Bone Morphogenetic Proteins-2 and -4 are Involved in the Retinoic Acid-Induced Differentiation of Embryonal Carcinoma Cells

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Bone morphogenetic proteins-2 and -4 (BMPs-2 and -4) are transforming growth factor β -related proteins that can induce bone formation in vivo. We observed that the level of endogenous BMP-2 mRNA increased an average of 11-fold on differentiation of F9 embryonal carcinoma cells into parietal endoderm after treatment with retinoic acid (RA) and cAMP, whereas the message for the closely related BMP-4 decreased 12-fold after this treatment. Therefore, the effects of exogenous recombinant BMP-2 protein on the RA-induced differentiation of F9 embryonal carcinoma cells were investigated. BMP-2 addition altered the growth and morphology of RA-treated but not untreated cells. Moreover, the abundance of several messages was affected by exogenous BMP-2 treatment. Notably, the BMP-2 and -4 messages themselves were reduced by the addition of exogenous BMP-2. The observations suggest that RA, which is known to affect bone morphogenesis, may regulate the osteoinductive proteins, BMP-2 and -4. Furthermore, BMP-2 and -4 may be involved in preimplantation embryogenesis.

INTRODUCTION

Retinoids, such as retinoic acid (RA),¹ are extremely teratogenic to developing vertebrate embryos, a fact that diminishes their clinical usefulness. In particular, specific malformations of skeletal structures are often observed. For instance, RA induces craniofacial abnormalities in developing avian (Wedden *et al.*, 1988) and mammalian embryos (Morris-Kay, 1991). The effects of RA on the developing chick limb and the regenerating amphibian limb provide striking examples of how RA can alter long-bone pattern. RA affects both developing and regenerating limbs by reproducibly inducing limb duplications (for review, see Brockes, 1989). Local application of RA to the anterior margin of the developing chick limb causes anterior-posterior duplication, whereas exogenous RA causes proximal-distal duplication in the

regenerating amphibian limb. These effects may result from alterations in a preexisting RA gradient, the existence of which is supported by measurements of endogenous chick limb retinoids (Thaller and Eichele, 1987) or by the induction of a specialized group of cells, the zone of polarizing activity, which subsequently organizes a new pattern (Noji *et al.*, 1991; Wanek *et al.*, 1991).

Bone morphogenetic proteins (BMPs) are molecules that can induce cartilage and bone growth in vivo (Urist, 1965; Urist *et al.*, 1973) and, as such, are potential targets for the teratogenic effects of retinoids. Moreover, many BMPs have been cloned and sequenced and shown to be members of the transforming growth factor beta (TGF β) superfamily (BMPs-2–6) (Wozney *et al.*, 1988; Celeste *et al.*, 1990). This is significant because many TGF β s are regulated by RA or act specifically on RA-treated cells (for review, see Sporn and Roberts, 1991).

It is likely that BMPs play roles other than in bone induction. In situ hybridization analyses indicate that the messages for BMP-2, -4, and -6 (Vgr-1) are expressed in many nonbony tissues, including the developing heart, hair and whisker follicles, central nervous system,

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† BMP, bone morphogenetic protein; CRABP I, cellular retinoic acid-binding protein I; RA, retinoic acid; RhBMP, recombinant human BMP; RACT, retinoic acid, dibutyl cAMP, and theophylline; RAR, retinoic acid receptor; TGF, transforming growth factor.

and preimplantation embryos (Lyons *et al.*, 1989; 1990; Jones *et al.*, 1991). Moreover, several members of the TGF β family have been shown to induce mesoderm in *Xenopus* animal cap explants (BMP-4 [Köster *et al.*, 1991], activin A and B [Thomsen *et al.*, 1990], and TGF β 2 [Rosa *et al.*, 1988] and 3 [Roberts *et al.*, 1990]) and axial structures in isolated chick epiblasts (PIF/activin [Mitrani *et al.*, 1990]). Many additional TGF- β -like factors are expressed in early embryos (e.g., Vg-1 and TGF β 5 (*Xenopus*) and BMP-6, mouse [Lyons *et al.*, 1989]) but have not, as yet, been demonstrated to induce mesoderm (for review, see Melton, 1991). Mitrani *et al.* (1990) have suggested that activin β_A is the endogenous axial mesoderm inducer, as it is expressed in the chick hypoblast, which is known to induce axial pattern in the overlying epiblast. As the mouse extraembryonic endoderm is thought to perform a similar function in the mouse embryo (Hogan *et al.*, 1986), similar mesoderm-inducing molecules might also be identified in this tissue.

