from wild-type mice. After 7 days in culture, het Col I–Wdr5 and hom Col I–Wdr5 osteoblasts maintained a significantly higher AP activity compared to the wild-type osteoblasts. However, the AP activity of the wild-type osteoblasts increased to that of the het Col I–Wdr5 and hom Col I–Wdr5 osteoblasts after 25 days in culture (data not shown).

The final stage of osteoblast differentiation is the formation of mineralized matrix nodules. Quantification of calcium deposited into the cultures revealed an increase in the calcium content of the het Col I–Wdr5 and hom Col I–Wdr5 cultures after 14 days compared to the wild-type cultures (Fig. 7B). However, by day 25, no statistically significant differences were observed among the cultures isolated from wild-type, het Col I–Wdr5 and hom Col I–Wdr5 mice. These data demonstrate that targeted overexpression of Wdr5 to osteoblasts accelerates osteoblast differentiation during skeletal development. To confirm the in vivo analyses demonstrating that osteoblast differentiation was associated with enhanced activation of the canonical Wnt pathway, Western analyses were performed to examine the level of β-catenin. As shown in Fig. 7C, β-catenin levels were higher in calvarial osteoblasts isolated from het Col I–Wdr5 and hom Col I–Wdr5 mice compared to calvarial osteoblasts isolated from wild-type mice, confirming that targeting expression of Wdr5 to osteoblasts results in enhanced activation of the canonical Wnt signaling pathway during osteoblast differentiation. It has been recently demonstrated that osteoprotegerin (OPG) is a target of the canonical Wnt signaling pathway (Glass et al., 2005). Confirming that the acceleration of osteoblast differentiation by Wdr5 is associated with the activation of the canonical Wnt signaling pathway, OPG protein levels were also higher

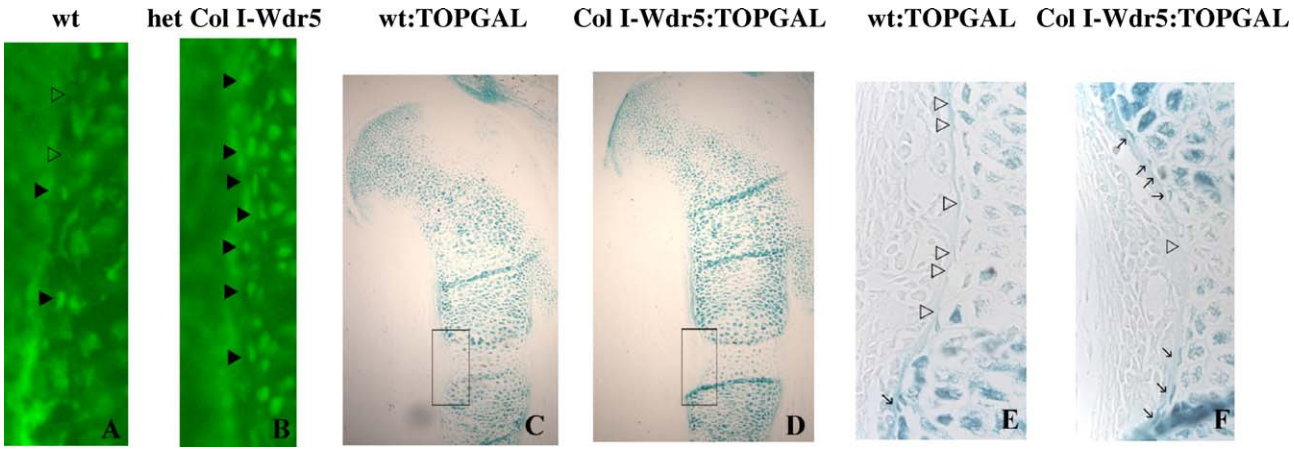

Fig. 6. Col I–Wdr5 transgenic mice demonstrate enhanced activation of the canonical Wnt signaling in osteoblasts. Panels A–B Fluorescent immunohistochemistry with an anti-β-catenin antibody. Nuclear β-catenin was evaluated in sections of humeri of wt and het Col I–Wdr5 embryos. Filled arrowheads indicate osteoblasts expressing β-catenin. Open arrowheads point to osteoblasts not expressing nuclear β-catenin. Panels C–F LacZ expression was evaluated in sections of humeri of Col I–Wdr5;TOPGAL and wt;TOPGAL embryos. (E and F) Higher magnification of boxed area shown in panels C and D. Arrows indicate X-gal staining of osteoblasts in the bone collar, unstained osteoblasts are indicated by arrowheads. Representative forelimbs from two 14.5 dpc Col I–Wdr5;TOPGAL and wt;TOPGAL littermates are shown.

