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Culturing and measuring fetal and newborn murine long bones.

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Dr. Anna Justis, Ph.D.
Editor, *JoVe*

January 17, 2019

Dear Dr Anna Justis

Thank you very much for your help with our submission of our manuscript 59509 and please find below our point-by-point responses to the editor comments.

With best regards,

Alberto Roselló-Díez and Veronica Uribe

Please order the steps properly so that the protocol can be followed in chronological order.

We have reorganized a few of the steps, so that now the protocol follows a chronological order.

Please note that the protocol text will be used to generate the script for the video and must contain everything that you would like shown in the video. Please review the protocol to ensure that there is enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We included a few small changes in the protocol, which we believe now has enough details to supplement the video. These changes include the possibility of using DMEM for dissection medium preparation (point 1.2), the storage conditions of the culturing medium (point 1.3), additional details on the preparations needed prior to embryo extraction (point 1.4), additional step of uterus transfer to a biosafety cabinet (point 2.5), the use of clean 60-mm petri dishes for fetus dissection (point 2.7), the importance of transferring the embryonic body to a clean dish (point 2.8), clarification on what bones are transferred and the pipette used (point 2.14), an additional Note suggesting change of cloudy medium, more detail on dissection medium removal (point 2.16), an additional step where the procedure for retinoic acid addition is explained (point 2.18) and a more detailed explanation on the fixation of the samples (point 2.21).

Please expand the section title to be more informative.

We expanded the titles of the three sections of the protocol as follows:

1. Preparations prior to the day of bone culture
2. Protocol: culture of fetus and newborn tibia and femur
3. Measurement and analysis of the full length of the bone and of the mineralized region

Peeling?

We corrected the spelling of the word to peeling

Tweezers or scissors?

The skin of the fetuses and newborns is soft enough to be cut with tweezors.

Please move the discussion of critical steps to the Discussion section.

The discussion of critical steps was moved to the Discussion section. The critical steps now look as follows:

Critical step: It is important to remove as much soft tissue as possible, taking special care in removing the tissue that connects the two cartilage poles, but avoiding damaging the cartilage, the perichondrium and the bones.
Critical step: Extra care should be taken when the bones are transferred to the wells, as they can easily stick to the pipette.

The discussion section was modified to include the discussion of the critical steps:
The most critical step of this method is the isolation of the bones, which have to remain intact and with as less soft tissue between the ends of the bone as possible. *Leaving soft tissue between the ends of the bones will prevent normal growth of the bone and induce bending, as is exemplified in Figure 1E. Soft tissue at the ends of the bones can be left, as it will eventually disappear. Another step, which requires extra care is the transfer of the bones to the culturing plate, as they easily can stick to the plastic pipette. One possible solution is pipetting up and down dissection medium containing blood and tissue pieces, as it creates a coating that helps to preclude the bones from sticking to the pipette.*

Which four bones? Please specify the size/capacity of the pipette.

We change the phrase to address these points:
Place the four bones (left and right tibiae and femurs) in the first well of the 24-well plate with a plastic 1-mL sterile pipette.

Purpose?

We changed the word *type* for *purpose* in the text.

Please describe how. Aspiration?

We change the phrase to address this point:
When all the desired bones are transferred to the wells, take the plate to the hood and remove the dissection medium with a plastic 1-mL sterile pipette by aspiration and taking extra care for not aspiring the bones.

Can this step be moved to section 2, i.e., after step 2.15?

This step was moved to the Note after new step 2.16.

10 µM? Please specify its concentration.

The phrase was modified to clarify this point:
2.20. To assess proliferation, add 5-ethynyl-2'-deoxyuridine (EdU) or 5-bromo-2'-deoxyuridine (BrdU) to the medium at a final concentration of 10 µM 1–2 h before fixation (EdU stock was 20 mM).

Is this step going to be filmed? If so, more specific details are needed.

This step is not intended to be filmed. We changed the phrase to remove all doubts:
2.23. Process the bones for desired downstream applications.

Can this step be moved to section 2, i.e., after step 2.17?

This step was moved to section 2, step 2.23.

Please also define growth rate and how it is calculated

We included an explaining point:
3.4. To calculate the growth rate, defined as the average increase in length per day, the difference between the final length of the bone and the initial one is divided by the number of days in culture.
And also included a new comment in the discussion regarding the measurement of the growth rate:
Apart from measuring the length of the bone, the growth rate (average increase in length per day) can be estimated easily in these conditions. However, this approach for growth rate calculation is not valid over longer culturing periods, where other methods, like calcein labeling, should be used.

How to identify the mineralized region?

We included two new points to clarify this:
3.2. To ensure easy and reliable measurements, it is important that the pictures are taken with a good contrast, to distinguish the mineralized part.
3.3. The mineralized region is characterized by the darker color and is easily distinguished from the cartilage. The measurements are done starting from the first dark cells in one of the ends till the last ones on the other end.

Please move such details to the Protocol section

The paragraph was moved to the protocol part as step 2.17.

Please specify the figure panels.

Figure panels were specified in the text

Please define P1 and P2. Please describe the top panel of panel D (P2).

The figure legend was changed to address this point:
*(**A-D**) Comparison between freshly extracted tibia (top) and tibia extracted at E14.5 (bottom) and cultured for 2 days (**A**), extracted at E15.5 and cultured for 2 days (**B**), extracted at E16.5 and cultured for 4 days (**C**) and freshly extracted at postnatal day 2 (P2) and extracted at postnatal day 1 (P1) and cultured for 1 day (**D**).*

Please spell out RA

RA spell out was added:
*Tibiae extracted at E15.5 and grown for 2 days with 0.1% DMSO (**A**) and retinoic acid (RA) (**B**).*

Do the three dots of control represent three replicates? Please explain

Explanation was included in the figure legend:
Comparison of the length of paired bones cultured for 2 days with either DMSO (right tibia) or RA (left tibia); each dot represents one of the 3 biological replicates per condition.

Please define error bars in the figure legend

Error bars were defined in the figure legend:
*(**C**) Graph showing the changes in the total length and only of the mineralized region over a period of 6 days. n=5 cultured tibiae; SD t=0 full length ± 0.0777, mineralized ± 0.0213; t=3d full length ± 0.1495, mineralized ± 0.056; t=6d full length ± 0.1193, mineralized ± 0.0521.*

If there are six or more authors, list the first author and then “et al.”

The format of citations was modified to fit this requirement.

We also included two new entries in the list of materials:

Dissection kit	Cumper Robbins	PFS00034
Eppendorf 2-mL tubes	Eppendorf	0030120094

TITLE:

Culturing and Measuring Fetal and Newborn Murine Long Bones

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KEYWORDS:

long bones, explant culture, tibia, femur, mouse models, bone growth

SUMMARY:

Here, we present a method for ex vivo culture of long murine bones at both fetal and newborn stages, suitable for analyzing bone and cartilage development and homeostasis in controlled conditions while recapitulating the in vivo process.

ABSTRACT:

Long bones are complex and dynamic structures, which arise from endochondral ossification via a cartilage intermediate. The limited access to healthy human bones makes particularly valuable the use of mammalian models, such as mouse and rat, to look into different aspects of bone growth and homeostasis. Additionally, the development of sophisticated genetic tools in mice allows more complex studies of long bone growth and asks for an expansion of techniques used to study bone growth. Here, we present a detailed protocol for ex vivo murine bone culture, which allows the study of bone and cartilage in a tightly controlled manner while recapitulating most of the in vivo process. The method described allows the culture of a range of bones, including tibia, femur, and metatarsal bones, but we have focused mainly on tibial culture here. Moreover, it can be used in combination with other techniques, such as time-lapse live imaging or drug treatment.

INTRODUCTION:

Organ growth has to be tightly tuned to prevent the appearance of growth disorders, and involves the regulation of multiple cell types, molecular pathways and crosstalk among different parts of the body. Imaging techniques are essential to address the changes occurring over time in a growing embryo, both in normal conditions, as well as after a perturbation is induced in the system. Embryos with intrauterine development, such as the widely used rodent models, present an additional challenge for live imaging and drug treatment, which can be partially overcome by using ex vivo culture techniques. To successfully recapitulate the in vivo processes and obtain meaningful results, it becomes crucial to find the right culturing conditions for each organ or tissue.

