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Safety profile and long-term engraftment of human CD31$^+$ blood progenitors in bone tissue engineering

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Abstract

Background. Endothelial progenitor cells (EPCs) participate in angiogenesis and induce favorable micro-environments for tissue regeneration. The efficacy of EPCs in regenerative medicine is extensively studied; however, their safety profile remains unknown. Therefore, our aims were to evaluate the safety profile of human peripheral blood–derived EPCs (hEPCs) and to assess the long-term efficacy of hEPCs in bone tissue engineering. *Methods.* hEPCs were isolated from peripheral blood, cultured and characterized. β tricalcium phosphate scaffold (βTCP, control) or 10^6 hEPCs loaded onto βTCP were transplanted in a nude rat calvaria model. New bone formation and blood vessel density were analyzed using histomorphometry and micro-computed tomography (CT). Safety of hEPCs using karyotype analysis, tumorigenecity and biodistribution to target organs was evaluated. *Results.* On the cellular level, hEPCs retained their karyotype during cell expansion (seven passages). Five months following local hEPC transplantation, on the tissue and organ level, no inflammatory reaction or dysplastic change was evident at the transplanted site or in distant organs. Direct engraftment was evident as CD31 human antigens were detected lining vessel walls in the transplanted site. In distant organs human antigens were absent, negating biodistribution. Bone area fraction and bone height were doubled by hEPC transplantation without affecting mineral density and bone architecture. Additionally, local transplantation of hEPCs increased blood vessel density by nine-fold. *Conclusions.* Local transplantation of hEPCs showed a positive safety profile. Furthermore, enhanced angiogenesis and osteogenesis without mineral density change was found. These results bring us one step closer to first-in-human trials using hEPCs for bone regeneration.

Key Words: *angiogenesis, biodistribution, blood endothelial progenitor cells, bone regeneration, chromosomal aberration, karyotype, safety, tumorigenecity*

Introduction

The need for improved bone regenerative techniques in all aspects of orthopedic and maxillofacial surgery cannot be over-emphasized. Currently, bone regeneration in cases of severe alveolar bone atrophy remains a major challenge. Cell-based therapy may be the key to overcome challenges in bone regeneration and has the potential to change current treatment modalities in many fields. However, research on the safety of cell-based therapy techniques is limited.

The current gold standard surgical technique for reconstruction of large bone defects involves the harvesting of autologous bone blocks from the fibula, iliac crest, scapula and radius [1,2]. These procedures require extended hospitalization, a secondary donor site with associated morbidity and complications, as well as significant graft resorption [3]. Alternatively, the use of guided bone regeneration procedures is less invasive [4].

Guided bone regeneration involves the maintenance of space between a rigid barrier (usually a membrane) and the underlying bone that prevents migration of epithelial and fibroblastic cells from the surrounding soft tissue, thus enabling the slower-moving bone-forming cells to migrate from the underlying bone to populate the space [3]. Guided bone regeneration is commonly used to enhance bone regeneration within the bony envelope (intra-bony) [5]. In contrast, the use of guided bone regeneration for achieving extra-cortical bone augmentation is very

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limited and still unpredictable with minimal extra-cortical bone gain [6]. These unexpected results can be attributed to insufficient blood supply to the graft, leading to inadequate amounts of osteoprogenitor cells. Therefore, angiogenesis plays a pivotal role in successful bone regeneration [7,8].

Numerous techniques to enhance vascularity to the graft have been investigated. One of these techniques is cell-based therapy that uses endothelial progenitor cells (EPCs). Circulating EPCs were discovered in 1997 by Asahara *et al.* [9]. EPCs participate in neovascularization [10], angiogenesis, vascular repair, blood-flow recovery after tissue ischemia, distraction osteogenesis [11], fracture healing [8] and bone regeneration [12–14]. EPCs are mainly located in bone marrow and mobilize into the circulation under the guidance of signals, such as granulocyte colony–stimulating factor (G-CSF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), placental growth factor, erythropoietin or stromal-derived factor-1 (SDF-1), to home to ischemic sites [15]. EPCs can be isolated from peripheral blood, cord blood or bone marrow [16] and are classically described as cells expressing a combination of an endothelial marker (CD31/VEGF receptor-2) and hematopoietic progenitor marker (CD34/CD133) [17].

The relatively easy isolation of EPCs from patients' own peripheral blood is an advantage, however, because EPC concentration in the blood is minimal [18]; *ex vivo* expansion is required to reach an adequate amount of cells viable for transplantation. Before EPCs can be used in clinical applications to treat bone defects, the safety profile of transplanted cells and long-term efficacy must be determined. Transplant safety issues include *in vitro* DNA stability during cell culture and *in vivo* unexpected migration of the transplanted cells [19,20] as well as the transformation of graft-derived progenitors into malignant tumor cells [21–24].

Therefore, the primary aim of the current study was to evaluate the safety profile of human peripheral blood–derived EPCs (hEPCs) *in vitro* and *in vivo*. The secondary objective was to assess long-term efficacy of hEPCs in extra-cortical bone regeneration.

Materials and methods

The study protocol was initially approved by the committee for the supervision of animal experiments at the faculty of Medicine, Technion, I.I.T. (approval # IL0530412), and by the Helsinki committee of Rambam Medical Center.

Isolation and expansion of hEPCs

For isolation of hEPCs, 50 mL blood was obtained from four healthy volunteers who signed an informed consent. Pooled peripheral blood was collected into sterile heparinized tubes and hEPCs were isolated as previously described for sheep EPCs [25] and rat EPCs [26]. Briefly, blood was diluted 1:1 with phosphate-buffered saline (PBS). Mononuclear cells (MNCs) were isolated with density gradient centrifugation (Lymphoprep, Axis-Shield) and pelleted cells were resuspended in Endothelial Basal Medium (EBM-2) containing 20% heat-inactivated fetal bovine serum (FBS), penicillin-streptomycin (Biological Industries Ltd.) and supplemented with Endothelial Growth Medium (EGM-2MV SingleQuote; Clonetics, Cambrex Bio Science) that includes: vascular endothelial growth factor, fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor-1 and ascorbic acid. Cells were seeded on six-well plates coated with 5 $\mu g/cm^2$ of fibronectin (Biological Industries Ltd.) and grown at 37°C with humidified 95% air/5% CO_2. After 4 days of culture, nonadherent cells were discarded by gentle washing with PBS, and fresh medium was applied. The attached cells were continuously cultured with complete EGM-2 medium. Cells were fed three times per week and were split when they reached ~80% confluent by brief trypsinization using 0.5% trypsine in 0.2% ethylenediaminetetraacetic acid (EDTA; Biological Industries Ltd.).

Characterization of hEPCs

EPC were characterized using flow cytometry (fluorescence-activated cell sorting, FACS) using fluorescein isothiocyanate-labeled antibodies specific for: CD14, CD34 (mouse anti-human, BD Biosciences) and CD31 (LifeSpan BioSciences), vascular endothelial growth factor receptor 2 (VEGFR-2) (mouse anti-human, BD Biosciences) and human/mouse pluripotent stem cells (R&D Systems). In this study, 5×10^5 cells in PBS were incubated 30 min with antibodies according to the manufacturers' recommendations. Negative controls were mouse immunoglobulin (Ig)G1 isotype (BD Biosciences). Following washings ×3, cells were resuspended in PBS and analyzed using FACScan and CellQuest software (Becton Dickinson & Co.).

