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A comparison of tissue engineering based repair of calvarial defects using adipose stem cells from normal and osteoporotic rats

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Abstract

Repairing large bone defects presents a significant challenge, especially in those people who have a limited regenerative capacity such as in osteoporotic (OP) patients. The aim of this study was to compare adipose stem cells (ASCs) from both normal (NORM) and ovariectomized (OVX) rats in osteogenic potential using both *in vitro* and *in vivo* models. After successful establishment of a rat OP model, we found that ASCs from OVX rats exhibited a comparable proliferation capacity to those from NORM rats but had significantly higher adipogenic and relatively lower osteogenic potential. Thirty-two weeks post-implantation with poly (lactic-co-glycolic acid) (PLGA) alone or PLGA seeded with osteogenic-induced ASCs for critical-size calvarial defects, the data from Herovici's collagen staining and micro-computed tomography suggested that the implantation of ASC-PLGA constructs exhibited a higher bone volume density compared to the PLGA alone group, especially in the NORM rat group. Intriguingly, the defects from OVX rats exhibited a higher bone volume density compared to NORM rats, especially for implantation of the PLGA alone group. Our results indicated that ASC based tissue constructs are more beneficial for the repair of calvarial defects in NORM rats while implantation of PLGA scaffold contributed to defect regeneration in OVX rats.

Keywords

adipose stem cells; osteoporosis; calvarial defect; osteogenesis; poly (lactic-co-glycolic acid)

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Competing Interest Statement

The authors do not have any conflicts of interest.

1. Introduction

Large bone defects often occur as a result of trauma, tumor resection, congenital malformation, or degenerative diseases. Bone tissue has a comparatively high regenerative capability, but it often fails when the defects are large or when the natural healing capabilities are insufficient or compromised [1]. Spontaneous calvarial reossification only occurs in infants younger than two years of age and adults lose the capacity to heal large calvarial defects [2,3]; thus massive calvarial bony defects remain a challenge for surgeons [4], especially in patients with limited regenerative capability, such as patients with osteoporosis (OP). Osteoporosis is the most common bone disease characterized by reduced bone mineral density (BMD), microarchitectural deterioration of the skeleton, and increased risk of fracture [5]. Approximately 44 million people in the United States are affected by OP and low bone mass [6]. As the population ages, this problem is expected to intensify, exacting a significant medical and financial toll on the United States each year. Other than age, female gender is the most significant risk factor; in fact, 80% of patients with OP are women. Estrogen deficiency increases the rate of bone remodeling and leads to an imbalance between bone resorption and formation, resulting in a net loss and OP.

Bone tissue engineering has become a promising approach for bone defect repair. Research on seed cell source is an important field in bone tissue engineering. Using embryonic stem cells as seed cells is compromised because of ethical controversy. Therefore, focus has shifted to adult stem cells. Bone marrow-derived stem cells (BMSCs) were considered the best candidate for bone repair and have been extensively studied [7]. However, practical issues including low stem cell yield per harvest, the need for expansion, and the morbidity imposed on the donor are hurdles for the application of BMSCs. A recent report found that the efficiency of autologous mesenchymal stem cell (MSC) transplantation from adipose, periosteum, and bone marrow following *ex vivo* cell expansion was not significantly different for the guided regeneration of porcine calvarial bone defects [8]. Chen and colleagues found that proliferation and osteogenic potentials of adipose stem cells (ASCs) were less affected by age and multiple passaging than BMSCs in humans [9]. Furthermore, the telomere length, telomerase activity, and osteogenic differentiation were maintained in OP derived ASCs but not BMSCs [10,11]. ASCs offer several advantages over other multipotent cells (such as BMSCs) for tissue engineering purposes. For example, ASCs are easier to obtain, have a relatively low donor site morbidity and a higher yield at harvest, and can be expanded more rapidly *in vitro* [12–14]. Moreover, the efficacy of using ASCs was recently reported for the healing of critical-size defects in a calvarial model [15–17].

Though ASC-based tissue engineering offers a promising strategy for successful repair of calvarial defects in normal (NORM) animals, there are no studies showing whether ASCs play a similar role in treating OP bone defects. To date, few biomaterials have been investigated in the repair of OP calvarial defects. For instance, Lin and coworkers found enhanced OP bone regeneration by strontium-substituted calcium silicate bioactive ceramics [18]; another report from Durão and colleagues showed that the implantation of a biocompatible Bovine-Bone Mineral (BBM) graft could heal critical-size calvarial defects in ovariectomized (OVX) rats [19]. However, it was unknown whether ASCs could contribute

to the repair of critical-size OP calvarial defects. In this study, we hypothesized that ASCs from OVX rats have a comparable osteogenic differentiation capacity to those from NORM rats. We compared ASCs from NORM and OVX rats in both proliferation and osteogenic and adipogenic capacity. We further attempted to determine whether osteogenic tissue constructs from ASCs and poly(lactic-co-glycolic acid) (PLGA) mesh could yield comparable regeneration after implantation for the repair of critical-size calvarial defects in an OVX rat model.