Most bones of the mammalian skeleton grow through endochondral ossification, where the embryonic cartilage (composed of cells called chondrocytes) drives longitudinal growth and is gradually replaced by bone. This process happens at the growth plates, located at the end of the long bones, where three zones can be distinguished: resting, proliferative, and hypertrophic[1,2]. First, the round progenitor chondrocytes in the resting zone transition into the cycling columnar chondrocytes in the proliferative zone. During the next stage of differentiation, these chondrocytes become hypertrophic and start secreting type X collagen. Hypertrophic chondrocytes orchestrate the subsequent steps of ossification: they secrete key signaling molecules, such as connective tissue growth factor, bone morphogenetic proteins and Indian hedgehog, and direct the mineralization of the matrix, recruit blood vessels to the central part of the bone, and, upon apoptosis, allow osteoblasts (bone-forming cells) to invade the matrix to form the primary ossification center[3,4]. The mineralized matrix facilitates the penetration of blood vessels through which osteoblasts migrate to replace this degraded cartilage with a bone matrix[5]. Most osteoblasts invade the cartilage matrix from the perichondrium, a fibrous layer that wraps the cartilage[6]. Alternatively, a proportion of hypertrophic chondrocytes are able to survive and transdifferentiate to osteoblasts[7–9]. The final length of the bone is due to the accumulated growth of the transient cartilage, whose growth rate in turn depends on the number and size of the hypertrophic chondrocytes, and their matrix production[10]. Additionally, it was recently shown that the duration of the last hypertrophy phase correlates with the final length of the bone[11]. Therefore, tight regulation of the proliferation and differentiation of these cells is required to ensure proper bone size.

Despite of the substantial knowledge acquired over the years on the organization and development of the growth plates, most of these conclusions are based on the observation of fixed histological sections. Tissue sectioning provides valuable information about this process, but can be ridden with technical artifacts, so it cannot be always reliably used to estimate morphological or size changes between different stages. Additionally, as bone growth is a dynamic process, the static two-dimensional (2D) images offer a limited insight into the movement of the cells in the growth plate, while time-lapse imaging on live tissue could offer valuable information on the behavior of the chondrocytes in the growth plate.

All these limitations can be potentially resolved using ex vivo bone cultures. While bone culture protocols have been developed some time ago, they were limitedly applied to murine long bones. Most of the studies use chick bones due to the technical advantages offered by the chick model[12,13]. Organotypic cultures (air/liquid interface) were applied to chick embryonic femurs, which were maintained in culture for 10 days[14]. The sophisticated genetic tools available in mouse make this model very appealing to be used in ex vivo bone culture. The studies that used mice to look into bone growth worked mostly with metatarsal bones[15], probably due to their small size and greater numbers obtained per embryo[16]. Although traditionally considered long bones, metatarsi enter senescence (characterized by reduced proliferation and involution of the growth plate[17]) earlier than other long bones in vivo, and therefore their continuous growth ex vivo does not really recapitulate the in vivo process. For the purposes of this article, we will use the term long bones for bones from the proximal and intermediate limb regions. Several previous studies used long murine bones, such as tibia, in ex vivo cultures and observed a

substantial growth of the cartilage but little ossification[18]. We also used tibial cultures recently, mainly to study chondrocyte dynamics[19]. Other studies used femoral heads from young mice[20] or only the distal part of the femur for culture[21]. Some more recent works successfully combine the ex vivo culture of full bones with time-lapse imaging to acquire three-dimensional (3D) movies of chondrocytes in living mouse tissue[22,23]. The authors managed to observe previously unnoticed events in the rearrangement of chondrocytes to the proliferative zone[23] in a good example of the potential application of bone ex vivo culture. The alternative, i.e., analyzing static images, requires indirect and complex techniques. This was exemplified by a recent study assessing the importance of transversally-oriented clones for cartilage growth, where genetic tracing with multicolor reporter mouse strains coupled with mathematical modeling were used[24]. Therefore, ex vivo culture might help gain insight into dynamic processes in a faster and more straightforward way.

Here, we present a method for murine long bones culture, which can be combined with different molecular treatments and/or with time-lapse live imaging. This protocol adapts the methods used in previous reports[15,18,25], but addresses some additional issues and focuses on long bones such as the tibia, rather than metatarsal bones. Finally, it explores the potential of using statistically powerful paired comparisons by culturing left and right bones separately in the presence of different substances.

PROTOCOL:

All the experiments should be carried out following the local governmental and institutional guidelines of ethical handling of laboratory animals.

1. Preparations prior to the day of bone culture

1.1. Set up timed mouse matings to obtain fetuses and pups from embryonic day 14.5 (E14.5) and onward.

NOTE: The culture of long bones can be successfully applied to different mouse strains; in the present protocol, outbred Swiss Webster wild-type mice are used.

1.2. Prepare dissection medium (adapted from Houston et al.[15]): dilute α-minimum essential medium (α-MEM) or Dulbecco's modified Eagle's medium (DMEM) 1/13 in phosphate-buffered saline (PBS) and 2 mg/mL bovine serum albumin (BSA) and filter sterilize through a 0.22 µm, 33 mm diameter syringe filter. Store aliquots at -20 °C.

1.3. The day before extraction of the fetuses, prepare the serum-free bone culture medium composed of DMEM containing 0.2% BSA, 0.5 mM L-glutamine, 40 U/mL penicillin/streptomycin, 0.05 mg/mL ascorbic acid, and 1 mM betaglycerophosphate. Filter sterilize through a 0.22 µm, 33 mm diameter syringe filter and store at 4 °C for up to 1 month.

1.4. On the day of the culture and prior to mouse culling, prepare 24-well plates and 60 mm dishes with dissection medium and keep them ice cold. Prewarm the bone culture medium in a

37 °C water bath. Spray 80% (v/v) ethanol on the tools (tweezers and small scissors) to be used for fetus handling. Transfer a binocular scope to a Class II biosafety cabinet.

2. Culture of fetus and newborn tibia and femur

2.1. Cull the pregnant mouse through cervical dislocation at the desired gestational stage (ranging from E14.5 to E18.5). If newborn pups are used, remove them from the mother one by one and cull by decapitation.

2.2. Place the mouse on her back and sterilize the abdominal region by spraying 80% (v/v) ethanol on its surface.

2.3. Cut the skin and the abdominal muscle with small scissors to access the uterine horns.

2.4. Extract the uterus from the abdominal cavity with the help of tweezers and small scissors, removing the mesometrium and cutting the base of the horns. Place the uterus in a 60 mm Petri dish with ice-cold dissection medium and keep the culture dish on ice during the whole procedure.

2.5. Transfer the Petri dish with the uterus to the biosafety cabinet and work there from now on.

2.6. Separate individual fetuses with scissors by cutting between the sacs.

2.7. Transfer individual sac under a dissection stereomicroscope in a clean 60 mm dish with dissection medium and open them up with tweezers to separate the fetuses from the placentas and clean them from membranes.

NOTE: Work with one embryo at a time, while keeping the rest on ice.

2.8. Decapitate the fetuses and transfer the body to a clean new 60 mm dish with a 1 mL cut sterile pipette.

2.9. Remove the skin of the fetuses or pups with tweezers starting from the back and peeling it out till the toes.

2.10. Separate the hindlimbs from the body by cutting with the tweezers close to the spine and transfer them to a clean dish with ice-cold dissection medium.

2.11. Separate the tibia from the femur with tweezers by carefully introducing them between the surface cartilage of distal femur and proximal tibia.

2.12. Remove the hip bones from the proximal femur and the calcaneus bone and the fibula from the tibia.

2.13. Carefully remove the soft tissue from the femur and the tibia by nipping and pulling it off.

NOTE: It is important to remove as much soft tissue as possible, taking special care in removing the tissue that connects the two cartilage poles, but avoiding damaging the cartilage, the perichondrium, and the bones.

2.14. Place the four bones (left and right tibias and femurs) in the first well of the 24-well plate with a plastic 1 mL sterile pipette. Alternatively, to compare the effect of different treatments on left and right limbs, place contralateral limbs in different wells.

NOTE: Extra care should be taken when the bones are transferred to the wells, as they can easily stick to the pipette.

2.15. Proceed the same way with as many fetuses as necessary.

NOTE: Change the dish with the dissection medium as soon as it gets too clouded, as it is important to see clearly the dissected bones.

2.16. When all the desired bones are transferred to the wells, remove the dissection medium with a plastic 1 mL sterile pipette and take extra care not to aspirate the bones.

