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Measuring Bone Remodeling and Recreating the Tumor Bone Microenvironment Using Calvaria Co-culture and Histomorphometry

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TITLE:

Measuring Bone Remodeling and Recreating the Tumor Bone Microenvironment Using Calvaria Co-culture and Histomorphometry

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KEYWORDS:

bone, bone formation, bone resorption, breast cancer, bone metastasis, calvaria, coculture

SUMMARY:

The ex vivo culture of bone explants can be a valuable tool for the study of bone physiology and the potential evaluation of drugs in bone remodeling and bone diseases. The presented protocol describes the preparation and culture of calvarias isolated from newborn mice skulls, as well as its applications.

ABSTRACT:

Bone is a connective tissue constituted of osteoblasts, osteocytes, and osteoclasts and a mineralized extracellular matrix, which gives it its strength and flexibility and allows it to fulfill its functions. Bone is continuously exposed to a variety of stimuli, which in pathological conditions can deregulate bone remodeling. To study bone biology and diseases and evaluate potential therapeutic agents, it has been necessary to develop in vitro and in vivo models.

This manuscript describes the dissection process and culture conditions of calvarias isolated from neonatal mice to study bone formation and the bone tumor microenvironment. In contrast to in vitro and in vivo models, this ex vivo model allows preservation of the three-dimensional environment of the tissue as well as the cellular diversity of the bone while culturing under defined conditions to simulate the desired microenvironment. Therefore, it is possible to investigate bone remodeling and its mechanisms, as well as the interactions with other cell types, such as the interactions between cancer cells and bone.

The assays reported here use calvarias from 5–7 day old BALB/C mice. The hemi-calvarias obtained are cultured in the presence of insulin, breast cancer cells (MDA-MB-231), or conditioned medium from breast cancer cell cultures. After analysis, it was established that

insulin induced new bone formation, while cancer cells and their conditioned medium induced bone resorption. The calvarial model has been successfully used in basic and applied research to study bone development and cancer-induced bone diseases. Overall, it is an excellent option for an easy, informative, and low-cost assay.

INTRODUCTION:

Bone is a dynamic connective tissue that has several functions, including supporting the muscles, protecting the internal organs and bone marrow, and storing and releasing calcium and growth factors[1,2]. To maintain its integrity and proper function, bone tissue is continuously under the process of remodeling. In general terms, a cycle of bone remodeling can be divided into bone resorption and bone formation[1]. An imbalance between these two phases of bone remodeling can lead to the development of bone pathologies. Also, diseases such as breast cancer often affect bone integrity; approximately more than 70% of patients in advanced stages have or will have bone metastases. When breast cancer cells enter the bones, they affect bone metabolism, resulting in excessive resorption (osteoclastic lesions) and/or formation (osteoblastic lesions)[3].

To understand the biology of bone diseases and develop new treatments, it is necessary to understand the mechanisms involved in bone remodeling. In cancer research, it is essential to investigate the bone metastasis process and its relation to the metastatic microenvironment. In 1889, Stephen Paget hypothesized that metastases occur when there is compatibility between the tumor cells and the target tissue, and suggested that the metastatic site depends on the affinity of the tumor for the microenvironment[4]. In 1997, Mundy and Guise introduced the concept of the "vicious cycle of bone metastases" to explain how tumor cells modify the bone microenvironment to achieve their survival and growth, and how the bone microenvironment promotes their growth by providing calcium and growth factors[5,6,7].

To characterize the mechanisms involved in bone remodeling and bone metastasis and to evaluate molecules with possible therapeutic potential, it has been necessary to develop in vitro and in vivo models. However, these models currently present many limitations, such as the simplified representation of the bone microenvironment, and their cost[8,9]. The culture of bone explants ex vivo has the advantage of maintaining the three-dimensional organization as well as the diversity of bone cells. In addition, experimental conditions can be controlled. The explant models include the culture of metatarsal bones, femoral heads, calvarias, and mandibular or trabecular cores[10]. The advantages of the ex vivo models have been demonstrated in diverse studies. In 2009, Nordstrand and collaborators reported the establishment of a coculture model based on the interactions between bone and prostate cancer cells[11]. Also, in 2012, Curtin and collaborators reported the development of a three-dimensional model using ex vivo cocultures[12]. The purpose of such ex vivo models is to recreate the conditions of the bone microenvironment as accurately as possible to be able to characterize the mechanisms involved in normal or pathological bone remodeling and evaluate the efficacy of new therapeutic agents.