Although the *in vivo* studies indicate quite clearly that RA can alter bone pattern, it is difficult to analyze the molecular mechanism of this effect in the models described above. Likewise, mesoderm induction is difficult to study in the small and inaccessible mouse embryo. F9 embryonal carcinoma cells can be induced to differentiate into cells that resemble extraembryonic endoderm in the early mouse embryo (Strickland *et al.*, 1980; Hogan *et al.*, 1981). RA alone induces primitive endoderm, and RA with agents that increase intracellular cAMP levels (e.g., dibutyryl cAMP and theophylline) induces parietal endoderm. This differentiation has been well characterized at the molecular level, and the steady-state levels of many messages increase or decrease during differentiation. Moreover, genes that are regulated by RA in F9 cells, for example, the RA receptor β (RAR β), are also regulated by RA in embryonic chick limbs (Noji *et al.*, 1991). Therefore, we have utilized this *in vitro* RA-responsive model to ask, first, whether BMPs-2 and -4 are regulated by RA and, second, whether these factors affect RA-treated cells.

We report here that the BMP-2 and -4 messages are expressed in a regulated manner during the RA-induced differentiation of F9 cells. In addition, we show that recombinant human BMP-2 (rhBMP-2) affects the morphology, growth, and gene expression of F9 embryonal carcinoma cells. The data suggest that RA and BMP-2 together may affect embryonic differentiation and bone formation.

MATERIALS AND METHODS

Cell Culture and Differentiation

Monolayer F9 embryonal carcinoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated calf serum and 2 mM glutamine and were induced to differentiate into primitive endoderm by the addition of 0.5 μ M RA and into parietal

endoderm by the addition of 0.5 μ M RA, 250 μ M cAMP, and 500 μ M theophylline. RhBMP-2 at >95% purity was provided by Genetics Institute, Cambridge, MA.

RNA Isolation and Analysis

Total cellular RNA was isolated by the guanidinium isothiocyanate method (Kingston, 1987), fractionated on 1% agarose/2.2M formaldehyde gels, transferred to nitrocellulose and hybridized to [32 P]-labeled probes (Feinberg and Vogelstein, 1983; LaRosa and Gudas, 1988a). Radioactive signal due to hybridization was measured directly from the nitrocellulose on a Betascope (Betagen, Waltham, MA) or indirectly by densitometry tracing of the autoradiograms using a Hewlett-Packard (Boston, MA) Scanjet Plus with Enhance 1.0.1 software.

Plasmids

All cDNA clones employed were of murine origin, except those for human RAR α , β , and γ . PAct-G (actin), PGemERA Bam 3 (Hox 1.6), pCI56 (laminin B1), pLamB2-1.5gem (laminin B2), and pCI5 (collagen IV[α 1]) are described in Rogers *et al.* (1990). P2-5 (Rex-1) is described in Hosler *et al.* (1989). cDNA plasmids encoding RAR α , β , and γ were a gift from Dr. P. Chambon (Institut de Chimie Biologique, Strasbourg, FR), and probes were prepared as described in Hu and Gudas (1990). pCRABP gem 10 cellular RA-binding protein I (CRABP I) (Stoner and Gudas, 1989) was constructed by Dr. C. Stoner. pMTM.2B2 (thrombomodulin) (Dittman *et al.*, 1988) was a gift from Dr. W. Dittman (Duke University, Durham, NC). Fragments of BMP-2 (BMP2-68) and BMP-4 (BMP4-40) cDNAs were subcloned into pGEM3. The regions of the BMP-2 and -4 transcripts chosen for probes are the least-conserved portions of the proregions. These probes detect distinct transcripts on Northern blots and *in situ* hybridized embryo sections and do not cross-hybridize to other BMPs.

