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Growth Factor Control of Otic Capsule Chondrogenesis

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Abstract

The otic capsule initially appears as condensations of mesenchyme around the developing otocyst, and serves as a template for the subsequent formation of the endochondral bony labyrinth of the inner ear. While it has long been known that the epithelial otocyst influences the induction and chondrogenic differentiation of the otic capsule, factors involved in epithelial control of otic capsule chondrogenesis have only recently been identified. This review discusses members of the fibroblast growth factor (FGF), transforming growth factor-beta (TGFß), and bone morphogenetic protein (BMP) families, and summarizes their roles in guiding the induction and cytodifferentiation of the otic capsule of the inner ear from periotic mesenchyme. The implications of this research to otosclerosis, a disease affecting the capsule of the inner ear, are discussed.

Introduction

The inner ear is derived from cephalic surface ectoderm that forms the otic placode. After thickening, the otic placode invaginates and vesiculates to form the otic vesicle (otocyst) (Figure 1), the epithelial primordia from which the vestibular and cochlear regions of the membranous labyrinth will form (Noden and Van De Water, 1986). The otocyst is surrounded by and closely apposed to mesenchyme of mesodermal origin (periotic mesenchyme) (Figure 1), and is essential to both the cytodifferentiation and morphogenesis of the otic capsule (McPhee and Van De Water, 1985, 1986). In response to induction by the otocyst epithelium, periotic mesenchyme aggregates, forming sites of cellular condensation. These regions of condensed mesenchyme synthesize a type II collagen-rich extracellular matrix and form a fully chondrified otic capsule (Figure 2) that serves as a template for the subsequent formation of the endochondral bony labyrinth.

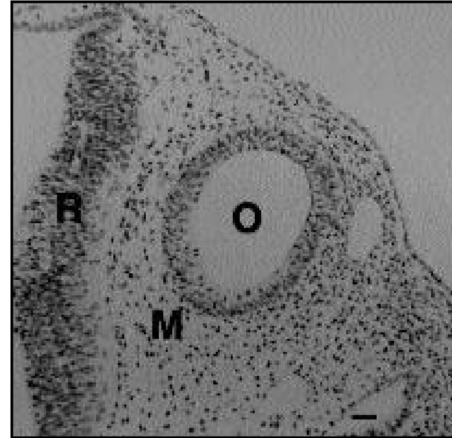

Figure 1: Transverse section through the developing otocyst (O) of a 10.5 day- old mouse embryo (E10.5). The otocyst is surrounded by loosely organized periotic mesenchyme (M) that, following induction by otocyst epithelium, will condense and later chondrify to form the cartilaginous otic capsule. Overt chondrogenesis begins at around day E12 and is completed by E16. The developing rhombencephalon is indicated (R). Bar line=100 μm.

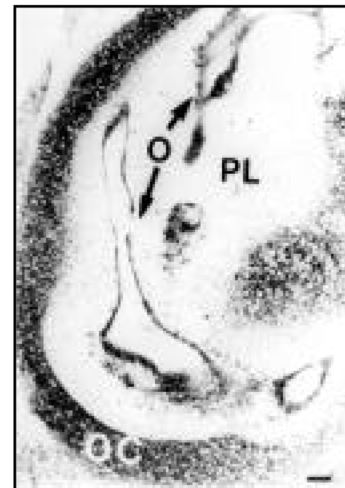

Figure 2: Transverse section through the developing inner ear of an E16 mouse embryo showing the contouring of the chondrified otic capsule (OC) around the anlagen of the epithelial-derived otocyst (O). Ossification begins shortly after E16 days. Perilymphatic spaces (PL) have begun to clear. Bar line=100 μm.

