

1 A Validated Preclinical Animal Model for Primary Bone Tumor Research

ABSTRACT

Background

Despite the introduction of 21st century surgical and neoadjuvant treatment modalities, survival of osteosarcoma (OS) patients has not improved in the last decades. **Advances will depend in part on the development** of clinically relevant and reliable animal models. This study describes the engineering and validation of a humanized tissue engineered bone organ (hTEBO) for preclinical research on primary bone tumors in order to minimize false positive results due to interspecies differences in xenograft models.

Methods

Pelvic bone and marrow fragments were harvested from patients when reaming the acetabular ground during hip arthroplasty. HTEBOs were engineered by embedding fragments in a fibrin matrix containing bone morphogenetic protein 7 (BMP-7) and implanted into NOD-*scid* mice. After ten weeks of subcutaneous growth, one group of hTEBOs was harvested to analyze the degree of humanization. A second group was injected with human luciferase-labelled OS cells (Luc-SAOS-2) and compared to tumors raised via intratibial injection. Tumor growth was followed *in vivo* via bioluminescence imaging. After 5 weeks osteosarcomas were harvested and analyzed.

Results

After 10 weeks of *in vivo* growth a new bone organ developed, containing human bone matrix as well as viable and functional human hematopoietic cells. Five weeks after injection of Luc-SAOS-2 cells into this humanized bone microenvironment, spontaneous metastatic spread to the lung was evident. Relevant prognostic markers like VEGF and periostin were found in this humanized OS but not in the conventional intratibial OS model. Hypoxia-inducible transcription factor 2 α (HIF-2 α) was detected only in the humanized OS.

Conclusions

27 We report an *in vivo* model that contains human bone matrix and marrow components in one
28 organ. BMP-7 made it possible to maintain viable mesenchymal and hematopoietic stem cells
29 and created a bone microenvironment close to the human physiology.

30 **Clinical Relevance**

31 This novel platform enables preclinical research on primary bone tumors in order to test new
32 treatment options.

INTRODUCTION

The survival of osteosarcoma (OS) patients has not significantly improved in the past 20 years, although the interplay between chemotherapy and surgical intervention has been continuously modified^{1, 2}. While new surgical techniques have led to an increase in quality of life and better local tumor control³⁻⁵, metastases remain the major cause of death and are present in 12.4% of patients at the time of diagnosis. Only 26.7% of these patients survive longer than 10 years^{6, 7}. The hampered efforts to increase survival rates of OS patients are due in part to a lack of clinically relevant animal models that can be used to reliably identify novel therapeutic agents. De facto, 80% of all substances that proved to be effective in preclinical testing, failed when finally trialed in humans⁸⁻¹¹. This is mainly due to species-specific incompatibilities in intercellular communication and intracellular signaling pathways¹². An ideal OS animal model should replicate all aspects of the human disease, specifically with regards to tumor biology, marker expression and response to therapeutic agents. As the prognosis of OS patients is significantly influenced by the presence of lung metastasis, it is a *conditio sine qua non* for an *in vivo* model to mimic the spontaneous development of pulmonary metastases¹³. Additionally, several studies have highlighted the importance of the tumor microenvironment for the initiation, progression and metastatic spread of solid tumors. Berlin et al. demonstrated that the human KRIB OS cell line only forms metastases in mice when injected into the mouse bone but not when inoculated subcutaneously¹⁴. Cells such as mesenchymal and hematopoietic stem cells (MSCs and HSCs), both residing in the bone microenvironment, have a considerable impact on tumor development and metastasis¹⁵⁻²⁰. Disseminated cancer cells directly compete with HSCs for occupancy of their niche and changes of the HSC microenvironment alter cancer cell dissemination²¹. Additionally, other cell types such as macrophages, present in bone and tumors, have been shown to be of prognostic significance in OS patients²².

Humanized xenograft models of osteotropic tumors that incorporate not only human extracellular bone matrix but also cellular components of the human **microenvironment** have emerged in recent years^{23, 24}. However, humanizing *in vivo* approaches for research on primary bone tumors have been neglected and failed to consider the importance of the hematopoietic niche. To our knowledge, we are the first to have developed and validated an animal model that utilizes recombinant human bone morphogenetic protein 7 (rhBMP-7) in combination with human pelvic bone and mimics the clinical aspects typically seen in OS patients.

