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hPSCs and NELL-1 Synergistically Enhance Spinal Fusion in Osteoporotic Rats

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Disclosure of Potential Conflicts of Interest

Drs. X.Z., K.T., and C.S. are inventors of Nell-1 related patents and K.T., B.P., and C.S. are inventors of perivascular stem cell-related patents filed from UCLA. Drs. X.Z., K.T., and C.S. are founders and/or board members of Bone Biologics Inc. which sublicenses Nell-1 patents from the UC Regents. Drs. K.T., and C.S. are founders of Scarless Laboratories Inc. which sublicenses perivascular stem cell-related patents from the UC Regents. Dr. Chia Soo is also an officer of Scarless Laboratories, Inc.

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Keywords

Spinal fusion; osteoporosis; perivascular stem cells; *NEL*-like protein-1; bone morphogenetic protein-2

Introduction

Autologous bone grafts are frequently required for successful spinal fusion [1]. However, Autologous bone grafts in osteoporotic conditions result in unsatisfactory results due to the lack osteogenic cells and reduced function of those cells [2–4]. Thus, the search for effective alternatives to autologous bone has led to the use of various bone graft substitutes such as allografts, growth factors, stem cells, and gene therapies [5]. Bone morphogenetic protein-2 (BMP-2) has been used in clinical spinal fusion with the approval of FDA as an autologous bone graft substitute [6]. However, side effects such as life-threatening inflammatory swelling and promotion of adipogenesis are apparent [7]. The search for an efficacious and safe modality to enhance spinal fusion outcomes in osteoporotic conditions is a field of ongoing research.

Nel-like protein-1 (NELL-1) has been found to induce osseous healing in small and large animal models including osteoporotic rat models without harmful side effects [8, 9]. Interestingly, we observed an additive effect of NELL-1 and human perivascular stem cells (hPSCs), a prospectively purified mesenchymal stem cell population with perivascular distribution and pro-osteogenic / pro-angiogenic properties, in an ectopic bone formation model [10, 11]. Additionally, hPSC alone has demonstrated pro-osteogenic effects in non-osteoporotic models including spinal fusion with non-osteoporotic rats [12].

The purpose of this study is to determine the efficacy of hPSCs combined with NELL-1 for enhancing spinal fusion in osteoporotic rats with the goal of ultimately developing an effective and safe therapeutic using hPSCs and NELL-1 to treat patients with osteoporosis.

Materials and Methods

Isolation of hPSCs

With the exception of one sample from autopsy, lipoaspirate was obtained from patients with and without osteoporosis undergoing liposuction under IRB exemption (Supplemental Table 1). The hPSCs consisting of two populations: pericytes (CD146+, CD34–, CD45–) and adventitial cells (CD34+, CD146–, CD45–) were purified as previously described [13].

In vitro assays for osteogenesis and adipogenesis of hPSCs

The hPSCs were cultured in osteogenic or adipogenic differentiation medium containing NELL-1, BMP-2 or phosphate buffered saline (PBS) to compare their effects on osteogenesis and adipogenesis of hPSCs [10].

Experimental animals, ovariectomy (OVX), and spinal fusion

To induce osteoporosis, 41 athymic rats were ovariectomized [14]. Induction of osteoporosis was confirmed by dual energy X-ray absorptiometry 4 weeks post-OVX. The implants were prepared using 2 different doses of hPSCs and NELL-1 based on our previous studies: (1) Regular dose: 0.25×10^6 cells/ml of hPSCs or 33.3 µg/ml of NELL-1 that demonstrated the successful fusion in non-osteoporotic models [12, 15]; (2) High dose: 0.75×10^6 cells/ml of hPSCs or 66.6 µg/ml of NELL-1. Finally animals were organized into the following 7 implant groups: (1) Regular P: Regular dose of hPSCs alone; (2) Regular N: Regular dose of NELL-1 alone; (3) Regular P+N: Combination of regular dose of hPSCs and NELL-1; (4) High P: High dose of hPSCs alone; (5) High N: High dose of NELL-1 alone; (6) High P+N: Combination of high dose of hPSCs and NELL-1; (7) Control. A detailed discussion of each of the implant constituents is presented in Table 1. Spinal fusion surgeries were performed as previously described [12]. Animals were harvested 4 weeks post-surgery.

