



Neural Cell Adhesion Molecule Regulates Osteoblastic Differentiation Through Wnt/ β -Catenin and PI3K-Akt Signaling Pathways in MC3T3-E1 Cells

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Neural cell adhesion molecule (NCAM) is involved in cell multi-directional differentiation, but its role in osteoblast differentiation is still poorly understood. In the present study, we investigated whether and how NCAM regulates osteoblastic differentiation. We found that NCAM silencing inhibited osteoblast differentiation in pre-osteoblastic MC3T3-E1 cells. The function of NCAM was further confirmed in NCAM-deficient mesenchymal stem cells (MSCs), which also had a phenotype with reduced osteoblastic potential. Moreover, NCAM silencing induced decrease of Wnt/ β -catenin and Akt activation. The Wnt inhibitor blocked osteoblast differentiation, and the Wnt activator recovered osteoblast differentiation in NCAM-silenced MC3T3-E1 cells. We lastly demonstrated that osteoblast differentiation of MC3T3-E1 cells was inhibited by the PI3K-Akt inhibitor. In conclusion, these results demonstrate that NCAM silencing inhibited osteoblastic differentiation through inactivation of Wnt/ β -catenin and PI3K-Akt signaling pathways.

Keywords: NCAM, osteoblast differentiation, Wnt/ β -catenin signaling, PI3K-Akt signaling, MC3T3-E1 cells

INTRODUCTION

Osteoporosis (OP) is one of the most common bone disorders in human beings, its incidence increases with age, especially in postmenopausal women because of the decrease in estrogen (1, 2). With the growth of the aging population in the world, the prevalence of OP is increasing dramatically, bringing a huge economic burden to the society and families (3, 4).

OP is characterized by a reduction in bone mineral density, which is mainly due to the imbalance of bone tissue formation and absorption (5, 6). Bone homeostasis depends on the balance between osteoblasts and osteoclasts, OP occurs as a result of the decrease of osteoblast-induced bone formation and the increase of osteoclast-induced bone absorption. Bone weakness and fracture are common in patients with OP because of their low bone mass and quality. Abnormal proliferation and differentiation of osteoblasts were shown involved in reduction of bone mass (7, 8). Study on the molecular mechanisms of osteoblastic differentiation is helpful to understand the pathogenesis of OP and develop new strategies for OP treatment.

Neural cell adhesion molecule (NCAM, CD56), a member of the immunoglobulin (IgG) superfamily, is widely expressed in the nervous system and plays multi-functional roles in neural regeneration, cell to cell adhesion, signal transduction and modulation of learning and memory (9–11). NCAM has been found to be expressed in other cell types such as epithelial cells, natural killer cells, skeletal muscle and mesenchymal stem cells (MSCs) (12, 13). In our previous studies, we demonstrated that NCAM enhances insulin sensitivity and promotes adipocyte differentiation, and we further found that NCAM inhibits hypertrophic chondrocyte differentiation and reduces chondrocyte hypertrophy in experimental osteoarthritis model (14, 15). Our results indicate that NCAM plays a key role in cell multi-directional differentiation, however, the function of NCAM in osteoblastic differentiation remains largely unknown.

In the present study, we evaluated the effects of NCAM on osteoblastic differentiation in mouse preosteoblast-like cells (MC3T3-E1) and in mouse MSCs. We also investigated the underlying mechanism involved in NCAM-regulated osteoblastic differentiation.

MATERIALS AND METHODS

Materials

LY294002, β -glycerophosphate, ascorbic acid-2-phosphate, and dexamethasone were obtained Sigma-Aldrich (St. Louis, MO, USA). XAV939 was obtained from EMD Millipore (Darmstadt, Germany). Lithium Chloride (LiCl) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA). The mammalian expression vector pcDNA4/Myc was obtained from Invitrogen (Carlsbad, CA, USA). siRNA expression vectors pSilencer 4.1-CMV neo was obtained from Ambion (Austin, TX, USA). Lipofectamine 2000, G418 and puromycin were purchased from Invitrogen (Carlsbad, CA, USA). Antibodies against RunX2, Osterix, β -catenin, phospho-Akt, and β -actin were purchased from Cell Signaling Technology, Inc. (Beverly, MD, USA). The secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). QuantiTect Reverse Transcription kit was purchased from Qiagen (Valencia, CA, USA). SYBR Green Master Mix was purchased from Bio-Rad Laboratories (Richmond, CA, USA). Other chemicals were purchased from Sigma-Aldrich.

NCAM-Deficient Mice and MSCs

The *Ncam*^{-/-} (knockout; KO) mice were generated on a C57/BL6 background as previously described (16). Wild-type (WT) and KO MSCs were obtained from 8-week-old male mice as previously described (14). Briefly, cells were harvested from mouse bone marrow and cultured in low glucose Dulbecco's modified Eagle's medium (DMEM-LG, with 1 g/L glucose; Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 g/mL streptomycin. Non-adherent hematopoietic cells were discarded after incubation for

7 days, and the adhered MSCs were purified by repeated passaging. MSCs with fibroblast-like morphology from passage 6 to 9 was used in this study.

