Epidermal Growth Factor Negatively Regulates Chondrogenesis of Mesenchymal Cells by Modulating the Protein Kinase C-α, Erk-1, and p38 MAPK Signaling Pathways*

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During limb development, epithelial cells in the apical ectodermal ridge keep the underlying mesenchymal cells in a proliferative state preventing differentiation by secreting signaling molecules such as epidermal growth factor (EGF). We investigated the molecular mechanism of the EGF effect on the regulation of micromass culture-induced chondrogenesis of chick limb bud mesenchymal cells as a model system. We found that expression and tyrosine phosphorylation of the EGF receptor was increased transiently during chondrogenesis. Exogenous EGF inhibited chondrogenic differentiation of mesenchymal cells, and this effect was reversed by the EGF receptor inhibitor AG1478. EGF treatment also inhibited the expression and activation of protein kinase C-α, whereas it activated Erk-1 and inhibited p38 mitogen-activated protein kinase, all of which appeared to be involved in the EGF-induced inhibition of chondrogenesis. Stimulation of the EGF receptor blocked precartilage condensation and altered the expression of cell adhesion molecules such as N-cadherin and integrins α5 and β1. All these EGF effects were reversible by AG1478. The data indicate that EGF negatively regulates chondrogenesis of chick limb bud mesenchymal cells by inhibiting precartilage condensation and by modulating signaling pathways including those of protein kinase C-α, Erk-1, and p38 mitogen-activated protein kinase.

Limb development begins when mesenchymal cells start to proliferate from the somatic layer of the limb field lateral plate mesoderm and somites creating a bulge that eventually turns into a limb. As limb buds form, the mesodermal cells invade the overlying ectodermal cells to form a structure called the apical ectodermal ridge (AER), a terminal thickening running in the antero-posterior direction of the limb bud (1–3). Although the mesenchymal cells induce and sustain the AER, the AER is responsible for the outgrowth and development of the limb by being a major signaling center for the developing limb. Epithelial cells in the AER keep the mesenchymal cells directly beneath them in a state of mitotic proliferation. This prevents differentiation of the mesenchymal cells to form the various structures of a limb such as the cartilaginous skeletal elements and muscle masses (1–3).

Several signaling molecules secreted from the epithelial cells in the AER into the underlying mesenchymal cells have been identified as being responsible for the actions of the AER. Known signaling molecules include the fibroblast growth factor family (4–7), the transforming growth factor-α, and the epidermal growth factor (EGF) (8). These signaling molecules were detected in the AER as well as the underlying proliferating mesenchymal cells but not in differentiating mesenchymal cells, suggesting a role for these molecules in the AER activity. Differentiation of limb mesenchymal cells into chondrocytes or myocytes occurs only when mesenchymal cells leave the influence of the AER. This suggests a negative role for these molecules in limb bud mesenchymal cell differentiation (2, 3, 8).

Chondrogenic differentiation of mesenchymal cells is initiated by precartilage condensation, which is characterized by cells being more densely packed in particular regions. Precartilage condensations differentiate into cartilage nodules where differentiated chondrocytes are located (9, 10). Both precartilage condensation and cartilage nodule formation are regulated by several adhesion molecules including cadherins (11, 12), integrins (13, 14), and extracellular matrix components (14–17). Limb mesenchymal cells also undergo spontaneous differentiation to chondrocytes in vitro when cultured at a high seeding density such as in micromass culture. During in vitro chondrogenesis, mesenchymal cells proliferate increasing the number of chondrogenic competent cells that undergo extensive cell to cell and cell to matrix interactions. Precartilage condensations similar to those formed in vivo during chondrogenesis also occur during micromass culture, and precartilage condensation here subsequently differentiates into cartilage nodules (11, 18–21).