2.16.1. Depending on the purpose of experiment, pictures can be taken of the bones before removing the dissection medium, as timepoint zero of the experiment. Take pictures with a microscope attached to a digital camera and annotate the exposure and scale used. To ensure easy and reliable measurements, take pictures with good contrast to distinguish the mineralized part.

2.17. Add 1 mL of culture medium to each well. If any treatment is intended on the bones (doxycycline, tamoxifen, growth factors, etc.), it should be added now.

2.18. To observe the effect of growth inhibition in the culture conditions, treat the left tibias with retinoic acid (RA, 500 nM), while incubating the right tibias with an equivalent volume of vehicle (dimethyl sulfoxide [DMSO], final concentration 0.1%) as a control.

2.19. Leave the bones to grow for two days or more in a cell culture incubator under standard cell culture conditions (at 37 °C in a 5% CO_2 incubator).

2.20. To assess proliferation, add 5-ethynyl-2'-deoxyuridine (EdU) or 5-bromo-2'-deoxyuridine (BrdU) to the medium at a final concentration of 10 µM 1–2 h before fixation.

NOTE: The stock concentration of EdU is 20 mM.

CAUTION: EdU and BrdU are thymidine analogues and can be toxic and mutagenic.

2.21. Thaw 4% paraformaldehyde (PFA) and fix the bones by immersion in PFA in individual 2 mL tubes.

CAUTION: PFA is toxic and designated as a probable human carcinogen. Avoid breathing paraformaldehyde powder and vapors. EdU and BrdU are thymidine analogues and can be toxic and mutagenic.

2.22. After a brief 10 min fixation in PFA at room temperature, transfer bones to PBS for picture acquisition at final timepoint. Then place bones back into PFA for overnight fixing at 4 °C.

2.23. After fixation bones can be processed for desired downstream applications.

3. Measurement and analysis of the full length of the bone and of the mineralized region

3.1. Use an image editing software to measure the length of the bones, taking into account the scale of the image. Measure both total length of the bone and the mineralized region. Start the measurements from the first dark cells at one end until the last ones at the other end.

NOTE: The mineralized region is characterized by the darker color and is easily distinguished from the cartilage.

3.2. To calculate the growth rate, defined as the average increase in length per day, divide the difference between the final length of the bone and the initial one by the number of days in culture.

REPRESENTATIVE RESULTS:

Bone culture can be performed starting from different stages. In **Figure 1A-D**, a comparison between cultured tibia and freshly extracted ones at equivalent stages is shown. The first observation is that up to two days of culture the size achieved is comparable to the in vivo bone growth for both cartilage and mineralized bone (**Figure 1A,B,D**). Longer culture periods lead to bigger differences between the cultured bones and the freshly extracted (**Figure 1C**). Additionally, as mentioned in the protocol, it is crucial to remove the soft tissue connecting both ends of the bones, as otherwise the bones will bend. **Figure 1E** shows an example of a tibia grown with incomplete removal of soft tissue versus a tibia without soft tissue.

Next, tibias were cultured for 2 days and their length was measured. As can be seen in **Figure 2A,B**, the measurement of the total length and of the mineralized part can be performed with an image analysis software. As shown by De Luca et al.[26], treatment with RA has a strong effect on the growth of the tibias already after 2 days of treatment and a similar result was observed in our cultures (**Figure 2B-D**). Importantly, the experiment was performed using paired bones, with the right bone as control and the left treated with RA (**Figure 2A,B,E**), which helps overcome the natural variability in bone size between different specimens. Thus, the culturing method described is suitable for assessing the effect of different compounds on bone growth.

Next, the growth rate of the bones after culture was assessed. Bones were extracted, measured and cultured at E15.5, 16.5 or P1, and fixed and measured again two days later. Both the total increase in length and the length of the mineralized part were measured (**Figure 3C**). An example of E15.5 tibia and femur before culture (**Figure 3A**) and at the end point of the experiment (**Figure 3B**) are shown. As can be observed from the graph and the table (**Figure 3C,D**), there is a consistent increase in the total length of the tibia, corresponding to an approximate increase of 9−29% from the initial length. This is less increase than the one observed in vivo; the main difference is likely due to the level of the proximal cartilage, bigger than the distal and less accessible to nutrients. Indeed, EdU labeling showed fewer positive cells in this region after culture compared to freshly extracted bones of equivalent stage (**Figure 4A,C**). The EdU incorporation in the distal tibia was similar in the cultured and freshly extracted bones (**Figure 4B,D**) The distal cartilage contributes approximately one third to the total growth of the tibia in vivo at this stage[27], comparable to the growth rate observed in culture, so we propose that the analysis should be focused on this part of the bone. Additionally, we assessed the mineralization of the cultured bones, and observe almost no increase in length of this region. The difference might be due to the absence of vessel and osteoblasts invasion in the ex vivo culture. This suggests that studies of cartilage growth can extend for several days, while if the region of interest is the ossified part of the bone, the period of culture should be shorter. This observation was confirmed by keeping tibia in culture for a longer period (up to 6 days). As can be seen in **Figure 5**, the total length of the skeletal element increases substantially, while almost no increase in the mineralized region is observed (**Figure 5C**).

Overall, these results suggest that the culture of long bones can be used to analyze the effect of different factors on overall bone growth and particularly to assess cartilage dynamics. While well-established metatarsal cultures can also be used with these purposes, we submit that both types of cultures complement each other, given the intrinsic differences between metatarsals and the rest of long bones.

FIGURE LEGENDS:

Figure 1: Example of cultured tibia for different time periods. (**A-D**) Comparison between freshly extracted tibia (top) and tibia extracted at E14.5 (bottom) and cultured for 2 days (**A**), extracted at E15.5 and cultured for 2 days (**B**), extracted at E16.5 and cultured for 4 days (**C**) and freshly extracted at postnatal day 2 (P2) and extracted at postnatal day 1 (P1) and cultured for 1 day (**D**). Note that, while cartilage growth remains quite physiological after different culturing periods, the ossified part shows a growth delay after culture time longer than 2 days compared to the freshly extracted bone at the corresponding stage. Scale bar = 1 mm. (**E**) Tibia cultured for 2 days starting at E15.5; note that the incomplete removal of the soft tissue between the two ends of the bones leads to the bending of the bone. Scale bar = 600 µm.

Figure 2: Measurement of the length of the bones upon retinoic acid (RA) treatment. (**A-B**) Tibias extracted at E15.5 and grown for 2 days with 0.1% DMSO (**A**) and RA (**B**). Note the difference in both total length and of the mineralized part. Scale bar = 1 mm. (**C-D**) Changes in

total length (**C**) or the mineralized part (**D**) of the tibia over a period of 6 days in control situation and in presence of RA. Results are shown as mean ± standard deviation (SD); comparison is done by two-way analysis of variance (ANOVA) with the type of treatment as variable (*p* values are shown in the graph). (**E**) Comparison of the length of paired bones cultured for 2 days with either DMSO (right tibia) or RA (left tibia); each dot represents one of the 3 biological replicates per condition. Paired two-tailed Student's *t*-test was used.

Figure 3: Comparison of total growth and mineralization of the tibia after 48 hours of ex vivo culture. (**A**) E15.5 tibias and femurs from left (top) and right (bottom) limbs prior to culturing. (**B**) The same tibias and femurs followed up after 2-day culture. Scale bar = 600 µm. (**C**) Graph representing the growth rate of tibias cultured at different developmental stages. Both the total growth and the growth of the mineralized part were assessed. (**D**) Table showing the initial and final length of the whole bone and the mineralized part before culturing at E15.5 and after 2 days in culture.

Figure 4: Bulky growth plates do not show much proliferation in culture. (**A-B**) EdU staining for proximal (**A**) and distal (**B**) tibial growth plates cultured from E15.5 for 2 days. (**C-D**) EdU staining for proximal (**C**) and distal (**D**) tibial growth plates freshly extracted at E17.5. Note the difference in the number of EdU(+) cells in the proximal tibia. Data include 6 cultured pairs of limbs and 3 freshly extracted.