RESULTS

BMP-2 and -4 mRNA Expression in F9 Cells

To determine if BMP-2 and -4 mRNAs were expressed in untreated F9 cells or in differentiated F9 cells, radioactive cDNA probes for BMP-2 and -4 were hybridized to Northern blots of total RNA isolated from F9 cells grown in monolayer for 1 or 3 d without drugs or with RA or RA + dibutyryl cAMP and theophylline (RACT) added to induce primitive and parietal endoderm, respectively (Figure 1A). The results indicate that BMP-2 mRNA levels are low in untreated F9 cells and are high in F9 cells induced to differentiate into parietal endoderm by RACT. In marked contrast, BMP-4 mRNA levels are high in untreated F9 cells and low in differentiated cells. Treatment of F9 cells with dibutyryl cAMP and theophylline alone or with retinol, retinyl acetate, or retinal (which do not induce differentiation) does not significantly affect the abundance of the BMP-2 and -4 mRNAs (Figure 1B). Quantitation of the signal intensities in Figures 1, A and B, and 4 indicate that, on average, after 3 d of drug treatment, the abundance of BMP-2 mRNA was increased 2-fold by RA and 11-fold by RACT, whereas the BMP-4 mRNA was decreased 6-fold by RA and 12-fold by RACT. Thus, expression of the BMP-2 and -4 genes is clearly modulated by RA in F9 cells.

Figure 1. Northern analysis of BMP-2 and -4 levels in untreated and drug-treated F9 cells. Thirty micrograms of RNA isolated from F9 cells treated as indicated in each figure were transferred to nitrocellulose and hybridized to radioactive probe for BMP-2 and -4 (top), followed by probe for actin, a constitutive message (bottom). (A) The presence or absence of each drug (RA or RA and CT, dibutyl-cAMP, and theophylline) is indicated by a + or -. The length of treatment is indicated in days (D) above each lane. (B) F9 cells were grown for 3 d in DME containing different drugs as follows: STEM, no drug; CT, 250 μ M dibutyl-cAMP and 500 μ M theophylline; trans-RA, *trans*-retinoic acid; RACT, *trans*-RA, dibutyl-cAMP, and theophylline; cis-RA, *cis*-retinoic acid; RAL, *trans*-retinal; ROH, *trans*-retinol; ROH Ac., *trans*-retinol acetate.

Exogenous rhBMP-2 Modulates F9 Cell Morphology and Growth in the Presence of RA

The finding that BMP-2 and -4 mRNAs are expressed in F9 cells suggests a role for these proteins in early embryonic cells. Moreover, RA treatment affects their expression. To attempt to understand the relevance of this observation to RA-regulated differentiation, the effect of rhBMP-2 protein on F9 embryonal carcinoma cells was investigated. RhBMP-2 was added to cultures of F9 cells grown in monolayer without drugs or with RA or RACT. F9 cells treated with rhBMP-2 alone appeared identical to untreated F9 cells (Figure 2, A and B). Although rhBMP-2 had only minor effects on the appearance of cells grown in RA without cAMP and theophylline (Figure 2, C and D), it caused a striking alteration in the morphology of RACT-treated cells (Figure 2, E and F). Whereas the control RACT-treated cells were rounded and highly separate, cells treated with rhBMP-2 and RACT were flat and distinctly resembled cells treated only with RA (Figure 2C). The effect was most pronounced at 100 ng/ml rhBMP-2 but was also visible at 10 ng/ml rhBMP-2. Thus, rhBMP-2 appears to prevent morphological changes associated with RACT-induced differentiation in F9 cells.

Others have previously reported that TGF β 1 and 2, which are 37% similar to BMP-2 and -4, inhibit the growth of RA-treated embryonal carcinoma and embryonic stem cells (Rizzino, 1987; Kelly and Rizzino, 1989; Mummery *et al.*, 1990). We have investigated the effect of rhBMP-2 on F9 cell growth in monolayer (Figure 3). Treatment of F9 cells with rhBMP-2 alone had little effect on growth. However, the simultaneous addition of rhBMP-2 and RA or RACT further inhibited cell growth by 60% and 27%, respectively, relative to cells treated only with RA or RACT. Growth repression (87%) by rhBMP-2 and RA, but not by rhBMP-2 alone, was also observed in another embryonal carcinoma cell line, P19.

