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Original Article

Parathyroid hormone related peptide mRNA expression during murine postimplantation development: evidence for involvement in multiple differentiation processes

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ABSTRACT In this study we describe the spatio-temporal expression of Parathyroid Hormone related Peptide (PTHrP) mRNA during murine postimplantation development from day 5.5 post coitum (pc) until day 12.5 pc. From day 5.5 pc and onwards PTHrP mRNA was detected in the trophoblast. In addition, at day 5.5 and 6.5 pc epithelial cells of the antimesometrial crypt and cells of the inner zone of the decidua directly adjacent to the implanted embryo expressed PTHrP mRNA. This supported a previous model in which parietal endoderm formation is regulated by a paracrine mechanism involving PTHrP expressing trophoblast cells and receptor expressing extra-embryonal endoderm cells. The first embryonal PTHrP mRNA expression was detected in the roof of the hindbrain at gestation day 10.5 pc. From day 11.5 pc and onwards PTHrP mRNA was detected in the otic vesicle, the semilateral channels, the roof of the hindbrain and later in the choroid plexus, in epithelial cells of the lung and heart ventricle, mesenchymal cells lining the nasal pit, the dermis of the snout and at all sites of endochondral bone formation. The widespread expression of PTHrP mRNA during embryogenesis in extra-embryonic and embryonic tissues suggests the involvement of the peptide in multiple growth and differentiation processes.

KEY WORDS: *parathyroid hormone related peptide, parathyroid hormone/parathyroid hormone related peptide receptor, extra-embryonal tissues, bone formation, postimplantation development*

Introduction

Parathyroid Hormone related Peptide (PTHrP) is a multifunctional peptide hormone that is involved in the regulation of various (patho)physiological processes. PTHrP is the major cause of malignancy associated humoral hypercalcemia by virtue of its parathyroid hormone (PTH)-like effects (reviewed in Mallette, 1991; Orloff *et al.*, 1994) and is involved in processes as skeletal development (Karaplis *et al.*, 1994), skin development (Wysolmerski *et al.*, 1994), smooth muscle relaxation (Williams *et al.*, 1994) and fetal calcium homeostasis (Rodda *et al.*, 1988).

Recently, we and others have proposed a role for PTHrP and its receptor (PTH/PTHrP receptor) in the formation of parietal endoderm (Chan *et al.*, 1990; van de Stolpe *et al.*, 1993; Karperien *et al.*, 1994). The formation of the parietal endoderm starts in the mouse blastocyst when cells of the inner cell mass (ICM) differentiate into primitive endoderm. Primitive endoderm cells migrating over the trophectoderm will form parietal endoderm, while cells which remain covering the growing egg cylinder will differentiate into visceral endoderm (reviewed in Gardner, 1983). Using F9 embryonal carcinoma (EC) and embryonal stem (ES-5) cells as *in vitro* model systems, it was shown that primitive endoderm-like cells obtained after retinoic acid treatment abundantly expressed PTH/PTHrP receptors. The subsequent addition of PTHrP efficiently induced further differentiation into parietal endoderm-like cells, suggesting that PTHrP might be an inducer of parietal endoderm differentiation *in vivo* (van de Stolpe *et al.*, 1993). This was corroborated by findings that the trophectoderm of the mouse blastocyst expressed PTHrP protein (van de Stolpe *et al.*, 1993) and that parietal endoderm expressed PTH/PTHrP receptor mRNA during the initial stages of postimplantation development (Karperien *et al.*, 1994).

Abbreviations used in this paper: PTHrP, parathyroid hormone related peptide; PTH, parathyroid hormone; PTH/PTHrP receptor, parathyroid hormone/parathyroid hormone related peptide receptor; ICM, inner cell mass; pc, post coitum.

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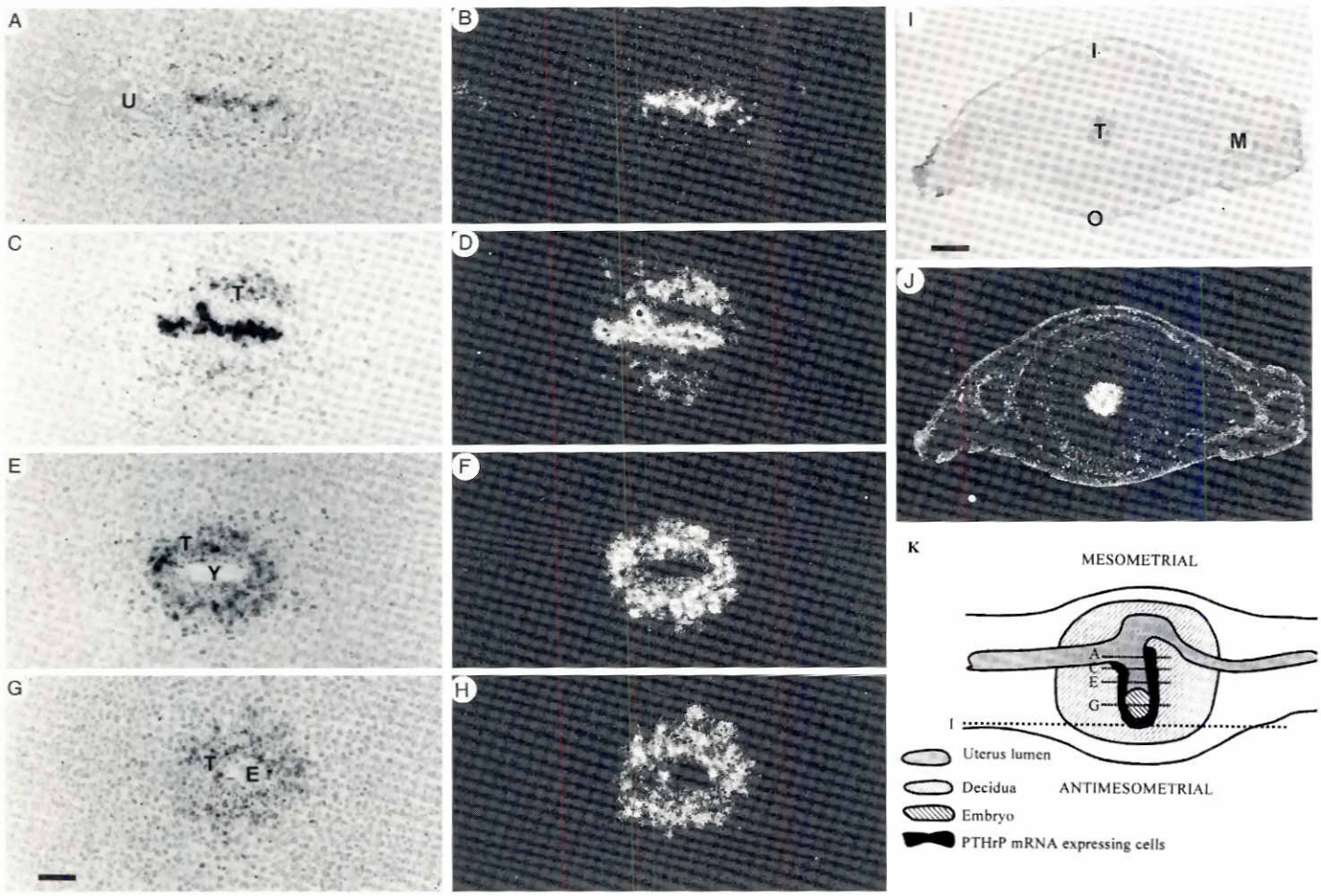


