pISSN 1738-2696 · eISSN 2212-5469
http://dx.doi.org/10.1007/s13770-014-0074-z
ORIGINAL ARTICLE

TERM

Wnt3a-Producing Fibroblasts in Ovariectomy-Induced Osteoporosis in a Rat Model

Je Hoon Jeong[1,2], Eun-Sun Jin[2,3], JoongKee Min[2], Sang Ryong Jeon[2,4], Kyoung Hyo Choi[2,5]*

[1]Department of Neurosurgery, Soonchunhyang University Bucheon Hospital, Soonchunhyang University College of Medicine, Bucheon, Korea
[2]Laboratory of Stem Cell Therapy, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea
[3]Department of Internal Medicine, Kyung Hee University College of Medicine, Seoul, Korea
[4]Department of Neurosurgery, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea
[5]Department of Rehabilitation Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea

We investigated the effect of *in vitro* cultured Wnt3a-producing fibroblasts on positive bone balance and regeneration of the osteopenic skeleton. Thirty-six female Wistar rats (250–300 g, aged 12 weeks) were randomized into three groups: a control group (sham-operated), ovariectomy (OVX) group, and OVX with Wnt-cell injection (OVX with Wnt) group. Wnt3a-producing fibroblasts were injected into the lateral tail vein at 2 and 4 weeks after OVX. Both tibias were removed at 5, 6, 7, and 8 weeks after OVX, and pathological and micro-CT evaluations were performed. We also evaluated β-catenin expression by immunohistochemical staining. For bone metabolism detection, we evaluated the expression of bone alkaline phosphatase (BALP), osteocalcin, and C-telopeptide of collagen type I. Bone mineral density, trabecular bone volume, trabecular number, trabecular thickness were higher, while trabecular seperation was lower, in the OVX with Wnt group than in the OVX group. BALP and osteocalcin levels were significantly higher in the OVX with Wnt group compared to the control and OVX groups. β-catenin expression was significantly lower in the OVX group than in the other two groups. Based on these results, we hypothesized that Wnt3a-producing fibroblasts may be effective for the induction of bone formation.

Tissue Eng Regen Med 2015;12(5):306-313

Key Words: Ovariectomy; Osteoporosis; Animal model; Wnt3a-secreting fibroblast

INTRODUCTION

Osteoporosis is a disease characterized by decreased bone mass, increased bone fragility, and increased risk of fractures [1-4]. There are two basic forms of osteoporosis, based on whether the disease occurs as the primary disorder or secondary to another identifiable medical condition or treatment. Primary osteoporosis is the result of the normal aging process. It is particularly prominent in post-menopausal women and is associated with the decreased estrogen levels in these women. We previously reported that cultured adipose-derived mesenchymal stem cells (MSCs) restore an overall positive bone balance in a rat model of ovariectomy-induced postmenopausal osteoporosis [5]. The Wnt/β-catenin signaling pathway is involved in the increase of positive bone balance resulting from adipose-derived MSCs. However, the physiological mechanisms underlying the evolution of the Wnt/β-catenin signaling pathway are not clear.

Wnt ligands belong to a family of glycoproteins that have diverse and essential roles in development, cell growth, and human diseases [6,7]. Wnt signaling is especially important during the early phases of embryonic nervous system development, playing a pivotal role in neural crest formation, neuronal differentiation, and synaptogenesis [8]. Furthermore, the Wnt/β-catenin signaling pathway is a major physiological pathway involved in the bone healing process; it induces osteoblastic differentiation and maturation of MSCs [9]. We investigated the effect of *in vitro* cultured Wnt3a-producing fibroblasts on positive bone balance and regeneration of the osteopenic skeleton.

Received: August 12, 2014
Revised: April 6, 2015
Accepted: May 12, 2015
***Corresponding author:** Kyoung Hyo Choi, Department of Rehabilitation Medicine, Asan Medical Center, University of Ulsan College of Medicine, 88 Olympic-ro 43-gil, Songpa-gu, Seoul 05505, Korea.
Tel: 82-2-3010-3797, Fax: 82-2-3010-6853, E-mail: khchoi@amc.seoul.kr

MATERIALS AND METHODS

Experimental design

Animal experiments were carried out in accordance with established practices as described in the National Institutes of

Health Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the Asan Institute for Life Sciences, Seoul, Republic of Korea. Thirty-six female Wistar rats (250−300 g, aged 12 weeks) were divided into three equal groups: control group (sham-operated), ovariectomy (OVX) group, and OVX with Wnt cell injection (OVX with Wnt) group. All rats in the OVX and OVX with Wnt groups were ovariectomized bilaterally, and rats in the control group were subjected to a sham surgery. Two million Wnt3a-secreting fibroblasts were injected into the lateral tail vein at 2 and 4 weeks after OVX in rats in the OVX with Wnt group. Body weight was measured once per week for 8 weeks during the experimental period. Rats in all three groups were sacrificed at 5, 6, 7, and 8 weeks, followed by immediate blood sampling and the extraction of the bilateral tibia.

