Parathyroid Hormone-related Peptide as an Endogenous Inducer of Parietal Endoderm Differentiation

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Abstract. Parathyroid hormone related peptide (PTHrP), first identified in tumors from patients with the syndrome of "Humoral Hypercalcemia of Malignancy," can replace parathyroid hormone (PTH) in activating the PTH-receptor in responsive cells. Although PTHrP expression is widespread in various adult and fetal tissues, its normal biological function is as yet unknown. We have examined the possible role of PTHrP and the PTH/PTHrP-receptor in early mouse embryo development. Using F9 embryonal carcinoma (EC) cells and ES-5 embryonic stem (ES) cells as in vitro models, we demonstrate that during the differentiation of these cells towards primitive and parietal endoderm-like phenotypes, PTH/PTHrP-receptor mRNA is induced. This phenomenon is correlated with the appearance of functional adenylate cyclase coupled PTH/PTHrP-receptors. These receptors are the mouse homologues of the recently cloned rat bone and opossum kidney PTH/PTHrP-receptors. Addition of exogenous PTH or PTHrP to RA-treated EC or ES cells is an efficient replacement for dBcAMP in inducing full parietal endoderm differentiation. Endogenous PTHrP is detectable at very low levels in undifferentiated EC and ES cells, and is upregulated in their primitive and parietal endoderm-like derivatives as assessed by immunofluorescence. Using confocal laser scanning microscopy on preimplantation mouse embryos, PTHrP is detected from the late morula stage onwards in developing trophectoderm cells, but not in inner cell mass cells. In blastocyst stages PTHrP is in addition found in the first endoderm derivatives of the inner cell mass. Together these results indicate that the PTH/PTHrP-receptor signalling system serves as a para- or autocrine mechanism for parietal endoderm differentiation in the early mouse embryo, thus constituting the earliest hormone receptor system involved in embryogenesis defined to date.

PARATHYROID hormone (PTH)[1] is a peptide hormone, that serves as a key regulator of calcium homeostasis in the adult organism, its prime target tissues being bone and kidney. It performs its action through binding to a specific receptor, which was recently cloned from opossum kidney and rat bone (Jüppner et al., 1991; Abou-Samra et al., 1992).

A second ligand for the PTH-receptor, Parathyroid hormone-related peptide (PTHrP), has been identified in tumors from patients with the syndrome of "Humoral Hypercalcemia of Malignancy" (reviewed in Orloff et al., 1989; Mallette, 1991). PTHrP competes with PTH for binding to the PTH-receptor (thus henceforward indicated as the PTH/PTHrP-receptor) and is, like PTH, able to activate both adenylate cyclase and phospholipase C–mediated signal transduction pathways (Abou-Samra et al., 1992). Although PTHrP is expressed in a broad range of adult and fetal tissues, little is known about its biological functions (Drucker et al., 1989; Campos et al., 1991; Moniz et al., 1990; Senior et al., 1991).

Murine embryonic stem (ES) cells, as well as some embryonal carcinoma (EC) cells provide suitable in vitro model systems to study regulatory mechanisms of early mouse development. A number of studies on the F9 embryonal carcinoma (EC) cells have indicated a possible role for PTHrP and the PTH/PTHrP-receptor in early mammalian development. Based on determinations of PTH inducible cAMP production, PTH/PTHrP-receptors were found to become expressed when F9 EC cells were induced to differentiate into parietal endoderm-like cells by the simultaneous treatment with retinoic acid (RA) and dibutyryl cAMP (dBcAMP) (Evain et al., 1981; Liapi et al., 1987). This finding was cor-

Drs. van de Stolpe and Karperien contributed equally to this work.

1. *Abbreviations used in this paper*: bPTH, bovine PTH; BRL, Buffalo rat liver; CLSM; confocal laser scanning microscopy; dBcAMP, dibutyryl cAMP; EC, embryonal carcinoma; ES, embryonic stem; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hPTHrP, human PTHrP; ICM, inner cell mass; PTH, parathyroid hormone; PTHrP, PTH-related peptide; RA, retinoic acid.

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roborated by Chan et al. (1990), who in addition showed that PTHrP mRNA was expressed upon RA treatment of F9 EC cells, and more importantly, that PTHrP was able to substitute dBcAMP in the differentiation towards parietal endoderm-like cells when added simultaneously with RA (Chan et al., 1990). These results suggested a possible function of PTHrP and the PTH/PTHrP-receptor in parietal endoderm differentiation.

In the present study we provide additional evidence for PTHrP as an endogenous inducer of parietal endoderm differentiation in the preimplantation mouse embryo. Using both ES-5 embryonic stem cells and F9 EC cells as in vitro models, we demonstrate that during the differentiation to primitive and parietal endoderm-like cells PTH/PTHrP-receptor mRNA is induced. The differentiation is correlated with the appearance of functional adenylate cyclase coupled PTH/PTHrP-receptors. The receptors are the mouse homologues of the recently cloned rat bone and opossum kidney PTH/PTHrP-receptors. Furthermore, we show that also in ES cells exogenously added PTHrP mimics dBcAMP in triggering the full differentiation from a primitive towards a parietal endoderm-like phenotype. In both F9 EC and ES-5 cells, this is accompanied by a further rise in PTH/PTHrP-receptor mRNA. Endogenous PTHrP is detectable by immunofluorescence at very low levels in undifferentiated EC and ES cells, and is upregulated in their primitive and parietal endoderm-like derivatives.

Using confocal laser scanning microscopy (CLSM), we show that in the preimplantation mouse embryos, PTHrP is detected from the late morula stage onwards primarily in developing trophectoderm cells. In blastocyst stages PTHrP is in addition found in the first endoderm derivatives of the inner cell mass (ICM). Together these results indicate that the PTHrP/PTH receptor signalling system may provide a para- or autocrine mechanism for parietal endoderm differentiation in the mouse embryo. Competence for this differentiation step is probably controlled at the level of PTH/PTHrP-receptor expression regulation. As such, PTHrP would constitute the first identified example of an embryonic inducer in preimplantation mammalian development.

Materials and Methods

Materials

Human PTHrP (hPTHrP) (1-34) and bovine PTH (bPTH) (1-34) were purchased from Peninsula Laboratories Inc. (Belmont, CA); affinity-purified polyclonal rabbit anti-human PTHrP(34-53) IgG and hPTHrP(34-53) from Oncogene Science Inc. (Mineola, NY); goat anti-rabbit IgG(Fab')$_2$-FITC were from Tago Inc. (Burlingame, CA). Rabbit anti-uvomorulin IgG was a kind gift of Dr. R. Kemler, Max Planck Institut für Immunologie, Freiburg, Germany. RA was prepared as a 10^{-2} M stock in DMSO and stored at −70°C. dBcAMP was stored as a 10^{-1} M stock at −20°C. hPTHrP(1-34) and bPTH(1-34) were stored as 100 μM stock in HBSS at −20°C. All plastic used for handling PTHrP was siliconized. Monensin was dissolved in 100% ethanol as 1 mM stock and stored at −20°C.