Karyotype analysis

Karyotyping was performed by the Cytogenomic Services Facility of Rambam Health Care Campus, Haifa, Israel, to analyze the karyotype of hEPCs. Cells were cultured and harvested at passage four and seven. Cell division was blocked in metaphase by adding 0.05 mg/mL colcemid (Invitrogen) for 1–2 h. The chromosomes were then observed by Giemsa produce G-banded-banding.

Coating of β-tricalciumphosphate with fibronectin

Based on our previous results [27], synthetic β-tricalciumphosphate (βTCP) granules (0.6–1 mm

grain size, 40% porosity and 100–200 μm pore size; Poresorb-TCP, Lasak Ltd.) were chosen as the scaffold for the present study. To enable attachment of cells, 0.2 g βTCP were coated with 50 μg fibronectin (Biological Industries Ltd.) [12].

hEPC local transplantation: a nude rat calvaria model

Twelve male nude (athymic) rats (Hsd: RH-FoxN1^{RNU}; 13 weeks; ~300 g) were used to allow xenogenic transplantation of human cells. Rats were anesthetized by intramuscular injection of 100 mg/kg body weight (bw) Ketamin (Ketaset) and 5 mg/kg bw Xylasin (Eurovet). Fifty mg/kg bw Cephalexin (Norbrook Laboratories) and 0.3 mg/kg bw Buprenorphine (Vetamarket) were injected subcutaneously (sc) preoperatively and 3 days postoperation. Surgical procedure was performed as previously described [26]. Briefly, a U-shaped incision served to raise a full thickness skin flap and exposure of the parietal bone. Five perforations (1 mm diameter) of the cortical bone were performed to allow passage of blood, cells and nutrients from the bone marrow into the space under the dome. Next, 10^6 hEPCs (n = 6) suspended in 50 μL medium or 50 μL medium without hEPCs (control, n = 6) were mixed with fibronectin-coated βTCP and transplanted immediately under rigid gold domes (7 mm radius; 5 mm height). The domes were secured to the calvarium using fixation screws. Surgical flaps were repositioned and horizontal mattress sutures were performed. Each rat was kept in a separate cage and fed rat chow and water *ad libitum* for 5 months. Then, rats were humanely killed by CO_2 asphyxiation and the domes were removed. Target internal organs (lungs, heart, liver, kidneys and spleen) were harvested and screened macroscopically for pathological changes and then fixated with 4% paraformaldehyde for further histopathologic examination. The part of the calvarium surrounding the regenerated area was sawed out and specimens were fixed immediately in 4% paraformaldehyde for 2 days.

Tail vein injection of hEPCs in SCID mice model

In this study, 10^8/kg hEPCs suspended in 100 μL medium were injected into the tail vein of two SCID mice. Mice were weighed and monitored for 5 months duration. At 5 months 1 mL blood was drawn directly from the heart and mice were humanely killed via a lethal dose of Ketamine. Target organs were collected and fixed in 4% paraformaldehyde for 2 days.

Histology

Fixed calvaria specimens were decalcified in 10% EDTA (Sigma-Aldrich) for 4 weeks, cut into two halves in the midline, embedded in paraffin and sectioned (5 μm). For determination of bone morphology, sections were stained with Masson's trichrome and hematoxylin and eosin (H-E). Fixed internal organs were embedded in paraffin, sectioned (5 μm) and stained with H-E.

Immunohistology

Sections obtained from nude rat calvaria were immunostained with human-specific mouse monoclonal antibody CD31 that do not react with rat CD31 (1:70, Thermo Scientific) and with rabbit anti-rat CD73 (1:1000, Novus Biologicals). H-E stain was used for general morphology. Internal organs were stained with CD31 (1:70, Thermo Scientific) and human nuclear antibody (1:400, abcam) to follow migration of the transplanted human cells into target organs. Quantitative analysis of immunohistochemistry results was performed using image-Pro software.

Histomorphometric analysis

Four Masson's trichrome–stained sections (~20 μm apart) from each calvaria specimen were scanned using a panoramic digital slide scanner (3DHISTECH panoramic MIDI). For each specimen we calculated the means of: (i) extra-cortical bone height: maximal gained bone height (in mm) measured from the top of the calvaria and (ii) bone area fraction: percentage of bone from the overall regenerated tissue under the dome. These parameters were analyzed morphometrically using image-Pro software.

Blood vessel density

Luminal structures perfused with red blood cells were identified as blood vessels. Ten sections were evaluated for each specimen. Blood vessels density was defined as mean number of blood vessels in microscopic field (260 × 444 μm).

Micro–computed tomography scanning

All specimens were scanned in a desktop micro–computed tomography (CT) system (Scanco μ40) at 80 V and 80 μA, with a 200 ms integration time. The image resolution was 20 μm/voxel. Each scan included a phantom containing regions of different hydroxyapatite densities for conversion of attenuation to mineral density (in mgHA/ccm). Specimen-specific thresholds to identify the βTCP and, separately, the mineralized tissue were chosen by identifying the peak in the attenuation histogram that corresponded to mineralized tissue and then setting the upper and lower thresholds as the local minima that bounded this peak. A region of interest (ROI) was defined in the

center of the specimens as follows: a cylinder (4 mm diameter) was defined in the middle of the dome extending from the calvaria to the apex of the tissue. This area was chosen because it represents the central area where dental implants are likely to be placed following such a regenerative procedure. Bone volume/total volume fraction (BV/TV), tissue mineral density (TMD) and βTCP volume fraction were calculated for the whole cylinder.

Statistical analysis

A StatPlus statistical package (AnalystSoft) was used. Descriptive statistics that included means and medians, ranges and standard deviation (SD) were initially tabulated. Comparisons between hEPCs and control groups were performed using unpaired Student *t* tests. A 5% significance level was chosen.

Results

Isolation, expansion and characterization of hEPCs

In this study, 50 mL of blood was drawn from healthy volunteers. MNCs were separated using ficoll density gradient and seeded on fibronectin-coated plastic plates. Then, 3–4 weeks after seeding, polyhedral cells appeared and rapidly replicated to form colonies (Figure 1a). Self renewal was preserved for at least 10 passages. FACS analysis revealed that >96% of the cells were CD31-positive and 48% were VEGFR-2–positive endothelial cell markers. Furthermore, 38.8% of the cells were CD34-positive early hematopoietic and vascular-associated tissue cell markers. Cells were also positive to pluripotent markers: SOX2 and OCT 3/4 (95.6% and 47.8%, respectively). Conversely and as important, cells were negative for CD14 (a hematopoietic cell marker; Figure 1b).