METHODS

Animal housing and handling.

All animal studies were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals and approved by the Animal Ethics Committee of *** **Blinded by JBJS** ***. Twenty four 4-week old male NOD-*scid* mice were purchased from the Animal Resources Centre (Canning Vale, WA, Australia).

Engineering of a humanized tissue-engineered bone organ (hTEBO)

Cancellous bone with marrow was obtained during reaming the acetabulum from 3 different otherwise healthy human donors undergoing hip arthroplasty as approved by the ethics committees of *** **Blinded by JBJS** *** under informed consent and in accordance with the World Medical Association's Declaration of Helsinki. The first two reamer heads were discarded to minimize the amount of cartilage remnants and cortical bone within the samples. The material was then positioned in a plastic cylinder (volume 0.5 cm³). RhBMP-7 (30 µL in 1 µg/µL, Olympus Biotech, USA) was embedded into 60 µL of fibrin glue (TISSEEL Fibrin Sealant, Baxter Healthcare International) and added to the graft material (Fig. 1). **Constructs were subcutaneously implanted at the left and right flank of 18 mice as previously**

described²³. Post implantation, hTEBOs were allowed to form a bone organ for 10 weeks. Six of the 18 animals were then euthanized **via CO₂ asphyxiation** in order to characterize the morphology and cellular composition of hTEBOs via μ CT, histology, immunohistochemistry and flow cytometry.

SAOS-2 cell culture

The human SAOS-2 cell line (Sigma-Aldrich, Castle Hill, NSW, Australia) was chosen to establish OS growth^{25, 26}. To assess tumor spread by bioluminescence imaging (BLI), luciferase-expressing human SAOS-2 cells (Luc-SAOS-2) were generated using a lentiviral gene expression system (Invitrogen, Mulgrave, Victoria, Australia) according to the manufacturer's instructions using blasticidine (Invitrogen) selection (5 μ g/mL). Standard culture was performed with McCoy's 5A modified media (Gibco, Life Technologies, Mulgrave, Victoria, Australia).

Development of a humanized OS platform

5x10⁵ Luc-SAOS-2 cells in 50 μ L PBS were transcutaneously injected into hTEBOs of mice (n = 12) using inhalational anesthesia with isoflurane. Correct injection was verified via radiographic and BLI control. To compare humanized OS growth with the conventional murine OS xenograft model, the same amount of Luc-SAOS-2 cells was inoculated intratibially in 6 mice²⁷. Bioluminescent imaging (BLI) was performed weekly to assess *in vivo* tumor growth and metastatic spread. After 5 weeks mice were sacrificed and hTEBOs as well as visceral organs were excised for *ex vivo* BLI and further tumor analysis.

Bioluminescence Imaging

For weekly BLI (IVIS Spectrum 200, Perkin Elmer, USA) animals were imaged 15 min post i.p. injection of 100 μ L (7.5 mg/mL) luciferin (PerkinElmer, USA). *Ex vivo* BLI was performed

20 min after luciferin injection to determine metastatic tumor burden of visceral organs. Images were analyzed with the Living Image Software (Perkin Elmer) using a threshold set at 10% around each bioluminescent source to determine the amount of photons emitted within a given time. Results were evaluated as average radiance [p/s/cm²/sr] \pm SEM (standard error of the mean)^{23,24}.

Micro-computed tomography (μ CT)

The hTEBOs were analyzed with a high-resolution μ CT scanner (μ CT 40, Scanco Medical AG, Switzerland) and scanned at a voxel size of 16 μ m. Samples were evaluated at a threshold of 150, a filter width of 0.8 and a filter support of 1.

Flow cytometry

For flow cytometry, ossicles were gently crushed with a mortar in ice-cold PBS with 2% FCS and then filtered on a 40 μ m cell strainer. Analyses of the murine hematopoietic system was performed as previously described²⁸. Aliquots of 5×10^6 bone marrow cells were stained with huCD45-APCCy7 (Biolegend, Karrinyup, Western Australia), mouse CD45-biotin (Biolegend), huCD34 APC (Biolegend), huCD19-PE (Biolegend), huCD20PE (Biolegend), huCD14-PeCy7 (Biolegend) and huCD3-V450 (BD Bioscience, North Ryde, New South Wales, Australia) and streptavidin brilliant violet 605 (Biolegend). Analysis was gated on viable cells following exclusion of dead cells with 7-amino actinomycin D (Life Technologies).

