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BMP-2 Induces the Expression of Activin βA and Follistatin In Vitro

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Abstract Activins are members of the transforming growth factor β (TGF-β) superfamily and have been shown to be multifunctional regulators of development and cell differentiation. Increasing evidence suggests activin βA is involved in skeletal development. Using differential display PCR we have identified activin βA as a gene associated with recombinant human bone morphogenetic protein-2 (rhBMP-2) induced differentiation of a mouse limb bud cell line, MLB13MYC clone 17, from a prechondroblastic to an osteoblastic phenotype. The expression of activin βA peaks at 24 h of rhBMP-2 treatment, before detection of osteocalcin mRNA expression. Cycloheximide treatment inhibits induction of activin βA, indicating a requirement for new protein synthesis. The induction of the mRNA encoding follistatin, an activin binding protein, was also examined. Follistatin mRNA increases within 18 h of rhBMP-2 treatment, as activin βA mRNA increases but before it peaks. Treatment of MLB13MYC clone 17 cells with purified activin βA concomitant with rhBMP-2 does not affect markers of chondrocyte or osteoblast differentiation, nor does treatment with purified activin βA alone. This suggests that activin βA exerts its effect via a paracrine mechanism. In situ hybridization analysis demonstrates that activin βA expression is localized to cells in the developing interphalangeal joints of embryonic mouse limbs. This is consistent with in vivo induction by BMP-2 which is also expressed in the developing joints. Activin βA, therefore, is downstream from BMP-2 in the cascade of events that result in skeletal development. J. Cell. Biochem. 79:80–88, 2000.

Key words: bone morphogenetic protein; BMP; activin; follistatin; bone

Activins exist as covalently linked heterodimers or homodimers of the two closely related βA and βB chains. First isolated from ovarian fluid, they were named for their ability to stimulate pituitary FSH release in vitro [Vale et al., 1986]. Follistatin, an activin binding protein, was also isolated from gonadal extracts and named for its ability to inhibit pituitary FSH release in vitro [Ueno et al., 1987]. In addition to regulation of the reproductive endocrine system, activins and follistatin have been implicated in the regulation of a variety of processes related to development, differentiation, and growth, including skeletal development.

Mesenchymal cells give rise to osteoblasts, chondrocytes, adipocytes, and myocytes. Commitment to one of these lineages is determined, in part, by properties of the local cellular milieu. The bone morphogenetic proteins (BMPs) and other TGF-β superfamily members have been shown to act as paracrine/autocrine signaling molecules in bone and limb development. BMPs can induce ectopic bone formation when injected subcutaneously or intramuscularly into rats [Urist, 1965], mimicking the normal developmental events of endochondral bone formation. Many members of the BMP subfamily are expressed during skeletal development [Chang et al., 1994; Helder et al., 1995; Macias et al., 1997; Vortkamp et al., 1996]. Disruption of BMP genes results in skeletal abnormalities in mice and in humans [Dudley et al., 1995; Hoffman et al., 1996; Kingsley et al., 1992; Storm et al., 1994; Thomas et al.,

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1997]. BMPs induce the expression of markers of osteoblast differentiation, including the PTH/PTHrP receptor, alkaline phosphatase, and osteocalcin, in a variety of immortalized cell lines [Rosen et al., 1994; Yamaguchi et al., 1991].

To identify other regulatory factors involved in endochondral bone formation, we used differential display PCR and an in vitro model of differentiation. The MLB13MYC clone 17 cell line was derived from 13-day post-coitus mouse limb buds immortalized with the v-myc oncogene. This prechondroblastic cell line responds to treatment with rhBMP-2 by decreasing expression of cartilage markers and increasing expression of markers of osteoblastic differentiation including osteocalcin [Rosen et al., 1994]. The ability of BMPs to induce osteoblast differentiation in vitro mimics their in vivo developmental role in endochondral bone formation. Therefore, dissection of the molecular events involved in rhBMP-2 induction of differentiation in MLB13MYC clone 17 cells will provide insight into this complex developmental process.

MATERIALS AND METHODS

Cell Culture

The MLB13MYC clone 17 cells and rhBMP-2 were kindly provided by Dr Vicki Rosen (Genetics Institute, Cambridge, MA). The cells were maintained in DMEM supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin/streptomycin. At confluence, cells were treated with 200 ng/ml rhBMP-2 in DMEM with 1% heat inactivated fetal bovine serum and 1% penicillin/streptomycin, adding fresh media and rhBMP-2 daily as previously described [Rosen et al., 1994].