Cell Culture and Osteogenic Differentiation

The mouse osteoblastic cell line MC3T3-E1 was purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were cultured in α -modified Eagle's medium (α -MEM; Hyclone, Logan, UT, USA) supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 g/mL).

WT and KO MSCs were cultured in DMEM-LG containing 10% FBS, 100 IU/mL penicillin and 100 g/mL streptomycin as previously described (14).

Cells were seeded in 12-well plates at a concentration of 1×10^5 cells/mL. The MC3T3-E1 cells and MSCs were respectively incubated in α -MEM and DMEM with 10 mM β -glycerophosphate, 25 μ M ascorbic acid-2-phosphate and 10 nM dexamethasone.

Plasmids and Transfection

siRNA vectors silencing NCAM and plasmids expressing full-length mouse NCAM were designed and constructed as previously described (14). Transfection was conducted using Lipofectamine 2000 following manufacturer's instructions. To obtain stable mixed cell lines, cells were selected with puromycin at 1 μ g/mL and G418 at 800 μ g/mL for 14 days. Gene silencing or overexpression of NCAM was validated by Western blotting with anti-NCAM antibody.

Real-Time PCR

Total RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's instructions, and cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The mRNA levels were measured using an ABI Prism 7500 Sequence detection system (Applied Biosystems) and SYBR Green qPCR Master Mix (KAPA Biosystems). The expression levels of *RunX2* and *Osterix* were normalized using the internal reference gene *GAPDH*. The PCR primers were shown in **Table 1**.

Western Blot

Cells were lysed with radio immuno-precipitation (RIPA) buffer and lysed on ice supplemented with proteinase and phosphatase inhibitors cocktail (Sigma). Protein concentrations were determined using BCA Protein Assay Kit (Pierce). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane. After blocking with 3% bovine serum albumin (BSA; Sigma) for 1 h, the membranes were incubated with primary

TABLE 1 | Primers for real-time PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
RunX2	CGAAATGCCTCCGCTGTTAT	TGAGGAATGCGCCCTAAATC
Osterix	TGACTACCCACCCCTTCCCTC	GCCTTGTACCAAGAGCCATA
GAPDH	CTTCAACAGCAACTCCCACT	GTCCAGGGTTTCTTACTCCT

antibodies against RunX2, Osterix, β -catenin, p-Akt, or β -actin overnight at 4°C. The membranes subsequently were washed three times with TBST and incubated with the secondary antibodies for 40 min at room temperature. The band density was scanned by ImageQuant LAS 4000 system (GE Healthcare) and analysed by ImageJ software.

Alizarin Red Staining and Quantification

The alizarin red staining was performed at 0, 7, or 14 days. Cells were fixed in 4% paraformaldehyde (PFA) for 30 min followed by staining with 40 mM Alizarin Red S (ARS; pH 4.2) for 30 min and then washed three times with deionized water. For the quantitative analysis of ARS, the stained wells were incubated with 10% cetyl pyridinium chloride monohydrate for 30 min at room temperature and then measured at 562 nm using a Molecular Devices microplate reader (15).

Statistical Analysis

All data are expressed as the mean \pm SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. Differences were regarded as statistically significant at $P < 0.05$. Each experiment was performed at least in triplicate.

RESULTS

NCAM Silencing Inhibits Osteoblast Differentiation of MC3T3-E1 Cells

To explore the function of NCAM in osteoblast differentiation, stable NCAM down-regulated MC3T3-E1 cells were developed using plasmid-based small interfering RNA (siRNA). The expression level of NCAM was determined using western blot. The result showed that NCAM expression in the Sh-NCAM cells was much lower compared with that of Sh-ctl cells and the interference rate reached 70% (**Figures 1A, B**). The gene expressions of osteogenic markers *RunX2* and *Osterix* were examined by quantitative real-time PCR, down-regulation of NCAM inhibited expressions of *RunX2* and *Osterix* during osteogenic differentiation (**Figures 1C, D**). Western blotting analysis showed that the protein levels of RunX2 and Osterix were also decreased in Sh-NCAM MC3T3-E1 cells (**Figures 1E-G**). In addition, calcium deposition was visualized by Alizarin Red staining and quantitative analysis, the calcium content was significantly decreased in Sh-NCAM cells as compared to Sh-ctl MC3T3-E1 cells (**Figures 1H, I**). These results demonstrated that gene silencing of NCAM inhibits osteoblast differentiation of MC3T3-E1 cells.

