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Nell-1 Enhances Bone Regeneration in a Rat Critical-Sized Femoral Segmental Defect Model

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Abstract

BACKGROUND—Effective regeneration of bone is critical for fracture repair and incorporation and healing of bone grafts used during orthopedic, dental, and craniofacial reconstructions. Nell-1 (Nel-like molecule-1) is a secreted protein identified from prematurely fused cranial sutures of craniosynostosis patients that has been found to specifically stimulate osteogenic cell differentiation and bone formation. To test the *in vivo* osteoinductive capacity of Nell-1, a critical-sized femoral segmental defect model in athymic rats was used.

METHODS—A 6-mm defect, which predictably leads to non-union if left untreated, was created in the left femur of each rat. Three treatment groups (n=8 each) were created consisting of (1) 1.5 mg/ml Nell-1, (2) 0.6 mg/ml Nell-1, and (3) PBS only as a Nell-free control. PBS or Nell-1 were mixed with demineralized bone matrix as carrier prior to implantation. All animals were euthanized 12 weeks post surgery, and bone regeneration was evaluated using radiographic, three-dimensional microCT, and histological analysis.

RESULTS—Both doses of Nell-1 treated groups had significantly greater bone formation compared to the Nell-free group, with bone volume increasing with increasing Nell-1 concentration.

CONCLUSIONS—Nell-1 in a demineralized bone matrix carrier can significantly improve bone regeneration in a critical-sized femoral segmental defect in a dose-dependent manner. The results of this study demonstrate that Nell-1 is a potent osteospecific growth factor that warrants further

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investigation. Results also support the potential application of Nell-1 as a bone graft substitute in multiple clinical scenarios involving repair of critical bone loss when autograft bone is limited or unavailable.

INTRODUCTION

Graft based bone regeneration has been widely used to repair bone defects caused by trauma, tumor resection, pathological degeneration, and congenital malformations. In 2005, over 2.2 million bone grafting procedures were performed by orthopedists, neurosurgeons, plastic surgeons, and dentists worldwide (1). Repair of critical sized bone loss poses a significant clinical problem, and requires application of adjuncts such as bone grafts in order to accelerate bone regeneration and thus fracture healing (2). Autologous bone is considered the gold standard bone graft material, but autologous harvest is an invasive procedure and is limited by host bone availability and associated complications such as significant donor site morbidity approaching 30% (3-5). In addition, variations in the osteogenic potential of the graft material make autograft harvest suboptimal (6). Numerous therapies based on osteoinductive growth factors have been developed to reduce the need for autogenous bone. Among them, bone morphogenetic proteins (BMPs) have demonstrated extraordinary osteogenic potential and have been extensively studied for the treatment of bone fractures and defects (7). However, large doses of BMPs are required to induce adequate bone formation in humans (8). In addition, functional heterogeneity and non-specificity lead to side effects such as bone resorption, ectopic bone formation, adipogenic differentiation, tumor growth, and life-threatening cervical swelling (9-11). Thus, for treatment of difficult bone repair scenarios, there is a need to discover new growth factors with equivalent potency as BMPs, but with greater osteospecificity leading to less adverse effects.

Nell-1 (Nel-like molecule-1; Nel—a protein highly expressed in neural tissue encoding epidermal growth factor like domain) is a novel growth factor that has been shown to specifically stimulate osteogenic cell differentiation and bone formation (12,13). Nell-1 was first discovered as an upregulated protein in surgically resected human cranial bones of patients with unilateral cranial synostosis (UCS), a congenital defect that results in premature fusion of sutures of the developing calvarium. Subsequent studies have demonstrated Nell-1's role in bone formation, including generation of N-ethyl-N-nitrosourea (ENU)-induced Nell-1 knockout mice that results in abnormalities to the skeletal system (14). Extensive *in vitro* studies have shown that Nell-1 acts as a critical downstream mediator of, and is directly regulated by, Runt-related transcription factor 2 (Runx2), a key regulator of osteoblast formation. This may explain the lack of ectopic bone formation and osteospecifity of Nell-1; BMPs, acting both upstream and downstream of Runx2 (15) are not osteospecific, and can direct non-osteoblastic cells toward an osteoblastic lineage, causing ectopic bone formation.