The Effect of rhBMP-2 on RA-Regulated Genes

The effect of rhBMP-2 on the morphology of RACT-treated cells suggested that it may influence the differentiation of F9 cells into parietal endoderm. To obtain a better understanding of how rhBMP-2 affects RA-induced differentiation at the molecular level, the expression of previously characterized RA-regulated genes was assayed in rhBMP-2 treated F9 cells.

Two genes that are induced rapidly by RA in F9 cells are *Hox 1.6* (LaRosa and Gudas, 1988a) and *RAR β* (Hu and Gudas, 1990). Both genes appear to be transcriptional regulators because *RAR β* has been shown to regulate transcription (Benbrook *et al.*, 1988; Brand *et al.*, 1988) and *Hox 1.6* contains a homeobox (LaRosa and Gudas, 1988b). The levels of these transcripts were elevated slightly by rhBMP-2 treatment in RA-treated cells but were unaffected in cells grown without RA or with

Figure 2. The morphology of rhBMP-2-treated F9 cells. F9 cells were grown for 4 d in DME alone (A), or DME with the following additions: 100 ng/ml rhBMP-2 (B), RA (C), RA + 100 ng/ml rhBMP-2 (D), RACT (E), and RACT + 100 ng/ml rhBMP-2 (F). Cells were photographed on a Nikon FG inverted microscope with Hoffman optics.

RACT (Figure 4, A and B). Three genes whose mRNA levels are decreased in RACT-treated cells, *RAR* α and γ (Hu and Gudas, 1990) and *Rex-1* (a putative transcription factor) (Hosler *et al.*, 1989), were unaffected by rhBMP-2. Thus, little, if any, effect was observed on the expression of genes that have been suggested to play regulatory roles in the early stages of differentiation.

The expression of genes that are thought to be involved in the latter stages of F9 differentiation, however,

was found to be repressed by BMP-2. The intracellular concentration of the CRABP I is thought to influence the response of cells to RA; specifically, high levels of CRABP inhibit the RA-induced differentiation of F9 cells (Boylan and Gudas, 1991). In F9 cells, CRABP I mRNA abundance is affected by RA and RACT treatment and by length of time in culture (Stoner and Gudas, 1989). The CRABP I protein and mRNA are also regulated by developmental and tissue-specific factors (Ruberte *et al.*, 1991). In light of the effect of CRABP levels on differentiation, it was important to ask whether rhBMP-2 affected CRABP levels. In comparison to untreated F9 cells, CRABP I mRNA abundance was reduced three- and fivefold by RA or RACT treatment, respectively; however, F9 cells treated with 100 ng/ml rhBMP-2 and RA or RACT had mRNA levels of 10- and 46-fold less (Figure 4). This further reduction in CRABP I mRNA level clearly indicates that rhBMP-2 treatment modulates CRABP I expression in this system.

In a pattern that is consistent with the effect of rhBMP-2 on the morphology of RACT-treated F9 cells, rhBMP-2 was found to alter the expression of genes that are activated more in RACT-treated cells than in RA-treated cells, for example, laminin B1 and collagen IV (α 1). Extracellular matrix plays an important role in regulating tissue differentiation and, in F9 cells, these mRNAs are induced threefold by RA treatment and eightfold by RACT treatment (Figure 4). The abundance of collagen IV (α 1) and laminin B1 (Figure 4) and B2

Figure 3. The effect of rhBMP-2 on F9 growth. Cells were grown 3 d in DME containing the drugs indicated under each bar and counted using a Coulter Counter. The number of cells is expressed as a percentage of the number of cells grown without drugs. The error bars indicate SD.