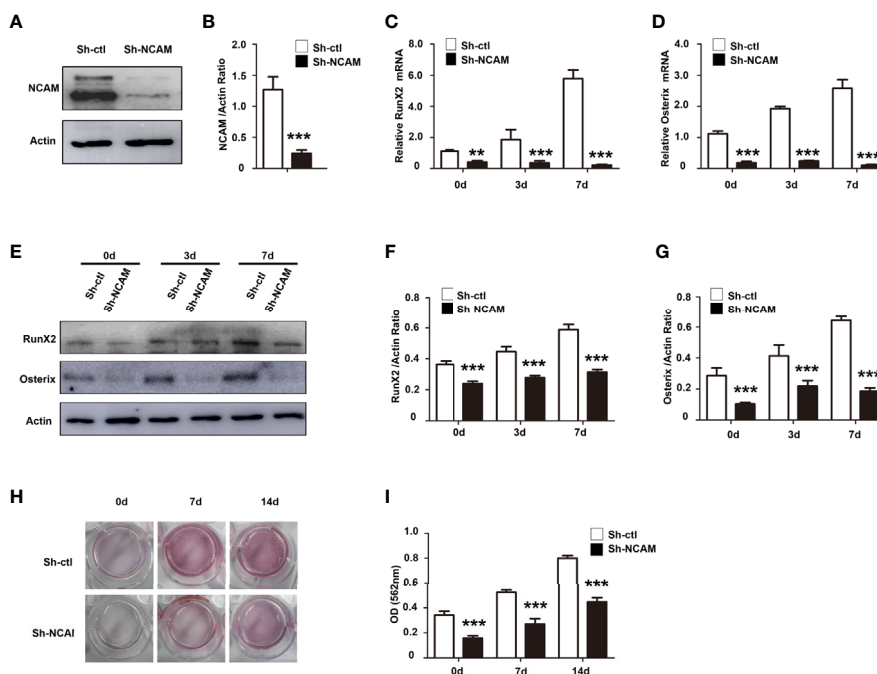


FIGURE 1 | NCAM silencing inhibits osteoblast differentiation of preosteoblast-like MC3T3-E1 cells. **(A)** Cells were transfected with pSilencer-4.1-based plasmid containing a scrambled sequence (control; Sh-ctl) or a 19-bp oligonucleotide insert targeting mouse NCAM (Sh-NCAM), and analysed by immunoblotting with anti-NCAM antibody. **(B)** Level of NCAM was quantified by densitometry and normalized to β -actin ($n=3$; mean \pm SEM; *** $p < 0.001$, compared with control siRNA group). The expression of RunX2 **(C)** and Osterix **(D)** was analysed by real-time PCR (The results are expressed as the mean \pm SEM of three independent experiments. ** $p < 0.01$, *** $p < 0.001$, compared with control siRNA-transfected cells). **(E)** The expression of RunX2 and Osterix was analysed by immunoblotting, β -actin was detected as a loading control. Levels of **(F)** RunX2 and **(G)** Osterix were quantified by densitometry and normalized to β -actin (Data are representative of three independent experiments and values are means \pm SEM. *** $p < 0.001$, compared with control siRNA-transfected cells). **(H)** Cells transfected with control or NCAM siRNA were induced with osteoblastic media and stained with Alizarin red. **(I)** The Alizarin red staining was extracted and quantified, the wavelength was measured at 562 nm (Data are representative of three independent experiments and values are means \pm SEM. *** $p < 0.001$, compared with control siRNA-transfected cells).

NCAM Deficiency Impairs Osteoblast Differentiation of Mouse MSCs

To further confirm the role of NCAM in osteoblast differentiation, we isolated bone marrow derived MSCs from wild-type (WT) and *Ncam*^{-/-} (KO) mice. The Western blot results confirmed the expression of NCAM in wild-type MSCs but not in *Ncam*^{-/-} cells (Figure 2A). To investigate whether NCAM deficiency affects osteoblast differentiation of MSCs, wild-type and *Ncam*^{-/-} MSCs were treated with osteogenic differentiation medium. The mRNA expressions of *RunX2* and *Osterix* were down-regulated in *Ncam*^{-/-} MSCs as compared to WT cells (Figures 2B, C). The protein levels of RunX2 and Osterix were detected by Western blotting. As shown in Figures 2D–F, the induction of RunX2 and Osterix in *Ncam*^{-/-} MSCs was also lower than that in wild-type cells. Alizarin red staining and quantitative analysis of the calcium content in *Ncam*^{-/-} MSCs showed decreased calcium deposition as compared to wild-type cells (Figures 2G, H). The results further confirmed the NCAM level involved in osteoblast differentiation of MSCs, suggesting that NCAM might play a key role in osteogenesis of various cell lines.

