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Short Running Title: ENDOCHONDRAL BONE REGENERATION WITH HUMAN iPSCs

Keywords: induced pluripotent stem cells, mesenchymal stem cells, endochondral ossification, bone regeneration

Abstract

The use of induced pluripotent stem cells (iPSCs) shows potential in bone regenerative strategies. Here, we investigated whether implantation of chondrogenically differentiated iPSC-derived mesenchymal stem cells (iMSCs) can lead to successful bone regeneration in nude mice with bone defects. Two human iPSC clones (201B7 and 454E2) were used. After generating iMSCs, chondrogenic differentiation was achieved by three-dimensional pellet culture. Thereafter, a 2-mm defect was created in the radius of nude mice, and chondrogenically differentiated iMSC pellets were transplanted in the defect. Micro-computed tomography imaging was performed 8 weeks post-transplantation to assess bone regeneration. All (100%) radii in the 201B7 cell-derived-pellet transplantation group and 7 of 10 (70%) radii in the 454E2 cell-derived-pellet transplantation group showed bone union. In contrast, 2 of 11 radii (18%) in the control group showed bone union. Thus, the experimental groups showed significantly higher bone union rates than the control group ($p < 0.05$). Histological analysis 2 weeks post-implantation in the experimental groups revealed hypertrophic chondrocytes within grafted iMSC pellets and the formation of woven bone around them. This hypertrophic chondrocyte transitioning to newly formed bone suggests that the cartilaginous template can trigger endochondral bone ossification (ECO). Four weeks post-implantation, the cartilage template was reduced in size; newly formed woven bone was predominant in the defect site. New vessels were surrounded by a matrix of woven bone, and hypertrophic chondrocytes transitioning to newly formed bone indicated the progression of ECO. Eight weeks post-implantation, the pellets were completely resorbed and replaced by bone; complete bone union was observed. Dense mature bone developed with evidence of lamellar-like bone formation. Collectively, our results suggest that using iMSC-based cartilage grafts recapitulating the morphogenetic process of ECO in the context of embryonic skeletogenesis is a promising strategy for repairing large bone defects.

Impact Statement

We investigated whether implantation of chondrogenically differentiated iMSCs could lead to the successful regeneration of bone defects *in vivo*. We implanted two different clones of human iPSCs into a radial bone defect model. Eleven of 11 (100%) and 7 of 10 (70%) radii in the 201B7 and 454E2 cell-derived-pellet transplantation groups, respectively, showed bone union, which were significantly higher than those in the control group [only 2 of 11 radii (18%)]. Overall, our results support the use of iMSC-based cartilage grafts recapitulating the morphogenetic process of ECO for repairing large bone defects.

Introduction

Treatment of bone defects caused by severe trauma, nonunion, osteomyelitis, or bone tumors remains challenging in orthopaedic surgery.[1] As a result, the quality of life and daily living activities of patients are disrupted. Bone grafting is the most commonly used surgical procedure for treating bone defects; autogenous bone grafting represents the clinical "gold standard." However, the amount of available autologous bone is limited, and the harvesting procedures are associated with donor site morbidity and various complications such as severe pain, hemorrhage, infections, fractures, and nerve damage. In fact, in over half of reported cases, autogenous bone grafts showed unsatisfactory results.[2] To overcome these limitations, bone tissue engineering using stem cells and scaffolds has emerged as a promising strategy for bone repair.[3]

