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Roles of notch signalling in mandibular condylar cartilage

Maria J. Serrano *, *Sarah So*, *Robert J. Hinton*

Department of Biomedical Sciences, Texas A&M University Baylor College of Dentistry, 3302 Gaston Ave, Dallas, TX 75246, USA

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ABSTRACT

Importance: Notch proteins are cell surface transmembrane spanning receptors which mediate critically important cellular functions through direct cell–cell contact. Interactions between Notch receptors and their ligands regulate cell fate decisions such differentiation, proliferation and apoptosis in numerous tissues. We have previously shown using immunohistochemistry that Notch1 is localized primarily to the prechondroblastic (chondroprogenitor) layer of the mandibular condylar cartilage (MCC).

Objective: To test if Notch signalling changes patterns of proliferation and differentiation in the MCC and to investigate if Notch signalling acts downstream of Fibroblast Growth Factor 2 (FGF-2).

Methods: Condylar cartilage explants were cultured over serum-free DMEM containing either 0 or 50 nM DAPT, a Notch signal inhibitor. Explants were used for RNA extraction and immunohistochemistry.

Results: Analysis of gene array data demonstrated that the perichondrial layer of the MCC is rich in Notch receptors (Notch 3 and 4) and Notch ligands (Jagged and Delta) as well as various downstream facilitators of Notch signalling. Disruption of Notch signalling in MCC explants decreased proliferation (Cyclin B1 expression) and increased chondrocyte differentiation (Sox9 expression). Moreover, we found that the actions of FGF-2 in MCC are mediated in part by Notch signalling.

Conclusion: These data suggest that Notch signalling contributes to the regulation of proliferation and differentiation in the MCC.

1. Introduction

The mandibular condylar cartilage (MCC) is a secondary cartilage that differs structurally from both limb growth plate and articular cartilage. This difference is most pronounced in its superficial layers, which comprise a perichondrium in which undifferentiated (prechondroblastic) cells secrete a matrix rich in type I collagen rather than the type II collagen matrix characteristic of chondrocytes.[1,2] Under normal functional conditions, it is these undifferentiated cells, rather than the chondrocytes in deeper layers, that proliferate and differentiate to effect growth at the MCC.[3] The Notch family of transmembrane receptors has been implicated as cell fate mediators in many tissues.[4–6] In a context-dependent manner, Notch signalling promotes or suppresses proliferation, cell death, acquisition of specific cell fates and activation of differentiation programs during development and during maintenance of self renewing adult tissues.[7] Because of their critical role in many fundamental processes and in a wide range of tissues, signalling

* Corresponding author. Tel.: +1 214 828 8976.
E-mail address: mserrano@bcd.tamhsc.edu (M.J. Serrano).
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via Notch receptors has been linked to human disorders and developmental syndromes.[8] In articular cartilage, Notch 1 is expressed in progenitor cells that exhibit phenotypic plasticity; blockage of Notch signalling decreases proliferation in these cells.[9] Notch receptors are also expressed in the early stages of chondrogenic differentiation, becoming confined to the perichondrium as differentiation proceeds.[10] We have previously localized Notch immunohistochemically to the prechondroblastic and upper chondroblastic layers of the MCC[11] and shown that FGF-2 and Tgfβ1 can upregulate and downregulate, respectively, Notch 1 expression in the MCC.[12] It is known that FGF-2 regulates proliferation and differentiation of growth-plate chondrocytes but the mechanisms underlying growth regulation in secondary cartilages such as the mandibular condyle have been less studied.[12] The goals of this study were: (1) to assess gene expression related to Notch signalling in the MCC perichondrium; (2) to test whether disruption of Notch signalling alters proliferation or differentiation in MCC; and (3) to investigate whether Notch signalling lies downstream of FGF-2 signalling.

