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Brief Report: Human Perivascular Stem Cells and Nel-Like Protein-1 Synergistically Enhance Spinal Fusion in Osteoporotic Rats

Soonchul Lee

Xinli Zhang

Jia Shen

Aaron W. James

Choon G. Chung

See next page for additional authors

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At the time of publication, author Chenshuang Li was affiliated with the School of Dentistry, University of California. Currently, (s)he is a faculty member at the School of Dental Medicine at the University of Pennsylvania.

Brief Report: Human Perivascular Stem Cells and Nel-Like Protein-1 Synergistically Enhance Spinal Fusion in Osteoporotic Rats

Abstract

Autologous bone grafts (ABGs) are considered as the gold standard for spinal fusion. However, osteoporotic patients are poor candidates for ABGs due to limited osteogenic stem cell numbers and function of the bone microenvironment. There is a need for stem cell-based spinal fusion of proven efficacy under either osteoporotic or nonosteoporotic conditions. The purpose of this study is to determine the efficacy of human perivascular stem cells (hPSCs), a population of mesenchymal stem cells isolated from adipose tissue, in the presence and absence of NELL-1, an osteogenic protein, for spinal fusion in the osteoporosis. Osteogenic differentiation of hPSCs with and without NELL-1 was tested in vitro. The results indicated that NELL-1 significantly increased the osteogenic potential of hPSCs in both osteoporotic and nonosteoporotic donors. Next, spinal fusion was performed by implanting scaffolds with regular or high doses of hPSCs, with or without NELL-1 in ovariectomized rats (n = 41). Regular doses of hPSCs or NELL-1 achieved the fusion rates of only 20%-37.5% by manual palpation. These regular doses had previously been shown to be effective in nonosteoporotic rat spinal fusion. Remarkably, the high dose of hPSCs+NELL-1 significantly improved the fusion rates among osteoporotic rats up to approximately 83.3%. Microcomputed tomography imaging and quantification further confirmed solid bony fusion with high dose hPSCs+NELL-1. Finally, histologically, direct in situ involvement of hPSCs in ossification was shown using undecalcified samples. To conclude, hPSCs combined with NELL-1 synergistically enhances spinal fusion in osteoporotic rats and has great potential as a novel therapeutic strategy for osteoporotic patients. © 2015 AlphaMed Press.

Keywords

Bone morphogenetic protein-2, NEL-like protein-1, Osteoporosis, Perivascular stem cells, Spinal fusion, Animals, Cell Differentiation, Disease Models, Animal, Humans, Mesenchymal Stem Cell Transplantation, Mesenchymal Stromal Cells, Nerve Tissue Proteins, Osteogenesis, Osteoporosis, Rats, Spinal Fusion, nel like protein 1, osteogenic protein 1, unclassified drug, Nell1 protein, rat, nerve protein, adipogenesis, animal cell, animal experiment, animal model, animal tissue, Article, bone density, bone development, bone mass, controlled study, female, human, human cell, immunohistochemistry, mesenchymal stem cell, micro-computed tomography, nonhuman, ossification, osteoporosis, palpation, perivascular stem cell, rat, spine fusion, stem cell transplantation, animal, cell differentiation, disease model, genetics, mesenchymal stem cell transplantation, mesenchymal stroma cell, metabolism, osteoporosis, pathology, procedures, spine fusion

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Comments

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Author(s)

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Brief Report: Human Perivascular Stem Cells and *Nel*-Like Protein-1 Synergistically Enhance Spinal Fusion in Osteoporotic Rats

SOONCHUL LEE,[a,b*] XINLI ZHANG,[c*] JIA SHEN,[c] AARON W. JAMES,[d] CHOON G. CHUNG,[c] REEF HARDY,[b] CHENSHUANG LI,[c] CAROLINE GIRGIUS,[c] YULONG ZHANG,[e] DAVID STOKER,[f] HUIMING WANG,[g] BENJAMIN M. WU,[b,e,h] BRUNO PEAULT,[b,i] KANG TING,[b,c] CHIA SOO[b,j]

Key Words. Spinal fusion • Osteoporosis • Perivascular stem cells • *NEL*-like protein-1 • Bone morphogenetic protein-2