Cell-based bone tissue engineering requires the large-scale production of homogeneous populations of lineage-restricted progenitor cells that can easily differentiate into specific tissues. Mesenchymal stem cells (MSCs) are considered as a promising source of progenitors for tissue engineering strategies.[4] MSCs are multipotent progenitors found in many connective tissues including the bone marrow and fat.[5-7] However, the use of MSCs is limited by their heterogeneity, availability, invasive and laborious harvest procedures, finite proliferation potential, and loss of differentiation capacity during expansion.[8-10] Recently, a major breakthrough in the generation of patient-specific pluripotent stem cells was reported by Yamanaka et al.; embryonic stem cell-like pluripotent stem cells, designated as induced pluripotent stem cells (iPSCs), were generated from adult somatic cells using reprogramming techniques.[11,12] iPSCs possess unlimited self-renewal capacity and the ability to differentiate into all somatic cell types. Importantly, iPSCs can be derived from the patient's own somatic cells, thereby avoiding immune rejection after transplantation as well as ethical concerns. Notably, several studies successfully achieved derivation of MSCs from iPSCs.[13,14] Therefore,

iPSC-derived MSCs, named as "iMSCs", represent a highly promising and attractive source of large populations of progenitors for use in bone tissue engineering.[15,16] However, the *in vivo* regenerative potential of iMSCs in the context of long-bone repair has not been clearly demonstrated.[16]

The bone repair process closely resembles normal development of the skeleton during embryogenesis, including intramembranous and endochondral ossification (IMO and ECO, respectively).[17-19] In IMO, MSCs differentiate directly into osteoblasts that form the bone. In ECO, MSCs differentiate into chondrocytes, which form a cartilage template; next, chondrocytes within this template undergo hypertrophy, secreting cytokines such as vascular endothelial growth factors (VEGF), enzymes such as alkaline phosphatase, and proteases (e.g., matrix metalloproteinases). These factors stimulate vascularization and mineralization of the cartilage template, which is subsequently resorbed by attracted osteoclasts and replaced by bone. Importantly, ECO is the dominant mode of ossification in the natural healing of bone fractures. Conventional bone tissue engineering approaches have typically recapitulated the process of IMO via direct *in vitro* osteogenic priming of MSCs within scaffolds before implantation. However, this approach is associated with insufficient vascularization of the graft following implantation, thereby preventing the necessary delivery of oxygen and nutrients.[20] As a result, such strategies have not been widely used for the clinical treatment of bone defects. Recently, studies have focused on strategies that recapitulate the process of ECO. In fact, several studies successfully demonstrated the capacity of *in vitro* tissue-engineered cartilage to undergo ECO and generate bone tissue *in vivo*.[17-19] Previous studies showed that chondrogenically primed constructs seeded with chondrocytes isolated from chick embryos,[21] human embryonic stem cells,[10] and human MSCs[10,22] act as osteoinductive templates and transform into bone tissues following implantation in rodent animal models. However, to date, bone repair using iPSCs through the process of ECO has not been attempted.

Here, we investigated whether implantation of chondrogenically differentiated human iMSCs could lead to the regeneration of radial bone defects in nude mice through ECO.

Methods

For more detailed procedures, please see the Supplementary Methods.

Derivation of human iMSCs

The culture of human iPSCs was approved by the institutional review board of Kobe University, Graduate School of Medicine (#26–41). Two clones of human iPSCs, 201B7[11] and 454E2,[23] were used in this study. MSCs were generated from iPSCs using a modified "direct-plating method" omitting the embryoid body (EB) formation step.[24-26] We referred to the cells obtained via this direct-plating method as iMSCs. The morphology of the different cell colonies is shown in Fig. 1A–C.

Surface antigens and multi-differentiation potential of iMSCs

The surface antigen profiles of iMSCs (n = 3 per iMSCs derived from 201B7 and 454E2) at passage 5 were characterized by flow cytometry. The multipotency of iMSCs was evaluated histologically. Differentiation (*in vitro*) into osteogenic, adipogenic, and chondrogenic lineages was investigated as previously reported.[9,10]

Immunohistochemistry of chondrogenically differentiated iMSC pellets

Chondrogenically differentiated iMSC pellet sections were stained with anti-human type collagen II (Col II), type X collagen (Col X), VEGF, and stromal cell-derived factor 1 (SDF-1) antibodies.