Interactions between epithelial and mesenchymal tissues often control the initiation of chondrogenic differentiation during embryonic development. In developing vertebrae, for example, somatic mesenchyme becomes chondrogenic in response to inductive influences from the notochord and neural tube (Holfreter, 1968; Lash *et al.,* 1968), while in the embryonic limb, the influence of ectoderm is required for the proliferation and differentiation of the limb mesoderm into cartilage (Gumpel-Pinot, 1980). Experiments in otic explants and in high-density cultures of periotic mesenchyme + otic epithelium have confirmed the importance of the otocyst epithelium in guiding the initiation and subsequent differentiation of the cartilaginous otic capsule (Frenz and Van De Water, 1991; McPhee and Van De Water, 1986), demonstrating that factors that are endogenous to the otocyst epithelium have a significant influence on otic capsule chondrogenesis (Frenz *et al.,* 1992, 1994,

1996a; Frenz and Liu, 1998). These factors, which include members of the fibroblast growth factor (FGF), transforming growth factor-beta (TGFß), and bone morphogenetic protein (BMP) families, and their functional roles in epithelial control of periotic mesenchyme chondrogenesis, will be reviewed in this article.

The role of fibroblast growth factors in initiation of otic capsule chondrogenesis

Recent attention has been directed at identifying the locally produced signal molecules that mediate the inductive effects of otocyst epithelium on periotic mesenchyme chondrogenesis. One growth factor family that has a significant involvement in early morphogenetic events, including the induction of chondrogenic differentiation in the developing capsule of the inner ear, is fibroblast growth factor (FGF). The FGFs constitute a multigene family comprised of structurally and functionally related members (Hebert *et al.,* 1990). Several of these genes, including FGF2 and FGF3, participate in the control of cartilage growth and differentiation during embryogenesis (Kato *et al.,* 1992), and are expressed in the epithelium of the embryonic mouse inner ear at a developmental stage coincident with the onset of capsule induction by the otocyst epithelium (Wilkinson *et al.,* 1989; Mansour *et al.,* 1993; McKay *et al.,* 1996). In accord with these findings, FGF2 protein demonstrates a pattern of distribution in inner ear mesenchyme and epithelium that is consistent with a role in initiation of capsule cartilage differentiation (Frenz *et al.,* 1994).

Studies in embryonic limb bud mesenchyme have shown that the chondrogenic events which occur *in vivo*, such as mesenchymal condensation formation and collagen type II accumulation, also occur during chondrogenic differentiation in high-density culture (Ahrens *et al.,* 1977). High-density (micromass) cultures of mouse periotic mesenchyme were developed to model the process of chondrogenesis in the developing capsule of the inner ear (Frenz and Van De Water, 1991). In accord with previous studies in organo-typic culture (McPhee and Van De Water, 1986), in high-density culture, periotic mesenchyme isolated from mouse embryos of embryonic age 10.5 days (E10.5) requires the presence of otocyst epithelium to differentiate into cartilage (Figure 3) (Frenz and Van De Water, 1991), raising the question whether the ability of periotic mesenchyme to respond to otocyst epithelium may be dependent on endogenous epithelial-derived signaling molecules. To address this, studies were performed in which exogenous FGF2 or FGF3 was substituted for otic epithelium and added to high-density cultures of E10.5 periotic mesenchyme (Frenz *et al.,* 1994; Frenz and Liu, 1998). The extent of chondrogenesis was assessed in culture first by the formation of mesenchymal cell condensations (Figure 3A) and subsequently, by spectrophotometric quantitation of matrix-bound Alcian blue stain, pH 1.0, a stain which binds specifically to sulfated glycosaminoglycans (S-GAG) in the extracellular matrix of chondrifying cells at this pH (Figure 4) (Lev and Spicer, 1960). Either FGF2 (1 ng/ml) or FGF3 (1 ng/ml) could initiate chondrogenesis. However, the extent of chondrogenesis induced by either FGF2 or FGF3, even at concentrations as high as 1µg/ml, was significantly less than that which occurs in the presence of the natural inductor tissue (i.e., otic epithelium). These findings, which indicate that signaling by either FGF2 or FGF3 is sufficient to induce otic capsule chondrogenesis, albeit to a limited extent, are in accord with previous studies demonstrating the induction of mesodermal differentiation by FGF during embryonic development (Slack *et al.,* 1987; Cohn *et al.,* 1995). Furthermore, FGF2 has been localized to sclerotic lesions of the otic capsule in the Palmerston North autoimmune strain mouse, suggesting that FGF2 plays a role not only in normal capsule formation and maintenance, but also in abnormal bone remodeling in autoimmune disease (Pederson *et al.,* 1993). In accord with these findings, in specimens of the stapedial footplate from patients with otosclerosis, a disease in which there is overgrowth of the bony otic capsule, osteoblast cells rich in FGF2 and FGF3 are present in regions of new bone formation.

