Induction of Bone Formation by 3D Biologically Active Scaffolds Containing RGD-NPs, BMP2, and NtMPCs

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It is demonstrated that nuclear transfer embryonic stem cells (NT-ESCs) can be transformed into mesenchymal progenitor cells (NtMPCs) via culture under appropriate conditions and it is proposed that NtMPCs can be used for bone regeneration instead of bone marrow-derived mesenchymal stem cells (BMSCs). In a 2-dimensional culture, NtMPCs undergo chondrogenic and adipogenic differentiation more readily than BMSCs upon culture in appropriate media. However, the osteogenic differentiation ability of NtMPCs is lower than that of BMSCs. 3-dimensional (3D) biologically active scaffolds (BAs) comprising poly(lactic-co-glycolic acid) (PLGA) microspheres are constructed to improve osteogenic differentiation. To generate BAs, nanoparticles containing an l-Arginyl-Glycyl-l-Aspartic acid (RGD) sequence (RGD-NPs) and dexamethasone (DEX), a drug used to control differentiation and inflammation, are simultaneously encapsulated by PLGA to form particles measuring 150–250 µm. After that, bone morphogenetic protein 2 (BMP2) is immobilized on the surface of polyelectrolyte (PEI)-coated microspheres via formation of ionic bonds between O-sulfate and N-sulfate in heparin with lysine/arginine residues of BMP2. Gene expression profiling is performed via QuantSeq 3’ mRNA-sequencing in mice transplanted with BAs containing NtMPCs and BMSCs. Expression of the osteogenic differentiation-related genes collagen Type I Alpha 2 (COL1A2), Homeobox protein MSX-2 (MSX2), Runt-related transcription factor 2 (RUNX2), and bone morphogenetic protein 2 (BMP2) is 2–3-fold higher in mice transplanted with BAs containing NtMPCs than in mice transplanted with BAs containing BMSCs.

1. Introduction

Regenerative medicine aims to treat incurable human diseases using technologies such as genomics, cell biology, pharmacology, and 3-dimensional (3D) printing, traditional materials, and cell-oriented approaches.[1] It is widely used to treat muscle damage, spinal cord injury, and cancer. In some types of regenerative medicine, cells are used to repair damaged or defective areas.[2] Research of cell replacement therapy using stem cells has remarkably progressed in recent years and raises the possibility of treating incurable diseases.[3] However, it is true that the development of a therapeutic agent using stem cells is limited in its use due to the issue of the immunogenicity of stem cells. To overcome this problem, customized stem cell lines can be established using cloned human stem cell lines and somatic cell nuclear transfer (SCNT) technology, and stem cell lines with reduced immunogenicity can be generated.[4] Using these approaches, the immunogenicity of cells introduced into damaged sites is minimized, which is considered to be the most important factor in the development of cell therapy products.[5]

Support materials that maximize the function of established stem cells are...
desperately needed to repair damaged tissues and organs. Such materials must be biocompatible, have adequate mechanical properties and porosity, and not cause immune responses, pathogen transmission, or inflammatory responses. Damaged tissues can be regenerated using scaffolds loaded with biologically active molecules capable of enhancing adhesion, proliferation, and differentiation of stem cells. Unfortunately, incorporating a large number of cells into a scaffold, inducing proliferation of these cells, and maintaining their phenotypes are problematic with tissue engineering techniques. To overcome these difficulties, a technology that generates mesenchymal precursor cells by replacing the culture medium during the culture process has been developed using a customized cloned human stem cell line. These cells are called NT-ESCs-derived mesenchymal progenitor cells (NtMPCs), and have the potential to undergo mesenchymal and endothelial differentiation. They readily differentiate into almost all adult cell types and are useful for tissue and organ replacement. These advantages have the advantage of maintaining the phenotype of stem cells that occur simultaneously with mass culture.

In this study, a series of processes for large-scale cultivation of NtMPCs was standardized. NtMPCs lacked characteristics of ESCs. A sufficient number of NtMPCs was acquired and used as a cell therapy source. NtMPCs were compared with bone marrow-derived mesenchymal stem cells (BMSCs). NtMPCs and BMSCs were loaded onto a biologically active scaffolds (BAs) containing physiologically active substances and their differentiation abilities were investigated. To prepare BAs, nanoparticles (NPs) containing an L-Arginyl-Glycyl-L-Aspartic acid (RGD) sequence (RGD-NPs) were generated to facilitate adhesion and encapsulated by poly(lactic-co-glycolic acid) (PLGA) microspheres together with dexamethasone (DEX), which promotes osteogenic differentiation. The surface of PLGA microspheres was coated with the growth factor bone morphogenetic protein 2 (BMP2). Proliferation and differentiation of NtMPCs and BMSCs were examined. Increased calcification of the extracellular matrix (ECM) induced by delivery of various bioactive factors accelerated bone formation by NtMPCs attached to BAs. Adhesion, growth, and differentiation of NtMPCs and BMSCs loaded onto BAs were examined by gene expression profiling using QuantSeq 3′ mRNA-seq (mRNA-Seq). Bone repair was examined in mice following transplantation of BAs containing NtMPCs and BMSCs (Scheme 1).