In vivo experiments

All animal procedures were performed under the approval and guidance of the Animal Care and Use Committee of our institution. BALB/cAJcl-nu/nu mice (CLEA, Tokyo, Japan) were used. A 2-mm nonunion defect was created in the radius of each mouse, as previously described (Fig. 2A).[27,28] Thereafter, chondrogenically differentiated iMSC pellets were transplanted into the defects; animals in the control group were treated with saline (Fig. 2B).

Reverse transcription polymerase chain reaction

At week 2, the newly generated tissues around implanted pellets in the defect site were harvested. The expression of human osteocalcin (*OC*), type I collagen (*Col I*), *Col II*, *Col X*, *VEGF*, and *SDF-1* was analyzed by reverse transcription polymerase chain reaction (RT-PCR). Human glyceraldehyde 3-phosphate dehydrogenase was used as an internal control for normalization.

Tissue staining

Histological, immunofluorescence, and immunohistochemistry analyses were carried out 2, 4, and 8 weeks post implantation using tissue sections from the radii of the different groups of animals.

Analysis of bone regeneration via micro-computed tomography

Bone regeneration was assessed by micro-computed tomography (μ-CT) imaging at 8 weeks post-implantation by three examiners blinded to the experimental details. Bone union was defined as the absence of a bone gap or presence of a bridging callus at three of four cortices in three dimensional μ-CT images.[29] Bone formation parameters, including the total callus

volume (TV), bone surface (BS), tissue mineral density (TMD), and volumetric bone mineral density (vBMD), were also determined.

Statistical analysis

Fisher's exact test was used to compare the bone union rates between the 201B7 or 454E2 group and control group. Kruskal-Wallis test followed by Bonferroni-corrected Mann-Whitney U post-hoc analysis was used to compare the capillary density and μ-CT measurements among the three groups. Statistical significance was set at $p < 0.05$.

Results

In vitro study

Characterization of human iMSCs

Flow cytometry analysis revealed that iMSCs were consistently positive for the MSC-related markers CD29, CD44, CD105, and CD166 but negative for the hematopoietic stem cell markers CD31, CD34, CD45, and CD133 (Table 1). Notably, iMSCs showed low expression of the pluripotent markers TRA-1-60 and TRA-1-81, suggesting the loss of pluripotency.

At 21 days after osteogenic induction of iMSCs, Alizarin Red S staining revealed a mineralized matrix rich in calcium deposition (Fig. S1A). Additionally, Oil red O staining showed fat vacuole accumulation in iMSCs at 21 days after adipogenic induction (Fig. S1B), whereas, at 21 days after the chondrogenic induction of 3-D cultures, the iMSC pellet was spherical (1–2 mm in diameter) and glistening transparent in appearance with a uniform distribution of proteoglycan deposition as revealed by Safranin-O staining (Fig. 3A). These results suggest that iMSCs can differentiate into osteogenic, adipogenic, and chondrogenic cells with multi-differentiation potential.

Immunohistochemistry of chondrogenically differentiated iMSCs

Further evidence of chondrogenesis in the context of iMSCs was provided by Col II immunohistochemistry staining (Fig. 3B). The patterns of Col II deposition corresponded to those observed by Safranin O staining (Fig. 3A). Additionally, Col X deposition within the pellets was observed as weak staining, indicating a certain degree of hypertrophy in the pellets (Fig. 3C). Notably, positive staining for VEGF and SDF-1 was observed in and around the chondrocytes (Fig. 3D, E).

In vivo study

RT-PCR analysis of bone defect sites

In the grafted groups, RT-PCR analysis revealed the positive expression of human *Col II*, *Col X*, *VEGF*, and *SDF-1*, but not of human *OC* and *Col I* in the grafted bone defect sites (Fig. 4). In contrast, in the control group, no expression of human genes was detected. In all groups, the positive expression of mouse counterpart genes was detected (Fig. S2).