Figure 4. Northern analysis of genes expressed in rhBMP-2-treated F9 cells. (A) Autoradiographs of northern blots of RNA isolated from F9 cells grown for 3 d in DME containing the drugs indicated below each lane. STEM indicates untreated cells. The messages detected are indicated beside each panel and three are abbreviated as follows: Coll. IV, collagen IV ($\alpha 1$); TM, thrombomodulin; Lam B1, laminin B1. (B) Bar graphs indicating the transcript levels normalized to actin levels and expressed as a percentage of the most abundant transcript level. Because the actin signal varied only 2.6-fold or less between lanes, these autoradiographs are not shown.

messages was reduced by rhBMP-2 and RACT cotreatment as compared with RACT treatment alone. Likewise, the thrombomodulin message, which is induced specifically by RACT, but not by RA (Imada *et al.*, 1987, 1990), was induced less in F9 cells treated with rhBMP-2 and RACT than in cells treated only with RACT (Figure 4). The steady-state level of thrombomodulin mRNA was threefold less in F9 cells grown in RACT and 100 ng/ml rhBMP-2 than in cells grown in

RACT alone. In conclusion, rhBMP-2 treatment apparently does not affect the early RA-regulated genes that we examined but suppresses the induction of several late RACT-regulated genes.

The Effect of rhBMP-2 on BMP-2 and -4 mRNA Abundance

Finally, experiments were performed to determine if the BMP-2 and -4 genes were autoregulated in F9 cells (Fig-

ure 4). The abundance of BMP-2 mRNA in 100 ng/ml rhBMP-2 and RACT-treated F9 cells was 4.7-fold less than in cells treated only with RACT (i.e., the mRNA was increased by only 5.8-fold instead of the 28-fold observed in RA-treated cells in this experiment). One hundred nanograms per milliliters of rhBMP-2 protein alone reduced the steady-state level of BMP-4 message in F9 cells 3.2-fold relative to untreated cells, whereas the addition of rhBMP-2 to RA-treated cells reduced the BMP-4 message by 12-fold relative to cells treated with RA alone (i.e., a 77-fold decrease instead of the 6.6-fold decrease observed in RA-treated cells relative to untreated cells). No further decrease in BMP-4 mRNA abundance was observed in rhBMP-2 and RACT-treated cells relative to those treated with RACT. Thus, rhBMP-2 protein can regulate the abundance of the murine BMP-2 and -4 mRNAs in F9 cells.

DISCUSSION

The data presented here indicate that the messages for two osteoinductive proteins, BMP-2 and -4, are synthesized by F9 cells and that RA regulates the mRNA abundance of these factors in F9 cells. Moreover, rhBMP-2 protein can alter the growth characteristics and morphology of RA-treated embryonal carcinoma cells and can regulate the abundance of many messages, including those for BMP-2 and -4, in these cells.

Undifferentiated F9 cells resemble cells of the blastocyst inner cell mass and differentiated F9 cells resemble extraembryonic endoderm. Although BMPs were first identified as bone-inducing factors, the findings that BMP-2, -4, (this work), and -6 (*Vgr-1* [Lyons *et al.*, 1989]) messages are developmentally regulated in F9 cells suggest multiple roles for BMPs in mouse embryogenesis. This idea is further supported by Jones *et al.* (1991) and Lyons *et al.* (1990), who reported the presence of these mRNAs in many tissues other than bone. Furthermore, as detailed earlier, BMP-4 can induce mesoderm in *Xenopus* embryos. By analogy to chick development, the extraembryonic endoderm and inner cell mass are likely to interact in the formation of axial pattern in the mouse embryo. Thus, the observations that BMP-2 and -6 are expressed in differentiated F9 cells make them strong candidates for mesoderm-inducing factors synthesized by extraembryonic endoderm in the mouse embryo.

The differential regulation of BMP-2 and BMP-4 messages in F9 cells is puzzling. BMP-4 mRNA is present in untreated F9 cells and repressed in RACT-treated cells, whereas BMP-2 is absent in untreated F9 cells and induced in RACT-treated cells. Although we have not yet determined whether the effect of RACT is direct or indirect, this is intriguing because BMP-2 and -4 are 92% similar at the amino acid level and exhibit virtually identical osteoinductive activities. An understanding of the significance of this observation will require addi-

tional study, but the tissue-specific regulation of these two messages suggests subtle and as yet unidentified differences between BMP-2 and -4.

