

Differential expression of BMP receptors in early mouse development

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ABSTRACT Bone morphogenetic proteins (BMPs) are members of the transforming growth factor- β family of polypeptide signaling molecules. They function via binding to two types of transmembrane serine/threonine kinase receptors, type I and type II receptors, that are both necessary for signaling. The expression patterns of the type II BMP receptor (BMPR-II) and three type I BMP receptors (ActR-I, BMPR-IA and BMPR-IB) were examined in preimplantation embryos by means of heminested reverse transcription-polymerase chain reaction (RT-PCR). BMPR-II mRNA was detected in one-cell, two-cell and blastocyst stage embryos. ActR-I exhibited a similar expression pattern. BMPR-IA mRNA however was only detected in blastocysts, whereas BMPR-IB transcripts were detected at all stages from the one-cell zygote to the uncompact morula, but not in the compacted morula and blastocyst. If translated into proteins, this suggests that different receptor complexes can be formed at different developmental stages. Transcripts for BMPs were not detected in preimplantation embryos, but were detected in the maternal tissues surrounding the embryos. BMPR-II, BMPR-IA and BMPR-IB mRNAs were also detected in undifferentiated and differentiated embryonal carcinoma and embryonic stem cells. In postimplantation embryos BMPR-II transcripts were first detected from 6.0 days post coitum. *In situ* hybridization analysis revealed that BMPR-II mRNA is ubiquitously expressed in the entire embryo at least until midgestation.

KEY WORDS: BMP, TGF- β , serine/threonine kinase receptors, mouse embryogenesis, RT-PCR, *in situ* hybridization

Introduction

The bone morphogenetic proteins (BMPs) are a group of signaling molecules belonging to the transforming growth factor- β (TGF- β) superfamily of growth factors. Other members of this superfamily are the TGF- β s, activins and inhibins, and Müllerian inhibiting substance (Kingsley, 1994). The BMP subgroup includes members such as *Drosophila* decapentaplegic (dpp; Padgett *et al.*, 1987), *Xenopus* Vg1 (Weeks and Melton, 1988), BMP-2, BMP-4 (Wozney *et al.*, 1988), BMP-6 (Vgr-1; Lyons *et al.*, 1989a) and BMP-7 (OP-1; Özkaynak *et al.*, 1990). Substantial evidence has accumulated that these gene products are important regulators of development and growth (Hogan, 1996). Dpp for instance is present in the dorsal ectoderm of the *Drosophila* embryo, where it functions as a dorsalizing morphogen (Irish and Gelbart, 1987; Ferguson and Anderson, 1992). Vg1 is restricted to the vegetal hemisphere in *Xenopus* embryos and is able to induce dorsal mesoderm (Dale *et al.*, 1993; Thomsen and Melton, 1993). Although originally identified as factors that can induce ectopic bone formation *in vivo* (Urist *et al.*, 1983; Wozney *et al.*, 1988), BMP-2 and BMP-4 were also found to induce ventral mesoderm, including erythrocytes, in *Xenopus* animal caps (Koster *et al.*, 1991; Dale *et al.*, 1992; Hemmati-Brivanlou and Thomsen, 1995). Moreover, BMP-4, but not

BMP-2, ventralizes dorsal mesodermal explants (Hemmati-Brivanlou and Thomsen, 1995) and inhibits neuralization in *Xenopus* (Wilson and Hemmati-Brivanlou, 1995). In murine embryonic stem (ES) cells, BMP-4 was found to enhance differentiation of posteroventral mesoderm including hematopoietic development (Johansson and Wiles, 1995). In addition, BMP-2 and BMP-4 can regulate differentiation and induce apoptosis in embryonal carcinoma (EC) cells (Rogers *et al.*, 1992; Glozak and Rogers, 1996).

TGF- β s and members of the TGF- β superfamily of growth factors function via binding to two types of distantly related transmembrane serine/threonine kinase receptors, denominated type I and type II receptors (Lin and Lodish, 1993; Massagué *et al.*, 1994). It has been shown that the type II receptors selectively bind ligand. The type I receptors in general cannot bind free ligand, but can recognize ligand bound to the type II receptors and thus become incorporated into the complex (Wrana *et al.*, 1992). The type II receptors are constitutively active kinases, so that recruitment

Abbreviations used in this paper: ALK, activin receptor like kinase; BMP, bone morphogenetic protein; EC, embryonal carcinoma; ES, embryonic stem; dBcAMP, dibutyryl cyclic AMP; dpc, days post coitum; dpp, decapentaplegic; PAI-I, plasminogen activator inhibitor-I; RA, retinoic acid; RT-PCR, reverse transcription polymerase chain reaction; TGF- β , transforming growth factor- β .

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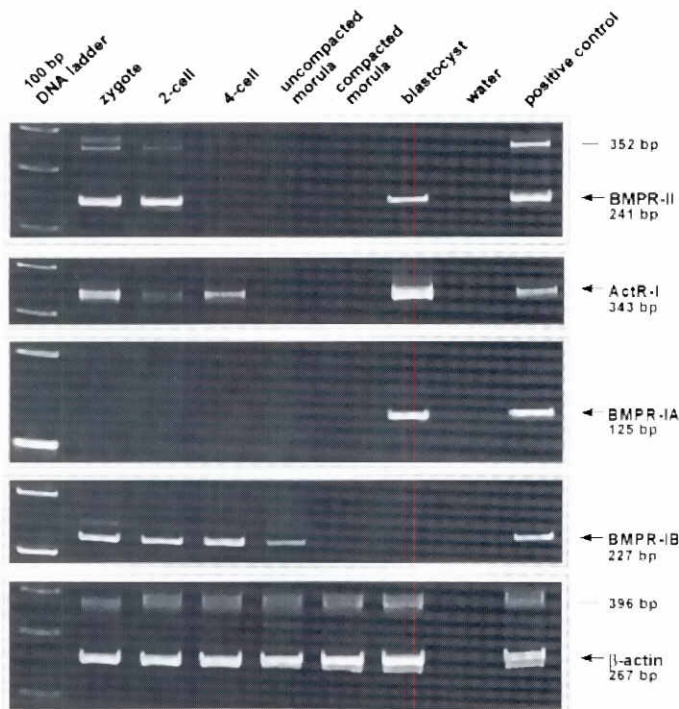