Manual palpation and micro-computed tomography (microCT)

Spinal fusion was determined by manual palpation of 3 blinded observers (Supplemental Table 2). MicroCT analyses including bone volume (BV) and bone mineral density (BMD) were performed using CT-Analyzer software (SkyScan 1172, Belgium) as described previously [12].

Histology and immunohistochemistry on decalcified tissue

After decalcification in 19% ethylenediaminetetraacetic acid, the samples were embedded in paraffin. H&E and immunohistochemistry for bone sialoprotein (BSP) (Chemicon) were performed as previously described [16].

Bone dynamic labeling and hPSCs tracking on undecalcified tissue

To visualize bone-forming activity, the selected animals were injected with Calcein/Alizarin prior to sacrifice. Frozen sections were cut following Kawamoto's procedure [17]. Immunofluorescent staining for human-specific major histocompatibility complex (hMHC) class I antigen (Santa Cruz Biotechnology) was performed and analyzed using the Olympus image system.

Statistics

A paired *t*-test was used to test significance when only two groups were tested after normality test. Kruskal–Wallis test with post hoc tests of Bonferroni was used to test the significance of data to compare more than two groups. The statistical software, SPSS for Windows Version 18.0 (SPSS, Chicago, IL, USA) was used for all statistical analyses. Statistical significance was determined $p < 0.05$.

Results

Similar osteogenic capacity of hPSCs from osteoporotic and non-osteoporotic conditions

No significant difference in osteogenic differentiation was observed between the healthy and osteoporotic donors. Interestingly, the addition of NELL-1 enhanced mineralization of

hPSCs from both types of donors without significant differences (Fig. 1). The additional experiment under adipogenic induction revealed that, in contrast to BMP-2, hPSCs treated with NELL-1 did not undergo more adipogenic differentiation compared to PBS control ($p>0.05$), although hPSCs from osteoporotic donors displayed inherently higher adipogenic differentiation compared to its healthy counterpart (Supplemental Fig. 1). Notably, the average yield of hPSCs from listed donors did not differ significantly from each other (Supplemental Table 1).

hPSC+NELL-1 increased fusion rate in the osteoporotic rats

Post-OVX, the average BMD of the L5 vertebrae significantly decreased by 10.2% compared to its preoperative state ($p<0.01$) (Supplemental Fig. 2). Among seven groups, High P+N group exhibited significantly increased palpation scores (4.7) with the highest fusion rate at 83.3% compared to the other study groups ($p<0.01$). Notably, neither the regular dose which was effective for healthy rats [12, 15] nor high dose of hPSCs or NELL-1 alone could produce a significant fusion rate in OVX rats, with only 20–28.6% spinal fusion (Table 1).

Robust bone formation promoted by hPSC+NELL-1 in the osteoporotic rats

Three dimensional micro-CT images showed that High P+N formed new bony masses between the transverse processes resulting in solid fusion. In contrast, the control group demonstrated clear clefts between the two transverse processes with minimal bone formation. Quantitatively, the High P+N group exhibited a significant increase in BV of $82.6\pm 1.97\text{ mm}^3$ compared to any other groups ($p<0.01$) (Fig. 2). However, the samples with regular dose did not exhibit a significant difference (Supplemental Fig. 3). Histologically, the fibrous tissue formation was prevalent in the control, High N and High P samples. In contrast, we observed large areas of chondroid matrix with bone formation, increased vascularization and complete bony bridging in High P+N specimens. Additionally, BSP immunohistochemistry demonstrated increased staining in new bone and cartilaginous tissue in High P and High P+N samples compared to the control samples. (Fig. 3A).

Tissue engraftment revealed involvement of hPSCs in active ossification

We observed a wider band of Calcein/Alizarin labeling in High P+N than other groups, suggesting more robust active ossifications along the edges of the demineralized bone matrix (Fig. 3B). We merged the images of hMHC Class I immunofluorescent staining with Calcein/Alizarin labeling and found that hPSCs and new bone formation were co-located in the same region, confirming the direct involvement of hPSCs *in situ* of the active ossification (Fig. 3C).