RhBMP-2 had little appreciable effect on untreated F9 cells; however, the addition of rhBMP-2 to media that normally induces the differentiation of F9 cells into primitive (RA alone) or parietal (RACT) endoderm resulted in additional growth inhibition. Moreover, the morphology of RACT-treated F9 cells was strikingly altered by rhBMP-2 treatment. The differentiation-specific growth inhibition is very similar to previously reported observations using TGF β 1 and 2 (Rizzino, 1987; Kelly and Rizzino, 1989; Mummery *et al.*, 1990). RhBMP-2 also inhibited the expression of many differentiation-specific mRNAs. In F9 cells late RACT-induced genes (BMP-2, extracellular matrix proteins, and thrombomodulin) were most affected. This difference is unlikely to be due to the absence of BMP receptors in undifferentiated F9 cells because they respond to rhBMP-2 by repressing the BMP-4 message. With regard to morphology and gene expression, rhBMP-2 rendered RACT-treated F9 cells more similar to RA-treated cells. Thus, rhBMP-2 may act by inhibiting the effect of cAMP or by delaying the maximum expression of late RACT-induced genes. The resulting cells would thus be more similar to primitive endoderm.

The striking induction of the endogenous BMP-2 mRNA in RACT-treated F9 cells appears contradictory to the observation that exogenous rhBMP-2 represses F9 cell differentiation in response to RACT. This finding is not likely to be a result of functional differences between the human and mouse BMP-2s as they are identical in sequence. One possible explanation may be related to the observation that TGF β s are often secreted in latent form (e.g., Lyons *et al.*, 1988). Thus, the BMP-2 synthesized by extraembryonic endoderm may require activation by another embryonic cell type, e.g., embryonic ectoderm (epiblast). Consequently, in the homogeneous parietal endoderm population derived from F9 cells, any endogenous BMP-2 might be inactive and not capable of regulating differentiation, whereas the *in vitro* synthesized rhBMP-2 is active.

The teratogenic effects of retinoids on developing and regenerating limbs suggest that retinoids may influence the expression of osteoinductive proteins or alter the response of skeletal precursor cells to growth factors. This report clearly establishes that RA can regulate the expression of the osteoinductive proteins, BMP-2 and -4, and that rhBMP-2 can influence the growth of RA-treated, but not untreated, cells. RA, RARs, and CRABPs, as well as the messages for BMP-2 and -4 (Lyons *et al.*, 1990; Jones *et al.*, 1991), are localized in the developing limb in distinct, but overlapping, patterns. The effect of rhBMP-2 on the RA-induced differentiation of F9 cells suggests that BMPs and RA may interact to influence limb pattern, but this remains to be determined.

