NELL-1 Promotes Cartilage Regeneration in an *In Vivo* Rabbit Model

Ronald K. Siu, M.S.,^{1,2} Janette N. Zara, M.D.,^{1,2} Yaping Hou, Ph.D.,^{1,3} Aaron W. James, M.D.,^{2,4,5}
Jinny Kwak, D.D.S.,^{2,5} Xinli Zhang, M.D., Ph.D.,² Kang Ting, D.M.D., D.M.S.,^{2,4,5}
Benjamin M. Wu, D.D.S., Ph.D.,¹ Chia Soo, M.D.,^{4,*} and Min Lee, Ph.D.^{1,3,*}

Repair of cartilage due to joint trauma remains challenging due to the poor healing capacity of cartilage and adverse effects related to current growth factor-based strategies. NELL-1 (Nel-like molecule-1; Nel [a protein strongly expressed in neural tissue encoding epidermal growth factor like domain]), a protein first characterized in the context of premature cranial suture fusion, is believed to accelerate differentiation along the osteochondral lineage. We previously demonstrated the ability of NELL-1 protein to maintain the cartilaginous phenotype of explanted rabbit chondrocytes *in vitro*. Our objective in the current study is to determine whether NELL-1 can affect endogenous chondrocytes in an *in vivo* cartilage defect model. To generate the implant, NELL-1 was incorporated into chitosan nanoparticles and embedded into alginate hydrogels. These implants were press fit into 3-mm circular osteochondral defects created in the femoral condylar cartilage of 3-month-old New Zealand White rabbits ($n = 10$). Controls included unfilled defects ($n = 8$) and defects filled with phosphate-buffered saline-loaded chitosan nanoparticles embedded in alginate hydrogels ($n = 8$). Rabbits were sacrificed 3 months post-implantation for histological analysis. Defects filled with alginate containing NELL-1 demonstrated significantly improved cartilage regeneration. Remarkably, histology of NELL-1-treated defects closely resembled that of native cartilage, including stronger Alcian blue and Safranin-O staining and increased deposition of type II collagen and absence of the bone markers type I collagen and Runt-related transcription factor 2 (Runx2) as demonstrated by immunohistochemistry. Our results suggest that NELL-1 may produce functional cartilage with properties similar to native cartilage, and is an exciting candidate for tissue engineering-based approaches for treating diverse pathologies of cartilage defects and degeneration.

Introduction

OSTEoarthritis, a leading cause of pain and debility, affects about 43 million American adults, and is the most prevalent chronic condition among Americans 15 years and older.¹ Half a million Americans require cartilage repair surgery every year.² Counting reconstructive and cosmetic cartilage replacement surgeries, the number of cartilage repair surgeries totals 1 million Americans per year.^{3,4} Articular cartilage heals poorly due to poor vascularization and limited mobility of chondrocytes to reach sites of injury.⁵ The inability of articular cartilage to heal, combined with its loss over time, frequently results in pain and osteoarthritis at load-bearing joints.² Additionally, joint trauma results in acute injury to articular cartilage and can

progress to osteoarthritis as a more severe chronic condition.⁶ Thus, there is a significant biomedical burden for developing an alternative for restoring the function of injured, diseased, or aging cartilage.

Osteochondral grafting is an attractive treatment option for articular cartilage injuries, but is associated with significant complications including limited donor availability and donor site morbidity.⁷ Cell-based tissue engineering therapies for cartilage repair are limited by the need for large numbers of chondrocytes and their propensity to rapidly differentiate away from their chondrocytic phenotype *in vitro* toward other mesenchymal cell fates such as osteoblast or fibroblast lineages.^{3,8,9} To counteract this, growth factors such as bone morphogenetic proteins (BMPs) and transforming growth factor-betas (TGF- β s) have been used to

¹Department of Bioengineering, ²Dental and Craniofacial Research Institute, and ³Division of Advanced Prosthodontics, Biomaterials, and Hospital Dentistry, School of Dentistry, University of California, Los Angeles, California.

⁴UCLA and Orthopaedic Hospital, Department of Orthopaedic Surgery and the Orthopaedic Hospital Research Center, University of California, Los Angeles, California.

⁵Section of Orthodontics, School of Dentistry, University of California, Los Angeles, California.

*Co-senior author.

expand smaller samples of chondrocytes before implantation and prevent their inappropriate de-differentiation.^{10–13} Factors such as BMPs and TGF- β s are critical signaling elements for chondrogenesis during development, but they can also act pleiotropically to produce adverse effects, thus reflecting their wide range of expression during development.¹⁴ For example, BMP-2 has been found to promote cartilage formation in a mouse cartilage defect model, but also increases the formation of osteophytes (ectopic bone) at the defect site.^{15,16} In addition, injection of TGF- β 1 into mouse knee joints has been shown to induce fibrosis and formation of large osteophytes.^{17–20} Thus, there is a need to identify alternative chondroinductive factors with more specific effects on cartilage.