2. Materials and Methods

2.1. Establishment of rat OP model

Female Sprague Dawley (SD) rats were obtained from Hilltop Lab Animals, Inc. (Scottsdale, PA) and housed in the Research Animal Facility. This project was approved by the Institutional Animal Care and Use Committee (IACUC) and conducted in compliance with National Advisory Committee for Laboratory Animal Research Guidelines. The facility, accessed by authorized personnel only, is temperature, ventilation, and illumination controlled. The animals had access to feed (Teklad Global 18% protein rodent diet) and water *ad libitum*. Before transportation, six-month-old female rats with similar body weights underwent either bilateral ovariectomy (n=5, OVX rats) or a sham operation (n=5, NORM rats) (<http://hilltoplabs.com/public/ovx.html>). Four months later, rat weight was collected and dual-energy X-ray absorptiometry (DEXA, Norland Corporation, Fort Atkinson, WI) was used to measure the percent fat in the spine region and spine BMD to confirm the OP model.

2.2. Cell culture

Adipose stem cells were isolated from minced inguinal fat pads of either OVX rats or NORM rats *via* 0.075% type I collagenase digestion at 37°C for 60 min. The digested fat tissue was centrifuged at 1200 g for 10 min to obtain a high-density stromal vascular fraction (SVF). The SVF collection was treated with red blood cell lysing buffer (0.3 g/L ammonium chloride in 0.01 M Tris-HCl buffer, pH 7.5) for 5 min, centrifuged at 600 g for 10 min, and filtered through a 100- μ m nylon mesh to remove undigested tissue. Cells were resuspended in Growth Medium [Minimum Essential Medium – Alpha Modification (α -MEM) containing 10% fetal bovine serum (FBS), 100 mg/mL streptomycin, and 100 U/mL penicillin], and plated at 40,000 cells/cm² in T75 culture flasks with the medium changed twice a week. When 80–90% confluence was reached, the cells were sub-cultured.

2.3. In vitro proliferation capacity of ASCs

Passage 2 ASCs from either OVX rats or NORM rats were seeded at 3000 cells/cm² and evaluated for proliferation capacity using the measurement of DNA content and proliferation index. DNA content was measured every two days from day 0 to day 8. Briefly, the collected cells from T75 flasks (n=4) were digested at 60°C for 4 h with 125 μ g/mL papain in PBE buffer (100 mM phosphate, 10 mM ethylenediaminetetraacetic acid, pH 6.5) containing 10 mM cysteine. To quantify cell density, the amount of DNA in the papain digestion was measured using the Quant-iTTMPicoGreen[®] dsDNA assay kit (Invitrogen, Carlsbad, CA) with a CytoFluor[®] Series 4000 (Applied Biosystems, Foster City, CA). To

measure proliferation index, before cell expansion, passage 2 ASCs were labeled with CellVue® Claret (Sigma-Aldrich, St. Louis, MO) at 2×10^{-6} M for 5 min according to the manufacturer's protocol. Expanded cells at day 4 and day 8 were measured using a BD FACS Calibur™ flow cytometer (dual laser) (BD Biosciences, San Jose, CA). Twenty thousand events of each sample were collected using CellQuest Pro software (BD Biosciences) and cell proliferation index was analyzed by ModFit LT™ version 3.1 (Verity Software House, Topsham, ME).