2. Results

2.1. Characterization of BA-Scaffolds and NtMPCs

To safely deliver cells for therapeutic purposes, a BA-scaffold containing a drug, an adhesion molecule, and a growth factor was designed and manufactured. RGD-NPs conjugated with fluorescein isothiocyanate (FITC) were prepared. These RGD-NPs were combined with DEX to prepare PLGA microspheres containing DEX and RGD-NPs. Next, the surface of these microspheres was coated with polyethyleneimine (PEI) to make it positively charged. Finally, BMP2 was complexed with negatively charged heparin via an electrostatic interaction and subsequently bound to the positively charged surface of PLGA microspheres (Figure 1A).

BAs were imaged using scanning electron microscopy (SEM) and confocal laser microscopy (Figure 1B,C). This confirmed that BAs were coated with BMP2. The surface of BMP2-coated groups was uneven. This confirmed that negatively charged heparin complexed with BMP2 associated with positively charged

Scheme 1. Schematic diagram of NtMPC and BMSC differentiation in BAs. A) Preparation of BAs containing RGD-NPs and BMP2. B) Mounting of (a) BMSCs and (b) NtMPCs on BAs. C) Enhanced adhesion and bone regeneration of BMSCs and NtMPCs mounted on BAs.****
Figure 1. Characterization of BAs and different type of stem cells. A) Schematic diagram of BAs containing RGD-NPs and BMP2. B) SEM images of deferent scaffolds. (i–j) Enlarged images of BAs. C) Confocal images of deferent scaffolds. (e) An enlarged image of BAs. D) (a) Schematic diagram of the protocol used to differentiate NT-ESCs into NtMPCs. (b) RT-PCR analysis of ESCs markers in NT-ESCs, NtMPCs, and BMSCs. (c) Immunostaining of BMSCs for ESCs and MSCs markers. Scale bar: 50 µm. E) Flow cytometric analysis of the MSC markers. F) (a) Morphologies of NT-ESCs, NtMPCs, and BMSCs. (b) Representative G-banding images. Scale bar: 100 µm. G) Chromosomal microarray results.

PEI on the surface of PLGA microspheres. It was confirmed that FITC-conjugated RGD-NPs were encapsulated by PLGA microspheres. Green fluorescence representing FITC was detected within/on PLGA microspheres by confocal laser microscopy (Figure 1C). Small aggregated FITC-conjugated RGD-NPs were observed within/on PLGA microspheres. RGD-NPs measured 121 nm and were almost spherical. They were successfully introduced into MSCs and uniformly distributed in the cytoplasm (Figure S1, Supporting Information). A large number of RGD-NPs and BMP2 was initially released from BAs, and their release was sustained for more than 21 days (Figure S2, Supporting Information). BMP2 on the surface of BAs remained bioactive for up to 21 days (Figure S3, Supporting Information).

The microspheres containing the drug, the adhesion molecule RGD and the growth factors that form differentiation induction were called bioactive scaffolds (BA-scaffold). Here, instead of bone marrow-derived mesenchymal cells, mesenchymal progenitor cells (MPCs) derived from nuclear transfer embryonic cells (SCNTs) were used as a replacement cell source for damaged tissues.

The ability of NtMPCs to replace damaged tissues instead of BMSCs was tested. To produce NtMPCs on a large scale, NT-ESCs were cultured as embryoid bodies (EBs) in ESC culture medium for 5 days and then converted to NtMPCs by culture in mesenchymal stem cell (MSC) culture medium (Figure 1D, (a)). Aggregated NT-ESCs disassembled into single cells after the medium was changed.

To determine whether these single cells had characteristics of MSCs, expression of the typical ESC markers NANOG, octamer-binding transcription factor 4 (OCT4), and sex determining region Y-box 2 (SOX2) was examined. RT-PCR (Figure 1D, (b)) and immunofluorescence (Figure 1D, (c)) analyses demonstrated that ESCs expressed these markers, but BMSCs and NtMPCs did not.