Capillary density in bone defect sites

Visualization of the capillaries via immunohistochemistry staining of isolectin B4 in tissue samples collected at 2 weeks post-implantation revealed greater angiogenesis surrounding the implanted pellets in the grafted groups (Fig. 5A), with the capillary density significantly higher than that in the control group ($p < 0.05$; Fig. 5B). There was no significant difference between the 201B7 and 454E2 groups.

Immunolocalization of mouse OC (mOC) in bone defect sites

At 2 weeks post-implantation, immunoreactivity against m*OC* was detected around the implanted pellets (Fig. 5C). Moreover, in the control group, immunoreactivity of m*OC* was

245 detected at cortical bones along the edge of bone defect sites.

246

247 *Radiographic assessment of bone regeneration*

248 μ-CT analysis performed at 8 weeks post-implantation showed striking bone formation in
249 the defect sites in the grafted versus control groups. Regarding the rate of bone union, 11/11
250 (100%) radii in the 201B7 group and 7/10 (70%) radii in the 454E2 group showed bone union.
251 In contrast, only 2/11 radii (18%) showed bone union in the control group, a significantly lower
252 rate than in the experimental groups ($p < 0.05$; Fig. 6A). Quantification of bone formation
253 revealed that the TV and BS in the defect sites in the 201B7 and 454E2 groups were higher
254 than those in the control group ($p < 0.05$), whereas no significant differences was observed
255 with respect to the TMD and vBMD among the three groups (Fig. 6B).

256

257 *Histological assessment of bone regeneration*

258 At 2 weeks post-implantation, the grafted pellets were still visible in the defect sites,
259 indicating their survival (Fig. 7A); they formed a cartilage template that bridged the defect.
260 Within the pellets, chondrocytes differentiated into hypertrophic chondrocytes, as indicated by
261 larger lacunae with smaller uniform nuclei. At the outer rim of the pellets, the extracellular
262 matrix was not stained with Safranin-O, suggesting the beginning of chondrocyte maturation.
263 Additionally, around the pellets, newly formed woven bone was observed. The presence of
264 remnants of hypertrophic cartilage next to the newly formed bone suggested that bone
265 formation occurred through an ECO process. In contrast, in the control group, the defect site
266 was filled with fibrous tissue.

267 Furthermore, at 4 weeks post-implantation, the cartilage template was reduced in size in
268 the experimental groups; newly formed woven bone was predominant in the defect site. New
269 vessels were surrounded by a matrix of woven bone and the hypertrophic chondrocytes

transitioning into newly formed bone indicated the progression of ECO. In contrast, in the control group, fibrous tissue remained in the defect site (Fig. 7B).

At 8 weeks post-implantation, the pellets were completely resorbed and replaced by bone in the experimental groups; complete bone union was observed overall, with evidence of lamellar-like bone formation. We even observed bone marrow-resembling tissues in some bone lacunae. In contrast, in the control group, the remaining gap was filled with fibrous tissue (Fig. 7C).

Overall, similar histological findings were observed in both the 201B7 and 454E2 groups. No evidence of teratoma formation was found in any mice implanted with iMSC pellets (Fig. 7A–C). The cartilage area at week 2 was significantly higher in the experimental groups than in the healthy group ($p < 0.05$; Fig. S4).

Donor-derived bone formation: immunohistochemistry analysis

To determine the contribution of the transplanted human chondrogenically differentiated iMSCs to new bone formation, immunohistochemistry using human-specific anti-osteocalcin and anti-nuclear monoclonal antibodies that do not cross-react with murine antigens was performed (positive control images are shown in Fig. S3). At 2 weeks post-implantation, immunoreactivity against human nuclei was not detected outside of the pellets in the experimental groups (Fig. 8A). However, at 4 weeks post-implantation, immunoreactivity against human nuclei was detected in the newly formed bone around the pellets (Fig. 8B). This was also evident at 8 weeks; immunoreactivity was observed in osteocytes within the newly formed bone (Fig. 8C).