Cell preparation and labeling

All cell lines were provided by the Department of Life Science, The University of Seoul, Seoul, Republic of Korea. Wnt3a-secreting fibroblasts from Mouse L929 (ATCC No. CCL1) cell line were established as previously described [10,11]. In briefly, Wnt cells were fed every 3 days with 10 mL of complete DMEM (Gibco, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Gibco, Invitrogen, Carlsbad, CA, USA). When Wnt3a-secreting fibroblasts reached 80% confluence, cells were harvested using 0.25% Trypsin-EDTA, and washed in 10 mL of phosphate-buffered saline. Then, the cells were used in the next experiment. For the labeling, the Wnt3a-secreting fibroblasts (3×10^5 cells) were seeded onto 6-well plates and allowed to grow for 24 h. Cells were labeled by adding Cell Stalker (Biterials, Korea) at a final concentration of 0.2 mg/mL. After centrifugation, 15 μL of an aqueous solution containing 2×10^6 labeled Wnt3a-secreting fibroblasts was injected into the lateral tail vein at 2 and 4 weeks after OVX in rats in the OVX with Wnt group.

Blood sampling and tibia extraction

Zoletil and Rompun were mixed in a 3:1 ratio, and 200 μL was injected into each rat as intra-abdominal anesthesia. Blood (3 mL) was sampled from each rat by transcardiac puncture and transferred into a serum separator tube (SST tube, BD, Franklin Lakes, NJ, USA). Samples were then centrifuged for 5 min in 1000×g at 4°C. After centrifugation, the supernatant was collected and stored at -200°C. The tibias were removed from the rats, cleaned with normal saline, and wrapped with saline-soaked gauze prior to cryopreservation in -70°C.

Micro-computed tomography measurements

The extracted femur samples were unfrozen, and the proximal metaphysis was scanned by micro-CT (SKYSCAN 1172 high-resolution micro-CT, SKYSCAN, Kontich, Belgium). The micro-CT scans were acquired with a rotation step of 0.4° at a tube voltage of 70 kV, a tube current of 141 μA, and pixel size of 18.81 μm. The image reconstruction and analysis were performed using SKYSCAN software (SKYSCAN N.V., SKYSCAN, Kontich, Belgium). The parameters evaluated included bone mineral density (BMD; g/cm^2), trabecular bone volume (BV/TV; %), trabecular number (Tb.N; /mm), trabecular thickness (Tb.Th; mm) and trabecular separation (Tb.Sp; mm). The volume of interest was the mean value of the maximal horizontal area of the cancellous bone region of the tibial head below the upper-most rudimentary growth plate for two hundred 1.5-mm sliced CT images in the analysis.

Pathological evaluation

Decalcification

After 12 weeks, all extracted and scanned femur specimens were fixed in 10% neutral buffered formalin for up to 7 d and washed in slowly running tap water for at least 30 min. Decalcification was performed with 8% hydrochloric acid stock solution, 8% formic acid solution, working solution (8% hydrochloric acid stock solution, 8% formic acid solution), and ammonia solution. All specimens were saturated with fresh solution every 3 d. After decalcification, the specimens were washed and neutralized in ammonia solution for 30 min. The final washing step was performed using tap water for 24 h.

H&E staining

The decalcified femurs were embedded in paraffin and longitudinally cut into 4-μm sections using a microtome (Leica Microsystems, Leica RM2255, Wetzlar, Germany). Sectioned slides were kept in an oven for 1 h and then deparaffinized in three washes of xylene followed by dehydration in three changes of 100% alcohol, and one change each of 95% alcohol, 80% alcohol, and 70% alcohol, with all incubations lasting 5 min. The slides were then rinsed in running tap water for 1 min. Sections were incubated in hematoxylin (Harris Hematoxylin-I, YD-Diagnostics.com, Yongin, Korea) for 5 min and washed in running tap water for 1 min. Sections were then rehydrated in a series of ethanols (70% to 100%) and stained with eosin (Eosin Y solution, Sigma-Aldrich Co., St. Louis, MO, USA) for 30 s. Excess eosin was washed with running tap water, and sections were mounted (Shandon Synthetic Mountant, Thermo Fisher Scientific, Waltham, MA, USA). The H&E-stained sections were observed by light microscopy.

