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Downregulation of ErbB3 by Wnt3a Contributes to Wnt-Induced Osteoblast Differentiation in Mesenchymal Cells

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ABSTRACT

Mesenchymal stem cells (MSC) can differentiate into osteoblasts upon activation of Wnt signaling. Identifying targets of Wnt signaling in MSC may help promote MSC osteoblast differentiation for bone regeneration. In this study, using microarray analysis we found that Wnt3a upregulates neuregulin 1 (NRG-1) during Wnt3a-induced osteoblast differentiation in primary human MSC and murine C3H10T1/2 mesenchymal cells. Western blot and qPCR analyses confirmed that NRG-1 is upregulated by Wnt3a, and that this effect was counterbalanced by decreased expression of the NRG-1 receptor ErbB3. Consistently, exogenous NRG-1 had no effect on alkaline phosphatase (ALP) activity, an early marker of osteoblast differentiation. In contrast, small interfering RNA-mediated silencing of endogenous NRG-1 increased basal and Wnt3a-induced ALP activity in MSC. We showed that short hairpin (sh) ErbB3 and Wnt3a additively increased β-catenin transcriptional activity and ALP activity in MSC. These effects were abrogated by DKK1, indicating that cross-talk between Wnt3a and ErbB3 control MSC osteoblast differentiation via Wnt/β-catenin signaling. Furthermore, ErbB3 silencing decreased Src expression. Pharmacological inhibition of Src signaling promoted ErbB3- and Wnt-induced ALP activity, suggestive of a role of Src signaling in the modulation of osteoblast differentiation by ErbB3 and Wnt3a. The results indicate that downregulation of ErbB3 induced by Wnt3a contributes to Wnt3a-induced early osteoblast differentiation of MSCs through increased canonical Wnt/β-catenin signaling and decreased Src signaling. J. Cell. Biochem. 113: 2047–2056, 2012.

KEY WORDS: Wnt SIGNALING; ErbB3; OSTEOBLAST; MESENCHYMAL CELLS

Mesenchymal stem cells (MSC) are an important source for tissue therapy in regenerative medicine [Caplan, 2007; Phinney and Prockop, 2007]. Notably, expanding osteogenic MSC may be of potential interest for bone regeneration in skeletal diseases [Kimelman et al., 2006; Marie and Fromigué, 2006; Kassem and Abdallah, 2008]. The osteogenic differentiation process is highly dependent on environmental, local, and hormonal factors that activate signaling pathways in MSC [Bianco et al., 2001; Marie and Fromigué, 2006; Phinney and Prockop, 2007]. However, the molecular mechanisms underlying osteoblast differentiation in MSC are not fully eludicated. Wnt signaling is an important pathway controlling osteoblastogenesis [Johnson et al., 2004; Bodine and Komm, 2006]. Activation of Frizzled and LRP5/6 co-receptors by Wnt ligands leads to inhibition of the activity of glycogen synthase kinase 3β, followed by the accumulation and nuclear translocation of unphosphorylated β-catenin, which in turn triggers TCF/LEF transcriptional activity [Nusse, 2008]. Inactivation of β-catenin in osteoblast progenitors inhibits osteoblast differentiation, indicating that Wnt/β-catenin controls osteoblastogenesis [Day et al., 2005; Rodda and McMahon, 2006]. Wnt signaling promotes MSC osteogenic differentiation through Runx2, the main osteoblast transcription factor, and increases timely expressed genes such as alkaline phosphatase (ALP) followed by the expression of osteoblast genes and extracellular matrix formation [Day et al., 2005; Gaur

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et al., 2005; Bodine and Komm, 2006; Gordon and Nusse, 2006]. Identifying the mechanisms by which Wnt signaling targets MSC may therefore help in the development of tools that promote MSC osteoblast differentiation.

In this study, we found that neuregulin 1 (NRG-1) is upregulated during Wnt3a-induced early osteoblast differentiation in murine MSC. NRG-1 is known to control cardiac, neuronal, and mammary differentiation [Britsch, 2007; Birchmeier, 2009; Pentassuglia and Sawyer, 2009; Xu et al., 2009]. The cellular effects of NRG-1 are mediated by its interaction with ErbB receptors, a family of tyrosine kinases, consisting of the EGF receptor, ErbB2, ErbB3, and ErbB4 [Carraway and Sweeney, 2001; Citri et al., 2003]. Notably, NRG-1 interacts with ErbB3 or ErbB4 [Sweeney and Carraway, 2000; Wiley, 2003]. The role of NRG-1/ErbB signaling in osteoblast differentiation remains poorly understood. A previous report indicated that a truncated soluble form of ErbB3 can induce osteoblast differentiation [Lin et al., 2008]. ErbB signaling was also found to be required for periosteal cell proliferation and differentiation [Fisher et al., 2007]. However, the mechanisms involved in these effects are unknown. Here, we present studies investigating the role of NRG-1/ErbB signaling in osteoblast differentiation in mesenchymal cells. We show that inhibition of ErbB3 by Wnt3a contributes to Wnt3a-induced osteoblast differentiation in murine mesenchymal cells through increased canonical Wnt/β-catenin signaling and decreased Src signaling.

