Original Paper

NEL-Like Molecule-1 (Nell1) Is Regulated by Bone Morphogenetic Protein 9 (BMP9) and Potentiates BMP9-Induced Osteogenic Differentiation at the Expense of Adipogenesis in Mesenchymal Stem Cells

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Key Words

BMP-9 • Nell1 • Mesenchymal stem cells • NEL-Like Molecule • Osteogenic differentiation • Adipogenesis

Abstract

Background: BMP9 induces both osteogenic and adipogenic differentiation of mesenchymal stem cells (MSCs). Nell1 is a secretory glycoprotein with osteoinductive and anti-adipogenic activities. We investigated the role of Nell1 in BMP9-induced osteogenesis and adipogenesis in MSCs. **Methods:** Previously characterized MSCs iMEFs were used. Overexpression of BMP9 and NELL1 or silencing of mouse Nell1 was mediated by adenoviral vectors. Early and late osteogenic and adipogenic markers were assessed by staining techniques and qPCR analysis. *In vivo* activity was assessed in an ectopic bone formation model of athymic mice.

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Results: We demonstrate that Nell1 expression was up-regulated by BMP9. Exogenous Nell1 potentiated BMP9-induced late stage osteogenic differentiation while inhibiting the early osteogenic marker. Forced Nell1 expression enhanced BMP9-induced osteogenic regulators/markers and inhibited BMP9-upregulated expression of adipogenic regulators/markers in MSCs. *In vivo* ectopic bone formation assay showed that exogenous Nell1 expression enhanced mineralization and maturity of BMP9-induced bone formation, while inhibiting BMP9-induced adipogenesis. Conversely, silencing Nell1 expression in BMP9-stimulated MSCs led to forming immature chondroid-like matrix. **Conclusion:** Our findings indicate that Nell1 can be up-regulated by BMP9, which in turn accelerates and augments BMP9-induced osteogenesis. Exogenous Nell1 may be exploited to enhance BMP9-induced bone formation while overcoming BMP9-induced adipogenesis in regenerative medicine.

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Introduction

Bone morphogenetic proteins (BMPs) belong to the TGF- β superfamily, and play critical roles in skeletal development, bone formation and stem cell differentiation [1-6]. At least 15 different BMPs have been identified in humans, and disruptions in BMP signaling result in a variety of skeletal and extraskeletal anomalies differentiation [3, 4, 7]. Mesenchymal stem cells (MSCs) are multipotent progenitor cells with the capacity of differentiating into osteoblastic, chondrogenic, and adipogenic lineages [8-13]. MSCs have attracted significant attention for their potential role in stem cell biology and regenerative medicine [12-18]. Through a comprehensive analysis of the 14 types of BMPs' osteogenic activities, we demonstrated that BMP9 is among the most osteogenic BMPs that induce osteoblastic differentiation of MSCs by regulating a distinct set of downstream mediators of osteogenic signaling [19-27], as well as through extensive crosstalk with other major signaling pathways [28-35].

BMP9 (also known as growth differentiation factor 2, or GDF-2) is a relatively less characterized member of the BMP family, which was first isolated from fetal mouse liver [27, 36]. BMP9 has been shown to stimulate hepatocyte proliferation, to induce and maintain the cholinergic phenotype within basal forebrain neurons, to inhibit hepatic glucose production and critical enzymes of lipid metabolism, and to help maintain the homeostasis of iron metabolism [36-42]. BMP9 is also a synergistic factor for hematopoietic progenitor cell generation [43] and more recently shown to act as an important regulator of endothelial cells, angiogenesis and lymphatic vessel formation [44-48]. Through a comprehensive analysis, we demonstrated that osteogenic BMPs, such as BMP9, effectively induce adipogenic differentiation of MSCs [4, 27, 49], which was further supported by other groups [50-52]. However, the detailed mechanism underlying BMP9-induced adipogenesis remains to be defined.