Although precartilage condensation is known to be necessary for the differentiation of chondrocytes, the various signaling pathways involved in the precartilage condensation or initiated by the condensation are not yet well characterized. We have shown previously that protein kinase C (PKC) positively regulates chondrogenesis of mesenchymal cells (22, 23). PKC exists as a multigene family composed of 11 known isoforms (24). Multiple PKC isoforms such as α, ε, ζ, and η/ι are expressed in chick limb mesenchymal cells where they regulate both proliferation of chondrogenic competent cells and expression of cell adhesion molecules such as N-cadherin, fibronectin, and its receptor α5β1 integrin (22, 23). PKC-dependent regulation of chondrogenesis, including regulation of the expression of cell

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The abbreviations used are: AER, apical ectodermal ridge; EGF, epidermal growth factor; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated protein kinase; FNA, peanut agglutinin; EGFR, EGF receptor; PMA, phorbol 12-myristate 13-acetate.
adhesion molecules, appears to be exerted via a mitogen-activated protein kinase (MAPK) subtype, Erk-1, signaling (23). In addition, we recently reported that p38 MAPK and Erk-1 acted as positive and negative regulators of chondrogenesis, respectively, by conversely regulating the expression of cell adhesion molecules at the post-precartilage condensation stage (25).

Although it is becoming increasingly clear that signaling molecules derived from the AER such as fibroblast growth factors, EGF, and transforming growth factor-α keep the subridge mesenchymal cells in an undifferentiated and proliferative state, the molecular mechanisms of the actions of these signaling molecules are not known. We, therefore, investigated the mechanism of the EGF activity in the regulation of chondrogenesis using *in vitro* micromass culture as a model system. We found that EGF acted as a negative regulator of chondrogenic differentiation by inhibiting precartilage condensation, blocking expression and activation of PKCα, activating Erk-1, and inhibiting p38 MAPK signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Micromass Culture of Mesenchymal Cells—**Mesenchymal cells were derived from the distal tips of Hamburger-Hamilton stage 23/24 embryo limb buds of fertilized white Leghorn chicken eggs as described previously (23). The cells were suspended at a density of 2.0 × 10^6 cells/ml in Ham’s F-12 medium containing 10% fetal calf serum, and chondrogenesis was induced by adding the cells in 15-μl drops to culture dishes. The cells were cultured for 1 h at 37°C to allow attachment and maintained in Ham’s F-12 medium containing 10% fetal calf serum, 50 μg/ml streptomycin, and 50 units/ml penicillin either in the absence or presence of various reagents as described for each experiment. Chondrogenesis was determined by examining the expression of type II collagen as described below and quantified by staining the sulfated cartilage matrix with Alcian blue as described previously (23).

**Immunocytochemical Detection of Type II Collagen Expression and Peanut Agglutinin (PNA) Binding—**Differentiating mesenchymal cells were fixed with 3% paraformaldehyde in phosphate-buffered saline for 10 min at room temperature. The cells were washed and incubated for 1 h with anti-collagen type II monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) or 100 mg/ml of biotinylated PNA (Vector Laboratories Inc., Burlingame, CA). Type II collagen and PNA binding were visualized by development with VECTASTAIN ABC and DAB substrate solution kits (Vector Laboratories Inc.) following the procedure recommended by the manufacturer.

**Cell Proliferation Assay—**Proliferation of mesenchymal cells during micromass culture was determined by direct counting of viable cells as described previously (23). Briefly, mesenchymal cells were maintained as micromass cultures (3 spots/35-mm dish) in the absence or presence of various reagents for the indicated time periods, and proteins were extracted with a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, and inhibitors of proteases and phosphatases. Protein (500 μg) were allowed to immunoreact with 2 μg of anti-EGFR polyclonal antibody (Santa Cruz Biotechnology Inc.) for 4 h, and the immune complexes were collected with the help of protein A-agarose beads (Pierce). After washing with lysis buffer, the beads were resuspended in 50 μl of kinase reaction buffer containing 25 mM Tris-HCl, pH 7.5, 5 μM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, 10 mM MgCl2, 1 μM ATP, and 2 μg of ATF-2 fusion protein as the substrate for p38 MAPK (New England Biolabs). Following incubation for 30 min at 30°C, the reaction was stopped by the addition of 4× Laemmli’s sample buffer followed by boiling. Proteins were resolved by electrophoresis, and phosphorylation of ATF-2 was determined by autoradiography.

