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N-cadherin regulates osteogenesis and migration of bone marrow-derived mesenchymal stem cells

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Abstract N-cadherin, a calcium-dependent cellular adhesive protein, plays important roles during embryonic development and bone formation. The potential of mesenchymal stem cells (MSCs) in osteoblast differentiation and homing to the sites of injury make it a promising cell resource for tissue engineering. However, the role of N-cadherin in MSCs osteoblast differentiation and migration remains still obscure. In the present study, our results showed that prolonged N-cadherin overexpression inhibited osteogenic differentiation of MSCs through negatively regulating β-catenin and ERK1/2 signaling pathways. The mRNA expression levels of osteogenesis-related genes (Osteopontin, Osteocalcin, runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP) and bone morphogenetic protein 2) were significantly inhibited by N-cadherin, as well as the ALP activity and calcium deposit as stained by Alizarin Red S. While, silencing N-cadherin using shRNA reversed this effect. Furthermore, ectopic bone formation conducted in nude mice verified that N-cadherin significantly inhibited ectopic bone formation of MSCs in vivo. In addition, we also found that the N-cadherin overexpression could promote the migration potential of MSCs. These findings reveal that N-cadherin inhibits osteogenesis but promotes migration of MSCs. The underlying mechanism of N-cadherin inhibiting osteogenesis may through suppressing β-catenin and ERK1/2 signaling pathways.

Keywords N-cadherin · Mesenchymal stem cells · Osteogenesis · Migration

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Introduction

Mesenchymal stem cells (MSCs) are non-hematopoietic cells that can differentiate into osteoblasts, adipocytes and chondrocytes under certain conditions [1]. They also have the ability of homing to the sites of injury and inflammation. The properties of MSCs make them a promising cell source for tissue engineering, particularly for bone repair. MSCs differentiation into mature functional osteoblasts is a complex process involving many transcriptional factors and signaling pathways. The Wnt/β-catenin signaling pathway is one of the most important pathways involved in osteogenesis and bone formation. The accumulation of β-catenin in the cytoplasm results in its translocation to the nucleus to initiate osteogenesis-related gene expression, such as Runx2 which is a master transcription factor of

osteogenesis [2]. The ERKs signaling pathway also plays a key role in regulating osteogenic differentiation, including induces ALP activity and *Osterix* gene expression [3, 4], as well as phosphorylates Runx2 [5].

N-cadherin, a calcium-dependent cellular adhesive protein, is known to be expressed in neural tissues [6]. But it is also found in other non-neural cells, such as fibroblasts, myoblasts, endothelial cells and mesenchymal stem cells (MSCs) [7–9]. Recent study also found N-cadherin was strongly expressed by osteoblasts in bone, although at various level of expression [10]. N-cadherin has been shown to play important roles during embryonic development, the embryos died in the middle of gestation in mice carrying a null mutation for N-cadherin [11]. As a hemophilic adhesion molecule, N-cadherin has also been shown to affect the morphology and adhesion properties of embryonic stem cells [12]. In addition, N-cadherin has also been found to play a key role in cell migration. In migrating chick neural crest cells, disruption of N-cadherin function specifically could reduce their migratory velocity [13]. More recently, N-cadherin has been found to control zebrafish granule cell migration by continuously coordinating cell–cell contacts and cell polarity [14].

The role of N-cadherin in MSCs differentiation in to osteoblasts has not been studied, and even its role in osteoblasts is still controversial. N-cadherin mediated adherens junctions play a role in mediating signal transduction events during bone development, osteoblast differentiation would be inhibited if N-cadherin was blocked with neutralizing antibody [15]. Haiyan Li et al. [16] have reported that when human bone marrow stromal cells (HBMSCs) were co-cultured with umbilical vein endothelial cells, N-cadherin concentrated in the membrane of co-cultured HBMSCs and resulted in upregulated osteoblastic differentiation of HBMSCs. On the other hand, N-cadherin has been shown to interact with Wnt co-receptor LRP5 to negatively regulate Wnt/β-catenin signaling in osteoblasts, leading to decreased osteoblast gene expression and osteogenesis; and the N-cadherin transgenic mice also showed defective osteoblast function, reduced bone formation and delayed bone mass acquisition [17].