Wnt/ β -catenin Signaling Contributes to NCAM-Mediated Osteoblast Differentiation

It has been shown that the Wnt/ β -catenin signaling pathway plays an important role in osteogenic lineage (17). To investigate

the mechanism of osteoblast differentiation mediated by NCAM, the expression of β -catenin was examined in Sh-ctrl and Sh-NCAM MC3T3-E1 cells. The Western blotting results showed that β -catenin level was significantly decreased in Sh-NCAM cells (Figures 3A, B). To determine the role of β -catenin, we applied the Wnt signaling inhibitor XAV939 during osteogenic differentiation in MC3T3-E1 cells. The β -catenin expression was almost completely inhibited by XAV939 ($\geq 5 \mu\text{M}$) (Figures 3C, D). As a result of β -catenin suppression, the mRNA expressions of *RunX2* and *Osterix* were down-regulated (Figures 3E, F). Accordingly, XAV939 at $5 \mu\text{M}$ totally inhibited the protein levels of RunX2 and Osterix in MC3T3-E1 cells (Figures 3G–I). In addition, calcium deposition was also significantly blocked by XAV939 (Figures 3J, K). These results indicate that Wnt/ β -catenin signaling is involved in NCAM-mediated osteoblast differentiation.

To further determine the contribution of Wnt/ β -catenin signaling to NCAM-mediated osteoblast differentiation, the β -catenin level was increased by the Wnt pathway activator LiCl during osteogenic differentiation in Sh-NCAM MC3T3-E1 cells (Figures 4A, B). As shown in Figures 4C, D, inducing of β -catenin restored the mRNA expressions of *RunX2* and *Osterix* down-regulated by NCAM silencing. The protein levels of RunX2 and Osterix were also recovered in LiCl-treated Sh-NCAM MC3T3-E1 cells, compared with that in control cells (Figures 4E–G). Besides, Alizarin red staining and quantitative

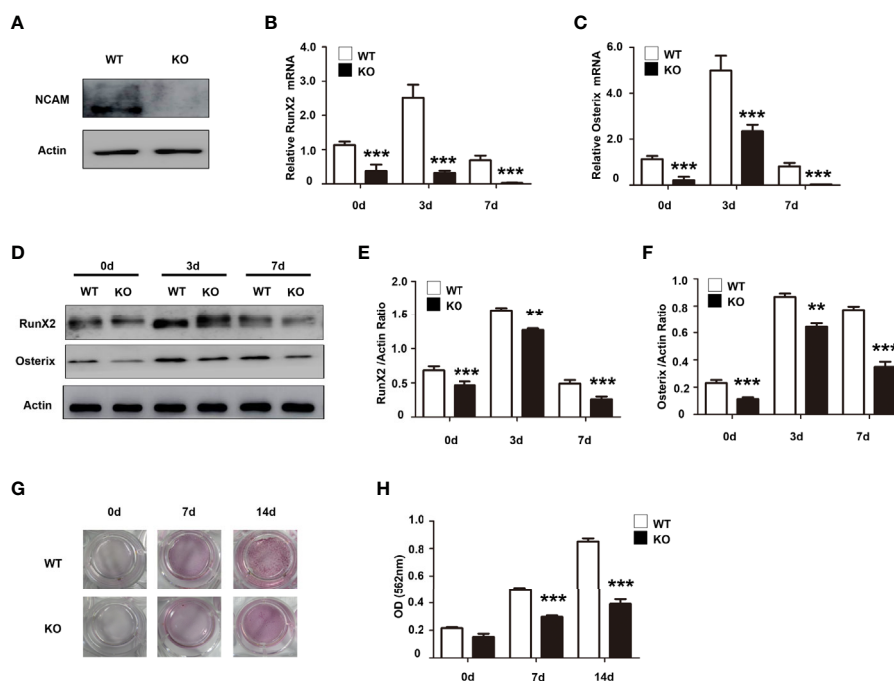


FIGURE 2 | NCAM deficiency inhibits osteoblast differentiation of mouse MSCs. (A) Wild-type (WT) and *Ncam*^{-/-} (KO) MSCs were examined with anti-NCAM antibody. β -actin was detected as a loading control. The expression of RunX2 (B) and Osterix (C) was analysed by real-time PCR (The data are expressed as the mean \pm SEM of three independent experiments. *** $p < 0.001$, compared with WT MSCs). (D) The expression of RunX2 and Osterix was analysed by immunoblotting, β -actin was detected as a loading control. Levels of RunX2 (E) and Osterix (F) were quantified by densitometry and normalized to β -actin (Data are representative of three independent experiments and values are means \pm SEM. ** $p < 0.01$, *** $p < 0.001$, compared with WT MSCs). (G) Calcium deposition of differentiated WT and KO MSCs was assessed by Alizarin red staining. (H) The Alizarin red staining was extracted and quantified, the wavelength was measured at 562 nm (Data are representative of three independent experiments and values are means \pm SEM. *** $p < 0.001$, compared with WT MSCs).