2.4. In vitro osteogenic and adipogenic capacity of ASCs

For osteogenic induction, ASCs (n=4) cultured for 21 days in osteogenic medium (Growth Medium supplemented with 0.1 μ M dexamethasone, 10 mM β -glycerol phosphate, 50 μ M ascorbate-2-phosphate, and 0.01 μ M 1,25-dihydroxyvitamin D3) were collected for alkaline phosphatase (ALP) activity assay with a reagent kit by measuring the formation of p-nitrophenol (PNP) from p-nitrophenyl phosphate (PNPP) following the manufacturer's instructions (Sigma-Aldrich). PNP was quantified based on the spectrophotometric absorbance at 405 nm and enzymatic activity was expressed as millimoles of PNP/min/ μ g DNA. For evaluation of calcium deposition, induced cells (n=4) were fixed with 70% ice-cold ethanol for 1 h and then incubated in 40 mM Alizarin Red S (ARS) at pH 4.2 for 20 min with agitation. After two intensive rinses with deionized water, matrix mineral-bound staining was photographed under a Nikon TE300 phase-contrast microscope (Nikon, Tokyo, Japan). TaqMan® real-time polymerase chain reaction (PCR) was used to measure osteogenic marker genes runt-related transcription factor 2 (*RUNX2*), secreted phosphoprotein 1 (*SPP1*), and bone gamma-carboxyglutamic acid-containing protein (*BGLAP*).

For adipogenic induction, ASCs (n=4) were cultured for 21 days in adipogenic medium (Growth Medium supplemented with 1 μ M dexamethasone, 0.5 mM isobutyl-1-methylxanthine, 200 μ M indomethacin, and 10 μ M insulin). The cultures (n=4) were fixed in 4% paraformaldehyde and stained with a 0.6% (w/v) Oil Red O (ORO) solution (60% isopropanol, 40% water) for 15 min. Intracellular lipid-filled droplet-bound staining was photographed under a Nikon TE300 phase-contrast microscope. Quantification of staining was performed by incubating cells with 1 mL of isopropyl alcohol for 5 min to extract the intracellular lipid-filled droplet-bound ORO stain. A two hundred microliter extract was placed in a transparent 96-well culture plate and the absorbance of the extract was measured using a spectrophotometer at 510 nm. Absorption values were normalized to total DNA amount for standardization. TaqMan® real-time PCR was used to measure adipogenic marker genes CCAAT-enhancer-binding protein beta (*CEBP*), peroxisome proliferator-activated receptor gamma (*PPAR*), and lipoprotein lipase (*LPL*).

2.5. TaqMan® real-time PCR

Total RNA was extracted from either osteogenically or adipogenically induced ASCs (n=4) using an RNase-free pestle in TRIzol® (Invitrogen). Two μ g of mRNA was used for reverse transcription with the High-Capacity cDNA Archive Kit (Applied Biosystems) at 37°C for 120 min. Adipogenic marker genes [*CEBP* (Assay ID Rn00824635_s1), *PPAR* (Assay ID Rn00440945_m1), and *LPL* (Assay ID Rn00561482_m1)] and osteogenic marker genes

[*RUNX2* (Assay ID Rn01512298_m1), *SPP1* (Assay ID Rn01449972_m1), and *BGLAP* (Assay ID Rn00566386_g1)] were customized by Applied Biosystems as part of their Custom TaqMan® Gene Expression Assays. Eukaryotic 18S rRNA (Assay ID HS99999901_s1) was carried out as the endogenous control gene. Real-time PCR was performed with the iCycler iQ™ Multi Color RT-PCR Detection and calculated by computer software (PerkinElmer, Wellesley, MA). Relative transcript levels were calculated as $X = 2^{-Ct}$, in which $Ct = E - C$, $E = Ct_{exp} - Ct_{18s}$, and $C = Ct_{ct1} - Ct_{18s}$.

2.6. Preparation and characterization of cell-scaffold constructs

Three-dimensional disc-shaped (5 mm diameter by 2 mm thickness) PLGA was obtained from Synthecon Inc. (Houston, TX). After sterilization with ethylene oxide, PLGA discs were immersed in 100% ethanol, 70% ethanol, and phosphate buffered saline (PBS, without Ca^{2+} and Mg^{2+}). Two million cells were resuspended in 30 μ L of Growth Medium and placed directly onto the scaffold for 3 h (30 μ L of medium without cells was used for PLGA alone as a control) [20]. The cell-scaffold constructs or scaffolds alone were subsequently submerged in 100 μ L of Growth Medium for 24 h. Before implantation, cell-seeded scaffolds were induced in osteogenic medium for 14 days; medium was changed every 3 days. At days 3 and 14 post-seeding, cell morphology and extracellular matrix were evaluated by scanning electron microscopy (SEM) as described previously [21]. Briefly, representative samples ($n=2$) were primarily fixed in 2.5% glutaraldehyde (Sigma-Aldrich) for 2 h, followed by secondary fixation in 2% osmium tetroxide (Sigma-Aldrich) for another 2 h. The samples were then dehydrated in a gradient ethanol series, in hexamethyldisilazane (HMDS, Sigma-Aldrich) at a ratio of 1:1 with ethanol twice for 1 h each time, in HMDS at a ratio of 1:2 with ethanol overnight, and in HMDS three times for 4 h each time. The samples were air-dried for 24 h and gold sputter was added. The images were recorded by an SEM (Hitachi, Model S 2400, San Jose, CA).