Fig. 1. PTHrP mRNA expression in the uterus at 5.5 days post coitum detected by *in situ* hybridization. (A-G) *Brightfield pictures of transversal sections through decidua and antimesometrial crypt marking the implantation site. The place of sections in the decidua is schematically depicted in* **(K)**. **(B-H)** *Corresponding darkfield illumination.* **(I-J)** *Bright- and darkfield illumination, respectively, of a transversal section through the uterus containing an implantation site. Location of the section is schematically depicted in* **(K)**. **(K)** *Schematic representation of the uterus at day 5.5 of gestation indicating the location of the sections* **(A-I)**. *Abbreviations: im, inner circular layer of myometrial smooth muscle; om, outer longitudinal layer of myometrial smooth muscle; m, endometrium; t, cells of the inner zone of the decidual reaction; u, uterine lumen; e, embryonic ectoderm; y, remnant of uterine lumen near the implanted embryo. Bar: 100 mM A-H and 400 mM I-J.*

At present, data on the expression of PTHrP mRNA and protein during murine postimplantation development are lacking. In rat and human fetuses PTHrP is expressed in multiple tissues derived from all 3 germ layers during organogenesis (Moniz *et al.*, 1990; Campos *et al.*, 1991; Senior *et al.*, 1991; Lee *et al.*, 1995). Embryonal PTHrP mRNA expression in the rat was first detected at gestation day 13.5 (Senior *et al.*, 1991).

In this study, we provide a systematic survey of PTHrP mRNA expression during murine postimplantation development starting at day 5.5 post coitum (pc) until day 12.5 pc using radioactive *in situ* hybridization. In the 5.5 day-old conceptus, PTHrP mRNA expression was found in uterine epithelial cells lining the antimesometrial crypt, in cells of the inner decidual zone surrounding the implanted embryo and in the trophoblast. The mRNA was detectable in cells of the trophoblast until at least day 11.5 pc, while the expression in the uterine epithelial and decidual cells gradually disappeared. This expression pattern supported a previous model in which parietal endoderm differentiation is regulated by the paracrine interaction between PTHrP producing trophoblast and decidual cells and receptor-expressing extra-embryonal endoderm cells (van de Stolpe *et al.*, 1993; Karperien *et al.*, 1994). The embryo proper did not express detectable amounts of the message until day 10.5 pc. Thereafter, high PTHrP mRNA expression levels were found at sites of endochondral bone formation which started in the mesenchyme of precartilage condensations at day 11.5 pc and in the dermis of the snout from day 11.5 pc and onwards. Additional PTHrP mRNA expression was detected in the roof of the hindbrain from day 10.5 pc, epithelial cells of the lung buds and the ventricle, the dermis, the otic vesicle from day 11.5 pc and the choroid plexus from day 12.5 pc and onwards, in line with and extending previous observations in rat and human fetuses.

Results

Recently, we have provided evidence that PTHrP is expressed in the trophectoderm of the preimplantation mouse blastocyst, as demonstrated by immunofluorescence (van de Stolpe *et al.*,

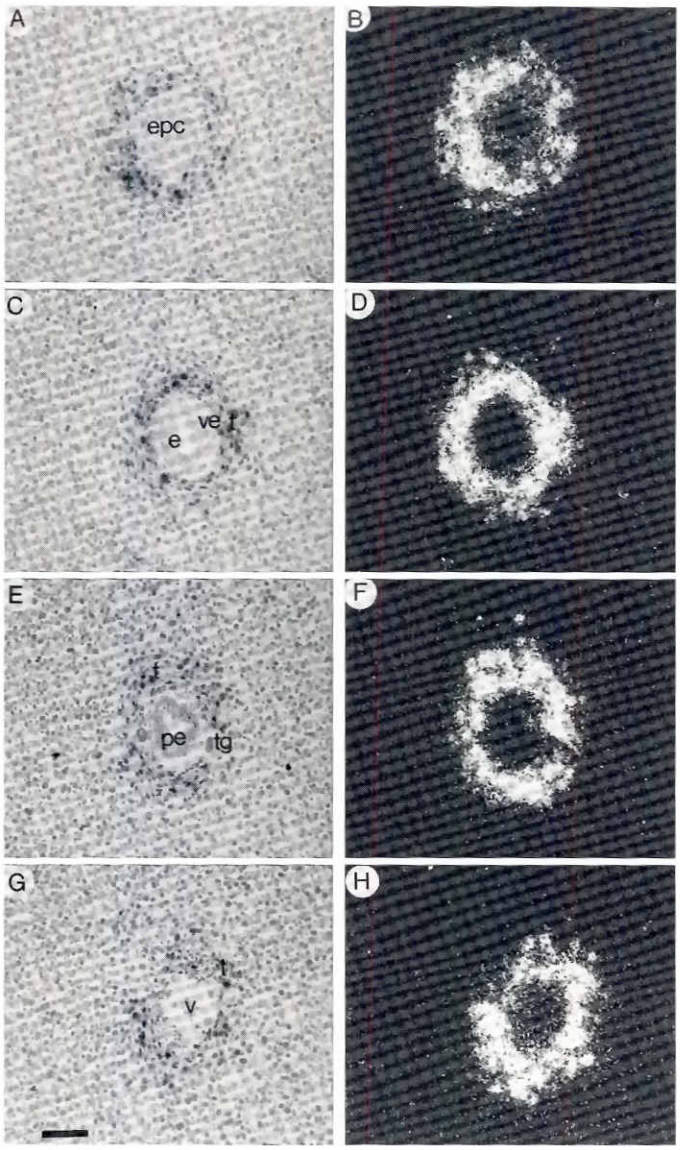


Fig. 2. PTHrP mRNA expression in the decidua and embryo at day 6.5 of gestation detected by *in situ* hybridization. (A-G) *Sequential brightfield illuminations of transversal sections at height of the ectoplacental cone* **(A-B)**, *the ectoplacental cavity* **(C-D)**, *and the embryo proper* **(E-H)**. **(B-H)** *Corresponding darkfield illuminations. Abbreviations: e, extra-embryonic ectoderm; epc, ectoplacental cone; pe, embryonic ectoderm; t, cells of the inner decidual zone; tg, mural trophoblast giant cell; v, visceral embryonal endoderm; ve, visceral extra-embryonal endoderm. Bar, 80 mM.*

