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Three-Dimensional Bone Extracellular Matrix Model for Osteosarcoma

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TITLE:

Three-dimensional Bone Extracellular Matrix Model for Osteosarcoma

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KEYWORDS:

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SHORT ABSTRACT:

The bone extracellular matrix (BEM) model for osteosarcoma (OS) is well established and shown here. It can be used as a suitable scaffold for mimicking primary tumor growth in vitro and providing an ideal model for studying the histologic and cytogenic heterogeneity of OS.

LONG ABSTRACT:

Osteosarcoma (OS) is the most common and a highly aggressive primary bone tumor. It is characterized with anatomic and histologic variations along with diagnostic or prognostic difficulties. OS comprises genotypically and phenotypically heterogeneous cancer cells. Bone microenvironment elements are proved to account for tumor heterogeneity and disease progression. Bone extracellular matrix (BEM) retains the microstructural matrices and biochemical components of native extracellular matrix. This tissue-specific niche provides a favorable and long-term scaffold for OS cell seeding and proliferation. This article provides a protocol for the preparation of BEM model and its further experimental application. OS cells can grow and differentiate into multiple phenotypes consistent with the histopathological complexity of OS clinical specimens. The model also allows visualization of diverse morphologies and their association with genetic alterations and underlying regulatory mechanisms. As homologous to human OS, this BEM-OS model can be developed and applied to the pathology and clinical research of OS.

INTRODUCTION:

Osteosarcoma (OS) usually occurs in actively growing areas, the metaphysis of long bones, during adolescence. More than 80% of the OS-affected sites have preference for the metaphysis of proximal tibia and proximal humerus as well as both distal and proximal femur, corresponding to the location of the growth plate[1]. OS comprises multiple cell subtypes with mesenchymal properties and considerable diversity in histologic features and grade. Evidences support mesenchymal stem cells (MSCs), osteoblasts committed precursors and pericytes as the cells of origin[2-5]. These cells can accumulate genetic or epigenetic alterations and give rise to OS under the influence of certain bone microenvironmental signals. Both intrinsic and extrinsic mechanisms result in the genomic instability and heterogeneity of OS, with multiple morphological and clinical phenotypes[6, 7]. For individualized therapies or screening of new drugs, novel models need to be generated to against heterogeneity or other clinical disorders.

OS is an intra-osseous malignant solid tumor. The complexity and activity of surrounding microenvironment elements confer phenotypic and functional differences upon OS cells in different locations of a tumor. Bone extracellular matrix (BEM) provides a structural and biochemical scaffold for mineral deposition and bone remodeling. The organic portion of extracellular matrix (ECM) mainly consists of type I collagen secreted by osteoblastic lineage cells, while its mineralized portion is composed of calcium phosphate in the form of hydroxyapatite[8]. The dynamic role of ECM networks is to regulate cell adhesion, differentiation, cross-talk and tissue function maintenance[9].

Demineralized BEM and ECM hydrogels have been successfully used in cell culture and can enhance cell proliferation[10, 11]. Synthesized bone-like ECM can regulate the pool size, fate decisions and lineage progression of MSCs[12-14]. Moreover, results evidence its clinical significance to provide osteogenic activity by stimulating cellular processes during bone formation and regeneration[15-17].

In this article, our group establishes a modified model and favorable alternative for three-dimensional long-term culture. OS cells injected into the tissue-derived BEM present a heterogeneously mesenchymal phenotype readily as compared to plastic two-dimensional cultures. BEM derived from site-specific homologous tissue show its dramatic advantage as being a native niche for OS cells in vitro and has great potential in OS theoretical and clinical research. This characterized BEM platform is simple but efficient for in vitro research and may be extended in modeling multiple cancers.

PROTOCOL:

Animal care and use are conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication NO.80-23, revised in 1996) after approval from the Animal Ethics Committee of Sun Yat-sen University.

1. Bone preparation

1.1) Obtain 4 to 6-week-old BALB/c mice (without sex-specific requirement). Euthanize a mouse aseptically by cervical dislocation and cut off fresh fibula, tibia and femur from a hindlimb with sterile surgical scissors. Peel off the epithelial tissue and then remove as much of the soft tissue as possible using scissors and tweezers.

1.2) Rinse the leg bones with sterile 10 mM phosphate buffered saline (PBS) twice to remove blood in a 6 cm dish. Immerse the bones in 75% ethanol for 3 min, then rinse with PBS twice. The clean bones can be stored in a sterile 50 mL centrifuge tube at -80 °C for months until required.

NOTE: PBS used in all the following steps has 10 mM PO_4^{3-}.

2. Bone demineralization and decellularization

2.1) Thaw the frozen bones at room temperature, and then freeze again at -80 °C for 1 h. Subject the bones to more than 2–3 freeze-thaw cycles for cell lysis and tissue breakdown.

2.2) Incubate the bones in a sterile 50 mL centrifuge tube with 0.5 N HCl overnight at room temperature on a rocking platform or orbital shaker with gentle rocking/shaking to ensure complete and even coverage of bones.

NOTE: Make sure the bones are entirely immersed during motion in the acid and do not settle during the process. The volume of HCl solution should be more than ten times as that of the bones.