2.2. Characterization of NtMPCs and BMSCs In Vitro

The phenotype of NtMPCs was compared with that of BMSCs. More than 97% of NtMPCs were positive for the MSC markers CD29, CD44, CD105, and CD166, similar to BMSCs (Figure 1E). The morphologies of BMSCs and NtMPCs did not differ during cell culture and differentiation, and both cell types had a normal karyotype upon extended cell culture (Figure 1F). Neither BMSCs...
Figure 2. Multilineage differentiation of NtMPCs and BMSCs. A) (a) In vitro differentiation of NtMPCs and BMSCs. (b) Morphologies of NtMPCs and BMSCs after induction of differentiation under appropriate culture conditions. B) In vitro chondrogenic differentiation of NtMPCs and BMSCs. (a) RT-PCR, (b) quantitative graph of mRNA expression, (c) Safranin-O staining, and (d) immunostaining of NtMPCs and BMSCs for SOX9 (red) and COL II (green). Nuclei were stained with DAPI (blue). C) In vitro adipogenic differentiation of NtMPCs and BMSCs. (a) RT-PCR, (b) quantitative graph of mRNA expression, and (c) Sudan black B staining. (d) Immunostaining of NtMPCs and BMSCs for aP2 (red) and c/EBP\(\alpha\) (green). Nuclei were stained with DAPI (blue). D) In vitro osteogenic differentiation of NtMPCs and BMSCs. (a) RT-PCR, (b) quantitative graph of mRNA expression, and (c) Von Kossa staining. (d) Immunostaining of NtMPCs and BMSCs for ALP (red) and Runt-related transcription factor 2 (RUNX2) (green). Nuclei were stained with DAPI (blue).

nor NtMPCs exhibited submicroscopic genomic gains or losses in a chromosomal microarray (Figure 1G).

The ability of NtMPCs to undergo chondrogenic, adipogenic, and osteogenic differentiation upon culture in appropriate media was next investigated and compared with that of BMSCs as a control. Chondrogenesis, adipogenesis, and osteogenesis were compared between NtMPCs and BMSCs in detail (Figure 2A). SRY-Box Transcription Factor 9 (SOX9) and collagen II (COL II), which are markers of chondrocytes, were used to assess cartilage formation. Upon induction of chondrogenic differentiation, mRNA expression of SOX9 and COL II increased in both NtMPCs and BMSCs, demonstrating that both cell types differentiated into chondrocytes, and was higher in NtMPCs than in BMSCs (Figure 2B). Safranin-O staining demonstrated that NtMPCs secreted more proteoglycan into the ECM than BMSCs. Immunofluorescence staining showed that the protein levels of SOX9 and COL II were higher in NtMPCs than in BMSCs. NtMPCs exhibited lacunae, which are characteristic of chondrocytes.

aP2 and c/EBP\(\alpha\) are important for adipogenic differentiation. Upon induction of adipogenic differentiation, mRNA expression of these two markers was higher in NtMPCs than in BMSCs.
2.3. Differentiation of NtMPCs and BMSCs in BA-Scaffolds In Vitro

The abilities of NtMPCs and BMSCs seeded on BAs to undergo osteogenic differentiation were examined. Cells were attached to BAs, and release of encapsulated RGD-NPs was examined upon decomposition of microspheres. Osteogenic differentiation of adherent and proliferating cells induced by RGD and BMP2 on the surface of BAs was investigated. As the RGD (Arg–Gly–Asp–Ser) sequence was combined with the cell integrin (RGD ligand), cell differentiation was increased and ossification was induced. The receptor (integrin) present in the cell membrane recognizes the RGD sequence and transmits intracellular signals. Fibronectin, which was increased by the interaction with the RGD sequence, was expressed during cell differentiation and proliferation, and was involved in the extracellular matrix and early stage of adhesion. BMP2 induces expression of RUNX2 via the Smad signaling pathway, which promotes expression of molecules related to osteogenic differentiation such as ALP, COLI, and osteocalcin (OCN) (Figure 3A).

NtMPCs and BMSCs were seeded on BAs and observed by confocal laser microscopy. Both cell types adhered well to the surface of BAs compared with control-scaffolds, which were not coated or loaded with any active molecules (Figure 3B). In addition, release of RGD-NPs facilitated adhesion of cells to the surface of BAs. Adhesion of cells to scaffolds and cell proliferation were examined by SEM. While NtMPCs and BMSCs adhered to both control-scaffolds and BAs, they only proliferated on the latter. Moreover, contacts between cells on BAs created pseudopodia that connected microspheres to each other, creating a true scaffold structure (Figure 3C). Figure S4 is show that RGD-NP-loaded microspheres and BAs were had superior cell adherent than control and BMP2-coated PLGA microspheres. Adhesion and growth differed between cells seeded on different types of PLGA microspheres (Figure S5, Supporting Information). Cells were clustered on the surface of control and BMP2-coated microspheres, but were uniformly distributed on the surface of RGD-NP-loaded microspheres and BAs, and grew in an interconnected fashion.

