Response to Reviewer 1 Comments

We thank for the supportive comments on the manuscript and the thoughtful advice and suggestions to improve or clarify some aspects. We specifically addressed each of the points raised with our responses in red below the relevant comment. Also, each action that we have taken to the text is in red characters in the manuscript itself.

Point 1: Can you explain why you chose agarose to make spheroids? Would other techniques give the same results?

Response 1: Multicellular spheroids can be prepared via several techniques including hanging drops, spinner flask cultures, overlay on non-adhesive surfaces, and microwells. Among these techniques, the use of hydrogel has proven to be particularly suitable for controlling the size of spheroids while maintaining high cell viability during/after spheroid formation due to the ECM-mimicking biophysical properties of hydrogels These platforms have been prepared using a variety of gels such as agarose, chitosan, poly (ethylene glycol) and gelatin methacrylate. Among these hydrogels, agarose is particularly attractive as it is natural, low-cost, non-toxic, not biologically active, and cell-repellent, which induces spheroid formation and inhibits random cell adhesion. Moreover, agarose permeability to gas and small biomolecules allows for diffusion of nutrients and drugs, which is critical for spheroid studies (Langhans SA. Three-Dimensional *in Vitro* Cell Culture Models in Drug Discovery and Drug Repositioning. Front Pharmacol. 2018; 23; 9:6. Slawny N, Labant M. Physiologically relevant spheroid models for three-dimensional cell culture. In Technology Platforms for 3D Cell Culture. 2018. S. Ed Przyborski.).

Additionally, both primary cultures and cell lines form spheroids with this liquid overlay technique. Finally, the Young's modulus of hydrogels is an important characteristic that is related to their ability to induce spheroid formation. For example, Young's modulus of collagen hydrogels is between 0.01 and 6 kPa, and that of agarose is between 5 and 100 kPa, a characteristic that induces a cell-specific mechanotransduction response (Vining KH, Mooney DJ. Mechanical forces direct stem cell behaviour in development and regeneration. Nat Rev Mol Cell Biol. 2017 Dec;18(12):728-742).

We have obtained hGF spheroids using ultra-low binding surface plates. We have no knowledge of other reports about the formation of hGF spheroids from individuals with cleft lip and/or palate using this technique or even using any of the other types mentioned above.

Point 2: Can you detail the immunofluorescence protocol on the spheroids: is it on sections or on the whole spheroids?

Response 2: The immunofluorescence protocol was performed on whole spheroids. The detailed protocol was included in the manuscript along with the immunofluorescence protocol performed in 2D (lines 144 to 166).

Point 3. Show immunofluorescence for all markers in spheroids. I am not convinced by the staining of Stim1. ¿Can you improve the images?

Response 3: Immunofluorescence of the spheroids for all the markers evaluated initially in 2D was also performed. This was included in the manuscript (lines 323 to -347) as well as an improved image of the Stim1 staining.

Point 4: To show the junctions, you could do TEM and immunofluorescence with the junction proteins like ZO1, occludin, connexin...

Response 4: We agree with this comment. Unfortunately, we were not able to include this analysis at this point. However, we will take it into account for future studies of these spheroids.

Point 5: Have you observed signs of necrosis or apoptosis in the spheroids?

Response 5: We have carried out the follow-up of the spheroids using optical microscopy for up to a month. We observed that they are viable for this period of time. In some cases, cells detached from the periphery of spheroids of more than 15 days of culture; however, we have not yet carried out live-dead assay or apoptosis assay.

Point 6: Regarding figure 5, the C is missing.

Response 6: We apologize. This was a typing error. Figure 5 only has 2 images (A and B). This was corrected in the manuscript.

Point 7: How to explain the presence of nanoparticles on the surface of spheroids?

Response 7: We hypothesize that intracellular calcium mobilization, evidenced by the expression of Stim1 in the spheroids, allows this ion and other ions to be encapsulated and transported in microvesicles. In fact, recent research on the process of early mineralization mediated by osteoblasts during intramembranous ossification of the calvaria in mice (Hara ES, et al. Re-Evaluation of Initial Bone Mineralization from an Engineering Perspective. Tissue Eng Part B Rev. 2022;28(1):246-255.), a process that is similar to the mineralization of the human palate, describes high compaction of cells, followed by deposition of amorphous calcium phosphate in "matrix vesicles" into the intercellular space by means of SEM, EDS, and TEM analyses. This is a point of great interest to continue our investigation.

In addition, as stated in the manuscript (lines 661 to 667), our results are in line with the findings of the most recent research on the osteogenic differentiation of human MSCs in 3D culture with or without the addition of external factors, but by using differentiation media or inducing molecules (references 38 and 39 of the manuscript) in which it is shown that cell condensation occurring in 3D culture is equal to, or even more important than, chemical induction of cells to undergo osteogenic differentiation, by recapitulating the mesenchymal condensation process during ossification. In the same way, hypoxic conditions, and actin depolymerization favored the cell differentiation process. Similar processes occurred during the 3D culture of hGF. Lastly, the ultrastructure of the hGF spheroids is like the surface of the spheroids of an osteoblast cell line generated by using the magnetic

levitation technique. In this case, SEM showed close contact between cells and the formation of a three-dimensional network of small structures indicating the deposition of a mineralized ECM (lines 668 to 672, and reference 40 of the manuscript).