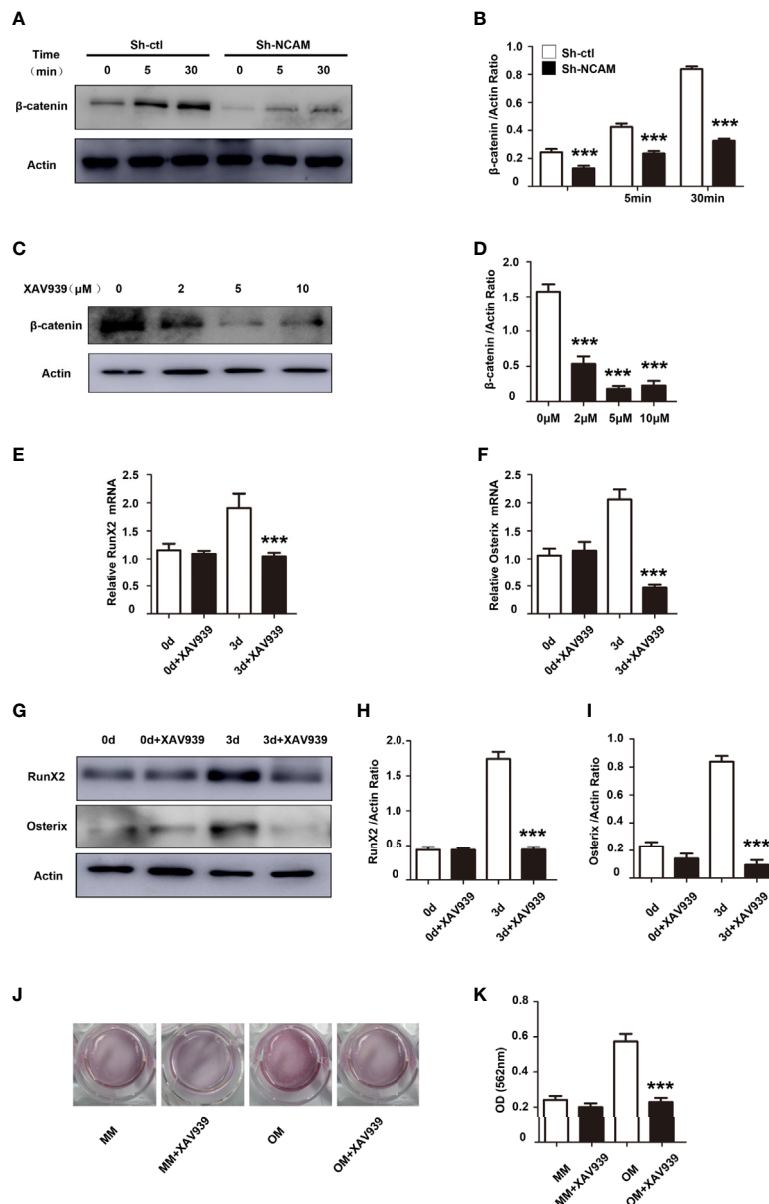


FIGURE 3 | Inhibition of Wnt/β-catenin signaling inhibits osteoblast differentiation. **(A)** Sh-ctl and Sh-NCAM MC3T3-E1 cells were treated with differentiation media for 0, 5, and 30 min, the expression of β-catenin was analysed by immunoblotting. β-actin was detected as a loading control. **(B)** Level of β-catenin was quantified by densitometry and normalized to β-actin ($n=3$; mean \pm SEM; $***p < 0.001$, compared with control siRNA group). MC3T3-E1 cells were pretreated with the Wnt inhibitor XAV939. **(C)** The expression of β-catenin was analysed and **(D)** quantified by densitometry and normalized to β-actin ($n=3$; mean \pm SEM; $***p < 0.001$, compared with cells without XAV939). The expressions of **(E)** RunX2 and **(F)** Osterix were examined by real-time PCR (The results are expressed as the mean \pm SEM of three independent experiments. $***p < 0.001$, compared with cells without XAV939). **(G)** The expressions of RunX2 and Osterix were analysed by immunoblotting, β-actin was detected as a loading control. Levels of **(H)** RunX2 and **(I)** Osterix were quantified by densitometry and normalized to β-actin (Data are representative of three independent experiments and values are means \pm SEM. $***p < 0.001$, compared with cells without XAV939). **(J)** Calcium deposition was assessed by Alizarin red staining. **(K)** The Alizarin red staining was extracted and quantified, the wavelength was measured at 562 nm (Data are representative of three independent experiments and values are means \pm SEM. $***p < 0.001$, compared with cells without XAV939).

analysis revealed that β-catenin up-regulation reversed the lower accumulation of calcium content induced by NCAM silencing in Sh-NCAM MC3T3-E1 cells (**Figures 4H, I**). The results further confirmed the contribution of Wnt/β-catenin signaling to NCAM-mediated osteoblast differentiation.