Here we investigate the effect of Nell1 (Nel-like molecule 1) on BMP9-induced osteogenic versus adipogenic differentiation of MSCs. Nell1 is a large secretory glycoprotein, and was originally identified in craniosynostosis patients as being specifically upregulated within prematurely fusing sutures [53]. *Nell1* deficiency severely disrupts bone growth, as mice with nonsense mutations in *Nell1* die perinatally with major skeletal anomalies in the craniofacial complex, spine, and long bones [54, 55]. Conversely, transgenic *Nell1*-overexpressing mice recapitulate craniosynostosis-like phenotypes [56]. Accordingly, several recent studies indicate that Nell1 exerts osteoinductive activity [55, 57].

In this study, we investigate the effect of Nell1 on BMP9-induced osteogenic differentiation and demonstrate that Nell1 synergizes with BMP9's osteogenic activity while effectively suppressing BMP9-induced adipogenic differentiation of MSCs. Our findings suggest that BMP9 and Nell1 may be further explored as a pair of synergistic bone forming factors for efficacious bone regeneration.

Materials and Methods

Cell culture and chemicals

The reversibly immortalized mouse embryonic fibroblasts (iMEFs) were previously characterized and used as mesenchymal stem cells [58, 59]. HEK-293 (from ATCC, Manassas, VA) and its derivative line 293pTP cells [60], as well as the iMEFs, were maintained in the completed Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO₂ as described [61-64]. Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA).

Construction of recombinant adenoviruses expressing BMP-9, NELL1 and simNell1

Recombinant adenoviruses were generated using our previously reported AdEasy technology [65, 66]. Briefly, the coding regions of human BMP9 and human NELL1 were PCR amplified and cloned into an adenoviral shuttle vector, and subsequently used to generate recombinant adenoviruses in HEK-293 or 293pTP cells [60]. The siRNA target sites against mouse Nell1 coding region were selected by using Dharmacon's siDESIGN program, and the siRNA oligonucleotide pairs were cloned into the pSES or pAdTrace-OK adenoviral shuttle vector to generate recombinant adenoviruses [67, 68]. The resultant adenoviruses were designated as Ad-BMP9, AdR-NELL1 and AdR-simNell1, which Ad-BMP9 also expresses GFP, whereas AdR-NELL1 and AdR-simNell1 co-express RFP as markers for monitoring infection efficiency [31, 33, 34, 69]. Analogous adenovirus expressing only RFP or GFP was used as controls [28, 61, 70]. In order to increase adenovirus infectivity, polybrene was added to all adenovirus infections as described [71].

RNA isolation, semi-quantitative RT-PCR (sqPCR), and TqPCR analyses

Total RNA was isolated using TRIzol Reagents (Invitrogen) and used to generate cDNA templates by reverse transcription using random hexamer and M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA). The cDNA products were further diluted 5 to 10 fold and used as PCR templates. The PCR primers (usually 18-20mers, product size ranged 120bp to 200bp; Table 1) were designed by using Primer3Plus [72]. The sqPCR were carried out as described [73-78]. Briefly, a touchdown cycling program was as follows: 94°C for 2 min for 1 cycle; 92°C for 20 s, 68°C for 30 s, and 72°C for 12 cycles decreasing 1°C per cycle; and then at 92°C for 20 s, 57°C for 30 s, and 72°C for 20 s for 20 to 25 cycles, depending on the abundance of target genes. PCR products were resolved on 1.5% agarose gels. All samples were normalized by the expression level of Gapdh.

The TqPCR analysis was also carried out as described [62, 79, 80]. Specifically, TqPCR reactions were carried out using the following conditions, 95°C × 3" for one cycle; 95°C × 20", 66°C × 10", for 4 cycles by