As a result of targeted mutation of the murine FGF3 gene, anomalies of the epithelial-derived anlagen (i.e., developing structures) of the inner ear are produced; however, development of the otic capsule appears normal (Mansour *et al.,* 1993). Hence, the necessity for FGF in the signaling of otic capsule formation was questioned. As an approach to define a role for FGF3 in the mediation of otic capsule differentiation and development, endogenous FGF3 was blocked in high-density culture using antisense oligonucleotides complementary to various regions of the murine FGF3 gene (Frenz and Liu, 1998). Cultures of E10.5 periotic mesenchyme containing otic epithelium (E10.5 periotic mesenchyme + otic epithelium) were treated with antisense oligonucleotide (30 µg/ml; 0.2 µM) complementary to the ATG initiation site of the murine FGF3 gene. The normally robust staining pattern for endogenous FGF3 in the periotic mesenchyme and otic epithelium of untreated control and FGF3 sense oligonucleotide-treated control cultures was reduced to a faint pattern of immunostain in the FGF3 antisense oligonucleotide-treated cultures (Frenz and Liu, 1998). Correspondingly, a marked suppression of chondrogenesis, as assessed by mesenchymal condensation number and binding of Alcian blue stain, ensued (Frenz and Liu, 1998), suggesting a compromised chondrogenic response by the cultured periotic mesenchyme in response to decreased levels of FGF3. This compromised chondrogenic response was restored to normal by supplementation of FGF3 antisense oligonucleotide-treated cultures with excess FGF3 (20 ng/ml), confirming the specificity of FGF3 inhibition. In accord with these findings, FGF3 antisense oligonucleotide inhibited the formation of the otic vesicle in explants of the developing chick otic placode (Represa *et al.,* 1991). Furthermore, abnormalities of FGF3 expression in *kreisler* mutant mice may account for abnormalities in their developing inner ears (McKay *et al.,* 1996).

To ascertain the necessity for endogenous FGF2 in initiation of otic capsule chondrogenesis, FGF2 specific antibody was added to high-density cultures of E10.5 periotic mesenchyme + otic epithelium (Frenz *et al.,* 1994). Treatment of cultures with neutralizing anti-FGF2 antibody suppressed chondrogenesis, and resulted in a marked decrease in the mesenchymal