2.7. Creation of calvarial bone defects

For *in vivo* experiments (Table 1), ten-month-old female SD rats (Hilltop Lab Animals, Inc.) were used in this study. At the end of the study period, 13 OVX and 17 NORM rats survived for data analysis. The rats were anesthetized with intra-peritoneal injection of ketamine (90 mg/kg, Phoenix, Bioniche Teoranta, Inverin, Ireland) and xylazine (10 mg/kg, Akorn, Inc., Decatur, IL). Prior to making the incision, bupivacaine (0.25%, 1–2 mg/kg dosage) was used for local pain relief. A 1 cm midline incision was made between the animal's ears and dissection was taken down to the calvarium. The periosteum was removed at least 3 mm away from the area where the defects were created. The defects were made on the lateral ridge about 5 mm below the ear on either side of the suture line. A round fast cutting stainless steel burr (diameter 5 mm, Stryker Instruments, Kalamazoo, MI) in a 3M Mini Driver (Henry Schein, Melville, NY) was used in combination with manual use of drill bits until full thickness defects were made in the calvaria. The defects were irrigated with 0.9% sodium chloride before the constructs or scaffolds were inserted. Following surgery, Buprenorphine SR (1 mg/ml, 1.2 mg/kg dosage) (ZooPharm, Windsor, CO) was used for systemic relief and provided 72 h of analgesia. The skin incision was closed with a running subcuticular 4-0 Vicryl suture (Ethicon, Johnson & Johnson, Somerville, NJ). Tissue adhesive (3M Vetbond, 3M Animal Care Products, St. Paul, MN) was applied.

The design for both OVX rats and NORM rats included three groups: “Construct”, “Scaffold”, and “Empty”. In the Construct group, defects were filled with ASC/PLGA constructs; the side receiving the construct was chosen randomly. In the Scaffold group, defects were filled with PLGA alone, also randomly chosen. In the Empty group, defects were left untreated. The animals were allowed to recover on a heating pad for a period up to 6 h before being returned to the vivarium. Thereafter, animals were observed once daily for three days and weekly thereafter to ensure post-operative recovery. The rats were euthanized at 6 weeks (n=10 rats) and 32 weeks (n=20 rats) post-implantation. For euthanasia, rats were anesthetized with intra-peritoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg) followed by 1 mL of Euthasol (Virbac AH, Inc., Ft. Worth, TX) injected by intra-cardiac puncture.

2.8. Bone mineral density and micro-computed tomography analysis

Six weeks post-operatively, DEXA was used to measure the BMD of the skull defect sites and surrounding area with the ventral side of the skull removed (n=6 for NORM rats and n=4 for OVX rats). Thirty-two weeks post-operatively, bone regeneration within the calvarial bone defects (n=11 for NORM rats and n=9 for OVX rats) was evaluated using micro-computed tomography (μ CT, viva CT 40, Scanco Medical, Scanco USA Inc., Wayne, PA) with the following settings: energy 70 kVp, intensity 114 μ A, integration time 200 ms, isotropic voxel size 35 μ m, and threshold 163. After obtaining two-dimensional image slices of the skulls, the view of interest (VOI) was uniformly delineated. Three-dimensional reconstructions were created using an appropriate threshold that was kept constant throughout the analyses. The newly regenerated bone volume was measured using evaluation software provided by the manufacturer. The bone morphometry including volume (mm^3) and density [mg hydroxyapatite (HA)/ cm^3] measurements regarding the nomenclature, symbols, and units followed the guidelines set by the American Society of Bone and Mineral Research [22]. The bone defect area was measured with Image J software (<http://rsbweb.nih.gov/ij/>) and the percentage of bone healing was determined by dividing the area covered by the newly regenerated bone 32 weeks post-implantation by the original defect area measured on day 1.

2.9. Histology staining

The calvarial samples were fixed in a 10% formalin solution for 48 h and decalcified in a solution of 10% ethylenediaminetetraacetic acid plus 1% sodium hydroxide solution. Radiographs were used to evaluate the progress of decalcification. After decalcification, the samples were dehydrated in graded ethanol solutions and embedded in paraffin. Tissue sections (cut along the coronal plane at the midline of the defect) of 5- μ m-thickness were stained using Herovici's Collagen Staining kit following the manufacturer's instructions (American MasterTech, Lodi, CA).