Cell Lines and Culture Techniques

F9 EC and parietal yolk sac-derived PYS-2 cells were cultured on gelatinized surface in medium consisting of a 1:1 mixture of DME and Ham's F12 containing 7.5% FCS, buffered with bicarbonate (DF-Bic, 7.5% FCS). Embryonic stem cells (ES-5) (Mummery et al., 1989) were cultured on gelatinized surface in medium containing 80% MEM conditioned by Buffalo rat liver cells (BRL) with β-mercaptoethanol (0.1 mM) and 20% heat-inactivated FCS, buffered by bicarbonate. BRL-conditioned medium contains leukaemia inhibitory factor (Smith et al., 1988; Williams et al., 1988) which prevents spontaneous differentiation of the ES cells. Characteristics of the ES cell line have been described in detail previously (Mummery et al., 1989). In short it is a pluripotent cell line derived from the mouse embryo inner cell mass, which is capable of forming chimeric mice after aggregation with eight-cell stage embryos. The primitive endoderm-like cell line, 1H5, was kindly provided by Eileen Adamson (La Jolla Cancer Research Foundation, La Jolla, CA) and cultured as described in Adamson et al. (1985).

Induction of Differentiation

Unless denoted otherwise, F9 EC cells were induced to differentiate for a total of 7 d and ES-5 cells for 5 d. To induce differentiation to primitive endoderm-like cells F9 EC and ES-5 cells were cultured in monolayer in the presence of 10^{-6} or 10^{-7} M RA for 5 and 3 d, respectively. To obtain parietal endoderm-like cells this treatment was followed by 2 d 10^{-3} M dBcAMP. In aggregation studies, the cells were treated as described in Hogan et al. (1981).

RNA Isolation and Northern Blot Analysis

Total cellular RNA was isolated by the guanidine isothiocyanate method followed by phenol extraction and precipitation (Chomczynski and Sacchi, 1987). 15 μg of total RNA was denatured for 10 min at 68°C in 50% (vol/vol) formamide, 2.2 M formaldehyde, 20 mM MOPS, pH 7.0, 5 mM sodium acetate, 1 mM EDTA, separated by electrophoresis on 0.8% agarose/2.2 M formaldehyde gels, and subsequently transferred to Hybond C-extra (Amersham Corp., Arlington Heights, IL) in 10× SSC. RNA was immobilized by cross-linking and baking at 80°C for 2 h under vacuum. Hybridization was performed in 50% formamide, 5× SSC, 50 mM sodium phosphate, pH 6.8, 10 mM EDTA, 0.1% SDS, 0.1 mg of sonicated herring sperm DNA per ml, 2× Denhardt solution (1× Denhardt solution contains 0.02% BSA, 0.02% ficoll, 0.02% polyvinylpyrrolidone) at 42°C overnight. ^{32}P-labeled probes were generated using a multiprime DNA labeling kit (Amersham Corp.). After hybridization and washing, filters were exposed to Kodak XAR-5 films (Eastman Kodak Co., Rochester, NY) at −70°C using intensifying screens. Filters were hybridized with a 1.4-kb rat PTH/PTHrP-receptor probe (Abou-Samra et al., 1992), a 1.1-kb mouse laminin B1 probe (Barlow et al., 1984) and a 1.4-kb fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Fort et al., 1985). Quantification of the amounts of probe hybridizing to the immobilized RNA was done using a phosphoimager (Molecular Dynamics Inc., Junction City, OR).

Mouse Embryos

Embryos were obtained as described before from both normal and superovulated Swiss mice and F1 crosses between C57B16 female and CBA male mice (Slager et al., 1991).

Immunofluorescence

The presence of PTHrP was determined using indirect immunofluorescence with affinity-purified polyclonal anti-PTHrP rabbit IgG in a concentration of 25 μg/ml. The anti-PTHrP antibody has been shown to be specific for PTHrP (Atillasoy et al., 1991).

Cells were fixed in 95% methanol/5% acetic acid, incubated for 15 min in PBS containing 0.2% PBS/BSA, incubated for 1 h with the first antibody or control serum at RT, rinsed extensively, and incubated with FITC-conjugated goat anti-rabbit IgG(Fab)$_2$ for 1 h at RT, again extensively rinsed, and mounted in Moviol (Hoechst). Whole embryos were fixed immediately after collection without removing the zona pellucida for 40 min in 2% paraformaldehyde solution in phosphate buffer (pH 7.4), quenched with 50 mM NH_4Cl for 5 min, permeabilized with 0.1% Triton-X 100 for 10 min and incubated for minimal 1 h in PBS/BSA. Subsequent steps were as described above. Staining with rabbit anti-uvomorulin IgG (dilution 1:60) (Vestweber and Kemler, 1984) was used as a control for proper penetration of the IgG antibody in the embryo (Slager et al., 1991). To show that the binding of the anti-PTHrP antibody was specific, competition experiments were performed in which the antibody was pre-incubated for 1 h with excess (1.7 μM) hPTHrP(34-53). In some immunofluorescence experiments 1 μM monensin was added to the cells 1-2 h before fixation to inhibit exocytosis of secretory vesicles (Mollenhauer et al., 1990).

Cell specimens were viewed either with a standard fluorescence microscope or with a CLSM (Lasersharp MRC-500; Bio-Rad Laboratories, Cambridge, MA). Embryos were studied using the CLSM, which allows

optional sectioning of the samples (White et al., 1987). Microscope settings were kept identical when comparing test and control samples.

Intracellular cAMP Levels

Cells were grown in 12-well plates and incubated for 10 min at 37°C in serum-free medium containing 0.1% BSA, 1 mM IBMX and 100 nM hPTHrP(1–34) or bPTH(1–34). The reaction was stopped by removing the medium and adding ice-cold propanol. Samples were freeze dried and cAMP levels were determined in triplo using a cAMP detection kit (Amersham Corp.).

Results

PTHrP Induces Differentiation of ES-5- and F9 EC-derived Primitive Endoderm-like Cells towards Parietal Endoderm

In the mouse blastocyst the pluripotent ICM cells lining the blastocoelic cavity differentiate into primitive endoderm, which in turn yields visceral and parietal endoderm. Characteristically, the parietal endoderm cells will migrate along the trophectoderm cells, and can be recognized by their position, morphology, and expression of high levels of extracellular matrix proteins, such as laminin (Gardner, 1981, 1985).