Fig. 1. Expression of BMP receptor types I and II mRNA in preimplantation mouse embryos. Heminested PCR was performed on cDNA from embryonic stages between the one-cell zygote and the advanced blastocyst. Detection of β -actin mRNA ensured correct RNA extraction and cDNA synthesis. cDNA (1000-fold diluted) from 6.5 dpc decidual tissue (BMPR-II and β -actin) or 12.5 dpc embryonal heart (ActR-I, BMPR-IA and BMPR-IB) served as positive controls for amplification. The samples were electrophoresed on 6% polyacrylamide gels and visualized by ethidium bromide staining, with a 100 bp DNA ladder as size marker. The amplified products with expected sizes are indicated on the right of the gels. The upper bands obtained with BMPR-II and β -actin primers indicated on the right represent first round carry over.

results in type I receptor phosphorylation. The activated type I receptor kinase propagates the signal to downstream substrates (Wrana *et al.*, 1994).

Recently, a human type II BMP receptor has been identified based on its ability to interact strongly with the cytoplasmic region of the type I TGF- β receptor (T β R-I) in a yeast two-hybrid system (Kawabata *et al.*, 1995; Liu *et al.*, 1995). This receptor, referred to as BMPR-II or T-ALK, was found to bind BMP-2 and BMP-7 with high affinity, but not to bind TGF- β 1 or activin-A (Liu *et al.*, 1995). The type II BMP receptor can form heteromeric complexes with four of six known mammalian type I receptors: ActR-I (ALK-2/Tsk 7L; Attisano *et al.*, 1993; Ebner *et al.*, 1993; ten Dijke *et al.*, 1993), BMPR-IA (BRK-1/ALK-3; ten Dijke *et al.*, 1993; Koenig *et al.*, 1994), T β R-I (ALK-5; Attisano *et al.*, 1993; Franzén *et al.*, 1993) and BMPR-IB (ALK-6; ten Dijke *et al.*, 1994a,b). However, a functional BMP receptor complex able to activate a p3TP-Lux reporter construct, containing a TGF- β -responsive element from the plasminogen activator inhibitor-1 (PAI-1) gene, is only formed with ActR-I, BMPR-IA and BMPR-IB (Liu *et al.*, 1995; Rosenzweig *et al.*, 1995). This indicates that only three of the four type I receptors in this case signal.

Mice lacking BMP-4 die between 6.5 and 9.5 dpc with little or no mesoderm being formed, suggesting that this protein is important for gastrulation and mesoderm formation (Winnier *et al.*, 1995). Mice lacking BMP-7 develop normally until midgestation, but display abnormalities in kidney, eye and hindlimb development (Dudley *et al.*, 1995; Luo *et al.*, 1995). In these embryos, early development is normal but maternal transfer of BMP-4 and BMP-7 protein, or rescue by related proteins, may mask early aberrant phenotypes. An inventory of BMP receptor expression in pre- and early postimplantation embryos to identify potential target cells could be helpful in finding out whether BMPs have an early function in development and if so by which receptor types they might signal. Northern hybridization data have shown high expression of BMPR-II mRNA in human heart and liver, but low levels of the transcript in other tissues (Kawabata *et al.*, 1995). Recently, the distribution of the type I BMP receptors IA and IB during postimplantation mouse development was described (Dewulf *et al.*, 1995). BMPR-IA mRNA was ubiquitously expressed from 6.5 dpc onwards, whereas BMPR-IB gene expression could only be detected from 9.5 dpc. At midgestation, BMPR-IA and BMPR-IB mRNA are both present in mesenchymal precartilaginous condensations, epithelium and neural tissue, but the distribution of BMPR-IB mRNA is more restricted than that of BMPR-IA (Dewulf *et al.*, 1995). In the