MATERIALS AND METHODS

CELLS AND MATERIALS

Human primary mesenchymal stromal cells (hMSCs) were obtained from PromoCell GmbH (Heidelberg, Germany). Murine pluripotent mesenchymal C3H10T1/2 cells were from ATCC (Rockville, MD). Cells were routinely cultured in Dulbecco's Modified Eagles Medium (DMEM; Invitrogen Corporation, Paisley, Scotland) supplemented with 5% heat inactivated fetal calf serum (FCS), 1% penicillin/streptomycin (10,000 U/ml and 10,000 μg/ml, respectively), at 37°C in humidified atmosphere containing 5% CO_2 in air. Culture media were changed every 2 days. Wnt3a conditioned medium (CM) and control CM were prepared as described previously [Haÿ et al., 2005]. Briefly, Wnt3a-producing L cells were cultured in DMEM with 2% FCS for 3 days. After harvesting, Wnt3a-CM was centrifuged at 1,000*g* for 10 min and filtered through a nitrocellulose membrane. The activity of Wnt3a-CM was assayed on L-cells by examining the increase in β-catenin [Haÿ et al., 2005]. Anti-NRG-1, anti-ErbB3, anti-epidermal growth factor receptor (EGFR), anti-ERK1/2 and anti-p-ERK1/2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Other antibodies (anti-PI3K p85, anti-p-PI3K p85) were from Cell Signaling (Danvers, MA). The β-actin antibody was from Sigma–Aldrich (Saint-Louis, MO). The Src pharmacological inhibitor PP2 was from Calbiochem (San Diego, CA).

Micoarray analysis. Subconfluent primary human MSCs were incubated with or without Wnt3a CM for 1, 3, 7, or 14 days. Total RNA was prepared from two separate experiments using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and examined with the Agilent Bioanalyzer (Agilent Technologies, Böblingen, Germany) and NanoDrop spectrophotometer (NanoDrop products, Wilmington, DE). Hybridization on Affymetrix HG-U133Plus-2.0 GeneChip arrays was performed as described previously [Häupl et al., 2007]. In brief, cDNA was synthesized and in vitro transcribed (ENZO, New York, NY) to generate biotin-labelled complementary RNA. After fragmentation, 15 μg of complementary RNA were hybridized for 16 h at 45°C. Arrays were washed and stained under standardized conditions and scanned on an Affymetrix Genechip Scanners 3000 7G. Raw gene expression data were processed with the GCOS 1.2 software for signal calculation and pair-wise comparison analysis followed by algorithms for group comparisons implemented in the BioRetis database (www.bioretis.de). Cellular pathways were analyzed using statistical tools (www.ingenuity.com).

TRANSFECTIONS

Specific NRG-1 and ErbB3 siRNA were designed as indicated (Electronic Supplementary Material 1). Control (scramble) siRNA was from Santa Cruz Biotechnology. For transfection, cells were seeded in 24-well plates and transfected at 50–60% confluence. siRNA (1 μg) was used and transfected with the Oligofectamine™ reagent following manufacturer's instructions (Life Technologies SAS, Courtaboeuf, France).

PLASMIDS AND INFECTION

Specific ErbB3 shRNA was designed as indicated (Electronic Supplementary Material 1) with *Eco*RI and *Age*I ends. ErbB3 shRNA was cloned into the pLKO.1-Puro plasmid (Addgene, Cambridge, MA) after *Eco*RI and *Age*I double-digestion following Addgene's pLKO.1 procedure for cloning (www.addgene.org/tools/protocols/plko). *Eco*RI and *Age*I were FastDigest® Restriction Enzymes from Fermentas (St. Leon-Rot, Germany). Lentiviral particles containing pLKO.1 (empty vector, EV) or pLKO.1-anti-ErbB3 shRNA (shErbB3) were produced as previously described [Orosco et al., 2007]. For infection, C3H10T1/2 cells were grown in 75 cm^2 flasks and transduced at 60% confluence with 10 ml HEK-293T medium containing virions, in the presence of polybrene (10 μg/ml). After 48 h, transduced cells were selected in DMEM medium containing 10% FCS, 1% penicillin/streptomycine and 5 μg/ml puromycine (Sigma–Aldrich) for 2 weeks.

CELL PROLIFERATION AND APOPTOSIS ASSAYS

For the cell proliferation assay, transduced C3H10T1/2 cells were cultured in 24-well plates (5,000 cells per well) and the number of cells was evaluated by cell counting at the times indicated. Additionally, cells were cultured in 96-well plates (800 cells per well) and BrdU incorporation was evaluated by ELISA (Biotrak System v2, Amersham) at day 2. For the apoptosis assay, transduced C3H10T1/2 cells were cultured in the presence of 0.5 or 5% FCS and apoptosis was detected using the Tunel assay at day 3 and the activity of caspases 3, 6, 7 was determined at day 2, as described previously [Fromigué et al., 2008].

ALP ACTIVITY AND STAINING

ALP activity was assayed using an Alkaline Phosphatase kit (Bio-Rad, Hercules), and ALP staining was performed using Sigma Fast

BCIP/NBT kit (Sigma–Aldrich) as described previously [Hamidouche et al., 2008].

QUANTITATIVE RT-PCR ANALYSIS

Total RNA was isolated at the times indicated using the Trizol reagent (Invitrogen, France). Total RNA (1 μg) from each sample was reverse transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) following manufacturer's instructions. Relative mRNA levels were evaluated by quantitative PCR (LightCycler; Roche Applied Science, Indianapolis, OH) using Absolute Blue qPCR kit (Thermo Fisher Scientific, Waltham, MA) and specific primers (Electronic Supplementary Material 2). Signals were normalized to 18S or GAPDH, used as internal controls.

WESTERN BLOT AND IMMUNOPRECIPITATION ANALYSES

Cell lysates were prepared at the indicated times as described previously [Hamidouche et al., 2009]. Briefly, proteins (30 μg) were separated on 4–12% SDS–PAGE and transferred onto PVDF nitrocellulose membranes (Millipore Corporation, Bedford,). Filters were incubated for 2 h in 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.1% (v/v) Tween-20, 0.5% (w/v) bovine serum albumin (TBST/BSA), then overnight at 4°C on a shaker with the indicated primary antibody (1/500–1/1,000 in TBST/BSA). Membranes were washed twice with TBST and incubated for 2 h with appropriate HRP-conjugated secondary antibody (1/10,000–1/20,000 in TBST/BSA). After the final washes, the signals were visualized with enhanced chemiluminescence Western blotting detection reagent (ECL, Amersham Biosciences, Piscataway, NJ) and autoradiographic film (X-OMAT-AR, Eastman Kodak Company, Rochester, NY). Densitometric analysis using ImageQuant software was performed following digital scanning (Agfa, Artigues, France). Representative images of immunoblots are shown. Immunoprecipitation analysis was performed using microMACS protein A/G microbeads magnetic separation (Miltenyi Biotech Auburn CA) according to manufacturer's recommendations. Briefly 100 μg of total protein were incubated on ice for 30 min with 2 μg of the indicated antibody or immunoglobulin fraction negative control (Dako, Glostrup, Denmark) and 20 μl of A/G magnetically labeled protein samples. The magnetically labelled immune complex was passed over a micro-column placed in a magnetic field. The complex bound was washed with lysis buffer, and the immuno-precipitated protein was eluted from the column with SDS gel loading buffer ready for Western blot assay.