The protein NELL-1 (Nel-like molecule-1; Nel [a protein strongly expressed in neural tissue encoding epidermal growth factor like domain]) was first identified from a screen of differentially expressed genes between normal and prematurely fusing craniosynostotic sutures,²¹ and has been shown to be able to drive differentiation and growth of bone and cartilage tissue *in vivo*.^{22–26} This ability to accelerate osteochondral differentiation derives in part from its direct regulation by Runt-related transcription factor 2 (Runx2),²⁷ the master regulator gene for osteochondral differentiation.²⁸ Mice deficient in *Nell1* exhibit abnormalities in vertebral disc fibrocartilage and other connective tissue defects, further underscoring the role of *Nell1* in skeletal and cartilage development.²⁹

In this study, we utilize a well-described rabbit femoral condyle defect model³⁰ to evaluate the ability of NELL-1 to promote articular cartilage regeneration *in vivo*. In our previous manuscript, we have already demonstrated that NELL-1 has stimulatory effects on rabbit chondrocyte proliferation and cartilage-specific extracellular matrix deposition in three-dimensional alginate gels *in vitro*.³¹ In the current study, a natural progression of our previous work, we describe the construction of an alginate composite system containing chitosan nanoparticle carriers to deliver the growth factor NELL-1 in a sustained manner to the site of an *in vivo* cartilage defect in rabbit femoral cartilage. After characterizing the release kinetics of NELL-1 from the nanoparticles *in vitro*, we evaluated the ability of experimental hydrogels containing NELL-1-loaded chitosan nanoparticles to promote articular cartilage regeneration in a critical-sized 3-mm full-thickness rabbit femoral condyle defect model. The quality and differentiated phenotype of regenerated cartilage was evaluated by histology and immunohistochemistry (IHC).

Materials and Methods

Chitosan (molecular weight 400,000; 85% deacetylated), pentasodium tripolyphosphate (TPP), chondroitin-4-sulfate (CHS) from bovine trachea, and sodium alginate (viscosity 250 cP, 2% w/v) were purchased from Sigma-Aldrich (St. Louis, MO). Calcium chloride was purchased from EMD Chemicals (Gibbstown, NJ). Bioactive recombinant human NELL-1 protein (rhNELL-1) was produced and purified from Chinese hamster ovary cells (Aragen Bioscience, Morgan Hill, CA).

Preparation of chitosan nanoparticles and alginate gels

Chitosan nanoparticles were prepared by ionic gelation of chitosan solution with polyanion TPP and CHS solution.³²

Polyanion solutions were prepared by dissolving TPP (0.1% w/v) or CHS (0.1% w/v) in deionized water. Chitosan solution (0.1% w/v) was prepared by dissolving chitosan in 0.05% aqueous acetic acid. All solutions were sterile filtered through a 0.22 μ m polyethersulfone membrane (Nalgene, Penfield, NY). Nanoparticles were formed by dropping TPP/CHS solution (0.1% w/v) into chitosan solution (0.1% w/v) with magnetic stirring at 1000 rpm. Twenty micrograms of rhNell-1 was loaded onto the nanoparticles by dissolving NELL-1 in the TPP/CHS solution. The ratio of chitosan:TPP:CHS was 5:1:1.5 (w/w/w). Nanoparticles were collected by centrifugation at 14,000 g for 30 min and washed twice with distilled water. Nanoparticles were mounted on aluminum, and the surface morphology was observed by using scanning electron microscopy (SEM, NOVA 230; FEI, Hillsboro, OR).

Alginate (1% w/v) solution was prepared by dissolving sodium alginate in distilled water. Collected chitosan nanoparticles were re-suspended in 50 μ L of alginate solution. Alginate gelation was initiated by adding 1 mL of 100 mM calcium chloride solution to the particle suspension. The particle/alginate gel constructs were washed with phosphate-buffered saline (PBS). All implants are acellular.

In vitro release of NELL-1

NELL-1 or NELL-1-loaded chitosan nanoparticles were encapsulated into alginate hydrogels. The amount of NELL-1 associated with the nanoparticles was indirectly determined by measuring the difference between the initial amount of NELL-1 dissolved in the TPP/CHS solution and the amount of NELL-1 remaining in the supernatant after centrifugation. NELL-1 content was quantified by using the 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) protein assay (Invitrogen, Carlsbad, CA). The amount of NELL-1 associated with alginate gels was similarly determined by measuring the difference between the initial amount of NELL-1 loaded into alginate gels and the amount of NELL-1 remaining in the CaCl₂ solution. Alginate gels containing 20 μ g NELL-1 were immersed in 1 mL PBS at 37°C with gentle shaking. At various time points, the incubating solution was taken and replaced with 1 mL of fresh solution. The amount of released NELL-1 protein in the supernatant was measured by using the CBQCA protein assay. Measurements were performed in triplicate, and the amount of protein release was expressed as a percentage of the initial amount of dissolved NELL-1.

Rabbit cartilage defect model

Approval from the UCLA Chancellor's Office of Animal Research Oversight was obtained before commencing animal experiments. Thirteen male New Zealand White rabbits, weighing ~3.5 kg and aged ~6 months, were used in this study. Animals were randomized into 3 groups, defined according to the implant contents (Table 1): no implant (control), alginate implant containing PBS-loaded chitosan nanoparticles (control), or alginate implant containing chitosan nanoparticles loaded with 200 μ g/mL NELL-1 protein, a dose we had previously established, robustly maintains the chondrogenic phenotype of chondrocytes *in vitro*.³¹ Animals were premedicated 30 min before surgery with subcutaneous injections of 0.05 mg/kg buprenorphine and 1 mg/kg acepromazine, and anesthetized by an intramuscular injection

TABLE 1. LIST OF EXPERIMENTAL GROUPS

Group	Implant contents	Number of animals	Number of samples
1	No implant	4	8
2	Alginate gels with PBS-loaded chitosan	4	8
3	Alginate gels with 200 μ g/mL Nell-1 protein-loaded chitosan	5	10

NELL-1, Nel-like molecule-1; Nel (a protein strongly expressed in neural tissue encoding epidermal growth factor like domain); PBS, phosphate-buffered saline.

of 35 mg/kg ketamine/5 mg/kg xylazine, with anesthesia maintained by 2% isoflurane. Since all implants were acellular, no immunosuppressant medications were used.