Figure 5: Longer periods of tibia culture show substantial cartilage growth but little mineralization. Freshly extracted tibia at E15.5 (t = 0) (**A**) and after 6 days in culture (**B**). Note the differences between cartilage growth and the growth of the mineralized part. Scale bar = 1 mm (**C**) Graph showing the changes in the total length and the mineralized region over a period of 6 days. n = 5 cultured tibias; SD at each time point is as follows: t = 0, full length = 0.0777, mineralized = 0.0213; t = 3 d, full length = 0.1495, mineralized = 0.056; t = 6 d, full length = 0.1193, mineralized = 0.0521.

DISCUSSION:

Bone ex vivo culture methods have been used for some time to assess the biology of bone growth[28], but have been seldom applied to murine long bones. With the development of imaging techniques, ex vivo bone culture offers an attractive way to study bone growth in real time in a setting closely resembling the in vivo conditions. In this scenario, it is important to define the conditions in which the growth of long bones is comparable to their growth in vivo.

In the present study, we describe a simple and affordable protocol for long bone culture, addressing the limitations and possible applications. The most critical step of this method is the isolation of the bones, which have to remain intact and with as less soft tissue between the ends of the bone as possible. Leaving soft tissue between the ends of the bones will prevent normal growth of the bone and induce bending, as is exemplified in **Figure 1E**. Soft tissue at the ends of the bones can be left, as it will eventually disappear. Another step that requires extra care is the transfer of the bones to the culturing plate, as they can easily stick to the plastic pipette. One possible solution is pipetting up and down dissection medium containing blood

and tissue pieces, as it creates a coating that helps to preclude the bones from sticking to the pipette.

Once extracted, bones reach comparable size to the corresponding in vivo stage when cultured for up to 48 hours. Apart from measuring the length of the bone, the growth rate (average increase in length per day) can be estimated easily in these conditions. However, this approach for growth rate calculation is not valid over longer culturing periods, where other methods, such as calcein labeling[29], should be used. Treatment with retinoic acid, which promotes premature chondrocyte differentiation, leads to a substantial reduction in the growth of the bones, suggesting that this time for culturing is enough to observe the effect that different substances might have on bone growth. Importantly, the comparison was also done on paired bones from the same specimen, so that the left tibia received the RA treatment and the right was the control. This is an advantage of the bone culture model, as it allows performing paired comparisons, which are statistically more powerful.

Culturing for longer time shows significant growth of the cartilage part, but a delay in the growth of the mineralized one, and it is important to consider this result when choosing the application of the technique. Similar results were observed in chick femurs cultured for up to 10 days which show an enlarged epiphyseal region and a reduced diaphyseal bone collar[14], as well as in mouse tibia cultured for 6 days[18]. Additionally, it is important to mention that in the cartilage region the growth is also not homogenous: the bulky distal region of the femur and proximal region of the tibia do not receive nutrients efficiently by simple diffusion and cannot grow properly. In this context, the studies should focus on the opposite growing plates, which were estimated to contribute to one-third of total growth[27], similar to the observed growth in culture. The delay in growth of cultured bones was also described for metatarsal cultures[15].

An important consideration of this type of culture is the absence of growth of the ossified part of the bone. It is well established that the osteoblasts invade the cartilage matrix from the periosteum together with blood vessels[3,4] and this process is obviously disrupted when bone is isolated. This might explain the absence of ossification under culture conditions. Hypertrophic chondrocyte transdifferentiation was also shown as an important source of osteoblasts[7–9], but whether this process also requires in part the presence of blood vessels, as it seems to do during fracture healing[30], or some other tissues not present in ex vivo cultures, needs further investigation. Additionally, it is well described the importance of the mechanical load in shaping bone growth[31,32], which influences long bones and metatarsal bones differently, but is absent in an ex vivo culturing setup. Nevertheless, the described culturing method is suitable for measuring chondrocyte dynamics and changes in longitudinal growth and, thus, can be used for certain applications.

Overall, the described protocol provides a simple and cheap method to culture long bones starting from different stages, which can be coupled with additional techniques to address key cellular and molecular mechanisms, such as live time-lapse imaging[13,23] or drug treatment.

ACKNOWLEDGMENTS:

We would like to thank Alexandra Joyner for her support when this protocol was being established, Edwina McGlinn and Yi-cheng Chang for sharing retinoic acid. The Australian Regenerative Medicine Institute is supported by grants from the State Government of Victoria and the Australian Government.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

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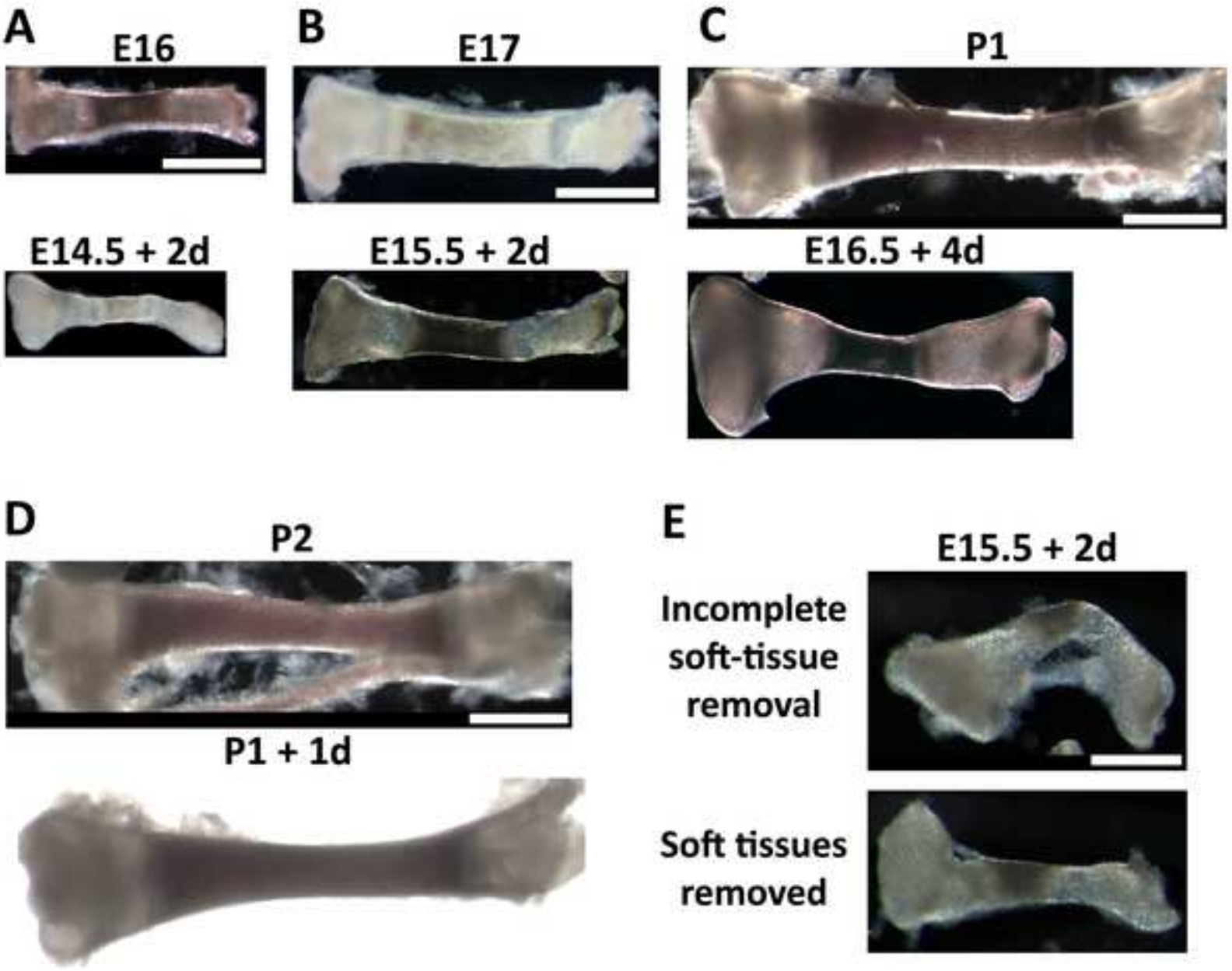