2.10. Statistical analysis

A two-factor ANOVA with contrast analysis and two-sample t-test were used for comparison in the assessment of the OP model (n=5), DNA amount (n=4), real-time PCR (n=4), and μ CT analysis (n=7 for the OVX rat group and n=8 for the NORM rat group). For

in vivo data analysis using μ CT, a minimum sample size of 7 per group is needed to achieve 80% power to detect a 25% difference between two groups at a two-sided 5% significance level, assuming the coefficient of variance is 0.15. Statistical analyses were performed with SPSS 13.0 statistical software (SPSS Inc., Chicago, IL) and R statistical software (Foundation for Statistical Computing, Vienna, Austria). All data are expressed as means $\pm$ standard deviation of the mean (SD). *p* values less than 0.05 were considered statistically significant.

3. Results

3.1. ASCs derived from OVX rats exhibited comparable proliferative potentials with ASCs from NORM rats

Four months after undergoing bilateral ovariectomy, the OP model was confirmed by comparing OVX rats with NORM rats (sham control) in rat weight (440.3 ± 25.5 g *versus* 321.1 ± 45.4 g, $p=0.002$) (Fig. 1A), % Fat (24.9 ± 4.3 *versus* 13.4 ± 2.8 , $p=0.010$) (Fig. 1B), and BMD data (0.22 ± 0.01 *versus* 0.26 ± 0.02 , $p=0.024$) (Fig. 1C). Passage 2 ASCs collected from both NORM and OVX rats were expanded at 3,000 cells per cm^2 for one passage. DNA content was measured every two days from day 0 to day 8 for both groups; though ASCs from OVX rats had lower DNA content at day 8, the difference between groups was not significant (Fig. 2A). Similarly, proliferation index was analyzed by flow cytometry at day 4 and day 8 after expansion; ASCs from both groups (NORM *versus* OVX) exhibited a similar proliferation index (8.96 *versus* 10.55 at day 4; 34.77 *versus* 32.17 at day 8) (Fig. 2B).

3.2. ASCs from OVX rats exhibited a potential decrease in osteogenic potential but an increased adipogenic capacity

After a 21-day osteogenic induction, a non-detectable difference in the staining of ALP (Fig. 3A) or ARS (Fig. 3B) was observed in ASCs isolated from NORM and OVX rats. Quantitative data showed that ALP activity (Fig. 3C) and *BGLAP* mRNA (Fig. 3D) in ASCs from OVX rats were significantly lower than those from NORM rats. Intriguingly, despite no difference at early time points (day 7 and day 14), *RUNX2* mRNA at day 21 in the OVX rats was higher than that from NORM rats (Fig. 3D). Similarly, *SPPI* mRNA at both day 14 and day 21 was also superior in the OVX group (Fig. 3D).

After a 21-day adipogenic induction, ASCs from OVX rats exhibited a high intensity of Oil Red O (ORO) staining compared to that from NORM rats (Fig. 4A). Quantitative data on the amount of ORO staining (Fig. 4B) and mRNA levels of *PPAR*, *CEBP*, and *LPL* (Fig. 4C) were significantly higher in ASCs from OVX rats than those from NORM rats.

3.3. ASC-PLGA construct morphology and 6-week bone formation after implantation

Our SEM data (Fig. 5) showed that cells were evenly distributed throughout the 3-day constructs (**A**: low magnification; **B**: high magnification). Since more matrices could be deposited during *in vitro* osteogenic induction, 14-day constructs (**C**: low magnification; **D**: high magnification) were chosen as implants for the *in vivo* repair study. After successful creation of bilateral critical-size 5 mm full thickness defects, the defects were either left

empty as a control (Fig. 6A) or filled with 14-day ASC/PLGA constructs or PLGA alone (Fig. 6B). Six weeks post-implantation, our DEXA data showed that the BMD ratio of repair tissue (R1 or R2) to surrounding tissue (R3–R7) (Fig. 6C) was 43.88 ± 6.23 versus 39.75 ± 8.17 ($p=0.348$, ASC/PLGA versus PLGA) for NORM rats (Fig. 6D) and 63.28 ± 12.22 versus 59.47 ± 9.05 ($p=0.634$, ASC/PLGA versus PLGA) for OVX rats (Fig. 6E). Interestingly, the repair tissue from OVX rats exhibited a higher BMD ratio than that from NORM rats in the ASC/PLGA implantation group ($p=0.010$) and the PLGA alone group ($p=0.007$).