In the present study, we showed that overexpression of N-cadherin in MSCs inhibited osteogenesis and ectopic bone formation. While, silencing N-cadherin in MSCs could promote osteogenesis in vitro. Our findings that overexpression of N-cadherin suppressed β-catenin and pERKs which coincided with increased osteogenesis, while N-cadherin silencing reversed this effect, strongly suggest that N-cadherin suppresses osteogenic differentiation by repression of β-catenin and ERK signaling pathways. In addition, we also found that the migration potential of MSCs was enhanced by overexpression of N-cadherin. This finding would be helpful for better understanding the regulation of osteogenesis and migration of MSCs.

Materials and methods

Chemicals

All the chemicals used were purchased from Sigma, except where specified.

Plasmid construction, transfection, lentivirus production and infection

The gene encoding rat N-cadherin was amplified and cloned into a lentiviral vector. The shRNA used for silencing N-cadherin was designed as published [18]. Pseudo-lentiviruses were produced by transient transfection of 293FT packaging cells (Invitrogen) using the calcium phosphate method. For transduction, 1×10^5 cells were seeded into 6-well plate and incubated with lentiviruses and 8 μg/mL polybrene in the incubator for 24 h. After 48 h, blasticidin (Invitrogen) was added into medium to select MSCs stably expressing N-cadherin.

Isolation and culture of BM-MSCs

All animal experiments were approved by the Animal Research Ethics Committee of the Chinese University of Hong Kong. The details of BM-MSCs isolation and culture have been described previously [19]. The cellular surface markers (CD90, CD44, CD31 and CD34) of MSCs were checked by flow cytometry (Supplemental Fig. 1).

Osteogenic differentiation and ALP activity assay

The MSCs were treated with osteogenic induction medium (OIM) containing 100 nM dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM L-ascorbic acid-2-phosphate. 7 days after osteogenic induction, the ALP activity assay was conducted using alkaline phosphatase (alp)-amp (Biosystems). The ALP activity was normalized to total protein concentration.

RNA isolation and quantitative RT-PCR

Total cellular RNA was isolated with RNA Mini Kit (Invitrogen), and then reverse transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. PCR amplification was performed using the primer sets outlined in Supplemental Table. 1. β-Actin was used as an internal control to evaluate the relative expression.