1993). This suggested that PTHrP might be expressed by cells of the trophoblast lineage during early postimplantation development and might act as a paracrine factor for parietal and visceral endoderm cells which, at these stages, express PTH/PTHrP receptors (Karperien *et al.*, 1994). In search for PTHrP mRNA expressing cells in the uterus at gestation day 5.5, uteri were isolated, fixed in paraformaldehyde and embedded in paraffin. Subsequently, 6 μM transverse sections containing an implantation site were cut and subjected to radioactive *in situ* hybridization using an antisense mouse α^{35}S-UTP labeled PTHrP riboprobe. Using a sense PTHrP probe as a negative control, no hybridization was observed at any of the gestation stages tested (data not shown). As shown in Figure 1, several cell types, both of embryonal and maternal origin, expressed high levels of PTHrP mRNA. PTHrP mRNA expression was detected in epithelial cells lining the antimesometrial crypt marking the implantation site, in contrast to epithelial cells covering other parts of the uterine lumen (Fig. 1A-B, see Fig. 1K for schematic representation of sections). In a more antimesometrial section, additional PTHrP mRNA expressing cells were detected in the inner zone of the decidual reaction surrounding the implantation crypt (Fig. 1C-D). Near the embryo, trophoblast cells, which had degenerated the uterine epithelium and maternal decidual cells, expressed PTHrP mRNA (Fig. 1E-F). At this stage the trophoblast is about one cell layer thick and begins to invade the decidua (Theiler, 1972; Schlafke and Enders, 1975). Both cell types were found expressing the message in the next section through the epiblast (Fig. 1G-H) and in a section just beneath the implanted embryo (Fig. 1I-J). In all sections, there were more maternal PTHrP expressing cells than embryonal PTHrP expressing trophoblast cells. The embryonic ectoderm did not express PTHrP mRNA (Fig. 1G-H). Interestingly, in an overview of the decidua and surrounding uterine tissues, other hybridization sites were also detected, although less intense than the hybridization signal present in the decidual cells lying in the inner zone and in the trophoblast cells. PTHrP mRNA was present in cell layers belonging to the outer zone of the decidua and in the inner circular and outer longitudinal myometrial smooth muscle layers (Fig. 1I-J).

In transverse sections through the decidua of a 6.5 day-old mouse conceptus, PTHrP mRNA expression was detected in decidual cell layers surrounding both extra-embryonic (Fig. 2A-D) and embryonic parts of the growing egg-cylinder (Fig. 2E-H). In addition, PTHrP mRNA was detected in most trophoblast cells. No PTHrP mRNA expression was detected in other parts of the egg-cylinder such as the embryonal and extra-embryonal endoderm (Fig. 2C-H), the primitive ectoderm (Fig. 2E-H) and the extra-embryonic ectoderm (Fig. 2A-D) except for some cells in the ectoplacental cone which faintly hybridized with the antisense PTHrP riboprobe (Fig. 2A-B). These cells are probably precursors of trophoblast cells. Primary giant cells of mural trophectodermal origin did not express detectable levels of PTHrP mRNA (Fig. 2E-F).

In the 7.5 day-old mouse conceptus, PTHrP mRNA was highly expressed by the invasive trophoblast cells and in some of the decidual cells adjacent to the embryo (Fig. 3A-B). No mRNA expression was detected in the embryo proper and other extra-embryonic structures, including the allantois and the parietal endoderm (Fig. 3A-B). The latter was in contrast with previous observations made in *in vitro* model systems, in which PTHrP mRNA and protein were detected in parietal endoderm-like cells (Chan *et al.*, 1990; van de Stolpe *et al.*, 1993). In the 8.5d and 9.5d pc embryos PTHrP mRNA expression was solely detectable in the trophoblast giant cells which completely surrounded the developing embryo (Fig. 3C-F; data of 8.5d pc embryos not shown), while neighboring decidual cells did not express detectable mRNA levels. In addition, single scattered cells in the outer zone of the decidua expressed high levels of PTHrP mRNA (Fig. 3C-D and for magnification Fig. 3E-F). According to their position these cells are probably of maternal origin.