In contrast, immunoreactivity against human OC was not detected in the experimental groups at 2 weeks post-implantation (Fig. 9A). However, at 4 weeks, osteoblast lining cells in the newly formed bone around the pellets were positive for human OC (Fig. 9B), whereas at 8

295 weeks, immunoreactivity against human OC was observed in osteocytes within the newly
296 formed bone (Fig. 9C).
297
298 **Discussion**
299 Recently, several *in vitro* methodologies have been developed to derive functional,
300 multipotent MSCs from human iPSCs and embryonic stem cells, including an intermediate step
301 using EB formation,[14,28] co-culture,[30] and cell sorting.[30,31] In this study, using a simple direct-
302 plating method without the need for EB formation or cell selection,[24-26] we successfully
303 generated MSCs from human iPSCs; these cells showed typical MSC characteristics.[14,28]
304 iMSCs exhibited a uniform fibroblastic morphology and expressed high levels of typical MSC
305 cell surface markers (CD29, CD44, CD105, and CD166) but did not express hematopoietic
306 lineage markers (CD31, CD34, CD45, and CD133). iMSCs also possessed multilineage
307 potential, differentiating toward the osteogenic, adipogenic, and chondrogenic lineages when
308 cultured in the presence of lineage-specific differentiation factors.[8,9] These results contrast
309 those of Xu et al.,[32] who observed marked reduction of the *in vitro* chondrogenic differentiation
310 potential of iMSCs (compared with that of bone marrow-derived MSCs). This discrepancy is
311 likely related to the different methods used for iMSC derivation. Xu et al.[32] used an EB
312 outgrowth method, as EBs are known to be composed of cells with different degrees of
313 multipotency that may limit the differentiation potential.[33] Additionally, the iMSCs obtained
314 in the present study did not express the pluripotency-related markers TRA-1-60 and TRA-1-
315 81; this fact, coupled with the observation that no teratomas were formed for up to 8 weeks
316 post-implantation *in vivo*, suggests the loss of pluripotency and a reduced tumorigenic risk. As
317 iPSCs can be expanded indefinitely, the direct-plating method has considerable potential for *in*
318 *vitro* generation of large numbers of homogeneous MSCs for bone regeneration.
319 In the last decade, the "developmental engineering" paradigm: engineering "processes"

should recapitulate embryonic events rather than "tissues," was proposed in regenerative medicine.[34] Importantly, ECO during bone repair recapitulates the key process of ECO during development. Therefore, the scientific community suggested performing bone regeneration via the ECO pathway.[17-19] ECO involves a complex series of highly regulated steps, in which MSCs condense, differentiate into chondrocytes, mature to the hypertrophic state, and stimulate vascular invasion and mineralization before transforming into bone.[19] A potential advantage of using cartilage grafts via the ECO (vs. IMO) route to promote bone healing is that cartilage cells are physiologically adapted to survive under avascular, hypoxic conditions. Thus, ECO-based bone formation can circumvent the issues associated with the supply of oxygen and nutrients to large, engineered constructs. Chondrogenically primed MSCs have an inherent tendency to undergo hypertrophy.[35] Indeed, our results revealed that iMSC pellets after differentiation *in vitro* developed a cartilage matrix expressing *Col X*, a hypertrophic marker (Fig. 3C). RT-PCR analysis *in vivo* confirmed the expression of human *Col X* (Fig. 4), and histological analysis revealed hypertrophic chondrocytes within grafted iMSC pellets and the formation of woven bone around them in the experimental groups. In addition, the cartilage area in the defect site at week 2 was significantly higher in the experimental groups than in the control group (Fig. S4). These results suggest that the cartilaginous template can trigger the process of ECO following exposure to the *in vivo* environment. Collectively, from a bone-developmental engineering perspective, iMSC-based cartilage grafts recapitulating the morphogenetic process of ECO in the context of embryonic skeletogenesis is a promising strategy for repairing large bone defects.