Murine ES cell lines as well as some EC cell lines provide suitable in vitro models for the study of the molecular nature of the mechanism underlying the regulation of these initial differentiation steps in mouse embryogenesis. We have made use of both ES-5 and F9 EC cells as two independent models that, in vitro, display the differentiation of pluripotent cells into a primitive endoderm-like and, subsequently, into a parietal endoderm-like phenotype (reviewed in Hogan et al., 1983; Mummery et al., 1990). ES cells are derived directly from the mouse inner cell mass (ICM) without an intervening tumorigenic phase. Therefore they provide a closer equivalent to the pluripotent ICM cells of the mouse embryo than EC cells (Evans and Kaufman, 1981; Martin 1981). In contrast to the usual differentiation protocols, which involve adding RA and dBcAMP simultaneously (Strickland et al., 1980; Evain et al., 1981; Chan et al., 1990), we used a stepwise protocol in which RA was added first.

Exposure of ES-5 and F9 EC cells to RA for 3 and 5 d, respectively, strongly enhances differentiation towards a cell type with primitive endoderm-like characteristics (Fig. 1, *A–D*) (Strickland, 1981, Hogan et al., 1981, 1983). The cells enlarge and flatten while laying in close apposition to their neighboring cells. In contrast to undifferentiated EC and ES cells, the primitive endoderm-like cells express low levels of laminin as assessed by immunofluorescence (data not shown). Differentiation of these primitive endoderm-like cells towards parietal endoderm is induced by the subsequent addition of 10^{-3} M dBcAMP to the medium. Within 48 h a new cell type appears in both cultures, which shows typical parietal endoderm characteristics. The cells become smaller and rounded with long cytoplasmic extensions and few intercellular contacts (Fig. 1, *E* and *F*). These morphological changes are associated with the appearance of increased immunofluorescence staining for laminin, which is considered to be characteristic for differentiation towards an endoderm-like phenotype (data not shown; Strickland et al., 1980; Hogan et al., 1983).

Previously it has been shown that parietal endoderm-like

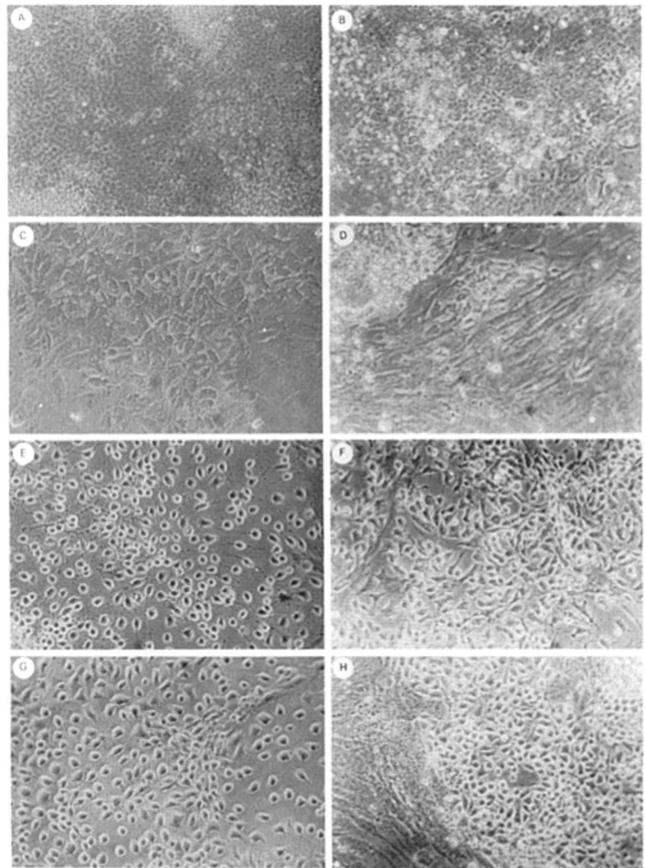

Figure 1. Morphology of differentiating F9 EC and ES-5 cells. Left side shows F9 EC cells, right side shows ES-5 cells. Culture conditions: (*A* and *B*) undifferentiated F9 EC and ES-5 cells; (*C* and *D*) 5 (F9 EC) or 3 (ES-5) d RA (10^{-6} and 10^{-7} M, respectively), followed by 2 d without treatment; (*E* and *F*) 5 (F9 EC) or 3 (ES-5) d RA (10^{-6} and 10^{-7} M, respectively), followed by 2 d dBcAMP (10^{-3} M); (*G* and *H*) 5 (F9 EC) or 3 (ES-5) d RA (10^{-6} and 10^{-7} M, respectively), followed by 2 d hPTHrP(1–34) (100 nM). Preparations were viewed with a standard microscope, magnification 250×. Bar, 250 μm.

derivatives of F9 EC cells express adenylate cyclase–coupled PTH/PTHrP-receptors, while undifferentiated F9 EC cells do not (Evain et al., 1981; Chan et al. 1990). The latter authors also showed that PTHrP, when added together with RA, could mimic dBcAMP in inducing differentiation towards a parietal endoderm-like phenotype. Therefore we investigated whether addition of PTHrP to the primitive endoderm-like derivatives of F9 EC and ES-5 cells could also mimic the action of dBcAMP. As before, ES-5 and F9 EC cells were exposed to RA and then cultured for another 2 d in the presence of 100 nM of either hPTHrP(1–34) or bPTH(1–34). This treatment of ES-5 and F9 EC cells indeed resulted in the typical changes in morphology and laminin expression as seen upon dBcAMP treatment (Fig. 1, *G* and *H*, results with PTH and laminin expression not shown). Apparently, activation of PTH/PTHrP-receptors in ES-5- and F9 EC-derived primitive endoderm-like cells is an efficient and sufficient trigger for parietal endoderm differentiation. Culturing undifferentiated F9 EC and ES-5 cells in the presence of hPTHrP(1–34) or dBcAMP alone does not result in changes in cell growth or differentiation (not shown), indi-