Figure2

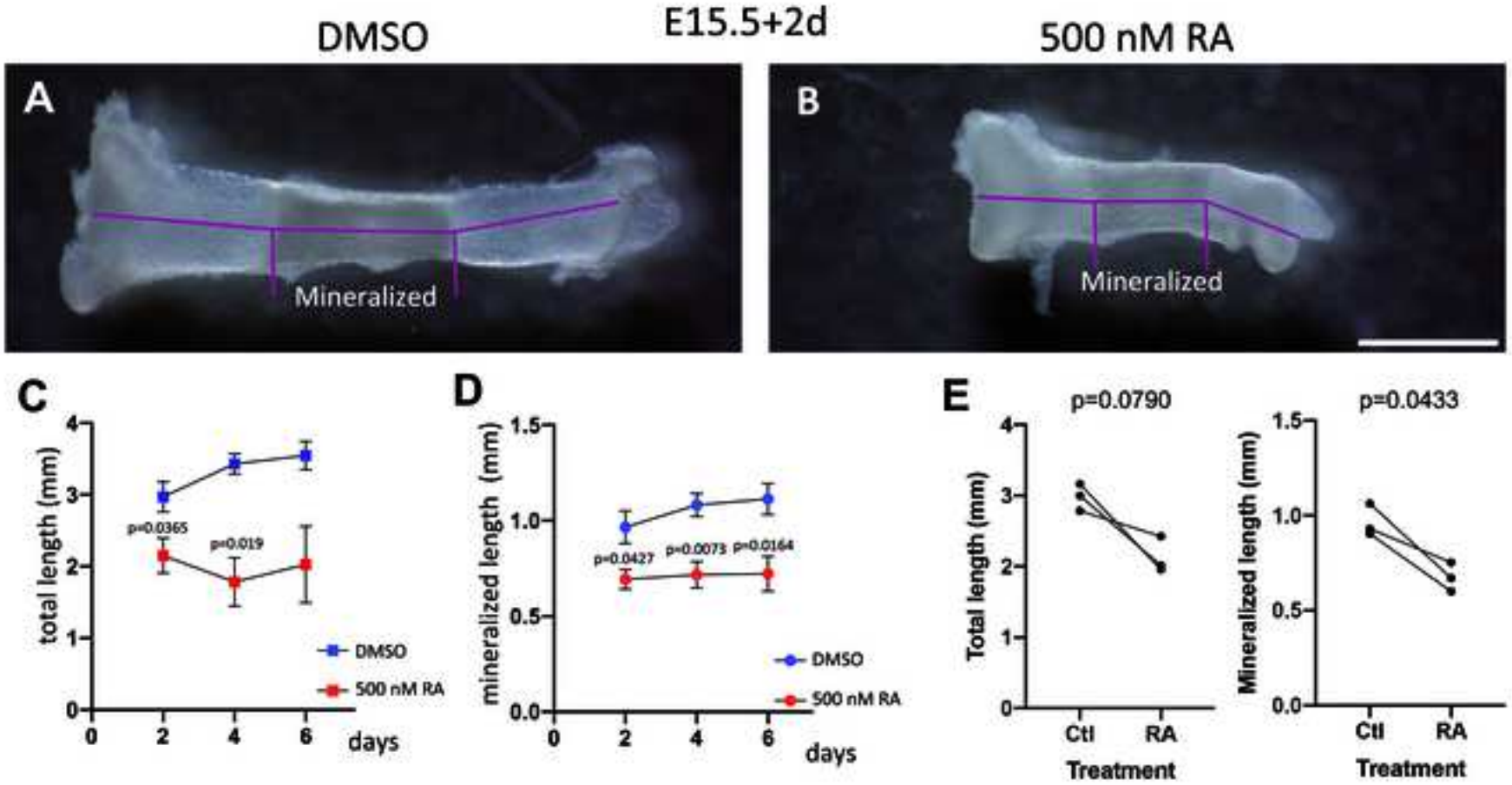

Figure3

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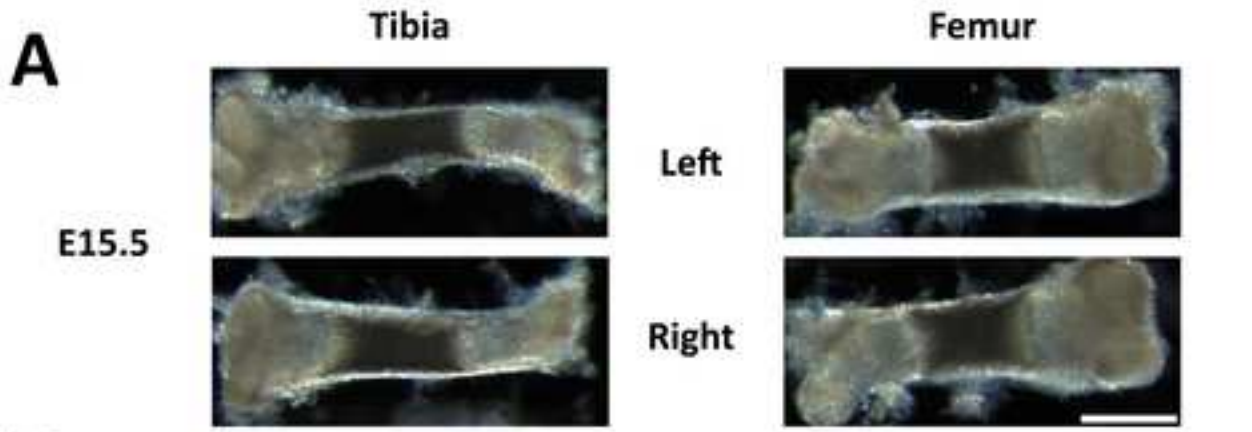