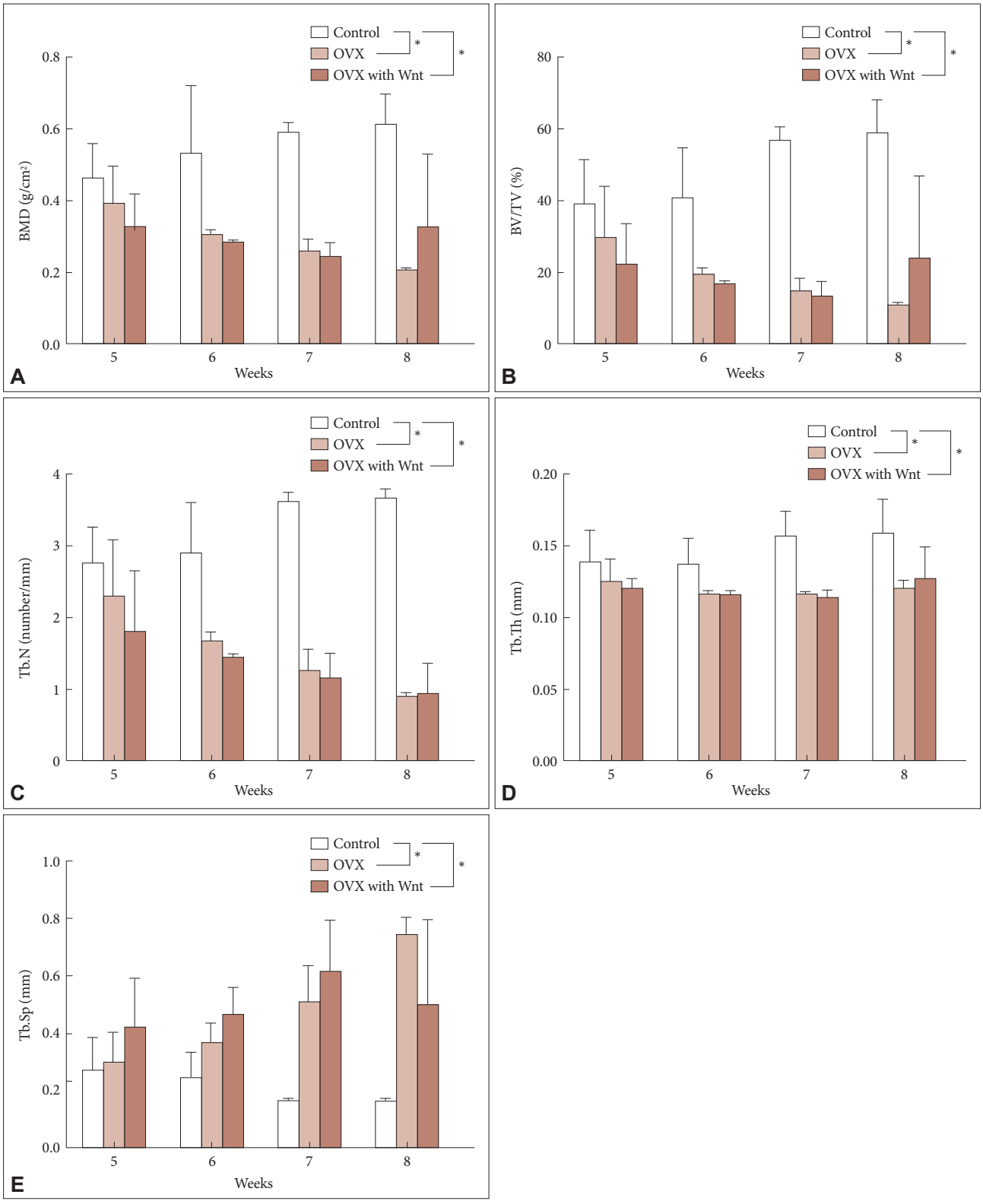

Figure 1. Micro-CT evaluation. The following were higher in the OVX with Wnt group than in the OVX group: (A) bone mineral density (BMD), (B) trabecular bone volume (BV/TV; %), (C) trabecular number (Tb.N), and (D) trabecular thickness (Tb.Th). (E) Trabecular separation (Tb.Sp) of the OVX group was higher than in the normal and OVX with Wnt groups. Normal: sham-operated group, OVX: ovariectomy group, OVX with Wnt: ovariectomy with Wnt cell injection group.

Biochemical assay

All samples were centrifuged at 600×g for 15 min at 4°C. Separated serum samples were analyzed with a rat osteocalcin enzyme-linked immunosorbent assay (ELISA) kit (Rat-MID™ Osteocalcin EIA, Immunodiagnostic System Inc., Boldon, UK), a rat bone-specific alkaline phosphatase (BALP) ELISA kit (Kamiya Biomedical Company, Seattle, WA, USA), and a rat C-telopeptide [Rat C-telopeptide of type I collagen (CTX-1)] ELISA Kit (Cusabio Biotech Co., Ltd., Hubei, P.R. China) according to standard protocols and the manufacturers' instructions.

