CONTROLLED APPLICATION OF MECHANICAL FORCE LEADS TO CALVARIA SUTURE FUSION

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Introduction Craniosynostosis, premature fusion of the skull bones at the cranial sutures (1.2 in 3000 live births), is treated by surgically separating the sutures (1). Studies have implicated mutations in the human homeobox gene MSX2 (Pro7His) in human fibroblast growth factor receptors-1,2,3, and Twist in the pathogenesis of craniosynostosis (2,3,4). Abnormal tensile and compressive mechanical forces caused by aberrant cranial base shape and intrauterine compression, respectively, have also been implicated as possible causes of premature fusion (5,6). The mechanisms by which mechanical force drives cellular, molecular, and genetic events affecting suture morphogenesis remain unknown. Therefore, the purpose of this study was to assess suture morphogenesis in cyclically loaded post-natal day 21 (PN21) wildtype mouse calvaria explants containing the sagittal sutures (which normally do not normally fuse in mice) *in vitro*. In doing so, we created a novel model of mechanical force induced craniosynostosis.

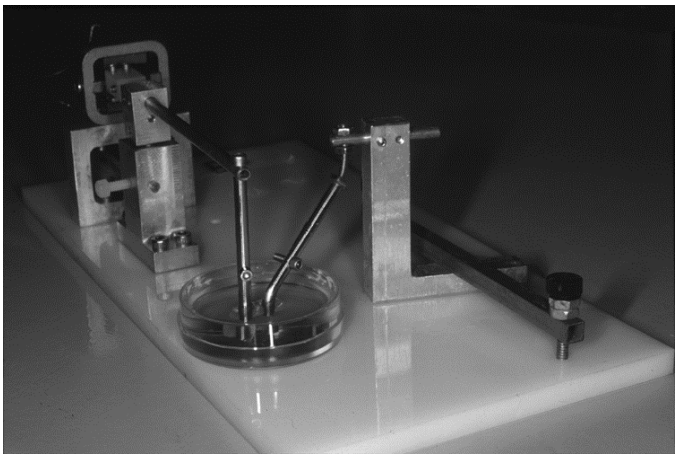

Figure 1: Loading device with specimen and culture dish

Methods Parietal bone specimens (size ≈ 4 x 12 mm^2) were harvested under sterile conditions and kept immersed in culture media. Specimens were clamped into the loading devices, which consisted of an electromagnetic actuator connected to a rotating brass armature (Fig. 1). Suture cross-section was placed perpendicular to the axis of loading. Specimens were cultured in a 35 mm well containing DMEM supplemented with 1 μg/ml gentamicin, 2 M glutamine, 1 mM non-essential amino acids, 1 mM ITS+, 3 mM inorganic phosphate, penstrep, and fungizone. All cultures were enriched daily with 100 μg/ml ascorbic acid and kept at 37° C, 98 % humidity, and 5 % CO_2. A trapezoidal load protocol of 0.3 g at 1 Hz for 30 min / day (20 % ramp, 20 % plateau) was controlled by customized Labview™ module and custom built power regulator. Six permutations of days clamped and loaded were performed: clamped either 0 (10 specimens) days, 5 (10) days, or 14 (20) days, loaded for 0 (17) days, 5 (12) days, or 14 (14) days. After 14 days in culture, specimens were harvested and fixed in paraformaldyhyde. Specimens were paraffin embedded and sectioned at 5 micron intervals. A subset was stained with hematoxylin and eosin. *In situ* hybridization analyses were undertaken to determine BMP4 expression (Boehringer Mannheim). To determine tissue level strains, PN21 sagittal suture specimens were subject to mechanical property analysis immediately following harvesting using a tension testing device and digitized specimen images (7).

Results Histologic analyses revealed that mechanical loading led to sagittal suture fusion, i.e. craniosynostosis, in 2 specimens loaded for 14 days (Fig. 2). When compared to unloaded control sutures (Fig. 3), the majority of specimens subjected to mechanical load exhibited a significant increase in cell density, alterations in cell orientation, and increased eosinophillic appearance within the sutures consistent with osteoid formation (Fig. 4). Specimens loaded for 14 days (Fig. 4) exhibited these changes to a greater degree then sutures loaded for 5 days. Preliminary *in situ* hybridization analyses reveal high levels of BMP-4 expression in the sutures loaded for 2 days (Fig. 5), but no expression in unloaded sutures (Fig. 6). Initial tissue moduli calculations predict a suture modulus of 0.2 - 1.2 MPa and strains near 1.0% during loading.

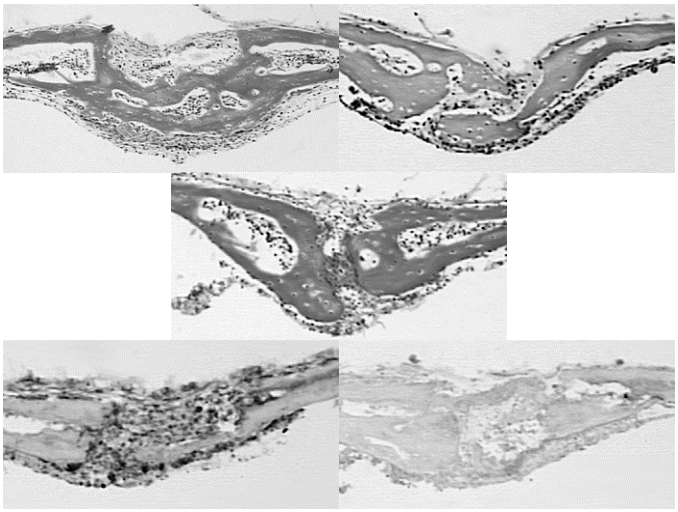

Figure 2: (Top left) H&E 14 day fused loaded specimen; **Figure 3**: (Top right) H&E 14 day unloaded clamped control; **Figure 4**: (Center) H&E 14 day loaded specimen; **Figure 5**: (Bottom left) 2 day loaded *in situ* BMP4 expression; **Figure 6**: (Bottom right) 2 day unloaded *in situ* BMP4 expression

Discussion Here we report a novel experimental system to study the effects of mechanical load on cranial suture morphogenesis *in vitro*. This is the first study to report craniosynostosis as a result of the application of controlled mechanical forces. Within the suture, we have demonstrated increased cell density, altered cell orientation, increased eosinophillic uptake, and high levels of BMP-4 expression, all consistent with bone formation as a result of applied mechanical load. Future studies will elucidate the molecular mechanisms responsible for the premature suture fusion created by the controlled application of mechanical load.

Acknowledgments This study is supported by grants from the NIH (AR20557), the Fredrick Fisher Fund, and the GAANN and Rackham Merit Fellowships. The authors sincerely thank Sandra Chang, Andrew Fribley, and Gary J. Brouhard.

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