The expression levels of osteogenic differentiation markers were investigated by RT-PCR. Expression of the adhesion molecule fibronectin was high in cells attached to BAs. COLII, an
ECM marker in cartilage tissue, was not highly expressed in any groups. Collagen I (COLI), a marker of bone tissue, was highly expressed by NtMPCs and BMSCs attached to BAs. Expression of COLI, ALP, RUNX2, and OCN, which are osteogenic differentiation markers, was high in NtMPCs attached to BAs (Figure 3D). Quantitative analysis found that cells attached to BAs expressed almost all osteogenic differentiation markers (Figure 3E).

2.4. mRNA-Seq Analysis of Bone Differentiation Gene Expression in NtMPCs and BMSCs

Gene expression was assessed by mRNA-Seq analysis at 4 weeks after transplantation of BAs containing BMSCs and NtMPCs into mice (Figure 4A). Collagen proteins (ECM) and noncollagen proteins (non-ECM) secreted by stem cells are linked with mineralization and bone remodeling. Expression of the ECM markers fibronectin, COLI, and COLII was first examined by RT-PCR to confirm mineralization in control-scaffolds and BAs containing BMSCs and NtMPCs. Expression of fibronectin and COLI was significantly higher in BAs than in control-scaffolds, whereas COLII expression was lower and did not significantly differ between these two types of scaffolds. Thus, fibronectin and COLI highly expressed in BAs containing BMSCs and NtMPCs promoted osteoblast proliferation and bone formation (Figure 4B). BA-scaffold containing BMP2, DEX, and RGD NP promoted the differentiation of BMSCs and NtMPCs into bone cells (osteoblast cells), produced collagen ECM, and induced fibrosis from cells.

BAs promoted differentiation of BMSCs and NtMPCs into osteoblasts, production of collagen, and fibrosis. ECM-related gene expression in control-scaffolds and BAs containing BMSCs and NtMPCs was analyzed by mRNA-Seq. Genes whose expression was up/downregulated by more than 2.0-fold versus the control-scaffolds were determined. Increased, decreased, and unchanged expression is shown in red, blue, and white, respectively. Expression of ECM-related genes was upregulated in BAs containing NtMPCs and BMSCs (Figure 4C). In the ECM receptor category, which is related to interactions with RGD-NPs, the expression patterns of upregulated genes were very similar in BAs containing NtMPCs and BMSCs. Expression of insulin like growth factor binding protein 7 (IGFBP7), Discoidin domain receptor tyrosine kinase 1 (DDR1), DDR2, and duchenne muscular dystrophy (DMD), which belong to the ECM receptor category, was compared between control-scaffolds and BAs containing NtMPCs and BMSCs. The integrin subunit alpha X (ITGAX) and integrin subunit alpha 2b (ITGAX2B) genes are colored white, pale red, or pale blue, indicating that their...
expression patterns did not differ between these two groups. In the results of hierarchical clustering, similar groups c and d, c–d to b were formed, and b–d to a were formed as similar groups again. This demonstrates that the expression patterns of ECM, ECM receptor, and cell–ECM adhesion genes were similar in NtMPCs and BMSCs mounted on control-scaffolds and BAs. NtMPCs, which underwent osteogenic differentiation less readily than BMSCs in vitro, mounted on BAs exhibited significantly increased expression of specific genes. BAs promoted bone formation by both NtMPCs and BMSCs and thereby accelerated mineralization by inducing deposition of mineral crystals between collagen fibers constituting the bone stroma.

In gene ontology (GO) analyses, genes belonging to five categories were extracted. The percentages of genes belonging to these five GO categories among genes whose expression significantly differed in BAs containing NtMPCs relative to control-scaffolds containing NtMPCs, BAs containing BMSCs relative to control-scaffolds containing BMSCs, and BAs containing NtMPCs relative to BAs containing BMSCs were determined. Among genes whose expression was more than 1.5-fold higher or lower in BAs containing NtMPCs than in BAs containing BMSCs, 2.51%, 5.03%, 2.75%, 2.54%, and 4.00% belonged to the ECM, cell adhesion molecule, cell adhesion, cell–cell junction, and collagen binding categories, respectively.

Masson’s trichrome staining was performed to detect cells, collagen, and ECM related to bone formation. Blue staining, representing collagen formation and mature bone cells, was more intense in BAs than in control-scaffolds (Figure 4D). Collagens and the ECM were examined by immunofluorescence and western blotting. Immunofluorescence staining of COL I was more intense in BAs containing cells than in control-scaffolds containing cells (Figure 4E). Western blotting demonstrated that expression of fibronectin and COL I was higher in BAs containing cells than in control-scaffolds containing cells.