in calvarial osteoblasts isolated from transgene positive mice compared to calvarial osteoblasts isolated from wild-type mice (Fig. 7D).

Discussion

Characterization of skeletal development in embryos over-expressing Wdr5 in osteoblasts demonstrates that targeted overexpression of this WD repeat-containing protein to osteoblasts results in accelerated endochondral bone formation during skeletal development.

Wdr5 is a member of a family of structurally conserved proteins, the WD-40 repeat proteins (Gori et al., 2001). These proteins contain 4 or more copies of a conserved Trp-Asp motif, the so-called WD-40 repeat (Neer et al., 1994; Neer and Smith, 1996, 2000; Smith et al., 1999). Each of the WD-repeats are though to fold into four antiparallel β strands radiating outward from a central axis, leading to the formation of a "β-propeller" structure which forms a scaffold for binding of other proteins. Several WD-40 proteins interact with histone deacetylases and regulate the expression of genes involved in the cell cycle and chromatin structure (Ahmad et al., 1999; Mitsuzawa et al.,

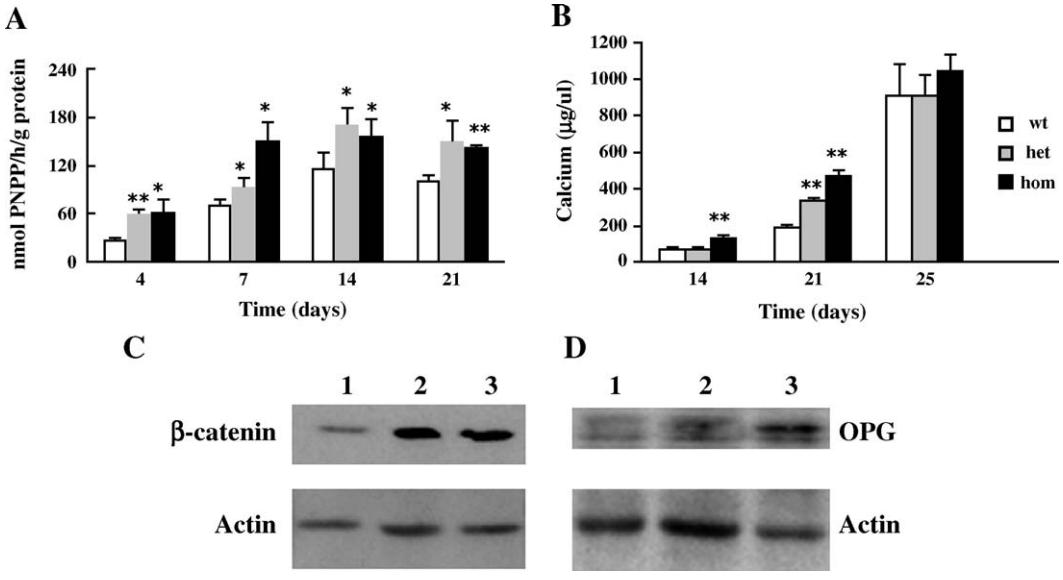

Fig. 7. Wdr5 enhances calvarial osteoblast differentiation. (A) Alkaline phosphatase activity and (B) calcium content of calvarial osteoblasts isolated from wt (open bar), het Col I–Wdr5 (gray bar) and hom Col I–Wdr5 (black bar) mice were assessed from 4 to 25 days in culture. Data shown are representative of that obtained with three independent calvarial osteoblast preparations. The results are the mean ± SEM. *$P<0.05$, **$P<0.005$ by Student's t test. (C) β-Catenin levels in calvarial osteoblasts isolated from wt (1), het Col I–Wdr5 (2) and hom Col I–Wdr5 transgenic mice (3) assessed by Western analysis at 4 days in culture. (D) OPG levels in calvarial osteoblasts isolated from wt (1), het Col I–Wdr5 (2) and hom Col I–Wdr5 transgenic mice (3) assessed by Western analysis at 7 days in culture. Control with an actin antibody confirms equal protein loaded. Data shown are representative of that obtained with two independent calvarial isolations.