TCF/TOP ASSAY

Cells seeded in 24-well plates were co-transfected at 50–60% confluence with 365 ng/well of TopFlash, 135 ng/well of pcDNA-TCF4 plasmid, a TCF4 expression plasmid [Haÿ et al., 2009] and 10 ng/well of phRL-SV40, a Renilla expression plasmid used as internal transfection control. Empty pGL3-Basic served as control for reporter activity. Forty-eight hours after transfection, Firefly and Renilla activities were measured sequentially using the Luciferase Reporter Assay System (Promega, Madison, WI). Luciferase activity was normalized both to Renilla activity as transfection control and to the values obtained with cells transfected with the empty pGL3-Basic as control for the variations induced by shRNA. Results are expressed as relative luciferase units.

STATISTICAL ANALYSIS

The data are the mean +/− SEM of an average of 4–6 samples and are representative of at least three distinct experiments. The data were analyzed by Student's *t*-test and a minimal level of $P < 0.05$ was considered significant.

RESULTS

Wnt3a UPREGULATES NRG-1 IN MESENCHYMAL CELLS

To determine the gene expression profile in hMSC that were induced to differentiate, the cells were treated at various time points (1–6 days) with 15% Wnt3a CM to promote osteogenic differentiation which was verified by increased ALP activity. The bioinformatics analysis of normalized data from three different cultures revealed a limited number of transcripts that were upregulated more than 2-fold in Wnt3a-treated cells compared to untreated cells. Notably, we found that two genes, NRG-1 and endothelin-1 (ET-1) were upregulated by Wnt3a in hMSC at all time points in two separate experiments (www.bioretis.de). Quantitative RT-PCR analysis confirmed that Wnt3a increased NRG-1 and ET-1 mRNA levels in hMSC at all time points, validating the microarray analysis (Fig. 1A,B). This effect was not specific to human MSC since a similar effect was found in murine C3H10T1/2 mesenchymal cells (Fig. 1C,D), suggesting that NRG-1 and ET-1 are direct or indirect targets of Wnt signaling in these cells. The increase in NRG-1 did not result from increased ET-1 since exogenous ET-1 had no significant effect on NRG-1 mRNA expression in these cells (data not shown). Based on these results, we investigated the potential role of the

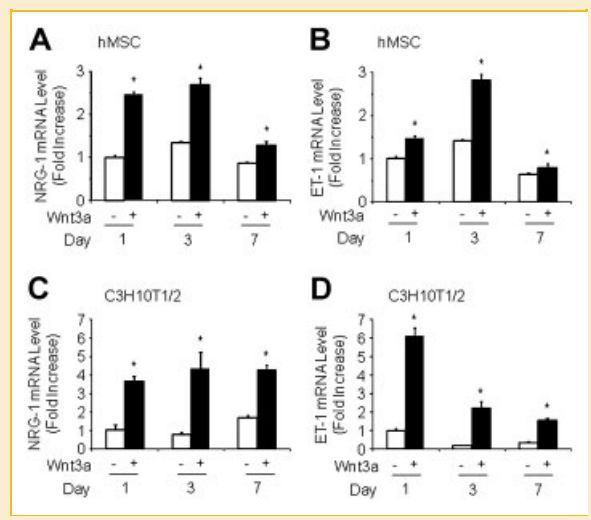

Fig. 1. Validation of the microarray analysis in MSCs. RT-qPCR analysis showing the expression of NRG-1 and ET-1 mRNA in human primary mesenchymal cells (A,B) and C3H10T1/2 murine cells (C,D) treated with 15% Wnt3a CM or control CM. Total mRNA was analyzed by qPCR. Data are mean ± SEM. *: indicates a difference compared to -Wnt3a cells ($P < 0.05$).

identified molecules in Wnt3a-induced osteoblast differentiation in murine MSC.

LACK OF EFFECT OF EXOGENOUS ET-1 AND NRG-1 ON OSTEOBLAST DIFFERENTIATION

ET-1 was previously shown to increase osteoblast proliferation [von Schroeder et al., 2003] and to modulate osteoblast differentiation markers, depending on the cellular context [Niger et al., 2008]. ET-1 was found to act in part via activation of Wnt signaling in bone [Clines et al., 2007]. To determine the potential role of ET-1 in osteoblast differentiation, C3H10T1/2 cells were treated with ET-1 and we analyzed ALP activity because it is an established early osteoblast marker. As shown in Electronic Supplementary Material 1A, ET-1 (100 nM) had no effect on ALP activity in basal conditions. Furthermore, the addition of ET-1 (100 nM) had no significant effect on Wnt3a-induced ALP activity (Electronic Supplementary Material 1A). ET-1 is known to act via type A and B receptors in osteoblastic cells [Suzuki et al., 1997]. In bone, the ET-1/ET-1A receptor was recently found to be an important regulator of osteoblast activity [Clines et al., 2011]. Here, we found that pharmacological inhibition of the ET-1 receptors A and B using selective antagonists did not affect ALP activity in C3H10T1/2 cells treated with Wnt3a, compared to untreated cells (Electronic Supplementary Material 1B), confirming the lack of a detectable role of exogenous ET-1 on Wnt3a-induced MSC osteoblast differentiation in this system.