2.3) After decalcification, decant the HCl solution completely and rinse under running water for 1 h. Then, wash the bones twice for 15 min per wash with distilled water on a rocking platform or orbital shaker.

NOTE: Make sure to completely remove the solution or water between washes and after the final wash with distilled water.

2.4) Extract the lipids in the demineralized bones with a 1:1 mixture of methanol and chloroform in a 50 mL centrifuge tube wrapped with tin foil for 1 h at room temperature[10].

2.5) Then, transfer the bones into another tube of methanol for 30 min. Remove the methanol completely and rinse with distilled water twice for 15 min with gently shaking. Decant final wash water and proceed with the following steps under sterile condition.

NOTE: During the extraction step, light must be avoided to prevent chloroform decomposition. The mixture can be stored in light-resistant container or a centrifuge tube wrapped with tin foil. Perform all treatment and wash steps under modest rotation or rocking motion.

2.6) Add 40 mL sterile 0.05% trypsin-EDTA (TE) into the tube and incubate bones for 24 h in a CO_2 incubator at 37 °C[18].

2.7) Discard the TE solution and rinse twice with sterile PBS supplemented with 90 µg/mL ampicillin and 90 µg/mL kanamycin. After decanting the final wash PBS completely, replenish with 40 mL sterile PBS. Wash thoroughly for 24 h at room temperature with gentle rocking or shaking for antibiotic soak.

NOTE: All the sterile PBS used in this and the following steps contains 90 µg/mL ampicillin and 90 µg/mL kanamycin. Overnight wash under rotation or rocking motion are performed for long periods thorough immersion with antibiotics to achieve effective sterilization of pore spaces.

2.8) Remove the PBS and transfer the bones into a fresh 50 mL centrifuge tube filled with sterile PBS. The prepared demineralized and decellularized bones are called bone extracellular matrix (BEM) and can be stored at 4 °C for 2 months until required.

3. Cell seeding and culture

3.1) Take out the BEM from 4 °C refrigerator and immerse it in 75% ethanol for 30 s, then rinse with PBS twice. Transfer the BEM onto a clean 6-well cell culture plate. Add 2 mL complete culture medium (Dulbecco's modified Eagle's medium/F12 (DF12) containing 5% fetal bovine serum, 90 µg/mL ampicillin and 90 µg/mL kanamycin). Incubate the BEM overnight in a CO_2 incubator at 37 °C.

3.2) Obtain human OS cell lines (MNNG/HOS and MG-63). Suspend approximately 1.0 x 10^5 OS cells with 100 µL PBS containing phenol red as indicator.

NOTE: To better track and observe multi-layer cells within the three-dimensional BEM model, MNNG/HOS and MG-63 are infected with lentiviral vector expressing fluorescent mCherry and green fluorescent protein (GFP).

3.3) After the BEM is fully soaked in the medium, inject OS cells into BEM from proximal or distal epiphysis when the needle reaches the medullary cavity of BEM. Incubate the OS-BEM model for a minimum of 2 h in a humidified 5% CO_2 atmosphere at 37 °C to ensure the injected cells firmly adhere to BEM.

NOTE: Pre-warm all the media used for cell culture. The incubator used for cell culture has a humidified 5% CO_2 atmosphere at 37 °C.

3.4) Add 1 mL complete culture medium into the plate to completely coat the surface of the BEM culture overnight in a CO_2 incubator at 37 °C.

3.5) Gently transfer the OS-BEM model into a new well of 6-well plate with a sterile tweezer and refeed 1 mL fresh culture medium. Culture the model for 14 days in a CO_2 incubator at 37 °C and refresh the culture medium according to the proliferation situation of OS cells.

3.6) Keep monitoring medium color and cell status under the inverted fluorescence microscope during the culture process. When OS cells expand to plate, gently transfer the OS-BEM model to another new well with sterile tweezer.

NOTE: The culture medium is bright red at pH 7.4, which is the optimum pH value for most mammalian cell culture. If the medium turns into orange or even yellow, immediately refresh the medium to maintain a healthy environment for OS cells.

3.7) Transfer the OS-BEM model into a new well with tweezers and gently rinse with PBS to remove the culture medium. Then, transfer into a 15 mL centrifuge tube and fix with 10% buffered formalin for histological identification.

REPRESENTATIVE RESULTS:

After demineralization and decellularization, BEM appears to be translucent with stronger resilience and tenacity compared to native mouse bone. A little muscle residue and the space of medullary cavity can be clearly observed (**Figure 1A,B**). To determine the effective decellularization of BEM, BEM is embedded in paraffin after fixation, and then sliced into 3–5 μm sections for hematoxylin-eosin (H&E) staining. The thorough removal of cell nuclei is shown by bright-field imaging. The natural porous structure and collagen network arrangement is well maintained in decellularized BEM (**Figure 1C,D**). Additionally, immunohistochemical (IHC) staining of predominant organic components of bone matrix, such as collagen I and collagen IV demonstrate no damage on ECM components in decellularized BEM compared to the native bone (**Figure 1E**). Therefore, BEM provides a suitable and promising scaffold with great biocompatibility for OS cell seeding and proliferation.