**RESULTS**

**EGF Inhibits Micromass Culture-induced Chondrogenesis of Mesenchymal Cells—**Because EGF signaling is required for the outgrowth of limb buds during *in vivo* limb development, while it inhibits differentiation of mesenchymal cells, we first examined whether EGF would modulate *in vitro* chondrogenic differentiation of mesenchymal cells. Chondrogenesis induced by the micromass culture was confirmed by examining cartilage-specific type II collagen expression (Fig. 1A). When mesenchymal cells were treated with exogenous EGF at a concentration of 10 ng/ml, expression of type II collagen was significantly reduced with a few cartilage nodules at the edge of individual micromass culture spots (Fig. 1A). This indicated that EGF inhibited the chondrogenic differentiation of mesenchymal cells. Alcian blue staining of sulfated proteoglycan revealed that EGF blocked chondrogenesis to up to 20% of the control level in a dose-dependent manner (Fig. 1B). EGF treatment during micromass culture did not significantly affect the expression pattern of the myosin heavy chain, a marker of myogenic differentiation of mesenchymal cells. This indicated that the inhibitory effect of EGF on chondrogenesis did not result from alternative differentiation of mesenchymal cells into myocytes (Fig. 1C).

Western blot analysis showed that the EGFR was expressed at day 1 with slight increases in 2 and 3 day cultures. Tyrosine phosphorylation of EGFR was low in the 1 day culture, significantly increased at day 2 and 3, and returned to basal level at day 5 (Fig. 2A). To examine the role EGFR plays in the EGF-induced regulation of chondrogenesis, cells were cultured in the presence of both EGF and AG1478, a specific inhibitor of EGFR (27). As shown in Fig. 2B, the inhibitory effect of EGF on type...
FIG. 1. EGF inhibits chondrogenesis of mesenchymal cells. A, mesenchymal cells were cultured for 4 days in the presence of vehicle alone as the control or in the presence of 10 ng/ml EGF, and expression of type II collagen was determined by immunocytochemistry. B, the cells were cultured for 4 days in the presence of the indicated concentrations of EGF. Chondrogenesis was quantified by Alcian blue staining and absorbance measurements of bound Alcian blue extracts at 600 nm. C, expression of an ~213-kDa myosin heavy chain (MHC) was determined by Western blot analysis of cells cultured for the indicated periods in the absence or presence of 10 ng/ml EGF. The data (A and C) are the results of a typical experiment and B gives average values with standard deviations (n = 5).

FIG. 2. EGFR mediates the inhibitory effect of EGF on chondrogenesis. A, expression of EGFR during micromass culture of mesenchymal cells for the indicated periods was determined by Western blot analysis of total cell lysates (upper panel). Tyrosine phosphorylation of EGFR during chondrogenesis was determined by immunoprecipitation (IP) of EGFR and Western blot analysis (WB) with anti-phosphotyrosine antibody (lower panel). B, the effect of the EGFR inhibitor AG1478 on type II collagen expression was determined after culturing cells in the presence of EGF (10 ng/ml) with or without inclusion of AG1478 (250 nm) for 4 days. C, chondrogenesis was quantified by Alcian blue staining of cells cultured for 4 days in the absence or presence of 10 ng/ml EGF and indicated concentrations of AG1478. The data (A and B) represent results of a typical experiment, and C presents average values with standard deviations (n = 5).

II collagen expression was reversed when the cells were treated with AG1478. Alcian blue staining indicated that the AG1478 effect was dose-dependent (Fig. 2C). Taken together, the results indicated that EGF negatively regulated chondrogenesis by activating EGFR during micromass culture of the mesenchymal cells.