Our previous *in vivo* studies have shown that Nell-1 successfully induced bone formation in multiple animal models, including rat calvarial defect and spinal fusion models, without significant side effects or ectopic bone formation (9-11,16-20). In this study, we evaluate the osteoinductive capacity of Nell-1 mixed with a demineralized bone matrix-based putty carrier in a critical-sized femoral segmental defect in rats. This model is a more stringent repair scenario where complete defect union is impossible without intervention (21). By itself, demineralized bone matrix has been shown to enhance bone regeneration (21-23), and has also been successfully used as a carrier for BMP-2 in previous studies of bone repair in similar rat femoral defect models (24). We expect that Nell-1 can enhance bone regeneration in this animal model over carrier alone, and to levels comparable to results using BMP-2.

MATERIALS AND METHODS

Preparation of Implants

To avoid xenograft compatibility issues, maximize consistency, and minimize variability in our demineralized bone matrix scaffold, a single batch of demineralized bone matrix putty (DBX®), derived from Rhesus monkey and manufactured to the same specifications for human DBX by the Musculoskeletal Transplant Foundation (Edison, NJ), was used as the carrier in all implants. Phosphate-buffered saline (PBS) or recombinant human Nell-1 were mixed with 75 μL of monkey DBX immediately prior to implantation.

Surgical Procedure and Study Groups

All surgical procedures were approved by the UCLA Chancellor's Animal Research Committee. Athymic rats were used to avoid immunological reaction to DBX. Twenty-four animals, ranging from 12-14 weeks of age, were randomly divided into three experimental implant groups: (1) DBX with 1.5 mg/ml of Nell-1; (2) DBX with 0.6 mg/ml of Nell-1; and (3) DBX with PBS only. The doses were selected based in part on previous reports citing efficacious BMP-2 doses in rodents and nonhuman primates (25), and our finding that equal mass doses of Nell-1 and BMP-2 produced equivalent bone healing (16). The surgical procedure was similar to that reported in the literature (21,26,27). The animals were anesthetized by isoflurane inhalation. With use of aseptic technique, a 25-30 mm longitudinal incision was made over the anterolateral aspect of the femur. The femoral shaft was then exposed by separating the vastus lateralis and biceps femoris muscles. To maximize bone regeneration consistency, the periosteum overlying the femoral defect was completely removed *en bloc* with the resected femoral segment. A polyethylene plate (length, 23mm; width, 4mm; height, 4mm) was placed on the anterolateral surface of the femur. The plate contained four pre-drilled holes to accommodate 0.9 mm diameter threaded Kirschner wires (Figure 1A). Taking the plate as a template, four threaded Kirschner wires were drilled through the plate and both cortices. Two 26-gauge stainless steel cerclage wires were tightened around the plate and bone for additional stability. With a small oscillating saw blade (Stryker, MI, USA), a 6-mm critical-sized mid-diaphyseal defect was created (Figure 1A). The volume of the defect was approximately 75 uL. An implant was placed into the defect, then the overlying muscle and fascia were closed with 4-0 Vicryl® absorbable suture to secure the implant in place.

Following surgery, the animals were housed in separate cages and allowed to eat and drink *ad libitum*. Weight bearing was started immediately postoperatively, with daily monitoring. Buprenorphine was administered for two days as an analgesic, and trimethoprim/sulfamethoxazole was administered for ten days as an antibiotic. All animals were sacrificed at 12 weeks post surgery.

Radiographic Evaluation

At 2, 4, 6, 8, 10, and 12 weeks after implantation, high-resolution lateral radiographs were obtained while the animals were under isoflurane sedation. Three observers, blinded to the treatment variables, scored the defect mineralization with a standardized scale adapted from others (28) as follows: 0, trace radiodense material in defect; 1, flocculent radiodensity and incomplete bridging of defect; 2, bridging of the defect at 1+ location; 3, bridging of the defect at cis and trans cortices, parent cortex visible; 4, one cortex obscured by new bone; 5, bridging of the defect by uniform new bone, cut ends of cortex not seen.

Three-Dimensional microCT Evaluation

At 12 weeks post surgery, all animals were euthanized. The femurs were dissected, harvested, and fixed in 10% buffered formalin (Fisher Scientific, Fair Lawn, NJ). Following

fixation for a minimum of 48 hours, the samples were scanned in the axial plane using a three-dimensional micro-computed tomography (microCT) scanner (μCT 40, Scanco Medical, Bassersdorf, Switzerland) at 20 μm resolution. After scanning, the μCT 40 software was used to analyze and measure the bone volume in the defect where the implant was placed. A constant volume of interest, 250 slices thick (approximately 5 mm) and centered over the defect site, was selected for analysis of all samples. The bone volume per defect was recorded as the measure of segmental defect bone regeneration.