Histology and Immunohistochemistry

Fixed samples were decalcified **and stained for immunohistochemical analysis using our a standard protocol**^{23,24}. Sections were incubated with the primary antibody **solutions according to Appendix Table 1. In order to detect human extracellular matrix sections**

were stained for human specific (hs) collagen I (hsCol-I) and Osteocalcin (OC). Human cells were detected by staining for nuclear mitotic apparatus protein 1 (hsNuMa). Human MSCs were stained for hsCD146 and human leucocytes for hsCD45. Proliferating tumor cell were detected by staining for Ki67. Proteins under current investigation for their application as OS tumor markers are vascular endothelial growth factor (VEGF), hypoxia-inducible transcription factor 1 α (HIF-1 α) and periostin. Sections were also stained for hypoxia-inducible transcription factor 2 α (HIF-2 α) as this protein plays an important role in tumor cell proliferation and metastasis^{31,33,50-55}. Human bone and OS tissue as well as mouse bone sections were used as positive and negative controls, respectively. The use of human OS tissue was approved by the Ethics Committee of the University of Regensburg, Germany (Approval No: 12-101-0013). Tartrate resistant acid phosphatase (TRAP) staining for osteoclasts has been performed according to our standard protocol²³.

Statistical analysis

IBM SPSS Statistics (version 21) was used for statistical analysis. Data was tested using the student t-test and Mann-Whitney-U test to evaluate differences between groups. The level of significance was set at $p < 0.05$.

Source of funding

***** Blinded by JBJS *****

RESULTS

Tissue engineering of vital humanized bone

Ten weeks post implantation hTEBOs developed calcified ossicles that appeared to be of hard structure and rich in blood (Fig. 2). μ CT and morphological H&E analysis demonstrated a trabecular network surrounded by an outer cortex-like structure. Viable osteocytes were found residing in the bone lacunae. Trabecular spaces were filled with hematopoietic cell clusters of different lineages.

Human bone marrow elements can be maintained in hTEBOs

Immunohistochemical staining revealed cells positive for human specific (hs) CD146 and identified as MSCs mainly located in direct proximity to sinusoids (Fig. 2). Furthermore, we identified human CD45⁺ leucocytes. Flow cytometry verified these findings and revealed that cells equivalent to human HSCs (hsCD34) can survive within the humanized microenvironment. Adding rhBMP-7 led to a distribution of bone marrow cells morphologically equivalent to those in corresponding mouse femurs (Appendix Fig. 1). Human B cells, T cells and monocytes were also present. Omitting rhBMP-7 led to bone constructs with non-viable matrix without human and hardly any murine bone marrow cells.

Direct cell injection into hTEBOs results in reproducible local and metastatic OS growth

Luc-SAOS-2 cells were injected directly into the newly formed humanized bone organs (Fig. 3). Positive BLI tumor signals within the hTEBC were found in 91.7 % of the mice (11/12) after 5 weeks. *Ex vivo* BLI indicated metastatic spread to the lung in 72.7% (8/11) at this experimental endpoint (Fig. 4). μ CT depicted osteoblastic regions within the hTEBOs, indicating the localization of a tumor (Fig. 3). Histology showed neoplastic tissue with a high amount of extracellular matrix (ECM) in these regions as indicated by the presence of hsCol-I and tumor osteoid, the latter being pathognomonic for OS. These areas were accompanied by regions of high cellular density and high proliferative activity as depicted by Ki67 staining.

Staining for hsNuMa proved that tumor cells were indeed of human origin. The overall histomorphological appearance was similar to patients with an osteoblastic subtype of OS. H&E staining of lung tissue demonstrated metastases with human osteoid as shown by the presence of eosinophilic extracellular deposits (Fig. 4). Metastases were positive for Ki67 and hsNuMa, demonstrating that the lung lesions were proliferating and of human origin.