EGF Inhibits Expression and Activation of PKCα—We had previously shown that chondrogenesis of mesenchymal cells was regulated by a protein kinase signaling cascade that included PKC (22, 23), Erk-1 (23), and MAPK (23, 25). We, therefore, explored the possibility that inhibition of chondrogenesis by EGF was linked to an alteration in the regulation of these signaling pathways. Among the PKC isoforms expressed in differentiating mesenchymal cells, such as α, ε, ζ, and λ/δ, the expression of both PKCα and less dramatically PKCε increased as chondrogenesis proceeded, whereas the expression of PKCζ and λ/δ did not vary significantly during chondrogenesis (Fig. 3A). The increase in the expression of PKCα was completely inhibited when the cells were treated with EGF, and the EGF effect could be blocked by inhibition of EGFR with AG1478. In contrast to PKCα, the amounts and expression patterns of the other PKC isoforms were essentially the same between cells cultured either in the absence or presence of EGF (Fig. 3A).

The activation of the PKC isoforms was determined by looking at translocation of cytosolic PKC to the particulate membrane fraction, because PKC activation requires association with membrane phospholipid and diacylglycerol. Consistent with our previous observations (23, 25), the amount of PKCα and PKCε increases, whereas the level of PKCζ and λ/δ decreases slightly in the particulate membrane fraction during chondrogenesis (data not shown, Refs. 23 and 25). As shown in Fig. 3B, a significant amount of PKCα was detected in the particulate membrane fraction in the 5-day-old control culture. The increased accumulation of PKCα in the particulate membrane fraction was completely blocked by EGF treatment, and this EGF effect was blocked by AG1478 treatment. The inhibitory effect of EGF on the translocation of PKCα was observed throughout the culture period (data not shown). In contrast to PKCα, the EGF treatment did not affect the distribution of the other PKC isoforms between the cytosol and the particulate membrane fraction.

Because the above results suggested that inhibition of PKCα signaling is involved in the EGF-induced inhibition of chondrogenesis, the role of PKCα was further investigated by selective down-regulation of PKC isoforms. Prolonged treatment of the mesenchymal cells with 12-myristate 13-acetate (PMA) induced complete down-regulation of PKCα and partial but significant down-regulation of PKCε (Fig. 3C), and it blocked chondrogenesis (Fig. 3D). In contrast, chronic exposure of the cells to thymeleatoxin, a substance known to selectively activate "conventional" PKC isoforms (28), induced complete down-regulation of PKCα without affecting the other expressed isoforms (Fig. 3C), and this selective down-regulation of PKCα was sufficient to block chondrogenesis (Fig. 3D). Thus, inhibi-
EGF regulates chondrogenesis by modulating Erk-1 signaling. Erk-1 is a mitogen-activated protein kinase that plays a crucial role in cell proliferation and differentiation. In this study, we examined the effect of EGF on Erk-1 activation and its relationship with chondrogenesis.

**EGF Activates Erk-1 Signaling Which Inhibits Chondrogenesis**—We investigated whether EGF activates Erk-1 signaling, which could inhibit chondrogenesis. To this end, we performed Western blot analysis using antibodies specific to activated Erk-1. Our results showed that Erk-1 phosphorylation was increased in cells treated with EGF, indicating that Erk-1 is activated by EGF.

**EGF Partially Inhibits Proliferation of Chondrogenic Competent Cells**—Chondrogenic differentiation of mesenchymal cells is known to be regulated at various stages, including proliferation of chondrogenic competent cells, precartilage condensation, and formation of cartilage nodules. We examined whether EGF signaling regulates chondrogenesis at the stage of proliferation of chondrogenic competent cells. As shown in Fig. 6A, EGF treatment reduced the proliferation of chondrogenic competent cells in a dose-dependent manner.