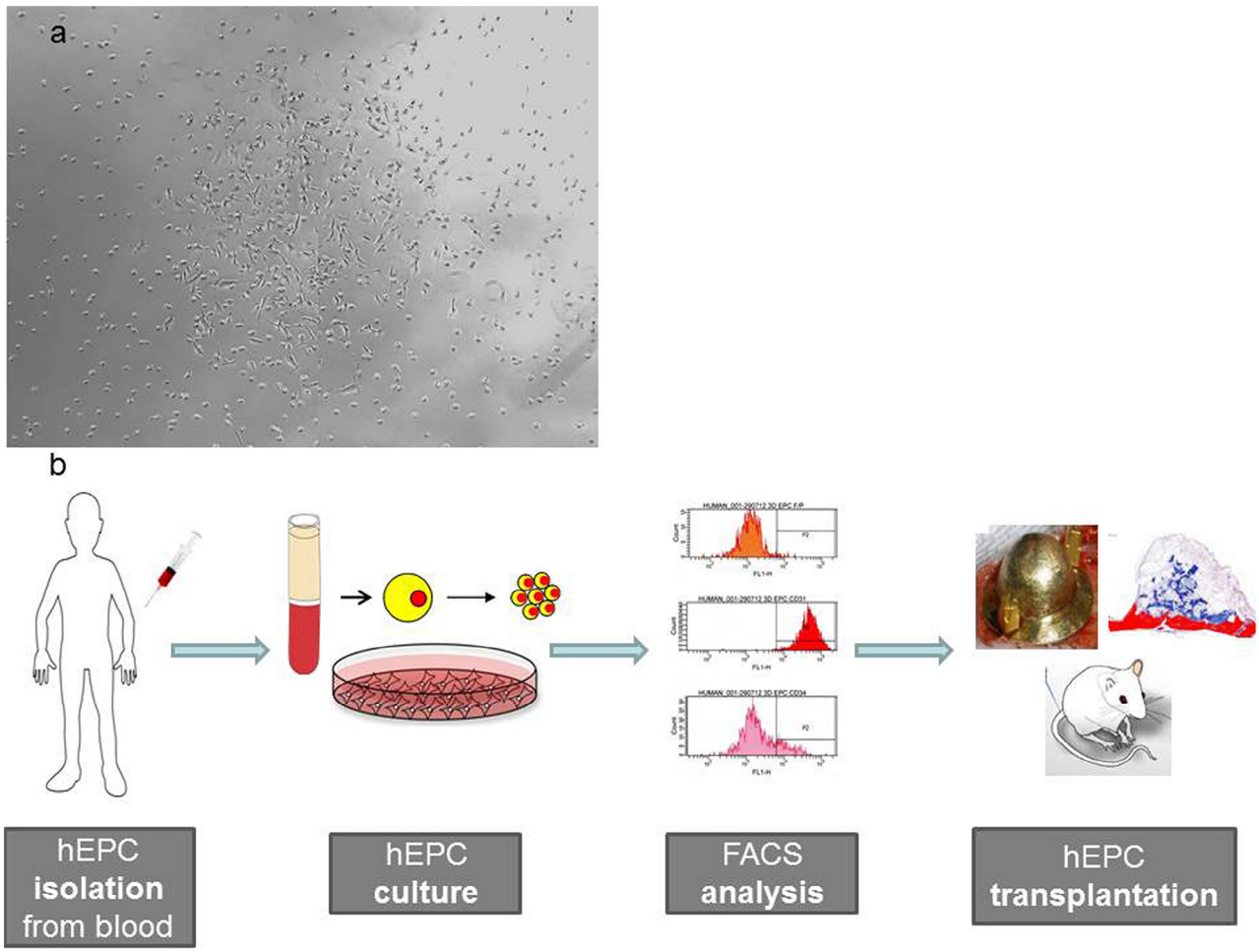

Figure 1. hEPC: culture, characterization and transplantation. (a) hEPCs colony was observed 17 days after isolation and culture. (b) Schematic of the study design: blood drawn from human volunteers, mononuclear fraction separated and seeded onto fibronectin-coated plates. Cells were then cultured and characterized using FACS. Cells mixed with TCP scaffold and transplanted under a gold dome anchored to rats' calvaria. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In vitro *and* in vivo *safety analyses*

DNA stability

hEPCs were expanded for seven passages and reserved their morphological characteristics. Moreover, karyotype analysis revealed that hEPCs retained their normal karyotype during cell expansion. Chromosomal aberrations were not found (Figure 2).

In vivo *tumorogenic potential and biodistribution of hEPCs following local transplantation*

Target organs collected from nude rats that were treated with local hEPC transplantation in a calvaria model were intact without any macroscopic signs for abnormal tissue growth or other pathological changes. Histopathologic examination of the target organs showed normal morphology in all the slides that were examined, therefore, excluding the presence of atypical cellular aggregations of pathological tissue growth. Specimens were also negative for anti-human CD31 and human nuclear antibody (HNA) ruling out migration of the transplanted cells to distant organs (Figure 3 and Figure 4a–4f).

In vivo *tumorogenic potential and biodistribution of hEPCs following intravenous injection*

SCID mice continued to gain weight and to display normal behavior during the 5-month follow-up period. Blood counts were within the normal ranges for SCID mice [28]. Histological analysis of target organs displayed no tumor formation, however, in one mouse micro-calcifications on the pericardium were observed. Immunohistochemical staining for human CD31 and HNA were both negative (Figure 4g–4j).

Bone formation

To evaluate the *in vivo* osteogenic potential of hEPCs, 10^6 cells were loaded onto βTCP scaffold and transplanted under a rigid gold dome that was fixed to the underlying calvaria. One rat (hEPCs group) did not survive the surgical procedure. For the remaining animals, healing was uneventful. All rats gained weight, presented normal behavior and survived the entire 5 months' duration of the study. Following humane killing, all rats presented with newly formed hard tissue connected to the underlining bone and filled the space under the dome.

Histological sections revealed that the space under the dome was filled with tissue composed of bone, residual scaffold and connective tissue. The newly formed bone was continuous with the original calvaria (Figure 5a and 5b) and presented normal bone architecture characterized by the presence of osteocytes in lacunae, osteoblasts, osteoclasts and reversal lines. Particles of residual scaffold were surrounded by mature lamellar bone (Figure 5c and 5d). Palisading of osteoblasts was observed adjacent to the newly formed bone and in close proximity to blood vessels.

Histomorphometric analysis of the mineralized tissue revealed that hEPCs significantly enhanced bone formation. In the control group (βTCP alone), newly formed bone did not reach the top of the dome in any of the specimens (dome height = 5 mm), whereas bone filled the entire dome in all specimens in the hEPC group. Bone height was increased by two-fold in the hEPCs group compared with the control group ($P \leq 0.001$; Figure 5e). In addition, overall bone area fraction (BA) was significantly higher in the hEPC group compared with the control group ($P \leq 0.01$; Figure 5f).

Similar to the histomorphometric findings, the micro-CT analysis revealed that the 3-D measurements of bone volume fraction in ROI was higher in the hEPC group compared with the control group (42.31 ± 4.49 vs 46.55 ± 4.38), however, these differences did not reach statistical significance (Figure 6a–6d). Bone mineral density and βTCP volume fraction were not affected by cell transplantation (Figure 6e–6g).

Angiogenesis

The angiogenic effect of hEPCs was also evident macroscopically; the newly formed blood vessels were evident on the surface of the mineralized tissue, whereas in the control specimens blood vessels were scarce.