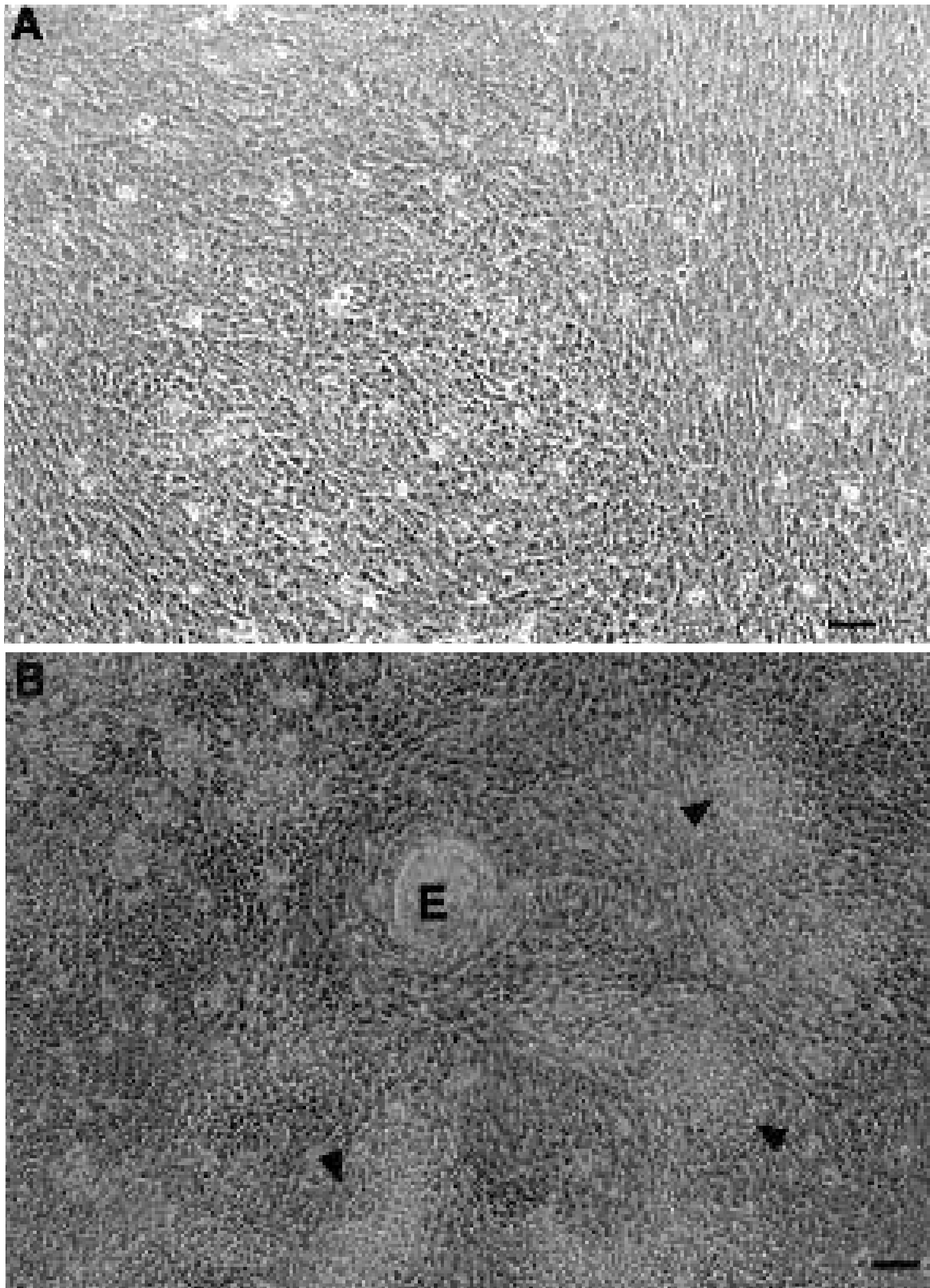

Figure 3: High-density cultures, day 3 *in vitro*, of (A) periotic mesenchyme and (B) periotic mesenchyme + otic epithelium (E) prepared from mouse embryos of age E10.5 days. Comparison of (B) to (A) demonstrates that in the presence of otic epithelium, periotic mesenchyme begins the chondrogenic process by forming sites of cellular condensation (arrowheads). By day 7 *in vitro*, cellular condensations differentiate into cartilage. Phase contrast micrographs; bar line=35 μm.