MNNG/HOS cells exhibit a highly atypical morphology with finely vacuolated cytoplasm, while MG-63 cells have fibroblast-like spindled shapes in monolayer culture (**Figure 2A,B**). The histological section from an OS patient displays significant cellular pleomorphism with rounded or polygonal cells, anisonucleosis and multiple mitoses (**Figure 2C**). To verify the viability and quality of the BEM model, both cell lines are injected into the medullary cavity of BEM and monitored via fluorescence imaging during the 14-day culture (**Figure 3A, B**). Histological sections

with H&E staining reveal that OS cells attach to muscle residues and grow into thick piles or adhere along bone matrix and proliferate. Both periosteum and endosteum are infiltrated by the expansion of OS cells. Strikingly, the cell growth patterns of OS-BEM model differ from two dimensional plate culture. As illustrated in **Figure 3C**, OS cells on the decellularized BEM show highly heterogeneous morphology similar to the cytopathologic features of an OS section. Some OS cells locating in cancellous bone and medullary cavity are spherical and partly spread out, whereas the cells resting along the periosteum and endosteum are extremely spread out into elongated cells accompanied by nuclear pleomorphism. Cell activity is determined using Ki67 immunostaining, which also shows great advantages in long-term cultures. Also, OS cells in BEM culture highly express bone matrix glycoprotein—secreted protein acidic and rich in cysteine (SPARC/osteonectin)—which is specific for osteoid matrix (**Figure 3D**).

FIGURE LEGENDS:

Figure 1: The structural characteristics of mouse decellularized BEM. (**A, B**) Overview of mouse native (A) and decellularized (B) bone. (**C, D**) Decellularization was assessed by H&E staining of mouse native tibia (C) and decellularized bone (D). Nuclei stained with hematoxylin could be observed in native mouse tibia, but not in the BEM. (**E**) IHC staining for collagen I and collagen IV to detect the main components of ECM that are preserved in mouse tibia after decellularization. Yellow arrow points out the abundant collagen I within cancellous bone and blue arrow points out the abundant collagen IV within compact bone. Scale bars = 50 μm.

Figure 2: The cytomorphological characteristics of OS. (**A, B**) Human OS cell lines MNNG/HOS (A) and MG-63 (B) expanded in plastic flask culture. Scale bar = 100 μm. (**C**) Histopathologic section with H&E staining of OS patient. Scale bar = 50 μm.

Figure 3: Characterization of OS cells in decellularized bone extracellular matrix model. (**A, B**) Representative mCherry expression (red) image of MNNG/HOS (A) and GFP expression (green) image of MG-63 (B) by fluorescence microscopy after seeding and culturing in BEM. Scale bar = 100 μm. (**C**) H&E analysis showing typical morphology of the injected MNNG/HOS and MG-63 cells after culturing in BEM. (**D**) IHC analysis on Ki67 and SPARC expression level of MNNG/HOS cells after culturing in BEM model for 14 days. The representative images are two sets of serial sections stained with Ki67 and SPARC. Scale bar = 50 μm.

DISCUSSION:

Generally, OS can be classified as osteoblastic, chondroblastic, and fibroblastic subtypes depending on its dominant histologic component. Its prognosis is dependent not only on histologic parameters but also on its anatomic site. It may occur inside the bones (in the intramedullary or intracortical compartment), on the surfaces of bones, and in extraosseous sites[19]. The emergence and heterogeneity of OS can be elucidated as a conjugation of oncogenic events and an adequate microenvironmental boost, followed by increasing development and

migration to distant organs[20-23]. Mystery during OS evolution might be deciphered with a proper model to outline clinical implications targeting the OS environment and niche.

Cultivation either on plastic dishes or in flasks in vitro can hardly recapitulate the dynamic and intricate biological microenvironment. Great strides of various pre-clinical models (e.g., mouse, zebrafish and dog) mimicking the osteosarcoma have been declared and applied to pathogenesis investigation and drug development[4, 24-28]. Still, researchers have concern for experimental animals due to their discomfort and suffering during experiments. In vitro three-dimensional models like our decellularized BEM model has the advantage due to its convenience, quick operation and cost saving; it provides long-term and easy maintenance of viable cells or tissues, and is also closer to the native biological situation than plastic culture. It has been used in our research demonstrating the phenotypic heterogeneity and regulatory mechanism of OS dedifferentiation with success[29].

This protocol clarified the feasibility to generate a tissue-derived BEM from mouse and might be used for tissues from human, rat and dog. The most critical steps for successful establishment and application of BEM are: (i) complete removal of cell debris; (ii) maintenance of a sterile, healthy culture condition; (iii) manual dexterity and gentle pipetting during injection, transfer and culture of OS-BEM model.

Other reported protocols generally employ pressurization or a combination of chemical and enzymatic treatments, such as Triton X-100, sodium dodecyl sulfate (SDS) and DNase/RNase solution to achieve potent decellularization[30, 31]. The cartilage tissue that undergoes decellularization with detergents has been shown to remove ECM components including glycosaminoglycans[32]. To recapitulate a more intact BEM to the greatest extent, a moderate yet powerful decellularization method is performed here to avoid the dissolution and damage of key components and native architecture of the bone environment.