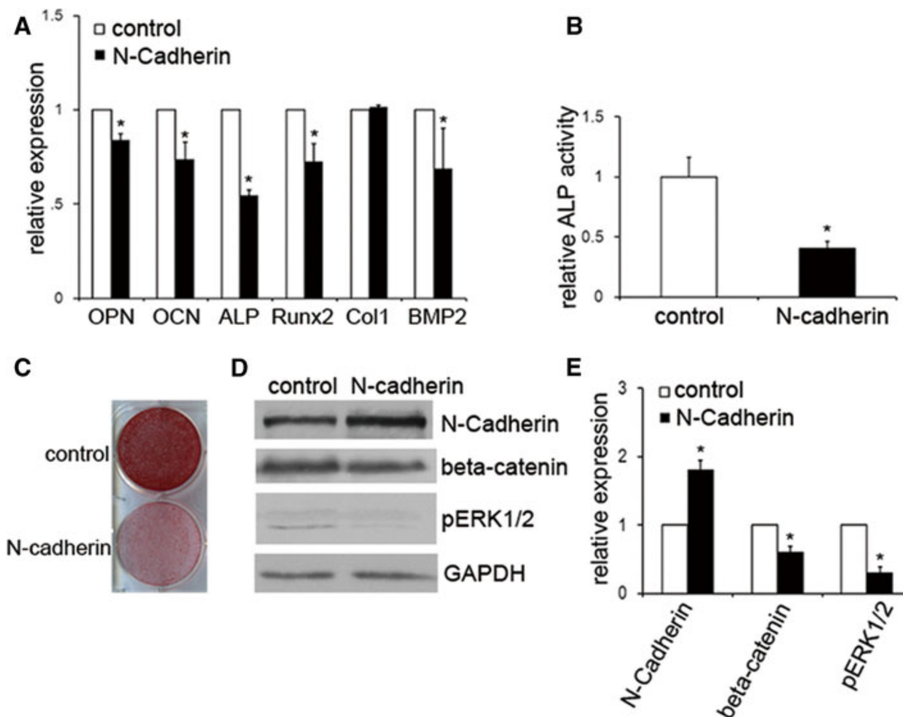

Fig. 1 N-cadherin inhibited osteogenesis through inhibiting β-catenin and ERK1/2 signaling pathways. BM-MSCs were transduced with N-cadherin or control (*empty vector*) using lentivirus. **a** The total RNA was extracted for quantitative PCR analysis after 7 days of osteogenic induction. The data are expressed as mean ± SD ($n = 3$). **b** The ALP activity assay was conducted after 7 days of osteogenic induction medium treatment. The data are expressed as mean ± SD ($n = 3$). **c** The mineralization of MSCs was showed by Alizarin Red S staining after 19 days of osteogenic induction. **d** Total proteins were extracted from MSCs transduced with N-cadherin or control, and then analyzed by western blot using indicated antibodies. GAPDH was used as loading control. The experiments were repeated three times. **e** Quantification of the bands intensity using ImageJ software. The protein level was normalized to GAPDH

Western blot

Equal proteins were loaded onto 10 % Tris/glycine gels for electrophoresis and then transferred to a PVDF membrane (Millipore). Anti-β-catenin (BD), anti-N-cadherin (BD) anti-p-ERK1/2 (BD) or anti-GAPDH (Santa Cruz) antibodies were used to in the analysis. After washing in TBST, the membrane was incubated with horseradish peroxidase-linked secondary antibodies (anti-mouse or anti-goat) for 1 h at room temperature. Following TBST washes, protein was detected with the enhanced chemiluminescence blotting reagents (Amersham Biosciences) according to the manufacturer's instruction. The band intensity was quantified using ImageJ software.

Cell migration

Migration was assayed using BD Falcon cell culture insert which is 8 μm in pore size. The upper of the insert was plated with 0.5×10^5 MSCs overexpressing N-cadherin or control in α-MEM. The lower chamber contained α-MEM supplemented with 10 % FBS as chemoattractant. Then MSCs were put into the incubator for 12 h. The MSCs remaining on the upper surface of the membrane were removed with a cotton swab. After washed with PBS, the membrane was fixed with 4 % paraformaldehyde for 15 min, and stained with 0.5 % crystal violet. The number of cells passed through the membrane was counted under microscope.

Ectopic bone formation assay

2×10^5 MSCs stably transduced with empty vector or N-cadherin were loaded onto sterilized Skelite® resorbable Si-TCP bone graft substitute, and incubated at 37 °C for 3 h to allow attachment. Four nude mice were under general anaesthesia and the substitutes with cells were then implanted subcutaneously at the dorsal sides. The transplants were harvested 8 weeks later and subjected to histological examination. The osteoid matrix areas were measured using ImageJ software, and measured as reported previously [20].

Statistical analysis

All experiments were performed at least three times. The data were analyzed by independent two-tailed Student's t