The present protocol is based on the procedures published by Garrett[13] and Mohammad et al.[14]. Neonatal mouse calvaria cultures have been used as an experimental model, as they retain the

three-dimensional architecture of the bone under development and bone cells, including cells at all stages of differentiation (i.e., osteoblasts, osteoclasts, osteocytes, stromal cells) that lead to mature osteoclasts and osteoblasts, as well as the mineralized matrix[14]. The ex vivo model does not represent the pathological process of bone diseases totally. However, effects on bone remodeling or cancer-induced bone osteolysis can be accurately measured.

Briefly, this protocol consists of the following steps: the dissection of calvarias from 5–7 day old mice, calvaria preculture, calvaria culture applications (e.g., culture in the presence of insulin, cancer cells or conditioned medium, and even agents with therapeutic potential, according to the aim of the investigation), bone fixation and calvaria decalcification, tissue processing, histological analysis, and result interpretation.

PROTOCOL:

All mice used in these assays were obtained from BALB/c mice strains, using male and female mice indiscriminately. Previous culture experiments have also been performed using other strains, such as FVB, Swiss mice, CD-1, and CsA mice[11,12,14]. All mice were housed according to National Institutes of Health (NIH) guidelines, Appendix Q. Procedures involving animal subjects have been approved by the Institutional Animals Care and Use Committee (IACUC) at the Center for Scientific Research and Higher Education at Ensenada (CICESE).

1. Calvarial dissection

1.1. Sterilize dissection instruments (e.g., 1 x 2 teeth tissue forceps, straight surgical scissors, dissecting scissors, fine-tipped tweezers, fine curved tip dissecting forceps, dissecting forceps, scalpel). Sterile distilled water can be used to clean the surgical tools between each dissection, and sterile 1x PBS can be used for cleaning each hemi-calvaria.

1.2. Place the sterile dissection instruments, water, 1x PBS, and required materials to carry out the procedure (e.g., micropipettes, precipitate glasses, sterile Petri dishes) in a laminar flow hood.

NOTE: Carry out the entire procedure under sterile conditions.

1.3. Add 12 mL of sterile 1x PBS into two 10 cm Petri dishes.

1.4. Select the pups and place them near the hood.

NOTE: Dissect the calvarias from 5–7 day old pups.

1.5. Pick and hold the mouse carefully using dissecting forceps.

1.6. Decapitate the mouse using dissecting scissors and place the head in a Petri dish with PBS.

NOTE: Decapitation is not suitable for older mice. If the experiment must be done with older mice, the method of euthanasia should be modified according to the animal experimentation rules.

1.7. Hold the head firmly by the nose area with 1 x 2 teeth tissue forceps and remove the skin over the skull until the calvaria is visible.

1.8. Identify the sutures (i.e., sagittal, coronal, and lambdoid) of the skull on the exposed calvaria.

1.9. Penetrate with the tip of the microscissors approximately 2 mm behind the lambdoid suture and make a straight cut alongside it.

1.10. Insert the tip of the microscissors on the rear side of the skull. Make a straight cut along the distal side of the lambdoid suture towards the coronal suture. Proceed similarly with the other lambdoid suture.

1.11. Make another cut (at a 45° angle) to connect the end of the cut made with the cut of the anterior fontanel.

1.12. Repeat steps 1.10 and 1.11 with the other lambdoid suture.

NOTE: It is important to identify the sutures to make appropriate cuts and to be able to perform adequate embedding and data analysis.