However, it is not to be neglected that this OS-BEM model rested in a plate without flowing medium, consequently leading to an uneven distribution of oxygen and nutrients. Vascular network and other cell subtypes that help regulating the communication and interaction of microenvironmental signals and bone homeostasis need to be taken into consideration[24, 33-35]. Hopefully, this model will be combined with other high-tech engineering techniques to shed light on OS research and guide precision medicine.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

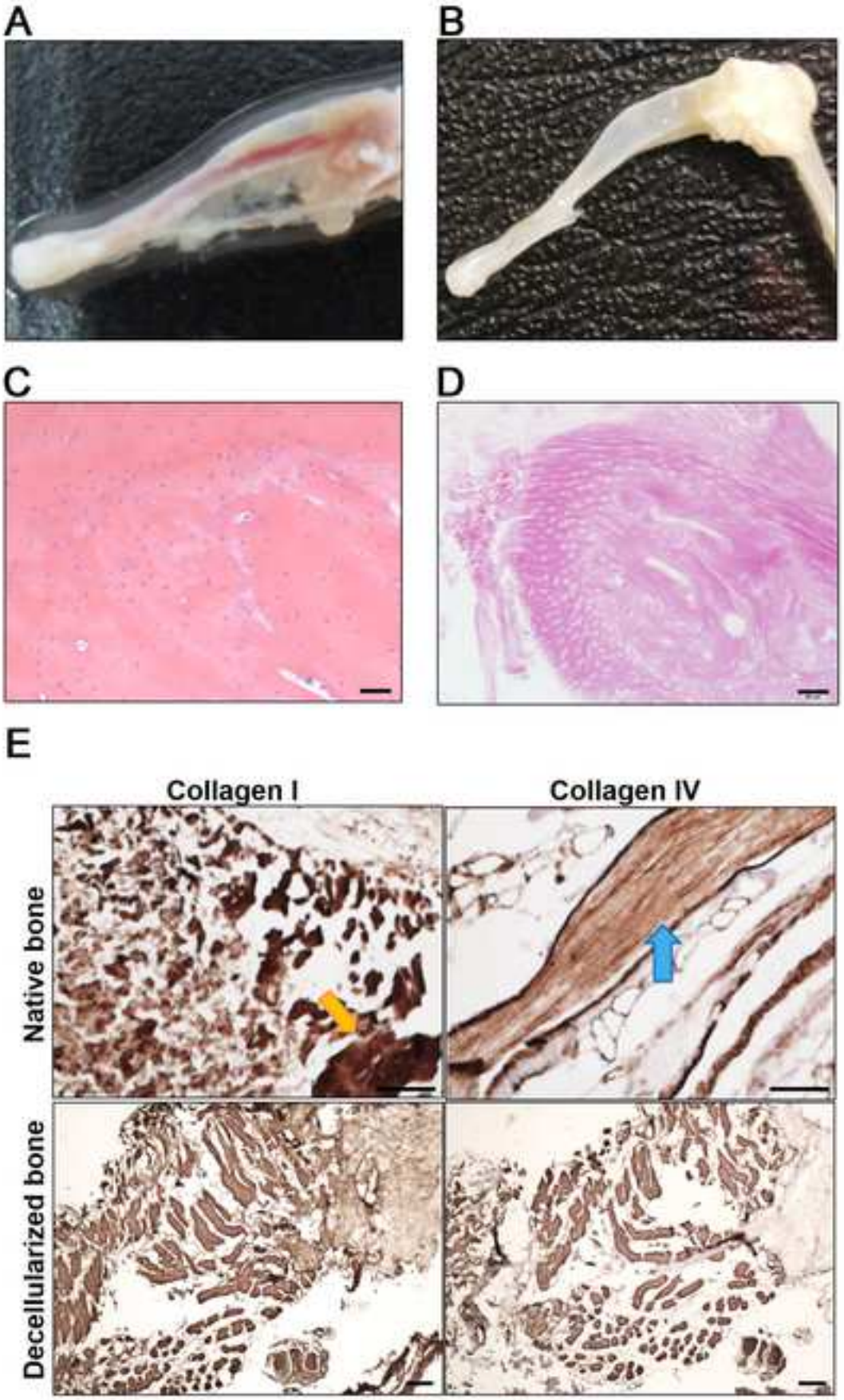


Figure 2

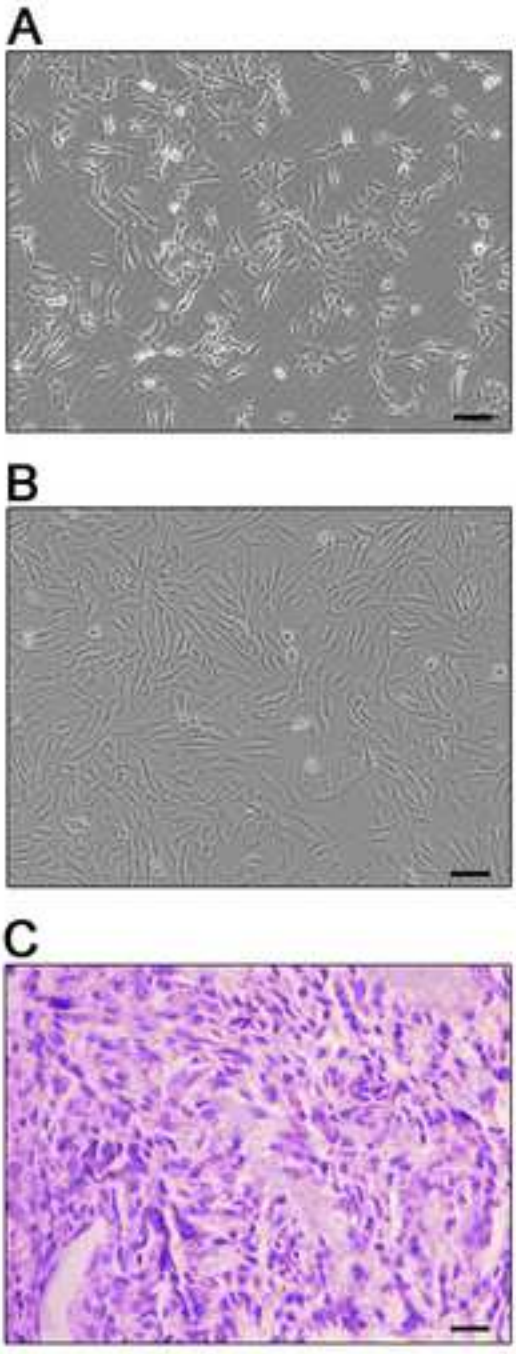
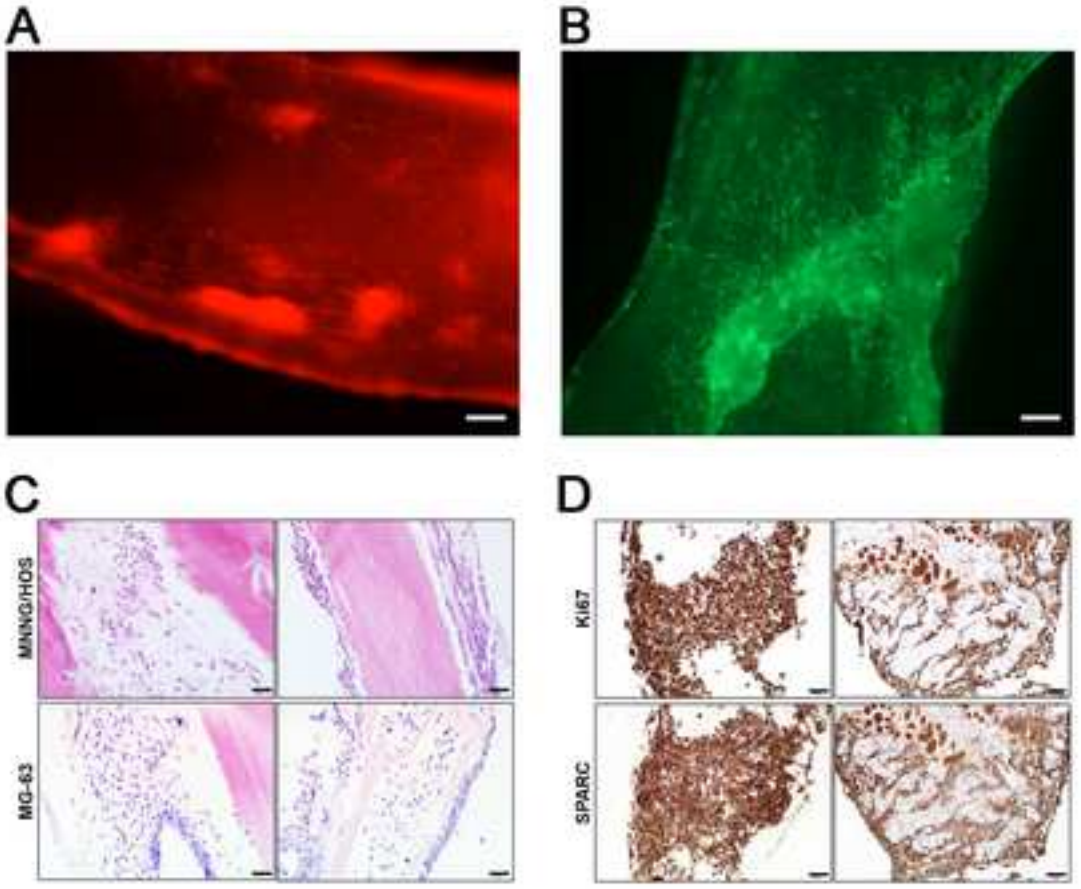


Figure 3



Name of Material/ Equipment	Company	Catalog Number
15 mL centrifuge tube	Greiner	188271
50 mL centrifuge tube	Greiner	227270
6 cm cell culture dish	Greiner	628160
6-well plate	Greiner	657160
Ampicillin	Sigma-Aldrich	A9393
C57-BL/6J mouse	Sun Yat-sen University Laboratory Animal Center	
CO_2 incubator	SHEL LAB	SCO5A
Dibasic sodium phosphate	Guangzhou Chemical Reagent Factory	BE14-GR-500G
DMEM/F12	Sigma-Aldrich	D0547
Fetal bovine serum	Hyclone	SH30084.03
Hemocytometer	BLAU	717805
Kanamycin	Sigma-Aldrich	PHR1487
MG-63	Chinese Academy of Science, Shanghai Cell Bank	
MNNG/HOS	Chinese Academy of Science, Shanghai Cell Bank	
Phenol red	Sigma-Aldrich	P4633
Potassium chloride	Sangon Biotech	A100395
Potassium Phosphate Monobasic	Sangon Biotech	A501211
Sodium chloride	Sangon Biotech	A501218

Comments/Description
Human osteosarcoma cell line
Human osteosarcoma cell line
A solution of phenol red is used as a pH indicator: its color exhibits a gradual transition from yellow to red over the pH range 6.6 to 8.0.