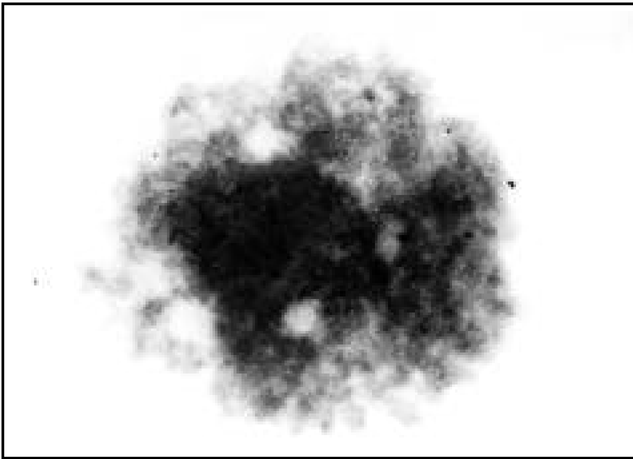

Figure 4: Low magnification view of a high-density culture of E10.5 periotic mesenchyme after 7 days *in vitro,* demonstrating binding of Alcian blue stain (pH 1.0) to the matrix of the chondrifying cells. As a measure of chondrogenesis, matrix-bound Alcian blue stain is extracted from culture, then quantitated spectrophotometrically.

accumulation of S-GAGs and cartilage-specific collagen type II. Since FGF2 protein is present in only the otic epithelium of the E10.5 mouse inner ear (Frenz *et al.,* 1994), suppression of otic capsule chondrogenesis in cultured E10.5 periotic mesenchyme + otic epithelium by neutralizing FGF2 antibodies suggests that this growth factor is directed extracellularly from the otic epithelium to elicit its inductive effects on periotic mesenchyme. However, the gene for FGF2 lacks a conventional signal peptide sequence (Gospodarowicz *et al.,* 1987; Folkman and Klagsbrun, 1987). Immunolocalization of endogenous FGF2 in the otic epithelium of cultured E10.5 periotic mesenchyme + otic epithelium on day 1, and in both the otic epithelium and its surrounding periotic mesenchyme on culture day 3, supports a paracrine mechanism of action of this signal molecule in otic capsule chondrogenesis. Moreover, the mitogenic effect of otic epithelial-conditioned culture medium in cultured human umbilical endothelial cells indicated the presence of secreted FGF-like factors (Frenz *et al.,* 1994).

Based on these findings, FGF2 and FGF3 are both necessary for the signaling of chondrogenic differentiation in cultured periotic mesenchyme and sufficient to the initiation of this process. Hence, although little evidence of defects in the otic capsule were demonstrated in FGF3 null mutant mice (Mansour *et al.,* 1993), this lack of capsular anomaly is likely to be attributed to mechanisms involving functional redundancy. Overlapping expression patterns of FGF2, FGF3, and FGF4 in the developing mouse inner ear (Wilkinson *et al.,* 1989; Frenz *et al.,* 1994; Mahmood *et al.,* 1996) are consistent with functional overlap between members of the FGF family. Moreover, since substitution of otic epithelium with either FGF2 or FGF3 can initiate chondrogenesis but only to a limited extent, factors other than FGF are also likely to be involved in signaling of otic capsule chondrogenesis.

The role of the Transforming Growth Factor-ß superfamily in regulation of otic capsule chondrogenesis

The extent of chondrogenic differentiation that occurs in the presence of either FGF2 or FGF3 is less than that which occurs in the presence of the natural inductor tissue, i.e., the otocyst epithelium. Hence, growth factors other than FGF were tested for their ability to mediate otic capsule chondrogenesis. Transforming growth factor-beta (TGFß) is a homodimeric molecule (Derynk *et al.,* 1985; Seyedin *et al.,* 1986; Jakowlew *et al.,* 1994; Chimal-Monroy and Leon, 1997) that is widely distributed in the mouse embryo, demonstrating a temporal-spatial pattern of distribution that correlates with sites of ongoing morphogenetic events (Heine *et al.,* 1987). Between embryonic ages E10-E16 days, a time period corresponding to initiation and completion of the development of the cartilaginous otic capsule, $TGFß_1$ protein is present at sites of ongoing epithelial-mesenchymal interactions in the developing mouse inner ear (Frenz *et al.,* 1992). Moreover, mRNA for $TGFß_1$ and $TGFß_2$ is present in the epithelial-derived sensory anlagen of the developing mouse and human inner ear (Paradies *et al.,* 1998; Frenz *et al.,* 1991; Gatherer *et al.,* 1990). Several TGFß isoforms, including $ß_1$, are implicated in control of mesenchyme proliferation and chondrogenic differentiation during embryonic development (Kulyk *et al.,* 1989; Leonard *et al.,* 1991; Chimal-Monroy and de Leon, 1997). Therefore, the ability of exogenous $TGFß_1$ to mediate epithelial control of periotic mesenchyme chondrogenesis was tested.