We then tested the effect of the other Wnt-induced gene, namely NRG-1, on MSC differentiation. NRG-1 interaction with ErbB3 or ErbB4 is known to stimulate tyrosine kinase activity [Sweeney and Carraway, 2000; Wiley, 2003]. Consistently, using an anti-phophotyrosine antbody, we found that NRG-1 (20 ng/ml) increased tyrosine phosphorylation in C3H10T1/2 cells at 5–10 min, indicating that these cells are sensitive to NRG-1 stimulation (Electronic Supplementary Material 2A). However, in repeated experiments, the addition of exogenous NRG-1 (20 ng/ml, 72 h) did not affect ALP activity. ALP activity was enhanced by Wnt3a with or without NRG-1 in C3H10T1/2 cells (Electronic Supplementary Material 2B). These results indicate that exogenous NRG-1 is ineffective at inducing ALP activity in C3H10T1/2 cells.

siRNA-MEDIATED SILENCING OF NRG-1 EXPRESSION PROMOTES OSTEOBLAST DIFFERENTIATION IN MESENCHYMAL CELLS

We next investigated whether endogenous NRG-1 may be effective at promoting MSC differentiation. To that goal, we used a selective small interference (si) RNA that was chosen to effectively reduce endogenous NRG-1 (Electronic Supplementary Material 1), as detected by Western blot analysis (Fig. 2A). We found that silencing NRG-1 transcripts using siRNA increased ALP activity in C3H10T1/2 cells compared to scramble siRNA used as control (Fig. 2B). Additionally, ALP activity was further increased by siNRG-1 compared to control (scramble siRNA) in the presence of Wnt3a and the two treatments had a synergistic effect (Fig. 2B). These results show that silencing endogenous NRG-1 exerts a positive impact on basal and Wnt3a-induced osteoblast differentiation in murine MSC measured by ALP activity, suggesting that endogenous NRG-1 negatively impacts osteoblast differentiation in MSC.

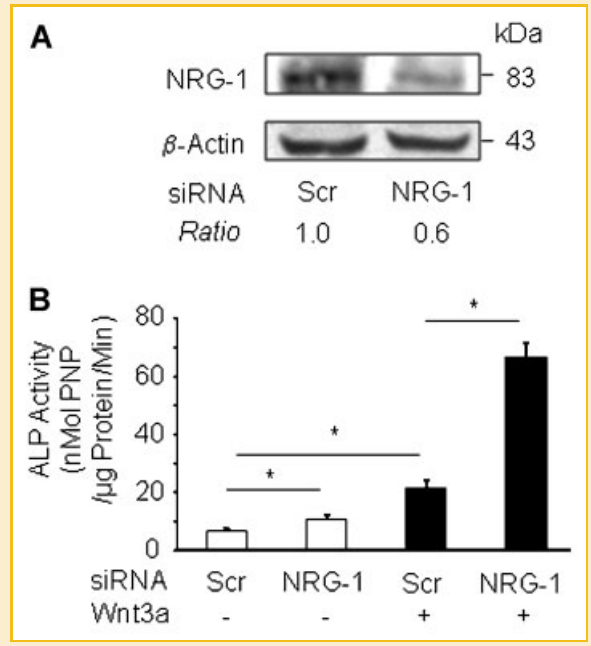

Fig. 2. Effect of endogenous NRG-1 on mesenchymal cell differentiation. A: Western blot analysis showing the effectiveness of inhibition of endogenous NRG-1 using siNRG-1 compared to scramble siRNA. Whole cell lysates were probed with the indicated antibody. B: Effective silencing of endogenous NRG-1 using siNRG-1 increased ALP activity compared to scramble siRNA and synergized with Wnt3a CM compared to control CM in C3H10T1/2 cells. Data are mean ± SEM. *: indicates a difference compared to the indicated group ($P < 0.05$).

Wnt3a DOWNREGULATES ErbB3 RECEPTORS IN MESENCHYMAL CELLS

Given the above results, we reasoned that the upregulation of NRG-1 induced by Wnt3a might result in alteration of NRG-1-mediated signaling in MSCs. We therefore investigated NRG-1 signaling in MSC treated with Wnt3a. The ErbB receptor family is composed of ErbB1 (EGFR) and ErbB2-4 receptors and NRG-1 is known to bind to the ErbB3 and ErbB4 receptors [Falls, 2003]. qPCR analysis showed that C3H10T1/2 cells express ErbB2 and ErbB3 mRNA (Fig. 3A,B). In contrast, ErbB4 mRNA was undetectable by qPCR analysis (data not shown). Interestingly, we found that Wnt3a slightly decreased ErbB2 mRNA and greatly decreased ErbB3 mRNA expression in C3H10T1/2 cells (Fig. 3A,B). Although we found a slight Wnt-induced decrease in ErbB2 mRNA expression, we found no change in ErbB2 at the protein level by Western blot analysis (data not shown). In contrast, Western blot analysis showed that Wnt3a markedly decreased ErbB3 protein levels in C3H10T1/2 cells (Fig. 3C). These results indicate that the increased NRG-1 expression induced by Wnt3a is associated with a strong attenuation of its receptor ErbB3 in MSCs.