Blood vessel density

To quantify the angiogenic effect of hEPCs, luminal structures perfused with red blood cells were counted on histology slides. Blood vessel density in the regenerated tissue was nine-fold higher in the hEPC group compared with the control group ($P \leq 0.0001$; Figure 7a–7c).

Engraftment of transplanted cells in the regenerated tissue

Immunohistological labeling of the transplanted (CD31+) cells revealed integration of hEPCs in blood vessel walls adjacent to the newly formed bone (Figure 8a and 8b). However, most of the vessels in the regenerated tissue lacked human antigens. CD31+ stained/unstained cells ratio was 0.028 ± 0.024 in the test and 0 in the control group.

Recruitment of resident CD73$^+$ cells to the regenerated tissue

Cells that stained positively for CD73 (one of the surface markers of mesenchymal stromal cells [MSCs]) were found in the test and control specimens. However, a stronger positive stain was observed in the test (hEPC) group compared with the control group

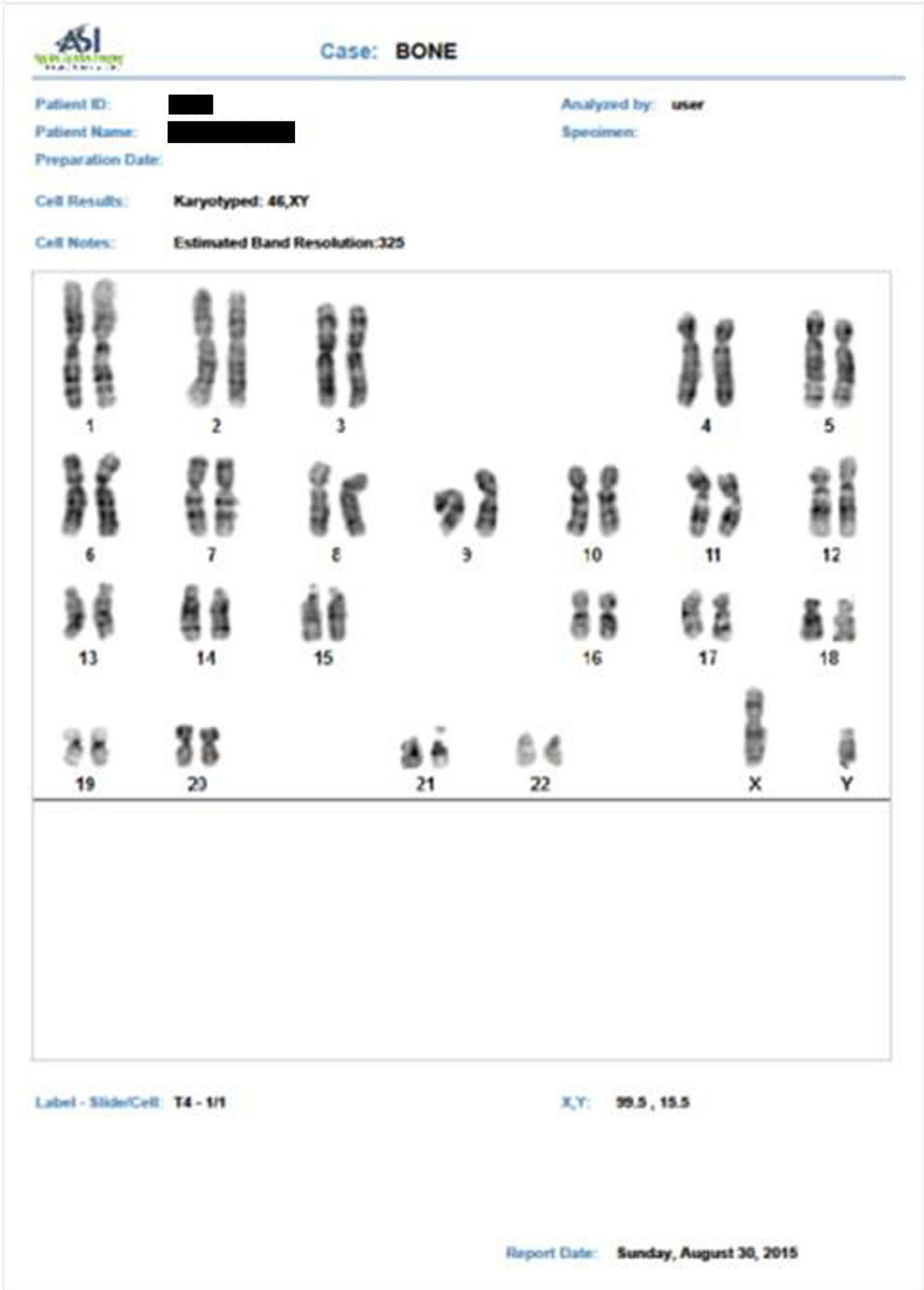

Figure 2. Karyotype analysis of cultured and expanded hEPC passages four and seven revealed that the cells retained their normal karyotype. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

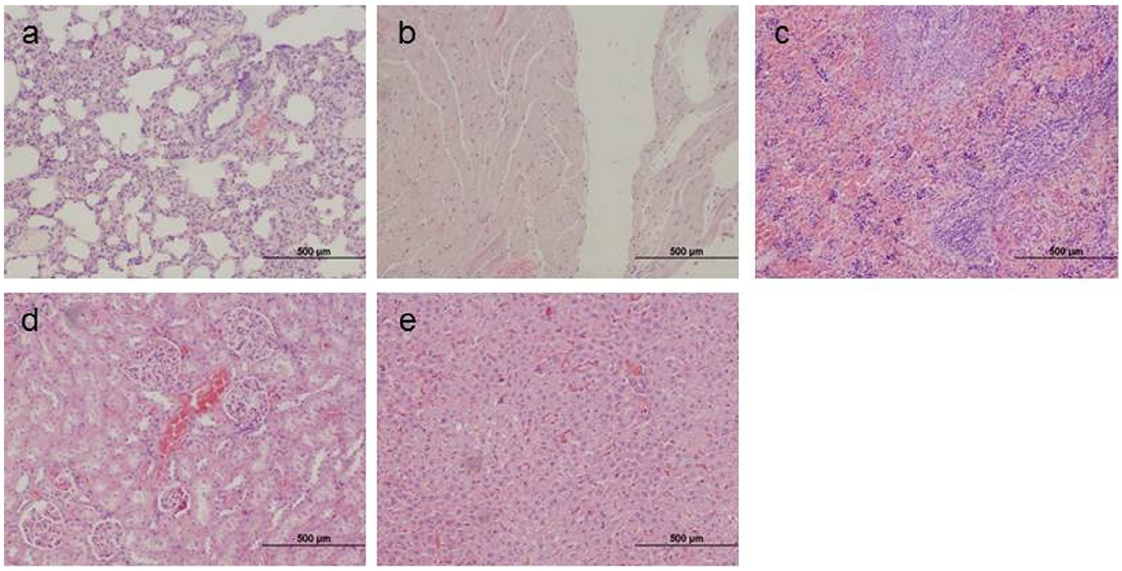

Figure 3. Histological evaluations of target organs. (a–e) Lung, heart, spleen, kidney and liver from local hEPC transplantation in nude rat calvaria model displayed normal morphology. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