Histological Assessment

Following microCT analysis, the specimen were decalcified using Cal-Ex solution (Fisher) for five to seven days, washed with running tap water for 3 to 4 hours and then transferred to 75% ethanol solution. The specimen were embedded in paraffin and 5 μm sagittal sections of each specimen were obtained and then stained with hematoxylin and eosin (H&E) or Masson's Trichrome stain.

Statistical Analysis

Student's t-test was used to compare radiographic scores and bone volume measurements between each group. $p < 0.05$ was considered statistically significant.

RESULTS

Radiographic Data

X-ray images (Figure 1B-C) show abundant bone formation bridging across the defect in the 1.5 mg/ml Nell-1 group at 12 weeks, with the parent bone indistinguishable from new bone. In the 0.6 mg/ml Nell-1 group, there was much more new bone traversing the proximal and distal ends of the defect compared to the Nell-free group; however, a bone bridge did not appear in either group. Semi-quantitative assessment of defect mineralization from 2 through 12 weeks revealed a significant ($p < 0.05$), progressive increase in mean defect mineralization with increasing Nell-1 concentration, demonstrating a dose-dependent response evident by week 12 (Table 1).

Three-Dimensional microCT Analysis

3D microCT evaluation of complete reconstructions (Figure 2A-C) revealed new bone formation in all groups. The Nell-free group exhibited minimal new bone formation, with the defect margins remodeling to form a rounded cap characteristic of non-union with an obvious defect at the middle of the diaphysis. In contrast, an increased amount of new bone filled the defect in the Nell-treated groups. The 1.5 mg/ml Nell-1 group demonstrated variable bridging of the defect and the 0.6 mg/ml Nell-1 group more commonly exhibited large voids between the regenerated bone of the two ends of the defect. In all cases, the new bone was limited to the area of the defect without obvious overgrowth. MicroCT-based quantitation of new bone regeneration revealed that both treatment doses of Nell-1 significantly increased bone volume compared to the Nell-free group, although no statistically significant difference was detected between the Nell-treated groups (Figure 2D).

Histological Analysis

Histological analysis of femurs confirmed the radiographic findings in our study (Figure 3). The 1.5 mg/ml Nell-1 group showed abundant new bone bridging the defect and integrated well with the parent diaphyseal bone. The 0.6 mg/ml Nell-1 group exhibited new bone formation extending the length of the two ends of the defect, but no bone bridge was seen. In the Nell-free group, the defect was still very evident and was mostly filled with loosely organized connective tissue and DBX particles (identified as bone particles with cell-free

lacunae) and only a small amount of bone. In contrast, osteocytes were present in the lacunae of newly formed bone matrix, with surrounding osteoblasts at the borders of the bone tissue. In the Nell-treated groups, the defect was connected by a bony bridge, which represented bone regeneration extending from the DBX implant. Higher magnification revealed that the DBX particles resembled remodeling bone, with bone marrow cavities forming around the particles. In contrast, there is less evidence of DBX remodeling in the Nell-free group, as the DBX particles were instead surrounded by fibrous tissue. Masson's Trichrome staining further revealed that Nell-treated groups contained more mature bone than the Nell-free group.

DISCUSSION

Our study shows that Nell-1, applied from a DBX carrier, is able to induce bone healing in a stringent repair scenario consisting of a critical-sized femoral segmental defect. Compared to the Nell-free group, both Nell-1 treated groups exhibited significantly greater bone formation as assessed by radiography. The higher the concentration of Nell-1 used, the greater the rate of union of the defect. There was no statistical significance between measured bone volumes of the Nell-treated groups; however, union was consistently achieved with the higher dose as assessed by radiography. As a carrier, DBX has the advantage that it is standardized, with well-defined material properties and baseline biological activity. Additionally, as a putty it can serve as a vehicle for Nell-1 delivery that conforms to differently shaped spaces, preventing escape of the implant from the implantation site, and broadening the bone repair applications for which Nell-1 can be applied in the clinic.

The rate of new bone formation achieved by Nell-1 shown here is comparable to previous results using approximately 0.06 and 0.44 mg/ml BMP-2 delivered from a rat DBX carrier in a similar segmental defect model in rats (29). The progressive bone formation within the defects demonstrated by radiographic evaluation was similar, with new bone evident as early as the first postoperative week in the Nell-1 and BMP-2 treated groups. In both studies, control groups treated with demineralized bone matrix alone failed to achieve significant bone regeneration. Histologic findings at the time of harvest showed bridging of cortical bone across the defect in both Nell-1 and BMP-2 treatment groups, while controls showed no formation of cartilage or bone. Although percent of union achieved by BMP-2 is greater than Nell-1, the osteoinductive capacity of Nell-1 is still significant and may be accounted for by differences between the two studies, including segmental defect size of 6 mm in this study versus 5 mm in the other, and differences in bone matrix origin and processing steps.