Addition of $TGFß_1$ to cultured E10.5 mouse periotic mesenchyme at concentrations ranging between 0.05-10 ng/ml did not initiate otic capsule chondrogenesis. However, when $TGFß_1$ (0.05-1ng/ml) was added to high density cultures of periotic mesenchyme of age E12 days, i.e., a developmental stage by which periotic mesenchyme is competent to differentiate into cartilage even in the absence of otic epithelium, chondrogenic differentiation was stimulated, as assessed by Alcian blue staining (pH 1.0) of cultures and incorporation of radioactive sulfate into S-GAG (Frenz *et al.,* 1992). In contrast, a suppression of chondrogenesis was evoked by $TGFß_1$ (0.05-1 ng/ml) in cultures of E14 periotic mesenchyme. This suppression of chondrogenesis *in vitro* is consistent with the turning off of chondrogenesis that occurs at E14 *in vivo* to allow for the clearing of perilymphatic spaces and modeling of the otic capsule. Furthermore, since this modulation (i.e., stimulation, suppression) of chondrogenesis by exogenous $TGFß_1$ mimicked the enhancement and suppression of chondrogenesis that occurred when periotic mesenchyme was cultured in the presence of otic epithelium (Frenz and Van De Water, 1991), $TGFß_1$ was implicated as an epithelial-derived regulatory molecule that can either stimulate chondrogenesis to promote otic capsule growth (E12) or selectively inhibit this process to permit perilymphatic space formation and capsular modeling (E14) (Frenz *et al.,* 1992). Similar effects were evoked in cultured periotic mesenchyme in response to $TGFß_2$.

BMP2, a member of the TGFß superfamily, also elicited modulatory effects on otic capsule chondrogenesis. However, the

periotic mesenchymal response to BMP2 differed from that evoked in response to either TGFß$_1$ or TGFß$_2$, suggesting distinct roles for different members of the TGF superfamily in mediating otic capsule chondrogenesis. Although exogenous BMP2 (1-10 ng/ml) could produce a stimulation of chondrogenesis in cultured E12 periotic mesenchyme, unlike TGFß, BMP2 did not evoke an inhibitory response in periotic mesenchyme of embryonic age E14 days (Frenz *et al.,* 1996a). Similar results were obtained when E12 and E14 periotic mesenchyme were cultured in the presence of the closely related molecule BMP4. In accord with these findings, in cultured chick limb bud mesenchyme, TGFß and BMP2 act by distinct mechanisms to regulate cartilage differentiation (Chen *et al.,* 1991; Roark and Greer, 1994). Furthermore, distinct expression patterns of TGFß$_1$ and BMP2 in developing mouse whisker follicles (Lyons *et al.,* 1990) and of BMP4, BMP5, and BMP7 in developing auditory and vestibular organs in the chick inner ear (Oh *et al.,* 1996) support unique roles for these related molecules in morphogenesis and pattern formation in the vertebrate embryo. Besides embryonic development, TGFß$_1$, BMP2, and BMP2, 4 are expressed in specimens of adult human otosclerotic bone consisting of the stapedial footplate (Figure 5), suggesting a role for these signal molecules in bone turnover and remodeling.