Under anesthesia, one 3-mm-diameter defect was made in the articular hyaline cartilage of the mid-trochlear region of the left and right femurs of each animal. The defects penetrated to a depth of 3 mm, which included the full thickness of the articular cartilage (~0.3 mm) and the underlying subchondral bone. The defect in each knee was made 1 cm distal to the fused growth plate. The alginate scaffolds were cored with a 4-mm trephine and press fit into the defect of one knee. Both knees of each individual animal received the same experimental treatment, as per the group definitions

in Table 1, for a total of 2 data points per animal. After implantation, the patella was reduced, the fascia incisions were closed by using absorbable 4-0 Vicryl sutures (Ethicon, Somerville, NJ), and the skin incisions were closed by using nonabsorbable 4-0 nylon monofilament sutures (Ethicon).

All animals were monitored until sternal after waking from anesthesia, and given 0.05 mg/kg buprenorphine twice per day for at least 2 days for pain management. As an antibiotic, 5 mg/kg enrofloxacin was prophylactically given once daily, starting intraoperatively and then 3 days postoperatively. Animals were allowed to eat and drink *ad libitum*. All animals uneventfully recovered and were healthy until sacrifice at 12 weeks postsurgery.

Histology and IHC

After sacrifice, knee tissues were harvested, fixed in 10% formalin, and embedded in paraffin as per standard protocol. Sagittal sections passing through the center of the cartilage defect were collected. Sections were deparaffinized and stained with hematoxylin and eosin (H&E) to examine cellular distribution, Alcian blue to assess glycosaminoglycan synthesis, and Safranin-O to evaluate proteoglycan accumulation as previously described.³¹ IHC was performed by using 0.05% bovine serum albumin/PBS blocking buffer, primary antibodies to type I (sc-25974; Santa Cruz Biotechnology, Santa Cruz, CA), type II (sc-7764; Santa Cruz Biotechnology), and type X collagen (RDI-COLL10abr;

FIG. 1. Implant characteristics and release kinetics. **(A)** Scanning electron microscopy of chitosan nanoparticles. Scale bar: 1 μ m. **(B)** Gross morphology of alginate gel implants. Approximate diameter and volume are 4.57 mm and 50 μ L, respectively. **(C)** *In vitro* release of NELL-1 (Nel-like molecule-1; Nel [a protein strongly expressed in neural tissue encoding epidermal growth factor like domain]) from alginate gels ($\blacktriangle$) or chitosan nanoparticles embedded in alginate gels ($\blacksquare$). Schematic diagrams depicting distribution of NELL-1 (yellow), chitosan (black), and alginate (green) in each construct are shown. Color images available online at www.liebertonline.com/tea

Fitzgerald Industries Int'l, Concord, MA), proliferating cell nuclear antigen (PCNA, M0879; Dako, Carpinteria, CA), and Runx2 (sc-10758, Santa Cruz Biotechnology), appropriate biotinylated secondary antibodies (Dako), the Vectastain ABC system (Vector Laboratories, Burlingame, CA), and AEC chromogenic substrate (Dako), as per manufacturers' instructions. Primary and secondary antibodies were used at dilutions of 1:100 and 1:200, respectively.

Scoring and quantitation of histology

The Mankin histological scoring system was used to evaluate overall structure, cellular morphology, proteoglycan accumulation, and tidemark integrity.³³ Scores were reported as an average $\pm$ SD of three independent observers, with a maximum score of 14. Proteoglycan deposition was quantified by image analysis of three randomly selected

fields of Alcian blue stained samples using Bioquant Nova software (Nashville, TN).

Statistical analysis

Statistical analysis was performed by using the two-tailed Student's *t*-test. Bonferroni adjusted significance levels of 0.025 per test ($0.05/2$) were applied to account for comparisons between the no implant and alginate with PBS-loaded chitosan groups and the no implant and alginate with NELL-1-loaded chitosan groups.

Results

In vitro release of NELL-1 from alginate hydrogel

To determine whether chitosan particles can sustain the release of NELL-1 from alginate gels, NELL-1 was

FIG. 2. Gross and hematoxylin/eosin (H&E) histology of postsacrifice knees. **(A)** Intraoperative photograph showing location and size of defect in the rabbit knee trochlear cartilage. **(B)** Schematic diagrams depicting implant contents of each group; yellow, NELL-1; black, chitosan; green, alginate. **(C)** Photographs of operated knees at sacrifice 3 months after surgery. Dashed lines indicate approximate margin of defect. Defects treated with no scaffold or phosphate-buffered saline (PBS)-loaded chitosan in alginate exhibited irregular surfaces clearly distinguishable from the surrounding unoperated cartilage. In contrast, the NELL-1-treated defect contained well-integrated cartilage tissue with a smooth, glistening surface. **(D, E)** H&E staining. Boxes in **(D)** represent magnified area shown in **(E)**. Defects with no implant exhibited a rough surface, the arrangement of cells was more lamellar than columnar, and the extracellular matrix was less dense and more resembles fibrous tissue than the dense cartilage matrix in the adjacent native cartilage. Defects containing PBS-loaded chitosan in alginate exhibited a smoother surface but also cellular and matrix morphology resembling fibrous tissue. In contrast, in NELL-1-treated defects, columns of chondrocytes resembling the adjacent native cartilage were observed. Arrowheads indicate approximate margins of defects. Original magnification: $40\times$ (low magnification), $200\times$ (high magnification). Scale bar: $125\mu\text{m}$ (both magnifications). Color images available online at www.liebertonline.com/tea

encapsulated into nanoparticles and subsequently incorporated into alginate gels. Chitosan nanoparticles were formed by ionic gelation methods by using TPP and CHS as chitosan crosslinkers. Particle sizes of the chitosan nanoparticles ranged from 100–300 nm, as observed by SEM (Fig. 1A). The association efficiency of NELL-1 with the nanoparticles was 85%, as determined by the CBQCA protein assay. PBS-loaded or NELL-1-loaded chitosan nanoparticles were suspended in alginate solutions, and alginate gelation was initiated in CaCl_2 (Fig. 1B). Gels had an average diameter of 4.57 mm and an average volume of 50 μL .

