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CELL FATE MEDIATORS *NOTCH* AND *TWIST* IN MOUSE MANDIBULAR CONDYLAR CARTILAGE

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Abstract

Objective—The objectives of this study were to examine if Twist and Notch 1 are present in the mandibular condylar cartilage (MCC) and whether their gene expression can be altered by exogenous FGF-2 and TGF- β 2.

Design—Half-heads from CD-1 mice pups harvested at embryonic day 17 (E17) were fixed, decalcified, and sectioned in the sagittal plane for immunohistochemical detection of Notch and Twist using confocal microscopy. Other mandibular condyles and adjacent ramus from E17 mice were cultured in serum-free DMEM containing 0, 3, or 30 ng/mL of FGF-2 (10–12 condyles per treatment group). This experimental design was repeated with medium containing 0, 3, or 30 ng/mL of TGF- β 2. After 3 days of culture, the pooled RNA from each group was extracted for examination of *Notch* and *Twist* gene expression using quantitative real-time RT-PCR.

Results—Immunohistochemical examination revealed that Notch and Twist were localized to the prechondroblastic and upper chondroblastic layers of the cartilage. Exogenous FGF-2 up-regulated *Notch1*, *Twist1* and *Twist2* gene expression in MCC explants from E17 mice, while TGF- β 2 had the opposite effect.

Conclusions—The gene expression data demonstrate that MCC explants are sensitive to growth factors known to affect Notch and Twist in other tissues. The subset of cells in which Twist and Notch immunoreactivity was found is suggestive of a role for FGF-2 and TGF- β 2 as regulators of cell differentiation of the bipotent MCC cell population, consistent with the role of Notch and Twist as downstream mediators of these growth factors in other tissues.

1. Introduction

The mandibular condylar cartilage (MCC) is a secondary cartilage that is structurally distinct 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 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 mature to effect growth at the MCC ^{3,4}. However, immobilization of the mandible or removal of the MCC from its normal functional environment produces a relatively rapid conversion of the prechondroblastic cells to an osteoblastic phenotype ^{3,5}

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Little is known about the molecular regulators controlling proliferation and differentiation in the prechondroblastic (also known as chondroprogenitor) MCC cells.

Twist is a helix-loop-helix transcription factor which in *Drosophila* regulates transcription of genes homologous to Fgfr genes ⁶. In cranial sutures, Twist shares an overlapping distribution with Fgfr2 and several FGF ligands ⁷. Osteoblastic cells overexpressing Twist stay in an undifferentiated state and increase their proliferation ⁸. In addition, Twist expression induced by canonical Wnt signaling strongly inhibits chondrocyte gene expression ⁹. Thus, Twist may be a negative regulator of differentiation in osteoprogenitor or chondroprogenitor cells. However, it is unknown whether Twist is present in MCC, or whether it can also influence proliferation and differentiation of prechondroblasts.

Notch 1 is a trans-membrane receptor that may be implicated as a cell fate mediator in many tissue ^{10, 11}. It is expressed in the early stages of chondrogenic differentiation, becoming confined to the perichondrium as differentiation proceeds ¹². There is also evidence that Notch 1 affects osteogenic differentiation, although studies differ regarding the direction of this influence ^{13, 14}. Mice in which Jagged 2 (a ligand of Notch 1) has been inactivated exhibit craniofacial defects including cleft palate ¹⁵. Moreover, Notch 1 is expressed in articular cartilage progenitor cells that exhibit phenotypic plasticity; blockage of Notch signaling decreases proliferation in these cells ¹⁶. In the MCC, Notch 1 intracellular domain (NICD) has been demonstrated immunohistochemically in proliferative cells at E15–E17, expanding its distribution to most layers of the MCC at later ages ¹². Immunoreactivity for the Notch ligands Jagged 1 and Jagged 2 showed a similar distribution and temporal sequence ^{17, 18}.

The growth factors, FGF-2 and TGF- β influence the gene expression of *Twist* and *Notch* in tissues other than the MCC. For example, treatment with FGF-2 increases Notch 1 expression in oligodendrites ¹⁹. Moreover, FGF-2-induced proliferation in mouse neural crest cells is effected by activating Notch signaling ²⁰. Twist is a well-known target of FGF signaling in cranial sutures ²¹, and suppression of Twist1 expression by TGF- β signaling is important for frontal bone development ²². Moreover, addition of exogenous TGF β 2 induces suture closure accompanied by elevated levels of cell proliferation ²³ in cells that have developmental and morphologic communalities with MCC prechondroblasts ²⁴.