Prognostic markers are expressed at the primary and secondary tumor site

Immunohistochemical staining for clinical relevant prognostic markers proved the presence of HIF-2 α in regions within the humanized tumors where high levels of VEGF expression coexisted³⁰⁻³⁴ (Fig. 5). **HIF-1 α was variably expressed (n = 2 of 5 analyzed samples). Staining for periostin was positive in the primary humanized tumor. Equivalent staining of an OS patient sample showed the same expression pattern for HIF-2 α , VEGF and periostin.**

Intratibial tumor growth depicts different OS patterns

To compare the humanized OS model with the conventional model, Luc-SAOS-2 cells were injected into the left tibia (Fig. 6). μ CT analyses demonstrated an exophytic growing tumor located at the proximal tibia. Immunohistochemical analysis showed positive staining for hsCol-I and hsNuMA. These cells were again highly proliferative as shown by Ki67 staining. Assessing the histological tumor morphology, intratibial tumors appeared more homogeneous with less osteoid-rich areas than the humanized OS and the OS found in patients (Fig. 3). Additionally, cell nuclei and cell shape appeared more spherical and uniform. After 5 weeks, a positive BLI signal was detected within the tibia of 50% of mice (n = 3/6). To compare the metastatic load of the lungs after 5 weeks, the average radiance of both groups was calculated via in vivo BLI. Although the average radiance was doubled in the hTEBO group ($4.00 \times 10^3 \pm 0.40 \times 10^3$ SEM [p/s/cm²/sr]) in comparison to the intratibial group (2.04×10^3

± 1.82 x10³ SEM [p/s/cm²/sr]), this difference was not significant (p = 0.648). Staining for HIF-2α, VEGF and periostin was negative in the analyzed intratibial OS samples (Fig. 5).

DISCUSSION

In the last decades, efforts to reduce the mortality rate in OS have remained disappointing as most novel therapeutic concepts have **failed**^{8,9}. **This might be in part due to the fact that conventional xenograft mouse models might produce false positive or negative results as functional species-specific cross-reactivity exists between inoculated human tumor cells and the murine host**^{12,35}. **Researchers have only recently started to realize the relevance of tissue microenvironments and the importance of extracellular structural cues for tumor development**^{23,36,37}. Humanizing animal models addresses both concerns by engrafting human tissues into immune-compromized mice prior to transplantation of human tumor cells and has been shown to provide a favorable microenvironment, in particular if focusing on aspects of the metastatic cascade^{24,38}.

In the past, attempts to humanize bone organs within mice by implanting human bone fragments without any growth factor support have failed as they did not recapitulate the morphological and functional features of a human organ bone (Appendix Fig. 1)³⁹⁻⁴¹. Therefore, we decided to utilize human cancellous bone in combination with rhBMP-7. Additionally, we did not use bone from the femur as described by others, but from the pelvis, which contains more red proliferative bone marrow and adult stem cell fractions⁴². This bone can be easily obtained from patients undergoing hip replacement during the reaming of the acetabular fossa. We demonstrated that human MSC and HSC populations were able to survive for at least 10 weeks and portrayed a humanized bone marrow compartment. Most humanized mouse models, which aim for a replacement of the mouse bone marrow have been accomplished by injection or implantation of human hematopoietic tissues^{12,43}. Although these

techniques are able to create a higher level of humanization of the hematopoietic system, they fail to provide a peripheral hematopoietic bone organ that can provide species-specific factors necessary for the maintenance of human HSCs and progenitor cells. The essential agent driving the development and maintenance of this fully functional bone organ is rhBMP-7^{23, 44}. Only recently, a small number of other groups have begun to hypothesize concerning the relevant role of BMP-7 in hematopoiesis⁴⁵⁻⁴⁸. Our study represents the *in vivo* validation of this work.

After the initial steps of characterization, we hypothesized that this model is highly suitable to study primary bone tumor growth and metastasis. We found a higher tumor take rate as well as a high rate of metastatic dissemination in our hTEBO carrying mice than in the animals with conventional intratibial OS. **Metastatic tumor load was not significantly different between the groups, which might be the result of low sample size. This and the utilization of one single cell line - not consistently described as a metastatic cell line – can be seen as a limitation of the study.** Nevertheless, the finding of a high metastatic potential in the humanized OS is of utmost importance, for the clinical relevance of an OS model^{6, 13}.