To determine the efficiency of the chitosan carrier/alginate gels as NELL-1 protein carriers, the release profile of NELL-1 from the gels was determined by incubating them in PBS. The release of NELL-1 directly from the gels exhibited a rapid burst release of $\sim 86\%$ over the first day, followed by a gradual release of $\sim 3\%$ per day and finally reaching 100% on the seventh day. The release of NELL-1 from the chitosan nanoparticles in alginate gel showed a burst release of $\sim 20\%$ over the first day, followed by a gradual release of $\sim 0.3\%$ per day up to 14 days (Fig. 1C). Thus, we expected that this delivery system could achieve a prolonged release of NELL-1 into an *in vivo* cartilage defect for optimized tissue repair.

In vivo cartilage defect model

Gross histology of the joint after sacrifice (Fig. 2C) revealed that the cartilage in NELL-1-treated knees contained well-integrated cartilage. In contrast, the defect sites of control groups exhibited rough surfaces that were morphologically distinct from the surrounding native cartilage. To observe differences in cellular structure and matrix composition between the different implantation groups, we performed microscopic histology with H&E (Fig. 2D, E), Alcian blue (Fig. 3A, B), and Safranin-O staining (Fig. 3C, D). Defects with no implant exhibited a rough surface corresponding with its gross histological appearance, and contained reduced Alcian blue and Safranin-O staining compared with the neighboring native cartilage. In defects containing alginate with PBS-loaded chitosan, the surface was smoother and more uniform, but still exhibited reduced Alcian blue and Safranin-O staining. In addition, defects with no implant or PBS-loaded chitosan contained cells morphologically distinct from chondrocytes and arranged in a lamellar pattern, surrounded by loose connective tissue resembling fibrous tissue and staining weakly for Alcian blue. In contrast, defects containing alginate with NELL-1-loaded chitosan exhibited tissue that more closely resembled the neighboring native cartilage in structure and Alcian blue and Safranin-O staining intensity, which was composed of chondrocyte-like cells arranged in columns, and surrounded

by Alcian blue and Safranin-O positive matrix. Quantitation of Alcian blue staining by image analysis of random fields within the defect (Fig. 3E) demonstrated that NELL-1-treated defects exhibited increased Alcian blue staining compared with no implant, thus confirming increased proteoglycan accumulation, though defects containing PBS-loaded chitosan exhibited reduced staining. Quantitation of Safranin-O staining yielded similar results (data not shown). Finally, semiquantitative evaluation of the quality of cartilage tissue regeneration using the Mankin histologic scoring system³³ revealed a significantly improved histologic score in the NELL-1-treated defects compared with defects with no implant or PBS-loaded chitosan (Fig. 3F).

To further characterize the composition of the regenerated tissue, we performed IHC for type II collagen, PCNA, and type X collagen to detect mature cartilage matrix, proliferating cells, and hypertrophic cartilage matrix, respectively (Fig. 4A–C). Type II collagen was expressed in the upper cartilaginous layer in the no implant and PBS-loaded chitosan groups, but remained intracellularly localized, with minimal deposition into the extracellular matrix. In contrast, NELL-1-treated defects exhibited a distribution of type II collagen consistent with native cartilage, with expression present in the matrix of the regenerated tissue within the defect. PCNA expression was observed in the no implant and PBS-loaded chitosan groups but not in the NELL-1-loaded chitosan group, thus indicating that the chondrocytes in the NELL-1-treated group were not in the proliferative phase. Finally, low levels of type X collagen expression was observed in all groups, but minimally expressed in the NELL-1-loaded chitosan group, thus consistent with the lack of hypertrophic chondrocytes in the upper layer.

To address possible osteoinduction by NELL-1, we also performed IHC for type I collagen, a major component of bone matrix, and the transcription factor Runx2, which is required for osteoblast differentiation (Fig. 4D, E). In the upper layer, type I collagen remained intracellularly localized in the no implant and PBS-loaded chitosan groups, but was not observed in the NELL-1-treated group; however, it was expressed in the subchondral bone in all groups as expected. Finally, nuclear Runx2 expression was observed in cells in the cartilaginous layers of the no implant and PBS-loaded chitosan groups, but not in any cells in the NELL-1-loaded chitosan group. Collectively, these results show that neither of the bone markers tested were expressed in the NELL-1-treated defect.

Discussion

Our group has studied the chondrogenic potential of the growth factor NELL-1, which we have previously shown to be

FIG. 3. Alcian blue and Safranin-O histology of cartilage at defect sites. Top panels, Alcian blue staining; bottom panels, Safranin-O staining. Boxes in upper panels represent magnified area shown in lower panels. (A, B) Defects with no implant or PBS-loaded chitosan in alginate exhibited reduced Alcian blue staining compared with NELL-1-treated defects, where Alcian blue-positive matrix surrounded the columns of chondrocytes. (C, D) Defects with no implant or PBS-loaded chitosan in alginate exhibited minimal Safranin-O staining compared with NELL-1-treated defects, consistent with Alcian blue results. Arrowheads indicate approximate margins of defects. Original magnification: 40 $\times$ (A, C), 200 $\times$ (B, D). Scale bar: 125 μm (both magnifications). (E) Quantitation of Alcian blue staining intensity by image analysis of random fields within the defects of each experimental group. Average $\pm$ SD shown. * $p < 0.02$, ** $p < 0.0005$ compared to no implant control. (F) Semiquantitative histologic evaluation of tissue at defect sites. Scoring scale was modified from Mankin *et al.* Average $\pm$ SD shown. # $p = 0.018$ compared with no implant control. Color images available online at www.liebertonline.com/tea

