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).

1. **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**.

1. **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.

1. **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.

1. **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.

1. **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).

1. **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).

1. **2.6: Please specify the concentration** **of trypsin-EDTA.**

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

1. **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).

1. **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).

1. **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).

1. **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).

1. **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).

1. **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).

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

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

1. **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).

1. **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).

1. **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.

1. **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.

1. **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).

1. **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).

1. **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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