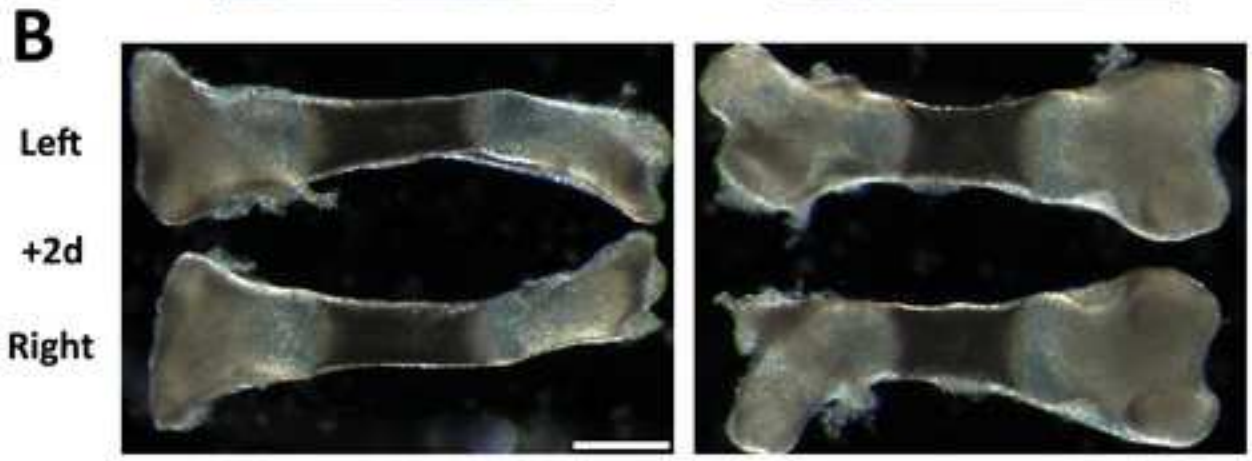

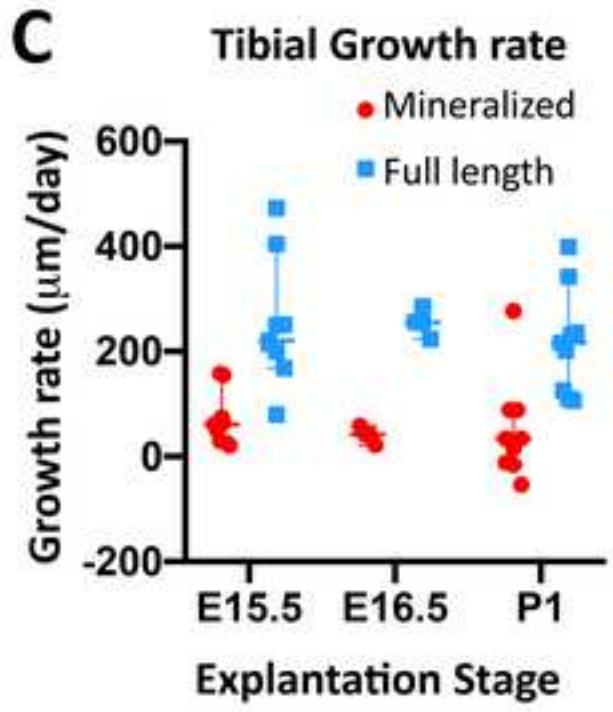

D

	Mineralized (mm)		Full length (mm)	
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tibia 2	0.617	0.739	1.7632	2.2629
tibia 3	0.533	0.587	1.7556	2.0919
tibia 4	0.722	1.033	2.316	3.2616
tibia 5	0.793	1.109	2.3448	3.1536
tibia 6	0.668	0.798	1.957	2.3864
tibia 7	0.602	0.703	1.8012	2.3028
tibia 8	0.509	0.551	1.8088	1.9684
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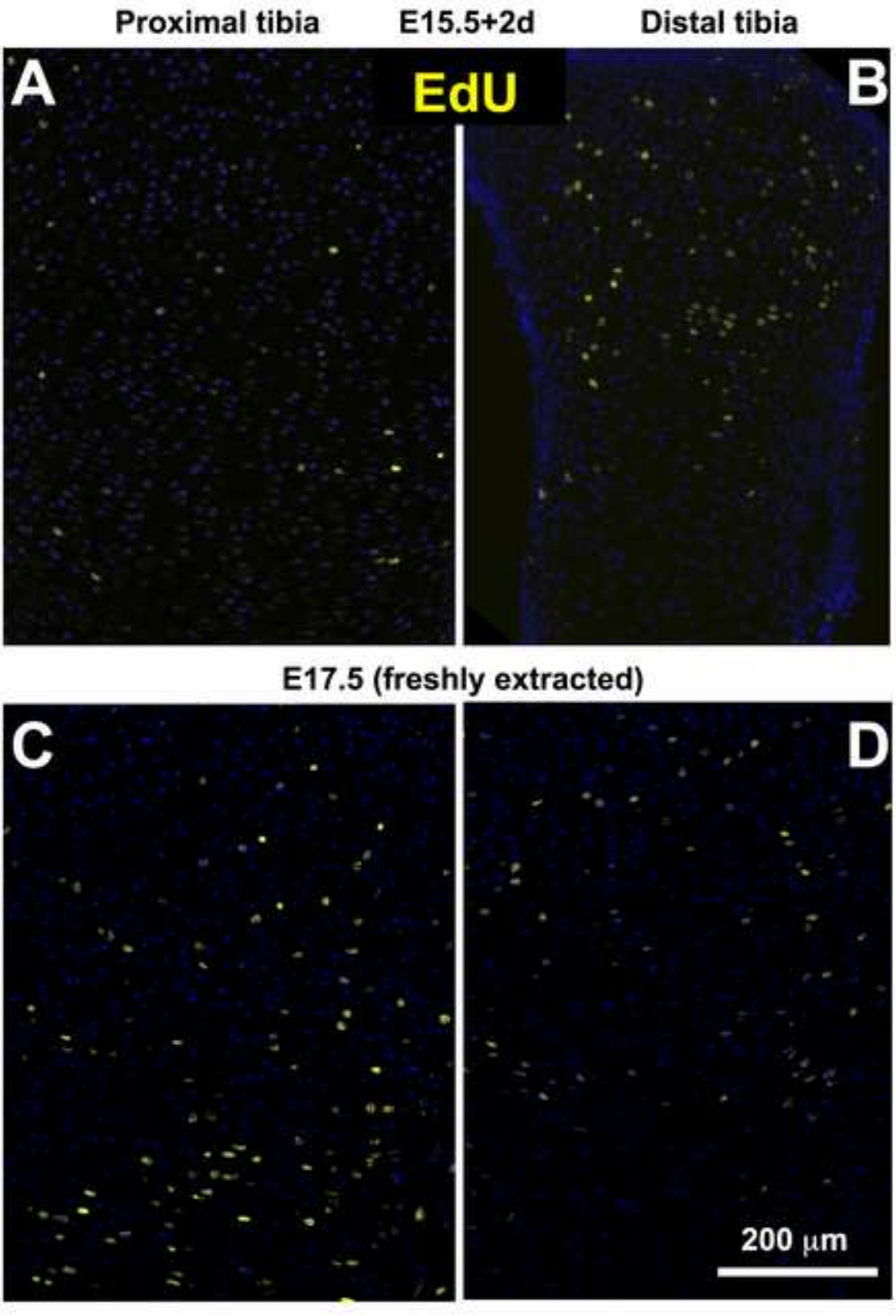