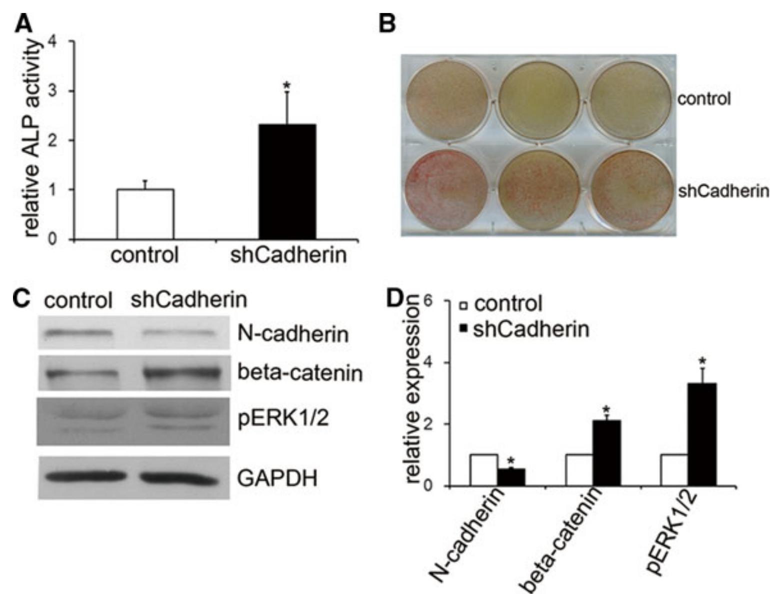

Fig. 2 Silencing N-cadherin using shRNA promoted osteogenesis. BM-MSCs were transduced with shCadherin or control (*empty vector*). **a** The ALP activity was greatly increased by N-cadherin silencing compared to control when the MSCs were treated for 7 days with osteogenic induction medium ($n = 3$). **b** The mineralization of MSCs was showed by Alizarin Red S staining after 14 days of osteogenic induction. **c** The total proteins were extracted from MSCs transduced with shCadherin or control, and then analyzed by western blot using indicated antibodies. GAPDH was used as loading control. The experiments were repeated three times. **d** Quantification of the bands intensity using ImageJ software. The protein level was normalized to GAPDH

test using SPSS (version 16.0; SPSS Inc, USA). $p < 0.05$ was regarded as statistically significant.

Results

N-cadherin overexpression inhibited osteogenesis of MSCs

In order to evaluate the role of N-cadherin in regulating osteogenesis of MSCs, the MSCs were infected with lentiviruses carrying N-cadherin and then subjected to osteogenic induction. After 7 days, quantitative RT-PCR result showed that the mRNA expression levels of osteogenesis-related genes (OPN, OCN, Runx2, ALP and BMP2) were significantly inhibited by N-cadherin (Fig. 1a). Also, the ALP activity assay indicated that the ALP activity was greatly inhibited by N-cadherin (Fig. 1b). 19 days after induction, Alizarin Red S staining showed that much less calcium deposit formed in MSCs overexpressing N-cadherin (Fig. 1c). Furthermore, the level of β-catenin and pERK1/2 in MSCs overexpressing N-cadherin was significantly down regulated, as shown in Fig. 1d, e.

N-cadherin silencing promoted osteogenesis of MSCs

To verify the inhibition of osteogenic differentiation by N-cadherin overexpression was specific, the endogenous N-cadherin gene was knocked down using shRNA technique. After 7 days of osteogenic induction, N-cadherin silencing was found to significantly increase the ALP activity (Fig. 2a). And after 14 days of osteogenic induction, much more calcium deposit was found in N-cadherin silencing MSCs (Fig. 2b). The western blot result showed that the level of N-cadherin was significantly reduced by shRNA targeting N-cadherin (shCadherin) compared with control (Fig. 2c, d). As expected, the level of β-catenin and pERK1/2 was greatly upregulated in MSCs transduced with shCadherin (Fig. 2c, d).

N-cadherin inhibited ectopic bone formation in vivo

Furthermore, to confirm the role of N-cadherin in osteogenesis of MSCs in vivo, the MSCs stably transduced with N-cadherin or GFP (empty vector) were loaded on to Si-TCP graft substitutes and implanted subcutaneously at the dorsal sides of nude mice. HE staining was used to detect the formation of osteoid matrix after 8 weeks of transplantation. The result showed that there was new bone formation in the pores of grafts, and the amount of osteoid matrix in N-cadherin overexpressing group was significantly less than that of control (Fig. 3).

N-cadherin promoted MSCs migration

We next examined the effect of N-cadherin overexpression on migration ability of MSCs using BD Falcon cell culture insert which is 8 μm in pore size. After incubation for 12 h,