2.5. mRNA-Seq Analysis of BA-Scaffolds Containing Cells Transplanted into Nude Mice

Osteogenic differentiation of BMSCs and NtMPCs seeded on BAs and control-scaffolds in vivo was investigated by RT-PCR. Expression of the osteogenic differentiation markers ALP, RUNX2, and OCN was compared between NtMPCs and BMSCs loaded on BAs and control-scaffolds at 4 weeks after implantation into nude mice. The expression levels of these three markers were significantly higher in BAs containing cells than in control-scaffolds containing cells (Figure 5A). In addition, mRNA-Seq data of genes associated with osteogenic differentiation were obtained (Figure 5B–D). This demonstrated that NtMPCs and BMSCs seeded on BAs underwent proliferation, differentiation, and calcification, and exhibited significantly increased expression of COL1A2 (Collagen Type 1 Alpha 2) and RUNX2. Expression of homeobox protein MSX-2 (MSX2) and BMP2, which are important for inducing osteogenic differentiation, was higher in BAs containing BMSCs than in control-scaffolds containing BMSCs. Expression of SMAD3, COL1A2, and IGF1 was higher in BAs containing NtMPCs than in BAs containing BMSCs, while expression of MSX2, RUNX2, and BMP2 was lower (Figure 5B). Among genes whose expression differed between control/BAs containing NtMPCs and BMSCs, those belonging to five GO categories were extracted (Figure 5C) and the results were presented in a volcano plot (Figure 5D).

In total, 22.06%, 12.87%, 11.72%, 4.38%, and 3.9% of genes belonged to the GO categories bone morphogenesis, stem cell proliferation, bone mineralization, osteoblast development, and bone maturation, respectively. Genes whose expression significantly differed between control-scaffolds containing NtMPCs and control-scaffolds containing BMSCs (Figure 5C), and between BAs containing NtMPCs and BAs containing BMSCs (Figure 5C, c), were identified. The percentages of genes belonging to the bone morphogenesis, stem cell proliferation, bone mineralization, osteoblast development, and bone maturation categories were 8.50%, 6.43%, 5.97%, 1.37%, and 1.60% in the comparison of control-scaffolds containing NtMPCs and control-scaffolds containing BMSCs, respectively, and 2.52%, 2.06%, 1.37%, 0.69%, and 0.46% in the comparison of BAs containing NtMPCs and BAs containing BMSCs, respectively. These percentages were lower in the latter comparison than in the former comparison, indicating that the gene expression patterns of NtMPCs and BMSCs were more similar when seeded on BAs.

2.6. Ability of Cells Seeded on BA-Scaffolds to Undergo Osteogenic Differentiation Following Transplantation into Nude Mice

BAs containing NtMPCs and BMSCs were transplanted into nude mice and the ability of these cells to undergo osteogenic differentiation was investigated (Figure 6A). Expression of ALP, RUNX2, and OCN, which are markers of osteogenic differentiation, was examined at 4 weeks after transplantation (Figure 6B). These markers were highly expressed in BAs containing NtMPCs and BMSCs. Quantitative analysis revealed that expression of ALP, RUNX2, and OCN was 2–10-fold higher in cells loaded on BAs than in cells loaded on control-scaffolds. Although expression of these markers was lower in NtMPCs than in BMSCs, it was significantly higher using BAs than using control-scaffolds, demonstrating that mounting on BAs effectively induced osteogenic differentiation.

von Kossa staining, which is used to observe calcium deposition indicative of osteogenic differentiation, yielded similar results (Figure 6C; Figure S6, Supporting Information). Black staining was observed in cells mixed with BAs, but not in cells mixed with control-scaffolds. ALP expression was approximately fourfold higher in BAs containing cells than in control-scaffolds containing cells (Figure 6D). Immunofluorescence staining of RUNX2 (red) and COL I (green) was intense in BAs containing cells (Figure 6F). Quantitative analysis revealed that expression of these two markers was at least twofold higher in BAs containing cells than in control-scaffolds containing cells (Figure 6E).

2.7. Confirmation of the Bone Differentiation Capacity of NtMPCs and BMSCs Loaded on BA-Scaffolds in an Animal Model

A defect was generated in the femur of a rat, control-scaffolds and BAs containing NtMPCs and BMSCs were transplanted, and the ability of these cells to undergo osteogenic differentiation was determined (Figure 7). Cells were mixed with BAs and
control-scaffolds and transplanted into the femoral bone defect. The defect was repaired at 2 weeks after transplantation (Figure 7B). The morphologies of scaffolds and cells transplanted into femoral bone defects were examined. The femoral bone defect was repaired over time following transplantation of BAs containing cells. Repair was faster following transplantation of BAs containing cells than following transplantation of control-scaffolds containing cells. Microcomputed tomography (micro-CT) demonstrated that both NtMPCs and BMSCs promoted bone defect repair (Figure 7C). To assess osteogenic differentiation of cells transplanted into femoral bone defects, micro-CT quantification of newly formed bones in and around the defect site was performed using the parameters of bone volume, bone mineral density (BMD), and trabecular thickness (Tb.Th). All these parameters were higher in mice transplanted with BAs containing cells than in control-scaffolds containing cells (Figure 7D).