Immunohistochemistry for β-catenin

For β-catenin immunohistochemistry, paraffin-embedded slides were heated at 60°C for 1 h, deparaffinized with xylene and rehydrated through a series of graded alcohol solutions. For antigen retrieval, the tissue samples were treated with 0.25% Trypsin-EDTA (Gibco, Invitrogen, Carlsbad, CA, USA) and incubated at 37°C for 5 min. We then followed a standard immunohistochemical method for the detection of β-catenin using rabbit monoclonal anti-β-catenin (Cell Signaling, Beverly, MA, USA) at a 1:100 dilution, followed by Dako REAL™ EnVision™ HRP rabbit/mouse secondary antibody (Dako, DK-2600 Glostrup, Denmark). For negative control slides, the primary antibody step was not performed. Antigen-antibody complexes were visualized using the DAB Substrate kit (Dako Liquid DAB+Substrate Chromogen System, K3468, Dako), and slides were counterstained with hematoxylin. β-catenin expression was evaluated by counting.

Statistical analyses

The SAS (version 9.3, SAS Institute Inc., Cary, NC, USA) statistical package was utilized for statistical analyses. Data were represented as means±standard deviations, and a value of $p<0.05$ was considered statistically significant. Two-way ANOVAs adjusting for time effects and Tukey's multiple comparison for post-hoc analysis were used to compare the results obtained for the three groups (the control, OVX, and OVX with Wnt groups).

RESULTS

Micro-CT measurements

All parameters were lower in the OVX group and the OVX with Wnt group than in the control group. BMD, BV/TV, Tb.N, and Tb.Th were higher in the OVX with Wnt group than they were in the OVX group. However, Tb.Sp was higher in the OVX group than in the normal and OVX with Wnt groups. However, only the difference between the control group and the OVX or OVX with Wnt groups was statistically significant (Fig. 1).

Histology

Over the 5–8 weeks after OVX, histologic analyses of stained sections of the proximal tibia showed that the bony trabeculae were thinner and the proportion of adipose tissue in the bone marrow was higher in the OVX and OVX with Wnt groups compared with that of the control. These changes were greater in the OVX group than in the OVX with Wnt group (Fig. 2).

Changes in the serum levels of biochemical markers

BALP levels were higher in the OVX with Wnt group than in the other two groups, with the lowest level observed in the OVX group ($p<0.05$). The osteocalcin levels were increased in the OVX with Wnt group and the OVX group than initial level. Finally, osteocalcin levels were highest in the OVX with Wnt group ($p<0.05$) and lower in the OVX group than in the control ($p<0.05$). CTX expression was highest in the OVX group ($p>0.05$) and lower in the OVX group than the control group ($p<0.05$) (Fig. 3).

Immunohistochemistry for β-catenin

In the negative control samples, as expected, no β-catenin expression was observed in the growth plate or in the cortical bone, and positive β-catenin expression was observed in all three groups. The β-catenin expression was lower in the OVX group than in the control and OVX with Wnt group ($p<0.05$). The OVX with Wnt group had more positive β-catenin expression than the control and OVX group ($p<0.05$) (Fig. 4).

DISCUSSION

Bone is a dynamic tissue, and the bone metabolism is maintained by a balance between the osteoblastic bone formation and osteoclastic bone resorption. Until skeletal maturation, the bone mass steadily increases. However, as people become older, there is gradual loss of bone mass, which results in conditions such as osteoporosis. We previously used the rat model to determine the effect of cultured adipose-derived MSCs on ovariectomy-induced postmenopausal osteoporosis. We verified the potential for adipose tissue derived MSCs to restore a positive bone balance in osteoporotic rats. Adipose-derived MSCs are a prospective source of osteoblastic precursors, and useful for bone regenerative medicine [12]. In this study, histological analysis by H&E staining of the proximal tibia showed a thinning of the bony trabeculae and an increased proportion of adipose tissue in the bone marrow in the OVX and OVX with Wnt groups compared with that of the control. Moreover, these changes were greater in magnitude in the OVX group than in the OVX with Wnt group. We also evaluated bone loss and trabecular architectural damage in rat tibia by *ex-vivo* micro-CT. The BMD, BV/TV, Tb.N, and Tb.Th were higher in the OVX with Wnt group than in the

OVX group. However, Tb.Sp was highest in the OVX group. Regarding biochemical marker changes, BALP and osteocalcin levels were higher in the OVX with Wnt group than in the other two groups. CTX expression was highest in the OVX group. Injected Wnt3a-producing fibroblasts restore overall positive bone balance in our rat model.

Wnt proteins are cysteine-rich glycoproteins secreted from cells. They regulate the expression of many types of genes by binding to cell surface receptors and are involved in various biological processes [7,13,14]. A Wnt gene initially named int-1 was commonly induced by the insertion of mouse mammary tumor virus in many types of tumor [15]. The Nusse group determined that the wingless gene, involved in segment polarity during morphogenesis in *Drosophila*, is homologous to int-1, which is involved in cancer and controls the communication between cells [16]. Since that finding, wingless and int-1 are both known as the Wnt genes [17].