ErbB3 SILENCING PROMOTES ALP ACTIVITY IN MESENCHYMAL CELLS

The above results are suggestive of a role of ErbB3 in Wnt-induced MSC osteoblast differentiation. To further investigate this hypothesis, we determined the impact of silencing ErbB3 on ALP activity in C3H10T1/2 cells. Silencing ErbB3 using short hairpin (sh) RNA

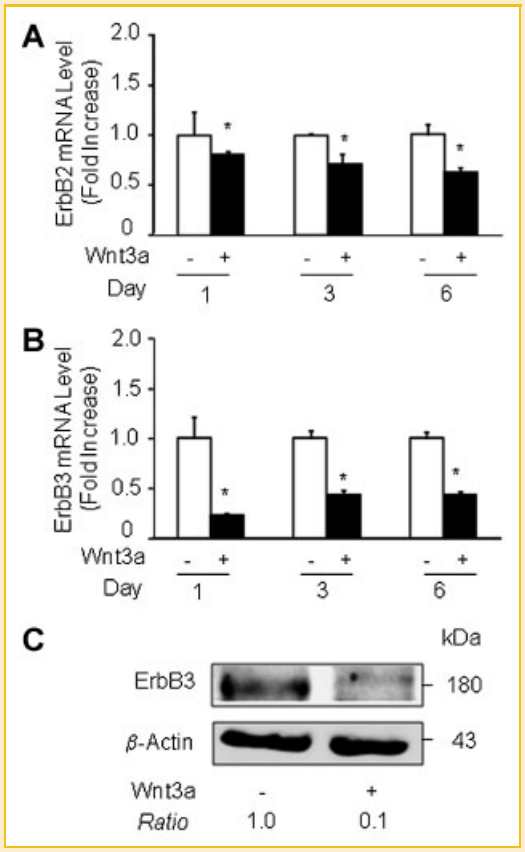

Fig. 3. Wnt3a decreases ErbB3 expression in mesenchymal cells. RT-qPCR analysis showing that Wnt3a CM slightly decreased the expression of ErbB2 (A) and markedly reduced ErbB3 mRNA levels (B) in C3H10T1/2 cells compared to control CM. Total mRNA from control and treated cells was analyzed by qPCR. C: Western blot analysis showing that Wnt3a CM dramatically decreased ErbB3 protein levels compared to control CM in C3H10T1/2 cells. Whole cell lysates at day 6 were probed with the indicated antibody. Data are mean ± SEM. * Indicates a significant difference compared to Wnt3a cells ($P < 0.05$).

effectively reduced ErbB3 mRNA and protein expression in C3H10T1/2 cells (Fig. 4A,B), but had no effect on other ErbB family members (data not shown), showing the effectiveness of the selected ErbB3 shRNA. Interestingly, we found that Wnt3a decreased ErbB3 expression levels and that shErbB3 and Wnt3a additively reduced ErbB3 mRNA expression in C3H10T1/2 cells (Fig. 4A). Consistent with this effect, we found that shErbB3 increased ALP activity and had a synergistic effect with Wnt3a on ALP activity in these cells (Fig. 4C). These results indicate that ErbB3 silencing promotes early osteoblast differentiation and suggest that Wnt3a-induced differentiation may involve a reduction of ErbB3 expression in murine mesenchymal cells.

Based on these findings, we investigated the role of ErbB3 in Wnt3a-induced MSC osteoblast differentiation. To this goal, we analyzed whether ErbB3 silencing may impact the Wnt/β-catenin transcriptional activity. As shown in Fig. 4D, silencing ErbB3 using shRNA had no detectable effect on basal β-catenin transcriptional activity but increased Wnt3a-induced transcriptional activity by twofold, as assessed by the TCF/TOP assay, indicating that ErbB3 acts in concert with Wnt3a to activate the canonical Wnt/β-catenin pathway (Fig. 4D). To further document this interaction, we determined whether the Wnt inhibitor DKK1 may reverse the ErbB3 inhibition induced by Wnt3a. We found that DKK1 partially abrogated the inhibitory effect of Wnt3a on ErbB3 expression (Fig. 4E), confirming the interaction between ErbB3 and canonical Wnt signaling. Consistent with this effect, DKK1 abolished the synergistic effect of shErbB3 and Wnt3a on ALP activity (Fig. 4F). These results indicate that downregulation of ErbB3 signaling induced by Wnt3a contributes to Wnt3a-induced early osteoblast differentiation in C3H10T1/2 cells at least in part via interaction with Wnt/β-catenin signaling.

ErbB3 CONTROLS MESENCHYMAL CELL PROLIFERATION BUT NOT SURVIVAL

ErbB receptors are known to be involved in cell proliferation [Blume-Jensen and Hunter, 2001]. To investigate the potential role of ErbB3 on mesenchymal cell proliferation, we tested the effect of ErbB3 silencing on C3H10T1/2 cell replication. As shown in Figure 5, Wnt3a slightly increased the cell number, and ErbB3 silencing reduced the cell number in the presence or absence of Wnt3a. The decreased cell number induced by ErbB3 silencing was due to reduced cell replication, as shown by BrdU analysis (Fig. 5B). shErbB3 had no effect on cell survival induced by Wnt3a, as shown by caspases and Tunel analyses in the presence or absence of serum (Fig. 5C,D). These findings indicate that ErbB3 signaling impacts MSC replication independently of Wnt3a in these assays, and has no effect on mesenchymal cell survival induced by Wnt3a, in contrast to the functional ErbB3/Wnt3a interaction on osteoblast differentiation reported above.

ErbB3 INTERACTS WITH Src SIGNALING

Besides the ErbB3/Wnt interactions, other mechanisms may be involved in the shErbB3-mediated osteoblast differentiation in mesenchymal cells. Notably, ErbB3 was found to interact with the EGFR in other cells [Samaga et al., 2009]. Consistently, immunoprecipitation analysis showed that ErbB3 interacts with EGFR in C3H10T1/2 cells (Fig. 6A). Western blot analysis showed that ErbB3 silencing using shErbB3 had no effect on EGFR protein levels in the absence of Wnt3a, but slightly increased EGFR expression in the presence of Wnt3a (Fig. 6B). To further study the potential role of EGFR in mesenchymal cell differentiation regulated by ErbB3 and Wnt, we analyzed the effect of shErbB3 and Wnt3a on PI3K and ERK1/2 MAP which are signaling pathways targeted by EGFR in MSC [Kratchmarova et al., 2005]. We found that Wnt3a did not significantly modulate ERK1/2 phosphorylation in C3H10T1/2 cells in these conditions. Wnt3a increased PI3K phosphorylation, but this effect was not affected by shErbB3 (Electronic Supplementary Material 3A,B). Consistently, pharmacological inhibition of PI3K using wortmannin (0.1–1 μM) did not affect the increased ALP activity induced by shErbB3 or Wnt3a (Electronic Supplementary Material 3C). These results suggest that EGFR signaling is not involved in Wnt3a- or shErbB3-mediated increased ALP activity in murine mesenchymal cells.