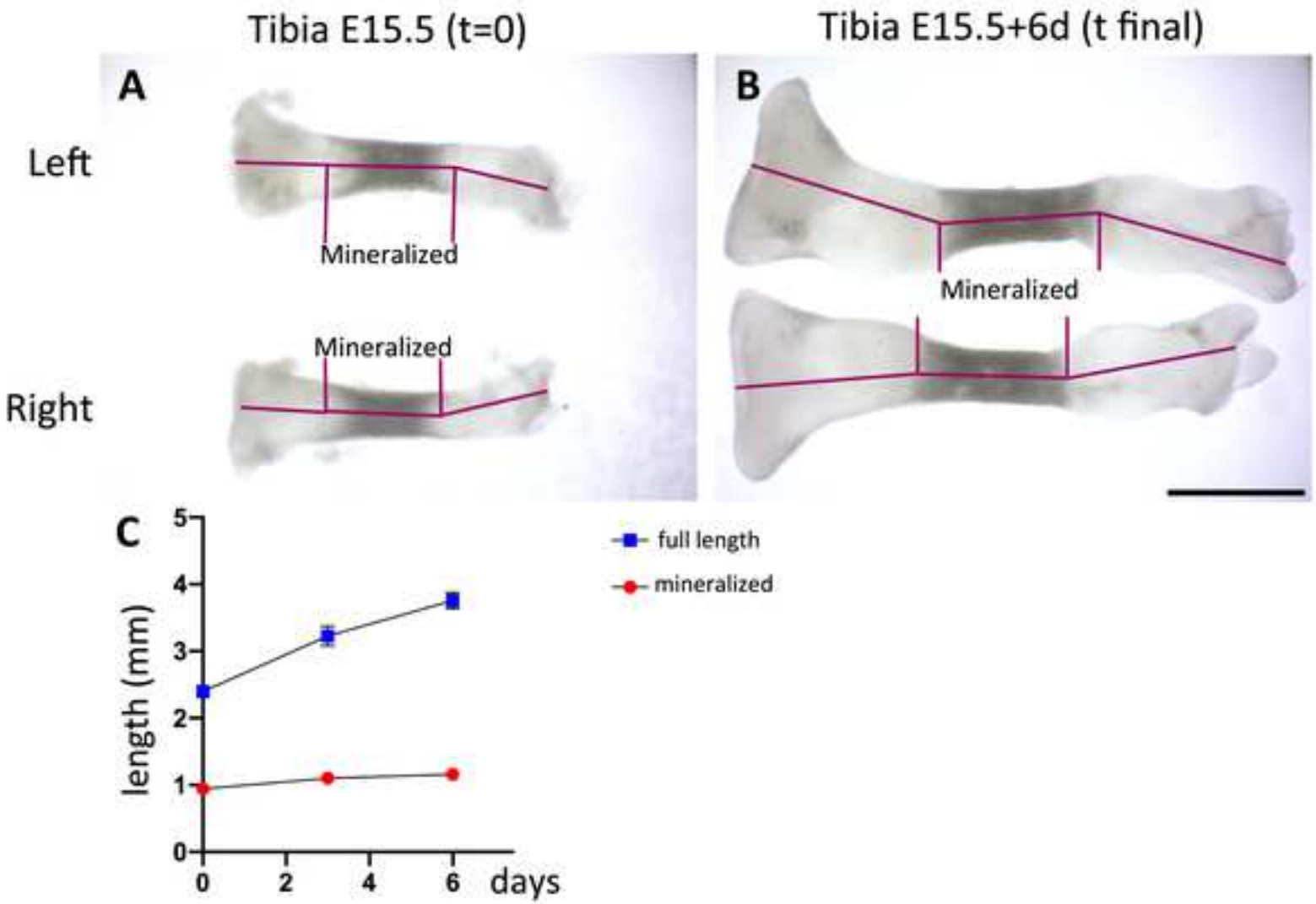

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Name of Material/ Equipment	Company	Catalog Number
5-Ethynyl-2'-deoxyuridine	Santa Cruz	CAS 61135-33-9
5-Bromo-2’-deoxyuridine	Sigma	B5002
50mL Conical Centrifuge Tubes	Falcon	352070
60 mm TC-treated Center Well Organ Culture Dish, 20/Pack, 500/Case, Ste	Falcon	353037
Adobe Photoshop	Adobe	CS4
Ascorbic acid	Sigma	A92902
Base unit for the scope	Zeiss	435425-9100-000
Betaglycerophosphate	Sigma	G9422
Binocular scope	Zeiss	STEMI-2000
Bovine Serum Albumin (BSA) fraction v	Roche/Sigma	10735086001
DigiRetina 500 camera	Aunet	
Dissection kit	Cumper Robbins	PFS00034
DMEM	Gibco	11960044
DMSO	Sigma	D8418
Eppendorf 2-mL tubes	Eppendorf	0030120094
Ethanol 96%	Merk	159010
Forceps Dumont#5 Inox08	Fine Science Tools	T05811
Heracell 150 CO2 incubator	Thermo Fisher	51026282
Minimum Essential Medium Eagle	Sigma	M2279
Multiwell 24 well	Falcon	353047
Paraformaldehyde	Sigma	158127
Penicillin-Streptomycin (10,000 U/mL)	Gibco	15140-122
Plastic pipettes 1mL Sterile Individually wrapped	Thermo	273
Syringe filter 0.2 um	Life Sciences	PN4612
Terumo syringe 20 mL	Terumo	DVR-5174
Tretinoin (retinoic acid)	Sigma	PHR1187-3X
Trinocular scope	Aunet	AZS400T

Comments/Description

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Reviewer #1:

Manuscript Summary:

In this manuscript, authors introduce their ex vivo mouse long bone culture method. As they cited, Houston DA et al. described similar method using mouse metatarsal in this journal (Houston DA et al. JoVE. 2016). Comparing to Houston's paper and this, using long bones is the major difference. It is interesting idea that long bone can grow ex vivo, and be useful method for researchers who have interest in cartilage differentiation / bone growth.

Major Concerns:

1. According to abstract, they "explore the potential of co-culturing bones from left and right limbs and separately to address the potential crosstalk between the left and right limbs after unilateral insult." I cannot find data or description relating to this topic

The reviewer is right. Because we finally decided not to include any information on this topic of crosstalk between left and right limbs, we removed this phrase from the abstract and any other references to this topic from the rest of the manuscript.

2. They only cited researches performed by other groups using ulna (Hirota et al. Sci Rep, 2018) or cranial bone growth plate (Romerein et al. Development, 2014) but not femur or tibia. Citing their original paper(s) using this method should be good help for readers who want to use this method.

We included a few additional references in the introduction (first paragraph, page 3) where long bones from both chicken and mouse were cultured. We also included the reference to our paper.

3. According to Fig.2C, it is useful for readers to show not only growth rate but also real length of tibia before and after culture. Description of the method how they measure the bone length is also helpful.

We included in Figure 2 an additional panel D with the length of the tibia before and after the culture. We also extended the protocol section including information on how the bone measurements were performed (page 5).

4. According to Fig.3, they described that "EdU labeling showed almost no positive cells" in proximal cartilage. I am not sure it is true or not as I can count more than 20 positive cells in Fig.3A.

To clarify this point, we include some images of EdU labeling in freshly extracted bones of E17.5 fetuses. The pregnant mouse was injected with EdU 1.5 hours before euthanasia and fetuses extraction. As can be observed from the Figure 4, while the amount of proliferative cells in the distal tibia is similar between the freshly extracted and cultured bones (Figure 4D), there is a clear difference in the case of the proximal tibia (Figure 4C), where the incorporation of EdU is much lower in the cultured samples. We include these data in the main text of the Results sections and also modify the phrase the reviewer refers to (second paragraph, page 6).

Minor Concerns:
1. Positions of scale bars should be reconsidered.

We changed the position of the scales in all the figures to the right bottom corner.

Reviewer #2:
The *ex vivo* culture of rodent long bones as a model to mimic endochondral ossification is not new and has been described in many papers and methods chapters. In most of these reports the murine embryonic metatarsal is the bone as choice as it can be maintained and growth can be manipulated (increased e.g. by growth factors and inhibited e.g. by cytokines) in culture for weeks if not months and research ethics perspective you get 6 metatarsals from 1 mouse pup which is helps satisfy the 3Rs. This present study reports an alternative model in which the authors propose to use of embryonic tibiae and femorae. The justification for this approach this is that metatarsals "enter growth plate senescence earlier than long bones". This may be true *in vivo* and would explain why some bones are bigger than others but its relevance to an *ex vivo* culture application is tenuous as metatarsals have been kept in culture for months and still grow. This justification is also hard to follow as metatarsals are also long bones.

We thank the reviewer for the feedback. We first wish to clarify that although we know that metatarsals are considered long bones, we reserve the term for the bones in stylopod and zeugopod, due to their different intrinsic properties (such as delayed senescence), regardless of what can be achieved in metatarsal culture. We have made this point clear in the text. We thus suggest that both culture systems complement each other, rather than being redundant. Choosing one or the other depends on the question addressed. Furthermore, we now show that growth of our bone cultures can be as easily manipulated as that of metatarsals, for example by treating them with retinoic acid (new figure 2).

Further limitations of the model are provided in the results. Data is limited to 2 days of culture, which is not long to follow the effects of a growth promoting or inhibititory substances. Growth data over a much longer period of time should be provided to allow others to determine if this model is of use to their particular study. The size of the bone probably limits diffusion of nutrients and the growth of the mineralization zone also appears to be limited. Neither of these are problematic for smaller metatarsal bones.

To address these points of the reviewer, we took several approaches. We included additional references where cultures of mouse tibia and chick femurs were performed for longer time (8 and 10 days, respectively) [1,2]. In both cases, they observe a substantial elongation of the epiphyseal region of the bone and almost no ossification. That is similar to what we observe after 4 days in culture, as shown in Figure 1C. We have also included new data of E15.5 tibias cultured for 6 days, and observe a similar result: the total length of the bone increase substantially, but the mineralized region shows very little growth (Figure 5).

Additionally, the statement that 2 days in culture are not enough to observe any effect of growth promoting and inhibitory substances, is not supported by previous publications. Treatment of fetal rat metatarsal cultures with retinoic acid (RA) leads to decreased length already detectable 2 days after culture [3]. We repeated this experiment in our tibial cultures. As can be seen in new Figure 2, already after 2 days in culture RA treatment leads to a significant decrease in the size of the bones. We included the data on RA treatment in the Results section (last paragraph, page 5).

More enthusiasm for the described methodology would have been forthcoming if the authors described some novel associated techniques as alluded to in the manuscript e.g . live time lapse imaging. Also in the text and the abstract the authors suggest that they explored co-culturing of bones to investigate cross talk. No data on this is provided.
Overall I am not sure what this procedure adds to what is already out there. I am not sure it will be of benefit as many of the advantages of the well described metatarsal culture method are lost with the use of the much larger long bones.

While we agree with the reviewer that metatarsal cultures proved to be a very useful and versatile method to study bone growth, we do not think it is enough reason for not expanding the culturing method to other types of bones. Besides, although the reviewer discarded that differences in the *in vivo* growth capacity between metatarsal and other bones impacts the outcomes of the *ex vivo* cultures, it was shown that treatment with IGF, BMP2 and noggin of 3-day cultures leads to different outcomes in terms of proliferation and hypertrophic chondrocytes height of metatarsal bones as compared to tibia or femur [4]. These results suggest that the data obtained from metatarsal cultures might not always be extrapolated to other types of long bones and stress the importance of developing *ex vivo* culture methods for other types of bones. Regarding live imaging, there are some examples in the literature already showing the feasibility of the approach. The co-culture of bones to study their crosstalk was merely mentioned as a possibility, but we have now removed it since we do not show related data.

Other points
1. In the introduction the description of the endochondral process needs some attention. (1) The mineralised matrix facilitates the penetration of blood vessels through which osteoblasts migrate to replace this degraded cartilage with a bone matrix (Mackie et al. 2011). This detail is missing and should be added.

We thank the reviewer for this comment. We included this phrase and the reference in the introduction in the corresponding section (paragraph 2, page 2).