ABSTRACT

Autologous bone grafts (ABGs) are considered as the gold standard for spinal fusion. However, osteoporotic patients are poor candidates for ABGs due to limited osteogenic stem cell numbers and function of the bone microenvironment. There is a need for stem cell-based spinal fusion of proven efficacy under either osteoporotic or nonosteoporotic conditions. The purpose of this study is to determine the efficacy of human perivascular stem cells (hPSCs), a population of mesenchymal stem cells isolated from adipose tissue, in the presence and absence of NELL-1, an osteogenic protein, for spinal fusion in the osteoporosis. Osteogenic differentiation of hPSCs with and without NELL-1 was tested in vitro. The results indicated that NELL-1 significantly increased the osteogenic potential of hPSCs in both osteoporotic and nonosteoporotic donors. Next, spinal fusion was performed by implanting scaffolds with regular or high doses of hPSCs, with or without NELL-1 in ovariectomized rats ($n = 41$). Regular doses of hPSCs or NELL-1 achieved the fusion rates of only 20%–37.5% by manual palpation. These regular doses had previously been shown to be effective in nonosteoporotic rat spinal fusion. Remarkably, the high dose of hPSCs+NELL-1 significantly improved the fusion rates among osteoporotic rats up to approximately 83.3%. Microcomputed tomography imaging and quantification further confirmed solid bony fusion with high dose hPSCs+NELL-1. Finally, histologically, direct in situ involvement of hPSCs in ossification was shown using undecalcified samples. To conclude, hPSCs combined with NELL-1 synergistically enhances spinal fusion in osteoporotic rats and has great potential as a novel therapeutic strategy for osteoporotic patients. STEM CELLS *2015;33:3158–3163*

SIGNIFICANCE STATEMENT

Spinal fusions performed in osteoporotic conditions result in poor prognosis due to its limited osteogenic cell numbers and function of the local bone micro-environment. In our study, a combination of hPSCs, a population of native mesenchymal stem cells prospectively isolated from adipose tissues, and NELL-1, an osteoinductive protein, was used. Our results showed successful spinal fusion in osteoporotic rats with minimal side effects such as inflammation and fat tissue formation. This combinational therapy using hPSCs combined with NELL-1 has the potential to be a novel stem cell / osteoinductive protein therapeutic for spinal fusion procedures in osteoporotic conditions.

INTRODUCTION

Autologous bone grafts (ABGs) are frequently required for successful spinal fusion [1]. However, ABGs in osteoporotic conditions result in unsatisfactory results due to the lack of 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

[a]Department of Orthopaedic Surgery, CHA Bundang Medical Center, CHA University School of Medicine, Gyeonggi-do, Republic of Korea; [b]UCLA and Orthopaedic Hospital Department of Orthopaedic Surgery and the Orthopaedic Hospital Research Center, [c]Division of Growth and Development, School of Dentistry, [d]Department of Pathology and Laboratory Medicine, [e]Department of Bioengineering, [h]Department of Materials Science and Engineering, and Division of Advanced Prosthodontics, and [j]UCLA Division of Plastic Surgery and Department of Orthopaedic Surgery and the Orthopaedic Hospital Research Center, University of California, Los Angeles, California, USA; [f]Marina Plastic Surgery Associates, Marina del Rey, California, USA; [g]Affiliated Hospital of Stomatology, Medical College, Zhejiang University, Hangzhou, People's Republic of China; [i]Center For Cardiovascular Science and MRC Center for Regenerative Medicine, University of Edinburgh, Edinburgh, United Kingdom

Correspondence: Kang Ting, M.D., D.Med.Sc., Chair, Section of Orthodontics, School of Dentistry, University of California, Los Angeles, 10833 Le Conte Avenue, CHS 30-117, Los Angeles, California 90095, USA. Telephone: 310-713-9979; Fax: 310-206-5349; e-mail: kting@ucla.edu; or Chia Soo, M.D., F.A.C.S., Vice Chair for Research, Division of Plastic and Reconstructive Surgery, Department of Orthopaedic Surgery, University of California, Los Angeles, 675 Charles E Young Dr South, MRL 2641A, Los Angeles, California 90095-1579, USA. Telephone: 310-794-5479; Fax: 310-206-7783; e-mail: bsoo@ucla.edu

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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 per 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)

[a]Regular P or regular N means that number of hPSCs or concentration of NELL-1 that demonstrates the successful fusion in nonosteoporotic models [12, 15]. High P or high N means the three times higher number of hPSCs compared to regular P or two 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.
[b]NELL-1 carried into the DBX after lyophilization onto β-TCP.
[c]Significantly higher palpation score than any other groups.
Abbreviations: DBX, dimineralized bone matrix; N, *NEL*-like protein-1 (NELL-1); P, human perivascular stem cells (hPSCs); SF, spinal fusion; β-TCP, beta tri-calcium phosphate particles.