Synergistic interactions between TGFß$_1$ and FGF2 in otic capsule chondrogenesis

The distinct roles that our culture studies suggested for FGF in induction of otic capsule chondrogenesis and for TGFß in modulation of this process prompted us to test if a combination of these factors could evoke a full chondrogenic response comparable to that which occurs in the presence of otic epithelium. To address this, growth factors were added in combination with FGF2 to cultured E10.5 periotic mesenchyme. Neither epidermal growth factor (EGF) (1 ng/ml) nor platelet derived growth factor (PDGF) (1 ng/ml) in combination with FGF2 (1 ng/ml) could potentiate the inductive effects of FGF2 on periotic mesenchyme chondrogenesis. However, simultaneous addition of FGF2 (1 ng/ml) and TGFß$_1$ (1 ng/ml) to cultured E10.5 periotic mesenchyme resulted in a chondrogenic response comparable to that which is evoked by otic epithelium, suggesting synergistic interactions between these growth factors during otic capsule formation (Frenz *et al.,* 1994). This synergistic response could be blocked by treatment of cultured periotic mesenchyme + otic epithelium with a combination of anti-TGFß$_1$ and anti-FGF2 antibodies, resulting in an inhibition of mesenchymal condensation formation and an inhibition of

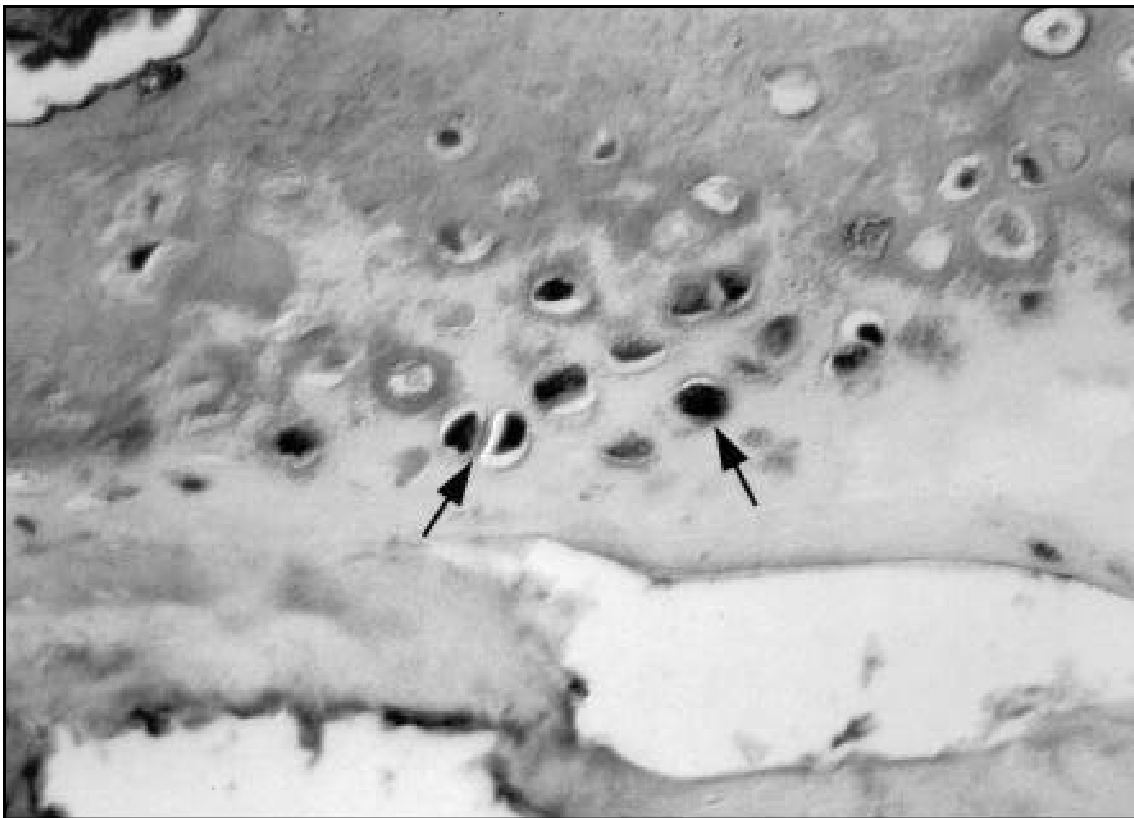

Figure 5: Specimen of human otosclerotic bone consisting of the stapedial footplate, immunostained for BMP2,4. Osteoblast cells rich in BMP2,4 (arrows) are present in regions of new bone formation.

both S-GAG and cartilage-specific type II collagen accumulation. Current studies are investigating the effects of growth factor combinations comprised of other FGF and TGFß isoforms, as well as BMPs, on otic capsule chondrogenesis.