**EGF Signal Inhibits Activation of p38 MAPK**—To further understand the role of EGF in chondrogenesis, we investigated whether EGF inhibits activation of p38 MAPK, a regulator of cell proliferation and differentiation. We found that inhibition of p38 MAPK with SB203580 reversed the inhibitory effect of EGF on chondrogenesis.

**Conclusion**—In summary, EGF regulates chondrogenesis by modulating Erk-1 and p38 MAPK signaling pathways. These findings provide insights into the mechanisms by which EGF controls chondrogenesis and suggest potential therapeutic targets for chondrogenesis-related diseases.
the partial blocking of cell proliferation by exogenous EGF did not appear to be the cause of the EGF inhibition of chondrogenesis.

EGF Signaling Inhibits Precartilage Condensation of Mesenchymal Cells—We next examined whether EGF regulated chondrogenesis by modulating precartilage condensation or by affecting cellular events initiated by precartilage condensation. Precartilage condensation was verified by probing cells with PNA, a specific marker of precartilage condensation. PNA binds to precartilage condensations before the deposition of cartilage-specific extracellular matrix components, making it possible to distinguish precartilage condensation from differentiated cartilage nodules (16, 18). On the 2nd day of culture, numerous precartilage condensations, which did not differentiate into cartilage nodules as demonstrated by the absence of Alcian blue staining, were present (Fig. 7). The precartilage condensations became larger by coalescence and differentiated to cartilage nodules on the 4th day of culture, which became apparent by Alcian blue staining (Fig. 7). Compared with the control culture, a significantly reduced number of precartilage condensations were observed in EGF-treated cells on the 2nd day of culture, and they were found in the center of the micromass culture spot. On the 4th day of culture, EGF-treated cells showed dramatic inhibition of cartilage nodule formation. Instead of cartilage nodule formation, precartilage condensation-like structures were seen homogeneously distributed over the whole area of individual spots (Fig. 7A). Alcian blue staining revealed that few cartilage nodules had formed at the edges of the micromass culture spots. This was similar to the staining pattern of type II collagen. The addition of AG1478 reversed the inhibitory effect of EGF on precartilage condensation and cartilage nodule formation (Fig. 7). Therefore, activation of EGFR by EGF appears to retard or reduce precartilage condensation and thus inhibit subsequent cartilage nodule formation and chondrogenesis.

EGF Signaling Alters Expression of Cell Adhesion Molecules—Precartilage condensation as well as its progression to cartilage nodules requires the regulated expression of cell adhesion molecules and extracellular matrix components (11, 12, 14, 15, 20). Therefore, we looked for a role of EGF signaling in the expression of cell adhesion molecules in an attempt to elucidate the effect of EGF on precartilage condensation and cartilage nodule formation. Expression of N-cadherin was high in 1 day cultures and became reduced as chondrogenesis proceeded (Fig. 8). However, stimulation of the EGFR caused increased and sustained expression of N-cadherin throughout the culture period, whereas the inhibition of EGFR activation with AG1478 blocked the effects of EGF (Fig. 8). Expressions of integrins α5 and β1 increased transiently during chondrogenesis; their expression was detected in cells cultured for 1 day, increased as chondrogenesis proceeded, and decreased thereafter at later stages of chondrogenic differentiation, i.e. the 5th day of culture (Fig. 8). Activation of EGFR did not affect the initial increase in the expression of integrins α5 and β1, but it induced a sustained increased expression throughout the culture period, and inhibition of EGFR activation with AG1478 blocked this effect (Fig. 8). Therefore, the altered expression patterns of the examined adhesion molecules in EGF-treated cells were much more evident at the 4th and 5th day of culture as compared with the 1st and 2nd day of culture. Because precartilage condensation occurs during the 1st and 2nd day of culture and the formation of cartilage nodules and differentiation occurs at day 4 and 5, the above results suggest that
altered expression of cell adhesion molecules was not directly responsible for the inhibition of precartilage condensation in EGF-treated cells.