1.13. Use fine-tip forceps to remove the calvaria and place it in a Petri dish. With a scalpel, make a straight cut from the posterior fontanel along the sagittal suture through the coronal suture and ending in the anterior fontanel to obtain two hemi-calvarias.

1.14. Pick up each of the hemi-calvarias with the fine-tipped tweezers and place them in a new Petri dish containing PBS.

2. Calvaria culture

2.1. Add 1 mL of high-glucose DMEM medium supplemented with 0.1% bovine serum albumin (BSA) and 1% antibiotic/antimycotic (i.e., penicillin and streptomycin) in the wells of a 24 well plate. With fine-tip forceps, pick up each hemi-calvaria and place it in a well.

NOTE: Put the hemi-calvarias concave side down.

2.2. Incubate the hemi-calvarias for 24 h at 37 °C with 5% CO_2.

2.3. Remove the culture medium from each well and add 1 mL of fresh media containing the compound to test or conditioned media from other cells.

NOTE: Use at least three hemi-calvarias for each treatment group and use negative and positive controls.

2.4. Incubate the hemi-calvarias for 7 days, changing the media every 2–3 days.

3. Culture with cancer cells

3.1. Select a Petri dish with cancer cells at 80–90% confluence and trypsinize them. To trypsinize, wash the cancer cells 1x using PBS (5 mL per 75 cm^2 flask) followed by incubation in an HBSS solution containing 0.05% trypsin and 0.53 mM EDTA (2 mL per 75 cm^2 flask).

3.2. Transfer the cell suspension into a 15 mL conical tube and centrifuge at 800 x *g* for 5 min at room temperature (RT).

3.3. Remove and discard the supernatant.

3.4. Resuspend the pellet in 2 mL of DMEM containing 2% fetal bovine serum (FBS) and 1% antibiotic/antimycotic. Count live cells.

3.5. Assign hemi-calvarias to each study group (i.e., the negative control and coculture group for RNA or histology analysis).

3.6. Remove the culture media from the hemi-calvarias and add 1 mL of DMEM containing 2% FBS and 1% antibiotic/antimycotic supplemented or not supplemented with cancer cells.

NOTE: The amount of cells to add can change depending on the cell line tested. With cancer cells like MDA-MB-231 or PC-3, 500,000 cells per well work appropriately.

3.7. Incubate for 24 h at 37 °C with 5% CO_2. Pass each hemi-calvaria to a new well to retain only cancer cells that adhered to the bone tissue.

3.8. Incubate for 7 days at 37 °C with 5% CO_2 and change the media every 2–3 days.

4. Fixation

4.1. Cut squares of tissue paper to wrap the hemi-calvarias.

NOTE: Biopsy foam pads or steel capsules for small biopsies can also be used.

4.2. Use straight fine-tip forceps to pick up each hemi-calvaria from the sagittal suture, place on a tissue paper, and wrap.

4.3. Place the wrapped hemi-calvaria inside a labeled embedding cassette.

4.4. Place the cassette into a container with 10% phosphate-buffered formalin.

4.5. Fix for 24 h at 4 °C.

5. Decalcification

5.1. Remove the formalin, add 1x PBS, and agitate the tissues for 30 min to rinse the hemi-calvarias.

5.2. Remove the PBS and add 10% EDTA (pH = 8, 0.34 M).

NOTE: Verify that the solution covers the tissues completely.

5.3. Decalcify the tissues for 48 h at 4 °C.

5.4. Discard the EDTA solution and add 1x PBS to rinse the tissue.

5.5. Store the hemi-calvarias in 70% ethanol until the histology processing.

6. Tissue processing

6.1. Dehydrate the tissues with rounds of 96% ethanol, 60 min (3x), followed by 100% ethanol, 60 min (3x).

6.2. Replace the ethanol by 100% xylene, 60 min (3x).

6.3. Incubate the cassettes in paraffin wax, 60 min (2x).

7. Embedding

7.1. Open the cassettes, carefully remove the tissue paper, and unwrap the calvaria.

7.2. Pick up each calvaria carefully and stack all the hemi-calvarias from the same group in the same orientation.

7.3. Put some paraffin in a mold.

7.4. Pick up all hemi-calvarias with the forceps and place them inside the mold with the sagittal suture down towards the base of the mold.

