The Impact of the *Eda* Pathway on Tooth Root Development

J.M. Fons Romero, H. Star, R. Lav, S. Watkins, M. Harrison, M. Hovorakova, D. Headon, and A.S. Tucker

Appendix

Supplementary methods:

Samples:

Postnatal heads were divided into upper and lower jaws, fixed in 4% paraformaldehyde and decalcified using EDTA, time depending on age of the specimen. Embryonic heads were fixed whole and did not require a decalcification step. After decalcification/fixation samples were dehydrated through an ethanol series before embedding in paraffin via histoclear. For histology a trichrome stain was used involving alcian blue, sirius red and haematoxylin.

In situ hybridization:

For in situ hybridization, sections were rehydrated, refixed in PFA 4% for 20min, permeabilized in 10µg/ml Proteinase K for 8 minutes, acetylated in triethanolamine (T58300 Sigma-Aldrich) plus acetic anhydride (100022M BDH) for 10 minutes and dehydrated again prior to the addition of anti-sense probe. The *Edar* probe was used at 1µg/ml and added in hybridization buffer (50% formamide, 20mM Tris/DEPC pH 7.5, 300mM NaCl/DEPC, 5mM EDTA/DEPC, 1x Denhardt's solution, 10% dextran sulphate, 0.5mg/ml tRNA) and incubated at 60ºC O.N. Samples were washed in 50%formamide-2x SSC, 2xSSC and 0.2xSSC each one twice for 30min at 60ºC. Sections were washed in TBS buffer (100mM Tris pH 7.5, 150mM NaCl) and blocked in TN buffer with 10% fetal bovine serum (F90665, Sigma) plus 1% BBR (Boehringer, 1096176) for 1 hour-RT and incubated with 1:1000 anti-DIG Alkaline Phosphatase

antibody (Boehringer, 1093274) overnight at 4 ºC in blocking solution. Samples were washed in TBS buffer for 1 hour and incubated in NTMT (100 mM Tris-HCl pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween 20) twice for 10 minutes. The colour reaction was developed with BM purple (11 442 074 001, Roche). Slides were then dehydrated and coverslipped using DPX. Images were taken on a Nikon light microscope.

Immunofluorescence:

For immunofluorescence, sections were rehydrated and treated with Tris-EDTA pH9 for 30 minutes at 90°C for antigen retrieval. Endogenous peroxidase was quenched with 3% hydrogen peroxide for 30min. Sections were blocked in TBS buffer plus 0.5% BBR, 10% Serum, 1% BSA and 0.05% Tween 20 for 1h at RT. Anti-Edar (Santa Cruz, sc-15289) or Anti-Shh (Santa Cruz,sc-9024) were added at 1:200 in blocking solution ON at 4°C. After washing in PBS 0.05% Tween 20, a biotinilated secondary antibody (E0432, Dako) was added at 1:300 for 2hr at RT. followed by SA-HRP at 1/150 and 1/150 TSA Fluorophore (Cy3). Slides were mounted with Fluoroshield^{TM} DAPI (ab104139, Abcam). For Ecad (Abcam, ab76319) and K14 (Abcam, ab7800) detection, a secondary Alexa-488 was used. Slides were imaged on a confocal microscope. Shh images used “fire luts” to indicate intensity where blue is the lowest and white the highest.

Proliferation:

Detection of proliferative cell nuclear antigen (PCNA) was achieved using anti-PCNA-Biotin (Abcam, ab113270) at 1/400 followed by SA-HRP at 1/250. The signal was developed using Vector DAB staining kit (SK-4100). Slides were weakly counterstained with Haematoxylin before dehydration and coverslipping. Images were taken on a Nikon light microscope.

Measurements and statistics:

For consistency all tooth measurements were made of the HERS on the buccal side of the tooth. Tooth angle measurements were made as illustrated in Figure 4A. Measurements were made by a researcher blind to the genotype of the tooth with no knowledge of HERS development to ensure unbiased recording. Proliferating cells were counted in different regions adjacent to the HERS as depicted in Fig4B and compared to total number of cells to give a percentage proliferation. Unpaired T-tests were performed with one tail and unequal variance for comparing the distance between the HERS (Fig3I), or two tails and unequal variance for the rest of the statistical analysis. Unequal variance was used due to the large variation between mutant molars compared to very small variation between controls.

microCT:

Specimens were immobilized using cotton gauze and scanned to produce 14μm voxel size volumes, using an X-ray tube voltage of 80kVp and a tube current of 80μA. An aluminium filter (0.05mm) was used to adjust the energy distribution of the X-ray source. To ensure scan consistency, a calibration phantom of known geometry (a dense cylinder) was positioned within the field of acquisition for each scan. Test reconstructions on this object were carried out to determine the optimum conditions for reconstruction, ensuring consistency in image quality, and minimising blurring.