To examine repair of the femoral defect, bone was sectioned after sacrificing mice (Figure 7E). Bone tissue was not repaired in the control group, but was repaired following transplantation of stem cells, and was repaired to a greater extent following transplantation of BAs containing cells (Figure 7E, (c,f)). Repair of the defect was assessed by H&E staining (Figure 7E; Figure S7, Supporting Information). The defect was barely visible following transplantation of BAs containing cells (Figures 7E, (c,f)), but was hardly repaired in the control-scaffold (Figure 7E, (a,b,d,e)). Masson’s trichrome staining was performed to detect collagen, which is a representative ECM molecule in repaired tissue (Figure 7F; Figure S8, Supporting Information). A large amount of collagen was detected in repaired tissues following transplantation of stem cells. The defect was not repaired in the control-scaffolds and consequently little collagen was detected. RUNX2 (red) and COL1 (green), which are related to bone differentiation, were detected by immunofluorescence staining (Figure 7G). These two proteins were secreted in the damaged region following transplantation of BAs containing NtMPCs and BMSCs, indicating that the transplanted cells underwent osteogenic differentiation (Figure 7G, (c,f)).

3. Discussion

Techniques to directly implant therapeutic cells into damaged or diseased sites have recently been developed. Many researchers have attempted to improve treatments for diseases that cannot be cured using human embryonic and adult stem cells. However,
technologies using these cells have limitations. There are many problems associated with stabilizing damaged tissues by directly transplanting cells. Stem cells are an important cell source in such technologies due to their multidifferentiation ability; however, their immunogenicity is thought to contribute to the instability of transplanted cells.\(^{[14]}\) To overcome these limitations, a technique to control the immune system of transplanted cells using NT-ESCs has been developed. In addition, a technique to secure a sufficient number of cells for transplantation was developed by standardizing a series of processes that ensure large-scale culture during generation of NtMPCs. In this study, NtMPCs, which did not readily undergo osteogenic differentiation in vitro, were induced to differentiate into bone cells using PLGA microspheres loaded with various drugs and adhesion molecules. NtMPCs shared many characteristics with BMSCs (Figure 1). Genomic analysis confirmed the safety of NtMPCs, demonstrating that they can be used for cell therapy products. The genetic safety of BMSCs is essential for their clinical use. Chromosomal abnormalities and genomic imbalances arise during extended cell culture, differentiation induction, and genetic manipulation.\(^{[15]}\) In the early stage of stem cell research, genetic safety was mainly tested via routine karyotyping. However, microarray and next-generation sequencing-based genomic analyses have been performed in recent years. In this study, we used the CytoScan 750K SNP-based CMA chip, which covers most online mendelian inheritance in man (OMIM) and cancer genes and can identify loss of heterozygosity and copy number variations of several kilobases.\(^{[16]}\) No pathogenic genomic abnormalities were identified, meaning that the genetic safety of NtMPCs is similar to that of existing cell sources (Figure 1G).\(^{[17]}\) NtMPCs underwent
chondrogenic and adipogenic differentiation more readily than BMSCs upon culture in appropriate media (Figure 2). However, NtMPCs differentiated into osteoblasts less readily than BMSCs. This indicates that NtMPCs cannot be used alone to repair damaged bone tissue and that auxiliary active substances capable of inducing bone tissue are needed. Therefore, PLGA microspheres were loaded with DEX and RGD-NPs and coated with BMP2 to continuously induce osteogenic differentiation (Figure S1, Supporting Information).

NtMPCs and BMSCs attached to BAs exhibited differences from those attached to control-scaffolds (Figure 3). This is because RGD-NPs loaded in BAs caused PLGA microspheres to degrade, and cells readily attached to BAs and proliferated due to stimulation by adhesion molecules. Consequently, cells attached to BAs formed a network that connected microspheres via communication between cells. We used BMSCs as a control and mounted these cells on BAs. In vitro and in vivo experiments demonstrated that NtMPCs differentiated more readily than BMSCs (Figure 3). Expression of ALP and RUNX2, which are important for bone differentiation, was higher in BAs containing NtMPCs than in BAs containing BMSCs and was more than twofold higher in BAs than in control-scaffolds.