NOTE: Orientation of the hemi-calvarias in the mold during inclusion is crucial to obtain consistent histological sections and reproducible results because this orientation will allow

posterior identification of the front and parietal bones from the calvarias and the coronal suture facilitating the quantitative assessment.

7.5. Release the forceps and verify that the hemi-calvarias stay in place.

7.6. Place the labeled cassette on top of the mold and fill it with more paraffin to cover the hemi-calvarias.

7.7. Move the molds to the cold surface.

8. Sectioning

8.1. Trim 500–600 µm of the sagittal suture with the microtome.

8.2. Cut 4 µm thick sections, and collect the sections needed.

8.3. Trim another 300 µm.

8.4. Cut and collect another six 4 µm thick sections.

8.5. Trim the block 300 µm further and collect more sections.

8.6. Mount the sections onto glass microscope slides.

8.7. Dry the slides at RT.

9. Staining

9.1. Immerse the sections in 100% xylene for 3 min.

9.2. Submerge in 100% ethanol for 1 min.

9.3. Merge in 96% ethanol for 1 min.

9.4. Immerse in 80% ethanol for 1 min.

9.5. Submerge in 70% ethanol for 1 min.

9.6. Rinse in water for 3 min.

9.7. Submerge in hematoxylin for 3 min.

9.8. Rinse in water until the section is clear.

9.9. Submerge in a saturated lithium carbonate solution for 10 sec.

9.10. Rinse in water for 3 min.

9.11. Submerge in 96% ethanol for 1 min.

9.12. Submerge in eosin for 3 min.

9.13. Immerse in 96% ethanol for 1 min.

9.14. Submerge in 100% ethanol for 1 min.

9.15. Submerge in 100% xylene for 3 min.

9.16. Add some per mount-mounting medium to the slide and protect it with a coverslip.

NOTE: You can complement the hematoxyline and eosin (H&E) staining with tartrate-resistant acid phosphatase (TRAP) staining to evaluate osteoclast and osteolysis.

10. Quantitative assessment: defining area for analysis

10.1. Examine the sections under low power (i.e., 4x) to identify the orientation and the sutures.

10.2. Define the coronal suture and identify the long bone surface on one side and the short surface on the other.

10.3. Identify the coronal suture under 40x magnification, and move two or three optical fields away from the suture along the long surface. Capture images of this area for analysis.

10.4. Define the old bone area and the new bone area. The eosin Y with orange G stains the old bone darker and the new bone lighter.

10.5. Measure the total bone area of the old and new bone with Image J software using the color threshold tool. Express the results as μm^2.

11. Data analysis

11.1. Analyze the results using statistical software. Significant differences between groups can be determined using appropriate tests (e.g., nonparametric Mann-Whitney U test as is done herel).

REPRESENTATIVE RESULTS:

To evaluate bone formation in the calvarial model, we cultivated the hemi-calvarias in media with or without 50 µg/mL of insulin. Tissue sections were prepared and stained with H&E. In

these conditions, the histology showed that the structural integrity of the calvarial bone was maintained, allowing the identification of its different components (**Figure 1**). The calvarias treated with insulin presented an increase in the amount of bone tissue compared to the control (**Figure 2A**). Quantitative histomorphometric analysis for the thickness and bone area of hemi-calvaria confirmed a significant increase for both parameters in the insulin-treated tissues compared to the control (**Figure 2B**). These data indicate the feasibility of the ex vivo model protocol to reproduce bone formation conditions.