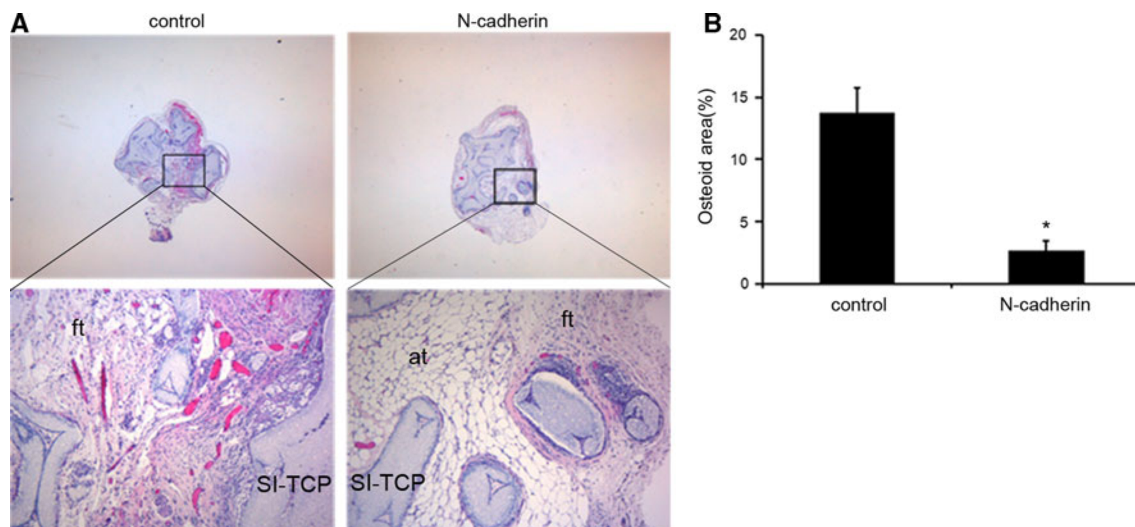

Fig. 3 Overexpression of N-cadherin in MSCs inhibited ectopic bone formation in vivo. **a** The sections were stained with routine hematoxylin and eosin, amorphous osteoid matrix could be seen in the pores of transplants. *at* adipose tissue; *ft* fibrous tissue. *Scale bar* = 200 µm. **b** Quantification of new bone area showed that there was much less osteoid matrix in N-cadherin overexpressing group. Five microscopic fields from each sample were used for measurement. Results are presented as mean ± SD

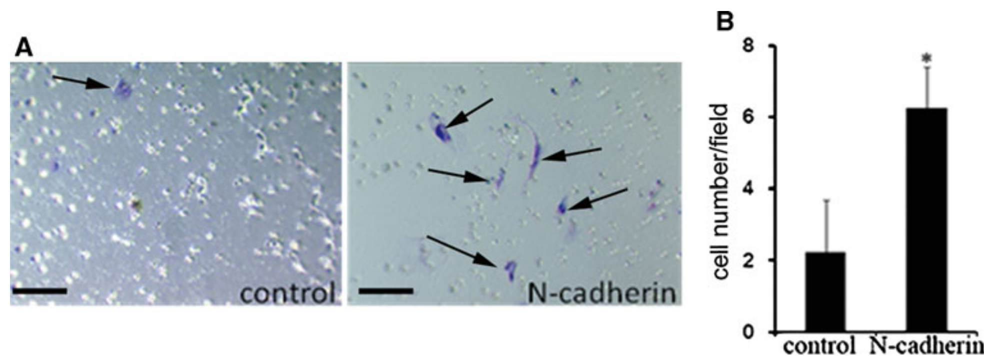

Fig. 4 N-cadherin overexpression promoted migration of MSCs. An equal number of MSCs transduced with N-cadherin or empty vector suspended in α-MEM were added into the upper layer of BD Falcon cell culture insert, respectively, and the MSCs migrated through the membrane were detected with crystal violet staining. **a** Representative images of crystal violet staining of MSCs crossed the membrane. The *arrowhead* indicates crystal violet staining of MSCs. **b** The number of MSCs that passed through the membrane was counted ($n = 3$). *Scale bar* = 100 µm

the number of MSCs that pass through the membrane was counted. Significant difference in the number of MSCs crossed the membrane was observed between N-cadherin overexpressing and control MSCs (Fig. 4).

Discussion

In the present study, we investigated the role of N-cadherin in regulating osteogenesis and migration of MSCs. The results indicated that N-cadherin overexpression negatively regulated osteogenesis through inhibiting β-catenin and pERK1/2 signaling pathways. However, the migration ability of MSCs was promoted by overexpression of N-cadherin. And when MSCs overexpressing N-cadherin combined with TCP material were transplanted in nude mice, the osteoid matrix formed after 8 weeks was much less than the control group.

β-Catenin is a downstream effecter of Wnt signaling pathway which plays an important role in osteogenesis and bone formation [21–23]. In β-catenin-deficient mice, the growth plate organization, endochondral and perichondrial bone formation have been shown to be defective [24]. We found that the level of β-catenin was significantly down regulated by N-cadherin overexpression, while N-cadherin silencing using shRNA greatly increased the level of β-catenin. Our result was consistent with the previous finding that N-cadherin down regulated Wnt/β-catenin signaling pathway by interacting with LRP5/6 to trigger β-catenin degradation in MC3T3E1 cells [17]. ERK1/2, a component of MAPK signaling pathway, is constantly activated during osteogenic differentiation, the study by Jaiswal et al. [25]

has suggested that the commitment of human MSCs into osteogenic or adipogenic lineages is governed by activation or inhibition of ERK1/2 respectively . In our study, we found that the level of pERK1/2 was also downregulated by N-cadherin overexpression.