Secretion of fibronectin and COLI during osteogenic differentiation of stem cells was examined by von Kossa staining. COLI secretion was increased when cells were loaded on BAs because they underwent osteogenic differentiation and formed a collagen matrix to generate hard bone tissue with accumulated calcium salts and phosphates. This demonstrates that cells loaded on BAs deposited more calcium than those loaded on control-scaffolds (Figure 4B). In addition, mRNA-Seq detected changes in expression of various genes encoding proteins that are secreted upon osteogenic differentiation of stem cells (Figure 4C). Expression of these genes was altered more in NtMPCs than in BMSCs. Such changes in gene expression are expected to affect protein secretion; therefore, secretion of these proteins was examined by western blotting and immunofluorescence staining. Secretion of COLII, which occurs upon chondrogenic differentiation, was lower in NtMPCs than in BMSCs, while secretion of COLI, which occurs upon osteogenic differentiation, was higher. This indicates that NtMPCs loaded on BAs undergo osteogenic differentiation more readily than chondrogenic differentiation.

NtMPCs and BMSCs loaded on BAs also underwent osteogenic differentiation in vivo (Figure 6). The ability of NtMPCs to undergo osteogenic differentiation was comparable with that of BMSCs when loaded on BAs. This was confirmed by von Kossa staining. As stem cells differentiate into bone cells, they secrete collagen substrates to accumulate calcium salts and phosphates, which is important for hardening of bone tissue. Black staining indicated accumulation of calcium around cells (Figure 6C). Cells loaded on BAs secreted large amounts of ALP, RUNX2, and OCN, which are important for osteogenic differentiation.

BAs containing NtMPCs and BMSCs were transplanted into an animal model with a femoral defect and bone tissue repair was examined. The femoral bone defect disappeared after transplantation of BAs containing NtMPCs and BMSCs (Figure 7B). Micro-CT revealed that the defect was not completely repaired,
but was filled with transplanted cells (Figure 7C). This was consistent with BMD measurements. BAs loaded with cells had a much improved repair ability, which highlights the need to use a scaffold loaded with a differentiation-inducing material when applying stem cells for therapeutic purposes.

4. Conclusion

In this study, SCNT-ESC-derived MPCs were mounted on PLGA microparticles called BAs into which various kinds of drugs and adhesion molecules were introduced, and the differentiation of these cells into bone cells was investigated. It was found that the bone differentiation of NtMPC and BMSC mixed with BAs differentiated significantly better than control PLGA microspheres in vitro and in vivo. Comparing NtMPC and BMSC, it was found that NtMPC has more bone differentiation than BMSC. Observing this fact with mRNA-seq, the changes in genes associated with bone differentiation showed the greatest difference in NtMPCs mounted on BAs. Consequently, it is thought that NtMPC is a crucial source in cell therapy instead of MSCs.

5. Experimental Section

Materials: Poly(ε-caprolactone) 50:50 Resomer RG 503 (PLGA) (MW 33 kDa) was purchased from Evonik (Essen, Germany). A FITC-labeled prototype RGD-containing peptide (AS-22946, sequence: FITC-LC–Gly–Arg–Gly–Asp–Ser–Pro-OH) was purchased from AnaSpec (San Francisco, USA). Recombinant human transforming growth factor (TGF) β1 (243-B3), BMP2 (355-BM), IGf (insulin-like growth factor) (291-G1), and basic fibroblast growth factor (bFGF) (234-FSE) proteins were purchased from R&D Systems (Minneapolis, USA). Alexa Fluor 647-conjugated phalloidin (ab176755) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Polyvinyl alcohol (PVA, MW 30–70 kDa), DEX, β-glycerophosphate, dimethyl sulfoxide (DMSO), chloroform, methylene chloride (DCM), and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents and chemicals were of analytical grade.

Production Process of NtMPCs: Attatch existing the ESCs (CHA-hES15 from Seongnam-si, CHA Group, Korea) to mouse fibroblasts (MEF). Knockout serum replacement (10% KSR; Invitrogen), bFGF (4 ng mL⁻¹; Invitrogen), 1% NEAA, 0.1 mmol L⁻¹ heparin for 2 h at 4°C and finally coated with 25 µg of BMP2.