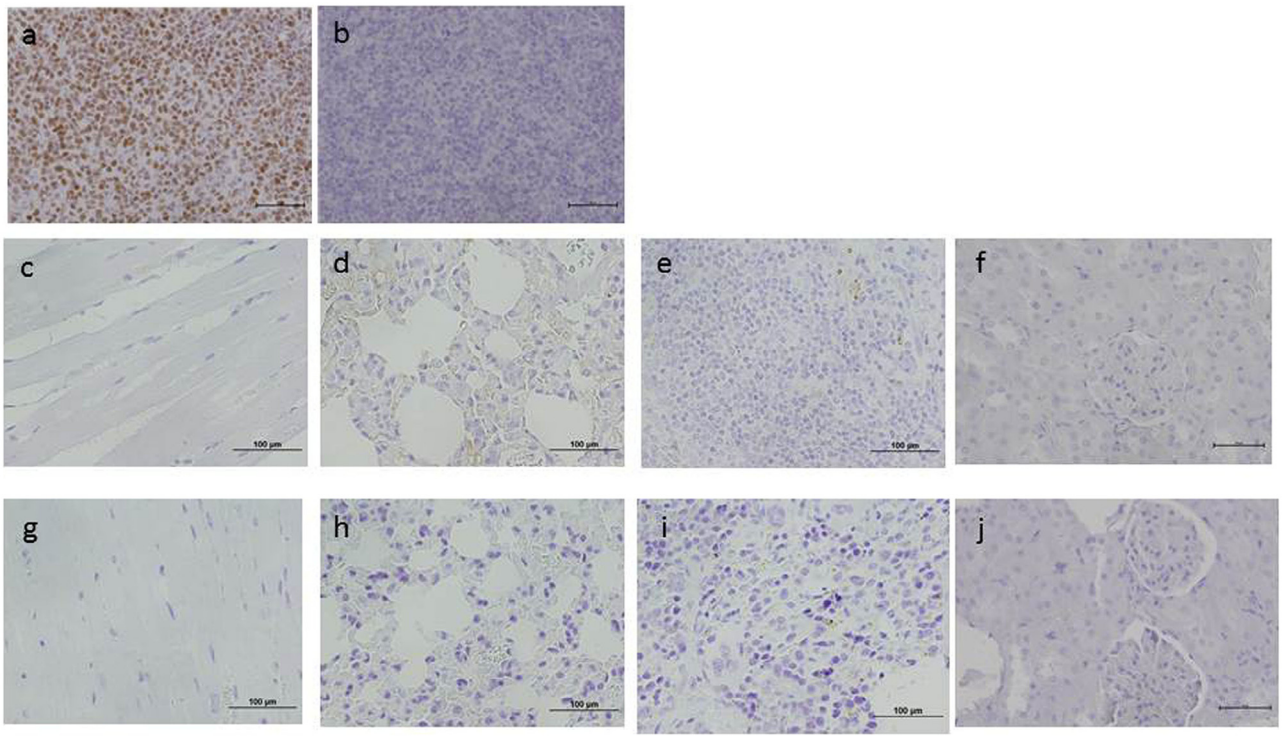

Figure 4. Evaluation of biodistribution of hEPC using human nuclear antibody immunohistochemistry. (a, b) Human tonsil tissue was used as positive control. (a) Primary and secondary antibodies. (b) Secondary antibody. (c–f) Organs obtained from rats treated with sc transplantation of hEPCs (primary and secondary antibodies). (c–f) Heart, lung, spleen and kidney, respectively. (g–j) Organs obtained from mice treated with intravenous injection of hEPCs (primary and secondary antibodies). (g–j) Heart, lung, spleen and kidney, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