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Article Title: Three-Dimensional Bone Extracellular Matrix Model for Osteosarcoma

Signature: Yan Zhang Date: Oct. 19. 2018

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Dear Editor:

Thank you very much for your letter and the reviewers' comments concerning our manuscript entitled **"Three-Dimensional Bone Extracellular Matrix Model for Osteosarcoma" (JoVE59271)**. We have studied these valuable and constructive suggestions carefully and made revision to strengthen our manuscript according to the comments. The main corrections and responses to the reviewer's comments are as following:

Responses to the comments:

Editorial comments:

Special thanks for your kind and helpful comments.

1. **Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.**

   Response: We have carefully checked and revised our manuscript, such as the usage of standard abbreviations for SI unit (e.g. ml) and time designation (e.g. hr).

2. **Keywords: Please provide at least 6 keywords or phrases.**

   Response: We are sorry for not providing keywords according to Manuscript Instructions for Authors. Please check the revised section of **Keywords**, which includes **osteosarcoma, extracellular matrix, bone, heterogeneity, three-dimensional culture, model construction**.

3. **Please rephrase the Introduction to include a clear statement of the overall goal of this method.**

Response: A clear statement of the overall goal of this method has been added in the Introduction (Page 2, Line 78-84). In this video, our group establish a modified model and favorable alternative for three-dimensional long-term culture. OS cells injected into the tissue-derived BEM present a heterogeneously mesenchymal phenotype vividly as compared to plastic two-dimensional cultures. BEM derived from site-specific homologous tissue show its dramatic advantage as being a native niche for OS cells in vitro and great potential in OS theoretical and clinical research. This characterized BEM platform is simple but efficient for *in vitro* research and might be extended in modeling multiple cancers.

4. **Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.**

   Response: An ethics statement has been included before the numbered protocol steps (Page 2-3, Line 88-90). Animal care and use are conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication NO.80-23, revised in 1996) after approval from the Animal Ethics Committee of Sun Yat-sen University.

5. **Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.**

Response: We are sorry for omitting detail information of protocol steps and make more clearer illustration and add references to published material according to the following Editorial comments.

6. **1.1: Please specify the age, gender and type of mouse used. Please also describe how to harvest fresh fibula, tibia and femur from hindlimb and specify all surgical instruments used.**

   Response: We are sorry for not providing detail information of laboratory animals. 4 to 6-week-old BALB/c mice (without gender-specific requirement) are purchased from Laboratory Animal Center of Sun Yat-sen University. Execute mouse aseptically by cervical dislocation and cut off fresh fibula, tibia and femur from hindlimb with sterile surgical scissors. Peel off the epithelial tissue and then remove the soft tissue using scissors and tweezers to the greatest extent. We have added age, gender as well as strain information and samples collected information as requested (Page 3, Line 93-97).

7. **2.4: Please specify how the extraction is done. What container is used? What are the wash steps?**

   Response: According to a published material specifying how the extraction is done, the lipid in the bone is able to be extracted with a 1:1 mixture of chloroform and methanol for 1 hr. We have added reference to this published article. To prevent chloroform decomposition during the extraction, we used light-resistant container or centrifuge tube wrapped with tinfoil, which is cheaper and easily obtained. The wash steps are performed as instructed in 2.4 that the bones are rinsed firstly in methanol and then distilled water (Page 3-4, Line 125-133).

8. **2.6: Please specify the concentration of trypsin-EDTA.**

   Response: We specify the concentration of trypsin-EDTA in the text (Page 4, Line 135).

9. **2.8: What does decellularized bone extracellular matrix (BEM) refer to?**

   Response: Decellularized bone extracellular matrix (BEM) are bone-specific integrated scaffold retaining abundant extracellular components after demineralization and decellularization (Page 4, Line 149-150).

10. **3.2: It is unclear where the OS cells are obtained. Please clarify.**

    Response: We are sorry for not providing detail information of the OS cells. Human OS cell lines (MNNG/HOS and MG-63) are obtained from cell bank of the Chinese Academy of Sciences (Page 4, Line 160-161).

11. **3.3: Please provide the composition of complete culture medium.**

    Response: The composition of complete culture medium is Dulbecco's modified Eagle's medium/F12 (DF12) supplemented with 5% fetal bovine serum, 90 μg/ml ampicillin and 90 μg/ml kanamycin (Page 4, Line 156-157).