OS is characterized as a mesenchymal malignancy producing tumor osteoid, the sole pathognomonic marker for OS to date. Histological analyses of the tumors grown in hTEBOs clearly met the histopathological criteria for the diagnosis of OS⁴⁹. The inoculated human OS cells showed extensive production of human ECM proteins a growth pattern characterizing the most common osteoblastic OS subtype. Intratibial tumors mimicked a different OS morphology, most likely a small cell subtype⁴⁹. **Similar histomorphological aspects can be found in studies describing intratibial injection of OS cell lines performed by Berlin et al. utilizing of KRIB cells and Dass et al. using SAOS-2 cells^{14,59}.**

To underline the clinical relevance of our model, we have studied the prognostic marker expression of the tumors. VEGF has been shown to be regularly expressed in OS patients

although its use as a prognostic marker is under debate^{50, 51}. We were able to find a high expression of VEGF in the humanized tumors, but not in the intratibial ones. This positive expression was accompanied by positive staining for HIF-2 α , which was also not present in the intratibial tumors. As far as we know, we are the first to describe an upregulation of HIF-2 α in OS and also could verify its presence in a patient sample. HIF-2 α has been shown to drive angiogenesis and proliferation as well as tumor metastasis on the basis of chronic hypoxia^{33, 52-55}. **Although we have stained tumor tissue from only one OS patient, this protein might represent a new prognostic marker as well as a therapeutic target as it has been shown in other studies that this protein plays a pivotal role in tumor development⁵².** Additionally, we could prove the presence of periostin within the humanized OS. High expression of periostin has been linked to a poor prognosis in OS patients mainly due to a higher rate of metastasis³¹. Again, periostin was not detected in intratibial OS. These findings underline the clinical relevance of our novel preclinical model, not only through their ability to reflect the histological appearance, but also the molecular marker expression of human lesions.

Our humanized bone microenvironment provides an advantage **over the conventional animal models** as new therapeutic options are targeted against the tumor cells as well as interfere with the interactions between tumor cells and their microenvironment. Recently, the modulation of natural killer cell or macrophage function through Cetuximab or muramyl tripeptide phosphatidylethanolamine (MTPE) has been shown to have potential efficacy against primary **OS as these cell types typically reside within the bone and tumor microenvironment^{56, 57}.** However, preclinical assessment of new drugs might incorrectly be interpreted as non-valuable because they are specifically directed against human cells, proteins or genes. For example, Denosumab is an antibody that inhibits bone resorption when binding to the human, but not

282 the murine, receptor activator NF- κ B Ligand (RANKL)⁵⁸. It now can be investigated in a
283 preclinical animal model. Hence, for the first time species-specific considerations can be
284 addressed in primary bone tumor research.

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Figure Legends

Figure 1: Experimental outline and differences between the presented and conventional tissue engineering approaches. We utilized a top-down approach by harvesting the acetabular reaming material from patients undergoing hip arthroplasty (**A [i]**). The first reaming portion was discarded in order to minimize the amount of cartilage and to achieve a high proportion of blood marrow (**A [ii]& [iii]**). The material was then positioned in a plastic cylinder. Then, rhBMP-7 was embedded into fibrin glue and added to the graft material (**A [iv]**). These grafts were subcutaneously implanted at the left and right flank of NOD-*scid* mice (**A [v]**). HTEBOs were allowed to form a humanized organ for 10 weeks. One group of mice was then euthanized and ossicles were harvested for bone matrix and marrow analysis (**A [vi]**); another group was injected with Luc-SAOS-2 cells (**A [vii]**). HTEBOs with tumors were harvested after additional 5 weeks for further analysis (**A [viii]**). Bottom-up approaches (**B**) relate to methods that generate tissues by scaffold printing (**B [i]**), cell isolation from humans (**B [ii]**), *in vitro* cell culture and cell differentiation (**B [iii]**) before implantation (**B [iv]**). Therefore, these techniques are dependent on time consuming bench work processes and significantly depend on host- and donor-derived stem cells, which organize and replace tissues during engraftment.