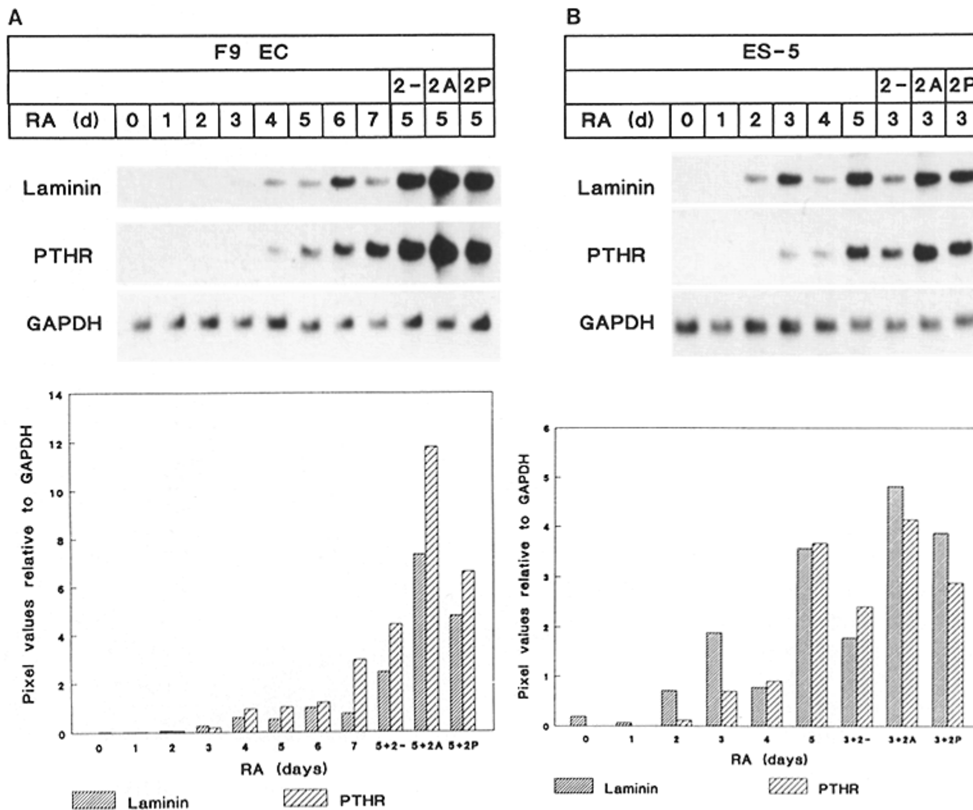

Figure 2. Northern blot analysis of differentiating F9 EC (*A*) and ES-5 cells (*B*). (*Top*) 15 μg of total RNA was blotted and hybridized with a mouse laminin Bl probe and a rat PTH/PTHrP-receptor probe. As a control for equal amounts of RNA loaded in each lane blots were hybridized with a GAPDH probe. F9 EC cultures were treated with 10^{-6} M RA for the indicated time or for 5 d with RA followed by 2 d of no treatment, 2 d of treatment with dBcAMP (10^{-3} M), or by 2-d treatment with 100 nM hPTHrP(1–34) (lanes marked *2-*, *2A*, and *2P*, respectively). ES-5 cultures were treated with 10^{-7} M RA for the indicated time or for 3 d with RA followed by 2 d no treatment, 2 d treatment with dBcAMP (10^{-3} M), or by 2 d of treatment with 100 nM hPTHrP-(1–34) (lanes marked *2-*, *2A*, and *2P*, respectively). (*Bottom*) Phosphoimager quantification of the Northern blot using imagequant software (Molecular Dynamics). Pixel values for PTH/PTHrP-receptor and laminin Bl are expressed relative to GAPDH.

cating that RA pretreatment is a prerequisite for PTHrP- or dBcAMP-mediated parietal endoderm differentiation. Dose-response analysis indicated that doses of PTHrP as low as 5 nM were also capable of mimcking the dBcAMP effect (not shown).

Primitive and Parietal Endoderm-like Derivatives of ES and EC Cells Express Adenylate Cyclase–Coupled PTH Receptors

The observation that PTHrP is able to substitute dBcAMP in inducing parietal endoderm differentiation and the recent cloning of the PTH/PTHrP-receptor prompted us to characterize the expression of PTH/PTHrP-receptors during the stepwise differentiation of F9 EC and ES-5 cells towards parietal endoderm in more detail. The presence of PTH/PTHrP-receptor mRNA was assessed by Northern blot (Fig. 2). Undifferentiated F9 EC and ES-5 cells express no PTH/PTHrP-receptor mRNA. Treatment with RA is accompanied by the appearance of a single PTH/PTHrP-receptor mRNA transcript of about 2.5 kb. In F9 cells low levels of mRNA can be detected after 3 d RA treatment, while in the next 24 h the level is strongly upregulated (Fig. 2 *A*, lanes *3* vs. *4*). In ES-5 cells low levels of PTH/PTHrP-receptor mRNA can first be detected at day 2 (Fig. 2 *B*, lane *2*). In both cell lines longer exposures to RA leads to increased mRNA expression. Subsequent differentiation of a primitive endoderm-like phenotype towards a parietal endoderm-like phenotype by 2 d of treatment with dBcAMP strongly increases the PTH/PTHrP-receptor mRNA expression, especially in F9 cells (Fig. 2 *A*, lanes *2-* vs. *2A*). Also in ES-5 cultures treated for 3 d with RA followed by 2 d dBcAMP compared with cultures treated with RA followed by 2 d no treatment (Fig. 2 *B*, lanes *2-* vs. *2A*) PTH/PTHrP-receptor mRNA expression is elevated. However, the increase is less pronounced compared to F9 cultures.

Differentiation of F9 EC- and ES-5–derived primitive endoderm-like cells to parietal endoderm by treatment with hPTHrP (1–34) results in an induction of PTH/PTHrP-receptor mRNA comparable with that induced by dBcAMP, albeit to a somewhat lower level. Thus, in these cells, PTHrP is able to upregulate the mRNA expression of its own receptor.

As a control for the differentiation of F9 EC and ES-5 cells towards parietal endoderm, the blots were hybridized with a laminin Bl probe. As can be seen in Fig. 2, laminin Bl mRNA expression is induced during the differentiation to and is highest in the cultures resembling parietal endoderm in accordance with literature data (Strickland et al., 1980; Hogan et al., 1983) and immunofluorescence data (not shown).

To test whether the PTH/PTHrP-receptor mRNA expression is correlated with the appearance of functional adenylate cyclase coupled PTH/PTHrP-receptors, we measured intracellular cAMP accumulation after stimulation with 100 nM hPTHrP(1–34), which is 10-fold the concentration

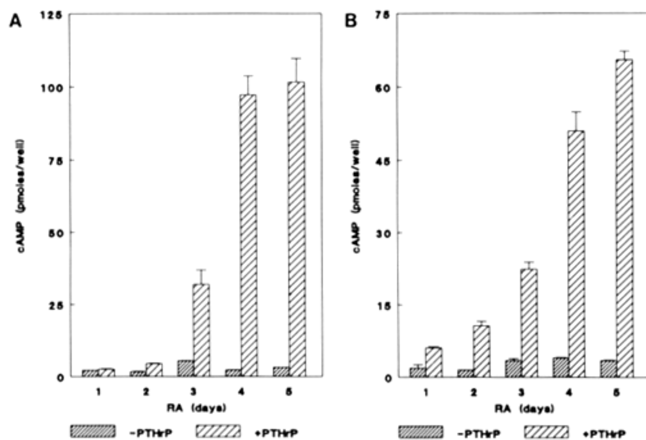

Figure 3. cAMP response to stimulation with hPTHrP (1–34) in differentiating F9 EC and ES-5 cells. (*A*) F9 EC cells; (*B*) ES-5 cells. Cells were grown in the presence of RA for 1–5 d (10^{-6} or 10^{-7} M, respectively) and subsequently incubated for 10 min with 100 nM hPTHrP(1–34) in the presence of IBMX. cAMP content is expressed in pmoles/well. Values represent the mean of three experiments + SEM.