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 nonosteoporotic models including spinal fusion with nonosteoporotic 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 (Supporting Information 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, 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 (ovariectomy). The implants were prepared using two different doses of hPSCs and NELL-1 based on our previous studies: (a) regular dose: 0.25×10^6 cells per milliliter of hPSCs or 33.3 μg/ml of NELL-1 that demonstrated the successful fusion in nonosteoporotic models [12, 15]; (b) high dose: 0.75×10^6 cells per milliliter of hPSCs or 66.6 μg/ml of NELL-1. Finally animals were organized into the following seven implant groups: (a) regular P: regular dose of hPSCs alone; (b) regular N: regular dose of NELL-1 alone; (c) regular P+N: combination of regular dose of hPSCs and NELL-1; (d) high P: high dose of hPSCs alone; (e) high N: high dose of NELL-1 alone; (f) high P+N: combination of high dose of hPSCs and NELL-1; (g) 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 Microcomputed Tomography

Spinal fusion was determined by manual palpation of three blinded observers (Supporting Information Table 2). Microcomputed tomography (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, Temecula, CA, http://www.chemicon.com) 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 complexone 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, Santa Cruz, CA, http://www.scbt.com) 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 Bonferoni 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,

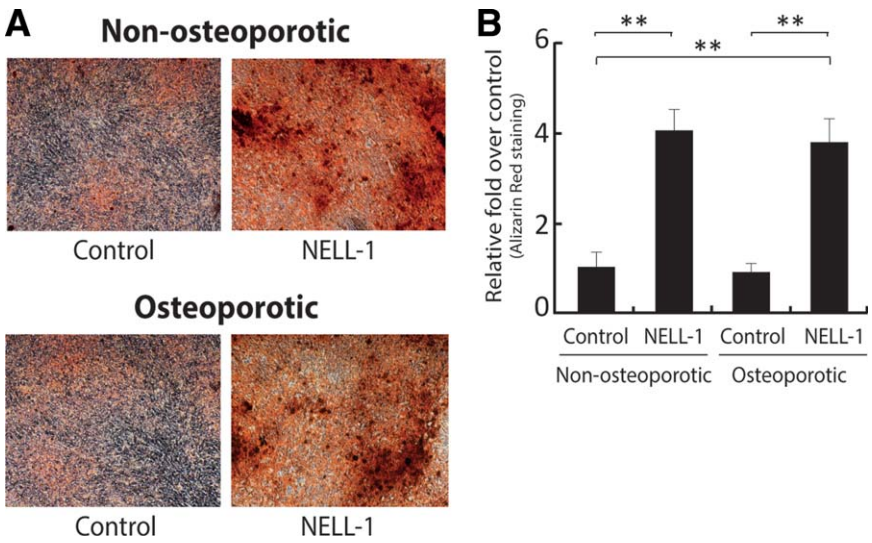

Figure 1. Adipose tissue derived human perivascular stem cells (hPSCs) retain their osteogenic potential and NELL-1 responsiveness with osteoporosis. The hPSCs underwent osteogenic differentiation over a time period of 15 days. Cells were seeded at 3×10^4 density, in 24 well plates with Dulbecco's modified Eagle's medium (DMEM) + 10% fetal bovine serum (FBS). Within 24 hours, cells were induced to osteogenic differentiation by NELL-1 (300 ng/ml) or phosphate buffered saline control in osteogenic differentiation medium (DMEM + 10% FBS + 50 μg/ml ascorbic acid, and 10 mM 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 nonosteoporotic 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 nonosteoporotic and osteoporotic patients. **, $p < .01$ compared to the control-treated hPSCs. Bars ±SD. Abbreviation: NELL-1: *Nel*-like protein-1.

IL) was used for all statistical analyses. Statistical significance was determined $p < .05$.

Results

Similar Osteogenic Capacity of hPSCs from Osteoporotic and Nonosteoporotic 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 ($p > .05$), although hPSCs from osteoporotic donors displayed inherently higher adipogenic differentiation compared to its healthy counterpart (Supporting Information Fig. 1). Notably, the average yield of hPSCs from listed donors did not differ significantly from each other (Supporting Information 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 ($p < .01$) (Supporting Information 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 ($p < .01$). 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 ± 1.97 mm^3 compared to any other groups ($p < .01$) (Fig. 2). However, the samples with regular dose did not exhibit a significant difference (Supporting Information 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 colocated 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.