PI3K-Akt Signaling Contributes to NCAM-Mediated Osteoblast Differentiation

The phosphatidylinositol 3'-kinase (PI3K)-Akt signaling plays a significant role in the osteoblast differentiation (18). To investigate the role of PI3K-Akt signaling in NCAM-mediated osteoblast

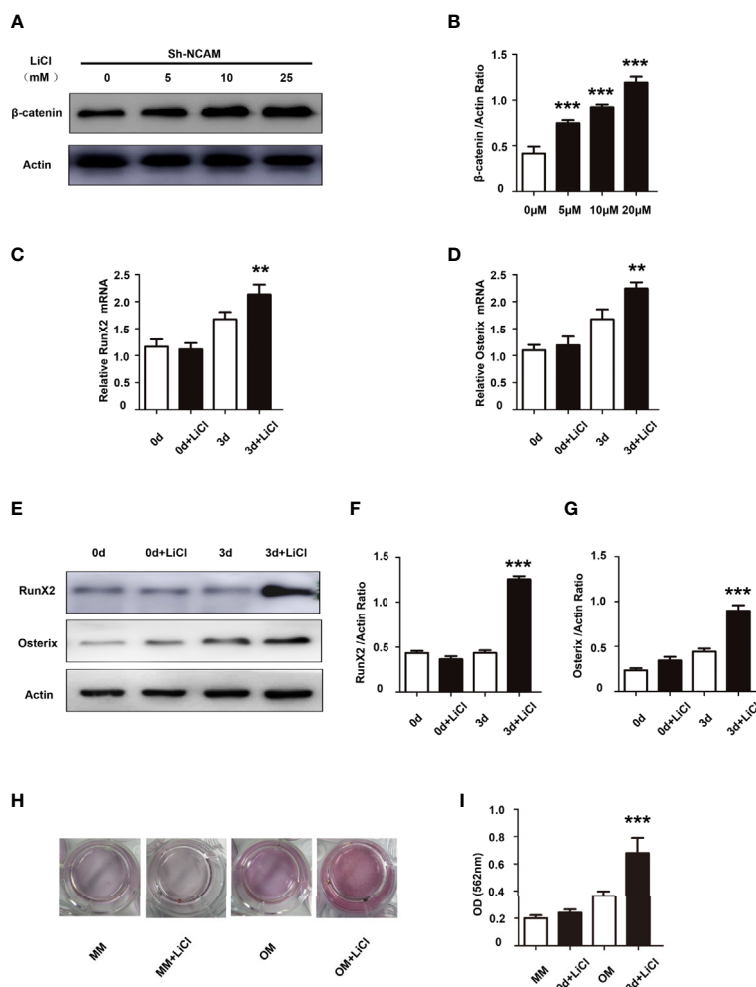


FIGURE 4 | Activation of Wnt/ β -catenin signaling recovers osteoblast differentiation inhibited by NCAM silencing. Sh-NCAM MC3T3-E1 cells were pretreated with the Wnt agonist LiCl. **(A)** the expression of β -catenin was analysed by immunoblotting at 0, 5, 10 and 25 min, β -actin was detected as a loading control. **(B)** Level of β -catenin was quantified by densitometry and normalized to β -actin ($n=3$; mean \pm SEM; *** $p < 0.001$, compared with cells without LiCl). The expressions of **(C)** RunX2 and **(D)** Osterix were examined by real-time PCR (The results are expressed as the mean \pm SEM of three independent experiments. ** $p < 0.01$, compared with cells without LiCl). **(E)** The expressions of RunX2 and Osterix were analysed by immunoblotting, β -actin was detected as a loading control. Levels of **(F)** RunX2 and **(G)** Osterix were quantified by densitometry and normalized to β -actin (Data are representative of three independent experiments and values are means \pm SEM. *** $p < 0.001$, compared with cells without LiCl). **(H)** Calcium deposition was assessed by Alizarin red staining. **(I)** The Alizarin red staining was extracted and quantified, the wavelength was measured at 562 nm (Data are representative of three independent experiments and values are means \pm SEM. *** $p < 0.001$, compared with cells without LiCl).

differentiation, the phosphorylation of Akt was examined during osteogenic differentiation. The results showed that the Akt phosphorylation was significantly decreased in Sh-NCAM MC3T3-E1 cells as compared to Sh-ctl cells (**Figures 5A, B**). The Akt inhibitor LY294002 was applied to determine whether the NCAM-mediated Akt inhibition is involved in osteoblast differentiation. The data revealed that 10 μ M of LY294002 was almost completely inhibited the phosphorylation of Akt (**Figures 5C, D**), and the levels of RunX2 and Osterix were significantly reduced in both mRNA expression (**Figures 5E, F**) and protein production (**Figures 5G–I**) during osteogenic differentiation in MC3T3-E1 cells. Alizarin red staining and quantitative analysis showed that the calcium deposition was also significantly decreased in LY294002-treated MC3T3-E1

cells (**Figures 5J, K**). These results indicated that Akt signaling is involved in NCAM-mediated osteoblast differentiation.