2001). The yeast homolog of Wdr5 is a subunit of the trithorax-related Set1/Ash2 histone methyltransferase (HMT) complex involved in activation of transcription via methylation of histone H3 lysine 4 (H3 K4) (Wysocka et al., 2003) and the human WDR5 has also been shown to be essential for H3 K4 methylation and for normal vertebrate development (Dou et al., 2005; Wysocka et al., 2005). WDR5 associates and regulates the circadian histone methylation of the clock protein, PERIOD-1 (Per 1), a key player in the circadian rhythms of mammalian cells (Brown et al., 2005). Taken together, these observations suggest that Wdr5, via its WD repeats, associates with nuclear proteins that play a role in chromatin remodeling thereby regulating gene expression. Interestingly, it has been recently shown that Per null mice display a higher bone mass due to an increase in bone formation (Fu et al., 2005), suggesting that accelerated osteoblast differentiation by Wdr5 may also be associated with regulation of the circadian rhythm genes.

Our analyses demonstrate that Wdr5 plays a role in endochondral and intramembranous ossification during development. Because skeletal development is regulated by several factors, it is likely that Wdr5 interacts with or modulates the expression of target genes that accelerate osteoblast differentiation. Several studies have demonstrated that the canonical Wnt signaling pathway regulates several key processes during skeletal development, including osteoblast differentiation and postnatal bone mass (Akiyama et al., 2004; Day et al., 2005; Gong et al., 2001; Hens et al., 2005; Hill et al., 2005; Hu et al., 2005; Johnson et al., 2004; Kato et al., 2002; Westendorf et al., 2004). Wnt proteins signal through the Frizzled family of receptors (Fzds) and the co-receptors Lrp5 and Lrp6 (low-density lipoprotein receptor-related proteins), leading to stabilization of β-catenin and activation of Lef/Tcf target genes. Interestingly, DasGupta et al. (2005), using a genome-wide RNAi screen in *Drosophila*, have recently identified WD repeat proteins as potential regulators of the canonical Wnt-Wingless pathway in *Drosophila*. Thus, the finding that Col I–Wdr5 transgenic mice display an earlier activation of canonical Wnt signaling in osteoblasts identifies Wdr5 as a potential positive regulator of the canonical Wnt signaling pathway in osteoblasts during mammalian skeletal development. In 293 cells, loss of WDR5 attenuates the expression of HoxC8, a transcription factor involved in anterior/posterior patterning during development (Wysocka et al., 2005). Since HoxC8 induces the expression of *Fzd2*, a Wnt co-receptor (Lei et al., 2005), it is possible that induction of *HoxC8* and thus *Fzd2*, enhanced the activation of the canonical Wnt signaling pathway observed with Wdr5 overexpression.

The overexpression of Wdr5 in Col I-expressing osteoblasts results in an expansion of the late hypertrophic chondrocyte layer that accounts for an increase in the length of humeri observed in transgene positive embryos. Longitudinal bone growth occurs by endochondral bone formation and is dependent upon the rate of chondrocyte proliferation and hypertrophic differentiation, a process regulated by several signaling pathways including the Ihh, PTHrP, BMP and FGF pathways (Kronenberg, 2003). Since the Col I–Wdr5 transgene is specifically expressed in osteoblasts, our findings suggest that Wdr5, overexpressed in the bone collar, regulates the activity of one or more of these pathways that in turn regulates chondrocyte maturation. Characterization of the molecular consequences of Wdr5 overexpression in osteoblasts is expected to elucidate which of these pathways underlie the paracrine actions of Wdr5 that regulate longitudinal skeletal growth.

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