A Tissue Engineering Model of Non-Syndromic Craniosynostosis for Identifying the Role of Microenvironmental Signals Leading the Premature Ossification of Calvarial Sutures

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INTRODUCTION: Craniosynostosis (CS) is a developmental condition that affects 1 in 2100 children worldwide, characterized by premature ossification of the calvarial sutures, causing skull deformation and eventually brain damage. The most common type of CS is the non-syndromic (NS) variant, which has been associated to microenvironmental causes. However, due to a lack of appropriate research models, little is known about the signalling pathways that govern the skull suture accelerated fusion. Therefore, we focused on investigating the role of microenvironmental cues by utilising tissue-engineering (TE) techniques that allow us to mimic the composition of the CS skull, in order to identify the effect of the extracellular matrix (ECM) biochemical composition and stiffness in directing premature suture ossification.

MATERIALS AND METHODS: Cells were isolated from calvarial bone, patent (unfused) and fused sutures of children (5-28 months) diagnosed with NS-CS, undergoing cranial vault remodelling (CVR) at the CHI at Temple Street, after parental consent and ethical approval were obtained [1]. Initially, cells were characterised by flow cytometry, including positive and negative markers for mesenchymal progenitors, to determine their native stemness. Subsequently, to establish the effect of biochemical cues in cell native behaviour, their intrinsic differentiation capacity towards bone tissue was assessed by quantifying alkaline phosphatase (ALP) activity and ECM mineralization. In addition, to understand how biophysical cues affect premature ossification, cells were cultured on soft (10 kPa) and stiff (300 kPa) collagen-coated polyacrylamide substrates, and cell morphology and bone formation capacity were evaluated. The effect of combining different biochemical and biophysical cues was further investigated with a 96 gene PCR array, to potentially identify differences in gene expression between the different cell populations [1]. Finally, cells were cultured on bone-like [2] or cartilage-like [3] collagen-based 3D scaffolds, mimicking fused and patent ECM, respectively [2,3], to determine variations in their native behaviour and differentiation potential when exposed to a CS microenvironment.

RESULTS: Cell characterization carried out by flow cytometry revealed that cells from patent sutures expressed higher levels of positive surface markers for MSCs (CD90 and CD44) and lower levels of negative markers (CD45, CD34, CD11, CD19 and HLA-DR) compared to cell from the calvarial bone and fused sutures. On the other hand, cells from patent and fused sutures showed similar expression of key markers of osteogenesis such as ALP activity and calcium deposition, when cultured with growth media (GM) for 7, 14 and 21 days. However, when cultured with osteogenic media (OM), cells from fused sutures exhibited the highest ECM mineralisation and ALP activity levels at the three different time-points evaluated. Subsequently, cells were cultured with GM on collagen-coated polyacrylamide substrates of different stiffness. Findings showed that cells from both patent and fused sutures exhibited morphological changes, such an increase in their spreading area, in a stiffness-increasing manner (Fig. 1). Particularly, cells from fused sutures showed a bigger size and rounded shape, resembling osteoblasts, while cells from patent sutures exhibited an elongated shape, resembling mesenchymal stem cells. Furthermore, when combining variations in the substrate stiffness and OM, cells from fused sutures showed a stiffness-dependent upregulation of genes mediating bone development (TSHZ2, IGF1) and activation of inflammatory response (IL1β) as well as genes involved in the breakdown of ECM (MMP9) and in the control of osteogenic differentiation (WIF1, BMP6, NOX1) [1]. Finally, cells were cultured on bone-mimicking [2] or suture-mimicking [3] collagen-based 3D scaffolds for 21 days. Data showed that cells from fused sutures cultured with OM on the suture-mimicking scaffolds exhibited higher ALP activity and ECM mineralization, compared to cells from calvarial bone and fused sutures (Fig.2).

DISCUSSION: Flow cytometry-based characterization demonstrated that NS-CS cell populations retained features of progenitor cells, with different levels of maturation between groups. However, cells from patent sutures showed the highest expression levels of progenitor markers, suggesting that they conserve the strongest multilineage differentiation potential. Furthermore, cells from fused sutures exhibited higher expression of key osteogenic markers demonstrating that these cells are the most sensitive to bone-forming biophysical signals and have the strongest osteogenic response when biochemically stimulated with osteoinductive growth factors. Mechanoresponse analysis also determined that the increased osteogenic potential of cells from fused sutures might be linked to activation of the BMP6, IGF1 and/or MAPK-associated non-canonical WNT pathways [1]. Finally, cells from fused sutures cultured on 3D suture-mimicking scaffolds were also able to undergo osteogenic differentiation quicker than cells from patent sutures, suggesting an increased sensitivity to changes in ECM biochemical and biophysical composition. In conclusion, this study identified differences in responsiveness between the cell populations isolated from calvarial bone, patent and fused sutures when exposed to variations on biochemical and biophysical signals, suggesting that NS-CS may be linked to an abnormal mechanical environment, confirming a microenvironment-dependent accelerated bone formation in cells from fused sutures.

SIGNIFICANCE/CLINICAL RELEVANCE: Understanding the effect of the ECM biochemical and biophysical composition and bone-forming factors at leading the premature suture ossification in CS, opens up avenues to not only understand better this developmental condition but may also help us to design novel TE-based therapeutic strategies to accelerate bone healing in adults.

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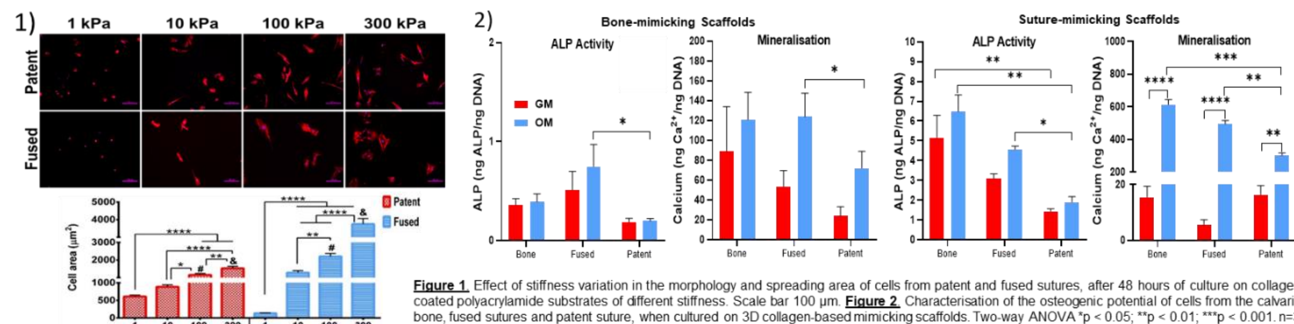

Figure 1. Effect of stiffness variation in the morphology and spreading area of cells from patent and fused sutures, after 48 hours of culture on collagen-coated polyacrylamide substrates of different stiffness. Scale bar 100 μm. **Figure 2.** Characterisation of the osteogenic potential of cells from the calvarial bone, fused sutures and patent suture, when cultured on 3D collagen-based mimicking scaffolds. Two-way ANOVA *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. n=3.