FIG. 4. Immunohistochemistry of tissue at defect sites. Expression of type II collagen (**A**), proliferating cell nuclear antigen (PCNA) (**B**), type X collagen (**C**), type I collagen (**D**), and Runt-related transcription factor 2 (Runx2) (**E**) in defects containing no implant, PBS-loaded chitosan in alginate, and NELL-1-loaded chitosan in alginate. Dashed line indicates the approximate tidemark between the upper cartilaginous layer and the underlying subchondral bone. Type II collagen is expressed intracellularly in defects containing no implant or PBS-loaded chitosan in alginate, but are not present in the matrix. Positive type II collagen staining is observed in the matrix of the upper cartilaginous layer of the defect treated with NELL-1. PCNA and type X collagen staining was observed in the no implant and PBS-loaded chitosan groups, but not in the NELL-1-loaded chitosan group. Type I collagen was expressed in the subchondral bone in all groups, appears intracellularly in defects containing no implant or PBS-loaded chitosan in alginate, but is specifically excluded in the cells and matrix of the upper cartilaginous layer of defects treated with NELL-1. In addition, Runx2 is not expressed in the upper cartilaginous layer of the defect treated with NELL-1. Original magnification: 200 $\times$. Scale bar: 125 μ m. Color images available online at www.liebertonline.com/tea

effective in various animal models of osteogenic regeneration.^{22,25,34} At the molecular level, cartilage and bone share a common developmental lineage, including regulation by the transcription factor Runx2, a key modulator of osteochondral development essential for normal chondrocyte maturation and proliferation during development.^{35–37} In addition, we have shown that Nell-1 could accelerate chondrocyte hypertrophy and endochondral ossification in an *ex vivo* maxillary distraction model, thus demonstrating that Nell-1 can affect differentiation of chondrocytes in adult tissues.²⁴ Finally, we have shown that Nell-1 induces proliferation of perivascular stem cells,³⁸ inhibits proliferation of goat bone marrow stromal cells,³⁹ and has no effect on the proliferation of mouse calvarial cells,^{40,41} thus suggesting that the effect of Nell-1 may be cell- or differentiation status dependent, and may drive proliferation or final differentiation of cells once their fates have been specified. Based on this hypothesis, we were able to demonstrate that Nell-1 induces proliferation of primary chondrocytes *in vitro*,³¹ but we also recently reported that Nell-1 inhibits chondrogenic differentiation of ATDC5 cells, a commonly used cell culture model of chondrocytes.⁴² This inhibition requires the transcription factor Nfatc2, which is induced by Nell-1 stimulation. Interestingly, the inhibition requires Runx2, thus suggesting that the effect of Nell-1 on chondrocytes and other cells may depend on the expression level of Runx2, possibly increasing the specificity of Nell-1, as Runx2 expression levels vary depending on chondrocyte maturity.^{35–37,43} Importantly, Nell-1 is a direct transcriptional target of Runx2,²⁷ and moreover, Nell-1 downregulates Osterix, a regulator specifically required for osteoblastic differentiation,^{23,44} thus suggesting that Nell-1 may preferentially direct chondrogenic differentiation of bipotential osteochondral progenitors from the subchondral bone or neighboring tissues. Future work will specifically elucidate the mechanisms by which Nell-1 influences chondrogenesis during both embryogenesis and tissue repair in adult tissues, especially in the context of the BMP and TGF- β pathways.

Higher concentrations of BMP-2 or TGF- β 1 were associated with a higher incidence of osteophytes without significant improvement of cartilage repair,^{15,16,19} indicating the requirement for controlled delivery of therapeutic molecules. Precise spatial and quantitative delivery of growth factors is required to minimize the amount of growth factor required to overcome the burst effect,⁴⁵ as well as to prevent diffusion of excess growth factor to nearby tissues that could cause adverse effects.⁴⁶ In this study, we achieved controlled release by devising a delivery system for NELL-1 where it was encapsulated within chitosan nanoparticles embedded in an alginate hydrogel. Alginates are biocompatible, biodegradable, and hypoimmunogenic scaffold biomaterials that liquefy over time and both release growth factors dispersed within and make room for newly formed repair tissue,^{47,48} and chitosan is a biodegradable polysaccharide that has been successfully used in cartilage repair.^{49–51} Pre-encapsulation reduced the amount of NELL-1 burst-released from the alginate gel during the first day (Fig. 1C), and, thus, more NELL-1 protein remained within the alginate gel instead of diffusing out of it. Efforts are ongoing to incorporate NELL-1 into the core of the chitosan nanoparticles to achieve controlled release over longer periods.

Using 200 μ g/mL NELL-1, a concentration that had been previously established to reliably maintain the chondrocyte phenotype,³¹ we demonstrated robust cartilage formation

in vivo, showing that an increased concentration above 200 μ g/mL was not necessary to overcome diffusion away from the defect. Although we have shown that NELL-1 has osteogenic effects in spinal fusion and femoral segmental defect models among others,^{22,25,26,34} we did not observe overgrowth of the subchondral bone within or adjacent to the defect. The absence of markers of proliferating (PCNA) or hypertrophic (type X collagen) chondrocytes suggest a stable hyaline phenotype in the NELL-1-treated group. In addition, lack of bone tissue in the upper cartilaginous layer, as observed by H&E histology and negative IHC staining for type I collagen and Runx2, indicate the absence of osteophyte formation. This suggests that the effects of NELL-1 were likely limited to the chondrocytes within the defect area, though the possibility of paracrine effects of Nell-1 on the adjacent and underlying bone cannot be completely excluded. Since we used a cell-free scaffold system to deliver Nell-1 to the defect site, cells within the defect space are likely derived from adjacent tissue. However, since osteoarthritis frequently involves large, chronic lesions that may exceed the reparative capacity of local cells, exogenous stem cells, including autologous and allogeneic chondrocytes,^{52–55} mesenchymal stem cells,^{56,57} and embryonic stem cells,^{58,59} in various delivery and scaffold systems, could be incorporated into our delivery system to augment repair of more extensive cartilage damage.