3.4. Evaluation of 32-week bone formation after implantation

For the 32-week repaired tissue, Herovici's collagen staining and μ CT data showed that the ASC/PLGA implantation group exhibited more regenerated tissue (blue color indicating new collagen and red color indicating old collagen) than the PLGA alone group; this finding applied to both the NORM and OVX groups with the empty control group having the least regenerated tissue (Fig. 7). The above morphology data were confirmed by the quantitative data measured by μ CT and Image J software (Fig. 8), in which the ratio of bone volume (BV) to total volume (TV) was higher in the ASC/PLGA group than that in the PLGA group for NORM rats (0.205 ± 0.061 versus 0.124 ± 0.052 , $p=0.013$) despite no significant difference for OVX rats (0.265 ± 0.078 versus 0.246 ± 0.076 , $p=0.640$). The implantation of ASC/PLGA constructs acquired a high ratio of BV to TV (but no significant difference) in the OVX rats compared to that in the NORM rats (0.265 ± 0.078 versus 0.205 ± 0.061 , $p=0.116$) while the implantation of the PLGA construct alone resulted in a significantly higher ratio of BV to TV in the OVX rats than that in the NORM rats (0.246 ± 0.076 versus 0.124 ± 0.052 , $p=0.003$).

4. Discussion

By utilizing the OVX rat model of OP with estrogen deficiency, in this study, we found that ASCs from both NORM and OVX rats exhibited comparable *in vitro* proliferation potentials. Not surprisingly, *in vitro* adipogenic potential was higher in OVX rats than in NORM rats. During osteogenic induction, ASCs from OVX rats exhibited a delayed osteogenic capacity with a peak at day 14. Implantation of osteogenically induced ASC/PLGA constructs in NORM rats yielded a significantly higher bone volume density for the repair of calvarial defects compared to the use of PLGA alone. However, this statistical difference did not exist in OVX rats though implantation of osteogenically induced ASC/PLGA constructs was better than the PLGA alone group. Interestingly, implantation of PLGA alone yielded a higher bone volume density for the repair of calvarial defects in OVX rats compared to that in NORM rats while implantation of osteogenically induced ASC/PLGA constructs yielded a high bone volume density but no significant difference in OVX rats compared to NORM rats.

Due to the OVX model, we were able to compare *in vitro* and *in vivo* osteogenic and adipogenic capacities of ASCs from both NORM and OVX rats. The success of establishing an OVX model was evidenced by a significant decrease of spine BMD accompanied by significant increases in both % fat in spine region and rat weight. These *in vivo* data were

consistent with *in vitro* evidence, in which ASCs from OVX rats exhibited a significant increase in all adipogenic marker genes, similar to the performance of BMSCs from OP patients [23], while resulting in a significant decrease in both *BGLAP* and ALP activity. Intriguingly, ASCs from OVX rats displayed higher levels of *RUNX2* and *SPP1* after a 21-day osteogenic induction compared to those from NORM rats. The above findings are in line with previous reports, in which the osteogenic potential of mouse ASCs was maintained with age by comparing cells from juvenile (6-day-old) and adult donors (60-day-old) [24]. Also, ASCs from OVX rats had higher osteogenic potential compared to BMSCs, evidenced by higher levels of ALP activity, *BGLAP*, collagen I (*COL1A1*), and bone morphogenetic protein 2 (*BMP2*) despite lower levels of bone sialoprotein (*BSP*) and *SPP1* [25]. Our data from cell counting and proliferation index also indicated comparable proliferation efficiency in ASCs from both OVX and NORM rats, which is in line with a previous report that human ASCs obtained from older donors appeared to have a slower rate of proliferation, but this relationship was not significant [26]. The above findings suggest that ASCs are a better candidate than BMSCs in treating OP patients with bone defects because BMSCs from OP donors exhibited a significant decrease in both proliferation [27] and osteogenic potential [27,28].

An early report showed that constructs made of PLGA and osteogenically differentiated human ASCs pre-cultured for 14 days before transplantation have better, more robust bone regeneration capability in critical-size nude rat calvarial defects than those with undifferentiated ASCs [17]. Di Bella and coworkers also found that osteogenically induced rabbit ASCs with fibronectin coated polylactic acid (PLA) scaffolds showed significantly higher bone formation for autologous critical-size skull defects compared to those with undifferentiated ASCs or no cells [29]. Fourteen-day osteogenic induction of ASCs from both NORM and OVX rats exhibited higher osteogenic differentiation contributing to our decision to use a tissue construct model in our *in vivo* experiment. Similar to our study, a short *in vitro* pretreatment of human ASCs followed by *in vivo* engraftment without recombinant protein may be just as advantageous without potentially stimulating neo-osteoclastogenesis [30,31]. Consistent with the findings from other groups [16,18,32], we found that the transplantation of PLGA scaffold seeded with osteogenically induced ASCs from NORM rats significantly promoted calvarial defect repair compared to implantation with PLGA alone.