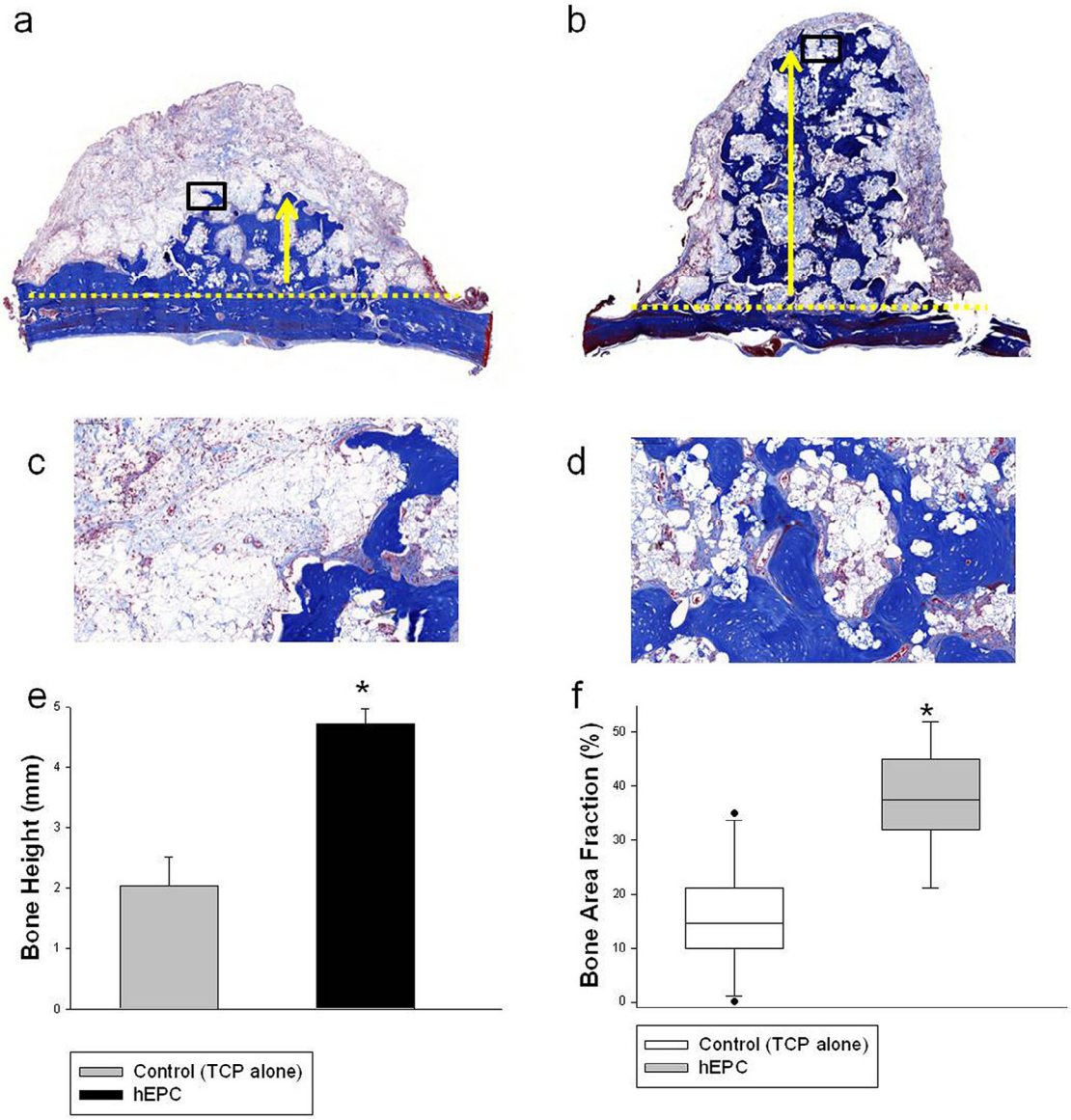

Figure 5. Histology and histomorphology. (a and b) Specimens stained with Masson's trichrome demonstrating new bone formation (bone stained blue). An arbitrary dotted line separates the original calvaria from the augmented tissue (×2 magnification). Extra-cortical bone height gain (arrows). In the hEPC group bone filled the entire space under the dome (b), whereas bone gain in control group (a) was limited. (c) Higher magnification of the insert in Figure 5a. In the control group the top of the regenerated tissue contains mainly connective tissue and scaffold. (d) Higher magnification of the insert in Figure 5b. In the hEPC group the top of the regenerated tissue contains mainly bone. (e and f) Histomorphometric measurement of bone height (e) and bone area fraction (f). $*P \leq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Figure 8c and 8d). CD73-positive cells were found adjacent to the newly formed bone and in the external borders of the residual βTCP scaffold.

Discussion

It is well established that EPCs initiate vasculogenesis [10], and, as such, these cells may play a critical role in pathological vasculature, such as in tumor growth [29] and metastasis [30]. Our perspective was to use the angiogenic potential of EPCs to enhance osteogenesis. To evolve therapeutic cell engineering techniques using autologous human peripheral blood cells, there is a need to evaluate not only the efficacy but also the safety of such treatment. While a concerning risk related to EPCs has been tumor formation, we did not find any evidence of this in our study. In the last decade promising results were demonstrated using sheep [25], rat [26] and human EPCs for bone reconstruction in several animal models [12,31]. However, these studies followed bone healing for a short period of up to 3 months and did not follow bio-distribution

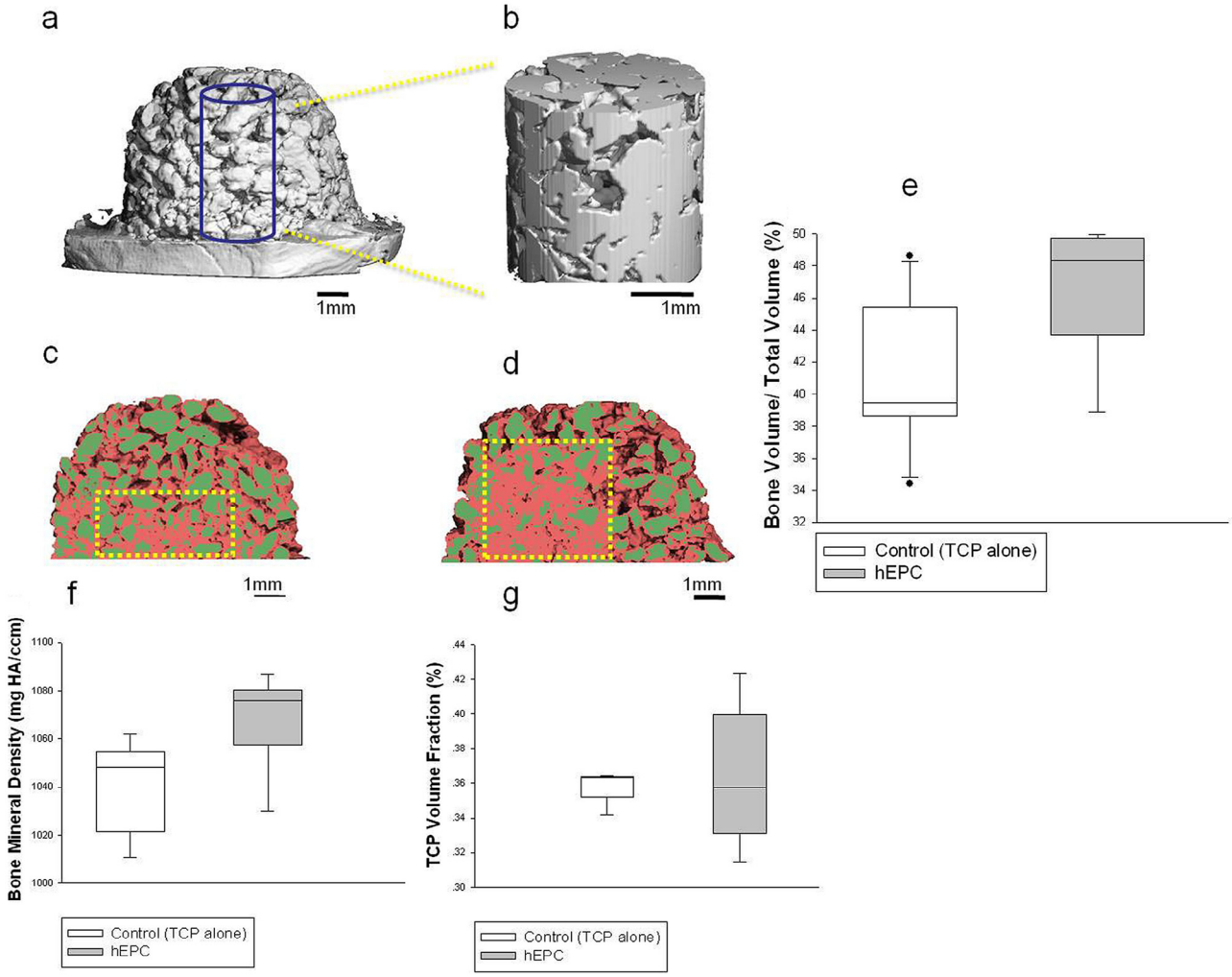

Figure 6. Micro-CT analysis. (a and b) A cylinder (4 mm diameter) was defined in the middle of the dome extending from the calvaria to the apex of the tissue. (c and d) Midsection of control (c) and hEPC (d) demonstrating new bone gain (red) and scaffold (green). (e, f and g) Measurements of bone volume fraction (BV/TV), bone mineral density (BMD) and βTCP volume fraction that were calculated for the whole cylinder. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and tumorigenesis following EPC transplantation. Recent clinical studies demonstrated an increase in the amount of circulating EPCs in patients suffering from malignant tumors and in cases of invasive or recurrence of the disease [32]. On the other hand, clinical trials that assessed the safety of hEPC transplantation have shown no severe adverse events during and after cell therapy [33]. However, these clinical trials were performed on G-CSF mobilized peripheral blood cells and without *ex vivo* cell expansion [34]. Although our study has a small sample size, with local hEPC transplantation, all target organs were intact without any macroscopic signs for abnormal tissue growth or other pathological changes. Histological examination of target organs displayed normal morphology, therefore, excluding the presence of atypical cellular aggregations of pathological tissue growth. Furthermore, immunohistochemical analysis revealed that all specimens were negative for CD31, ruling out migration of the transplanted cells into distant organs. No animals showed signs of infection and the only rat that did not survive the treatment died during the surgical procedure. Although our study could not evaluate immune reaction owing to the use of the nude athymic rat model (immune-deficient), nude rats are considered the ideal model for tumor investigation [35]. On a tissue level, tumor growth was not evident in any of these animals. On a cellular level, hEPCs kept their normal karyotype during expansion (up to seven passages). We were unable to perform karyotype analysis on passages higher than seven due to cell senescence. Unlike embryonic stem cells, MSCs and immortalized cells, EPCs (similar to human umbilical vein endothelial cells [HUVECs]) have more restricted