In conclusion, we have demonstrated significant improvement in healing of an *in vivo* model of cartilage degeneration by using the novel growth factor Nell-1, as NELL-1 treatment resulted in the regeneration of higher quality cartilage tissue than either no implant or PBS-loaded chitosan in an alginate implant, as characterized by gross histology, staining for cartilage matrix components, and IHC. In the current study, we observed healing in a single-site cartilage defect model, but we envision cartilage repair in more complex models of osteoarthritis such as anterior cruciate ligament transection and medial meniscectomy, which results in more extensive and chronic cartilage injury,^{60,61} by modifying the form factor of the scaffold and delivery particles or with stem cell augmentation. Overall, developing improved tissue engineering-based methods of cartilage repair and regeneration will advance the development of new clinical products that will significantly improve quality of life for patients suffering from arthritis and other cartilage disorders. Further successful applications of NELL-1 in *in vivo* cartilage repair would establish the use of NELL-1 protein as an important new paradigm for cartilage tissue engineering.

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Disclosure Statement

Nell-1 related patents were licensed to Bone Biologics, Inc. by UCLA. K.T., C.S., X.Z., and B.W. are co-founders of Bone Biologics, Inc. and inventors of the related patents.

References

1. From the Centers for Disease Control and Prevention. Arthritis prevalence and activity limitations—United States, 1990. *JAMA* **272**, 346, 1994.
2. Laurencin, C.T., Ambrosio, A.M., Borden, M.D., and Cooper, J.A., Jr. Tissue engineering: orthopedic applications. *Ann Rev Biomed Eng* **1**, 19, 1999.
3. Saraf, A., and Mikos, A.G. Gene delivery strategies for cartilage tissue engineering. *Adv Drug Deliv Rev* **58**, 592, 2006.
4. Jackson, D.W., Simon, T.M., and Aberman, H.M. Symptomatic articular cartilage degeneration: the impact in the new millennium. *Clin Orthop Relat Res* (**391Suppl**), S14, 2001.
5. McPherson, J.M., and Tubo, R. Articular cartilage injury. In: Lanza, R.P., Langer, R., Vacanti, J., eds. *Principles of Tissue Engineering*, 2nd ed. San Diego: Academic Press, 2000, pp. 697–709.
6. Hunziker, E.B. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. *Osteoarthritis Cartilage* **10**, 432, 2001.
7. Vinatier, C., Mrugala, D., Jorgensen, C., Guicheux, J., and Noel, D. Cartilage engineering: a crucial combination of cells, biomaterials and biofactors. *Trends Biotechnol* **27**, 307, 2009.
8. Benya, P.D., and Shaffer, J.D. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell* **30**, 215, 1982.
9. Benya, P.D., Padilla, S.R., and Nimni, M.E. Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. *Cell* **15**, 1313, 1978.
10. Chubinskaya, S., and Kuettner, K.E. Regulation of osteogenic proteins by chondrocytes. *Int J Biochem Cell B* **35**, 1323, 2003.
11. Yang, W.D., Gomes, R.R., Brown, A.J., Burdett, A.R., Alicknavitch, M., Farach-Carson, M.C., *et al.* Chondrogenic differentiation on perlecan domain I, collagen II, and bone morphogenetic protein-2-based matrices. *Tissue Eng* **12**, 2009, 2006.
12. Grunder, T., Gaismaier, C., Fritz, J., Stoop, R., Hortschansky, P., Mollenhauer, J., and Aicher, W.K. Bone morphogenetic protein (BMP)-2 enhances the expression of type II collagen and aggrecan in chondrocytes embedded in alginate beads. *Osteoarthritis Cartilage* **12**, 559, 2004.
13. Redini, F., Daireaux, M., Mauviel, A., Galera, P., Loyau, G., and Pujol, J.P. Characterization of proteoglycans synthesized by rabbit articular chondrocytes in response to transforming growth factor-beta (TGF-beta). *Biochim Biophys Acta* **1093**, 196, 1991.
14. Ducy, P., and Karsenty, G. The family of bone morphogenetic proteins. *Kidney Int* **57**, 2207, 2000.