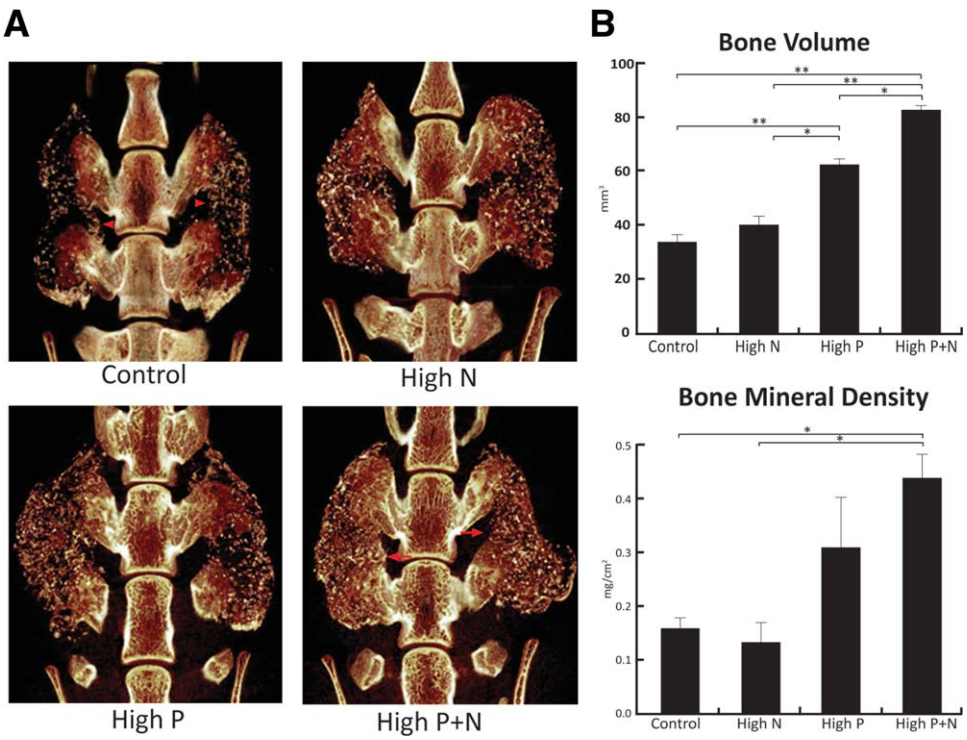

Figure 2. hPSCs+NELL-1 promotes solid bony fusion in osteoporotic rat. **(A):** Representative images of microcomputed tomography scanning of fusion mass with three-dimensional reconstruction from high P+N, high P, high N, and control groups 4 weeks after implantation. The high P+N group had marked bone 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 bone volume 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. *, $p < .05$; **, $p < .01$. Bars ± SD. Abbreviations: N, *Nel*-like protein-1; P, human perivascular stem cells.

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: (a) decreased proliferation and differentiation capacity of endogenous mesenchymal stem cells; (b) diminished formation of vasculature; (c) lower osteoinductive activity; and (d) changes in local and systemic signaling molecules [19].

In agreement with prior studies, we observed that the healing potency of osteoporotioc bone was severely impaired compared to its healthy counterpart (Supporting Information Table 3) [3, 4, 20–22]. Only 20% of fusion was achieved in osteoporotic rats using the same number (0.25×10^6 cells per milliliter, regular dose) of hPSCs that induced 100% fusion in nonosteoporotic 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 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 orthopedic clinical setting. The hPSCs induce bone formation via both direct osteogenic differentiation and indirect trophic effects. They secrete high levels of pro-osteogenic, provasculogenic 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: (a) NELL-1 inhibits BMP-2 induced inflammation by acting as an anti-inflammatory molecule [7]. (b) NELL-1 has antiosteoclastic effects [25]. (c) 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 this 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