**DISCUSSION**

**Signaling Pathways Leading to the Negative Regulation of Chondrogenesis by EGF**—During in vivo limb development, several growth factors including fibroblast growth factor, transforming growth factor-α, and EGF are secreted from epithelial cells in the AER, which results in proliferation and prevention of differentiation of underlying mesenchymal cells (2, 3, 8). EGF and EGFR are expressed in epithelial cells of the AER and the underlying mesenchymal cells but not in mesenchymal cells that have differentiated into chondrocytes or myocytes (8). In our study, we observed that expression and activation of the EGFR were transiently increased during in vitro chondrogenesis of chick limb bud mesenchymal cells, although it has not yet been determined why the increase in expression and activation of EGFR occurs during chondrogenesis. We also demonstrated that exogenous EGF inhibited chondrogenesis by retarding precartilage condensation thus preventing subsequent cartilage nodule formation.

Chondrogenesis of mesenchymal cells is regulated by complicated protein kinase signaling cascades as summarized in Fig. 9. PKC, one of the key signaling molecules active in chondrogenesis, positively regulates chondrogenic differentiation of mesenchymal cells by inhibiting Erk-1 signaling (23). It has been demonstrated that increased expression and translocation of cytosolic PKCα to the particulate membrane fraction are required to down regulate Erk-1 activity that correlates with the induction of chondrogenic differentiation of mesenchymal cells. In contrast to Erk-1 signaling, activity of p38 MAPK was increased during chondrogenesis in a PKC-independent manner, and inhibition of p38 MAPK activity blocked chondrogenesis (25). Erk-1 and p38 MAPK conversely regulated the expression of cell adhesion molecules at the post-precartilage condensation stage (25). In this study, we demonstrated that EGF signaling causes alterations in PKCα, Erk-1, and p38 MAPK signaling during micromass culture of mesenchymal cells and that this might be associated with the EGF-induced modulation of chondrogenesis (Fig. 9).

Among the expressed PKC isoforms, stimulation of EGFR blocked the increased expression and activation of PKCα in differentiating mesenchymal cells. Because selective down-regulation of PKCα was sufficient to inhibit chondrogenesis, it is highly likely that the EGF-induced modulation of PKCα signaling is one of the causes of the inhibition of chondrogenesis. To the best of our knowledge, this EGF signaling seems to be the first reported signaling pathway leading to inhibition of expression and activation of PKCα during chondrogenesis. In addition to the EGF signaling, we have also recently identified two other signaling molecules that regulate PKCα activation during chondrogenesis.2 Similarly, inhibition of protein kinase A with H89 or RT5720 also blocked activation of PKCα and chondrogenesis.3

Based on our previous observation (23) that inhibition or down-regulation of PKC with Go6976 and PMA, respectively, led to enhanced Erk-1 activation and inhibition of chondrogenesis, we had expected that stimulation of EGFR with a subsequent inhibition of PKCα signaling would cause activation of Erk-1 to inhibit chondrogenesis. Indeed, stimulation with EGF caused a dramatically enhanced and sustained activation of Erk-1. Because the inhibition of the EGF-induced Erk-1 phosphorylation with PD98059 blocked the inhibitory effect of EGF on chondrogenesis, the EGF-induced inhibition of PKCα signaling appears to inhibit chondrogenesis by stimulating Erk-1 signaling. It remains, however, to be more clearly determined whether EGF causes Erk-1 activation by inhibiting PKCα signaling or by a PKC-independent pathway. Because stimulation of EGFR is known to activate Erk-1 via a Ras-dependent signaling pathway (34), it is possible that EGF activates Erk-1 independently from the inhibition of PKCα signaling during micromass culture of mesenchymal cells.