Table 1. List of the Oligonucleotides for Cloning, siRNAs and qPCR

Genes	Species	Forward	Reverse	Accession No.	Use
Nell-1	mouse	GGCCTCTAGCTTGGCCCTAGT	GCTCAGCCTCGAATACCAG	NM_001037906	sqPCR or TqPCR
Gapdh		GAAGGTGCGGTGAACGGAT	ACTGTGCGGTGAATTTGCC	NM_008084	
Osteocalcin		CCTTCATGTCCAAGCAGGA	GGCGGTCTTCAAGCCATAC	NM_001032298	
Osteopontin		CCTCCGGTGAAAGTGAC	CTGTGGCGCAAGGAGATT	NM_001204201	
Osterix		GGGAGCAGATGCCAAGA	TACTCTGGCCATAGGG	NM_130458	
Runx2		CCGGTCTCTCCAGGAT	GGGAAGTCTGTGGCTTC	NM_001146038	
AggreCAN		TGGCTTCTGGAGACAGGACT	TTCTGCTGTCTGGGTCTCCT	NM_007424	
Col2a1		CCGAGTCACTCCAGGAT	TGCAGTCTGCCAGTTCA	NM_031163	
Col9a1		GGCTCTCTCTGGAAGGTAA	AGTCTGCGCTTTCCTATTCT	NM_007740	
Comp		GGACACAGCATCCAGGT	ACCGCACCTGATGTAGC	NM_016685	
Sox9		GGCAAGCAAGGAGACCA	GCAGGCAGACTCCAGCAT	NM_011448	
Ap2/Fabp4		GGTGACAGATGGGATGG	TGGCTCATGCCCTTTCAT	NM_024406	
C/ebpa		GCCTTGTGCAATGGCAGT	CTTCCCCAGCCGTTAGT	NM_007678	
C/ebpb		GGCCAAGGCCAAGAAGAC	GTGCTGCGTCTCCAGTT	NM_009883	
Pgc-1		ATGTGTCGCCCTCTTGCTCT	ATCTACTGCTGGGGACCTT	NM_008904	
Pparg2		ACTGCCGATCCACAAA	TCTCTCTCTGGCCTGTG	NM_011146	
Ucp1		AGAATGCAAGCCAGAGC	TAGGGGTGCTCCCTTTC	NM_009463	
		agCACCTAAGTGTGGAGAAAtttt	aTTTCTCCACACTTAGGTGCTttt		
Nell-1		agAGCCTGGTCAAGGAATAtttt	aTATCTCTTGAACAGGCTctttt	NM_001037906	siRNA
		aCAACTTAGCAGGAGGATTTttt	aAAATCCTCTGCTAAGTTGttt		
NELL-1	human	cgcgatgccaccatgggATGCCGATGGATTTGATTTAGTTG	cccaagctTCAATTATTTTGAAGACACTCAAAATC	NM_006157	cloning sqPCR or TqPCR
GAPDH		GGTGTCTGGAATGCACATG	AAGTGGATGGCTTCTGCTGT		
		GTCAGGCTGAGAACGGGAA	AAATGAGCCCCAGCCTTCTC	NM_002046	

decreasing 3°C per cycle; 95°C × 20", 55°C × 10", 70°C × 1", followed by plate read, for 40 cycles. Gapdh was used as a reference gene. All reactions were done in triplicate.

Alkaline phosphatase (ALP) activity assays

Exponentially growing iMEFs were seeded in 12 or 24-well culture plates and infected with indicated adenoviruses. At the indicated time points, ALP activity was assessed quantitatively with a modified assay using the Great Escape SEAP Chemiluminescence assay kit (BD Clontech, Mountain View, CA) and qualitatively with histochemical staining assay (using a mixture of 0.1 mg/ml naphthol AS-MX phosphate and 0.6 mg/ml Fast Blue BB salt) as described [22, 26, 49, 64, 78, 81, 82]. Each assay condition was performed in triplicate.

Matrix mineralization assay (Alizarin Red S staining)

iMEFs were seeded in 24-well cell culture plates and infected with Ad-BMP9 and/or AdR-NELL1 or AdGFP. The cells were maintained in the presence of ascorbic acid (50 mg/mL) and β-glycerophosphate (10 mM) [19]. At 10 days post infection, mineralized matrix nodules were stained for calcium precipitation using Alizarin Red S staining as described [19, 24, 32, 83]. Briefly, cells were fixed with 0.05% (v/v) glutaraldehyde at room temperature for 10 min and rinsed with distilled water. The fixed cells were incubated with 0.4% Alizarin Red S for 5 min, followed by being thoroughly washed with distilled water. The staining of calcium mineral deposits was recorded under a bright field microscope. Each assay condition was done in triplicate.