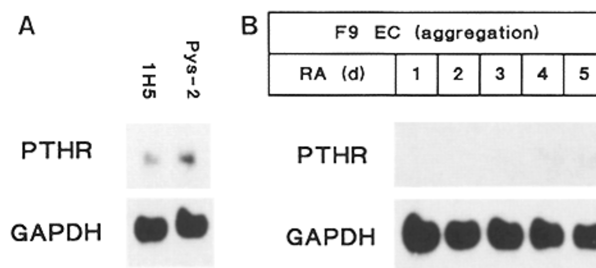

Figure 4. PTH/PTHrP-receptor mRNA expression in PYS-2, 1H5, and visceral endoderm-like cells. (*A*) 15 μg of total RNA of 1H5 and PYS-2 was isolated and blotted. The blot was hybridized with the rat PTH/PTHrP-receptor probe and quantified using GAPDH. (*B*) F9 EC cells were aggregated in the presence of 10^{-6} M RA for the indicated time. After 5 d of treatment the cultures resemble visceral endoderm-like cells (Hogan et al., 1981). 15 μg of total RNA was blotted and hybridized with the rat PTH/PTHrP-receptor probe and quantified using GAPDH.

needed to get a half-maximal response in these cells (not shown). As indicated in Fig. 3, in F9 EC cells cAMP responsiveness to PTHrP is detectable at day 3, the first day that PTH/PTHrP receptor mRNA can be detected (Fig. 3 *A*, lane *3*). Likewise, in ES-5 cells the cAMP response can be detected at day 2. In both cell types the PTHrP (or PTH) induced cAMP accumulation increases during the RA-mediated differentiation in concert with the PTH/PTHrP-receptor mRNA expression data. When bPTH(1–34) instead of hPTHrP(1–34) was used the same kinetics and stimulation values were found (data not shown).

Importantly, the expression of PTH/PTHrP-receptor mRNA and protein in both cell types coincides with the appearance of primitive endoderm-like cells, which renders it likely that this is the first cell type expressing PTH/PTHrP-receptors. Analysis of Northern blots of total RNA of two other cell lines, 1H5, a primitive endoderm-like cell line (Adamson et al., 1985), and PYS-2, a mouse parietal yolk sac embryonal cell line, supports the data obtained in F9 EC and ES-5 cells, in that both cell lines express PTH/PTHrP-receptor mRNA (Fig. 4 *A*). In marked contrast, aggregation of F9 EC cells in the presence of RA leading to differentiation towards visceral endoderm-like cells (Hogan et al., 1981), is accompanied by a very low level of PTH/PTHrP-receptor mRNA expression (Fig. 4 *B*). This result indicates that PTH/PTHrP-receptor expression is cell-type specific and not merely a general response to RA treatment.

PTHrP Is Expressed in Differentiating ES and EC Cells

For PTHrP to act as an endogenous inducer of parietal endoderm differentiation in the embryo it has to be produced by either primitive endoderm cells themselves or neighboring cells. In search for the presence of PTHrP we applied standard indirect immunofluorescence microscopy as well as CLSM, using an affinity-purified rabbit anti–PTHrP(34–53) IgG.

PTHrP is detectable in ES-5 as well as F9 EC cells. Undifferentiated ES-5 and F9 EC cells show a minimal but significant cytoplasmic staining with anti-PTHrP, especially at the border of groups of undifferentiated cells (Fig. 5, *A–C*). The staining is present in a granular pattern and found in cells which have a slightly different morphology, suggesting some early endoderm differentiation. The staining is specific since it can be competed away with excess hPTHrP(34–53) (Fig. 5 *C*).

Intense specific cytoplasmic staining for PTHrP is observed in primitive endoderm-like (Fig. 5, *D* and *E*) and parietal endoderm-like derivatives of ES-5 and F9 EC cells (Fig. 5, *F–H*). In these cells the staining pattern is again granular, with the granules distributed predominantly in the perinuclear area, but also detectable in peripheral extensions of the cells. Comparing the labeling intensities of primitive vs. parietal endoderm-like cells indicates that, at least in differentiating ES-5 cells, PTHrP levels are upregulated upon parietal endoderm differentiation.

The granular PTHrP staining pattern seen in primitive and parietal endoderm-like derivatives suggests that the peptide is present in vesicle-like structures, the diameter of the vesicles being ~1 μm, as determined with the CLSM. To obtain more information on the characteristics of putative vesicle-like structures we incubated the cells for various times with 1 μM monensin. Monensin is known to inhibit both the function of the Golgi apparatus and exocytosis of vesicle contents (Mollenhauer et al., 1990). Monensin treatment of ES-5–derived primitive endoderm-like cells resulted in a clearly detectable and specific increase in intracellular immunofluorescence for PTHrP, presumably caused by accumulation of PTHrP-containing vesicles in the cytoplasm (Fig. 6). This supports the idea that PTHrP is transported in vesicles and subsequently secreted by the cell.

Taken together, these results suggest that PTHrP is available to F9 EC- and ES-5–derived primitive endoderm-like cells as an endogenous activator of PTH/PTHrP-receptors by the time these receptors are expressed. However, although both PTHrP and functional PTH/PTHrP-receptors are present, we never observe spontaneous differentiation towards parietal endoderm in F9 EC-derived primitive endoderm-like cultures. It is conceivable that the balance between action of PTHrP and other unknown (growth?) factors inhibits