Additionally, the calvarial ex vivo model can be used to reproduce cancer and bone microenvironment conditions. The protocol was used to evaluate the effect of the breast cancer cells MDA-MB-231 on murine calvarias. To perform this, the calvarial bones were directly cocultured with the cancer cells or cultured only with the conditioned media of the cancer cells. After 7 days of culture, the calvarias were embedded in paraffin, and H&E staining was performed. The coculture with MDA-MB-231 breast cancer cells appeared to increase osteolysis, as shown by the decrease in bone and the damages to the calvarial structure when compared to the control calvarias cultivated only with media (**Figure 2A**). Bone area quantification of calvarias showed a significant decrease in the total bone area of the calvarias cultured with MDA-MB-231 cancer cells compared to the control (**Figure 2B**). These results demonstrated that the cocultures of cancer cells with calvarial tissue can recreate the cancer and bone microenvironment and could be used to investigate the mechanisms of osteolytic bone metastasis or to evaluate possible inhibitors of this process.

FIGURE LEGENDS:

Figure 1: Histological structure of the calvarial bone. Representative tissue section of hemi-calvaria after H&E staining.

Figure 2: Insulin increased the bone area and thickness of hemi-calvarias ex vivo while coculture of hemi-calvarias with MDA-MB-231 breast cancer cells induced osteolysis. For the bone formation model, the hemi-calvarias were cultured in the presence or absence of insulin (50 µg/mL), while for the coculture with cancer cells, hemi-calvarias were cultured in the presence or absence of 5 x 10^5 MDA-MB-231 cells for 7 days and histomorphometrical analysis was performed on H&E stained sections. (**A**) Representative tissue sections. (**B**) Histomorphometrical analysis of the bone area. Results are represented as the average ± SEM (n = 3). *P < 0.05, nonparametric Mann-Whitney U test.

DISCUSSION:

Here, we describe the protocol for a calvarial ex vivo model to evaluate bone formation or resorption and to study the interactions of cancer cells with calvarial mouse bone. The critical steps of this technique are the dissection, culture, embedding, and histomorphometrical analysis of the calvarias. During the dissection of the calvarias, it is crucial to cut the hemi-calvarias into a trapezoid, as it will strongly facilitate the orientation during the paraffin inclusion. When studying cancer cell interactions with the calvarias, it is important to use multiwell plates that are not treated for cell culture to prevent cancer cells from adhering and growing on the plastic instead of the bone. During the tissue inclusion, it is important to carefully

orient each hemi-calvaria. They need to be placed in a vertical manner with the sagittal border on the outer side of the paraffin block. The orientation of the bone is essential to obtain consistent histological sections and reproducible results. Similarly, it is critical for consistency during the histomorphometric analysis to define a specific region in the calvaria to measure bone remodeling. Here, we identified first the coronal suture and then the long bone surface where the pictures were taken and the measurements made.

To achieve the standardization and develop the protocols described, we modified some established methodologies slightly. During the dissection of the hemi-calvarias, PBS was used to rinse the mouse heads instead of culture media. To determine the number of cells necessary to produce an effect on the calvarias, the tissue was incubated with different numbers of breast cancer cells. In general, 5×10^5 cancer cells work well for a 7 day assay. Also, during fixation, tissue paper was used instead of sponges to protect the calvarias. The tissue was well-preserved within the paper.

The model described has some limitations. The calvarias from neonatal mice lack some of the components of the bones that are the recipient of metastases, whether structural (i.e., trabecular bone) or cellular components such as immune cells or other cells of the bone marrow, including hematopoietic stem cells that cancer cells interact with. The culture of the calvarias in vitro cannot simulate the complete physiopathology. Also, when it comes to bone metastases, breast cancer cells seldom metastasize to the calvaria, and tend to favor bones with more active bone remodeling, such as the vertebrae, the hip, or the long bones of the arms and legs. Besides, the coculture of hemi-calvarias with cancer cells only represents the latest steps of the metastatic cascade: the colonization of the bone and the growth of the metastasis. Furthermore, the number of samples is limited by the number of pups per litter. It is recommended to use one litter per experiment and not mix litters to avoid variations within the results. We obtained similar results in bone remodeling when using calvarias from BALB/c or FVB mice, but whether the strain affects the time response of bone remodeling in this assay remains to be determined.