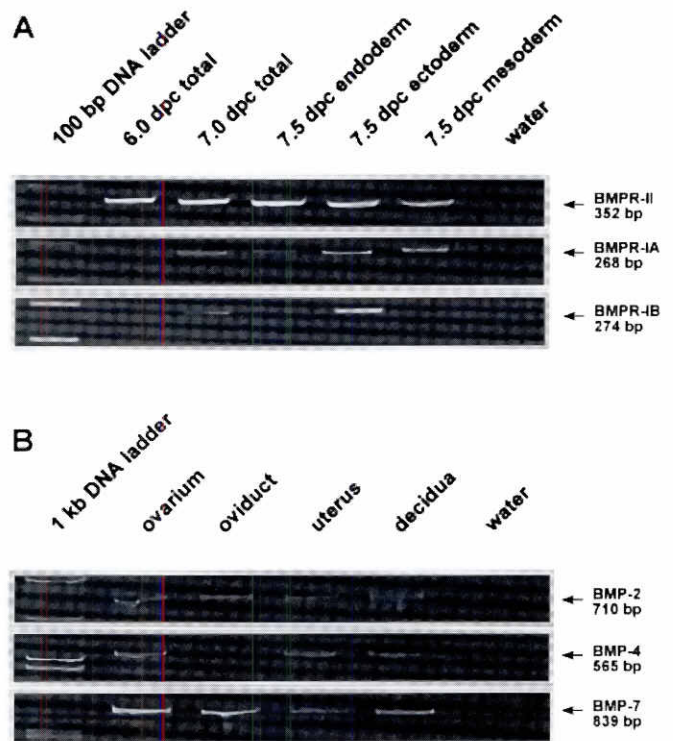


Fig. 2. Expression of BMPs and BMP receptors. (A) Detection of BMP receptor types I and II in 6.0 dpc and 7.0 dpc complete embryos and isolated embryonic endoderm, ectoderm and mesoderm from 7.5 dpc embryos. cDNA from 6.5 dpc decidua (BMPR-II) and 12.5 dpc heart tissue (BMPR-IA and BMPR-IB) served as positive controls for amplification. (B) RT-PCR assay for BMP-2, BMP-4 and BMP-7 in non-embryonic tissues. cDNA was obtained from ovary, oviduct, uterus and decidua of a 6.5 dpc pregnant mouse. The amplified products were electrophoresed on 6% polyacrylamide gels with a 100 bp DNA ladder as size marker and visualized by ethidium bromide staining. Expected product sizes are indicated on the right of the gels.

adult mouse, low levels of BMPR-IA mRNA are detected in various tissue types (Koenig *et al.*, 1994). In contrast, BMPR-IB mRNA is only present in brain and lung tissue of the adult mouse (ten Dijke *et al.*, 1994a).

The presence of type I BMP receptors in preimplantation embryos has not yet been described. In addition, the distribution of the type II BMP receptor in pre- and postimplantation embryos is unknown. We have studied the expression of these receptor types in preimplantation and early postimplantation embryos by means of RT-PCR. The expression of the type II BMP receptor was investigated in more detail in postimplantation embryos by *in situ* hybridization. The results showed that the expression of functional receptor complexes is developmentally regulated and that the likely composition of these complexes is highly stage dependent.

Results

Biphasic expression of BMPR-II and type I BMP receptors in preimplantation embryos

To examine the temporal patterns of gene expression in preimplantation embryos, we used the reverse transcription polymerase chain reaction (RT-PCR) on RNA isolated from one-cell zygotes, two-cell embryos, four-cell embryos, uncompact morulae (8-16 cell-stage), compacted morulae and late blastocysts. Since the cDNA of only 0.75 embryo-equivalents was used per PCR assay, a first round amplification of 40-cycles was followed by a second round 30-cycle heminested amplification. Not only sensitivity, but also specificity is greatly enhanced by this method.

BMPR-II mRNA was detected in zygotes and two-cell embryos; thus it probably represents maternal gene transcripts. No BMPR-II mRNA could be detected at the four-cell stage, nor at the morula stages. In contrast, BMPR-II gene expression was clearly detected at the blastocyst stage (Fig. 1). At all stages examined, β -actin transcripts were found, indicating successful RNA extraction and cDNA synthesis of these samples (Fig. 1).

For the type II BMP receptor to be functional, heteromeric complex formation with a type I receptor is required. Therefore, expression of all three known type I BMP receptors (ActR-I, BMPR-IA and BMPR-IB) was also studied in preimplantation embryos by heminested RT-PCR. ActR-I mRNA was detected at the zygote stage, two-cell stage, four-cell stage and at the late blastocyst stage, but not in uncompact or compacted morulae (Fig. 1). BMPR-IA mRNA expression on the other hand was not detected in any of the early preimplantation embryos. Only in late blastocysts was BMPR-IA mRNA present (Fig. 1). Interestingly, BMPR-IB gene expression showed a reciprocal pattern; this receptor was detected in the zygote, two-cell embryo, four-cell embryo and uncompact morula, but not in the compacted morula or late blastocyst (Fig. 1). Thus preimplantation embryos express either BMPR-IA or BMPR-IB mRNA but at no stage the mRNA expression patterns overlap. BMP receptor expression patterns were strictly stage dependent, and no differences in these patterns were observed whether embryos were used directly after collection from the oviduct/uterus for RT-PCR or were cultured *in vitro* from the two-cell stage to that required for experimentation (data not shown).

BMP-receptor expression in early postimplantation embryos

Expression of type I and type II BMP receptors was studied in postimplantation embryos at 6.0, 7.0 and 7.5 dpc by a combination

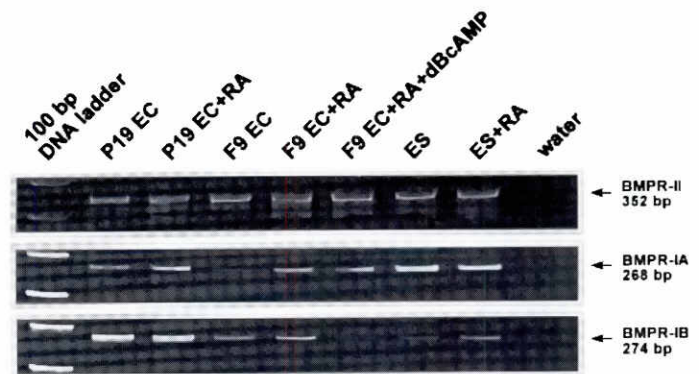


Fig. 3. RT-PCR for type II and type I BMP receptors in EC and ES cells. The amplified products were electrophoresed on 6% polyacrylamide gels with a 100 bp DNA ladder as size marker and visualized by ethidium bromide staining. Expected product sizes are indicated on the right of the gels.

of RT-PCR and *in situ* hybridization. The 6.0 and 7.0 dpc embryos were dissected from decidua and Reichert's membranes, but were otherwise collected complete, including both the embryonic and the extraembryonic parts. For the 7.5 dpc embryos, the embryonic part was collected and the three germ layers were separated as previously (Roelen *et al.*, 1994). RT-PCR was performed on the mRNA isolated from these embryos using a single round 40-cycle amplification.