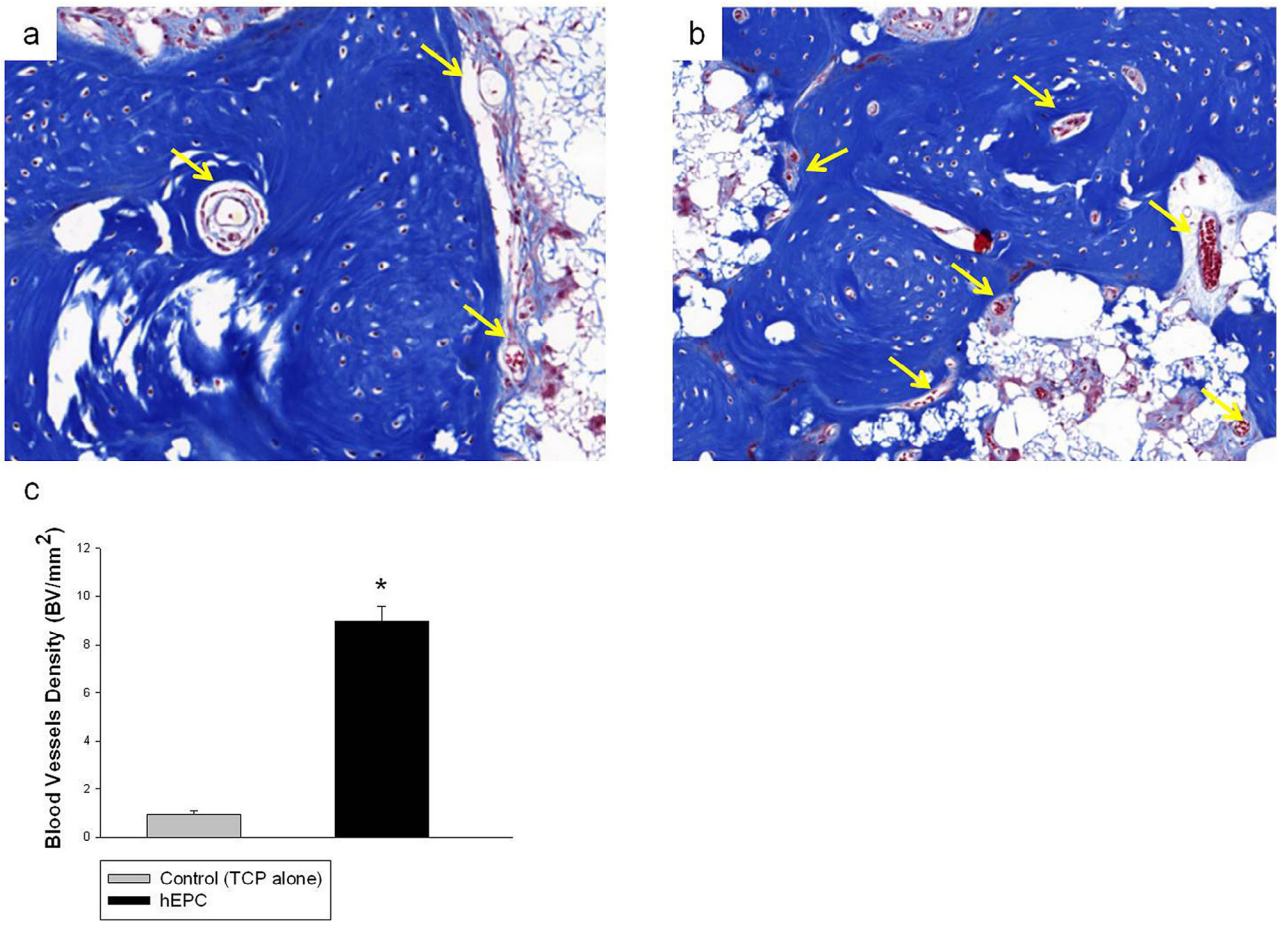

Figure 7. Blood vessel density. (a and b) Blood vessels (arrows) adjacent to the newly formed bone in control (a) and hEPC (b). (c) Quantification of blood vessel density BV/mm^2. *$P \leq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

self-renewal capacity and usually are used for research and clinical purposes in passages three to four. Because karyotype analysis requires the presence of at least 10 cells in the mitotic stage, it is impossible to perform this assay on EPCs on high passages (>seven). Previous studies that followed DNA stability among cord blood–derived EPCs showed altered karyotypes at very early passages (passage two). In contrast, other studies on peripheral blood (PB)–derived EPCs found normal and stable karyotype [36]. These results further express the safety of use of PB hEPCs.

Healing of bone defects and bone fractures involves complex and well-coordinated interactions between cells and cytokines that with mechanical stability and an adequate blood supply [37] can provide the optimal micro-environment required to generate new bone rather than a fibrous scar [10,38]. As with Giannoudis' "diamond concept," the current study emphasizes the importance of an adequate microenvironment. In the current extra-cortical bone formation model, EPCs seeded onto a scaffold assure nutrient and oxygen supply to the regenerated site, while the use of gold domes fixed to the calvaria provides space for bone growth and mechanical stability. In many fields, such as in orthopedics and maxillo-facial surgery, extra-cortical bone regeneration is imperative for patient rehabilitation and improved quality of life. Tooth extraction due to periodontal disease, root fracture or bone cysts are usually accompanied by severe alveolar bone resorption and insufficient bone available to support dental implants. Gaining extra-cortical bone formation is challenging due to limited supply of oxygen, nutrients and cells to the areas remote from the basal bone. The regenerative potential of the current clinical available methods to enhance extra-cortical bone formation are limited to a few millimeters (1–2 mm) [6]. Moreover, cases of severe alveolar bone atrophy are often treated with repeated surgeries to achieve an adequate amount of bone available for implant installation [6]. In the present study, a ninefold increase in blood vessel density in the hEPC samples compared with scaffold without cells was observed. Bone filled the entire dome in all specimens in the hEPC group, whereas in the control group