DISCUSSION

OP is a systemic skeletal disease characterized by low bone mineral density (19). Recent studies have shown that abnormality of osteoblast differentiation is one of the key factors leading to decreased bone mass (8, 20). In the present study, we revealed a novel role of NCAM in osteoblast differentiation. NCAM deficiency inhibits osteoblast differentiation in both mouse pre-osteoblast cells and mouse adult stem cells, in which Wnt/ β -catenin and Akt signaling pathways play important roles.

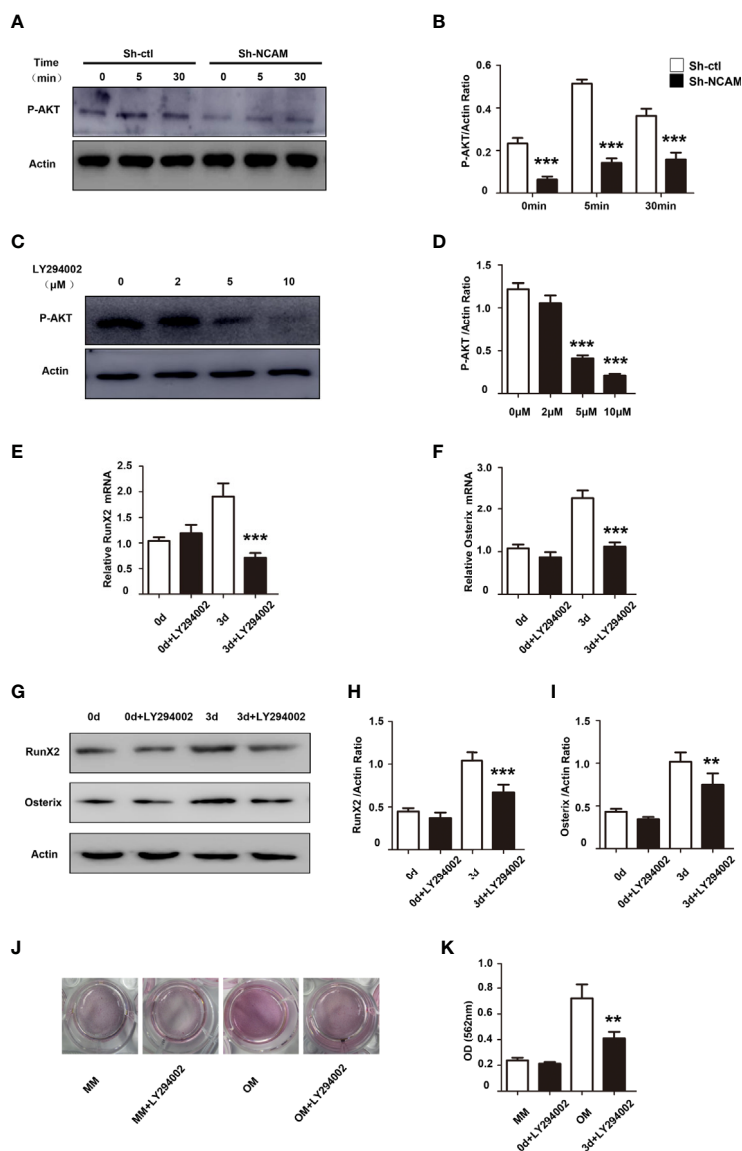


FIGURE 5 | PI3K-Akt signaling contribute to NCAM-mediated osteoblast differentiation. **(A)** Sh-ctl and Sh-NCAM MC3T3-E1 cells were treated with differentiation media for 0, 5, and 30 min, the phosphorylation of Akt was analysed by immunoblotting. β -actin was detected as a loading control. **(B)** Level of phosphorylated Akt was quantified by densitometry and normalized to β -actin ($n=3$; mean \pm SEM; $***p < 0.001$, compared with control siRNA group). MC3T3-E1 cells were pretreated with the PI3K-Akt inhibitor LY294002. **(C)** The expression of phosphorylated Akt was analysed and **(D)** quantified by densitometry and normalized to β -actin ($n=3$; mean \pm SEM; $***p < 0.001$, compared with cells without LY294002). The expressions of **(E)** RunX2 and **(F)** Osterix were examined by real-time PCR (The results are expressed as the mean \pm SEM of three independent experiments. $***p < 0.001$, compared with cells without LY294002). **(G)** The expressions of RunX2 and Osterix were analysed by immunoblotting, β -actin was detected as a loading control. Levels of **(H)** RunX2 and **(I)** Osterix were quantified by densitometry and normalized to β -actin (Data are representative of three independent experiments and values are means \pm SEM. $**p < 0.01$, $***p < 0.001$, compared with cells without LY294002). **(J)** Calcium deposition was assessed by Alizarin red staining. **(K)** The Alizarin red staining was extracted and quantified, the wavelength was measured at 562 nm (Data are representative of three independent experiments and values are means \pm SEM. $**p < 0.01$, compared with cells without LY294002).