VEGF plays a key role in ECO, affecting angiogenesis, extracellular matrix remodeling, and ossification.[36] In the later stage of ECO during bone development and repair, chondrocytes in the cartilaginous template stop proliferating, undergo hypertrophy, and synthesize Col X. Hypertrophic chondrocytes also express and release VEGF, inducing the invasion of blood

vessels into the cartilage template and facilitating bone formation.[37] In the current study, immunoreactivity against human VEGF was detected within iMSC pellets cultured for 3 weeks under chondrogenic conditions. Moreover, the expression of human VEGF was observed *in vivo* at 2 weeks post-implantation. We also found that the capillary density around the bone defect sites in the experimental groups was significantly higher than that in the control group (at week 2), suggesting that transplanted chondrogenically differentiated iMSC pellets enhanced angiogenesis and vasculogenesis. Overall, these data strongly support the initiation of ECO.

In fact, previous studies demonstrated that chondrogenically differentiated MSCs can form bone through ECO after ectopic subcutaneous implantation in rodents.[20-22] However, ectopic implantation does not fully reflect the complex environment of bone defects (e.g., nonunion); thus, this model is not ideal for assessing constructs for bone repair. Therefore, to fully determine the bone regenerative capacity in an orthotopic environment, we used a weight-bearing bone (radius) defect model.[27,28] This model has clinical relevance for treating human bone defects with large segment grafting. Several recent studies revealed endochondral bone regeneration after the transplantation of MSCs pre-differentiated into chondrogenic cells in orthotopic models.[38-42] For instance, Harada et al.[40] implanted PLGA scaffolds seeded with chondrogenically primed rat bone marrow-derived MSCs into critical-sized (5 mm) and massive full-thickness (15 mm) rat femoral defects; newly formed bone and full bone union were observed at 8 and 16 weeks post-implantation, respectively. Similarly, rapid healing was reported when chondrogenically primed human bone marrow-derived MSC pellets were implanted into a 6-mm rat femoral defect.[38] Additionally, Sheyn et al.[28] implanted undifferentiated BMP-6-overexpressing human iMSCs into 1.5-mm radial defects in mice and demonstrated complete bone regeneration by week 8. Jungbluth et al.[43] implanted undifferentiated human iSCs into 11-mm diameter and 25-mm depth “partial-thickness”

cylindrical defects in the proximal tibia of mini pigs (a model that does not mimic non-union or refractory fracture of the long bones in patients), with only 46% of bone defect consolidation by week 6. Importantly, in the current study, we demonstrated that human iMSCs can regenerate bones *in vivo* via ECO without gene-transfection in a pre-clinical orthotopic long bone defect model. We showed that in nude mice, implantation of chondrogenically differentiated iMSC pellets into 2-mm radial bone defects results in successful bone regeneration with a significantly higher bone union rate, TV, and BS in the defect sites in the grafted groups compared with those in the control group (Fig. 6).

It is unclear whether the implanted iMSCs contribute directly to bone regeneration in an autocrine manner or have an indirect effect, such as paracrine signaling-induced regeneration via the recruitment and activation of osteoprogenitor cells from host animals. In this study, paracrine-induced recruitment of host osteoprogenitor cells was suggested by the observation of mouse osteocalcin positive cells around the implanted pellets (Fig. 5C). In agreement with this, previous studies showed that the paracrine effects of growth factors and cytokines secreted by transplanted MSC pellets can promote tissue repair and regeneration.[44] One such candidate is SDF-1, which is among the most important molecules for ECO. SDF-1 plays a pivotal role in fracture healing, promoting the migration and differentiation of progenitor cells in fracture sites, and contributing to endochondral bone repair.[45,46] For instance, Kitaori et al.[45] reported that SDF-1 promotes ECO by recruiting MSCs to the injury site. Additionally, in experimental models of fracture healing, SDF-1 was expressed in pre-hypertrophic and hypertrophic chondrocytes within the fracture callus.[46,47] Murata et al.[47] also showed that SDF-1 was expressed at the growth plate and regulated chondrocyte differentiation during endochondral bone development, indicating that SDF-1 is crucial for hypertrophic conversion and the subsequent calcification of chondrocytes. In the current study, SDF-1 immunoreactivity was detected within chondrogenically differentiated iMSC pellets *in vitro* (Fig. 3E). In addition,