15. van Beuningen, H.M., Glansbeek, H.L., van der Kraan, P.M., and van den Berg, W.B. Differential effects of local application of BMP-2 or TGF-beta 1 on both articular cartilage composition and osteophyte formation. *Osteoarthritis Cartilage* **6**, 306, 1998.
16. Yeh, T.T., Wu, S.S., Lee, C.H., Wen, Z.H., Lee, H.S., Yang, Z., *et al.* The short-term therapeutic effect of recombinant human bone morphogenetic protein-2 on collagenase-induced lumbar facet joint osteoarthritis in rats. *Osteoarthritis Cartilage* **15**, 1357, 2007.
17. Blaney Davidson, E.N., van der Kraan, P.M., and van den Berg, W.B. TGF-beta and osteoarthritis. *Osteoarthritis Cartilage* **15**, 597, 2007.
18. Bakker, A.C., van de Loo, F.A., van Beuningen, H.M., Sime, P., van Lent, P.L., van der Kraan, P.M., *et al.* Overexpression of active TGF-beta-1 in the murine knee joint: evidence for synovial-layer-dependent chondro-osteophyte formation. *Osteoarthritis Cartilage* **9**, 128, 2001.
19. van Beuningen, H.M., van der Kraan, P.M., Arntz, O.J., and van den Berg, W.B. Transforming growth factor-beta 1 stimulates articular chondrocyte proteoglycan synthesis and induces osteophyte formation in the murine knee joint. *Lab Invest* **71**, 279, 1994.
20. van der Kraan, P.M., and van den Berg, W.B. Osteophytes: relevance and biology. *Osteoarthritis Cartilage* **15**, 237, 2007.
21. Ting, K., Vastardis, H., Mulliken, J.B., Soo, C., Tieu, A., Do, H., *et al.* Human NELL-1 expressed in unilateral coronal synostosis. *J Bone Miner Res* **14**, 80, 1999.
22. Siu, R.K., Lu, S.S., Li, W., Whang, J., McNeill, G., Zhang, X., *et al.* NELL-1 protein promotes bone formation in a sheep spinal fusion model. *Tissue Eng Part A* **17**, 1123, 2011.
23. Aghaloo, T., Cowan, C.M., Chou Y-F, Zhang, X., Lee, H., Miao, S., *et al.* NELL-1-induced bone regeneration in calvarial defects. *Am J Pathol* **169**, 903, 2006.
24. Cowan, C.M., Cheng, S., Ting, K., Soo, C., Walder, B., Wu, B., *et al.* NELL-1 induced bone formation within the distracted intermaxillary suture. *Bone* **38**, 48, 2006.
25. Li, W., Lee, M., Whang, J., Siu, R.K., Zhang, X., Liu, C., *et al.* Delivery of lyophilized NELL-1 in a rat spinal fusion model. *Tissue Eng Part A* **16**, 2861, 2010.
26. Li, W., Zara, J.N., Siu, R.K., Lee, M., Aghaloo, T., Zhang, X., *et al.* NELL-1 enhances bone regeneration in a rat critical-sized femoral segmental defect model. *Plast Reconstr Surg* **127**, 580, 2011.
27. Truong, T., Zhang, X., Pathmanathan, D., Soo, C., and Ting, K. Craniosynostosis-associated gene NELL-1 is regulated by Runx2. *J Bone Miner Res* **22**, 7, 2007.
28. Komori, T. Regulation of bone development and extracellular matrix protein genes by RUNX2. *Cell Tissue Res* **339**, 189, 2010.
29. Desai, J., Shannon, M.E., Johnson, M.D., Ruff, D.W., Hughes, L.A., Kerley, M.K., *et al.* Nell1-deficient mice have reduced expression of extracellular matrix proteins causing cranial and vertebral defects. *Hum Mol Genet* **15**, 1329, 2006.
30. Dragoo, J.L., Carlson, G., McCormick, F., Khan-Farooqi, H., Zhu, M., Zuk, P.A., and Benhaim, P. Healing full-thickness cartilage defects using adipose-derived stem cells. *Tissue Eng* **13**, 1615, 2007.
31. Lee, M., Siu, R.K., Ting, K., and Wu, B.M. Effect of NELL-1 delivery on chondrocyte proliferation and cartilaginous extracellular matrix deposition. *Tissue Eng Part A* **16**, 1791, 2010.
32. Gonzalez-Rodriguez, M.L., Holgado, M.A., Sanchez-Lafuente, C., Rabasco, A.M., and Fini, A. Alginate/chitosan particulate systems for sodium diclofenac release. *Int J Pharm* **232**, 225, 2002.
33. Mankin, H.J., Dorfman, H., Lippiello, L., and Zarins, A. Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. *J Bone Joint Surg Am* **53**, 523, 1971.
34. Lu, S.S., Whang, J., Zhang, X., Wu, B., Turner, A.S., Seim, H.B., *et al.* NELL-1 promotes bone formation in a sheep spinal fusion model. *J Bone Miner Res* **23**, S171, 2007.
35. Yoshida, C.A., Yamamoto, H., Fujita, T., Furuichi, T., Ito, K., Inoue, K., *et al.* Runx2 and Runx3 are essential for

- chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. *Genes Dev* **18**, 952, 2004.
36. Inada, M., Yasui, T., Nomura, S., Miyake, S., Deguchi, K., Himeno, M., *et al.* Maturation disturbance of chondrocytes in Cbfa1-deficient mice. *Dev Dyn* **214**, 279, 1999.
 37. Sato, S., Kimura, A., Ozdemir, J., Asou, Y., Miyazaki, M., Jinno, T., *et al.* The distinct role of the Runx proteins in chondrocyte differentiation and intervertebral disc degeneration: findings in murine models and in human disease. *Arthritis Rheum* **58**, 2764, 2008.
 38. Zhang, X., Peault, B., Chen, W., Li, W., Corselli, M., James, A.W., *et al.* The NELL-1 growth factor stimulates bone formation by purified human perivascular cells. *Tissue Eng Part A* 2011 [Epub ahead of print].
 39. Aghaloo, T., Jiang, X., Soo, C., Zhang, Z., Zhang, X., Hu, J., *et al.* A study of the role of NELL-1 gene modified goat bone marrow stromal cells in promoting new bone formation. *Mol Ther* **15**, 1872, 2007.
 40. Zhang, X., Carpenter, D., Bokui, N., Soo, C., Miao, S., Truong, T., *et al.* Overexpression of NELL-1, a craniosynostosis-associated gene, induces apoptosis in osteoblasts during craniofacial development. *J Bone Miner Res* **18**, 2126, 2003.
 41. Zhang, X., Kuroda, S., Carpenter, D., Nishimura, I., Soo, C., Moats, R., *et al.* Craniosynostosis in transgenic mice overexpressing NELL-1. *J Clin Invest* **110**, 861, 2002.
 42. Chen, W., Zhang, X., Siu, R.K., Chen, F., Shen, J., Zara, J.N., *et al.* Nfatc2 is a primary response gene of NELL-1 regulating chondrogenesis in ATDC5 cells. *J Bone Miner Res* **26**, 1230, 2011.
 43. Hinoi, E., Bialek, P., Chen, Y.T., Rached, M.T., Groner, Y., Behringer, R.R., *et al.* Runx2 inhibits chondrocyte proliferation and hypertrophy through its expression in the perichondrium. *Genes Dev* **20**, 2937, 2006.
 44. Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J.M., Behringer, R.R., *et al.* The novel zinc finger-containing transcription factor Osterix is required for osteoblast differentiation and bone formation. *Cell* **108**, 17, 2002.
 45. Uludag, H., D'Augusta, D., Palmer, R., Timony, G., and Wozney, J. Characterization of rhBMP-2 pharmacokinetics implanted with biomaterial carriers in the rat ectopic model. *J Biomed Mater Res* **46**, 193, 1999.
 46. Walker, D.H., and Wright, N.M. Bone morphogenetic proteins and spinal fusion. *Neurosurg Focus* **13**, 1, 2002.
 47. Langer, R., and Vacanti, J.P. Tissue engineering. *Science (New York, NY)* **260**, 920, 1993.
 48. Mierisch, C.M., Cohen, S.B., Jordan, L.C., Robertson, P.G., Balian, G., and Diduch, D.R. Transforming growth factor-beta in calcium alginate beads for the treatment of articular cartilage defects in the rabbit. *Arthroscopy* **18**, 892, 2002.
 49. Hoemann, C.D., Sun, J., Legare, A., McKee, M.D., and Buschmann, M.D. Tissue engineering of cartilage using an injectable and adhesive chitosan-based cell-delivery vehicle. *Osteoarthritis Cartilage* **13**, 318, 2005.
 50. Hoemann, C.D., Hurtig, M., Rossomacha, E., Sun, J., Chevrier, A., Shive, M.S., and Buschmann, M.D. Chitosan-glycerol phosphate/blood implants improve hyaline cartilage repair in ovine microfracture defects. *J Bone Joint Surg* **87**, 2671, 2005.
 51. Hoemann, C.D., Sun, J., McKee, M.D., Chevrier, A., Rossomacha, E., Rivard, G.-E., Hurtig, M., and Buschmann, M.D. Chitosan-glycerol phosphate/blood implants elicit hyaline cartilage repair integrated with porous subchondral bone in microdrilled rabbit defects. *Osteoarthritis Cartilage* **15**, 78, 2007.
 52. Moriya, T., Wada, Y., Watanabe, A., Sasho, T., Nakagawa, K., Mainil-Varlet, P., *et al.* Evaluation of reparative cartilage after autologous chondrocyte implantation for osteochondritis dissecans: histology, biochemistry, and MR imaging. *J Orthop Sci* **12**, 265, 2007.
 53. Fragonas, E., Valente, M., Pozzi-Mucelli, M., Toffanin, R., Rizzo, R., Silvestri, F., *et al.* Articular cartilage repair in rabbits by using suspensions of allogenic chondrocytes in alginate. *Biomaterials* **21**, 795, 2000.
 54. Lin, L., Zhou, C., Wei, X., Hou, Y., Zhao, L., Fu, X., *et al.* Articular cartilage repair using dedifferentiated articular chondrocytes and bone morphogenetic protein 4 in a rabbit model of articular cartilage defects. *Arthritis Rheum* **58**, 1067, 2008.
 55. Pei, M., He, F., Boyce, B.M., and Kish, V.L. Repair of full-thickness femoral condyle cartilage defects using allogeneic synovial cell-engineered tissue constructs. *Osteoarthritis Cartilage* **17**, 714, 2009.
 56. Chen, J., Wang, C., Lu, S., Wu, J., Guo, X., Duan, C., *et al.* *In vivo* chondrogenesis of adult bone-marrow-derived autologous mesenchymal stem cells. *Cell Tissue Res* **319**, 429, 2005.
 57. Yoo, J.U., Barthel, T.S., Nishimura, K., Solchaga, L., Caplan, A.I., Goldberg, V.M., *et al.* The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. *J Bone Joint Surg Am* **80**, 1745, 1998.
 58. Hwang, N.S., Varghese, S., Lee, H.J., Zhang, Z., Ye, Z., Bae, J., *et al.* *In vivo* commitment and functional tissue regeneration using human embryonic stem cell-derived mesenchymal cells. *Proc Natl Acad Sci U S A* **105**, 20641, 2008.
 59. Toh, W.S., Lee, E.H., Guo, X.M., Chan, J.K., Yeow, C.H., Choo, A.B., *et al.* Cartilage repair using hyaluronan hydrogel-encapsulated human embryonic stem cell-derived chondrogenic cells. *Biomaterials* **31**, 6968, 2010.
 60. Kamekura, S., Hoshi, K., Shimoaka, T., Chung, U., Chikuda, H., Yamada, T., *et al.* Osteoarthritis development in novel experimental mouse models induced by knee joint instability. *Osteoarthritis Cartilage* **13**, 632, 2005.
 61. Yoshioka, M., Coutts, R.D., Amiel, D., and Hacker, S.A. Characterization of a model of osteoarthritis in the rabbit knee. *Osteoarthritis Cartilage* **4**, 87, 1996.

Address correspondence to:

Min Lee, Ph.D.

Division of Advanced Prosthodontics, Biomaterials,
and Hospital Dentistry

University of California

School of Dentistry

10833 Le Conte Avenue, CHS 23-032B

Los Angeles, CA 90095-1668

E-mail: leemin@ucla.edu

Chia Soo, M.D.

UCLA and Orthopaedic Hospital Department
of Orthopaedic Surgery and the

Orthopaedic Hospital Research Center

University of California

675 Charles E Young Dr., South, MRL 2641A

Los Angeles, CA 90095-1579

E-mail: bs00@ucla.edu

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