There are also studies on mouse and human ASCs showing that pre-differentiation is not necessary for repairing critical-size calvarial defects [12,15,33,34]. The utilization of growth factors can be an efficient approach in promoting robust bone formation of calvarial defects. Direct application is *via* delivery of BMP2 either locally or systemically. The local and sustained *in vivo* release of BMP2 based on the biodegradable PLGA microsphere system [35] and apatite-coated PLGA/nanohydroxyapatite particulates [36] resulted in faster and more complete bone healing in critical-size calvarial defects. Subcutaneous injection of BMP2 for three days post-operatively enhanced undifferentiated human ASC-seeded PLGA constructs in the repair of critical-size calvarial defects in a nude mouse model [15]. Indirect application is *via* delivery of other factors activating BMP2. For instance, local delivery of alendronate for one week post-operatively promoted undifferentiated human ASC-seeded

PLGA constructs in bone regeneration for rat critical-size calvarial defects, probably due to an increased expression of BMP2 in ASCs [16]. A recent report demonstrated that BMP2 plays an important role in host dura mater stimulation of human ASC osteogenesis in the context of calvarial bone healing [37]. Implant of recombinant human parathyroid hormone and atelocollagen I effectively regenerated critical-size Wistar female rat calvarial defects compared to use of atelocollagen I alone [38].

Intriguingly, we found that implantation of PLGA seeded with or without osteogenically induced ASCs yielded better bone regeneration in OVX rats than in NORM rats despite no statistically significant difference for transplantation in the tissue construct group. This finding might be explained by increased levels of *RUNX2* (day 7: 0.88-fold; day 21: 1.19-fold) and *SPP1* (day 7: 0.91-fold; day 21: 1.56-fold) in ASCs from OVX rats compared to NORM rats during *in vitro* osteogenic induction. There is no consensus on osteogenic potential of ASCs from OVX donors. Veronesi and colleagues found that ASC clonogenicity, gene expression (*SPP1* and *BGLAP*), and subsequent protein production (ALP activity and osteocalcin) of important osteogenic markers are unaffected by estrogen deficiency; some of these markers were higher in ASCs from OVX rats, including matrix mineralization while the production of pro-inflammatory cytokines [interleukin 1 beta (IL-1 β) and IL-6] is similar to that of healthy cells [39]. These results provide a foundation for the use of autologous ASCs for bone pathologies, such as in OP women. However, despite a downregulation of *RUNX2*, *Sp7* (osterix), *COL1A1*, *SPARC* (secreted protein, acidic, cysteine-rich), and *SPP1* in patients with OP, Dalle Carbonare and colleagues found that circulating MSCs were increased in OP patients compared with normal donors [40], possibly indicating a major mobilization of MSCs in OP due to an increase in MSCs for the bone remodeling process. Considering that multiple signaling pathways contribute to the balance between osteoblastogenesis and adipogenesis, such as the mitogen-activated protein kinase pathway [41], AMP-activated protein kinase [42], and the Wnt/ β -catenin pathway [43], further investigation is needed to focus on the comparison of these signals in the donor-matched ASCs from both NORM and OVX rats after osteogenic and adipogenic induction.

5. Conclusions

In summary, we have demonstrated that ASCs from OVX rats exhibited comparable proliferation and osteogenic potential to those from NORM rats; implantation of either PLGA alone or with osteogenically induced ASCs promoted critical-size calvarial defect repair, especially in OVX rats. For the treatment of skeletal defects such as OP, ASCs may represent a viable stem cell-based therapeutic regimen.

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Highlights

1. ASCs from OVX rats exhibit a comparable proliferation capacity to those from NORM rats but have lower osteogenic potential
2. Implantation of ASC constructs exhibit a higher osteogenesis in the NORM rat group compared to the scaffold group
3. Implantation of PLGA scaffold exhibit a higher osteogenesis for the defects in OVX rats compared to NORM rats
4. ASC based tissue constructs are more beneficial for the repair of calvarial defects in NORM rats
5. Implantation of PLGA scaffold contributes to defect regeneration in OVX rats

Fig. 1. Establishment of an OP model using OVX rats. Six-month-old SD rats underwent either bilateral ovariectomy (OVX) or sham surgery (NORM). Four months post-operatively, the rats from both OVX and NORM groups were compared in weight (A), % fat in spine region (B), and spine bone mineral density (BMD) (C). Data are shown as average $\pm$ standard deviation for $n=5$. * $p < 0.05$ indicated a statistically significant difference.