During the progressive process of osteogenesis, the complex cell–cell and cell–matrix interactions are indispensible for osteoblast adhesion, communication and gene expression [26–28]. However, the role of N-cadherin in osteoblast differentiation is still not clarified as controversial evidences exist. For example, a direct evidence support N-cadherin could promote osteogenesis comes from the study that osteoblast differentiation would be inhibited if N-cadherin was blocked with neutralizing antibody in MC3T3E1 pre-osteoblasts [15]. On the other hand, N-cadherin has also been shown to interact with Wnt co-receptor LRP5 to negatively regulate Wnt/β-catenin signaling in MC3T3E1 pre-osteoblasts, leading to decreased osteoblast gene expression and osteogenesis [17]. So far, the evidence has suggested that N-cadherin is indispensible for osteogenesis as completely blocking it leads to impaired osteogenesis. While our result showed that partially silencing N-cadherin could promote osteogenesis, and N-cadherin overexpression showed the reversed effect. Taken together, the findings up to now suggest that N-cadherin, as a cellular adhesive protein, is indispensible for MSCs or osteoblast, but its expression level should be strictly regulated, too much or too little is bad for osteogenesis.

In addition, N-cadherin has been found to be upregulated in prostate, skin and breast cancers, which is associated with enhanced cell motility, invasion and metastasis [29, 30]. Silencing N-cadherin has been shown to suppress the long-term engraftment of hematopoietic stem cells [31]. In the present study, we found that overexpression of N-cadherin significantly promoted the migration ability of MSCs. As we know, MSCs have the capacity to migrate from the bone marrow to the sites of injury in response to injuries. N-cadherin may play a vital role in this process, as our unpublished data shows that the peripheral blood-derived MSCs expressed higher levels of N-cadherin compared with bone marrow-derived MSCs.

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References

1. Jiang YH, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR et al (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41–49
2. Dong YF, Soung DY, Schwarz EM, O'Keefe RJ, Drissi H (2006) Wnt induction of chondrocyte hypertrophy through the Runx2 transcription factor. J Cell Physiol 208:77–86
3. Takeuchi Y, Suzawa M, Kikuchi T, Nishida E, Fujita T, Matsumoto T (1997) Differentiation and transforming growth factor-beta receptor down-regulation by collagen-alpha 2 beta 1 integrin interaction is mediated by focal adhesion kinase and its downstream signals in murine osteoblastic cells. J Biol Chem 272:29309–29316
4. Salasznyk RM, Klees RF, Williams WA, Boskey A, Plopper GE (2007) Focal adhesion kinase signaling pathways regulate the osteogenic differentiation of human mesenchymal stem cells. Exp Cell Res 313:22–37
5. Xiao G, Jiang D, Thomas P, Benson MD, Guan K, Karsenty G et al (2000) MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. J Biol Chem 275:4453–4459
6. Inuzuka H, Redies C, Takeichi M (1991) Differential expression of R- and N-cadherin in neural and mesodermal tissues during early chicken development. Development 113:959–967
7. Knudsen KA, Myers L, McElwee SA (1990) A role for the Ca_2 (+)-dependent adhesion molecule, N-cadherin, in myoblast interaction during myogenesis. Exp Cell Res 188:175–184
8. Li WG, Xu XX (2005) The expression of N-cadherin, fibronectin during chondrogenic differentiation of MSC induced by TGF-beta(1). Chin J Traumatol 8:349–351
9. Salomon D, Ayalon O, Patel-King R, Hynes RO, Geiger B (1992) Extrajunctional distribution of N-cadherin in cultured human endothelial cells. J Cell Sci 102(Pt 1):7–17
10. Marie PJ (2002) Role of N-cadherin in bone formation. J Cell Physiol 190:297–305
11. Radice GL, Rayburn H, Matsunami H, Knudsen KA, Takeichi M, Hynes RO (1997) Developmental defects in mouse embryos lacking N-cadherin. Dev Biol 181:64–78
12. Karabekian Z, Gillum ND, Wong EW, Sarvazyan N (2009) Effects of N-cadherin overexpression on the adhesion properties of embryonic stem cells. Cell Adh Migr 3:305–310
13. Kasemeier-Kulesa JC, Bradley R, Pasquale EB, Lefcort F, Kulesa PM (2006) Eph/ephrins and N-cadherin coordinate to control the pattern of sympathetic ganglia. Development 133:4839–4847
14. Rieger S, Senghaas N, Walch A, Koster RW (2009) Cadherin-2 controls directional chain migration of cerebellar granule neurons. PLoS Biol 7:e1000240
15. Guntur AR, Rosen CJ, Naski MC (2011) N-cadherin adherens junctions mediate osteogenesis through PI3 K signaling. Bone 50:54–62
16. Li H, Daculsi R, Grellier M, Bareille R, Bourget C, Amedee J (2010) Role of neural-cadherin in early osteoblastic differentiation of human bone marrow stromal cells cocultured with human umbilical vein endothelial cells. Am J Physiol Cell Physiol 299:422–430
17. Hay E, Laplantine E, Geoffroy V, Frain M, Kohler T, Muller R et al (2009) N-cadherin interacts with axin and LRP5 to negatively regulate Wnt/beta-catenin signaling, osteoblast function, and bone formation. Mol Cell Biol 29:953–964
18. Ezzat S, Zheng L, Winer D, Asa SL (2006) Targeting N-cadherin through fibroblast growth factor receptor-4: distinct pathogenetic and therapeutic implications. Mol Endocrinol 20:2965–2975
19. Xu L, Song C, Ni M, Meng F, Xie H, Li G (2012) Cellular retinol-binding protein 1 (CRBP-1) regulates osteogenesis and adipogenesis of mesenchymal stem cells through inhibiting RXRalpha-induced beta-catenin degradation. Int J Biochem Cell Biol 44:612–619
20. Liu Y, Wang L, Kikuiri T, Akiyama K, Chen CD, Xu XT et al (2011) Mesenchymal stem cell-based tissue regeneration is