Treatment with all-trans retinoic acid decreases levels of TGFß$_1$ in the developing otic capsule

In utero exposure of the embryonic mouse to teratogenic levels of all-trans retinoic acid (atRA) at a critical period of development produced defects in the developing inner ear and its surrounding cartilaginous capsule (Frenz *et al.,* 1996b). Correspondingly, when cultures of mouse periotic mesenchyme + otic epithelium were treated with high doses of atRA (10^{-8}M), an inhibition of otic capsule chondrogenesis was produced (Frenz and Liu, 1997). Since many common congenital malformations occur in tissues in which TGFß appears to have morphogenetic action (Heine *et al.,* 1987), it was hypothesized that the inhibitory effects of *in utero* atRA exposure on otic capsule formation may be directed at TGFß signaling of otic capsule chondrogenesis.

Comparison of immunohistochemical staining patterns for endogenous TGFß$_1$ in atRA exposed and control inner ears demonstrated a marked reduction in TGFß$_1$ in prechondrogenic and condensing periotic mesenchyme following *in utero* exposure to teratogenic levels of atRA (Frenz and Liu, 2000). In accord with these *in vivo* findings, levels of TGFß$_1$ were also markedly reduced in cultured E10.5 periotic mesenchyme + otic epithelium following treatment with 10^{-10}M atRA (Frenz and Liu, 2000). Since supplementation of atRA-treated cultures with excess TGFß$_1$ restored chondrogenesis to an extent comparable to that in untreated control cultures, reduction in levels of this growth factor was suggested to be a contributing factor to atRA-induced chondrogenic inhibition, and was consistent with the abnormal patterns of chondrogenesis that were produced in the developing otic capsule by in utero atRA-exposure (Frenz *et al.,* 1996b). Furthermore, reduced levels of endogenous TGF1 may contribute to the compromised ability of *in utero* atRA-exposed inner ear tissues to mediate otic capsule chondrogenesis when interacted with normal (untreated) inner ear tissues in high-density culture (Frenz and Liu, 1998). The effect of *in utero* atRA exposure on expression levels of other growth factors endogenous to the inner ear, including FGF2, FGF3, and BMP4, is under investigation.

Conclusions

Based on our findings to date, it appears that members of the FGF, TGFß, and BMP families may interact both simultaneously and sequentially to regulate periotic mesenchyme cells through different stages of otic capsule differentiation. Since cells are rarely exposed to one signal at a time, but more typically to a barrage of signals, full understanding of the mechanisms of growth factor control in chondrifying tissues will require an understanding of not only what signal molecules act during the chondrogenic process, but also how these signal molecules interact to coordinate inductive processes and patterns of tissue differentiation. We have identified FGF, TGFß, and BMP in their individual roles as molecular mediators of otic capsule formation, comparable to isolating individual pieces of a large puzzle. The challenge is to now determine how these individual "pieces" fit together, yielding a greater understanding of their interrelatedness and insight into the complex processes that initiate and guide the development of the capsule of the mammalian inner ear. Furthermore, advances in our understanding of the functional role of signal molecules in normal otic capsule development will provide insights into the likely influence of these factors in the abnormal chondrification and bone remodeling of otosclerosis. In patients with otosclerosis, lesions to the otic capsule are produced as a result of inappropriate chondrification and bone remodeling (Jiang and Yi, 1994). Since chondrification of the otic capsule during development is dependent on the integrated action of signal molecules (e.g. FGF, TGFß, BMP), the abnormal remodeling and overgrowth of the otic capsule in otosclerotic patients may reflect mechanisms in which the action of these signal molecules is recapitulated.

Acknowledgments

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