In addition, studies comparing Nell-1 and BMP-2 in other animal models suggest that Nell-1 has osteoinductive capacity comparable to BMP-2. Live microCT evaluation in a rat calvarial defect model showed that Nell-1 and BMP-2 at the same concentration induced equivalent levels of bone formation with complete bridging of the defect on the endocranial side as early as 4 weeks after surgery. Moreover, bone volume continued to increase in Nell-1 and BMP-2 groups throughout the 12 week study period to fill in the calvaria on the exocranial surface (16). Interestingly, defects implanted with Nell-1 showed consistently greater mineralization at 2, 4, and 8 weeks after treatment versus those implanted with BMP-2, although the treatment groups were equivalent after 12 weeks. In addition, the molecular weight of Nell-1 is about 15 times that of BMP-2 (29,30); thus, on a molar basis, the dosage of Nell-1 used is about 15-fold less than that of BMP-2 for a given mg/ml concentration. We found that Nell-1 and BMP-2 function similarly in stimulating increased osteogenic differentiation, as evidenced by increased production of BMP-7, bone sialoprotein, and osteocalcin shown by immunohistochemistry. Notably, staining for Osterix (Osx), a marker of early osteoblastic differentiation (31), was reduced after Nell-1

stimulation, likely indicating increased progression to more mature osteoblastic differentiation, while BMP-2 increased Osx expression. These data suggest that Nell-1 and BMP-2 may promote osteogenesis through distinct signaling pathways (32,33).

Furthermore, our *in vitro* studies suggest that Nell-1 has an advantage over BMP-2 in its specificity for cells of the osteochondrogenic lineage. This osteospecificity is derived, in part, from direct promoter regulation of Nell-1 by Runx2, the essential transcription factor in osteoblast differentiation and bone formation (33,34). Nell-1 has three Runx2 binding sites (osteoblast-specific cis-acting element 2; OSE2) within its promoter and signals downstream of Runx2 (35) in comparison to BMP-2, which acts both upstream and downstream of Runx2 (15,36). Nell-1 upregulation has been shown to accelerate differentiation and bone formation, and its downregulation inhibits osteoblast differentiation (13,37-39). Whereas BMP-2 is known to induce proliferation and osteogenic differentiation of both osseous and nonosseous mesenchymal cells (40,41), Nell-1 primarily stimulates cells of the osteochondral lineage. This was demonstrated in a study in which C2C12 myoblasts were transduced with overexpression viruses of Nell-1, BMP-2, and Nell-1+BMP-2 (42). Results showed that Nell-1 alone did not induce osteogenic differentiation of myoblasts, demonstrating that Nell-1 alone cannot initiate osteoblastic differentiation of muscle cells to form ectopic bone. However, C2C12 myoblasts co-transduced with Nell-1 and BMP-2 showed greater alkaline phosphatase activity and osteopontin production compared to BMP-2 alone, suggesting a synergistic effect in promoting bone regeneration. Additionally, we have found that *in vivo* BMP-2 transduction increases the genesis of non-bone tissues such as adipose tissue, and that combotherapy of Nell-1 and BMP-2 in a femoral defect model increases bone formation while suppressing inappropriate adipogenesis (unpublished data), both intriguing leads for future study.

In summary, Nell-1 appears to be a novel growth factor with potent osteoinductive capacity. The current study shows that Nell-1 delivered from a DBX carrier can significantly enhance bone regeneration in a critical-sized femoral segmental defect model. Our ongoing work includes biomechanical analysis of the strength and durability of Nell-1-induced bone, optimization of Nell-1 delivery parameters and dose, and dissection of the molecular relationship between Nell-1 and other osteogenic factors, most notably BMP-2. Our findings indicate a potential use of Nell-1 as a bone graft substitute and adjunct treatment for repair of significant bone loss when autograft is limited or unavailable, with potentially less adverse effects compared to other growth factor-based therapies.

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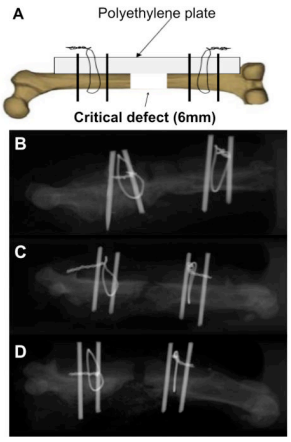

Figure 1.