12. **3.4: Please describe how the cell status is monitored. What is observed?**

    Response: Keep monitoring medium color and cell status under the inverted fluorescence microscope during the culture process. The culture medium is bright red for pH 7.4, the optimum pH value for most mammalian cell lines cell culture. If the medium turns into orange or even yellow, immediately refresh the medium to maintain a healthy environment for OS cells. Besides, When OS cells expand to plate, gently transfer the OS-BEM model to another new well with sterile tweezer (Page 5, Line 178-184).

13. **3.5: Please describe how to harvest the OS-BEM model.**

    Response: OS-BEM model is transferred into another new well with tweezer and gently rinse with PBS to remove the culture medium. Then, transfer into a 15 ml centrifuge tube with tweezer and fix with 10% buffered formalin for histological identification (Page 5, Line 186-188).

14. **Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.**

    Response: The most critical steps for successful establishment and application of BEM are: 1) complete removal of cell debris; 2) maintenance of a sterile, healthy culture condition; 3) manual dexterity and gentle pipetting during injection, transfer and culture of OS-BEM model. Other reported protocols generally employ pressurization or a combination of chemical and enzymatic treatments, such as Triton X-100, SDS and

DNase/RNase solution to achieve potent decellularization. The cartilage tissue that undergoes decellularization with detergents has been shown to remove ECM components including glycosaminoglycans. To recapitulate a more intact BEM to the greatest extent, a moderate yet powerful decellularization method are performed here to avoid the dissolution and damage of key components and native architecture of bone environment. However, it is not to be neglected that this OS-BEM model rested in the plate without flowing medium, and consequently leading to an uneven distribution of oxygen and nutrients. Vascular network and other cell subtypes that help regulating the communication and interaction of microenvironmental signals and bone homeostasis need to be taken into consideration (Page 6-7, Line 262-278).

15. **References: Please do not abbreviate journal titles.**

    Response: We have carefully checked and revised references as requested.

16. **Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.**

    Response: We have sorted the items in alphabetical order according to the name of material/equipment.

Reviewers' comments:

Reviewer #1:

Special thanks to Reviewer #1 for the constructive and professional comments.

1. **Page 4 line 136: The authors write: "Note: All the sterile PBS used in this and the following steps contains 90 μg/mL ampicillin and 90 μg/mL kanamycin. Overnight wash under rotation or rocking motion are performed for effective sterilization." Please clarify: what provides an effective sterilization of the matrix. The sterilization is key for the protocol.**

   Response: All the sterile PBS used in this and the following steps contains 90 μg/ml ampicillin and 90 μg/ml kanamycin, which are powerful antibiotics for aseptic manipulation and also supplemented in complete culture medium for cell culture in the following steps. Overnight wash under rotation or rocking motion are performed for long-time thorough immersion with antibiotics to achieve effective sterilization for pore spaces (Page 4, Line 143-146).

2. **In general figures are of low quality and ill described, for example for Figure 3 A and B describe how cells were stained. I am assuming details and quality will be better in the video.**

   Response: Sorry for the ill described of cells with expressing mCherry and GFP fluorescence. To better track and observe multi-layer cells within the three-dimensional BEM model, MNNG/HOS and MG-63 are infected with lentiviral vector expressing mCherry and GFP fluorescence (Page 4, Line 161-163). Figure 3 A and B are high resolution pictures captured under the inverted fluorescence microscope. However, this is a 2D picture that cannot show stereoscopic effect during the observation through adjusting the microscope focus. We believe details will be presented better in the video.

Reviewer #2:

Special thanks for your detail suggestions.

1. **The application potential of the model should be described in more detail. How could this model be used to study the histological heterogeneity when seeded with tumor cell lines? Or is the idea to use primary OS cells?**

   Response: Heterogeneity between cancers from different patients (inter-tumor heterogeneity) and within a single tumor (intra-tumor heterogeneity) is a key challenge in the field of cancer therapy[1, 2]. Even within one tumor, genomic and phenotypic diversity has long been recognized, for example, a subpopulation that proposed as cancer stem cell. Diverse phenotypic and functional features of OS result from the genetic or epigenetic alterations, multiple anatomic site and complex bone microenvironmental signals. Both intrinsic and extrinsic mechanisms result in the genomic instability and heterogeneity of OS, with multiple morphological and clinical phenotypes[3-6]. Appropriate models are needed for revealing intra-tumor heterogeneity and explore potential strategies for OS therapy[7]. OS cells cultured *in vitro* is also a heterogeneous population comprised of multiple subclones which could show their distinguishable features under certain condition. Unlike the more unified morphology presented by OS cells in 2D monolayer culture, OS cells located in different areas within BEM could display cellular pleomorphism. This heterogeneous morphology exactly resembles clinical OS histopathology, which make this BEM model to be an ideal system for studying the heterogeneity of OS. It has been used in our prior publication demonstrating the phenotypic heterogeneity and regulatory mechanism of OS dedifferentiation with success[8].

[1] Lipinski, K.A. et al. Cancer Evolution and the Limits of Predictability in Precision Cancer Medicine. Trends in Cancer. 2, 49-63 (2016).

[2] McGranahan, N. & Swanton, C. Clonal Heterogeneity and Tumor Evolution: Past, Present, and the Future. Cell. 168, 613-628 (2017).