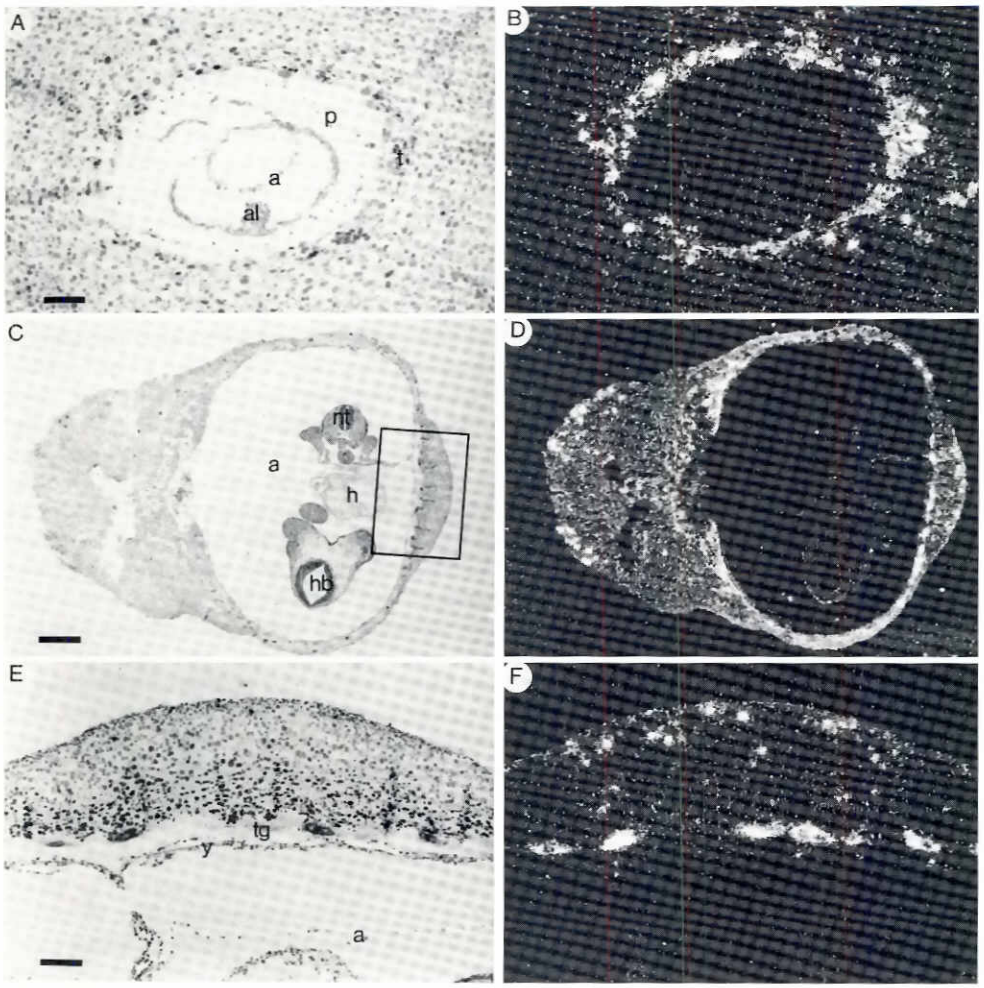


Fig. 3. PTHrP mRNA expression in 7.5 day-old and 9.5 day-old concepti detected by *in situ* hybridization. (A-B) *Transversal section through decidua and extra-embryonal structures of a 7.5d pc embryo.* **(C-D)** *Cross section through a 9.5d pc embryo and the surrounding decidua.* **(E-F)** *Higher magnification of box in* **(C)**. *Abbreviations: a, amnion; al, allantois; h, heart; hb, hindbrain; i, individually scattered cells expressing PTHrP in the outer zone of the decidua; nt, neural tube; p, parietal endoderm; t, trophoblast; tg, trophoblast giant cell; y, visceral yolk sac. Bar: 180 mM A-B, 400 mM C-D and 100 mM E-F.*

Summarizing, PTHrP mRNA expression was detected in cells of the trophoblast from day 5.5 pc onwards, until at least day 11.5 pc (data not shown). This was consistent with our previous observations in which we detected PTHrP protein in the trophectoderm of the mouse blastocyst (van de Stolpe *et al.*, 1993). Additionally, PTHrP mRNA was highly expressed by maternal uterine epithelial cells lining the antimesometrial crypt and in cells lying in the inner zone of the decidua in the 5.5d pc conceptus. The latter still expressed the message at day 6.5 but the mRNA expression in these cells gradually disappeared thereafter and was not detected in 8.5d pc and older embryos. The embryo proper and other extra-embryonic structures did not express detectable levels of PTHrP mRNA until gestation day 9.5. In addition, PTHrP mRNA was expressed in the smooth muscle layers of the uterus of gestation day 5.5 and in the 8.5 and 9.5d pc concepti also in scattered cells in the outer zone of the decidua.

PTHrP mRNA expression in embryonic tissues

Before day 10.5 pc no PTHrP mRNA was detected in the embryo proper. This was remarkable, since from day 9.5 pc onwards PTH/PTHrP receptor mRNA is expressed by the epithelium of the intestine (Karperien *et al.*, 1994). At day 10.5 pc the first faint embryonal PTHrP mRNA expression was found in the roof of the hindbrain (data not shown). From day 11.5 pc and onwards, PTHrP mRNA expression was detected in a wide variety of tissues representing derivatives of all 3 germ layers. In the 12.5 day-old mouse embryo, PTHrP mRNA was detected in the choroid plexus, in mesenchymal cells lining the nasal pit, in epithelial cells of the lung and in some epithelial cells of the ventricle, in the dermis, especially in the snout region, in the semilateral channels and at all sites of endochondral bone formation (Fig. 4A-D).

Around day 11.5 pc, endochondral bone formation begins with the condensation of mesenchymal cells. These cells differentiate into chondrocytes that eventually form cartilage which will be replaced by mineralized bone after vascularization (Cowell *et al.*, 1987). PTHrP mRNA was detected at sites of bone formation beginning at day 11.5 pc and onwards. In the 11.5d pc mouse embryos faint hybridization was observed in the precartilage condensations of the ribs and vertebrae (data not shown). In 12.5 day pc embryos, PTHrP mRNA expression was detectable at various sites of precartilage condensation of the vertebrae, the ribs, the mandible and the bones of the extremities (Figs. 4 and 5). At this stage some condensed mesenchymal cells of the ribs, vertebrae and bones of the extremities have differentiated into maturing chondrocytes which abundantly express PTH/PTHrP receptor mRNA (Lee *et al.*, 1993; Karperien *et al.*, 1994). PTHrP mRNA was not detectable in these cells but its expression was