BMPR-II mRNA was detected at all stages examined, and in all three germ layers at 7.5 dpc (Fig. 2A). BMPR-IA was expressed at 7.0 dpc and at 7.5 dpc in all three germ layers but not at 6.0 dpc (Fig. 2A). Similarly, BMPR-IB mRNA was first detected at 7.0 dpc, but at 7.5 dpc it was only present in the embryonic ectoderm, but not in the embryonic endoderm or embryonic mesoderm (Fig. 2A). In a previous study, we showed that ActR-I mRNA is present in 6.0 and 6.5 dpc embryos, and in the embryonic endoderm and embryonic mesoderm of 7.5 dpc embryos (Roelen *et al.*, 1994). These results are not completely in agreement with the results of Dewulf *et al.* (1995), who were not able to detect BMPR-IB mRNA before 9.5 dpc using *in situ* hybridization. However, this is most likely due to the higher sensitivity of the RT-PCR method for detecting gene expression.

BMP expression in preimplantation embryos and non-embryonic tissues

Using heminested RT-PCR we were not able to detect BMP-2, BMP-4 or BMP-7 transcripts in one-cell zygotes, two-cell embryos, four-cell embryos, uncompact morulae, compacted morulae or late blastocysts, whereas these transcripts were detected in a 1000-fold dilution of 12.5 dpc embryonic heart cDNA, indicating that the amplification process itself was successful (data not shown). To investigate whether BMPs would be available to preimplantation embryos from maternal tissues, we looked for the expression of BMP-2, BMP-4 and BMP-7 mRNA in ovary, oviduct, uterus and decidua by means of a single round 40-cycle RT-PCR amplification. In all tissues examined, mRNA for BMP-2, BMP-4 and BMP-7 could be detected, with the exception of BMP-4, which was not present in the oviduct (Fig. 2B). Thus, BMPs are not produced by preimplantation embryos, but are present in the maternal tissues surrounding these embryos.