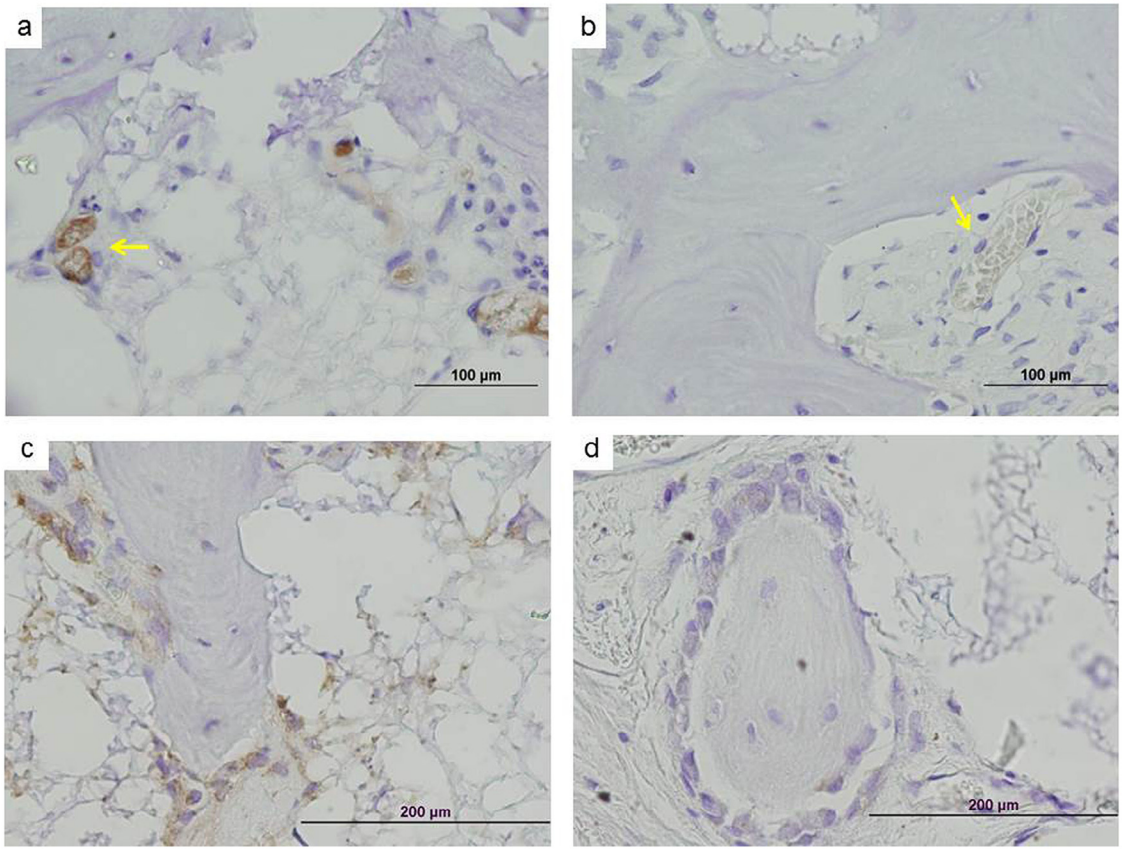

Figure 8. Engraftment of human CD31+ cells and recruitment of rat CD73+ cells into the regenerated tissue. (a) hEPC and (b) control–Engraftment of human CD31 cells into the walls of newly formed blood vessels adjacent to the regenerated bone (5 months post–cell transplantation). (c) hEPC and (d) control–Recruitment of CD73 rat cells into the regenerated tissue was higher in the hEPC group. CD73+ cells were found adjacent to the regenerated bone and residual scaffold (5 months post–cell transplantation). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(βTCP alone) newly formed bone did not reach the top of the dome in any of the specimens. The mean gained bone height reached 4.5 mm. Rat calvaria height ranges between 0.5 and 1 mm, emphasizing the significance of the newly regenerated bone. Histological and micro-CT analyses revealed normal bone architecture and bone mineral density. Therefore, these results are promising for cases that require significant extra-cortical bone regeneration.

Current research on the use of expanded EPCs in the field of bone regeneration is scarce and limited to pre-clinical trials. Most of the research is focused on the use of MSCs for bone regeneration. Indeed, animal and human clinical trials demonstrated ectopic bone and improved healing of bone defects following MSC transplantation [39–41]. However, several difficulties concerning the use of MSCs still exist: (i) their aspiration involves an invasive procedure and morbidity [42]; (ii) age-dependent decline exists in their proliferation and osteogenic differentiation potentials [43]; and (iii) inadequate neo-vascularization may cause malfunction and death of the transplanted cells [44]. To meet some of these difficulties, attempts are made to find new sources for MSC isolation that do not require complex surgical interventions, such as from adipose tissue [45] and periodontal ligament [46]. Numerous studies presented osteogenic [47] *in vitro* and *in vivo* [48] potential of adipose-derived mesenchymal cells; nevertheless, their osteogenic potential is low compared with mesenchymal cells derived from bone marrow [49]. Furthermore, MSCs have been reported to have a tumorigenic potential in *in vitro* and *in vivo* experiments within long-term expanded cell populations [50–52]. MSCs were also reported to induce tumor transformation of surrounding cells if loaded into bio-scaffold, possibly inducing a tumor niche [53]. This risk is continually monitored in human clinical trials and there have been reports with no systemic toxicity or neoplastic findings [54,55].

If cell therapy is to be avoided, recombinant protein administration is another approach to enhance bone tissue formation. Growth factors that participate in MSC recruitment and MSC osteogenic differentiation were investigated [56]. One of the most-studied

proteins in this field is bone morphogenetic protein 2, which was already approved by the US Food and Drug Administration for the treatment of spinal cord injuries and jaw bone reconstruction. Clinical long-term evaluations are still missing; however, preliminary results revealed uncontrolled bone formation and carcinogenesis in several cases [57]. Platelet-derived growth factor (PDGF) and VEGF were also tested for bone formation. Both presented promising results in pre-clinical animal models [58] but clinical trials are not yet available. Several disadvantages concerning growth factor use include the following: high production costs, inflammatory response to the administrated proteins and short-term effect of the protein [59]. Moreover, comparing growth factor application to cell transplantation, it is obvious that transplanted cells secrete an array of proteins that participate in multiple biological process as long as the cells are present and functional. Our results demonstrated direct engraftment of CD31-positive human cells into the vessel walls, suggesting that these cells actively participate in angiogenesis. However only a minority of the newly formed vessels (approximately 5%) stained positive for human antigens, therefore, we hypothesize that the major role of hEPCs in angiogenesis is by paracrine effect. Recruitment of resident rat $CD73^{+}$ cells to the regenerated tissue was also demonstrated in the hEPCs group, suggesting an additional paracrine role of hEPCs in MSC recruitment for bone regeneration. CD73 can be used as a marker of lymphocyte differentiation as well as a marker to phenotypically characterize MSCs [60]. Because we used athymic rats in this study, it is reasonable to presume that the majority of the stained cells are MSCs [61]. Direct incorporation of transplanted EPCs into intima layer of blood vessels was demonstrated in several *in vivo* models [62,63]. The indirect role of late EPCs to sustain MSC survival and function was demonstrated in an ectopic subcutaneous model [44]. According to this study, EPC-derived paracrine factors via PDGF-BB/PDGF receptor (PDGFR)-β signaling regulate MSC engraftment [44].

In conclusion, macroscopic, microscopic and chromosomal evidence revealed that local hEPC transplantation in a nude rat model is safe with no evidence of abnormal pathologies. At 5 months, vertical bone growth was doubled (5 mm) in the hEPCs group with substantial neo-vascularization. These results suggest that local hEPC transplantation in conjunction with a rigid barrier is an effective and safe treatment modality for extra-cortical bone regeneration.

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