Characterization of Human NT-ESCs, NtMPCs, and BMSCs: To compare the characteristics of NtMPCs with those of BMSCs and ESCs, expression of the ESC markers NANOG, OCT4, and SOX2 was examined by RT-PCR and immunostaining, and expression of the MSC surface markers CD29, CD44, CD105, and CD166 was examined by fluorescence-activated cell sorting (easyCyte 6HT, Luminex, TX, USA). Genetic Stability of Human NT-ESCs, NtMPCs, and BMSCs: To confirm the genetic stability of the cells used in the study, karyotyping and SNP array were performed. In order to observe the chromosomes of proliferating cells, KaryoMAX colcemid (Gibco, CA, USA) as a reagent that stops cell division, was treated for 2 h. Then, the cells were collected using a scraper and the supernatant was removed. The cells were treated with a hyperton solution and fixed by adding methanol/acetic acid solution (3:1). Then, the chromosomes were stained with Giemsa staining and observed under a microscope.

SNP arrays were performed to confirm CNVs (copy number variations). The genomic DNA of cells was extracted using the Exgene Cell SV mini kit (106-101) purchased from GeneAll (Seoul, South Korea). Then, PCR was performed with Affymetrix Cytoscan (Affymetrix, Santa Clara, CA, USA) through a 750K SNP-based CMA chip and analyzed.

Induction of Chondrogenic, Adipogenic, and Osteogenic Differentiation of NtMPCs and BMSCs: To induce chondrogenic differentiation, NtMPCs and MSCs were cultured for 14 days in DMEM/F12 or DMEM HG containing 1% antibiotic–antimycotic solution, 1.0 × 10⁻⁴ m DEX, 0.17 × 10⁻³ m ascorbic acid, 0.35 × 10⁻³ m l-proline, 1 × 10⁻³ m sodium pyruvate, 1 × ITS, 5 ng mL⁻¹ BMP2, and 5 ng mL⁻¹ TGFβ. To induce adipogenic differentiation, cells were cultured for 21 days in DMEM/F12 or DMEM HG containing 10% FBS, 1% antibiotic–antimycotic solution, 0.5 × 10⁻⁳ m DEX, 0.5 × 10⁻³ m IBMX, 100 × 10⁻⁶ m indomethacin, 5 ng mL⁻¹ bFGF, and 5 ng mL⁻¹ IGF. To induce osteogenic differentiation, cells were cultured for 21 days in DMEM/F12 or DMEM HG containing 10% FBS, 1% antibiotic–antimycotic solution, 0.1 × 10⁻⁶ m DEX, 10 × 10⁻³ m β-glycerophosphate, 0.05 × 10⁻³ m ascorbic acid, 5 ng mL⁻¹ BMP2, and 5 ng mL⁻¹ BMP7. The culture medium was replaced every 2–3 days. Technical and morphological changes were recorded using specific staining. Expression of specific genes was confirmed at the mRNA and protein levels.

Cell Seeding on BA-Scaffolds: NtMPCs or BMSCs were centrifuged in a 15 mL conical tube at 1300 rpm for 3 min. The cell pellet was cultured in media containing 10% FBS and 1% amino acids for 2 weeks and then in serum-free medium for another week. The culture medium was changed every 2–3 days. To confirm that cells adhered to BAs, cells were fixed with 4% parafomaldehyde (PFA), stained with phallolidine and DAPI, and
observed by confocal laser scanning microscopy. For SEM, cells were fixed with 4% PFA, incubated with 2.5% glutaraldehyde for 2 h, dehydrated with 50%, 70%, 80%, 90%, 95%, and 100% ethanol, dried overnight on HMDS (Bis(trimethylsilyl)amine), and imaged.

mRNA and Protein Analyses of NTMPCs and BMSCs Seeded on BA-Scaffolds: After 3 weeks of culture in the 3D system, mRNA and protein were extracted using TRIzol (Invitrogen, Waltham, USA) and radioimmunoprecipitation buffer, respectively, and analyzed by RT-PCR/quantitative PCR and western blotting, respectively.

In Vivo Femoral Defect Model: Seven-week-old SD rats were purchased from Rat Bio (Yongin-si, Korea). A hole measuring 2 × 2 × 2 mm was drilled in the femur while rats were anesthetized with isoflurane, and then scaffolds harboring NTMPCs or BMSCs were transplanted. Rats were sacrificed and femurs were harvested at 2 and 4 weeks after surgery. The animal study was approved by the Institutional Animal Care and Use Committee (IACUC) of CHA University in South Korea (Approval No. IACUC-180117). The study was approved by the Institutional Animal Care and Use Committee (IACUC) of CHA University in South Korea (Approval No. IACUC180117).

Statistical Analysis: Statistical analyses were performed using the Student’s t-test. *P < 0.05, **P < 0.01, and ***P < 0.001 were considered to be statistically significant.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Author Contributions
J.S.P., K.-H.P., and H.J.K. came up with concept. H.J.K., S.J.H., S.L., J.M.P., and J.-I.P. carried out the experiments. J.S.P., K.-H.P., H.J.K., and S.H.S. wrote the paper.

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