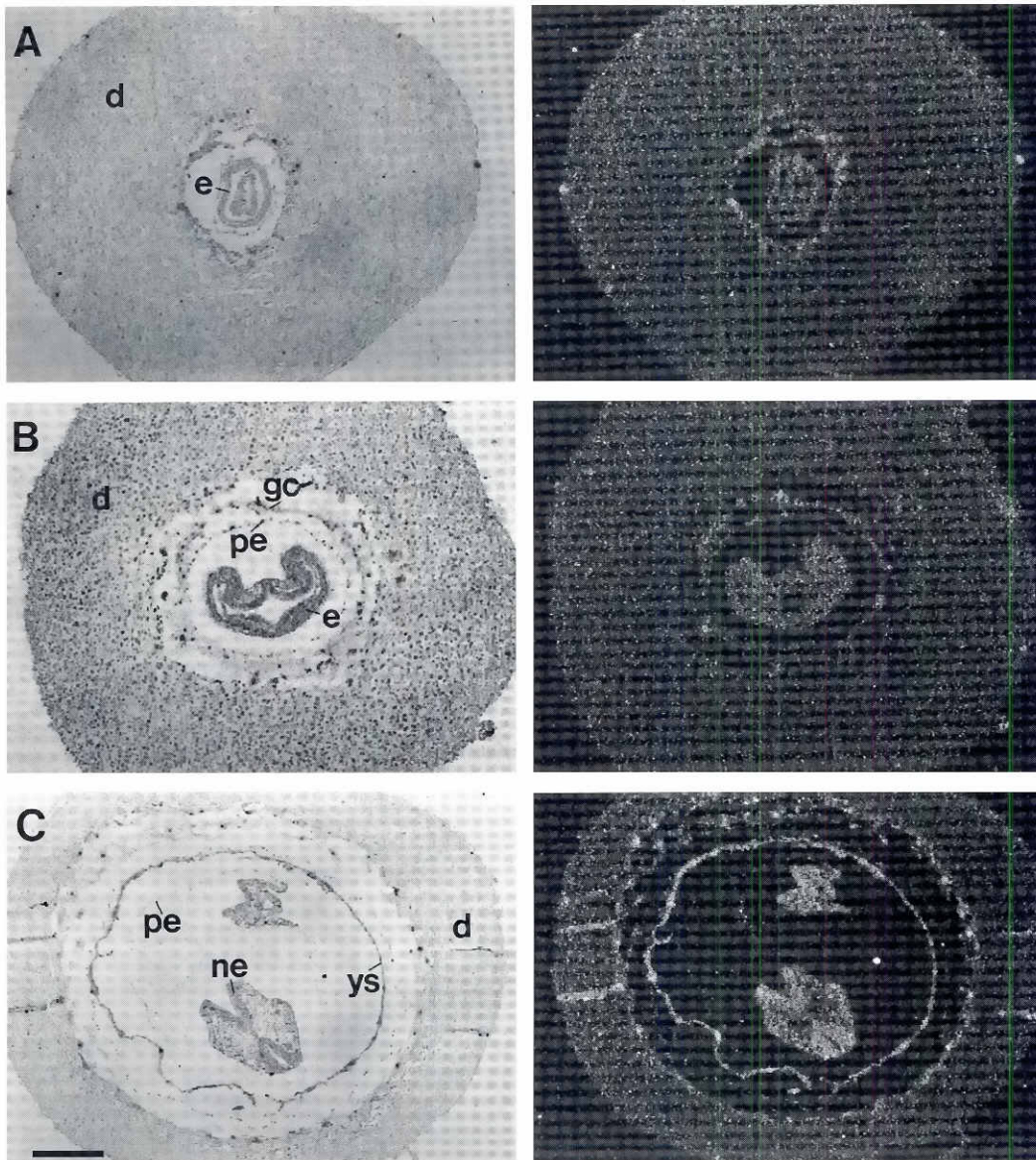


Fig. 4. BMPR-II expression in early postimplantation embryos analyzed with *in situ* hybridization. Transverse sections of 6.5 dpc (A), 7.5 dpc (B), and 8.5 dpc (C) embryos in the decidua. Brightfield pictures on the left with corresponding darkfield pictures on the right. Abbreviations: d, decidua; e, embryo; gc, trophoblast giant cell; ne, neuroepithelium; pe, parietal endoderm; ys, visceral yolk sac. Bar, 250 μ m.

BMP receptors in EC and ES cells

Murine ES cell differentiation to mesoderm is enhanced by BMP-4 (Johansson and Wiles, 1995), and addition of BMP-2 to F9 EC cells pretreated with retinoic acid (RA) or RA and cAMP to induce endoderm differentiation results in growth inhibition (Rogers *et al.*, 1992). Moreover, BMP-2 and BMP-4 can induce apoptosis in P19 EC cells (Glozak and Rogers, 1996). To examine the possible type II and type I receptor combinations in ES and EC cells, the mRNA expression patterns of these receptors were analyzed by RT-PCR. Undifferentiated F9 EC cells, as well as F9 EC cells differentiated to primitive endoderm-like cells by RA, or to parietal endoderm-like cells by RA and dibutyryl cyclic AMP (dBcAMP), were found to contain BMPR-II, BMPR-IA and BMPR-IB mRNA (Fig. 3). P19 EC cells also expressed BMPR-II, BMPR-IA and BMPR-IB both before and after differentiation induced by RA treatment (Fig. 3). Similarly, in ES cells all receptor types examined were detected before and after differentiation to an

endoderm-like derivative (Fig. 3). In these cell lines therefore, BMPR-II expression is ubiquitous as in embryos from the blastocyst stage onwards but expression of the type I receptors is not mutually exclusive as in cells of the embryos that might be considered at a similar stage of development.

BMPR-II expression in postimplantation development

Recently, the temporal and spatial expression patterns of ActRI (Feijen *et al.*, 1994), BMPR-IA and BMPR-IB (Dewulf *et al.*, 1995) have been described in detail. However, since these type I BMP receptors are not autophosphorylated, coexpression of a type II BMP receptor is essential for a functional BMP receptor complex. The expression pattern of BMPR-II mRNA was therefore analyzed in more detail in postimplantation embryos from 6.5-16.5 dpc using *in situ* hybridization.

In sections of 6.5 -8.5 dpc embryos, BMPR-II mRNA was ubiquitously expressed, and was detected in both the embryonic