Adipogenic differentiation assay (Oil red O staining)

The iMEFs were seeded in 12 or 24-well cell culture plates and infected with adenoviruses Ad-BMP9, AdR-NELL1 and/or AdR-simNell1 for 10 days. Oil Red O staining was performed as described [31, 49, 82]. Briefly, cells were fixed with 10% formalin at room temperature for 10min, and washed with PBS. The fixed cells were stained with freshly prepared and filtered Oil Red O solution (six parts saturated Oil Red O dye in isopropanol plus four parts water) at 37°C for 30-60 min, followed by washing with 70% ethanol and distilled water. The stained lipid droplets were recorded under a bright field microscope. Each assay condition was done in triplicate.

Stem cell implantation and micro-CT analysis

The use and care of the animals followed the guidelines approved by the institutional IACUC committee. The subcutaneous ectopic bone formation was performed as previously described [20, 30, 59, 84]. Briefly, iMEFs were infected with Ad-BMP9 & AdRFP, AdR-NELL1 & AdGFP, AdR-simNell1 & AdGFP, Ad-BMP9 & AdR-NELL1, and Ad-BMP9 & AdR-simNell1. At 24h post infection, cells were harvested and resuspended in PBS for subcutaneous injection (5×10⁶ cells in 80μl per injection) into the flanks of athymic nude (nu/nu) mice (5 mice/group, male, 4-6wk old; Harlan Research Laboratories/ENVIGO, Indianapolis, IN). At 4-week post implantation, animals were sacrificed. Implantation sites were retrieved for micro-CT analysis, histologic evaluation, and other stains. All retrieved specimens were fixed and imaged using the μCT component of the GE triumph (GE Healthcare) trimodality preclinical imaging system. All image data analyses were performed using Amira 5.3 (Visage Imaging, Inc.), and 3D volumetric data were determined as described [30, 58, 59, 83].

Hematoxylin & eosin and trichrome staining

Retrieved tissues were fixed, decalcified in 10% buffered formalin, and embedded in paraffin. Serial sections of the embedded specimens were stained with hematoxylin and eosin (H & E) and Masson's Trichrome staining as previously described [20, 33, 85, 86].

Statistical analysis

All quantitative assays were performed in triplicate and/or carried out in three independent batches. Statistical analysis was carried out using Microsoft Excel program. Data were expressed as mean ± SD. Statistical significances were determined by one-way analysis of variance and the student's t test. A value of p<0.05 was considered statistically significant.

Results

BMP9 induces Nell1 expression in MSCs, which can be effectively silenced by recombinant adenovirus-mediated RNAi

We first tested the effect of BMP9 on Nell1 expression in MSCs and found that BMP9 was able to induce Nell1 expression at as early as 24h and more robustly at 48h after Ad-BMP9 infection (Fig. 1A panel a). BMP9-induced Nell1 expression was further confirmed quantitatively by TqPCR and found statistically significant (Fig. 1A panel b). These results suggest Nell1 may function downstream of BMP9 signaling.

To further explore potential roles of Nell1 in BMP9-induced MSC differentiation, we constructed adenoviruses to effectively overexpress Nell1 or silence Nell1 in MSCs. As shown in Fig. 1B, Ad-NELL1 infected iMEFs were shown significantly elevated NELL1 expression, while Ad-simNell1 was able to silence BMP9-upregulated Nell1 expression in the infected iMEFs (Fig. 1B panel a). The NELL1 overexpression and silencing Nell1 expression were further quantitatively confirmed by TqPCR analysis (Fig. 1B panel b).