Differential Display PCR

The RNAImage Kit from GeneHunter was used for RT-PCR of RNA isolated from untreated and rhBMP-2 treated MLB13MYC clone 17 cells. The radioactive products were resolved by electrophoresis on a urea denaturing 6% polyacrylamide gel. After overnight autoradiography, differentially expressed bands were identified by comparison of the products obtained using RNA from the untreated and rhBMP-2 treated cells. Products found to be reproducibly differentially expressed were reamplified and subcloned. DNA sequencing was performed using a Sequenase Kit (United States Biochemical Corporation, Lake Placid, NY).

Northern Analysis

Total RNA was isolated from cultured cells using Trizol Reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. Electrophoresis of 10 μg total RNA was performed in formaldehyde agarose gels. The RNA was transferred to nylon membranes (Biotrans, ICN, Irvine, CA) by capillary action, and baked in a vacuum oven. Probes were synthesized using hexamer as random primer and $\alpha(^{32}P)$dATP. Hybridization was performed with QuikHyb Solution (GIBCO BRL). After washing, the blot was exposed to X-ray film at −80°C for 1 to 7 days. Density of bands was measured using a densitometer (E-C Apparatus Corp.) linked to an integrator (Hewlett-Packard, Corvallis, OR). Plasmids containing the cDNA for rat activin βA and rat follistatin were kindly provided by Dr Kelly Mayo (Northwestern University). Blots were also probed for osteocalcin and tubulin.

In Situ Hybridization

Hind limbs from day 15.5 post-coital mouse embryos were fixed in 10% buffered formalin followed by dehydration and paraffin embedding. Serial sections 6 μm thick were deparaffinized in xylenes. Following rehydration through incubation in graded ethanol concentrations and phosphate buffered saline, the sections were treated with proteinase K (10 μg/ml). The sections were then fixed in 4% paraformaldehyde. RNAse-free DNAse was used to digest DNA. Finally the sections were dehydrated through incubation in graded ethanol concentrations and air dried. A 300 bp DNA fragment from the coding region of the mouse activin βA (amino acids 309 to 399) was generated by reverse transcription PCR from RNA isolated from MLB13MYC clone 17 cells after rhBMP-2 treatment. The vector was linearized and run off transcription was carried out in the presence of ^{35}S-UTP to generate a labeled riboprobe. Following overnight hybridization at 55°C, the sections were washed and treated with RNAse. The sections were dipped in photoemulsion (Kodak, Rochester, NY) and exposed at 4°C for 4 weeks. The slides were developed and counter stained with hematoxylin and eosin. Bright-field and dark-field images

were obtained. Serial sections were also probed for Type II and Type X collagen.

Alkaline Phosphatase Assay

The alkaline phosphatase activity in cell lysates was quantified at 37°C in assay buffer containing 0.75 M 2-amino-2methyl-1-propanol, pH 10.3 using p-nitrophenylphosphate as substrate. The release of p-nitrophenol was monitored by measuring absorbance at 410 nm [Puzas and Band, 1985].

Statistical Analysis

All values are expressed as mean ± SEM. Analysis of variance was used to evaluate differences between the treated and untreated samples.

RESULTS

Confluent MLB13MYC clone 17 cells were induced to differentiate by treatment with 200 ng/ml of rhBMP-2 every 24 h for up to 72 h. This concentration and duration of treatment is sufficient for these cells to acquire markers of osteoblast differentiation, including expression of the osteocalcin gene. RNA isolated from rhBMP-2 treated and untreated cells was used for differential display PCR. After overnight autoradiography, bands representing differentially expressed mRNAs were identified (Fig. 1). The differentially expressed product designated AAP5 is an 87 base pair product whose sequence is 94% identical to the 3′ untranslated region of human activin βA (Genbank X57580; Fig. 2). Northern analysis confirmed that AAP5 is differentially expressed during rhBMP-2 treatment of MLB13MYC clone 17 cells (Fig. 3). AAP5 hybridized on northern to an mRNA species larger than the 28S rRNA band. A coding region probe for rat activin βA hybridized to an mRNA species of identical size and induction pattern as that observed with AAP5 (data not shown). Therefore, we conclude that AAP5 represents activin βA.