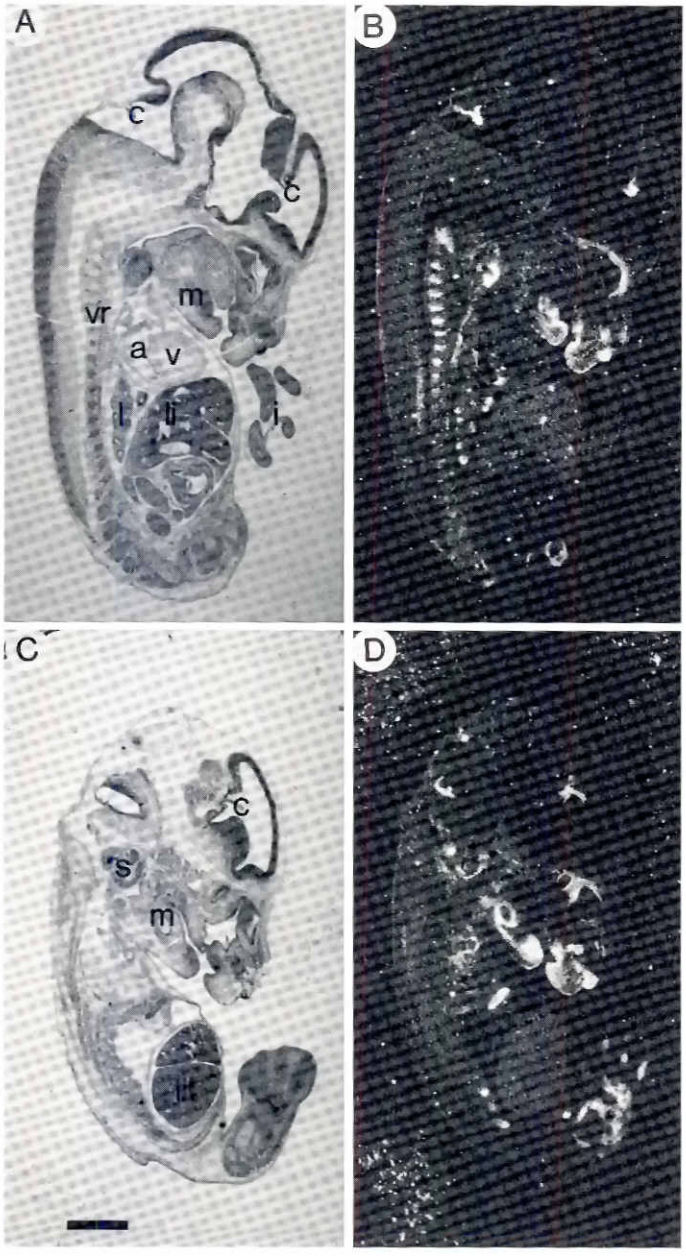


Fig. 4. PTHrP mRNA expression in embryos at gestation day 12.5. detected by *in situ* hybridization. (A-B) *Near mid-sagittal section of a 12.5 day mouse embryo.* **(C-D)** *Parasagittal section of a 12.5 day mouse embryo. Abbreviations: a, atrium; c, choroid plexus; i, intestine; l, lung; li, liver; m, mandible; s, semicircular canal; v, ventricle; vr, vertebra. Bar, 800 mM.*

restricted to the growth plate chondrocytes as shown in a parasagittal section through the hindlimb bud (Fig. 5A-B) and in a transversal section through the forelimb bud (Fig. 5C-D).

In the 11.5 and 12.5 day pc mouse embryos, strong hybridization was observed in the snout and in mesenchymal cells lining the nasal pit. The expression in the snout was localized in the dermis (Fig. 4A-D). Interestingly, highly localized expression of PTHrP mRNA was detected in two small clusters of dermal cells rostrally and caudally localized relative to the attached hind limb bud in the 11.5 and 12.5d pc mouse embryos. Similar clusters of PTHrP expressing cells were detected at both sides of the forelimb bud in a 12.5d pc mouse embryo. A cluster of PTHrP expressing cells is shown in Figure 5A-B (see arrow) representing a sagittal section through the hindlimb bud of a 12.5 day pc mouse embryo. As reported previously, expression of PTH/PTHrP receptor mRNA was particularly high in dermal regions near the hindlimb bud (Karperien *et al.*, 1994).

PTHrP mRNA expression was found in the developing head of 10.5, 11.5 and 12.5 day pc mouse embryos in several neuroectodermal derivatives. The first mRNA expression was found in the roof of the hindbrain at day 10.5 pc. One day later, mRNA expression was additionally detected in the epithelium of the otic vesicle. To better characterize PTHrP mRNA expression patterns in the brain of 12.5d pc mouse embryos, sequential transversal sections were subjected to *in situ* hybridization. PTHrP mRNA expression was detected in the roof of the third ventricle (Fig. 6A-B), and in forebrain structures giving rise to the choroid plexus such as the medial walls of the lateral ventricles (Fig. 6A-B) and the choroidal fissure (Fig. 6C-D). In addition, PTHrP mRNA was expressed by structures arising from the roof of and extending into the central part of the cavity of the fourth ventricle which will form the more voluminous part of the choroid plexus (Fig. 6C-H). At day 11.5 pc PTHrP mRNA was detected in the epithelium of the otic vesicle (data not shown). In the 12.5d pc embryo, the otic vesicle has differentiated into the semicircular canals which expressed the message and in the endolymphatic sac which did not (Fig. 6I-J). In addition, high PTHrP mRNA expression was detected in the mesenchymal precartilage condensation of the otic capsule surrounding the cochlea which will form the future petrous part of the temporal bone (Fig. 6K-L) and in the precartilage condensations of the first branchial arch (Meckels precartilage mass) (Fig. 6K-L). The latter sites were not reported to express detectable levels of PTH/PTHrP receptor mRNA until day 13.5 pc (Karperien *et al.*, 1994), when the precartilage condensations have differentiated into chondrocytes.

Discussion

Recently, we and others have proposed a role for PTHrP and the PTH/PTHrP receptor in the formation of extra-embryonic endoderm. The formation of extra-embryonic endoderm starts at the blastocyst stage in which ICM cells lining the blastocoel differentiate into primitive endoderm. Some primitive endoderm cells start to migrate over the trophectoderm and will form the parietal endoderm, while the remaining cells covering the growing egg cylinder differentiate into visceral endoderm (reviewed in Gardner, 1983; Hogan *et al.*, 1983). Evidence for a role of the PTHrP/receptor signaling system in this process comes from several observations made *in vitro* and *in vivo*. Using *in vitro* model systems, it was shown that retinoic acid treatment of F9 EC and ES-5 cells results in the differentiation of primitive endoderm-like cells. These cells abundantly express PTH/PTHrP receptors. The subsequent treatment of primitive endoderm-like cells with PTHrP induces parietal endoderm differentiation (Chan *et al.*, 1990; van de Stolpe *et al.*, 1993). This suggests that PTHrP and its receptor might act as an endogenous signaling system regulating parietal endoderm differentiation *in vivo*. This hypothesis was supported by the detection of PTHrP protein in the trophectoderm of the mouse blastocyst and by the expression of