Nell1 potentiates BMP9-induced late stage osteogenic differentiation while inhibiting early osteogenic marker ALP

We next examined the effect of Nell expression levels on BMP9-induced osteogenic differentiation of MSCs. While Nell1 overexpression alone did not induce any detectable ALP activity, Ad-NELL1-transduced MSCs exhibited reduced ALP activity upon BMP9 stimulation, when compared with that of the MSCs stimulated with BMP9 only (Fig. 2A). Further quantitative time-course studies revealed that Nell1 overexpression inhibited BMP9-induced ALP activity relatively in an adenovirus titer-dependent manner (Fig. 2B, panel a). Conversely, silencing Nell1 expression in MSCs increased BMP9-induced ALP activity (Fig. 2B, panel b). We also analyzed the effect of Nell1 expression on BMP9-induced late stage osteogenic differentiation and found that overexpression of Nell1 significantly enhanced BMP9-induced mineral nodule formation *in vitro* (Fig. 2C), while silencing Nell1 expression did not significantly affect BMP9-induced mineralization (data not shown). These results suggest that Nell1 overexpression may accelerate osteogenic differentiation of the BMP9-induced committed osteoblastic progenitors from MSCs.

Fig. 1. BMP9 induces Nell1 expression in mesenchymal stem cells, which can be effectively silenced by recombinant adenovirus-mediated RNAi. (A) Subconfluent iMEFs were infected with AdGFP or AdBMP9 for 24h or 48h. Total RNA was isolated and subjected to sqPCR (a) and TqPCR (b) using the primers for mouse *Nell1*. *Gapdh* was used as a reference gene. “*”, $p < 0.05$; “**”, $p < 0.001$. (B) Recombinant adenovirus-mediated effective silencing and overexpression of Nell1 in iMEFs. Subconfluent iMEFs were co-infected with AdBMP9/AdRFP, AdR-NELL1, or AdBMP9/AdR-simNell1 for 48h. Total RNA was isolated and subjected to sqPCR (a) and TqPCR (b) using the primers for mouse *Nell1* or human *NELL1*. *Gapdh* was used as a reference gene. “**”, $p < 0.001$.

Fig. 2. Nell1 potentiates BMP9-induced late stage osteogenic differentiation while inhibiting early osteogenic marker ALP. (A) & (B) Subconfluent iMEFs were infected with AdGFP, AdR-NELL1, AdBMP9, AdBMP9/AdR-NELL1, or AdBMP9/AdR-simNell1. At the indicated time points the infected cells were subjected to the qualitative histochemical staining (A, at day 5, two magnifications are shown for each group) or quantitative assay (B) of alkaline phosphatase (ALP) activity. For the Nell1 adenovirus titer-dependent assay, the cells were co-infected with varied titers of AdR-NELL1 at the MOIs (multiplicities of infection) of 10 (NELL1 Lo), 20 (NELL1 Med), and 30 (NELL1 Hi). All assay conditions were done in triplicate. “*”, $p < 0.05$; “***”, $p < 0.001$. (C) Subconfluent iMEFs were infected with the indicated adenoviral vectors and cultured in mineralization medium for 10 days. The cells were fixed and subjected to Alizarin Red staining. Each assay condition was done in triplicate, and representative images are shown.

We further analyzed the effect of Nell1 expression on BMP9-regulated chondrogenic and osteogenic lineage-specific differentiation markers in MSCs as chondrogenesis and osteogenesis are generally considered highly related to each other. Overexpression of Nell1 alone slightly induced Runx2 expression at day 3 and Osterix at day 5, but did not significantly affect the expression of late osteogenic markers osteocalcin and osteopontin, while BMP9 was shown to up-regulate the expression of Runx2, Osterix, osteocalcin and osteopontin at day 3 and/or day 5 (Fig. 3A, panel a). Furthermore, BMP9-induced expression of osteopontin and osteocalcin was shown to increase by Nell1 overexpression (Fig. 3A, panel a).

The effect of Nell1 overexpression on chondrogenic markers was less pronounced although Nell1 slightly induced Sox9 and aggrecan expression while repressing Comp expression (Fig. 3A, panel b). The Nell1 overexpression potentiated BMP9-induced expression of Sox9, Col2a1 and Col9a1 at early (day 3) time points (Fig. 3A, panel b).