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REFERENCES

- Benbrook, D., Lernhardt, E., and Pfahl, M. (1988). A new retinoic acid receptor identified from a hepatocellular carcinoma. *Nature* 333, 669–672.
- Boylan, J.F., and Gudas, L.J. (1991). Overexpression of the cellular retinoic acid binding protein-I (CRABP-I) results in a reduction in differentiation-specific gene expression in F9 teratocarcinoma cells. *J. Cell Biol.* 112, 965–979.
- Brand, N., Petkovich, M., Krust, A., Chambon, P., de Thé, H., Marchio, A., Tiollais, P., and Dejean, A. (1988). Identification of a second human retinoic acid receptor. *Nature* 332, 850–853.
- Brockes, J.P. (1989). Retinoids, homeobox genes, and limb morphogenesis. *Neuron* 2, 1285–1294.
- Celeste, A.J., Iannazi, J.A., Taylor, R.C., Hewick, R.M., Rosen, V., Wang, E.A., and Wozney, J.M. (1990). Identification of transforming growth factor β family members present in bone-inductive protein purified from bovine bone. *Proc. Natl. Acad. Sci. USA* 87, 9843–9847.
- Dittman, W.A., Kumada, T., Sadler, J.E., and Majerus, P.W. (1988). The structure and function of mouse thrombomodulin. *J. Biol. Chem.* 263, 15815–15822.
- Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132, 6–13.
- Hogan, B., Constantini, F., and Lacy, E. (1986). *Manipulating the Mouse Embryo*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Hogan, B.L.M., Taylor, A., and Adamson, E. (1981). Cell interactions modulate embryonal carcinoma cell differentiation into parietal or visceral endoderm. *Nature* 291, 235–237.
- Hosler, B.A., LaRosa, G.J., Grippo, J.F., and Gudas, L.J. (1989). Expression of REX-1, a gene containing zinc finger motifs, is rapidly reduced by retinoic acid in F9 teratocarcinoma cells. *Mol. Cell. Biol.* 9, 5623–5629.
- Hu, L., and Gudas, L.J. (1990). Cyclic AMP analogs and retinoic acid influence the expression of retinoic acid receptor α , β , and γ mRNAs in F9 teratocarcinoma cells. *Mol. Cell. Biol.* 10, 391–396.
- Imada, M., Imada, S., Iwasaki, H., Kume, A., Yamaguchi, H., and Moore, E.E. (1987). Fetomodulin: Marker surface protein of fetal development which is modulatable by cyclic AMP. *Dev. Biol.* 122, 483–491.
- Imada, S., Yamaguchi, H., Naguna, M., Katayanagi, S., Iwasaki, H., and Imada, M. (1990). Identification of fetomodulin, a surface marker protein of fetal development, as thrombomodulin by gene cloning and function assays. *Dev. Biol.* 140, 113–122.
- Jones, C.M., Lyons, K.M., and Hogan, B.L.M. (1991). Involvement of bone morphogenetic protein-4 (BMP-4) and Vgr-1 in morphogenesis and neurogenesis in the mouse. *Development*, 112, 531–542.
- Kelly, D., and Rizzino, A. (1989). Inhibitory effects of transforming growth factor- β on laminin production and growth exhibited by endoderm-like cells derived from embryonal carcinoma cells. *Differentiation* 41, 34–41.
- Kingston, R.E. (1987). Extraction of RNA from tissue. In: *Current Protocols in Molecular Biology*, ed. F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman, and K. Struhl, New York: Green Publishing and Wiley-Interscience, 423–425.
- Köster, M., Plessow, S., Clement, J.H., Lorenz, A., Tiedemann, H., and Knöchel, W. (1991). Bone morphogenetic protein-4 (BMP-4), a member of the TGF β family, in early embryos of *Xenopus laevis*: analysis of mesoderm inducing activity. *Mech. Ageing Dev.* 33, 191–200.
- LaRosa, G.J., and Gudas, L.J. (1988a). An early effect of retinoic acid: cloning of an mRNA (Era-1) exhibiting rapid and protein synthesis-independent induction during teratocarcinoma stem cell differentiation. *Proc. Natl. Acad. Sci. USA* 85, 329–333.
- LaRosa, G.J., and Gudas, L.J. (1988b). Early retinoic acid-induced F9 teratocarcinoma stem cell gene ERA-1: alternate splicing creates transcripts for a homeobox-containing protein and one lacking the homeobox. *Mol. Cell. Biol.* 8, 3906–3917.
- Lyons, K., Graycar, J.L., Lee, A., Hashmi, S., Lindquist, P.B., Chen, E.Y., Hogan, B.L.M., and Derynck, R. (1989). Vgr-1, a mammalian gene related to *Xenopus Vg-1*, is a member of the transforming growth factor β gene super family. *Proc. Natl. Acad. Sci. USA* 86, 4554–4558.
- Lyons, R.M., Keski-Oja, J., and Moses, H.L. (1988). Proteolytic activation of latent transforming growth factor-beta from fibroblast-conditioned medium. *J. Cell Biol.* 106, 1659–1665.
- Lyons, K.M., Pelton, R.W., and Hogan, B.L.M. (1990). Organogenesis and pattern formation in the mouse: RNA distribution patterns suggest a role for Bone Morphogenetic Protein-2A (BMP-2A). *Development* 109, 833–844.
- Melton, D. (1991). Pattern formation during animal development. *Science* 252, 234–241.
- Mitrani, E., Ziv, T., Thomsen, G., Shimoni, Y., Melton, D., and Bril, A. (1990). Activin can induce the formation of axial structures and is expressed in the hypoblast of the chick. *Cell* 63, 495–501.
- Morris-Kay, G. (1991). Retinoic acid, neural crest and craniofacial development. *Semin. Dev. Biol.* 2, 211–218.
- Mummery, C.L., Slager, H., Kruijer, W., Feijen, A., Freund, E., Koornneef, I., and van den Eijnden-van Raaij, A.J.M. (1990). Expression of transforming growth factor β 2 during the differentiation of murine embryonal carcinoma and embryonic stem cells. *Dev. Biol.* 137, 161–170.
- Noji, S., Nohno, T., Koyama, E., Muto, K., Ohshima, K., Aoki, Y., Tamura, K., Ohsugi, K., Ide, H., and Taniguchi, S. (1991). Retinoic acid induces polarizing activity but is unlikely to be a morphogen in the chick limb bud. *Nature* 350, 83–86.
- Rizzino, A. (1987). Appearance of high affinity receptors for type β transforming growth factor during differentiation of murine embryonal carcinoma cells. *Cancer Res.* 47, 4386–4390.
- Roberts, A., Kondaiah, P., Rosa, F., Watanabe, S., Good, P., Danielpour, D., Roche, N., Rebbert, M., Dawid, I., and Sporn, M. (1990). Mesoderm induction in *Xenopus laevis* distinguishes between the various TGF- β isoforms. *Growth Factors* 3, 277–286.
- Rogers, M.B., Watkins, S.C., and Gudas, L.J. (1990). Gene expression in visceral endoderm: a comparison of mutant and wildtype embryonal carcinoma cell differentiation. *J. Cell Biol.* 110, 1767–1777.
- Rosa, F., Roberts, A., Danielpour, D., Dart, L., Sporn, M., and Dawid, I. (1988). Mesoderm induction in amphibians: the role of TGF β 2-like factors. *Science* 239, 783–785.
- Ruberte, E., Dollé, P., Chambon, P., and Morris-Kay, G. (1991). Retinoic acid receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryos. *Development* 111, 45–60.
- Sporn, M.B., and Roberts, A.B. (1991). Interactions of retinoids and transforming growth factor-b in regulation of cell differentiation and proliferation. *Mol. Endocrinol.* 5, 3–7.