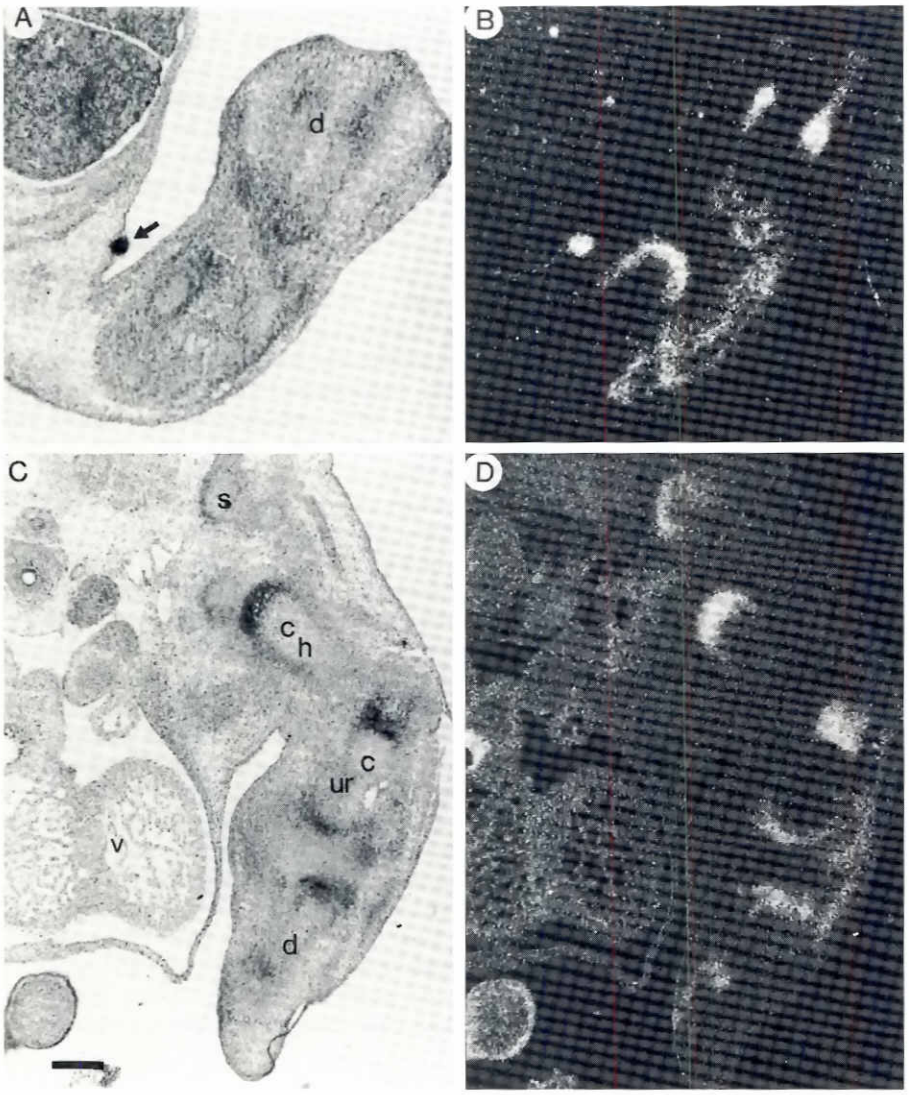
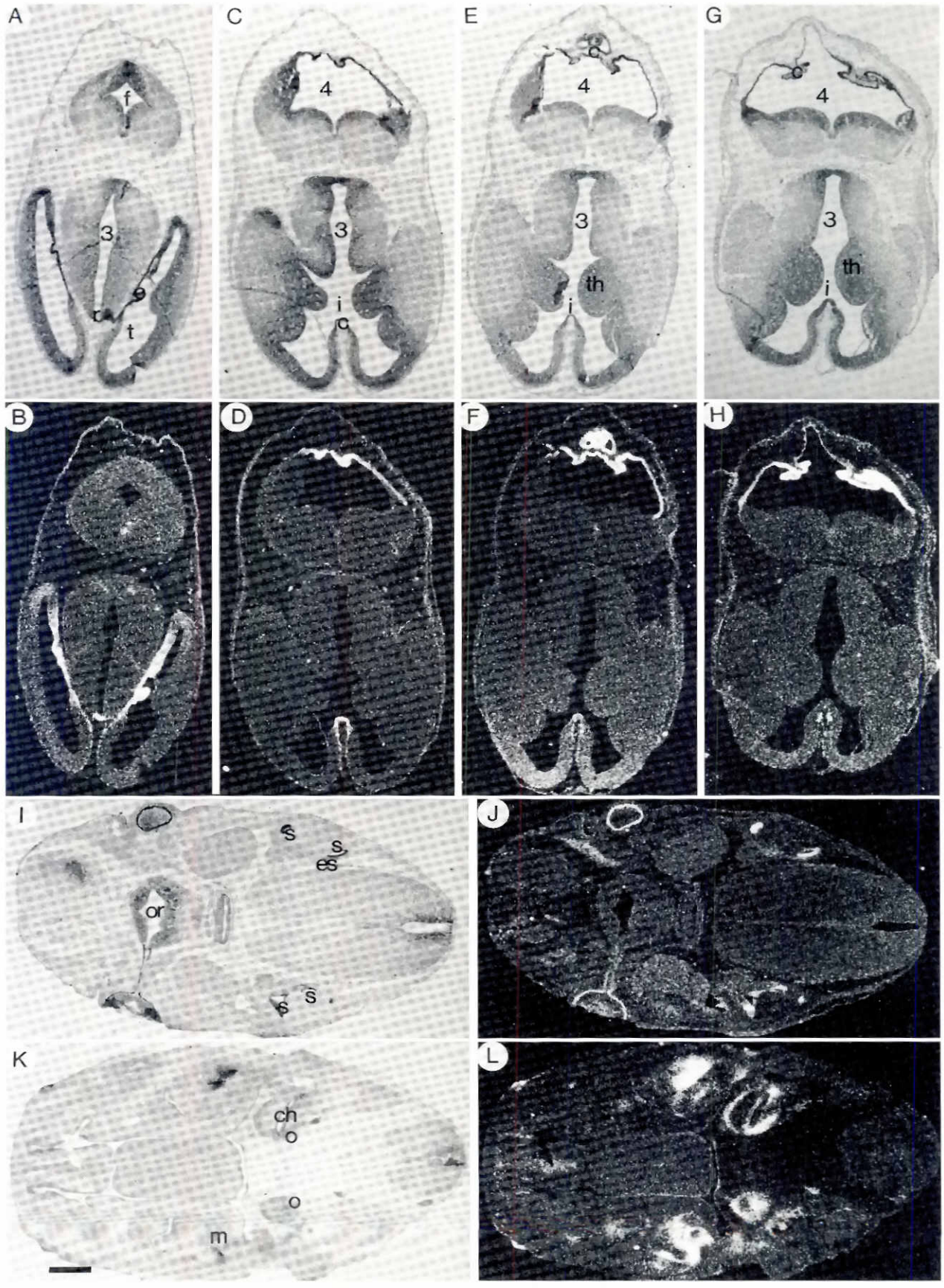


Fig. 5. PTHrP mRNA expression in precartilage condensations of the extremities at day 12.5 pc as detected by *in situ* hybridization. (A-B) *Sagittal section through the hind limb bud of a 12.5 day-old mouse embryo. Arrow marks cluster of highly localized dermal cells expressing PTHrP mRNA.* **(C-D)** *Transversal section through the forelimb bud of a 12.5 day pc mouse embryo. Abbreviations: c, chondrocytes; d, digital bones; h, humerus; l, liver; v, ventricle; ur, ulna and radius; s, precartilage primordium of shoulder. Bar, 200 mM.*