at 2 weeks after *in vivo* implantation, the transplanted iMSCs expressed human SDF-1 (Fig. 4). Overall, these results indicate that bone regeneration is at least partially due to the paracrine effects of implanted iMSC pellets expressing SDF-1 and consequent recruitment of host osteoprogenitor cells.

At 4 and 8 weeks post-implantation, positive immunoreactivity against human nuclei and OC was detected in the newly formed bone, indicating that the implanted iMSCs survived and directly contributed to bone regeneration (Fig. 8B–C and Fig. 9B–C). These results suggest that one of the mechanisms underlying bone regeneration is direct differentiation of iMSCs into osteoblastic cells. Such cells may have developed from the remaining iMSCs within pellets that did not undergo differentiation during chondrogenic pre-induction.[48] Another possibility is that not all chondrocytes became hypertrophic and underwent apoptosis, but rather that some cells retained the capacity to transdifferentiate into osteoprogenitor cells. Recent studies showed that during the end stages of ECO, hypertrophic chondrocytes can transdifferentiate into osteoblasts and osteocytes.[40,49] In contrast, at week 2, neither immunoreactivity against human OC (Fig. 8A) nor human *OC* expression (Fig. 4) was detected in the defect sites. Thus, at least at earlier time points, donor iMSCs are likely involved only in indirect bone formation via paracrine effects. Ultimately, the mode of action of implanted cells remains unclear. iMSC tracing experiments are required to investigate the autocrine effects of iMSCs. In the current study, we revealed the potential of implanted chondrogenically differentiated iMSCs for repairing bone defects via ECO in an autocrine/paracrine manner.

One potential limitation of our study is that although no teratoma formed during the 8-week period, iMSCs may still be tumorogenic *in vivo*. Further investigation (long-term observation) is necessary to support the safety of chondrogenically differentiated iMSCs *in vivo*. In addition, although we demonstrated successful bone regeneration using iMSCs in a murine bone defect model, further experiments in large animals are necessary before translation into human trials.

Additionally, we used scaffold-free pellet cultures to form cartilage grafts from iPSCs. iMSC pellets are inherently limited in size to approximately 1–2 mm in diameter and may not be sufficient to graft large bone defects in humans. Incorporation of iMSCs into appropriate scaffolds that can be scaled-up is required to repair larger, clinically relevant bone defects. In contrast, there was a discrepancy between the results of radiographic (μ-CT) and histological assessments at week 8. Although histology in the control group showed that the defect site was filled with fibrous tissue, there were no significant differences in the TMD and vBMD in μ-CT analysis among the three groups. In some control group samples, the radius at the resection stump was fused with the ulna. In such cases, the callus tissues were included in the region of interest, which may explain this discrepancy.

Conclusions

Overall, our data show that grafting chondrogenically differentiated human iMSCs into bone defects (in the radius) in nude mice leads to successful bone regeneration through the process of ECO. Therefore, endochondral bone tissue engineering using human iPSCs should be considered as a strategy to repair long bone defects.

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Authorship Confirmation Statement

M. Arakura: Study conception and design, Acquisition of data, Analysis and interpretation of data, Drafting and revising the article.

S.Y. Lee: Study conception and design, Analysis and interpretation of data, Drafting and revising the article.

T. Fukui: Analysis and interpretation of data, Drafting and revising the article.

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Disclosure Statement

No competing financial interests exist.

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