Discussion

Recent developments in regenerative medicine support the crucial role that stem cells play in bone regeneration. However, most studies are designed using a healthy animal to create disease models [12, 18]. In order to replicate clinical osteoporotic settings, it becomes critical to demonstrate the efficacy of these stem cell therapeutics in osteoporotic animals. To our knowledge, this is the first study that clearly demonstrated the great potential of

combinatorial application of stem cells (hPSCs) and osteogenic factor (NELL-1) in promoting successful spinal fusion in osteoporotic rats.

Although not yet fully understood, delayed fusion or nonunion in osteoporotic bone have been attributed to: (1) decreased proliferation and differentiation capacity of endogenous mesenchymal stem cells; (2) diminished formation of vasculature; (3) lower osteoinductive activity; and (4) changes in local and systemic signaling molecules [19].

In agreement with prior studies, we observed that the healing potency of osteoporotic bone was severely impaired compared to its healthy counterpart. (Supplemental Table 3) [3, 4, 20–22]. Only 20% of fusion was achieved in osteoporotic rats using the same number (0.25×10^6 cells/ml, regular dose) of hPSCs that induced 100% fusion in non-osteoporotic rats [12].

It was reported that stem cells from fat, even from osteoporotic patients, can undergo osteogenic differentiation at a similar rate to bone marrow stem cells (BMSCs) from younger patients [23]. Our study revealed similar osteogenic capacity of hPSCs from lipoaspirate between donors with and without osteoporosis. Considering the defects in osteogenic property of BMSCs from osteoporotic condition [24], these characteristics of hPSCs will be a good building block in the development of efficacious and safe therapy using autologous stem cells from adipose tissue in an orthopaedic clinical setting. The hPSCs induce bone formation via both direct osteogenic differentiation and indirect trophic effects. They secrete high levels of pro-osteogenic, pro-vasculogenic growth factors, such as vascular endothelial growth factor, fibroblast growth factor 2, and epidermal growth factor [11, 13].

There are several advantages to using NELL-1 in osteoporotic conditions over BMP-2: (1) NELL-1 inhibits BMP-2 induced inflammation by acting as an anti-inflammatory molecule [7]. (2) NELL-1 has anti-osteoclastic effects [25]. (3) NELL-1 inhibits adipogenic differentiation [26]. In previous studies [27], we observed NELL-1 could stimulate proliferation of hPSCs. Consequently we suggest that the administration of hPSCs+NELL-1 restores the reduced native osteoprogenitor cell and osteoinductive microenvironment in osteoporotic bone. In the current study, the direct involvement of hPSCs in active ossification was further validated by a novel cryostat sectioning technique using undecalcified samples.

However, further studies with larger sample size focusing on the mode of action of this promising therapy and on any differences of osteogenic capacities of hPSCs from obese and slim donors are warranted. It is unclear if differences in body mass index translate to differences in hPSC behavior, as has been previously reported in adipose derived stem cells [28]. The synergistic effects of hPSCs and NELL-1 in enhancing spinal fusion with osteoporotic condition shed light on possibility of developing hPSCs based therapy for osteoporotic patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Adipose tissue derived hPSCs retain their osteogenic potential and NELL-1 responsiveness with osteoporosis

The hPSCs underwent osteogenic differentiation over a time period of 15 days. Cells were seeded at 3×10^4 density, in 24 well plates with DMEM + 10% FBS. Within 24 hours, cells were induced to osteogenic differentiation by NELL-1 (300 ng/ml) or PBS control in osteogenic differentiation medium (DMEM + 10% FBS + 50 μ g/ml ascorbic acid, and 10mM β -glycerophosphate). Media was changed every 3 days. (A) Osteogenic differentiation was determined by Alizarin Red staining. (B) Quantification of osteogenesis of hPSCs derived from non-osteoporotic and osteoporotic patients showed that osteogenesis was significantly increased in both groups when the hPSCs were cultured with NELL-1, and there were no significant differences on basal and NELL-1-induced osteogenic properties between hPSCs from non-osteoporotic and osteoporotic patients. hPSCs: human perivascular stem cells, NELL-1: *Nr1*-like protein-1, PBS: phosphate buffered saline, DMEM: dulbecco's modified eagles medium, FBS: fetal bovine serum, ** $p < 0.01$ compared to the control-treated hPSCs. Bars, $\pm$ SD