PTH/PTHrP receptor mRNA in parietal and visceral endoderm during early stages of murine postimplantation development (van de Stolpe *et al.*, 1993; Karperien *et al.*, 1994). The observations made in this study provide additional evidence in support for a role of the PTHrP/receptor signaling system in the formation of extra-embryonic endoderm. PTHrP mRNA expression was detected in the trophoblast in the earliest stage we tested, the 5.5 day pc embryo, while, in contrast, the embryonic ectoderm did not express the message. PTHrP mRNA remained expressed by the trophoblast cells until at least day 11.5 pc. From day 8.5 pc onwards the expression was restricted to the trophoblast giant cells. PTHrP mRNA was not detected in the embryo proper until day 10.5 pc nor in extra-embryonic ectoderm and endoderm at any stage tested. Interestingly, PTHrP mRNA is also expressed in trophoblast giant cells of the rat conceptus from day 7.5 pc and onwards (Senior *et al.*, 1991; Beck *et al.*, 1993). These results indicate that PTHrP and its receptor have complementary expression patterns during the initial stages of murine postimplantation development and strongly support a model in which PTHrP and its receptor constitute an endogenous signaling system regulating parietal endoderm differentiation.

If this model holds true, one would expect PTHrP knock out embryos to die early in gestation due to defects in the formation of the parietal yolk sac. However, Karaplis *et al.* (1994) showed that PTHrP depleted embryos develop a macroscopically normal parietal endoderm and die around birth. Apparently, the absence of embryonal PTHrP does not inhibit parietal endoderm formation, although a detailed analysis excluding the existence of microscopic abnormalities is lacking. This indicates that: i) the PTHrP/receptor signaling system is not involved in extra-embryonic endoderm differentiation, or ii) other ligand receptor systems take over the role of the PTHrP/receptor signaling system, or iii) maternally derived PTHrP (or PTH) complements the absence of embryonic PTHrP. Data presented in this study support the possibility of the last option. In the 5.5 day pc conceptus, high PTHrP mRNA levels were expressed by maternal cells in close vicinity of the embryo such as the uterine epithelial cells of the antimesometrial crypt which have also been reported to express PTHrP mRNA in the rat uterus (Beck *et al.*, 1993) and cells of the inner zone of the decidua which remained expressing PTHrP until at least day 6.5 pc. Interestingly, the maternal PTHrP expressing decidual cells outnumbered the PTHrP mRNA positive trophoblast cells in these stages, which might suggest that PTHrP is mainly derived from the mother in early murine (post)implantation development. This is supported by the absence of detectable levels of PTHrP mRNA in mural trophectodermal giant cells in the 6.5 day pc embryos, in contrast to the trophoblast giant cells in later stages of development which express high levels of PTHrP mRNA. Remarkably, no PTHrP mRNA expression was detected in

Fig. 6. PTHrP mRNA expression in the developing head of a 12.5d pc embryo as detected by *in situ* hybridization. (A-K) *Bright field illuminations of sequential transversal sections through the head of a 12.5d pc mouse embryo.* **(B-L)** *Corresponding darkfield illuminations. Abbreviations: c, choroid plexus/choroidal fissure; ch, cochlea; e, epithalamus; es, endolymphatic sac; f, fourth ventricle; i, intraventricular foramen; m, precartilage primordium of Meckels cartilage; o, mesenchymal precartilage condensation of otic capsule; or, optic recess; r, roof of third ventricle; s, semicircular canal; t, telencephalic vesicle; th, thalamus; 3, third ventricle; 4, fourth ventricle. Bar, 400 mM.*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decidual cells of the rat uterus in comparable stages of gestation by *in situ* hybridization (Beck *et al.*, 1993). The significance of this species specificity is unclear at present. However, the presence of decidual cells expressing PTHrP in the mouse renders it likely that maternally derived PTHrP is able to complement the absence of PTHrP in the knock out mice via a paracrine mechanism. This requires transfer of maternal PTHrP to the embryo. Transfer of maternal growth factors to knock out embryos has also been described to occur for other growth factors such as TGFβ (Shull *et al.*, 1992). Therefore, to establish the role of PTHrP and its receptor in the formation of extra-embryonic endoderm, knock out embryos of the PTH/PTHrP receptor might prove to be more elusive, since in these mice maternal PTHrP will not be able to complement the lack of embryonal receptor expression.

Additional support for a role of PTHrP in the formation of parietal endoderm is presented in a recent paper by Behrendtsen *et al.* (1995). It was shown that isolated ICMs formed an outgrowth of parietal endoderm cells when plated on fibronectin. The outgrowth of parietal endoderm was significantly enhanced by the addition of exogenous PTHrP or medium conditioned by trophectoderm cells. The activity of the trophectoderm conditioned medium was abolished by a function-perturbing antibody to PTHrP. Moreover, the outgrowth of parietal endoderm on other substrata than fibronectin was critically dependent on the presence of exogenous PTHrP. This study suggests that besides the PTHrP/receptor signaling system also a cooperative interplay of extracellular matrix and integrins is required for the formation of parietal endoderm.

What other roles might be played by PTHrP expressed by maternal cells in the decidua and uterus? It is possible that the peptide is involved in the implantation of the embryo in the uterus, given its highly localized expression in the epithelial cells of the antimesometrial crypt. Several groups have shown that PTHrP is able to relax uterine smooth muscles and to inhibit uterus contractility before partus (Thiede *et al.*, 1991; Daifotis *et al.*, 1992; Paspaliaris *et al.*, 1992; Williams *et al.*, 1994). The expression of PTHrP mRNA in the longitudinal and circular smooth muscle layers of the uterus of 5.5 day pregnant mice is in line with and extends these observations to early postimplantation stages. PTHrP has been shown to act on the smooth muscles of the vasculature thereby increasing the blood flow (Davicco *et al.*, 1993; Smogorzewski *et al.*, 1993; Burton *et al.*, 1994). The implanted conceptus is surrounded by multiple blood vessels which are required for the efficient transportation of nutrients to the embryo. Locally produced PTHrP might stimulate the flow through these vessels. Furthermore, PTHrP might be involved in the regulation of fetal calcium metabolism as has been suggested by others (Rodda *et al.*, 1988; Care *et al.*, 1990). More studies are needed to define the role of PTHrP during the early stages of embryonal development.