The Wnt/β-catenin signaling pathway is a major physiological pathway involved in the bone healing process that induces osteoblastic differentiation, maturation of MSCs promoting osteoblast

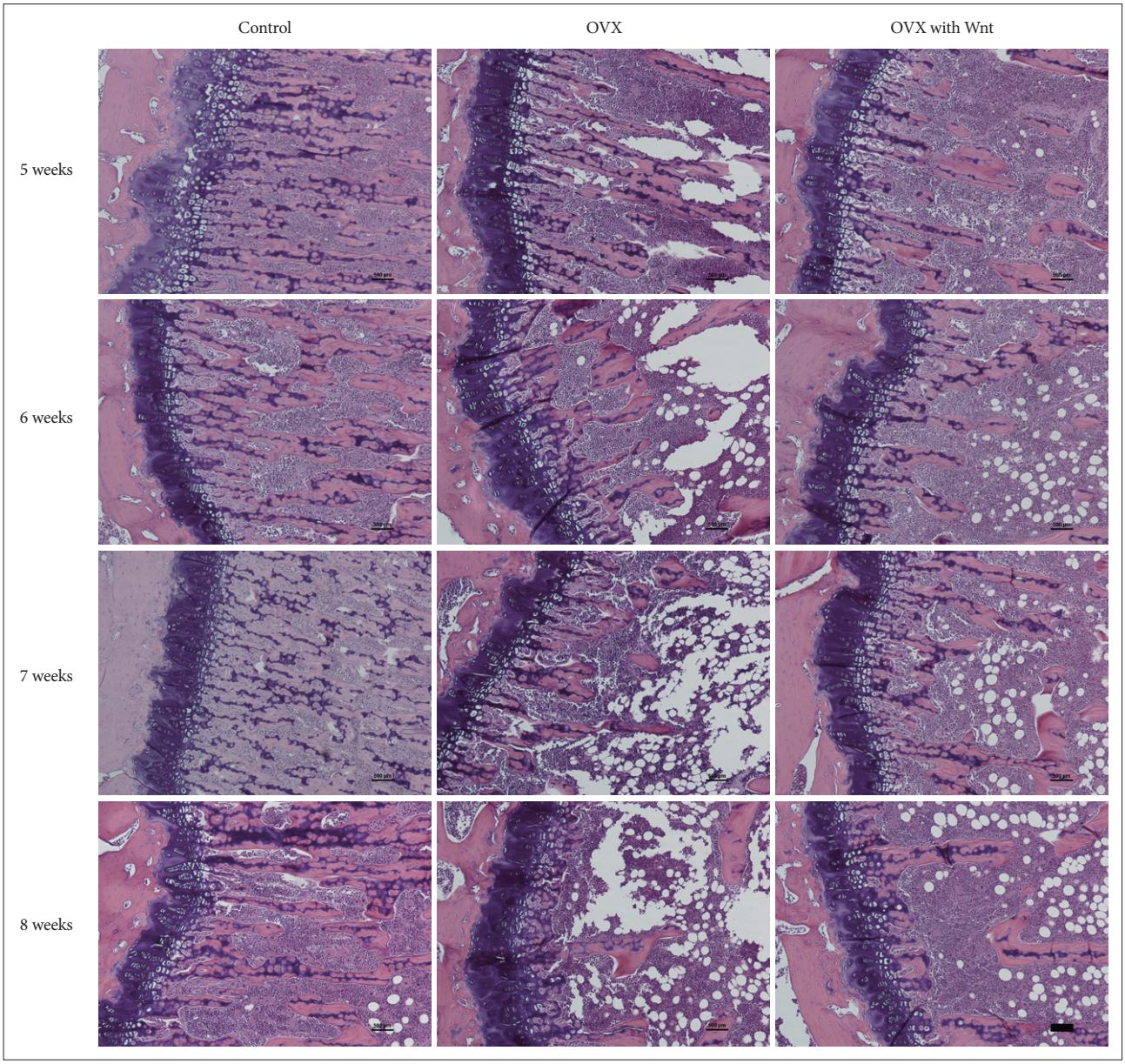

Figure 2. Hematoxylin & eosin staining of the extracted tibia. Photography of the stained sections of the proximal tibia shows thinning of the bony trabeculae and an increased adipose tissue proportion in the bone marrow in the OVX and OVX with Wnt groups compared with the control group. These changes were more prominent in the OVX group than in the OVX with Wnt group. Normal: sham-operated group, OVX: ovariectomy group, OVX with Wnt: ovariectomy with Wnt cell injection group. Scale bar=500 μm (×200).