ErbB3 was found to interact with the non-receptor tyrosine kinase Src in other cell types [Olayioye et al., 2001]. We therefore wanted to

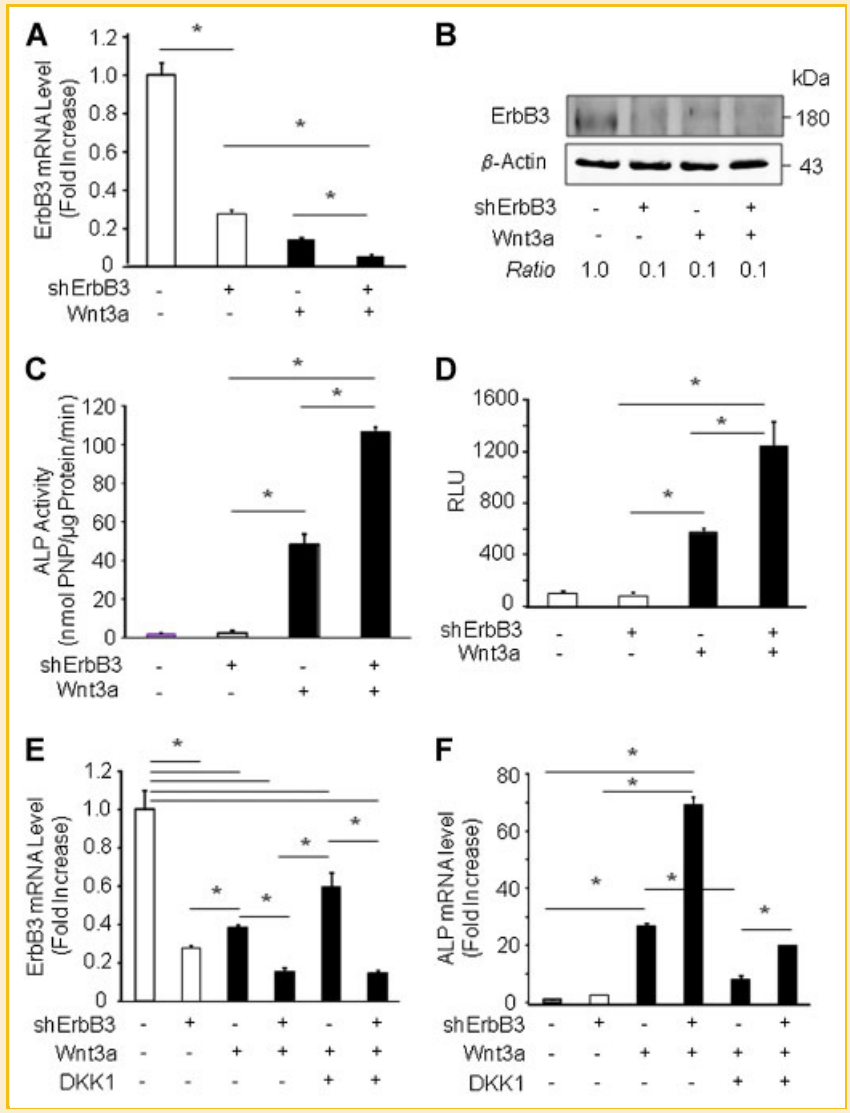

Fig. 4. ErbB3 silencing promotes osteoblast differentiation in mesenchymal cells. qPCR analysis at day 3 (A) and Western blot at day 6 (B) showing that shErbB3 and Wnt3a CM reduced ErbB3 protein expression in C3H10T1/2 cells compared to control vector and control CM, respectively. ErbB3 silencing using shRNA promoted basal and Wnt3a-induced ALP activity (C) and β-catenin transcriptional activity measured by the TCF/TOP assay (D) compared to the respective controls in C3H10T1/2 cells. DKK1 partially abrogated the inhibition of ErbB3 mRNA expression (E) and the increased ALP activity (F) induced by Wnt3a in C3H10T1/2 cells. Data are mean ± SEM. *: indicates a difference compared to the indicated group ($P < 0.05$).

determine the implication of Src signaling in the control of osteoblast differentiation by Wnt3a/ErbB3. Immunoprecipitation analysis showed that ErbB3 interacts with Src in C3H10T1/2 cells (Fig. 6A). Based on this finding, we determined the potential role of Src as a downstream signaling effector of ErbB3 in Wnt3a-induced osteoblast differentiation. We found that ErbB3 silencing decreased Src expression in the presence or absence of Wnt3a (Fig. 6B). This effect was functional since inhibition of Src signaling using the pharmacological inhibitor PP2 further increased Wnt3a-induced ALP activity and staining in C3H10T1/2 cells (Fig. 6C,D). These results suggest that ErbB3-induced inhibition of Src signaling concurs with Wnt3a to promote early osteoblast differentiation in mesenchymal cells. Taken together, our results reveal that Wnt3a downregulates ErbB3 in murine mesenchymal cells, which contributes to promote osteoblast differentiation through increased canonical Wnt/β-catenin signaling and decreased Src signaling (Fig. 7).