2. Materials and methods

2.1. Gene array

The mandibular condyle and adjacent ramus were dissected from CD1 mice at embryonic day 17 (E17). Under a dissecting microscope, the perichondrium (PC) was gently teased away from the underlying cartilage and the cartilage (C) was separated from the bone. The PC and C samples were then snap frozen in liquid nitrogen. RNA was extracted from pooled samples of approximately 50 tissues using the RNEasy Micro RNA Isolation Kit (Qiagen, Valencia, CA). The quantity and quality of mRNA were measured by an Agilent 2100 Bioanalyzer. The RNA samples were analysed using the Mouse Notch Signalling Pathway RT^2 Profiler^{TM} PCR Array (PAMM-059, SABiosciences, Frederick, MD), which profiles the expression of 84 genes involved in Notch signalling including binding and receptor processing genes as well as genes that cross-talk with Notch. Genes were considered to be differentially expressed if they were expressed at least two times higher in either the PC or C sample.

2.2. Condylar cartilage explant culture

Condylar cartilage explants were placed on a triangular piece of Poretics polycarbonate disc filter and metal grid suspended over 1 mL of serum-free DMEM (3 mM sodium phosphate, 0.1 mM non-essential amino acids, 2 mM L-glutamine, ITS + TM Premix (BD Biosciences), 500 μg/mL gentamicin, 100 μg/mL of ascorbic acid) containing either 0 or 50 nM DAPT, a Notch signal inhibitor (Sigma Cat # D5942-5MG) in a 60 × 15 mm centre well organ culture dish. After 3 days of culture, the mandibular condylar cartilages were separated from the bone under a dissecting microscope, and snap frozen in liquid nitrogen for RNA extraction. Five to six explants in each group were used for immunohistochemistry.

In a second set of experiments, other explants (5–6 per group) were incubated with media alone, media containing 50 nM DAPT, or media containing 50 nM DAPT plus 3 ng/mL of FGF-2. After 3 days of incubation, RNA was extracted for gene expression analysis.

2.3. Statistical analysis

Means and standard deviations were calculated for the relative quantities of gene expression in the control and experimental explant samples. Statistical differences were evaluated using the Kruskall–Wallis test to identify differences among the samples and the Mann–Whitney test to analyse specific sample pairs for significant differences.

2.4. Quantitative real-time PCR

Primers were designed using the SciTools software (Integrated DNA Technology). Each primer was evaluated in a primer matrix to determine what combination of sense (S) and anti-sense (AS) primer concentrations maximized the fluorescence response for real-time PCR. SYBR® Green Real-Time PCR Master Mix (Stratagene) was used for fluorescence. The primer sequences used were: Cyclin B1, (S: ACA GGG TCG TGA AGT GAC TGG AAA; AS: CTT GGG CAC ACA ACT GTT CTG CAT); Delta like 1 (S: ATG GAG CCGAGA AGA GCA GCT TTA; AS: ACT TGG TGT CAC GTT TGC TGT GTG); HES1, (S: AGC CTA TCA TGG AGA AGA GGC GAA; AS: TGG AAT GCC GGG AGC TAT CTT TCT). Samples along with primers and SYBR Green Master Mix were loaded in an eight tube strip and the reaction was run in the MX4000 Multiplex Quantitative PCR System (Stratagene). 18s II was used as an internal control. The primer sequences for 18s II were: S: CCG AAG CGT TTA CTT TGA; AS: GCC GTC CCT CTT AAT CAT.

The standard curve method was used to calculate the efficiency of the target genes. Serial dilutions of cDNA (1:10) were made for the calibration curve and loaded into an eight strip tube. Correlation coefficients for all standard curves were 0.92 or higher.

2.5. Immunofluorescent staining

Condyles were fixed in 4% neutral buffered paraformaldehyde, decalcified in 0.5 M EDTA, processed for routine histology, and embedded in paraffin. 6-μm sections cut in the sagital plane were taken for immunohistochemical staining. Immunohistochemistry was done by incubating at 4 °C overnight with primary antibody to Notch 1 (Santa Cruz) at 1:75 dilution. After three 5 min washes in 2% serum, the sections were incubated in Alexa Fluor 546 (Molecular Probes) goat anti-rabbit (Notch) secondary antibody for 1 h in the dark at RT. Finally, the sections were washed four times in PBS, incubated in To Pro 1:500 for 5 min, cover-slipped with slow fade mounting medium (Invitrogen), and examined using a Leica TCS SP2 confocal microscope.

3. Results

3.1. Notch-related gene expression in MCC perichondrium

To examine if differential expression of Notch pathway genes exists in the perichondrium, we analysed gene expression in