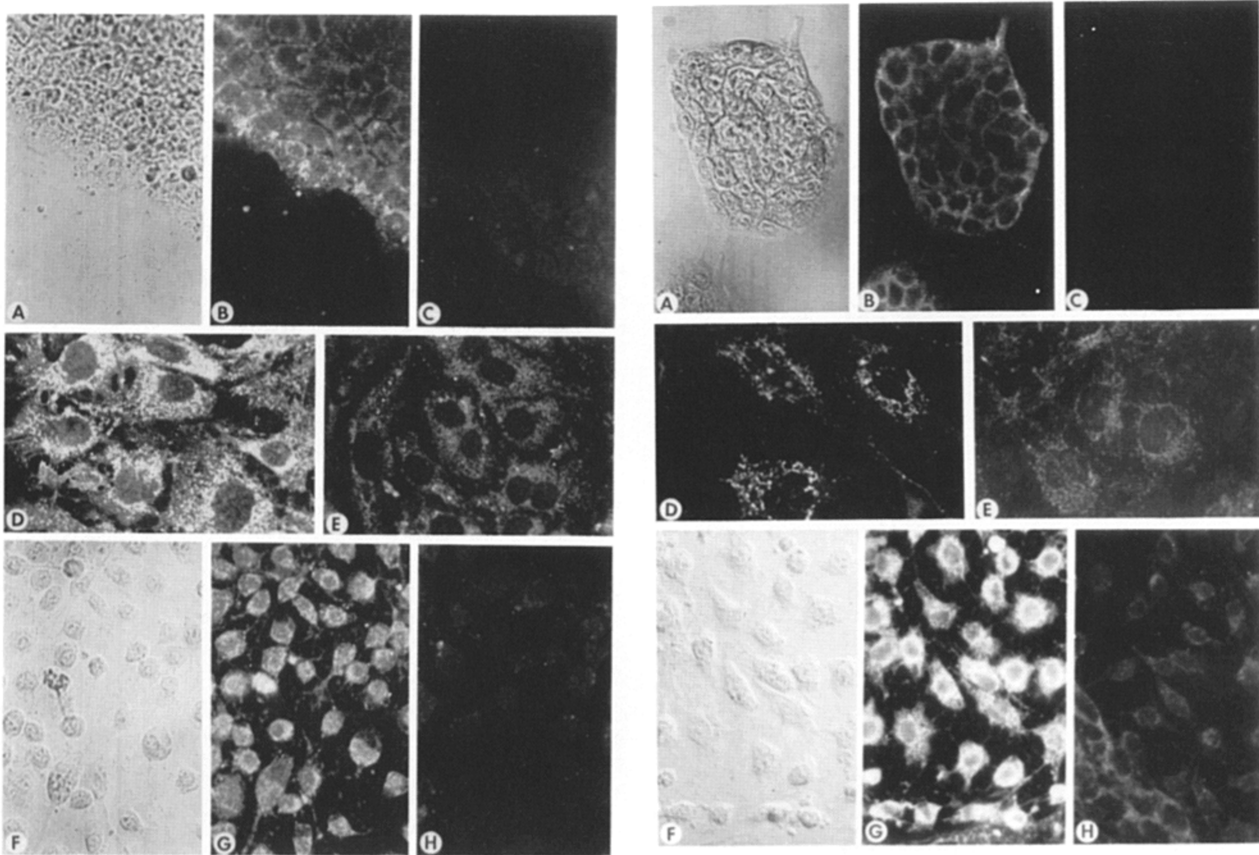

Figure 5. Immunofluorescent staining with anti-PTHrP during differentiation of F9 EC (*left*) and ES-5 cells (*right*). Culture conditions: (*A–C*) no treatment; (*D* and *E*) 5 (F9 EC) or 3 (ES-5) d with RA (10^{-6} or 10^{-7} M, respectively), followed by 2 d without treatment; (*F–H*) 5 (F9 EC) or 3 (ES-5) d with RA (10^{-6} or 10^{-7} M, respectively), followed by 2 d with dBcAMP (10^{-3} M). Samples were viewed with CLSM (oil immersion). Nomarsky image (*A* and *F*), fluorescent image (*B*, *D*, and *G*), fluorescent image of samples treated with preabsorbed antibody using hPTHrP(34–53) (*C*, *E*, and *H*). Controls with normal rabbit serum or IgG at proper dilution were negative (not shown). Bar, 10 μm.

this spontaneous differentiation. Disrupting this balance by adding exogenous PTHrP or chemical agents as dBcAMP directs the differentiation towards parietal endoderm. Alternatively we cannot exclude the possibility that the PTHrP immunoreactive material is not fully active. In marked contrast, we do observe spontaneous differentiation towards parietal endoderm in ES-5 derived primitive endoderm-like cultures. As described before, longer exposure to RA leads to changes in morphology characteristic of parietal endoderm differentiation and also to increases of PTH/PTHrP-receptor and laminin B1 mRNA expression (Fig. 2 *B*; Mummery et al., 1990). A relevant observation in this respect is that we have not been able to detect PTHrP bioactivity in ES-5 or F9 EC conditioned media. One explanation for this phenomenon could be immediate binding and processing of the hormone.

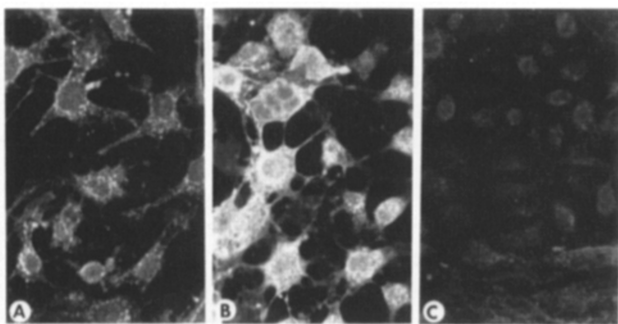

Figure 6. Effect of monensin treatment on immunofluorescent staining with anti-PTHrP antibody. (*A*) ES-5 cells treated for 3 d with RA (10^{-7} M), followed by 2 d dBcAMP (10^{-3} M) (*B*) ES-5 cells treated similarly, except for preincubation with 1 μM monensin for 1 h before fixation. (*C*) Similarly as in *B*-treated ES-5 cells, antibody preabsorbed with hPTHrP(34–53). Preparations were viewed with the CLSM (oil immersion). Bar, 10 μm.

PTHrP Is Present in the Preimplantation Embryo

The hypothesis that PTHrP may act as an endogenous inducer of parietal endoderm differentiation in the preimplantation mouse embryo predicts that the peptide should be present at the blastocyst stage or earlier. To test this, a systematic survey of the presence of PTHrP during preimplantation mouse embryogenesis was made. The results presented in Fig. 7 clearly demonstrate the presence and developmental regulation of PTHrP expression. PTHrP is first detectable in compacted morula stages, specifically in the outer cells that will become trophectoderm (Fig. 7, *A–C*). Only minimal PTHrP

staining could be found in the inner cells at this stage. In intact blastocysts high levels of PTHrP remain detectable in trophectoderm cells, but some cells lining the blastocoelic cavity are also positively stained (Fig. 7 *D*). Most likely these cells represent the first primitive endoderm cells formed at that stage (Gardner, 1981, 1985). Parietal endoderm cells cannot be distinguished at this stage of development. The staining pattern observed is not due to insufficient penetration of IgG's in the inner cell mass, since anti-uvomorulin IgG clearly stained all cells under the conditions used (Fig. 7, *E* and *F*). Anti-PTHrP staining was specific since it could be effectively competed away by addition of excess hPTHrP-(34-53), and since control experiments with affinity-purified normal rabbit serum at the appropriate concentration showed no fluorescence (not shown).