MC3T3-E1, an osteoblast precursor cell line derived from mouse calvaria, has been widely used as a useful model for osteoblast differentiation research (21). Here, we explored the function of NCAM in osteoblast differentiation in MC3T3-E1 cells. During the process of osteoblast differentiation, *RunX2* and *Osterix* are key genes required for osteoblasts. Previous studies reported that *Runx2*-deficient (*Runx2*^{-/-}) mice lack osteoblasts and

bone formation (22, 23). Knockout of *Osterix* study in mice also demonstrated a complete lack of osteoblasts (24). *Osterix* and *Runx2* initiate osteoblast differentiation by regulating the expression levels of various specific genes that characterize the osteogenic lineage, such as osteopontin, osteocalcin, bone sialoprotein, and alkaline phosphatase (25–28). In our study, we showed that NCAM silencing inhibited osteoblast differentiation

as demonstrated by decreased induction of RunX2 and Osterix, and decreased calcium deposition in MC3T3–E1 cells. This novel role of NCAM was further corroborated in mouse MSCs, the differentiation of osteoblast was also inhibited in *Ncam*^{-/-} MSCs. Our results demonstrated that either NCAM down-regulation in preosteoblast-like cells or NCAM deficiency in MSCs impaired osteoblast differentiation, indicating that NCAM plays a pivotal role in osteogenesis.

Wnt/ β -catenin signaling, as the canonical Wnt pathway, is crucial for osteogenic lineage at early stages of osteogenic differentiation (29). Activation of Wnt/ β -catenin signaling involves the binding of Wnt to surface receptors low-density lipoprotein receptor related protein 5/6 (LRP5/6) and Frizzled (30). In this study, we demonstrated that the β -catenin expression is decreased whereas the osteogenic markers and calcium deposition are recovered by Wnt activator in NCAM-silenced MC3T3–E1 cells. NCAM induces activation of FGFR and is an important interaction partner (31). Previous study (32) showed that NCAM-silencing represses β -catenin signaling *via* fibroblast growth factor receptor (FGFR) and glycogen synthase kinase-3 β (GSK-3 β). Other report has demonstrated that Wnt/ β -catenin pathway reduces expression of NCAM through miR-30a-5p (33). These findings suggest that the effect of NCAM on Wnt signaling appears to be bidirectional and indirect, which might be mediated by FGFR, GSK-3 β or miR-30a-5p. PI3K/Akt signaling pathway was also found to play a central role in osteoblast differentiation (34). We showed here that Akt activation is decreased in NCAM-silenced MC3T3–E1 cells and contributes to NCAM-mediated osteoblast differentiation. A previous study demonstrated that the critical osteogenic gene *Runx2* is a target of β -catenin/TCF1 for the regulation of osteoblast differentiation and bone formation (35). *Runx2* is also involved in Akt pathway activation, *Runx2* and PI3K-Akt signaling are dependent on each other in the control of osteoblast differentiation, PI3K inhibitor inhibits DNA binding of *Runx2* and *Runx2*-dependent transcription, and *Runx2* also up-regulates expression of Akt and p85 and p110 β subunits of PI3K (36). Another key osteogenic gene *Osterix* is a downstream osteoblastic gene of *Runx2* (24, 37). These previous studies are consistent with our results indicating that NCAM silencing inhibits the expressions of osteogenic genes *Runx2* and *Osterix* by regulation of Wnt/ β -catenin and PI3K-Akt signaling pathways.

OP and osteoarthritis (OA) are the two most common disorders of bone related to aging worldwide. OA is characterized by degeneration of cartilage and damage in subchondral and periarticular bone (38). Although some studies reported that OP may aggravates the degree of cartilage destruction in OA knee (39–41), the relationship between them is complex. In the present study, we found that NCAM silencing inhibits osteoblast differentiation,

indicating that NCAM plays a key role in OP. We also previously demonstrated that NCAM deficiency increases the severity of cartilage damage in experimental OA (15). Our data provide NCAM as a novel target insight into the relationship between OA and OP, suggesting that overexpression of NCAM can be used as a new potential strategy for the treatment of patients with OP and OA.

However, there are some limitations to the study, for example that it neither take into the role of NCAM in osteoblastogenesis *in vivo* studies, nor relates to further research on in-depth mechanism that how NCAM triggers the signaling pathways.

In conclusion, we demonstrate a novel role of NCAM in osteoblast differentiation in both pre-osteoblasts and MSCs. Wnt/ β -catenin and PI3K-Akt signaling pathways contribute to NCAM-mediated osteoblast differentiation. The results support NCAM as a new regulator of osteoblast differentiation and a potential therapeutic target for OP treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by The Ethics Committee of Xinxiang Medical University.

AUTHOR CONTRIBUTIONS

H-JY and B-FC conceived and designed the experiments. B-FC, XF, Y-XG, LW, Y-FX, S-RL, and MW performed the experiments. XF, Y-XG, S-QJ and Z-WF analyzed the data. B-FC and H-JY wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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