survival, and interacts with bone morphogenic protein [9]. Intracellular Wnt signal transduction can be divided into two pathways. First, the "canonical signaling pathway" passes through the key effector molecule, β-catenin. Second, the "non-canonical signaling pathway" influences the activity of protein kinase C and adjusts the concentration of intracellular calcium, or regulates the activation of c-Jun NH2-terminal kinase activity via Rac and Rho small G protein. Young autosomal-recessive osteoporosis-pseudoglioma syndrome (OPPG) patients have very low bone mass, and a very high frequency of skeletal fractures. Family trees of these patients were used to determine that Wnt signaling is important to bone formation and the LRP5 gene is associated with OPPG [18,19]. LRP5 is the co-receptor for Wnts in the canonical signaling pathway. Furthermore, LRP5 combined with the Wnts subfamily (Wnt1, Wnt3a, etc.) forms a ternary complex with the Frizzled receptor [20,21]. This complex activates the canonical Wnt signaling pathway by promoting osteoblastogenesis and eventually increases bone formation. In this study, β-catenin expression was lower in the OVX group than in the control and OVX with Wnt groups. The OVX with Wnt group had more positive β-catenin expression than the control and OVX groups. This result are same findings our previous study's finding suggests that the Wnt/β-catenin signaling pathway is involved in the ADMSC-induced increase in osteoblastogenesis to restore the osteoporotic effects of ovariectomy in rats [5]. Therefore, we thought that Wnt3a-producing fibroblasts produce Wnt3a protein, and activate the Wnt/β-catenin signaling pathway. Finally, increase the osteoblastogenesis.

Our study has some limitations. First, the *ex-vivo* micro-CT analysis did not produce continuous data from individual subjects. Second, intravenously administered stem cells preferentially engraft into bone marrow tissue and bone. This is consis-