Although our knowledge is incomplete, both FGF-2 and TGF- β and their receptors are present in the MCC. We and others have shown that the prechondroblastic (chondroprogenitor) cells of the MCC express Fgfr2 receptors ^{25, 26} in the same layer (prechondroblasts or chondroprogenitor cells) as BrdU immunoreactivity. FGF-2, a ligand for Fgfr2, has been demonstrated immunohistochemically in the perichondrium and chondroblastic layers of the MCC ^{27, 28}. Several TGF- β isoforms are present in the MCC ²⁹. Moreover, Oka and associates (2008) have shown that TGF- β signaling plays a role in cell fate determination between chondrogenic and osteogenic lineages as well proliferation in MCC cells.

The purposes of this study were twofold: 1) to establish whether Twist and Notch 1 are present in the MCC and their localization in its various layers; 2) to examine whether Notch 1 and Twist gene expression in MCC can be altered by exogenous FGF-2 and TGF- β 2.

2. Materials and Methods

2.1 Tissue Harvest

The mandibular condyle and adjacent ramus were dissected from CD-1 mice pups harvested at embryonic day 17 (E17). The E17 time frame was chosen because the basic structure of

the temporomandibular joint are largely developed in mice at this juncture. Three condyles were then placed into each well of a 24-well culture cluster plate containing 500µl of DMEM with 3mM sodium phosphate, 0.1mM non-essential amino acid, 2mM L-glutamine, ITS+™ Premix (BD Biosciences), 500µg/ml gentamicin, 100µg/mL of ascorbic acid with media changes every other day. In roughly one-third of the explants, the medium also contained 3 ng/mL of FGF-2; in another third, the medium contained 30 ng/mL of FGF-2. This experimental design was repeated with medium containing 0, 3, or 30 ng/mL of TGF-β2 for approximately ten to eleven condylar cartilages in each group. After 3 days of culture, the mandibular condyle and adjacent ramus in each specimen were cleaned of adherent muscle and connective tissue, and the cartilage separated from the bone under a dissecting microscope. The condylar cartilage was then snap frozen in liquid nitrogen.

2.2 RNA Extraction and Real-Time RT-PCR

In most instances, the RNA was extracted immediately using Ultraspec RNA Total RNA Isolation Reagent (Biotecx); otherwise, the tissue was stored at -80°C until isolation. The quantity and quality of mRNA were measured by an Agilent 2100 Bioanalyzer. mRNA was reverse-transcribed with SuperScriptII reverse-transcriptase (Invitrogen) and the resulting cDNA was diluted to 1:10 for quantified real-time PCR. The relative quantitation value was calculated by $2^{-\Delta\Delta C_t}$ method. All quantifications were normalized to 18s (SuperArray). For each primer set the running conditions were optimized. The primer sequences used were: *Notch 1*, (S: GAA GAT GCA CCT GCT GTC; AS: GTG GAG TTG TGC CAT CAT; *Twist 1*, (S: TGG ACT GGC TCC ATT TTA; AS: ATG CAT TTA GAC ACC GGA; *Twist 2*, (S: TCA GCT AGC CGT GTT TTC; AS: TAT TGT TCC TGG GTG TGG). *18s*, S: CCG AAG CGT TTA CTT TGA; AS: GCC GTC CCT CTT AAT CAT.

2.3 Confocal Microscopy

Heads from E17 pups were fixed in 4% neutral buffered paraformaldehyde, decalcified in 0.5M EDTA, processed for routine histology, and embedded in paraffin. 6-micron sections in the sagittal plane were obtained and the temporomandibular joint identified in hematoxylin and eosin stained sections. Sections were deparaffinized in xylene, hydrated in a decreasing ethanol series and washed in 1x PBS. Following 30 minutes in 2% blocking serum at 4°C, the sections were incubated at 4°C overnight in the appropriate primary antibody: Notch 1 (Santa Cruz Biotechnology, sc-6014-R) at 1:75; Twist (generously donated by C. A. Glackin) at 1:100. After three 5 min washes in 2% serum, the sections were incubated in Alexa Fluor 488 donkey anti-rabbit secondary antibody (Molecular Probes) for one hour in the dark at room temperature. Finally, the sections were washed four times in 1x PBS for 5 minutes and cover slipped using propidium iodide with mounting medium (Vector Labs) in the dark. All sections were imaged using a Leica TCS SP2 confocal microscope.