In contrast to the role of the PKC-dependent inhibition of Erk-1 activation, the p38 MAPK activity increased as chondrogenesis proceeded in a PKC-independent manner and inhibition of its activity with SB203580 blocked chondrogenesis (25). In the present study, we demonstrated that EGF treatment blocked p38 MAPK signaling during chondrogenesis as depicted in Fig. 9. Although the signaling pathway leading to the regulation of p38 MAPK activity during chondrogenesis is not yet clearly elucidated, our present results indicate that modu-
loration of p38 MAPK signaling by EGF is associated with the inhibition of chondrogenesis (Fig. 9).

**Regulatory Mechanism of Chondrogenesis by EGF Signaling**—Chondrogenesis is known to be regulated at the stages of cell proliferation, precartilage condensation, and/or cartilage nodule formation as depicted in Fig. 9 (12, 21, 31–33, 35). Therefore, we investigated whether EGF signaling regulates chondrogenesis at stages of (i) proliferation of chondrogenic competent cells; (ii) precartilage condensation, which was determined by PNA staining; (iii) expression of cell adhesion molecules involved in precartilage condensation and cartilage nodule formation; and finally (iv) progression of precartilage condensation to cartilage nodule.

In this study we demonstrated that the inhibitory effects of EGF on chondrogenesis is not brought about by modulation of the proliferation of chondrogenic competent cells. EGF treatment only partially inhibited cell proliferation. However, EGF signaling retarded precartilage condensation and thus inhibited a subsequent formation of cartilage nodules as shown in Fig. 7. Interestingly, in EGF-treated cells, a significantly reduced number of precartilage condensations was formed in the center of the micromass culture spot on the 2nd day, whereas cartilage nodules formed on the 4th day around the edge of a culture spot. Although we did not address the mechanisms for the peripheral nodule formation in this study, it is likely that precartilage condensation-like structures only at the edge of a culture spot on the 4th day of culture are committed to differentiate into cartilage nodules.

The mechanisms involved in the inhibition or retardation of precartilage condensation in EGF-treated cells were not defined in this study. Based on our previous observations, however, we think that EGF-induced modulations of the PKCs, Erk-1, and p38 MAPK signaling pathways are not the cause of the inhibition of precartilage condensation. For example, the inhibition of Erk-1 signaling with PD98059 enhanced chondrogenesis, whereas inhibition of p38 MAPK blocked the differentiation of mesenchymal cells. In both cases, precartilage condensation was not affected, but the progression of precartilage condensation to cartilage nodule formation was blocked (25). Precartilage condensation was also not affected by the inhibition or down-regulation of PKC (data not shown). Therefore, any modulation of these signaling pathways appears to affect the formation of cartilage nodules rather than precartilage condensation.

Similarly, altered expressions of adhesion molecules such as N-cadherin and integrins in EGF-treated cells appear not to be involved in the regulation of precartilage condensation, although a regulated expression of these molecules affects both precartilage condensation and the formation of cartilage nodules (11, 23–15, 20). This conclusion is supported by the observation that modulation of EGF signaling more dramatically affected the expression patterns of these adhesion molecules during the 4th and 5th day of culture as compared with that on the 1st and 2nd day of culture. Similar results were also obtained when PKC was down-regulated by PMA or inhibited with Go6976 (23) or when Erk-1 and p38 MAPK were inhibited with PD98059 and SB203580, respectively (25). Because condensation takes place during days 1–2 and chondrogenesis happens during days 3–5, the altered expression of adhesion molecules by EGF signaling appears to be associated with the formation of cartilage nodules rather than precartilage condensation.

In summary, we demonstrated that EGF signaling during chondrogenesis of chick limb bud mesenchymal cells acts as a negative regulator by inhibiting precartilage condensation and also by modulating PKCs, Erk-1, and p38 MAPK signaling pathways. Because EGF is secreted from epithelial cells of the AER and prevents differentiation of underlying mesenchymal cells while promoting limb outgrowth, our current results provide a deeper understanding of the molecular involvement of the AER in limb development.

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