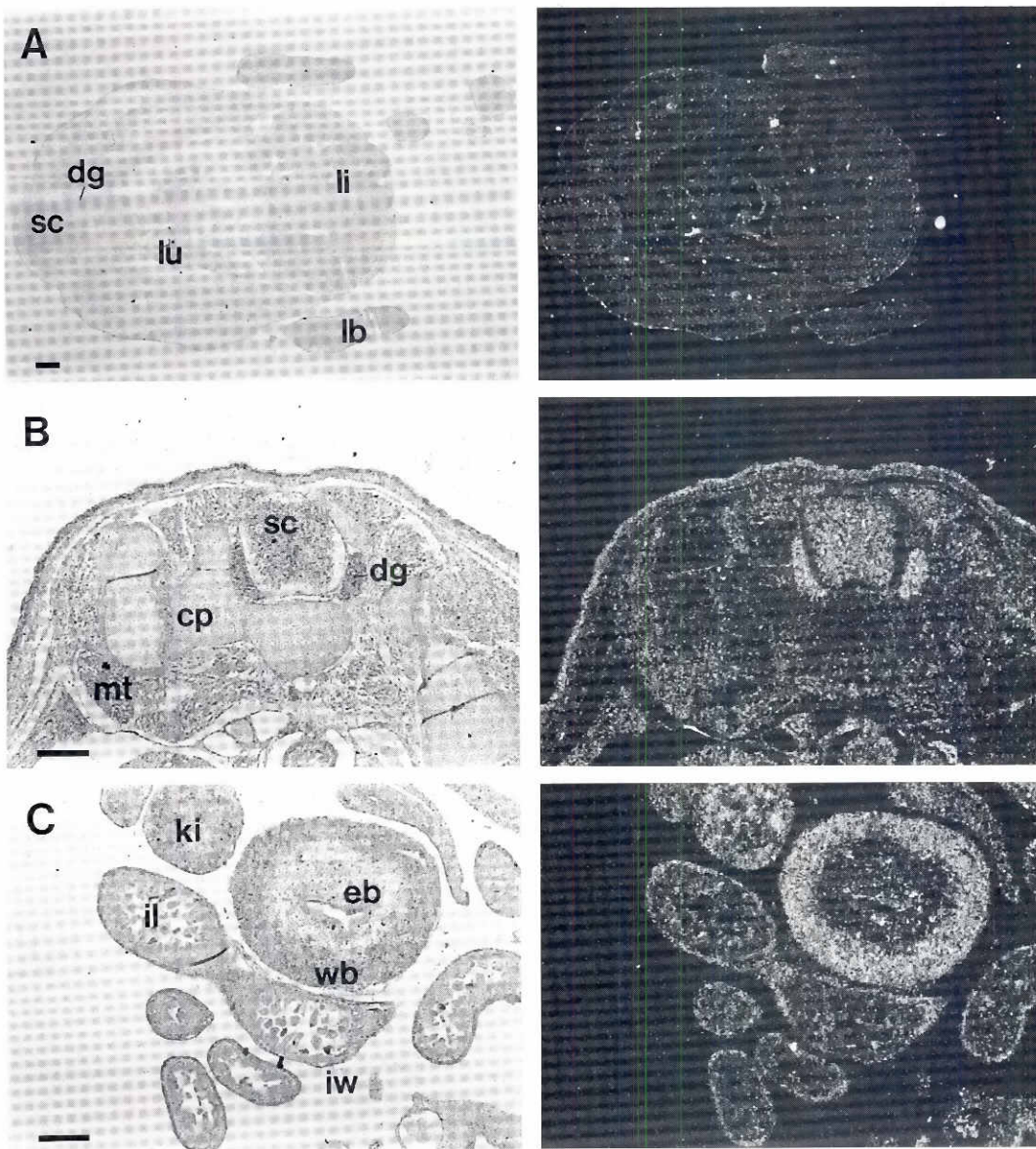


Fig. 5. BMPR-II expression in 12.5 and 16.5 dpc embryos detected with *in situ* hybridization. Brightfield pictures on the left with corresponding darkfield pictures on the right. **(A)** Transverse section of a 12.5 dpc embryo. **(B,C)** Transverse sections of the trunk region of a 16.5 dpc embryo. Abbreviations: cp, cartilage primordium; dg, dorsal root ganglia; eb, endodermal lining of the bladder; il, intestinal wall; ki, right kidney; lb, hind limb bud; li, liver; lu, lung; mt, muscle tissue; sc, spinal cord; wb, wall of bladder. Bars, 250 μ m.

and extraembryonic parts of the conceptus and in the non-embryonic tissues of the pregnant reproductive tract, in agreement with the RT-PCR data. Particularly high expression levels were observed in the trophoblast giant cells, the visceral yolk sac and the developing neural tube (Fig. 4). At 12.5 dpc overall expression of BMPR-II was detected with no obvious differences in expression levels between the various tissues and organs (Fig. 5A). However, the hybridization pattern observed was specific and not due to general non-specific hybridization of the riboprobe, since at 16.5 dpc areas with distinctly high levels of signal were observed using the same probe. Most notably the dorsal root ganglia, muscle tissue, kidney, skin and the walls of the intestines and bladder expressed BMPR-II mRNA significantly above the lower ubiquitous levels (Fig. 5B,C). Remarkably, hybridization was absent or very low in the cartilage primordia (Fig. 5B). No hybridization was detected using the sense probe (data not shown).

Discussion

In many animal species, members of the TGF- β superfamily of growth factors, to which the BMPs belong, play crucial roles in specific developmental events. Initial studies to identify the functions of BMPs in mammalian development analyzed the distribution of ligands (Lyons *et al.*, 1989b, 1990, 1995) or type I receptors (Dewulf *et al.*, 1995) in postimplantation mouse embryos. Here we have extended these studies and examined the expression of a type II BMP receptor, three type I BMP receptors, and BMPs in preimplantation and early postimplantation mouse embryos. In addition we complemented existing studies on the distribution of type I receptors in mid- to late gestation embryos with a description of the expression patterns of the type II BMP receptor at later stages using *in situ* hybridization.

We show that mRNA for BMPR-II is present in one-cell zygotes, two-cell embryos, and later in advanced blastocysts. The

expression at the one- and two-cell stages most likely represents a product from the maternal genome, since embryonic gene transcription only starts in the mouse at the two-cell stage (Flach *et al.*, 1982). Of the type I receptors capable of binding BMPs, ActR-I parallels the type II receptor most closely in its expression pattern, being present in the one-cell zygote, two- and four-cell embryo, and also in the advanced blastocyst. BMPR-IA signal was only detected in advanced blastocysts. It thus represents a product of embryonic gene transcription, whereas BMPR-IB expression was detected at all stages from the one-cell zygote to the uncompact morula, but not in advanced blastocysts. Since maternal gene products are generally degraded rapidly after the start of zygotic transcription (Flach *et al.*, 1982), BMPR-IB mRNA at the one- and two-cell stage is probably of maternal origin, but at the four-cell and uncompact morula stages may be of embryonic origin. These results demonstrate that one- and two-cell stage embryos could have the capacity to respond to BMPs, either through an ActR-I/BMPR-II receptor complex, or by forming a BMPR-IB/BMPR-II receptor complex. In advanced blastocysts, transcripts are present that may form an ActR-I/BMPR-II complex, or a BMPR-IA/BMPR-II complex. These results imply that at different developmental stages, different receptor complexes can be formed, which might be a possibility for multiple responses to be generated by one protein type.

The expression of ActR-I and BMPR-IB at stages when no type II BMP receptor is present, is interesting. Since type I receptors have been shown to be rather promiscuous in interacting with type II receptors it could be that these type I receptors interact via other type II receptors. For ActR-I it is known that it can also form a functional activin receptor complex with the type II activin receptors ActR-II and ActR-IIB (Attisano *et al.*, 1993) and since ActR-II mRNA is present in blastocysts (van den Eijnden-van Raaij *et al.*, 1992) a functional activin receptor complex could also be formed at this stage. Moreover, BMP-7 has been shown to bind to and signal via the complexes of ActR-I/ActR-II, ActR-I/ActR-IIB, BMPR-IB/ActR-II and BMPR-IB/ActR-IIB (Yamashita *et al.*, 1995).