Femoral fixation and x-ray images at 12 weeks post-operation. (Top) Schematic diagram of femoral fixation to polyethylene plate using two Kirschner wires and a cerclage wire on either side of the defect. The 1.5 mg/ml Nell-1 group shows a bone bridge across the defect with the parent bone invisible. The 0.6 mg/ml Nell-1 group exhibits more bone regeneration than Nell-1 free group; however, neither group formed a bone bridge. Radiographs are presented in descending dose from top to bottom.

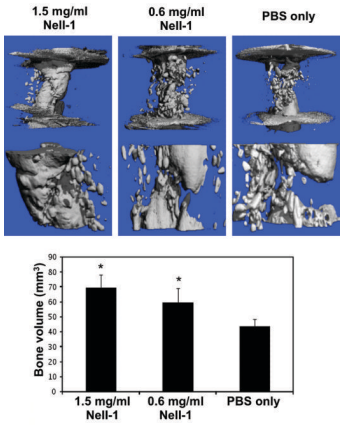

Figure 2.

microCT evaluation of complete reconstructions and coronal sectional cuts. An increased amount of new bone filled the defect in the Nell-treated groups. The 1.5 mg/ml and 0.6 mg/ml Nell-1 groups demonstrated variable bridging of the defect. In contrast, Nell-free femurs exhibited minimal new bone formation. Figures are presented in descending dose from left to right. Bar graph, quantitative microCT measurement demonstrated that bone volume in each Nell-1 treated group was significantly greater than Nell-free controls. $p = 0.0001$ ($F = 21.14$, $df = 2$) between three groups, * $p \leq 0.01$ between control and either Nell-1 group, no significance between Nell-1 groups (one-way ANOVA, with Tukey's HSD test).

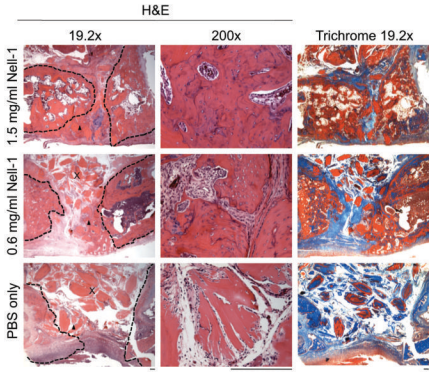

Figure 3.

Histological staining of femoral defect tissue slices. In the Nell-1 treated groups (top and middle rows), new bone bridged the defect and integrated well with the parent diaphyseal bone, and DBX particles were well remodeled and surrounded by mature bone. In the Nell-free group (bottom row), the defect space contains mostly unremodeled DBX particles (X) and fibrous tissue. Staining with Masson's Trichrome (right column) causes mineralized tissue to appear red and connective tissues to appear blue. In the Nell-1 treated groups, mineralized tissue spans the defect space, while in the Nell-free control group, only diffuse connective tissue, likely representing fibrous tissue, spans the defect space. Dashed lines indicate cut edges of bone. Magnification is indicated on top axis; arrowheads indicate magnified area. Bar: 125 μm.

Table 1

Semi-quantitative radiographic assessment of defect mineralization. Increasing Nell-1 concentration produced a progressive increase in mean defect mineralization. Radiographic score for the Nell-treated groups are most significantly elevated from Nell-free controls starting at 4 weeks post-operation.

Treatment	Postoperative week					
	2	4	6	8	10	12
PBS only	1 ± 0	1.125 ± 0.52	1.5 ± 1.07	2 ± 0.83	2.125 ± 0.92	2.25 ± 0.64
0.6 mg/ml Nell-1	1 ± 0	2 ± 0.93*	2.625 ± 1.06**	2.75 ± 0.89	2.875 ± 0.83	3.375 ± 0.52**
1.5 mg/ml Nell-1	1 ± 0	1.625 ± 0.35	2.5 ± 0.76**	3.125 ± 0.76**	3.375 ± 0.35**	4.125 ± 0.46**§
Median score (P25-P75)	1 (1-1)	1 (1-2)	2 (1.75-3)	3 (2-3)	3 (2-3)†	3 (2.75-4)†

* $p \leq 0.05$,

** $p \leq 0.025$ compared to PBS control;

§ $p \leq 0.05$ between Nell-1 groups (Mann-Whitney U).

† $p < 0.02$ between groups (Kruskal-Wallis).