Northern analysis of RNA from MLB13MYC clone 17 cells treated with rhBMP-2 revealed that activin βA message is present at low levels in untreated cells, is markedly induced within the first 24 h of treatment, and declines with longer treatment (Fig. 3). The peak of activin βA expression precedes detectable osteocalcin message by 24 h. To understand the regulation of activin βA during differentiation of

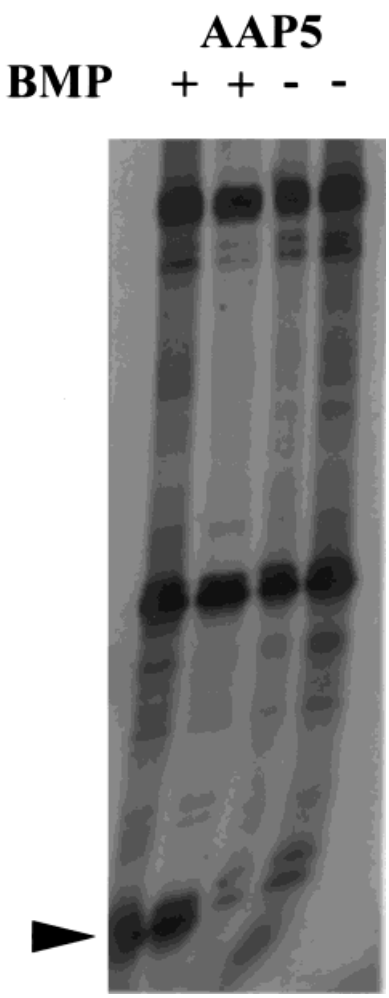

Fig. 1. Differential display of mRNA from MLB13MYC clone 17 cells. Autoradiograph of PCR products generated from mRNA of rhBMP-2-treated cells (+) compared to PCR products generated from mRNA of untreated cells (−) separated by polyacrylamide gel electrophoresis. Duplicate experiments are shown. The arrow marks a product identified as differentially expressed, and designated AAP5.

MLB13MYC clone 17 cells, the expression of follistatin during rhBMP-2 treatment was investigated. Follistatin is an activin binding and inactivating protein that has been shown to regulate the actions of activins in a variety of physiologic systems. On Northern analysis, a rat follistatin cDNA probe hybridized to the appropriately sized RNA species (2.7 kb) and showed peak induction at 18 h of rhBMP-2 treatment (Fig. 3). Follistatin, therefore, is likely to be involved in modulating the function of activin βA in the developing skeleton.

To assess whether induction of activin βA and follistatin expression is a direct effect of BMP

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AAP5          87 AGTAGGCAGTATTCAGCAATGCCCGATAGCTTCTTTGGCCTTATGTTAAATAAAAAGACCTGTTTGGGATGTA
                 ||| | ||||||||||||||| ||| ||||||||||||||||||||||||||||||||||||||||||||||||
Activin βA    28 AGTGGACAGTATTCAGCAACGCCTGATAGCTTCTTTGGCCTTATGTTAAATAAAAAGACCTGTTTGGGATGTA
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Fig. 2. Sequence alignment of AAP5 and 3′ untranslated region of human activin βA (GenBank # X57580). Vertical lines indicate sequence identity.