PTHrP expression during organogenesis

PTHrP mRNA expression was detected in the embryo proper from day 10.5 pc onwards. At day 10.5 and 11.5 pc the expression was restricted to the roof of the hindbrain and from day 12.5 onwards specifically detected in fore- and hindbrain structures forming the choroid plexus. mRNA expression in the choroid plexus was in line with and extends the observations made by others in rat and human embryos using immunohistochemistry (Moniz *et al.*, 1990; Campos *et al.*, 1991). From day 11.5 pc PTHrP expression was detected in the epithelium of the otic vesicle and at day 12.5 pc in the epithelium of the semilateral channels. So far no reports have been published on the expression of PTHrP mRNA or protein in these structures. The role which PTHrP plays during the ontogenesis of these structures is unclear. The expression itself was rather surprising given the observations that embryos of similar age do not express PTH/PTHrP receptor mRNA in the brain (Karperien *et al.*, 1994).

PTHrP mRNA expression was detected from the earliest stage of endochondral bone formation and onwards in mesenchymal precartilage condensations, even before the appearance of maturing chondrocytes which abundantly express PTH/PTHrP receptor mRNA (Lee *et al.*, 1993; Karperien *et al.*, 1994), but not PTHrP mRNA. PTHrP expression was restricted to chondrocytes within the resting zone of the growth plate (this study; Amizuka *et al.*, 1994). The complementary expression pattern suggests that the PTHrP/receptor signaling system is involved in bone formation via epithelial-mesenchymal-like interactions. The importance of PTHrP in the ontogenesis of the skeleton is underscored by findings of Karaplis *et al.* (1994), who showed that PTHrP knock out embryos developed severe bone malformations.

Complementary expression patterns were also found in the developing lung and the skin. In the lung, PTHrP mRNA expression was found in the epithelial cells while the underlying mesenchyme expressed PTH/PTHrP receptor mRNA (this study; Karperien *et al.*, 1994). Recently, it has been shown that PTHrP is able to regulate the synthesis of lung surfactants via an epithelial-mesenchymal interaction (Rubin *et al.*, 1994). In the skin PTHrP expression was particularly high in the dermis of the snout and in highly localized small clusters of dermal cells at both sites of the extremities attachment sites. The significance of the high PTHrP mRNA expression in these regions is unclear at present. However, since PTHrP has profound effects on both keratinocyte proliferation and differentiation (Holick *et al.*, 1994; Kaiser *et al.*, 1994; Wysolmerski *et al.*, 1994), it seems likely that the peptide is involved in the regulation of these processes. Its effect might be mediated by the PTH/PTHrP receptor, of which the mRNA is abundantly expressed in the dermis in the snout and near the hind and forelimb bud (Karperien *et al.*, 1994).

During organogenesis, PTHrP mRNA was detected in several organs which have been shown not to express detectable levels of PTH/PTHrP receptor mRNA. These tissues include the hindbrain, choroid plexus, otic vesicle and the heart. In the latter organ, PTHrP is expressed in epithelial cells of the ventricle and might exert chronotropic effects on the embryonal heart, similarly as has been reported in the perfused adult rat heart (Nichols *et al.*, 1989). This opens the possibility that in these tissues PTHrP effects are mediated via, yet unknown, PTHrP receptors. On the other hand, tissues have been identified which do not express detectable levels of PTHrP mRNA but, in contrast, do express PTH/PTHrP receptor mRNA such as the intestine and the mesonephros (Karperien *et al.*, 1994). The PTH/PTHrP receptor mRNA expression in these organs was already detected in embryonal stages which do not have parathyroid glands. Since the first PTH mRNA expression is detected in the parathyroid gland anlage at gestation day 13.5 in rat embryos (Senior *et al.*, 1991), it seems unlikely that PTH functions as the ligand for these receptors. This

raises the interesting possibility that embryonal PTHrP regulates PTH/PTHrP receptor activity in the intestine and mesonephros by working as a classical endocrine hormone in contrast to its auto- or paracrine mode of action in most other tissues.

PTHrP depletion in gene knock out embryos has proven to be lethal (Karaplis *et al.*, 1994). The embryos have severe malformations in the skeleton. These morphological malformations are fully in line with the high PTHrP mRNA expression at all sites of bone formation as described in this study. This underscores the importance of the correct spatio-temporal PTHrP expression in osteogenesis. By contrast, the PTHrP depleted embryos developed macroscopically normal lungs, brain and skin in spite of the fact that in normal embryos, these tissues express high levels of PTHrP mRNA during their formation as shown in this study. Whether these structures are altered on a microscopic level remains to be determined. Interestingly, targeted overexpression of PTHrP rather than gene depletion in the epidermis of transgenic mice leads to epidermal hyperkeratosis and to interference with normal hair follicle development (Wysolmerski *et al.*, 1994). Furthermore, addition of exogenous PTHrP to rat fetal lung explants changes the constitution of lung surfactants (Rubin *et al.*, 1994). More studies are required to unravel the role of PTHrP and its receptors in the genesis of these and other organs.

Materials and Methods

Embryos

F1 embryos were obtained from C57B16 females mated to CBA males. The embryos were kept on a 14/10 h light/dark rhythm. Gestation was assumed to have begun in the middle of the dark At noon, the day the plug was first detected, zygotes were assumed to be 1/2 day old. Postimplantation embryos of 5.5d, 6.5d, 7.5d, 8.5d, and 9.5d pc were left in the decidua or left in the uterus. Older embryos were taken out of the decidua. Embryos were fixed in 4% PBS buffered paraformaldehyde at 4°C overnight. After dehydration in graded alcohols embryos were embedded in paraffin (Wilkinson, 1992).

In situ hybridization

In situ hybridization experiments were carried out on 6 μM sections of paraffin-embedded embryos according to Wilkinson *et al.* (1992) as modified by Vogels *et al.* (1990). A pGEM-vector (PROMEGA) containing a 354bp insert encoding exon 3 of the mouse PTHrP gene was used as a template to generate the riboprobes and was kindly provided by Dr. A. Broadus (Mangin *et al.*, 1990). After linearizing the template, α^{35}S-UTP labeled sense or antisense RNA probes were generated using the appropriate RNA-polymerase. The full-length riboprobe was degraded to obtain fragments with an average length of 100 nucleotides by treatment with 80 mM $NaHCO_3$, 120 mM Na_2CO_3 pH 9.6 during 65 min at 65°C as previously described (Wilkinson, 1992). Whole embryos were sectioned and sequential sections were subjected to *in situ* hybridization. Some sections were hybridized with a sense PTHrP labeled riboprobe as a negative control. No specific hybridization was observed in these cases.

After high stringency washing and autoradiography, slides were counterstained with hematoxylin. Slides were exposed 14 to 21 days at 4°C depending on the age of the embryos. All hybridizations were repeated at least 3 times.

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