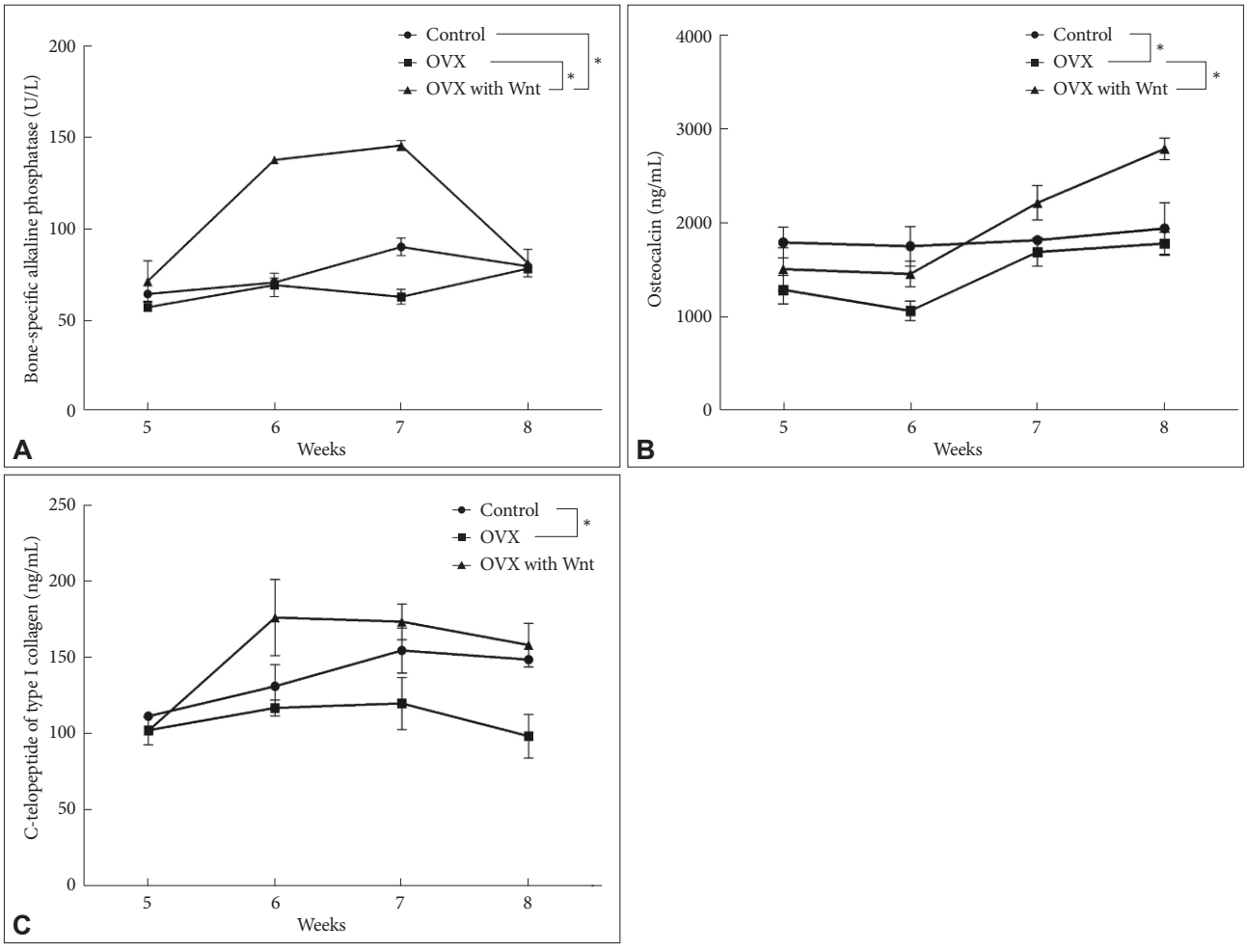

Figure 3. Changes in the levels of serum markers. (A) BALP levels were higher in the OVX group than in the other two groups, with the lowest level observed in the control group. (B) Osteocalcin levels were highest in the OVX with Wnt group and lowest in the OVX group. (C) CTX expression was highest in the OVX with Wnt group and lowest in the OVX group. Normal: sham-operated group, OVX: ovariectomy group, OVX with Wnt: ovariectomy with Wnt cell injection group. BALP: bone alkaline phosphatase.

tent with other previous work suggesting the homing potential of stem cells. In addition, using an intravenous transplantation technique, another study showed that cells are immediately distributed over the entire body, instead of directly engrafting into bone. We used fibroblast (not stem) cells. We attempted to determine the cellular distribution of PKH26 (red fluorescence dye) labeled Wnt3a-producing fibroblasts by cell trafficking methods such as OPTIX and IVIS. Unfortunately, we could not definitely detect the migration of the Wnt3a-producing fibroblasts to the bone tissue. Third, we only evaluated the immunohistochemical