We also quantitatively analyzed the expression of important osteogenic and chondrogenic markers in BMP9 and/or Nell1-transduced MSCs and found that BMP9-induced expression of Runx2, Osterix, and osteopontin was significantly potentiated by Nell1 overexpression, while BMP9-induced Sox9 expression was not significantly affected by Nell1 overexpression (Fig. 3B). These results suggest that Nell1 may potentiate BMP9-induced expression of osteogenic regulators and markers in MSCs.

Forced expression of Nell1 suppresses BMP9-induced adipogenic differentiation of mesenchymal stem cells

We and others demonstrated that BMP9 is a potent inducer of adipogenic differentiation of MSCs [49-52]. Here we investigated the effect of Nell1 overexpression on BMP9-induced

Fig. 3. Nell1 augments BMP9-induced expression of osteogenic differentiation markers. Subconfluent iMEFs were infected with the indicated adenoviral vectors for 3 or 5 days. Total RNA was isolated and subjected to sqPCR (A) for osteogenic markers (a) or chondrogenic markers (b). Several osteogenic and chondrogenic regulators/markers were further quantitatively analyzed using TqPCR (B). *Gapdh* was used as a reference gene. “**”, $p < 0.01$.

adipogenic differentiation and found that forced Nell1 expression inhibited BMP9-induced adipogenesis as indicated in oil-red O staining assay, while silencing Nell1 expression did not significantly affect BMP9-induced adipogenesis (Fig. 4A).

We further analyzed the expression of the regulators and markers of adipogenesis in BMP9-stimulated MSCs. BMP9 up-regulated the expression of *Pparγ2*, *C/ebpα*, *C/ebpβ*, *Pgc1* and *Ap2/Fabp4*, which was inhibited by forced Nell1 expression at day 3 and/or day 7 after AdBMP9 infection (Fig. 4B). However, the expression of *Ucp1* was not slightly affected by Nell1 overexpression (Fig. 4B). Quantitative analysis further confirmed that BMP9-induced expression of *Pparγ2*, *C/ebpα*, *C/ebpβ*, *Pgc1* and *Ap2/Fabp4* was significantly inhibited by Nell1 overexpression, while silencing Nell1 expression did not affect BMP9-induced expression of these adipogenic regulators and markers (Fig. 4C). These results demonstrate that forced expression of Nell1 would significantly inhibit BMP9-induced adipogenic differentiation of MSCs.

Forced Nell1 expression enhances the mineralization and maturity of BMP9-induced ectopic bone formation, while inhibiting BMP9-induced adipogenesis

To further confirm the *in vitro* results, we conducted *in vivo* experiments to analyze the effect of Nell1 expression on BMP9-induced osteogenesis and adipogenesis of MSCs. At 4 weeks after subcutaneous implantation, the MSCs transduced with adenoviruses expressing

Fig. 4. Nell1 inhibits BMP9-induced adipogenic differentiation of mesenchymal stem cells. (A) Oil Red O staining. Subconfluent iMEFs were infected with the indicated adenoviral vectors. At 10 days post infection, the cells were fixed and subjected to Oil-Red O staining. Each assay condition was done in triplicate, and representative images are shown. (B) & (C) Subconfluent iMEFs were infected with the indicated adenoviral vectors, and RNA was isolated at days 3 and 7. The expression of adipogenic regulators/markers were assessed by sqPCR (B) and TqPCR (C). Each assay condition was done in triplicate, and *Gapdh* was used as a reference gene. “***”, $p < 0.01$.

Nell1, simNell1, or GFP alone did not form any detectable masses (data not shown). However, the MSCs transduced with Ad-BMP9 & AdGFP, Ad-BMP9 & AdR-NELL1, or Ad-BMP9 & AdR-simNell1 all formed readily detected masses at the injection sites. The retrieved masses from the three groups did not show any significant volumetric differences in 3-D reconstruction analysis even though the BMP9/Nell1 group seemingly formed smaller masses ($p > 0.05$) (Fig. 5A panels *ab*). However, the bony masses retrieved from BMP9/Nell1 group exhibited higher average bone mineral density, while silencing Nell1 reduced the mineral density of BMP9-induced bone masses (both $p < 0.001$), when compared with that of BMP9-transduced MSCs group (Fig. 5A panel *c*).