[3] Poos, K. et al. Genomic Heterogeneity of Osteosarcoma - Shift from Single Candidates to Functional Modules. PLoS One. 10, e123082 (2015).

[4] Martin, J.W., Squire, J.A. & Zielenska, M. The Genetics of Osteosarcoma. Sarcoma. 2012, 1-11 (2012).

[5] Klein, M.J. & Siegal, G.P. Osteosarcoma: Anatomic and Histologic Variants. American Journal of Clinical Pathology. 125, 555-581 (2006).

[6] Alfranca, A. et al. Bone Microenvironment Signals in Osteosarcoma Development. Cellular and Molecular Life Sciences. 72, 3097-3113 (2015).

[7] Brown, H.K., Schiavone, K., Gouin, F., Heymann, M. & Heymann, D. Biology of Bone Sarcomas and New Therapeutic Developments. Calcified Tissue International. 102, 174-195 (2018).

[8] Zhang, Y., Pan, Y., Xie, C. & Zhang, Y. MiR-34a exerts as a key regulator in the dedifferentiation of osteosarcoma via PAI-1–Sox2 axis. Cell Death & Disease. 9 (2018).

2. What was the reason to use mCherry and GFP fluorescent transgenic cells? I guess it were transgenic cells as it is not mentioned in material and methods at all. Tracking of the cells within the bone matrix? The quality of figure 3A is not sufficient to recognize cells.

Response: Thanks for this helpful comment, the Reviewer #1 also gives us the same suggestion. Sorry for the ill described of cells with expressing mCherry and GFP fluorescence. To better track and observe multi-layer cells within the three-dimensional BEM model, MNNG/HOS and MG-63 are infected with lentiviral vector expressing mCherry and GFP fluorescence (Page 4, Line 161-163). Figure 3A is a high resolution picture captured under the inverted fluorescence microscope. However, this is a 2D picture that cannot show stereoscopic effect during the observation

through adjusting the microscope focus. We believe details will be presented better in the video.

3. **Was there a difference in the distribution within the bone matrix between these two cell lines? Was there a difference between the different bones used?**

   Response: There is no difference in the distribution within the bone matrix between these two cell lines. We suggest OS cells present heterogeneous morphology depending on the structural features and surrounding extracellular matrix components of different areas within the bones, but not the different bones used. This characterization could be well duplicated in another bone after the same demineralization and decellularization procedure.

4. **What was the rationale for choosing the antibodies for collagen I, collagen IV, fibronectin and laminin? The immunohistochemistry shows no positive controls which would allow judging the specificity of the antibody binding. I doubt the results, as all four images (Fig 1. E-H) look the same. Moreover, the histology figure does not show bone tissue but muscle tissue.**

   Response: Native bone is the complex hierarchical composites, consisting of around 65 wt % mineral phase, 25 wt % organic fiber (mostly collagen I), and 10 wt % water. Collagen is the predominant organic component in the extracellular matrix (ECM) of natural bones and plays an important role in the process of bone regeneration and remodeling[1, 2]. Consequently, we present IHC results of collagen and retract the staining of fibronectin and

laminin, although these are also main components of bone ECM. To dispel this concern on specificity of the antibody binding, we also add the IHC staining on native bone for positive control, which also presents a high expression level of four key ECM components (Figure 1E). Preserved ECM components in decellularized BEM provide a suitable microenvironment for OS cells that is similar to that of native bones. Besides, muscle tissue is already peeled off and removed using scissors and tweezers and no muscle tissue exists inside the bone. All four images are serial section therefore they look quite the same.

[1] Triffit, J.T. The organic matrix of bone tissue. J.B. Lippincott Philadelphia (1980).

[2] Long, T. et al. Fabrication of three‐dimensional porous scaffold based on collagen fiber and bioglass for bone tissue engineering. J Biomed Mater Res B Appl Biomater (2015).

5. **The H&E stained images are examples of OS cells grown in bone matrix for 14 days. The cells seem to be viable and well distributed. A detailed examination of the cells grown within the bone matrix would be necessary. An immunohistochemical staining of the four antibodies on these samples would have been interesting to see if osteogenic matrix, a marker for osteosarcoma, is produced and if and how cells are differentiated.**

Response: Cell activity is determined using Ki67 immunostaining, which also show great advantage on long-term *in vitro* culture of OS cells. Also, OS cells in BEM culture highly express secreted protein acidic and rich in cysteine (SPARC/osteonectin), which is a bone matrix glycoprotein that binds calcium and specific for osteoid matrix (Figure 3D). SPARC also shows affinity for collagen in addition to bone mineral calcium[1]. This result also confirm that the histology figures are bone tissue.

[1] Young, M.F. et al. Structure, expression, and regulation of the major noncollagenous matrix proteins of bone. Clinical Orthopaedics and Related Research (1992).

6. **Some of the figures are not in focus (for example Fig. 1B, Fig. 3A), scale bars in Figs 1E-H are double in different colors.**

   Response: We have replaced Figure 1B with another picture of higher quality. Scale bars in Figure1E are corrected in all black.

We sincerely hope that the corrections will meet with approval. Once again, we truly appreciate your comments and helpful suggestions.

Best regards.

Yours sincerely,

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