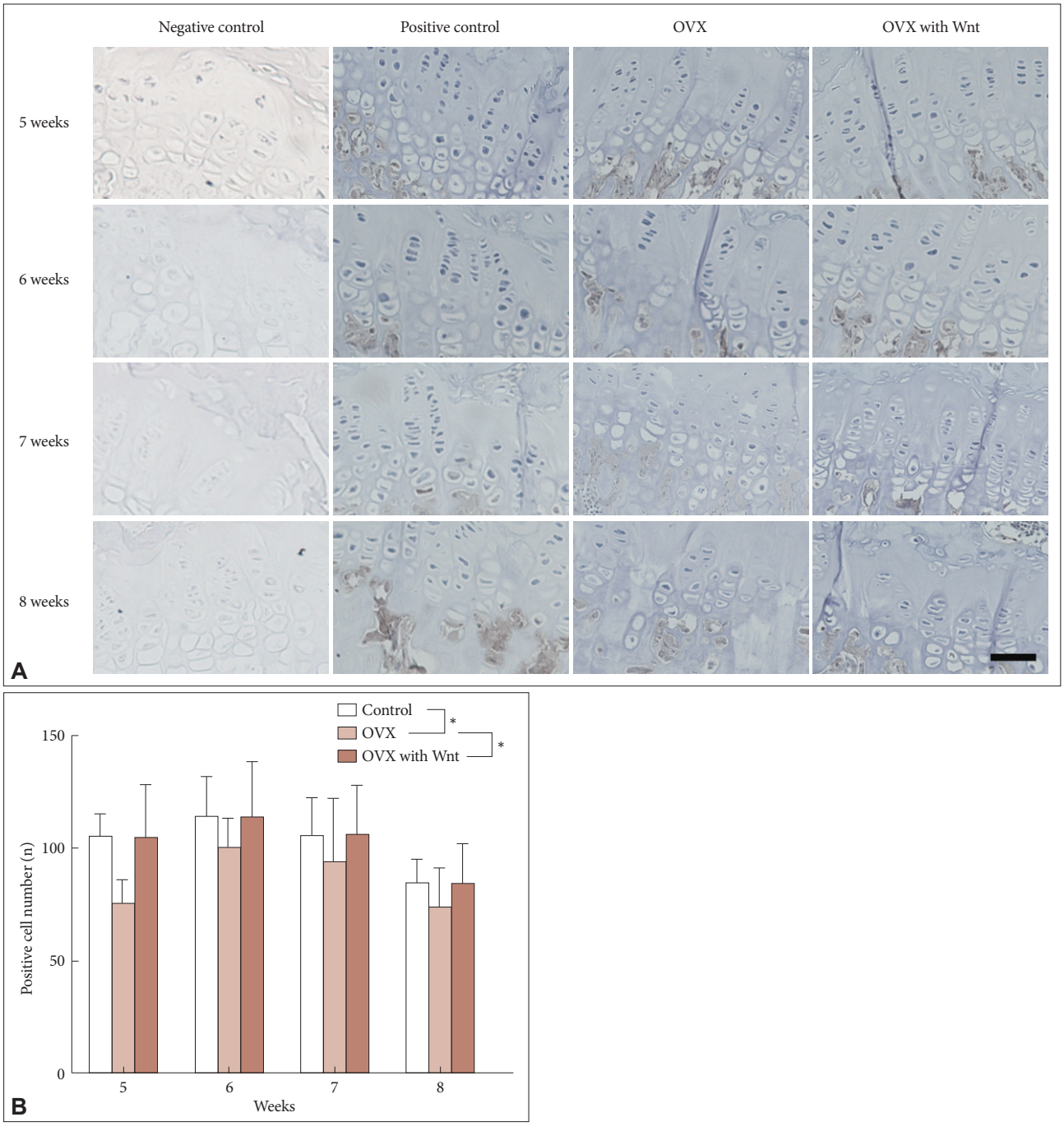

Figure 4. Immunohistochemistry for β-catenin. (A) Positive β-catenin expression was observed in all three groups. (B) The β-catenin expression was lower in the OVX group than in the control and OVX with Wnt group. The OVX with Wnt group had more positive β-catenin expression than that in the control and OVX group. Normal: sham-operated group, OVX: ovariectomy group, OVX with Wnt: ovariectomy with Wnt cell injection group. Scale bar=500 μm (×200).

staining of the bone and could not evaluate the precise intracellular physiologic mechanism of the Wnt/β-catenin signaling pathway. Therefore, further studies is needed. In conclusion, we demonstrated that transplantation of Wnt3a-secreting fibroblasts significantly improved bone quality of the ovariectomized osteoporosis rat model.

Acknowledgements

This work supported by a grant from the Han-kok Medical Board (2012) and this study was supported by a grant (2012-303) from the Asan Institute for Life Sciences, Seoul, Korea.

Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

Animal experiments were carried out in accordance with established practices as described in the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the Asan Institute for Life Sciences.

REFERENCES

1. Khosla S, Riggs BL. Pathophysiology of age-related bone loss and osteoporosis. Endocrinol Metab Clin North Am 2005;34:1015-1030, xi.
2. Voskaridou E, Terpos E. New insights into the pathophysiology and management of osteoporosis in patients with beta thalassaemia. Br J Haematol 2004;127:127-139.
3. Christenson RH. Biochemical markers of bone metabolism: an overview. Clin Biochem 1997;30:573-593.
4. Kanis JA. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis: synopsis of a WHO report. WHO Study Group. Osteoporos Int 1994;4:368-381.
5. Jeong JH, Park JR, Jin ES, Min JK, Jeon SR, Kim DK, et al. Adipose tissue-derived stem cells in the ovariectomy-induced postmenopausal osteoporosis rat model. Tissue Eng Regen Med 2015;12:28-36.
6. Huelsken J, Behrens J. The Wnt signalling pathway. J Cell Sci 2002;115(Pt 21):3977-3978.
7. Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 2004;20:781-810.
8. Toledo EM, Colombres M, Inestrosa NC. Wnt signaling in neuroprotection and stem cell differentiation. Prog Neurobiol 2008;86:281-296.
9. Im GI. Intracellular signal transduction pathways and transcription factors for osteogenesis. J Korean Rheum Assoc 2008;15:1-10.
10. Im J, Kim H, Kim S, Jho EH. Wnt/beta-catenin signaling regulates expression of PRDC, an antagonist of the BMP-4 signaling pathway. Biochem Biophys Res Commun 2007;354:296-301.
11. Yun S, Rim Y, Jho EH. Induced expression of the transcription of tropomodulin 1 by Wnt5a and characterization of the tropomodulin 1 promoter. Biochem Biophys Res Commun 2007;363:727-732.
12. Cho SW, Sun HJ, Yang JY, Jung JY, Choi HJ, An JH, et al. Human adipose tissue-derived stromal cell therapy prevents bone loss in ovariectomized nude mouse. Tissue Eng Part A 2012;18:1067-1078.
13. Moon RT, Kohn AD, De Ferrari GV, Kaykas A. WNT and beta-catenin signalling: diseases and therapies. Nat Rev Genet 2004;5:691-701.
14. Reya T, Clevers H. Wnt signalling in stem cells and cancer. Nature 2005;434:843-850.
15. Nusse R, Varmus HE. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell 1982;31:99-109.
16. Rijsewijk F, Schuermann M, Wagenaar E, Parren P, Weigel D, Nusse R. The Drosophila homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless. Cell 1987;50:649-657.
17. Nusse R, Brown A, Papkoff J, Scambler P, Shackleford G, McMahon A, et al. A new nomenclature for int-1 and related genes: the Wnt gene family. Cell 1991;64:231.
18. Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, et al. LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. Cell 2001;107:513-523.
19. Gong Y, Vikkula M, Boon L, Liu J, Beighton P, Ramesar R, et al. Osteoporosis-pseudoglioma syndrome, a disorder affecting skeletal strength and vision, is assigned to chromosome region 11q12-13. Am J Hum Genet 1996;59:146-151.
20. Pinson KI, Brennan J, Monkley S, Avery BJ, Skarnes WC. An LDL-receptor-related protein mediates Wnt signalling in mice. Nature 2000;407:535-538.
21. Tamai K, Semenov M, Kato Y, Spokony R, Liu C, Katsuyama Y, et al. LDL-receptor-related proteins in Wnt signal transduction. Nature 2000;407:530-535.