3. Results

3.1 Tissue Localization of Notch 1 and Twist

Notch 1 protein was most prominent in the superficial layers of the MCC, beginning to be apparent around 3 cell layers from the surface (Fig. 1). The most intensive immunoreactivity was in the prechondroblastic layer of cells. A marked diminution of Notch 1 immunoreactivity occurred in the deeper chondroblastic and hypertrophic layers, where only an occasional cell showed reactivity. Expression was not seen in the nucleus, but adjacent to the cytoplasm in a discontinuous distribution. Sections in which the primary antibody was replaced by PBS showed only propidium iodide fluorescence.

Twist protein was present predominantly in the prechondroblastic layer but also to a considerable extent in the deeper chondroblastic layer (Fig. 2A). It was not evident in the hypertrophic layer. In the majority of cells, Twist was localized to the cytoplasm (Figure 2B).

3.2 Gene Expression in Response to Exogenous Growth Factors

Quantitative RT-PCR showed that Notch 1 expression increased in dose-dependent fashion following treatment with FGF-2 (Fig. 3A), with a 3X increase over controls at 30 ng/mL FGF-2. By contrast, Notch 1 was strongly down-regulated (3-fold) by increasing concentrations of TGF- β 2 (Fig. 3B).

Twist 1 expression was elevated somewhat (1.5X) by 3 ng/mL exogenous FGF-2 (Fig. 4A), while Twist 2 expression was elevated almost 3X at the same concentration (Fig. 4B). Interestingly, the higher concentration of both growth factors (30 ng/mL) had no effect. Exogenous TGF- β 2 slightly elevated Twist 1 expression at 3 ng/mL (Fig. 5A), but down-regulated it 2-fold at 30 ng/mL. Twist 2 expression was more strongly down-regulated by TGF- β 2, decreasing its expression around 2-fold at 3 ng/mL and 13-fold at 30 ng/mL (Fig. 5B).

4. Discussion

The mandibular condylar cartilage is recognized as an important site at which growth of the mandible takes place via endochondral bone formation. It is a secondary cartilage, which differs from primary cartilage in several morphological and molecular characteristics ^{2, 30, 31}. Our data provide support for the role of both Twist and Notch in regulating MCC development.

Our immunohistochemical results demonstrate that both Twist and Notch are localized largely to the prechondroblastic and early chondroblastic layers of the MCC. This localization is consistent with the pattern of immunoreactivity for Notch 1 intracellular domain (NICD) for E17 mice in a previous study by Shimizu and coworkers (2008); in similar studies, the Notch ligands Jagged 1 and 2 had a similar age-specific distribution ^{17, 18}. Because it is a transcription factor, Twist would be expected to be located in the nucleus. However, our observations indicate that, while there is occasional expression of the molecule in the nucleus, much of the Twist immunolocalization is in the cytoplasm. Demontis and co-workers ³² have suggested that the cytoplasmic form may represent molecules undergoing ubiquitination for ultimate degradation. Nevertheless, other investigators have reported cytoplasmic (and nuclear) Twist immunolocalization ^{33, 34}. Twist-1 gene expression has been demonstrated in Runx-2 expressing perichondrial cells of mouse embryos ³⁵, where it may inhibit premature osteoblast differentiation by interaction with the DNA-binding domain of Runx2. Runx2 a transcription factor that is required to initiate differentiation along the osteogenic pathway ³⁶. Under regulation by Twist, Runx2 is also essential for the regulation of chondrocyte maturation via FGF-18 ³⁶. In recent years, the MCC has been shown to be reduced in size or organization in mice with conditional inactivation of Tgfbr2 in neural crest cells ³⁷ or knockout of Indian hedgehog and eliminated altogether in Runx2-deficient mice ³⁹. Oka and associates have suggested their reduced phenotype may be due to downstream effects of TGF- β on Indian hedgehog ³⁷ or alternately to downstream effects on Sox 9 ⁴⁰. They have postulated that a competitive interaction of osteogenic (Runx2) and chondrogenic (Sox9) factors regulates MCC development ⁴⁰. The co-localization of both Runx2 and Sox 9 to the prechondroblastic layer of the MCC ³⁹ where Twist and Notch were found in this study supports this scenario. In light of the likely regulation of Runx2 by Twist, the reduction of Twist1 and 2 in MCC explants by increasing concentrations of TGF-B2 demonstrated in this study may reflect this dynamic interaction.