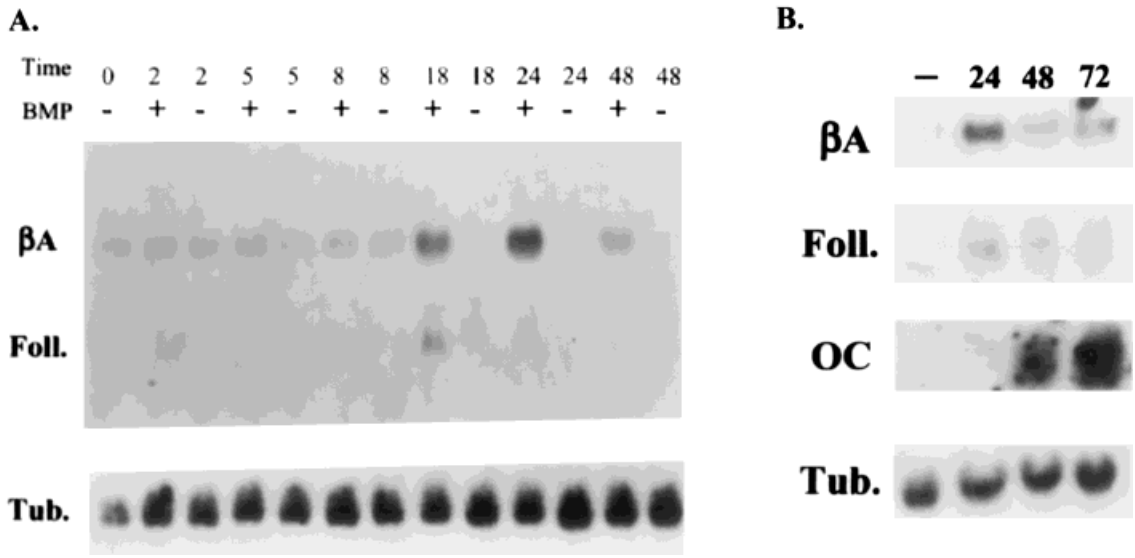

Fig. 3. Time course of activin βA and follistatin expression in MLB13MYC clone 17 cells following treatment with rhBMP-2. RNA from cells was isolated at various times (hours) following treatment with rhBMP-2 treatment. Northern blots were probed with labeled cDNA fragments for activin βA (βA), follistatin (Foll), osteocalcin (OC), and tubulin (Tub), used as control for RNA loading. Experiments were performed three times and a representative autoradiograph is shown. **A**: Autoradiograph of short time course shows peak of follistatin at 18 h and activin βA at 24 h. **B**: Autoradiograph of long time course shows that induction of activin βA and follistatin expression precedes induction of osteocalcin expression. Duration of rhBMP-2 treatment (hours) is indicated above each lane (−) = untreated.

signaling, protein synthesis was inhibited by cycloheximide. MLB13MYC clone 17 cells were treated with cycloheximide (1 μg/ml) 2 h before rhBMP-2 treatment and for the duration of rhBMP-2 treatment. At this concentration of cycloheximide greater than 90% of protein synthesis was inhibited as measured by ^{35}S-methionine incorporation (data not shown). Induction of activin βA expression by rhBMP-2 was inhibited by cycloheximide as determined by Northern analysis (Fig. 4). Therefore, new protein synthesis is required. However, induction of follistatin expression was not reduced by cycloheximide treatment (Fig. 4). The increase in follistatin mRNA by rhBMP-2 is independent of new protein synthesis. Superinduction of follistatin mRNA in the presence of cycloheximide suggests that its transcription is negatively regulated in this cell line, and that the silencer(s) decrease with cycloheximide treatment. Alternatively, cycloheximide may inhibit the degradation of follistatin mRNA.

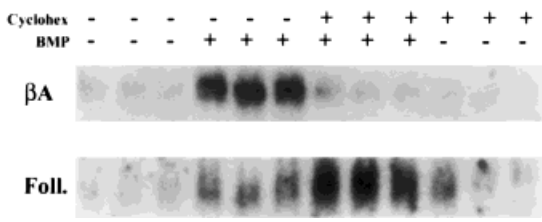

Fig. 4. Effect of cycloheximide treatment on induction of activin βA and follistatin expression. MLB13MYC clone 17 cells were treated (+) or untreated (−) with 200 ng/ml rhBMP-2 (BMP) in the presence (+) or absence (−) of 1 μg/ml cycloheximide (cyclohex) for 24 h. RNA was isolated and Northern blots probed for activin βA (βA) and follistatin (Foll). Autoradiograph of three identical experiments demonstrates that cycloheximide inhibits induction of activin βA expression but does not inhibit induction of follistatin expression.

To learn more about the role of activin βA in osteoblast differentiation, MLB13MYC clone 17 cells were treated with activin βA alone and in combination with rhBMP-2 (200 ng/ml) for 24 to72 h. Purified recombinant activin βA