Fig. 2.

Evaluation of *in vitro* proliferative potential of ASCs from both OVX and NORM rats. Passage 2 ASCs were expanded at 3000 cells/cm² for one passage. Expanded cells were collected at days 0, 2, 4, 6, and 8 for the measurement of DNA content using the Quant-iTTMPicoGreen[®] dsDNA assay kit with a CytoFluor[®] Series 4000 (A). Expanded cells at days 4 and 8 were also measured for proliferation index using a BD FACS CaliburTM flow cytometry (B).

Fig. 3.

Evaluation of *in vitro* osteogenic potential of ASCs from both OVX and NORM rats. After a 21-day-osteogenic induction, ASCs were evaluated for osteogenic differentiation using ALP staining (A) and activity (B) as well as ARS staining (C) and osteogenic marker genes including *BGLAP*, *RUNX2*, and *SPPI* (D). Data are shown as average $\pm$ standard deviation for $n=4$. $*p<0.05$ indicated a statistically significant difference.

Fig. 4. Evaluation of *in vitro* adipogenic potential of ASCs from both OVX and NORM rats. After a 21-day-adipogenic induction, ASCs were evaluated for adipogenic differentiation using ORO staining for lipid-filled droplets (A) and chemical quantitation of staining (B) as well as real-time PCR for adipogenic marker genes including *PPAR*, *CEBP*, and *LPL* (C). GM: Growth Medium; AM: Adipogenic Medium. Data are shown as average $\pm$ standard deviation for n=4. * $p < 0.05$ indicated a statistically significant difference.

Fig. 5. ASC morphology and extracellular matrix as well as PLGA mesh. SEM was used to characterize morphological properties of PLGA mesh and ASCs and secreted matrix at 3 days (A/B) and 14 days (C/D) post-cell seeding and osteogenic induction. Scale for low magnification is 1 mm (A/C); scale for high magnification is 300 μ m (B/D).

Fig. 6. Calvarial defect model and early-stage bone regeneration evaluation. Calvarial defects of 5 mm created bilaterally in both OVX and NORM rats were either left empty (A) or implanted with PLGA plus osteogenically induced ASCs or PLGA alone (B). Six weeks post-operatively, the defects were evaluated for bone formation using DEXA (C). Defect BMD ratio was defined as $(R1 \text{ or } R2) / [(R3+R4+R5+R6+R7)/5]$. Data are shown as the average for $n=6$ defects in the NORM rat group (D) and $n=4$ defects in the OVX rat group (E).

Fig. 7. Evaluation of late-stage bone regeneration of calvarial defects in SD rats using histological analysis and μ CT. Calvarial defects from NORM (A) and OVX rats (B) were implanted with PLGA plus osteogenically induced ASCs or PLGA alone for 32 weeks with untreated groups as empty controls (C and D, respectively). Representative images of calvarial bone sections were with Herovici's collagen staining (10 $\times$ original magnification). Arrows point to the defect margins. In the defect area, blue indicates new collagen while red indicates old collagen.

Fig. 8.

Quantitative μ CT analysis of 32-week bone formation *in vivo* using the ratio of bone volume (BV) to total volume (TV). The implantation with PLGA plus osteogenically induced ASCs and PLGA alone was evaluated for bone regeneration in NORM rats and OVX rats. Data are shown as average $\pm$ standard deviation for $n=8$ in the NORM rat group and $n=7$ in the OVX rat group. $*p<0.05$ indicated a statistically significant difference.

Table 1

Study design for animal experiments.

Rat OP Model		6-month-old female SD rats			
Surgery 4-month post-operation	Ovariectomy f OVX rats (n=5 rats)	Sham f NORM rats (n=5 rats)	NORM ASCs		
			PLGA alone	PLGA Empty + ASCs untreated	PLGA Empty + ASCs untreated
In Vivo Study (30 rats=60 defects)	f OVX ASCs		PLGA alone	PLGA alone	PLGA Empty + ASCs untreated
			n=4 defects	n=4 defects	n=6 defects
			n=7 defects	n=7 defects	n=8 defects
6-week post-implantation			n=4 defects	n=4 defects	n=6 defects
32-week Post-implantation			n=7 defects	n=4 defects	n=8 defects