- Stoner, C.M., and Gudas, L.J. (1989). Mouse cellular retinoic acid binding protein: cloning, complementary DNA sequence, and messenger RNA expression during the retinoic acid-induced differentiation of F9 wildtype and RA-3-10 mutant teratocarcinoma cells. *Cancer Res.* 49, 1497–1504.
- Strickland, S., Smith, K.K., and Marotti, K.R. (1980). Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP. *Cell* 21, 347–355.
- Thaller, C., and Eichele, G. (1987). Identification and spatial distribution of retinoids in the developing chick limb bud. *Nature* 327, 625–628.
- Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W., and Melton, D. (1990). Activins are expressed early in *Xenopus* embryogenesis and can induce axial mesoderm and anterior structures. *Cell* 63, 485–493.
- Urist, M.R. (1965). Bone: formation by autoinduction. *Science* 150, 893–899.
- Urist, M.R., Iwata, H., Ceccotti, P.L., Dorfman, R.L., Boyd, S.D., McDowell, R.H., and Chien, C. (1973). Bone morphogenesis in implants of insoluble bone gelatin. *Proc. Natl. Acad. Sci. USA* 70, 3511–3515.
- Wanek, N., Gardiner, D.M., Muneoka, K., and Bryant, S.V. (1991). Conversion by retinoic acid of anterior cells into ZPA cells in the chick wing bud. *Nature* 350, 81–83.
- Wedden, S., Ralphs, J., and Tickle, C. (1988). Pattern formation in the facial primordia. *Development* 103 (Suppl.), 31–40.
- Wozney, J.M., Rosen, V., Celestes, A.J., Mitsock, L.M., Whitters, M.J., Kriz, R.W., Hewick, R.M., and Wang, E.A. (1988). Novel regulators of bone formation: molecular clones and activities. *Science* 242, 1528–1534.