governed by recipient T lymphocytes via IFN-gamma and TNF-alpha. Nat Med 17:1594–1601
21. Day TF, Guo X, Garrett-Beal L, Yang Y (2005) Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Dev Cell 8:739–750
22. Gaur T, Lengner CJ, Hovhannisyan H, Bhat RA, Bodine PVN, Komm BS et al (2005) Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. J Biol Chem 280:33132–33140
23. Mbalaviele G, Sheikh S, Stains JP, Salazar VS, Cheng SL, Chen D et al (2005) Beta-catenin and BMP-2 synergize to promote osteoblast differentiation and new bone formation. J Cell Biochem 94:403–418
24. Nagayama M, Iwamoto M, Hargett A, Kamiya N, Tamamura Y, Young B et al (2008) Wnt/beta-catenin signaling regulates cranial base development and growth. J Dent Res 87:244–249
25. Jaiswal RK, Jaiswal N, Bruder SP, Mbalaviele G, Marshak DR, Pittenger MF (2000) Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase. J Biol Chem 275:9645–9652
26. Civitelli R (1995) Cell-cell communication in bone. Calcif Tissue Int 56(Suppl 1):S29–31
27. Globus RK, Moursi A, Zimmerman D, Lull J, Damsky C (1995) Integrin-extracellular matrix interactions in connective tissue remodeling and osteoblast differentiation. ASGSB Bull 8:19–28
28. Franceschi RT (1999) The developmental control of osteoblast-specific gene expression: role of specific transcription factors and the extracellular matrix environment. Crit Rev Oral Biol Med 10:40–57
29. Hazan RB, Kang L, Whooley BP, Borgen PI (1996) N-cadherin promotes adhesion between invasive breast cancer cells and the stroma. Cell Adhes Commun 4:399–411
30. Nagi C, Guttman M, Jaffer S, Qiao R, Keren R, Triana A et al (2005) N-cadherin expression in breast cancer: correlation with an aggressive histologic variant-invasive micropapillary carcinoma. Breast Cancer Res Tr 94:225–235
31. Hosokawa K, Arai F, Yoshihara H, Iwasaki H, Nakamura Y, Gomei Y et al (2010) Knockdown of N-cadherin suppresses the long-term engraftment of hematopoietic stem cells. Blood 116:554–563