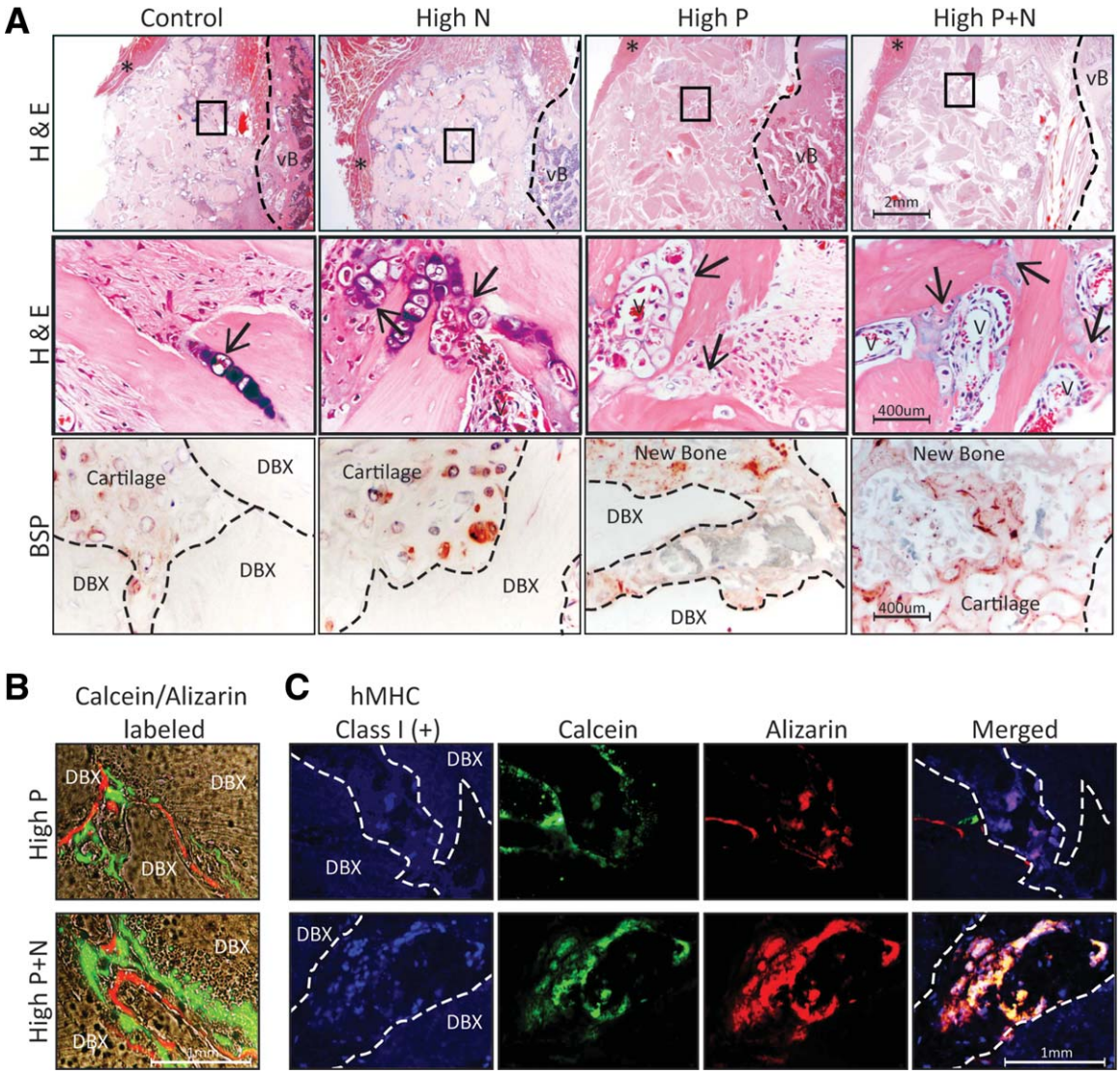

Figure 3. Histologic evidence of new bone formation and direct involvement of hPSCs in active ossification. **(A):** Top panel: H&E staining in low magnification images showing a dash line divides the bone mass formed over the transverse processes of vertebral bones (vB). The asterisk indicates the capsule 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 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 colocalized/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). 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. Abbreviations: BSP, bone sialoprotein; DBX, demineralized bone matrix; H&E, hematoxylin and eosin; hMHC, human major histocompatibility complex; hPSCs, blue by Aminomethylcoumarin streptavidin or pink by merging with green and red; N, *Nel*-like protein-1 (NELL-1); P, human perivascular stem cells (hPSCs).

possibility of developing hPSCs based therapy for osteoporotic patients.

Conclusion

Human adipose tissue derived hPSCs from both nonosteoporotic and osteoporotic conditions exhibited similar osteogenic capacity and responsiveness to osteoinductive factor in vitro. The hPSC combined with NELL-1 synergistically enhances spinal fusion in osteoporotic rats and has great potentialas a novel therapeutic strategy for osteoporotic patients.

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Author Contributions

S.L.: collection and/or assembly of data, data analysis and interpretation, and manuscript writing; X.Z.: conception and design, collection and/or assembly of data, manuscript writing, and administrative support; J.S., A.W.J., C.C., R.H., and C.L.: collection and/or assembly of data, data analysis and interpretation; C.G. and H.W.: data analysis and interpretation; Y.Z., D.S., and B.W.: provision of study material or patients; B.P., K.T., and C.S.: conception and design and final approval of manuscript. S.L. and X.Z. contributed equally to this work.

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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