The main advantages of the calvarial ex vivo model compared to in vivo models include lower cost, simplicity of the assay, and shorter experimental time to obtain a bone remodeling response. The coculture of cancer cells and calvarias allows studying the cell-contact dependent interactions as well as the effect of secreted factors on bone remodeling, while the use of conditioned media allows focusing on the effect of soluble factors. In addition, the direct contact model can facilitate the characterization of intercellular interactions and molecular mechanisms of the bone metastasis microenvironment, as well as the study and evaluation of new drugs for the treatment of skeletal complications of malignancies.

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DISCLOSURES:

The authors declare no competing interests.

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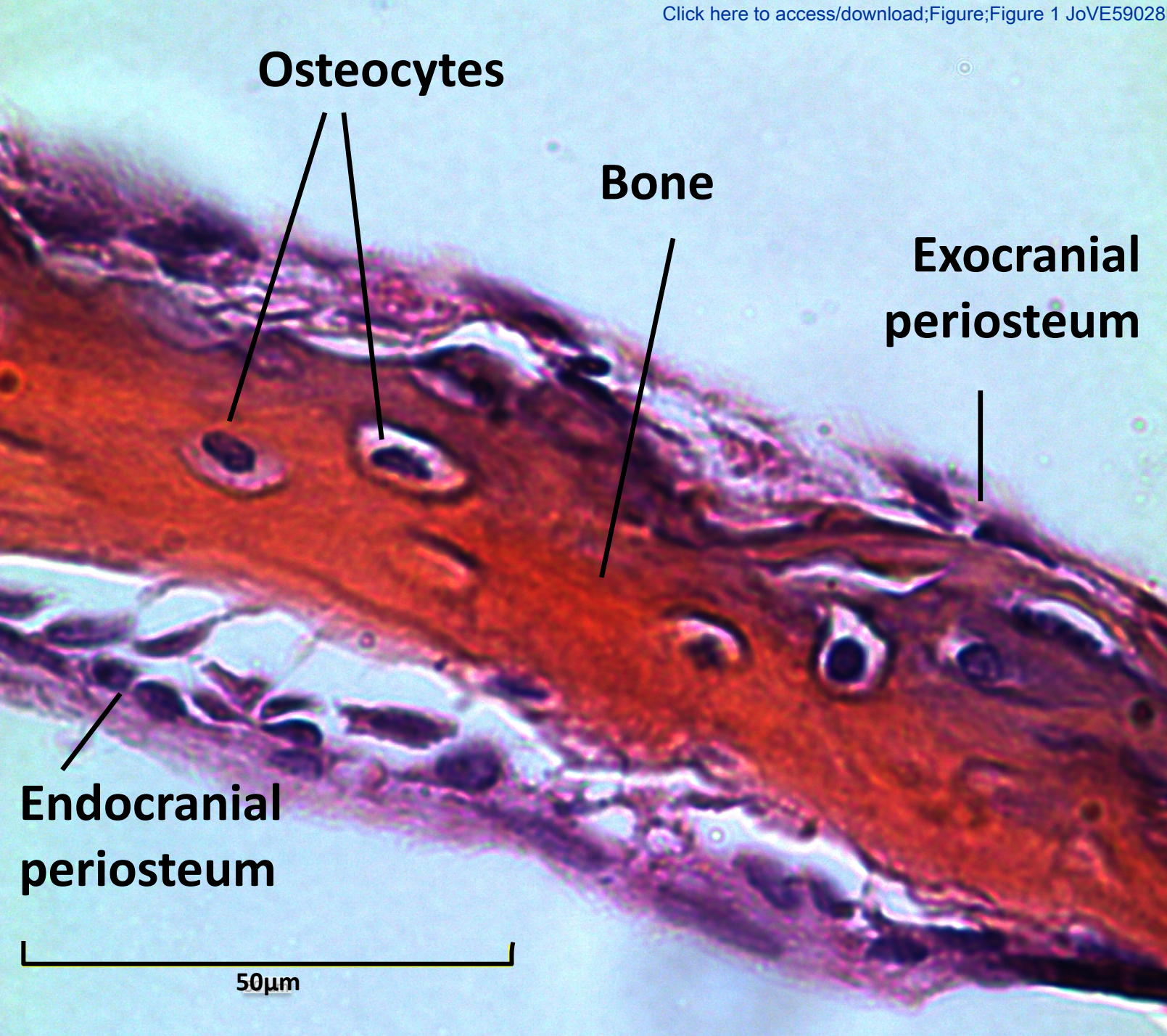