In the condylar cartilage explants, the distribution of immunoreactivity for Notch is fairly similar to that of Twist. Although Notch has been less studied in calcified tissues than has Twist, Notch1 gene expression has been localized mainly to the perichondrium and periosteum in E16.5 mouse embryos, with some expression in the deeper chondroblastic zones of limb cartilage¹². Notch1-expressing cells that exhibit considerable phenotypic plasticity have been identified near the surface of articular cartilage¹⁶. These reports reflect a presumed role of Notch as an inhibitor of cell differentiation, thereby maintaining a pool of progenitor cells^{41, 42}. In addition, our work using targeted gene arrays (osteogenesis, stem cell) to identify genes preferentially expressed in the micro-dissected perichondrium and the cartilaginous portions of the MCC in perinatal mice has shown that the perichondrium is enriched in Notch isoforms (especially Notch 3 and 4) and their ligands compared to the cartilaginous portion of the MCC⁴³. Taken together, these observations are consistent with our localization of Notch to the MCC prechondroblastic cells, which do not express the protein profile of differentiated chondrocytes^{1,2}. The down-regulation of Notch receptors observed during chondrogenesis in human bone-marrow stromal cell⁴² aggregates is consistent with the reduction in reactivity in cells in the deeper (chondroblastic) MCC layers.

Notch and *Twist* expression were affected similarly by exogenous FGF2 and by exogenous TGF- β 2, but the nature of this effect differed between the growth factors. FGF-2 increased the expression of both *Notch* and *Twist*; whereas, TGF- β 2 decreased *Notch* and *Twist* expression. The role of Twist in FGF-mediated signaling during limb morphogenesis has been well-documented⁴⁴. Moreover, beads soaked in FGF-2 upregulate *Twist* expression in the midsutural mesenchyme of cranial sutures while inhibiting bone sialoprotein expression⁷. The role of Notch1 activation in mediating the effects of FGF2 is just now beginning to be appreciated^{20,45}. The demonstration that normal frontal bone development requires TGF- β signaling to down-regulate Twist expression²² suggests a similar role for TGF- β in reducing Twist to allow differentiation to proceed in MCC.

In addition to their role in inhibiting differentiation, both Twist and Notch are instrumental in increasing proliferation. Forced expression of Twist-1 has been linked to uncontrolled proliferation in several experimental systems^{46, 47}. Notch has also been linked with increased proliferation in a variety of cell types^{48,49}. In MCC explants, FGF-2 increases proliferation²⁶, and *Fgfr2* expression in MCC changes dramatically following altered mandibular position⁵⁰ in concert with changes in proliferation. Thus, it is possible that either Notch, Twist or both may play a downstream role in growth-factor-mediated changes in proliferation in MCC.

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Figure 1.

Fig. 1A Immunolocalization of Notch1 in mandibular condylar cartilage (MCC); articular surface is inferior. Cell nuclei are in red, Notch1 in green. Upper left: low power (10X) view showing strong localization of Notch1 to periosteum and perichondrium., upper right: Propidium iodide only; lower left: merged image. Fig. 1B: Higher power (40X) view of condylar cartilage showing primary localization of Notch 1 to prechondroblastic and upper chondroblastic layers of the MCC. Tissue at lower border of image is the articular disc, separated from the articular surface by the inferior joint space.

Figure 2.

Fig. 2A Immunolocalization of Twist in mandibular condylar cartilage (articular surface is at right). Cell nuclei are in red, Twist in green. A low power view showing localization to prechondroblastic and upper chondroblastic layers. Fig. 2B: High power view showing predominantly cytoplasmic reactivity of Twist in MCC cells.

Figure 3.

Fig. 3A: Notch1 expression with increasing concentration of FGF-2; Fig. 3B: Notch1 expression with increasing concentration of TGF-β2. Y-axis shows the relative expression of mRNA levels in experimental and control groups. All calculations were normalized to 18S, and then the control values normalized to 1.0. Each bar represents the mean ± SEM of the RT-PCR in triplicate.

Figure 4.

Fig. 4A: Twist1 expression with increasing concentration of FGF-2.; Fig. 4B: Twist1 expression with increasing concentration of TGF- β 2.

Figure 5.

Fig. 5A: Twist2 expression with increasing concentration of FGF-2.; Fig. 5B: Twist2 expression with increasing concentration of TGF-β2.