A **B**

Figure 2. hPSCs+NELL-1 promotes solid bony fusion in osteoporotic rat

(A) Representative images of microCT scanning of fusion mass with three-dimensional reconstruction from High P+N, High P, High N, and control groups four weeks after implantation. The High P+N group had marked bone formation around the transverse processes of L4 and L5 (arrow). In contrast, the control group demonstrated radiolucent spaces (arrow head). (B) Histomorphometric analyses of the fusion mass showed a significant increase in BV in rats treated with High P+N. The region of interest was defined as starting from the lower border of transverse process of L5 to the upper border of the transverse process of L4. Only graft material and new bone formation between the transverse processes of L4 and L5 were analyzed and quantified. P: human perivascular stem cells, N: *Nel*-like protein-1, microCT: micro-computed tomography, BV: bone volume, * $p < 0.05$ and ** $p < 0.01$. Bars, $\pm$ SD

Figure 3. Histologic evidence of new bone formation and direct involvement of hPSCs in active ossification

(A) Top panel: HE staining in low magnification images showing a dash line divides the bone mass formed over the transverse processes of vertebral bones (vB). The asterisk indicates the capsule of bone mass over the transverse processes. Middle panel: H&E staining in high magnification of black box area of corresponding top panel image reveals more active and mature bone formation areas (arrows) and blood vessels (V) in High P+N group over either High P or High N. Bottom panel: Immunohistochemical staining demonstrated positive brown staining for the BSP. In control and High N groups, the BSP positive chondrocytes were shown without bone formation. New bone and cartilage tissues

were stained BSP positive in High P and High P+N samples. Overall, more positive cells were revealed in High P+N samples. (B) The active ossifications were observed along the edge of DBX particles (dark gray) using Calcein (green) and Alizarin red complexone (red) dynamic labeling in the superimposed images of bright light and fluorescent fields. Samples from High P+N revealed more robust activity of new bone formation in cryosection of undecalcified tissue. (C) The hMHC class I positive hPSCs were co-localized/embedded in mineralized matrix in cryosection of undecalcified tissue. Higher numbers of hPSCs positive of MHC class I (blue) were observed in High P+N group compared to High P. When the images were merged, bone formation was specifically correlated with the area of hPSCs (pink). H&E: hematoxylin and eosin, P: human perivascular stem cells (hPSCs), N: *Nel*-like protein-1 (NELL-1), BSP: bone sialoprotein, DBX: demineralized bone matrix, hMHC: human major histocompatibility complex, hPSCs: blue by Aminomethylcoumarin streptavidin or pink by merging with green and red. Images were acquired at 40× magnification for the top panel of (A) and 200× magnification for middle and bottom panel of (A) and (B, C) originally, and the relevant scale bars were provided.

Table 1
Composition of different implant groups and summary of the manual palpation score, fusion rate

Group(n) ^a	Implant materials and dose (per side)				Palpation score	Fusion rate at 4 weeks post SF(n)
	hPSCs (cells/ml)	NELL-1 (µg/ml)	β-TCP ^b (mg)	DBX (µl)		
Regular P(5)	0.25 × 10 ⁶	0	50	300	3.2	20%(1/5)
Regular N(5)	0	33.3	50	300	3.1	20%(1/5)
Regular P+N(8)	0.25 × 10 ⁶	33.3	50	300	3.4	37.5%(3/8)
High P(7)	0.75 × 10 ⁶	0	50	300	3.5	28.6%(2/7)
High N(5)	0	66.6	50	300	3.5	20%(1/5)
High P+N(6)	0.75 × 10 ⁶	66.6	50	300	4.7 ^c	83.3%(5/6)
Control(5)	0	0	50	300	2.2	0%(0/5)

^aRegular P or Regular N means that number of hPSCs or concentration of NELL-1 that demonstrates the successful fusion in non-osteoporotic models [12, 15]. High P or High N means the 3 times higher number of hPSCs compared to Regular P or 2 times higher concentration of NELL-1 compared to Regular N in each. P+N means the combination of hPSCs and NELL-1 with regular or high dose. Control means the implant without hPSCs and NELL-1.

^bNELL-1 carried into the DBX after lyophilization onto β-TCP.

^cSignificantly higher palpation score than any other groups.

P: human Perivascular stem cells (hPSCs), N: *NEL*-like protein-1 (NELL-1), DBX: demineralized bone matrix, β-TCP: beta tri-calcium phosphate particles, SF: spinal fusion.