DISCUSSION

The identification of genes that are involved in Wnt-induced early osteoblast differentiation in mesenchymal cells is an important issue for promoting osteoblastogenesis and bone formation. Here, we report the discovery of novel genes that are upregulated by Wnt signaling in MSC. We focused on NRG-1, which we found to be

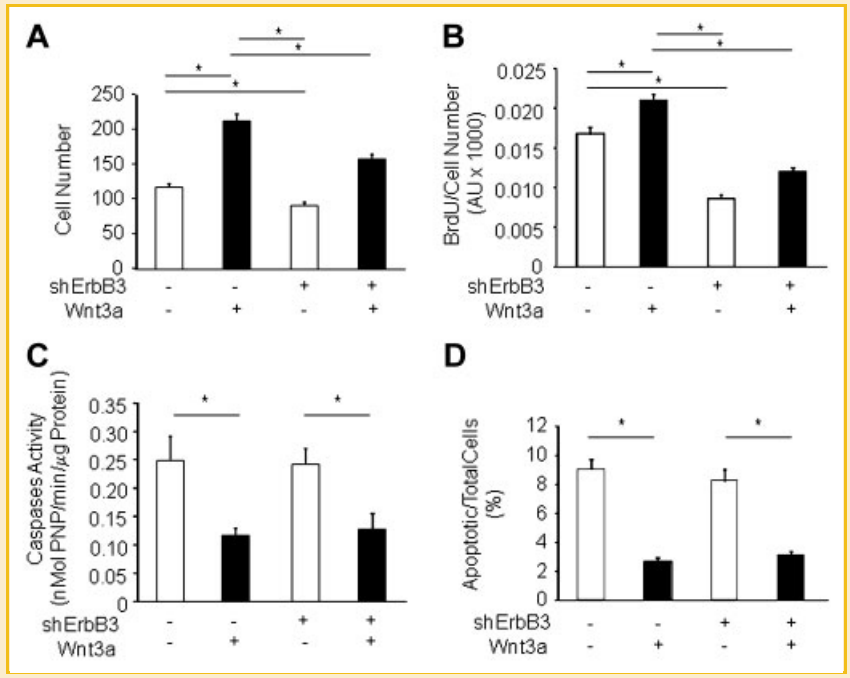

Fig. 5. ErbB3 silencing reduces basal and Wnt3a-induced cell proliferation but not survival in mesenchymal cells. C3H10T1/2 cells transduced with shErbB3 or control vector (EV) were cultured in the presence or absence of Wnt3a CM or control CM, and the cell number at 3 days (A), BrdU incorporation at 2 days (B), effector caspase activity (C) and Tunel analysis (D) at 3 days in the presence or absence of serum, were determined. Data are mean ± SEM. * Indicates a difference compared to the indicated groups ($P < 0.05$).

upregulated by Wnt3a in a microarray assay. We confirmed the results of the microarray analysis and showed that NRG-1 is upregulated at all time points by Wnt3a in both human and mouse mesenchymal cells. Unlike other members of EGF family ligands such as amphiregulin, EGF or epiregulin, the neuregulin-1 promoter region does not show TCF/LEF binding sites [Katoh and Katoh, 2006]. Thus, NRG-1 does not seem to be a direct target gene of Wnt/β-catenin signaling. Using siRNA-mediated gene knockdown of NRG-1, we found that silencing NRG-1 has a positive impact on ALP activity, suggesting a negative role of endogenous NRG-1 in early osteoblast differentiation in mesenchymal cells. Importantly, we found that Wnt3a induced a strong attenuation of the NRG-1 receptor ErbB3 in mesenchymal cells. A similar decrease in ErbB3 gene expression induced by Wnt3a has been reported in human mesenchymal cells [Si et al., 2006], further suggesting that ErbB3 is a negative target of the Wnt3 pathway. We found that the decreased ErbB3 expression functionally contributes to Wnt3a-induced osteoblast differentiation in mesenchymal cells. In support of this concept, our siRNA-mediated gene knockdown experiments showed that the positive effect of canonical Wnt signaling on osteoblast differentiation was due in large part to ErbB3 downregulation. Our analysis using the Wnt inhibitor DKK1 further indicates that ErbB3 inhibition by Wnt3a contributes to Wnt3a-induced osteoblast differentiation. Although ErbB3 silencing reduced cell number, we did not find evidence that ErbB3 may interact with Wnt3a to control mesenchymal cell proliferation or survival, suggesting that the interaction between Wnt and ErbB3 mainly impacts early osteoblast differentiation in mesenchymal cells. Taken together, these results provide novel evidence for a crosstalk between canonical Wnt and ErbB3 signaling which contributes to Wnt-induced osteoblast differentiation in mesenchymal cells.

Having shown that Wnt3a downregulates ErbB3 expression and enhances canonical Wnt3a-induced osteoblast differentiation, we analyzed whether other signaling mechanisms may be involved in this effect. EGFR is known to interact with ErbB receptors through heterodimerization [Samaga et al., 2009] and EGFR signaling plays a role in osteoblastogenesis [Schneider et al., 2009]. Notably, EGFR signaling was found to enhance MSC and osteoblastic cell proliferation in vitro [Canalis and Raisz, 1979; Tamama et al., 2006] and to promote bone formation in vivo [Chan and Wong, 2000; Marie et al., 1990; Zhang et al., 2012]. Although we found that ErbB3 interacts with EGFR in C3H10T1/2 cells, our data do not support an important role of the possible ErbB3/EGFR interaction in Wnt3a-induced MSC osteoblast differentiation since the downstream signaling events of EGFR were unaffected by ErbB3 silencing in mesenchymal cells. We also investigated the role of Src because Src interacts with ErbB3 signaling [Olayioye et al., 2001; Ishizawar et al., 2007] and Src proteins were found to control osteoblast differentiation [Marzia et al., 2000; Kaabeche et al., 2004] and survival [Almeida et al., 2005]. Interestingly, we found that ErbB3/Src interaction may play a role in Wnt3a-induced osteoblast differentiation. This is supported by our finding that ErbB3 silencing reduced Src levels. Furthermore, pharmacological inhibition of Src activity increased ErbB3- and Wnt3a-induced ALP activity in mesenchymal cells, suggesting a role for Src in the modulation of early osteoblast differentiation by Wnt3a/ErbB3 interaction. All together, the results indicate that downregulation of ErbB3 acts in concert with Wnt3a to induce early osteoblast differentiation in