Histological evaluation revealed that BMP9 alone induced robust bone formation and adipogenesis, while Nell1 overexpression promoted the formation of more mature and trabecular bone-like structures (Fig. 5B, panel *a*). Interestingly, silencing Nell1 expression in MSCs inhibited BMP9-induced ossification and enhancement of chondrogenesis (Fig. 5B, panel *a*). The maturity and mineralization status of the retrieved bony masses were further assessed by trichrome staining. The BMP9 & Nell1 co-expression group exhibited highest level of mineralization, while silencing Nell1 expression in BMP9-stimulated MSCs led to the formation of immature, less mineralized chondroid matrix, when compared with that of the BMP9 alone group's (Fig. 5B, panel *b*). Taken together, these *in vivo* results are consistent with the *in vitro* findings, and demonstrate that Nell1 potentiates the mineralization and maturity of BMP9-induced ectopic bone formation, while inhibiting adipogenesis (Fig. 5C).

Fig. 5. Nell1 enhances the mineralization and maturity of BMP9-induced ectopic bone formation, while inhibiting adipogenesis. (A) Ectopic bone formation. Subconfluent iMEFs were infected with AdGFP, AdR-NELL1, AdR-simNELL1, AdBMP9/AdGFP, AdBMP9/AdR-NELL1, or AdBMP9/AdR-simNell1 for 16h and collected for subcutaneous injections into the flanks of athymic nude mice for 4 weeks. Masses were retrieved and subjected to μ CT imaging (A) for 3D reconstruction (a) and analysis of average bone volume (b) and bone density (c). “***”, $p < 0.01$. (B) Histologic evaluation. The retrieved masses were fixed, decalcified and subjected to paraffin-embedding and sectioning. The tissue sections were stained with H & E (a), as well as trichrome staining (b). Representative images are shown. (C) A proposed working model that depicts an important role of Nell1 in mediating BMP9-induced osteogenic differentiation of mesenchymal stem cells.

Discussion

In this study, we have shown that BMP9-induced osteogenic differentiation of MSCs can be effectively augmented by forced expression of Nell1, which leads to the inhibition of BMP9-induced adipogenesis. A proper control of the balance between osteogenesis and adipogenesis of MSCs is critical to maintain the homeostasis of bone and marrow stromal microenvironment [87]. Disruption such balance may lead to disorders such as osteoporosis. Interestingly, through a comprehensive *in vitro* and *in vivo* analysis we found the osteogenic BMPs, such as BMP9, BMP2, BMP6 and BMP7, also exert strong adipogenic activity and that the BMP-induced osteogenesis and adipogenesis were shown mutually exclusive [49]. Thus, it is conceivable that tipping the balance towards osteogenesis by suppressing adipogenesis should be beneficial for BMP9-induced bone regeneration and tissue engineering involved in the use of MSCs and osteoblast progenitor cells. Our results demonstrate that Nell1 expression effectively accelerates BMP9-induced bone formation from MSCs.

Several studies have shown Nell1 is capable of inducing osteogenic differentiation and bone formation in various bone defect animal models. An earlier study reported that Nell1 stimulated ALP activity in rat calvarial cells and pre-osteoblast MC3T3 cells [56]. It was shown that the Nell1 modified goat bone marrow stromal cells promoted new bone formation [88]. Exogenous Nell1, in most cases through the co-delivery with BMP2, was shown to facilitate or promote osseous healing of critical-sized calvarial defects, femoral segmental defects, and spinal fusion models [89-93]. A shorter isoform containing the N-terminal 240 amino acid residues of Nell1 was able to stimulate MSC proliferation and osteogenic differentiation [94], although it was shown that Nell1 itself has a limited ability to induce *de novo* bone formation from myoblastic cells [95]. Nonetheless, in our studies we found that overexpression of Nell1 alone did not yield any significant osteogenic differentiation in the iMEF cells. While