These results demonstrate that PTHrP is present in the preimplantation mouse embryo, and that its expression is subject to precise regulation. Trophectoderm cells may provide a major source of PTHrP from their earliest formation onwards. During further development PTHrP is also detectable in primitive endoderm cells. Whether this is indeed due to primitive endoderm-linked expression, or possibly the result of PTH/PTHrP-receptor-mediated internalization remains to be established. Taken together, our results provide strong support for the hypothesis that PTHrP is an endogenous inducer of parietal endoderm differentiation in the early mouse embryo.

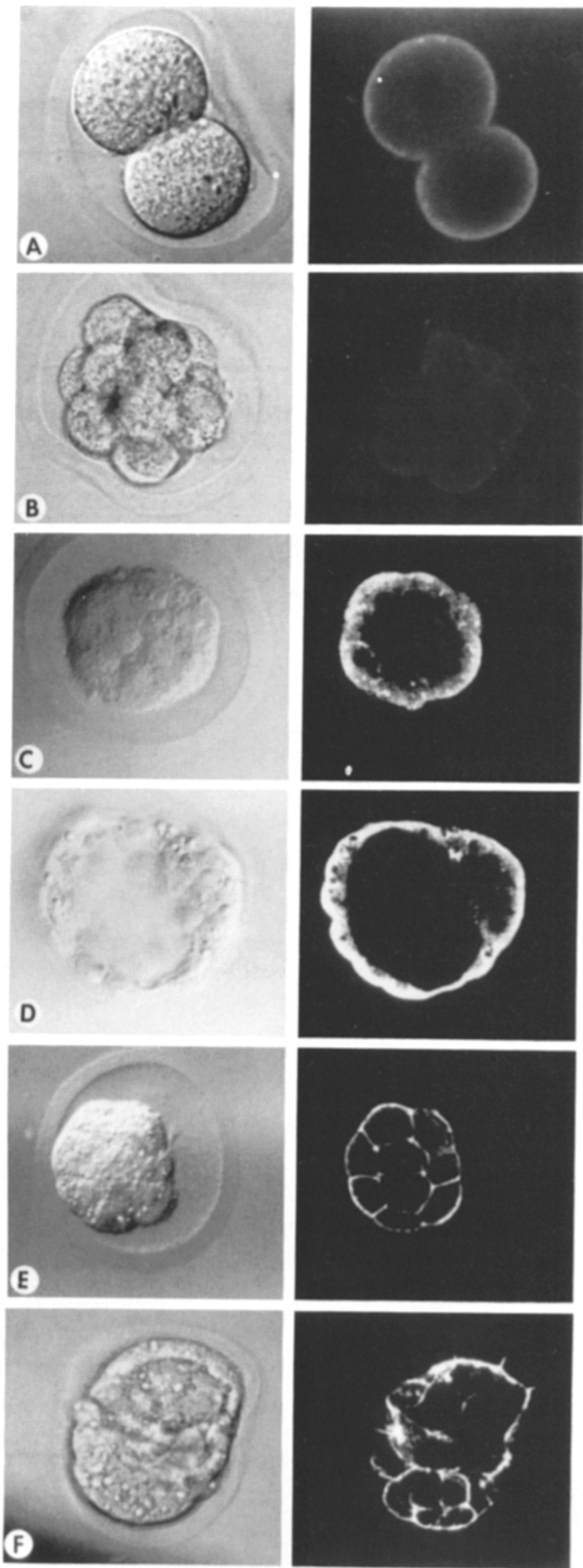

Figure 7. Immunofluorescent staining with anti-PTHrP and anti-uvomorulin antibody in whole preimplantation embryos. (*A*) Two-cell stage, staining with anti-PTHrP. (*B*) Eight-cell stage, anti-PTHrP. (*C*) Morula, anti-PTHrP. (*D*) Blastocyst, anti-PTHrP. (*E*) Morula, staining with anti-uvomorulin. (*F*) Blastocyst, anti-uvomorulin. Left photograph shows Nomarsky image, right photograph shows the corresponding fluorescent image. Control experiments with normal rabbit serum or IgG and with antibody preabsorbed with excess hPTHrP(34-53) were negative (not shown). Fluorescent staining with anti-uvomorulin shows complete penetration of IgG antibodies in preimplantation embryos. All preparations were viewed with the CLSM (oil immersion). Bar, 10 μm.

Discussion

Cumulating data on the widespread expression of PTHrP during normal postimplantation development in rat and human suggest an important role for PTHrP in embryonal growth and differentiation processes (Moniz et al., 1990; Campos et al., 1991; Senior et al., 1991). In addition a role for PTHrP in early preimplantation development has been suggested by Chan et al. (1990), based upon their findings that differentiated F9 EC cells express mRNA for PTHrP, and that culturing F9 EC cells in the presence of retinoic acid and hPTHrP(1-34) results in differentiation to parietal endoderm-like cells.

Here we present evidence that PTHrP may indeed act as an endogenous inducer of parietal endoderm differentiation in the preimplantation mouse embryo by activating the mouse homologue of the recently cloned rat bone and opossum kidney PTH/PTHrP-receptor (Jüppner et al., 1991; Abou-Samra et al., 1992). This evidence is provided by the following data: (*a*) Functional adenylate cyclase coupled PTH/PTHrP-receptors are expressed on the precursor cells for parietal endoderm, i.e., primitive endoderm-like cells; (*b*) exogenous PTHrP is a sufficient trigger to convert primitive endoderm-like cells into parietal endoderm-like cells;

and (*c*) endogenous PTHrP is detectable at the proper stage in the preimplantation embryo.

We used ES-5 cells as the closest available in vitro equivalent of the pluripotent inner cell mass cells of the mouse embryo, as well as F9 EC cells, to analyze the expression of functional PTH/PTHrP-receptors. Our differentiation protocol, in contrast to that used by others (Strickland et al., 1980; Evain et al., 1981; Chan et al., 1990), allowed us to distinguish in both model systems between primitive and parietal endoderm differentiation (Strickland, 1981). In this way it became clear that the earliest detectable differentiation towards a primitive endoderm-like phenotype is associated with the onset of expression of adenylate cyclase coupled PTH/PTHrP-receptors, and that these receptors are upregulated upon subsequent differentiation towards a parietal endoderm-like phenotype. The onset of expression of functional PTH/PTHrP-receptors is correlated with the induction of a single 2.5-kb mRNA transcript encoding the PTH/PTHrP-receptor. We have recently isolated cDNA clones encoding the PTH/PTHrP-receptor from an EC-cell derived mouse cDNA library (M. Karperien, manuscript in preparation). This cDNA encodes a protein that is 98.5% identical in amino acid sequence to the rat PTH/PTHrP-receptor. The differences detected are likely to reflect species rather than functional differences. The expression of PTH/PTHrP-receptors seems specific for the parietal endoderm lineage, since visceral endoderm-like derivatives of F9 EC cells express only very low levels of PTH/PTHrP-receptor mRNA, probably due to the presence of some primitive endoderm-like cells, the precursor cells of both parietal and visceral endoderm.