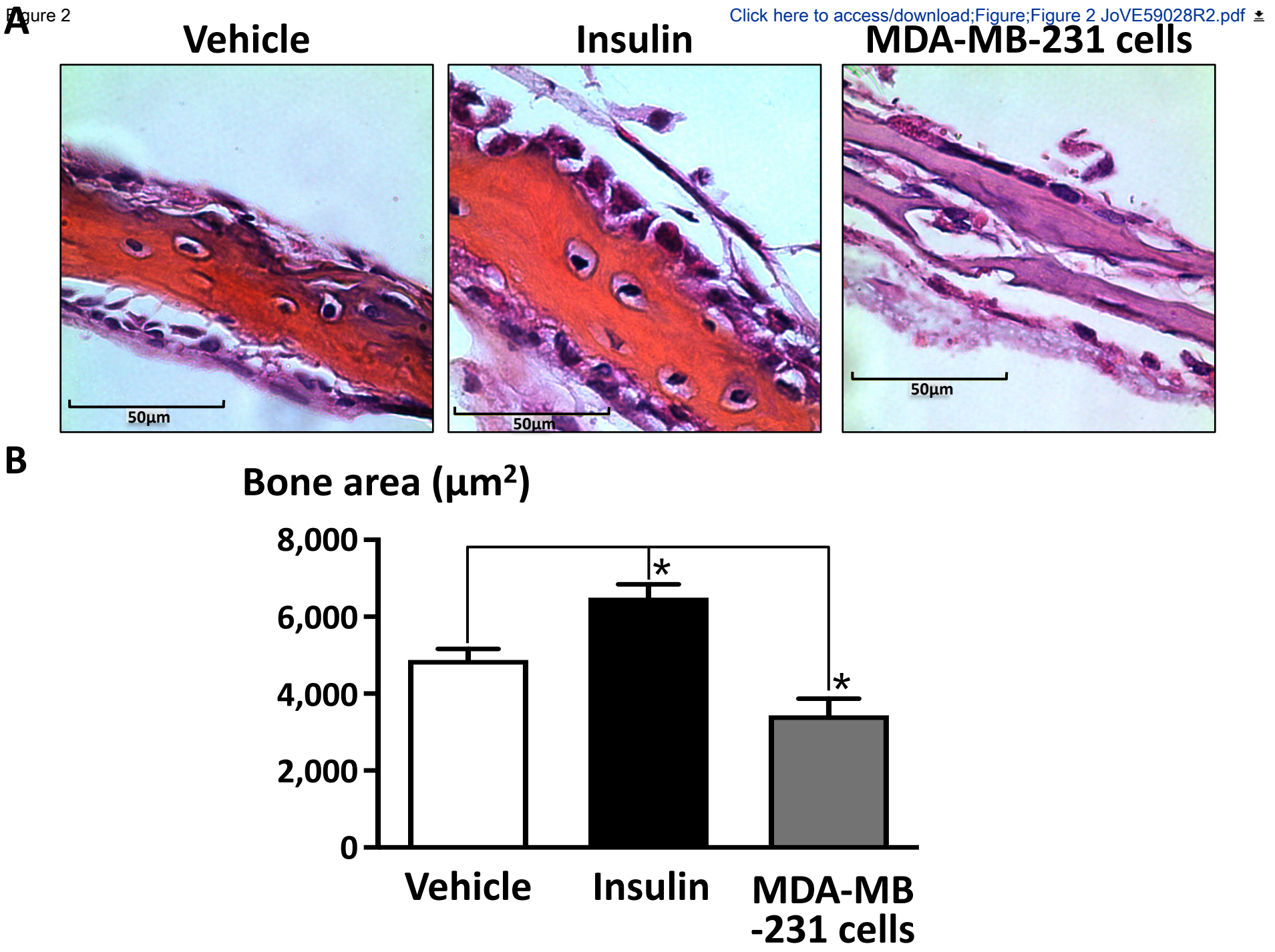

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
24 well cell culture	Corning	CLS3524	
24 well non tissue culture	Falcon	15705-060	
2 mL cryovial	SSI	2341-S0S	
Antibiotics-Antimycotic	Corning	30-004-CI	
BSA	Biowest	P6154-100GR	
Centrifugue	Eppendorf	22628188	Centrifuge 5810R
Coverslips	Corning	2935-24X50	
Cytoseal resin	Richard Allen	8310-10	
DMSO		D2650-100ML	
Dulbecco´s Modification of Eagles Medium, with 4.5 g/L glucose and L-glutamine, without sodium pyruvate	Corning	10-017-CV	
Dulbecco's PBS (10X)	Corning	20-031-CV	
Ebedding Cassettes	Sigma	Z672122-500EA	
EDTA	Golden	26400	
Embedding Workstation	Thermo Scientific	A81000001	
Eosin	Golden	60600	
Ethanol absolute	JALMEK	E5325-17P	
Fetal Bovine Serum	Biowest	BIO-S1650-500	
Filters	Corning	CLS431229	
Forceps and scissors	LANCETA HG	74165	
Formalin buffered 10%	Sigma	HT501320	
Glass slides 25 x 75 mm	Premiere	9105	
Harris's Hematoxylin	Jalmek	SH025-13	
High profile blades	Thermo Scientific	1001259	
Histoquinet	Thermo Scientific	813150	STP 120
Insulin from bovine pancreas	Sigma	16634	
Microscope	ZEISS	Axio Scope.A1	
Microtome	Thermo Scientific	905200	MICROM HM 355S
Mouse food, 18% prot, 2018S	Harlan	T.2018S.15	

Neubauer	VWR	631-0696
Orange G	Biobasic	OB0674-25G
Paraffin	Paraplast	39601006
Paraffin Section Flotation Bath	Electrothermal	MH8517X1
Petri dish	Corning	CLS430167
Phloxin B	Probiotek	166-02072
Trypan Blue	Sigma	T8154
Trypsin-EDTA	Corning	25-051-CI
Wax dispenser	Electrothermal	MH8523BX1