(2) The authors are encouraged to read the papers by Farnum and Wlisman as the size of the hypertrophic chondrocytes is correlated to the rate of bone growth and not the final length.

Our point was that the size of the hypertrophic chondrocytes, as it influences growth rate, when integrated over time it also influences the final length of the bone. We

included in the introduction a reference citing the authors mentioned and tried to clarify the sentence (paragraph 2, page 2).

Also, Line - 63 - remove "closer to the central…… bone". This adds nothing.

We removed this part of the phrase.

Line 64/65 "secrete the substances that trigger…..". More detail is required to what they mean substances - matrix proteins, phosphatases, Calcium, phosphate etc?????????

We added additional information to this point (paragraph 2, page 2).

2. The authors should expand on what they mean by "tissue sectioning is frequently ridden by technical artefact" but they should bear in mind that many significant advantages have been made on the back of growth plate histomorphometry. They also end this paragraph with reference to time-lapse imaging. Again this study would have benefited by the demonstration of this procedure to the organ culture studies described. Intriguingly, in line 170 the authors recommend that the cultured bones should be fixed and cut for histological sectioning!!!

We changed the phrase referencing the tissue sectioning (paragraph 3, page 2). We only propose tissue sectioning after the *ex vivo* characterization has been done, as a complementary source of information.

3. Line 104 (and elsewhere) - metatarsals are long bones!
As stated above, we have now clarified the distinction we do between metatarsals and the rest of long bones.

4. Lines 104-106 - not addressed in this study.

We removed the phrase referencing to the studies of crosstalk between paired limb bones.

5. The authors must have a good look at the protocol and where appropriate make the methods and procedures clearer to allow these techniques to be used by others. More detail is required in certain places

We included additional information in the protocol to address this point. We mention the mouse strains used (Protocol point 1.1, page 3), the stages at which the bones are collected (Protocol point 2.1, page 4), more detail on how the uterus is removed form the mother (Protocol point 2.4, page 4), the fetuses handling before removing the limbs (Protocol point 2.7 and 2.8, page 4), and added an extra critical step alert related to limb transfer to the wells (end of page 4). We also clarified the fixation procedure before the final pictures are taken (Protocol point 2.19, page 5) and added a few new steps describing how the measurement of the bones was done (page 5).

6. Lines 143/144. Introducing them between the two growth plates? Unclear. Do you mean between the surface cartilage of the tibia and femur?

The reviewer is right about this imprecise terminology. We changed the sentence to the following: *Separate the tibia from the femur with fine tweezers by carefully introducing them between the surface cartilage of distal femur and proximal tibia.*

7. Line 149. Are the growth plates formed in these embryonic bones - unlikely?

A sandwiched plate has indeed not formed yet. We changed the sentence to the following: *It is important to remove as much soft tissue as possible, taking special care in removing the tissue that connects the two cartilage poles, as this will prevent normal growth of the bone, but avoiding damaging the cartilage, the perichondrium and the bones*.

8. Line 161- growth factors

We thank the reviewer for noticing this error; we corrected it in the main text.

9. How are the bones measured and how much do they grow (compared with published data from metatarsals)?

We included a new section in the protocol where we explain in detail how the measurement of the bones was done. We included new data with longer 6-day culture of the tibiae (Figure 5) and observe that the average increase in full length is around 1.3 mm. The increase described in metatarsal cultures over the same time period was around 0.9 mm [5]. In both cases, the growth is delayed compared to their growth *in vivo* [5,6].

10. Line 197. Area?? Did you measure area? If so how?

We meant region of the bone (corrected now in the main text, paragraph 2, page 6).

11. Fig 2C growth rate is over 2 days only - is this really meaningful. Data over a longer period would be required to get a useful model which could be challenged.

As argued above, 2 days can already be meaningful. Moreover, we have now included growth data over a longer period (Figure 5).

Reviewer #3:
Manuscript Summary:
The summary does not state the specific species and age/ gestational stage used.

We agree with this point of the reviewer. We modified the summary to include these details (lines 27-29):
Here, we present a method for *ex vivo* culture of long murine bones at both fetal and newborn stages, suitable for analyzing bone and cartilage development and

homeostasis in controlled conditions while recapitulating the in vivo process.

Major Concerns:
The title doesn't reflect well the content of the article (see other comments below).

We changed the title, so now it refers to all the stages analyzed in the protocol.

Minor Concerns:
Title:
Line 3: The details in long bone measurement are not explained in the protocol.

We included a new section in the protocol where the procedure to image and measure bones is explained (page 5).

The title highlights the fetal mouse but representative results also show the result from postnatal mouse (P1 and P2)?

We changed the title, so now it refers to all the stages analyzed in the protocol.

Introduction:
Line 70: The statement here need to be supported by relevant reference(s).

We changed this statement to a more accurate description of bone growth and introduced relevant citations (paragraph 2, page 2).

Protocol:
Line 121: Correct sub-numbering? 1.4?

This was corrected in the main text.

Line 129: Is the same protocol applies to extract long bones from postnatal mouse?

The reviewer is right that the protocol can be applied to both fetal and newborn mouse bones. We included this information in the protocol (Protocol point 2.1, page 4).

Representative results:
Line 191: Is the data shown in the results?

We include a new panel in new Figure 3, in which the full length of the bone is shown before the culturing and 2 days after. As can be observed from the numbers, the median increase in length is 18.5%.

Line 192: Is any data shown/ any supporting reference(s)?

This data is inferred based on the results obtained by EdU labeling. We included in new Figure 4 EdU labeling of freshly extracted E17.5 bones after injection in the mother 90

minutes before sample collection. As can be observed in this new figure, incorporation of EdU is seriously compromised in the proximal tibia compared to the *in vivo* situation (Figure 4A,C), while the number of EdU(+) cells is similar in the distal. These new results are included in the main text.

Line 194: Is this statement based on the current study or reference(s)? What are the references

We now included a reference in the result section in which we base this statement (paragraph 2, page 6).

Figure and table legend
Line 205: The freshly extracted tibia refers to the figures on the top for each A, B, C, D panels of Figure 1?

The reviewer is right about this point. We included a clarification in the figure legend (paragraph 3, page 6).

Line 218: The total growth and mineralized part were assessed after 2 day-cuture?

Yes, the results presented show the growth after two days in culture. We now also include a table where the total length and the mineralization are shown at t=0 and at t final (Figure 2D).

Line 222: The same scale bar applies for both A and B in Figure 3?

Yes, the same scale applies to all the panels in Figure 3. We realized that there was a mistake in the number of the scale and now it is corrected.

Discussion:
Line 237: The statement here seems to be not fully substantiated by the result (line 192).

We now included two lines of evidence that support this statement (also mentioned above): the length of the tibiae before and after the 2-day culture and the EdU incorporation in freshly extracted proximal and distal tibia versus cultured. We hope these new data clarify this point.

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2 Kanczler, J. M., Smith, E. L., Roberts, C. A. & Oreffo, R. O. A novel approach for studying the temporal modulation of embryonic skeletal development using organotypic bone cultures and microcomputed tomography. *Tissue engineering. Part C, Methods.* **18** (10), 747-760, (2012).

3 De Luca, F., Uyeda, J. A., Mericq, V., Mancilla, E. E., Yanovski, J. A., Barnes, K. M., Zile, M. H. & Baron, J. Retinoic acid is a potent regulator of growth plate chondrogenesis. *Endocrinology.* **141** (1), 346-353, (2000).
4 Lui, J. C., Jee, Y. H., Garrison, P., Iben, J. R., Yue, S., Ad, M., Nguyen, Q., Kikani, B., Wakabayashi, Y. & Baron, J. Differential aging of growth plate cartilage underlies differences in bone length and thus helps determine skeletal proportions. *PLoS biology.* **16** (7), e2005263, (2018).
5 Houston, D. A., Staines, K. A., MacRae, V. E. & Farquharson, C. Culture of Murine Embryonic Metatarsals: A Physiological Model of Endochondral Ossification. *Journal of visualized experiments : JoVE.* 10.3791/54978 (118), (2016).
6 Stern, T., Aviram, R., Rot, C., Galili, T., Sharir, A., Kalish Achrai, N., Keller, Y., Shahar, R. & Zelzer, E. Isometric Scaling in Developing Long Bones Is Achieved by an Optimal Epiphyseal Growth Balance. *PLoS biology.* **13** (8), e1002212, (2015).