In undifferentiated ES and EC cells, BMPR-II and all three type I BMP receptors are detectable by RT-PCR. These results are consistent with a similarity between undifferentiated ES and EC cells and the inner cell mass (ICM) of the blastocyst, since in the blastocyst transcripts for BMPR-II, ActR-I and BMPR-IA are also detectable. However, in the blastocyst we were not able to detect BMPR-IB mRNA. This could mean that in the cell culture a subpopulation of the cells has started to differentiate, even though they look morphologically undifferentiated. If so, BMPR-IB mRNA levels would be very low in these samples. Of the ligands, it is known that BMP-2 mRNA is absent in undifferentiated F9 cells, but is expressed upon differentiation towards parietal endoderm induced by RA and dbcAMP. BMP-4 mRNA levels, on the other hand, show the opposite pattern, being high in untreated F9 cells and low in differentiated cells (Rogers *et al.*, 1992). The current study suggests that the shift in BMP subtypes upon differentiation is not accompanied by changes in possible receptor combinations.

Embryos in which the BMP-4 gene has been inactivated by homologous recombination develop morphologically normally until 6.5 dpc. Growth in most of these embryos is arrested at the egg cylinder stage, with little or no mesoderm being formed (Winnier *et al.*, 1995). From these results, it was concluded that BMP-4 first becomes indispensable for the embryo during the

proliferation of epiblast cells and mesoderm formation. Mice homozygous for a defective BMPR-IA die during embryogenesis at about day 8.5 pc with severe abnormalities already apparent at day 7.0 pc. The mutant embryos are characterized by a thickened epiblast and the absence of mesoderm (Mishina *et al.*, 1995). By RT-PCR we could detect BMPR-II and ActR-I as early as 6.0 dpc, but BMPR-IA and BMPR-IB were first detected at 7.0 dpc. However, since mouse embryos already have messengers to form functional BMP receptor complexes at the preimplantation stages, an earlier role for BMP-4 cannot be excluded. Although transcripts for BMPs could not be detected in preimplantation embryos themselves, the BMP proteins could be provided by maternal tissues such as ovary, oviduct and uterus. In postimplantation embryos, BMPR-II is ubiquitously expressed until midgestation. Only later in development does BMPR-II expression become more spatially restricted. This expression pattern coincides with that of BMPR-IA, of which mRNA is detected throughout the embryo at least until 15.5 dpc (Dewulf *et al.*, 1995), indicating that in principle many cells could be responsive to BMPs. However, BMP responsiveness is not only dependent on receptor expression but also on the presence of more downstream signaling molecules, some of which (the Smad proteins) have recently been identified (Niehrs, 1996). The localization of these proteins may shed more light on the many different roles BMPs play in the developing mouse embryo.

Materials and Methods

Embryo and tissue collection

Preimplantation embryos were obtained from superovulated (intraperitoneal injection of 5 IU PMSG, 46 h later followed by injection of 5 IU hCG) F1 crosses between C57Bl6 females and CBA males. Zygotes were isolated from the oviducts in M2 culture medium and treated with 0.33 mg/ml hyaluronidase to remove cumulus cells. Two-cell embryos, four-cell embryos and uncompact morulae were isolated from the oviducts, compacted morulae and blastocysts were collected from the uterus. In addition, two-cell embryos were isolated from the oviducts in M2 medium, and cultured in groups of 20 embryos in M16 culture medium covered with paraffin oil at 37°C with 5% CO₂ until later stages. All embryos were rinsed several times before mRNA isolation took place.

Postimplantation embryo collection and germ layer isolation essentially took place as described before (Roelen *et al.*, 1994). Non-embryonic tissues were isolated from 6.5 dpc pregnant C57Bl6 females.

Cell culture

EC and ES cells were cultured as described previously (Mummery *et al.*, 1990). To induce differentiation, P19 EC, F9 EC and ES-5 cells were cultured in monolayer in the presence of 10⁻⁷ M RA for 5 days. To obtain parietal endoderm-like cells, F9 EC cells were cultured in monolayer in the presence of 10⁻⁷ M RA for 5 days followed by 2 days 10⁻³ M dbcAMP.

RNA isolation, cDNA synthesis and PCR

Cultured cells were lysed with guanidine thiocyanate directly after aspiration of the culture medium. Subsequent RNA isolation was performed according to Chirgwin *et al.* (1977). For the embryos, embryonic tissues or in case of preimplantation embryos groups of 15 embryos, were placed in 90 µl Ultraspec solution (Biotecx) and homogenized either by shaking vigorously (embryos) or using a polytron (non-embryonic tissues). After addition of 15 µg polyI (Sigma) as a carrier, the homogenate was kept at 4°C for 5 min, after which 20 µl of chloroform was added. The samples were then shaken vigorously, kept on ice for 5 min and centrifuged at 12,000g for 30 min at 4°C. The aqueous phase was precipitated