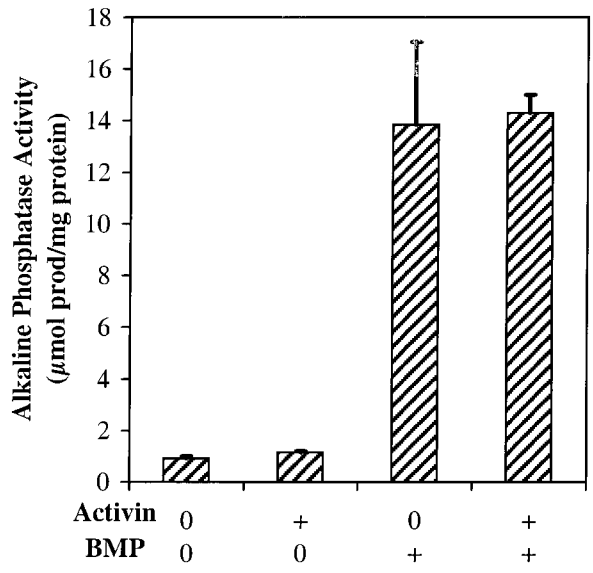

Fig. 5. Alkaline phosphatase activity of MLB13MYC clone 17 cells. Alkaline phosphatase activity was measured in cell lysates treated (+) with activin βA (Activin) 360 ng/ml, rhBMP-2 (BMP) 200 ng/ml, or both, or untreated (0) for 48 h. Data are expressed as mean ± SEM of three experiments. No statistical difference is observed between untreated and activin βA, or between BMP and BMP + activin βA.

(National Hormone and Pituitary Program) was used at concentrations from 0.36 ng/ml to 360 ng/ml. This concentration range includes the linear portion of the in vitro pituitary cell FSH release assay and includes the concentration used by other investigators [Jiang et al., 1993; Luyten et al., 1994]. Northern analysis was then carried out to determine effects of activin βA on expression of osteoblast markers, including osteocalcin and type 1 collagen, and on expression of chondrocyte markers, type II collagen, and cartilage proteoglycan. Activin βA had no demonstrable effect either alone or in combination with rhBMP-2 (data not shown). Assays for alkaline phosphatase activity revealed no significant effect of activin βA treatment on activity induced by rhBMP-2 or in the absence of rhBMP-2 (Fig. 5). These data suggest that, although activin βA expression is induced during endochondral bone formation, it acts as a paracrine rather than autocrine factor.

To examine where activin βA is expressed during skeletal development, we performed in situ hybridization in embryonic mouse limbs. Hind limbs from day 15.5 post-coitus mouse embryos were used because at this stage of limb development nearly every stage of endochondral bone formation is represented in a single limb. A 300 bp cDNA fragment from the coding region of mouse activin βA (amino acids 309 to 399) was generated by reverse transcription PCR from RNA isolated from MLB13MYC clone 17 cells after rhBMP-2 treatment. This region of activin βA has been used by other investigators for in situ hybridization analyses and has been shown to be specific for activin βA [Roberts and Barth, 1994]. As shown in Figure 6, activin βA expression is observed in the cells between the developing phalanges of the paw. No hybridization signal was detected in osteoblasts or in the long bones. Type II collagen expression is seen in the neighboring chondrocytes (Fig. 6). Type X collagen expression, a marker of hypertrophic chondrocytes, is not observed in the paw (Fig. 6) but is detected in the long bones (not shown) at this stage of limb development. Therefore, activin βA is produced by cells in the interphalangeal region that do not express type II collagen. BMP-2 expression has also been localized to the articular interspaces during chick limb development [Macias et al., 1997], suggesting it induces activin βA in vivo also.

DISCUSSION

Although BMPs regulate the early commitment of mesenchymal cells to the chondrocyte and osteoblast lineage, other members of the TGF-β superfamily may affect the subsequent differentiation of committed mesenchymal cells. Our data is the first to demonstrate that activin βA expression is induced by rhBMP-2 in an in vitro model of endochondral bone formation. The peak of activin expression is after 24 h of rhBMP-2 treatment and before detectable osteocalcin expression. The requirement for new protein synthesis indicates that induction of activin βA is not a direct effect of the BMP signaling SMAD pathway. Therefore, activin βA expression is an intermediate step in the coordinated cascade of cytokines and growth factors that act in a paracrine or autocrine manner to regulate skeletal differentiation.

One mechanism by which the function of BMPs and activins are modified is by the presence of antagonists such as noggin, chordin, and follistatin. Follistatin is an activin binding protein. The affinity of activin for follistatin is nearly identical to its affinity for the activin receptors [Mathews and Vale, 1991; Schneyer