the exact cause(s) of such discrepancies among these studies are not known, the different responsiveness to Nell1 may be attributable to the different stages of MSCs involved in these studies. It is conceivable that Nell1 may exert significant osteogenic activity on the committed or more differentiated pre-osteoblasts, such as calvarial cells, bone marrow strom cells, or MC3T3 cells. However, Nell1 has a limited ability to induce osteogenic differentiation from the MSCs in higher stemness hierarchy, such as the iMEFs we used in this study. This may also explain why Nell1 is able to synergize with osteogenic BMPs (such as BMP2 and BMP9) in bone formation.

Interestingly, exogenous Nell1 was also shown to promote chondrocyte proliferation and deposition of cartilage-specific extracellular matrix materials, and regulate chondrogenesis [96-98]. Meanwhile, several studies revealed that exogenous Nell1 exhibits anti-adipogenic differentiation activity [99, 100], probably through Nell1 activated canonical Wnt signaling pathway to repress BMP2-induced adipogenesis [101].

Nell1 is a large 810-amino acid secretory glycoprotein and belongs to the Nell family of proteins, together with Nell2 [53, 102]. Interestingly, even though *Nell1* and *Nell2* genes are highly expressed in brain and share 72% similarity in amino acid sequence level, the biological functions of Nell1 and Nell2 proteins differ significantly. Nell2 was shown to support the survival of neurons from hippocampus and cerebral cortex and to function as a negative regulator of neuronal activity and as *Nell2* deficient mice had a significant enhancement of long-term potentiation in the dentate gyrus [103-105]. It was recently shown that Nell2 can regulate axon guidance by functioning as a ligand for Robo3 receptor [106]. On the other hand, Nell1 plays a key role as a regulator of craniofacial skeletal morphogenesis as *Nell1* deficient mice show cranial and vertebral skeletal defects [54].

Nell1 protein is structurally similar to thrombospondin-1 and contains several structural motifs, including an N-terminal thrombospondin-1-like (TSPN) domain, coiled-coil (CC) domain, four von Willebrand factor type C (VWC) domains, and six epidermal growth factor (EGF)-like domains [57, 105, 107]. Mechanistically, Nell1 was shown to transduce osteogenic signals through the Ras-MAPK and Runx2 pathways [108] and Runx2 [109, 110] although Nell1 was also found as a direct target of Osterix [111]. More recently, Nell1 was shown to activate canonical Wnt signaling [101]. Nonetheless, the cognate receptor(s) for Nell proteins have yet to be identified although Nell1 was shown to form oligomers and interact with heparin and integrins [105, 112-115]. Thus, the molecular mechanisms underlying NELL1-induced osteogenic differentiation remain to be fully understood.

Taking together, we demonstrate that potent osteogenic BMP9 induces Nell1 expression in MSCs. Overexpression of Nell1 potentiates BMP9-induced late stage osteogenic differentiation while inhibiting early osteogenic marker ALP, suggesting that Nell1 overexpression may accelerate osteogenic differentiation of the BMP9-induced committed osteoblastic progenitors from MSCs. Nell1 overexpression enhances BMP9-induced expression of Runx2, Osterix, osteopontin, and osteocalcin in MSCs, while BMP9-upregulated expression of Ppar γ 2, C/ebp α , C/ebp β , Pgc1 and Ap2/Fabp4 is inhibited by forced Nell1 expression. Furthermore, the *in vivo* ectopic bone formation assay demonstrates that exogenous Nell1 expression enhances the mineralization and maturity of BMP9-induced bone formation, while inhibiting BMP9-induced adipogenesis in MSCs. Conversely, silencing Nell1 expression in BMP9-stimulated MSCs led to the formation of immature, less mineralized chondroid matrix. Thus, our findings suggest that Nell1 may be exploited as a co-osteogenic factor to augment BMP9-induced bone formation while overcoming BMP9-induced adipogenesis in regenerative medicine.

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Disclosure Statement

The authors declare no competing financial interests.

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