TABLE 1

OLIGONUCLEOTIDE PRIMERS USED FOR RT-PCR

product		oligonucleotide sequence	bp position	annealing temp.	reference
BMPR-II (human)	forward	5'-CGCAGAATCAAGAACGGCTATG-3'	80-101	65°C	Kawabata <i>et al.</i> (1995)
	reverse	5'-TGAATGAGGTGGACTGAGTGGT-3'	411-432		
	nested (r)	5'-TTGAGGGAGGAGTGGTAGTTAC-3'	300-321		
ActR-I (human)	forward	5'-AGATGAGAAGCCCAAGGTCAAC-3'	169-190	52°C	ten Dijke <i>et al.</i> (1993)
	reverse	5'-AGTGCCATACTCCACGTCTCGG-3'	580-601		
	nested (f)	5'-GGCTTCCACGTCTACCAGAAAG-3'	287-308		
BMPR-IA (mouse)	forward	5'-TCGTCGTTGTATTACAGGAG-3'	1323-3242	60°C	Dewulf <i>et al.</i> (1995)
	reverse	5'-TTACATCCTGGGATTCAACC-3'	1572-1591		
	nested (r)	5'-ACGATTGGCCGCAAGCGTTT-3'	1429-1448		
BMPR-IB (mouse)	forward	5'-TGGAGCAGTGATGAGTGTCT-3'	1555-1574	60°C	ten Dijke <i>et al.</i> (1994a)
	reverse	5'-TCTGGGTTCTCTGTGTCTG-3'	1810-1829		
	nested (r)	5'-TGAACACTGGGCAGTAGG-3'	1763-1782		
BMP-2 (mouse)	forward	5'-AGACGTCCTCAGCGAATTTG-3'	1879-1898	60°C	Feng <i>et al.</i> (1994)
	reverse	5'-GTTTGTGTTTGGCTTGACGC-3'	8589-8608		
	nested (r)	5'-GACGACCTGTGTTCACTTTG-3'	8500-8519		
BMP-4 (mouse)	forward	5'-TGTGAGGAGTTTCCATCACG-3'	7156-7175	60°C	Kurihara <i>et al.</i> (1993)
	reverse	5'-TTATTCTTCTTCTGGACCG-3'	8707-8726		
	nested (f)	5'-TTCCTTCAACCTCAGCAG-3'	8230-8249		
BMP-7 (mouse)	forward	5'-GACATGGTCATGAGCTTCGT-3'	491-510	60°C	Özkaynak <i>et al.</i> (1991)
	reverse	5'-GTCAAGTAGAGGACAGAGA-3'	1311-1320		
	nested (r)	5'-TGGCGTTCATGTAGGAGTTC-3'	1201-1220		
β -actin (mouse)	forward	5'-TGAACCCTAAGGCCAACCGTC-3'	409-429	56°C	Tokunaga <i>et al.</i> (1986)
	reverse	5'-GCTCATAGCTCTTCTCCAGGG-3'	784-804		
	nested (r)	5'-TGTAGCCACGCTCGGTCAGGA-3'	655-675		

for 30 min with isopropanol at -20°C and centrifuged at 12,000g for 30 min at 4°C. The RNA pellet was subsequently washed with 75% ethanol, dried under a vacuum and dissolved in distilled water. After DNase treatment with 5 U DNase (Promega) for 1 h at 37°C, the RNA was phenol/chloroform extracted and precipitated overnight with 100% ethanol at -20°C. cDNA synthesis took place as described before (Roelen *et al.*, 1994). For the preimplantation embryos the equivalent of 7.5 embryos, and for postimplantation embryos, tissues, EC and ES cells, 1 µg RNA was used for cDNA synthesis. PCR was carried out using 2 µl of the cDNA product. For the preimplantation embryo material this is 0.75 embryo-equivalents for each reaction. Amplification took place in a total volume of 50 µl containing 75 mM Tris-HCl pH 9.0, 0.1% (w/v) Tween 20, 20 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 0.2 mM dGTP, dATP, dTTP, and dCTP each (Gibco-BRL), 1 µM of each specific oligonucleotide primer and 1.25 U Goldstar polymerase (Eurogentec). PCR was performed in a Perkin-Elmer 2400 thermal cycler. After 5 min denaturation at 94°C, 40 cycles of PCR were performed with each cycle including 15 sec at 94°C, 30 sec annealing at a primer specific temperature (Table 1), and 45 sec primer extension at 72°C. After 40 cycles, samples were kept at 72°C for 5 min and then chilled to 4°C. In order to enhance sensitivity of detecting gene expression in preimplantation embryos, a heminested PCR of 30 cycles followed the first 40-cycle round PCR amplification. Two µl of the first-round PCR product was used for a second 30-cycle round of PCR with one oligonucleotide primer identical to the first-round PCR and one oligonucleotide primer internal to the first-round PCR product. As a control for genomic DNA contamination, RNA samples in which reverse

transcription had been omitted were included. Also in every PCR amplification a water control and a positive control (cDNA from tissue known to express significant levels of the gene of interest) were included. Amplification was repeated on several independent samples to ensure reliability of the data. The PCR products were run on 6% polyacrylamide gels which were stained for 5 min with 0.4 µg/ml ethidium bromide after electrophoresis. Documentation took place with a CCD camera coupled to a computer-linked imaging system (The Imager, Appligene).

Probe synthesis and in situ hybridization

With the oligonucleotide primers as indicated in Table 1 a 352 bp BMPR-II specific DNA fragment was amplified from mouse uterus cDNA using PCR. The DNA fragment was ligated into a PGEM-T vector (Promega), transformed into DH5 α bacteria after which its identity was confirmed by sequencing. The vector was linearized by *Sa*I digestion and a single stranded ³⁵S labeled RNA probe was generated with T7 polymerase (antisense probe) or Sp6 polymerase (sense probe) as described before (Feijen *et al.*, 1994). *In situ* hybridization took place overnight at 55°C, and washing at 65°C at high stringency essentially as described before (Feijen *et al.*, 1994).

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