Figure 2. Establishment of a humanized tissue-engineered bone organ (hTEBO). After 10 weeks of subcutaneous *in vivo* growth in NOD-*scid* mice, ossicles (**A**) with trabecular formations and an outer cortex-like structure have formed as seen in μ CT (**B**) and H&E staining (**C**), the latter showing a viable **bone** marrow compartment (**n=6 of 6 samples**). Viable osteocytes were still residing within the lacunae (**D**, \rightarrow) and bone formation was still ongoing as depicted by staining for human osteocalcin (OC, antibody not reactive to mouse; **n=6/6**). Immunohistochemistry using human-specific (hs) antibodies showed hsCD146⁺ cells located in direct proximity to sinusoids and therefore were identified as MSCs (**E**, \rightarrow ; **n=6/6**). Anti-hsCD45 is a panleukocytic antibody and stained in particular parasinusoidal cells (**F**, \rightarrow ;

n=6/6). Flow cytometric analysis of 2 ossicles (G) verified the presence of human HSCs by staining for hsCD34 and hsCD45 after excluding murine cells with muCD45. Tr, trabeculae; Cx, Cortex; BM, bone marrow;

Figure 3. Establishment of a humanized tissue-engineered OS model. Five weeks after injection of Luc-SAOS-2 cells into hTEBO (A) 91.7 % of the mice showed tumor take as verified by BLI (B). μ CT showed a mainly osteoblastic tumor formation (C, \rightarrow), which was identified as a neoplasm producing human collagen-I depicted by immunohistochemistry (G, \rightarrow ; n=6/6). H&E staining showed areas with extensive ECM formation (D) next to tumor regions of high cell density (*) with areas of osteoid (\rightarrow). The areas with high cellularity showed an increased proliferation as depicted by Ki67 staining (E; n=6/6) and cells within these areas were shown to be human as verified by positive hsNuMA staining (F; n=6/6). The tissue morphology of humanized OS (D&G) looked similar to the histological appearance of a human osteosarcoma (H&I).

Figure 4. Evaluation of the metastatic potential of the model. Lung metastasis originating from humanized OS showed the production of osteoid (A, \rightarrow). Cells within the lesion proved to be human when stained for hsNuMA (B), therefore identifying these as an OS metastasis. *Ex vivo* BLI analysis of harvested lungs (C) determined lung metastasis in 72.7% of mice after 5 weeks (8/11 mice). Scale bars depict 100 μ m.

Figure 5. Evaluation of marker expression. Immunohistochemical staining showed islets with an increased expression of VEGF (A; n=4/5) accompanied by a high expression of HIF-2 α (D; n=4/5). Periostin was detected in humanized OS (G; n=4/5). Intratibial tumors were all negative for these markers (C,F,I; n=0/3) in comparison to the humanized OS and an OS patient sample (B,E,H). Scale bars depict 100 μ m.

Figure 6. Comparison to the conventional intratibial mouse model. Intratibial injections of Luc-SAOS-2 cells were performed under radiographic control (A). μ CT revealed an

exophytic growing tumor **(B)**, which also produced human-specific ECM as shown by positive staining for hsCol-I **(C)**. The cells also proved to be of human origin **(E)** and were proliferating **(F)**. **The tissue morphology of intratibial OS (D) showed less osteoid formation and a more homogeneous growth pattern than humanized OS (Figure 3D)**. BLI showed a tumor take rate of 50 % (3/6 mice, **G**). Although the means of the average radiances of lung metastasis were doubled in the humanized OS group compared to animals with intratibial tumors, this difference was not significant ($p = 0.648$) **(H)**.

Appendix Figure 1: Morphological evaluation of human bone marrow cell fractions. The fractions of human blood marrow cells within the hTEBOs without **(A&B)** and with rhBMP-7 **(C&D)** were analyzed after 10 weeks of *in vivo* growth. H&E stainings of the hTEBO with rhBMP-7 **(C&D)** and the mouse femur **(E&F)** suggested equal bone marrow cell morphology, whereas hTEBOs, growing without rhBMP-7 were only filled with fibrous and adipose tissue **(A&B)**. Flow cytometric analysis was performed in 2 hTEBOs without rhBMP-7 **(Panel G)** and 2 with rhBMP-7 **(Panel H)**. Human (hu) CD45+ leucocytic cells **(G&H [i])** and huCD34+ HSCs **(G&H [ii])** were only present in the rhBMP-7 ossicles **(H [i]&[ii])**. HuCD3+ T cells and huCD19+/CD20+ B cells as well as huCD14+ monocytes also were only detected within the hTEBOs with added rhBMP-7 **(H [ii]&[iii])**. Graphs show results of representative hTEBOs. Tr, trabeculae; Cx, cortex; BM, bone marrow; GP, growth plate; FT, fibrous tissue.