P8-ALTERATION OF TOOTH DEVELOPMENT IN TWO-PHASE ORGANOTYPIC CULTURES BY TRANSIENT GLYCOGEN SYNTHASE KINASE-3 (GSK-3) INHIBITION

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Introduction

Regulation of intercellular signalling pathways is a key question in tooth organogenesis. Wnt pathway is essential for patterning tooth development, and for controlling the shape of individual teeth (Liu *et al*., 2008). Moreover, conditional overexpression of β-catenin leading to an overactivation of the canonic Wnt pathway of the dental epithelium, (Järvinen *et al*., 2006), or conditional inactivation of Apc (Wang *et al*., 2009) produces a phenotype whereby multiple extra teeth are formed. In addition, Lef1 (a Wnt activator) knockout or Wnt inhibitor-Dkk1 overexpressing mouse models present altered tooth development characterized by the accumulation of dental germs at bud stage (Andl *et al*., 2002). In the absence of Wnt signals, a multiheteromeric destruction complex formed by protein GSK-3 (Glycogen Synthase Kinase 3), among others, phosphorylates and triggers β-catenin degradation in the proteasome (Seidensticker and Behrens, 2000). All together data support the importance of the Wnt pathway on odontogenesis. The aim of this work was to characterize the implication of the Wnt signalling pathway on tooth development by means of Wnt activation in mouse first molar cultures.

Methodology

In order to specifically determine the role of Wnt pathway activation at different stages of dental development, we performed organotypic cultures of 14.5 and 17.5 day-old embryos mouse first molar, at the onset of cap and early bell stages, respectively. In a first phase, we cultured tooth germs for 24 hours, 48 hours and 6 days, in the absence or the presence of pharmacological inhibitor of GSK-3 activity, Lithium Chloride (LiCl; 2mM and 10mM). In situ hybridization for mRNA of Lef1 (Lymphoid enhancer-binding factor-1) was performed on these samples together with detection of odontoblast and ameloblast markers (nestin and amelogenin, respectively), and cell proliferation by immunohystochemistry. In a second phase, we used *in vitro* cultured molars to transplant them to testicles of surrogate male mice, to eventually achieve full-term tooth development. This two-phase procedure culminated with the production of a fully calcified molars and the analysis by stereomicroscope and X-ray.

Results and discussion

Increased expression of Lef1 on LiCl□ treated murine molar: Lymphoid-enhancing factor 1 (LEF-1)/T-cell factor (TCF) transcription factors can bind to the β-catenin and the complexes formed transactivate the target genes (Cadigan and Nusse, 1997; Barker et al., 2000). In our experiments, E17 5 and E14 5 molars were cultured for 24 or 48h in the presence or the absence of different LiCl concentration. In situ hybridization revealed an importrant increase of Lef1 expression on 10mM LiCl-treated molars (not shown), indicating an overactivation of the Wnt pathway. Lef1 overactivation was mainly located on the dental mesenchyme.

Alterations in the morphology of molar development upon LiCl treatment: Controls and treated E14.5 and E17.5 molars cultured for 6 days progressed in their development, but samples treated shown an atypical morphology (Figure 1A-D). In the presence of 2mM LiCl the enamel organ showed anomalous invaginations towards the underlying mesenchyme, that resulted in the formation of very pronounced dental ridges. These morphological changes can reveal fundamental events in the abnormal development of the tooth crown**.** Additionally, the cervical loops of the treated sample is indicative of possible malformations at the tooth root.

LiCl-mediated overactivation of the Wnt pa-

thway delays ameloblast and odontoblast differentiation: Nestin has been described as an odontoblast differentiation marker (About et al., 2000). In control E14.5 molars cultured for 6 days, we detected that nestin labeling covers mesenchymal cells facing the inner enamel epithelium (IEE, not shown). In the LiCl treatment, nestin was restricted to some pre-odontoblastic unpolarized cells, which is an indicative of a delay of cell differentiation. When examining amelogenin as an ameloblast marker in E17.5+6 days control molars, we observed that some areas of the IEE were positive, whereas in 2mM LiCl samples the immunohistochemical reaction was negative, indicating a full inhibition of the differentiation process to ameloblasts (Figure 1E-F). Regarding cell proliferation, the number of cells in the DNA synthesis phase was significantly higher in the dental mesenchyme in LiCl-treated molars when comparing to untreated control samples (not shown).
These changes seem to be related to the delay in the differentiation of dental papillae cells into odontoblasts. Undifferentiated cells do not leave cell cycle, similarly to cementoblast precursor cells when they are treated with LiCl (Nemoto, 2009). This could explain that cell prollifferation was very high in the dental mesenchyme.

LiCl-mediated overactivation of the Wnt pathway affects the mineralization of the dental samples: E14.5 and E17.5 control and treated molars for 6 days, mineralized after transplantation in testicles for 21 days. This indicates that the delay in tooth development induced by LiCl during organ culture was not permanent, and teeth were able to recover their growing when LiCl was removed and samples transplanted. Despite dentin and enamel were formed in LiCl-treated samples after transplantation, mineralization was clearly reduced in first molars as seen by X-Ray analysis (Figure 1G-J).

We can conclude that continuous activation of Wnt signaling by LiCl drastically affects the number of dental ridges, proliferating cells, odontoblast and ameloblast differentiation and mineralitation of first molars

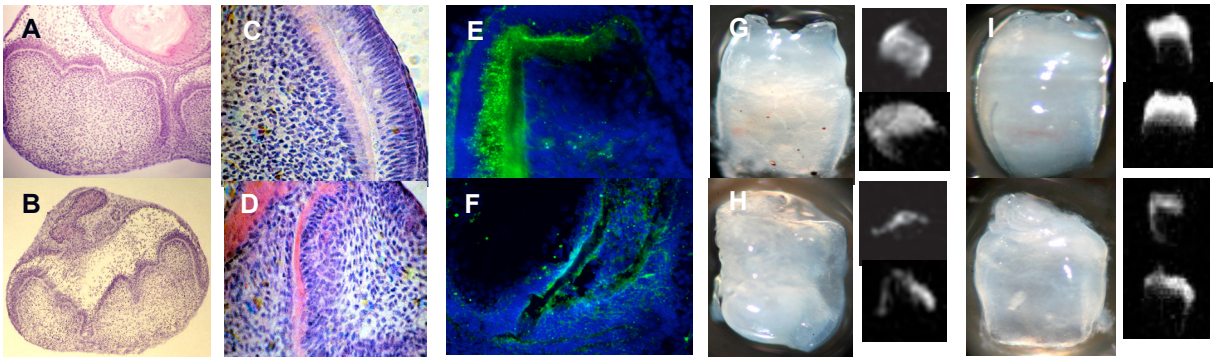

Figure 1. A-B: E14.5 molars cultured for 6 days in the absence (A) or presence of 2mM LiCl (B); C-D: E17.5 molars cultured for 6 days in the absence (C) or presence of 2mM LiCl (D); E-F: immunohistochemistry for amelogenin of E17.5 molars cultured for 6 days in the absence (E) or presence of 2mM LiCl (F); G-J: E14.5 control (G) and treated (H) molars cultured for 6 days and transplanted for 21 days in testicles. E17.5 control (I) and treated (J) molars cultured for 6 days and transplanted for 21 days in testicles. Two X-ray samples are seen close to each case.

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