Xylene	Golden	534056-500ML

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DIVISION DE BIOLOGIA EXPERIMENTAL Y APLICADA

Feb 12, 2020
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Phillip Steindel
Review editor
JoVE

Dear Editor,

We appreciate the valuable editorial comments for our work entitled "**Measuring Bone Remodeling and Recreating the Tumor Bone Microenvironment Using Calvaria Co-culture and Histomorphometry**". Your comments were addresses as follow:

Editorial comments:

1) H&E images in the video still have 'Endostium' labels, despite this being changed in the figures. Please correct.

The H&E images in the video were modified accordantly.

2) in 'Calvarial dissection', step 7, you mention the possibility of leaning on the lid of a Petri dish; can you clarify this a bit (e.g., what part of the body)?

In Calvarial dissection, the most important is to reduce the movement of the mouse- head to be precise when cutting the bone. To do that, holding the head by the nose area helps a lot. Sometimes when the hand of the person is small, it can help a little to put the mice leaning on the lid, not in a specific position, it depends on the person, and it is to have more support. However, we believe that this phrase can be confusing for the reader, and we do not want to miss lead it. For this reason, we decide to remove it from step 7.

Thank you for your consideration.

Sincerely,

Patricia Juárez, Ph.D.
Associate Professor
Biomedical Innovation Department