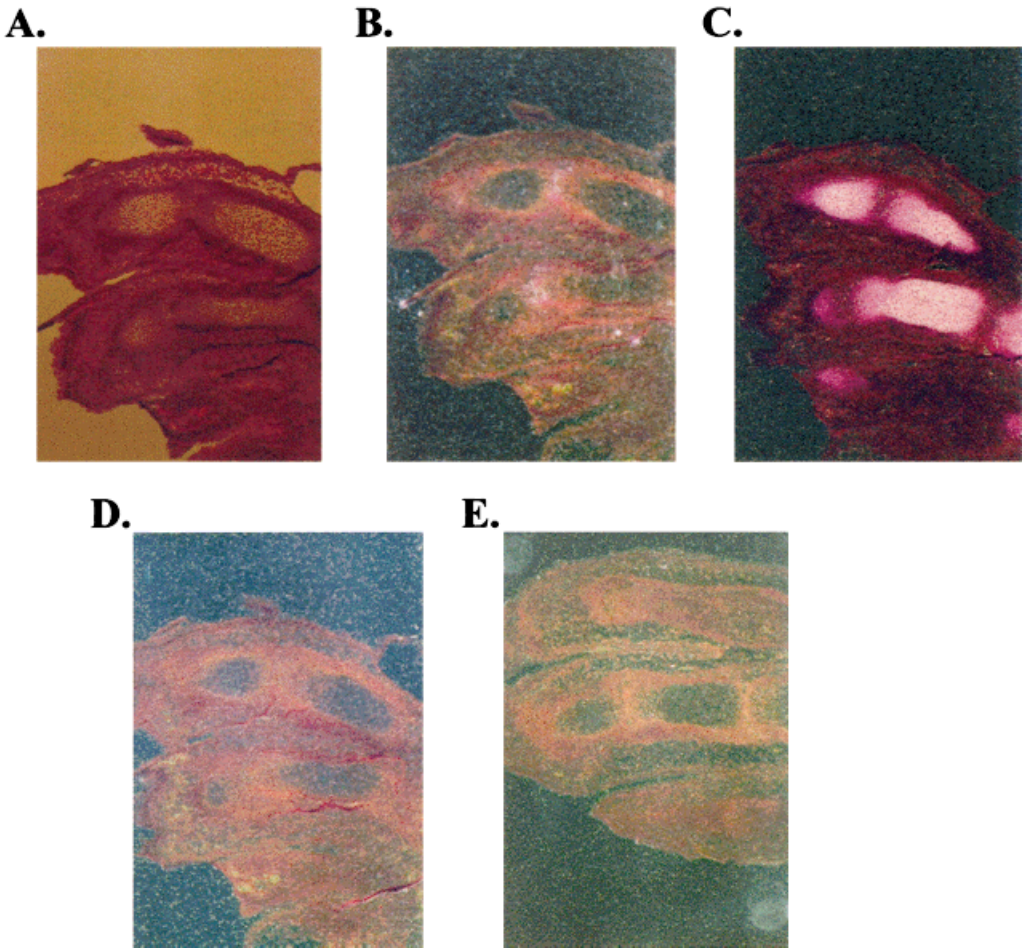

Fig. 6. In situ hybridization of developing mouse paw. Light-field (**A**) and dark-field photographs of day 15.5 mouse paw hybridized with riboprobes for mouse activin βA (**B**, antisense; **D**, sense), type II collagen (**C**), and type X collagen (**E**). Dark-field images reveal signal with antisense activin βA probe is localized to the developing interphalangeal joint space, in cells distinct from those expressing type II collagen. No signal is observed with sense probe, as expected. Hybridizations were performed on sections from three limbs and representative results are shown. Magnification = 10×.

et al., 1994]. Once bound to follistatin activins are unable to interact with their receptors [de Winter et al., 1996; Hashimoto et al., 1997]. To better understand regulation of activin action during skeletogenesis we examined the expression of follistatin in our in vitro model. Follistatin expression is also increased during acquisition of the osteoblastic phenotype in response to rhBMP-2 treatment. Like activin βA, follistatin message peaks at a time when chondrocyte markers are decreasing but still detectable (data not shown) [Rosen et al., 1994] and before detectable osteocalcin message. The coordinated expression of follistatin and activin βA suggests that the function of activin βA is regulated by both induction of its gene expression by rhBMP-2 and modulation of its protein activity by follistatin. Follistatin protein is found in demineralized bone extracts [Funaba et al., 1996], and in osteoblastic MC3T3-E1 cells [Hashimoto et al., 1992]. In vivo, follistatin increased chondrocyte content and decreased mineralization in ectopic bone induced by demineralized bone matrix in rats [Funaba et al., 1996]. Similarly, the decreased osteogenic activity of demineralized bone from aged spontaneously hypercholesterolemic rats is postulated to be due to the higher levels of follistatin present in the bone extract [Funaba et al., 1997]. Follistatin can also bind BMP complexed with its receptor and inhibit BMP action during *Xenopus laevis* development [Ie-

mura et al., 1998]. Therefore, in addition to its role in regulating activin effects, follistatin may be involved in modulating the actions of BMPs by inactivating the BMP-receptor complex. Such a function for follistatin has yet to be investigated in skeletal development.