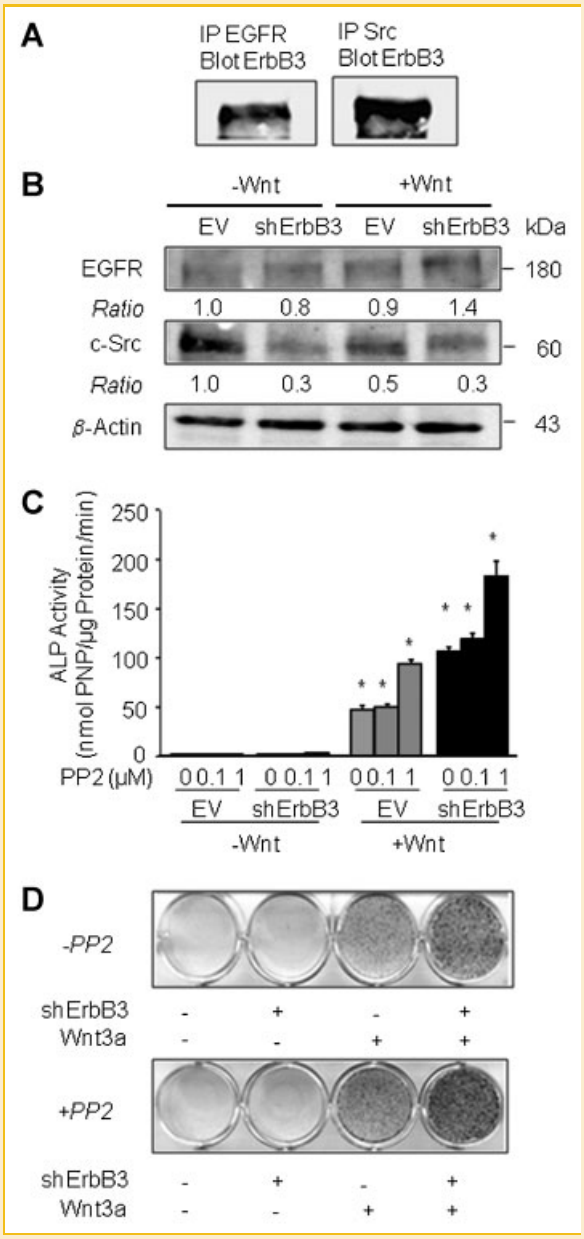

Fig. 6. ErbB3 interacts with Src to promote osteoblast differentiation in mesenchymal cells. Immunoprecipitation analysis (A) showing that ErbB3 co-immunoprecipitates with EGFR and c-Src in C3H10T1/2 cells. Cell lysates immunoprecipitated with EGFR or Src (same input) were probed with an anti-ErbB3 antibody. Western blot analysis (B) showing that shErbB3 decreased Src protein levels in C3H10T1/2 cells. Whole cell lysates (same input) were probed with the indicated antibody. Pharmacological inhibition of Src using PP2 potentiated Wnt3a- and shErbB3-induced increase in ALP activity (C) and staining (D) compared to the solvent in C3H10T1/2 cells. * Indicates a difference compared to the respective Wnt3a groups ($P < 0.05$).

mesenchymal cells in part through Wnt/β-catenin signaling and Src signaling inhibition (Fig. 7).

Our results may have significant therapeutic implications as they reveal a novel pathway that could be targeted in order to promote early osteoblast differentiation in mesenchymal cells. The finding

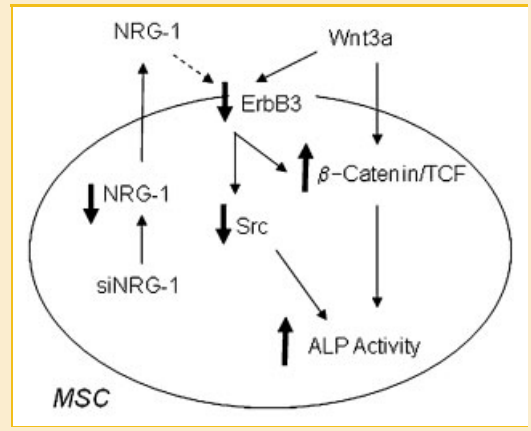

Fig. 7. Proposed model by which downregulation of ErbB3 by Wnt3a contributes to Wnt-induced early osteoblast differentiation in mesenchymal cells (MSC). Wnt3a decreases ErbB3 expression, which in turn enhances ALP activity, an early osteoblast differentiation marker, by interacting with β-catenin activity and by decreasing Src signaling whereas endogenous NRG-1 negatively regulates ALP activity in mesenchymal cells.

that downregulation of ErbB3 signaling may contribute to Wnt-induced osteoblast differentiation suggests that ErbB3 inhibitors could be used to promote osteoblastogenesis. Several ErbB inhibitors are currently used in oncology to reduce tumorigenesis in cancers associated with aberrant ErbB signaling [Citri et al., 2002; Denny, 2002; Krause and Van Etten, 2005]. In this context, we found in preliminary experiments that the molecule AZD8931 (1–10 μM), an equipotent reversible inhibitor of ErbB2 and ErbB3signaling [Hickinson et al., 2010], increases ALP activity in C3H10T1/2 cells, suggesting that pharmacological inhibition of ErbB2/3 signaling is effective at promoting early osteoblast differentiation in vitro. Since we identified the NRG-1/ErbB3 axis as a Wnt target involved in early commitment to the osteoblast lineage, inhibition of ErbB3 signaling using specific inhibitors may prove to be useful for promoting osteoblast differentiation and bone formation.

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