In the clonal cell line used, we were unable to demonstrate an effect of activin βA on markers of chondrocyte and osteoblast differentiation when administered alone or in combination with rhBMP-2. We observed no effect on alkaline phosphatase activity over an activin βA concentration range of four orders of magnitude. This range of concentrations should be sufficient to overwhelm endogenous follistatin binding capacity. Our findings are consistent with other reports. Activin βA treatment of a murine bone marrow stromal cell line (BMS-2) did not alter alkaline phosphatase activity and had no effect on proliferation [Ogawa et al., 1992]. In osteoblastic MC3T3 cells, activin βA treatment actually decreased alkaline phosphatase activity induced by retinoic acid [Hashimoto et al., 1992]. Despite the reports of activin receptors on osteoblasts in vivo [Nagamine et al., 1998; Shuto et al., 1997] and immunohistochemical detection of activin βA on osteoblasts [Funaba et al., 1996; Nagamine et al., 1998], no cell culture studies to date have demonstrated a definitive role for activin βA in osteoblast differentiation. The relative simplicity of monolayer cells in culture may not be ideally suited to understanding the role of activin βA in skeletogenesis. More complex cell-cell interactions are not recapitulated which may explain the lack of effect with activin βA treatment in vitro in monolayer cell culture model systems [Jiang et al., 1993; Ogawa et al., 1992]. In contrast, in vivo activin βA enhanced formation of ectopic bone in rat subcutis when implanted in combination with partially purified bovine BMP [Ogawa et al., 1992]. In another similar ectopic bone study activin βA increased cartilagenous area and content of C propeptide of type II procollagen [Funaba et al., 1996]. Taken together with our data, there is growing evidence that during skeletal development activin βA acts in a paracrine rather than autocrine fashion. However, the lack of a major skeletal defect in activin βA knockout mice suggests a redundancy for activin βA in endochondral bone formation [Matzuk et al., 1995].

By in situ hybridization, we demonstrate expression of activin βA in vivo in the developing interphalangeal joints in the paws of 15.5-day post-coital embryonic mouse limbs. BMP-2 expression is a positive marker for the articular interspaces in developing chick limbs [Macias et al., 1997]. Therefore it is likely that in vivo activin βA expression is also downstream of BMP-2 which is consistent with our in vitro data. Also, the localization of activin βA expression to developing joint space in vivo and the timing of its expression in our model in vitro both coincide with down regulation of cartilage differentiation. The formation of joints constitutes a complex process beginning with arrest of chondrogenic differentiation in the prospective joint interzone and disappearance of collagen type II [Hurle and Colombatti, 1996]. This is accompanied by cell death and by deposition of specific matrix proteins [Pitsillides et al., 1995; Ros et al., 1995]. Concomitantly with the process of joint formation the cartilaginous anlage of the bones undergoes differentiation to form the diaphyses and epiphyses.

The down regulation of BMP activity by interaction with the antagonist noggin is important for initiation of joint formation in mice [Brunet et al., 1998]. The excess BMP activity, a consequence of the absence of noggin, resulted in a failure of joint formation with fusion of joints, and in an enhancement of cartilage growth [Brunet et al., 1998]. Noggin may also regulate joint formation by its ability to bind and inhibit the activity of GDF-5, another member of the BMP family critical for joint formation [Merino et al., 1999]. In chondrocyte and osteoblast cultures, BMP induced expression of noggin, and this negative feed back loop is postulated to be responsible for precise localization of BMP activity [Gazzerro et al., 1998; Kameda et al., 1999]. By analogy to BMP antagonism by noggin, activin βA activity in the developing limb may be spatially restricted by follistatin, thereby limiting long range effects. Our finding that both activin βA and follistatin are induced in vitro suggests a feed back mechanism similar to that proposed for BMP and noggin, and that described for activin βA and follistatin in tooth development [Ferguson et al., 1998]. In addition, as discussed above, follistatin may regulate not only activin βA but also BMP, analogous to noggin's ability to regulate both BMP and GDF-5.

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