Using our differentiation protocol, we could demonstrate that PTHrP (or PTH) can fully mimic dBcAMP in the induction of differentiation from a primitive towards a parietal endoderm-like phenotype. Since the concentrations required for this effect are similar to those that induce a significant accumulation of intracellular cAMP, we assume that parietal endoderm differentiation is a direct consequence of PTH/PTHrP-receptor–mediated cAMP production. The differentiation of a primitive endoderm-like phenotype towards a parietal endoderm-like phenotype by PTHrP or dBcAMP coincides with a strongly enhanced expression of PTH/PTHrP-receptor mRNA, suggesting that, at least in our system, PTHrP is able to upregulate the expression of its own receptor. It will be of interest to investigate whether this is a direct consequence of the activated cAMP signal transduction pathway or a result of the differentiation towards parietal endoderm. Importantly, our model systems confirm the notion that the expression of the cloned PTH/PTHrP-receptor is not restricted to the classical target tissues of PTH, namely bone and kidney (Üreña, P., K. Lee, D. Weaver, X. F. Kong, D. Braun, A. T. Bond, A.-B. Abou-Samra, and G. V. Segre. 1992. Abstract. American Society for Bone and Mineral Research meeting. Minneapolis, MN). Its presence in model systems for early mouse embryogenesis suggests that it might be a natural target for PTHrP action, which is locally present in a wide variety of tissues.

Since we found a close correlation between PTH/PTHrP-receptor expression and the first appearance of primitive endoderm-like cells in differentiating ES-5 and F9 EC cells, it was of particular interest to characterize the timing of PTHrP production during this process. Chan et al. (1990) reported that PTHrP mRNA is not detectable in undifferentiated F9 EC cells, while treatment with retinoic acid induces the expression of low amounts of PTHrP mRNA within 24 h. So far, data on the production of PTHrP protein were lacking. We applied immunofluorescence techniques combined with CLSM to detect PTHrP at high resolution in ES-5- and F9 EC-derived cells. In accordance with the available mRNA data on F9 cells (Chan et al., 1990), PTHrP seems to be one of the earliest detectable marker proteins of (primitive) endoderm differentiation, as PTHrP staining was seen at the outer border of groups of undifferentiated ES-5 or F9 EC cells, before any clear morphological change occurred. These are probably cells that start to differentiate to primitive endoderm-like cells.

In differentiated primitive and parietal endoderm-like F9 and ES-5 cells, as well as in the established parietal endoderm PYS-2 cells (not shown), PTHrP is present in a granular, predominantly perinuclear pattern. These granules are ~1 μm in diameter, and they accumulate upon addition of monensin to the cells. This suggest that these granules represent secretory vesicles (Mollenhauer et al., 1990), which renders it likely that PTHrP is secreted by these cells.

It is of interest to consider the differences observed between ES-5 and F9 EC cells in further detail. F9 EC cells will not differentiate into parietal endoderm unless they are exposed simultaneously (Strickland et al., 1980; Chan et al., 1990), or successively (Strickland, 1981; and this study), to retinoic acid and cAMP-elevating agonists for prolonged periods. In ES-5 cells a more subtle regulatory mechanism seems to be involved. Prolonged exposure of ES-5 cells to retinoic acid alone also results in the appearance of a subpopulation of parietal endoderm-like cells, as judged by their morphology and high levels of laminin B1 expression (this study; Mummery et al., 1990). Since ES-5–derived primitive endoderm-like cells express high levels of PTHrP, secretion of PTHrP and subsequent activation of the PTH/PTHrP-receptors in a para- or autocrine fashion may account for this spontaneous differentiation.

Our data derived from ES and EC cells provide strong, albeit indirect, support for a role of PTHrP and the PTH/PTHrP-receptor in parietal endoderm differentiation. Direct support for this hypothesis came from PTHrP localization studies in the preimplantation mouse embryo itself. In agreement with our findings in ES-5 and F9 EC cells, PTHrP production appeared to be tightly regulated during early development. Surprisingly, PTHrP is already detectable from the compacted morula stage onwards, days before the onset of endodermal differentiation. The first cells that express PTHrP are the future trophectoderm cells; PTHrP seems to be in fact one of the earliest markers of trophectoderm differentiation. Trophectoderm cells remain positive for PTHrP, at least until the blastula stage is reached, while the inner cell mass hardly shows any staining for PTHrP at all stages studied. In complete agreement with the pattern observed in ES-5 and F9 EC cells, PTHrP is also found in the first derivatives of the inner cell mass in blastocysts, that probably represent primitive endodermal cells. A recent study has shown that trophectoderm derived cells remain positive for PTHrP mRNA until 13.5 d after gestation in the developing rat fetus (Senior et al., 1991).

In summary, our findings support the hypothesis that PTHrP acts as an autocrine or paracrine factor, which may

be capable of enhancing differentiation to parietal endoderm in the preimplantation embryo by activating adenylate cyclase. We propose a model in which the primitive endoderm cells lining the blastocoelic cavity start expressing the PTH/PTHrP-receptor. PTHrP, expressed and secreted by the trophectoderm, subsequently induces differentiation of primitive endoderm cells to form parietal endoderm while they migrate along the trophectoderm cells. During this differentiation process the primitive and parietal endoderm cells themselves start expressing and secreting PTHrP conceivably leading to recruitment of additional primitive endoderm cells to form parietal endoderm. It has been suggested by others that contact between trophectoderm and extraembryonic endoderm is important for full parietal endoderm differentiation (Hogan and Tilly, 1981; Hogan et al., 1983). The presence of large amounts of immunoreactive PTHrP in the trophectoderm may account for this phenomenon. Since PTHrP is expressed days before the parietal endoderm differentiation takes place, we suggest that competence for PTHrP-mediated parietal endoderm differentiation is at the level of PTH/PTHrP-receptor expression. Direct proof that the recently cloned PTH/PTHrP-receptor is indeed expressed in the preimplantation embryo itself is so far lacking. At present we are carrying out in situ hybridization studies to solve this issue. Since our model predicts an autocrine loop, it also predicts the existence of (*a*) factor(s) that should interfere with this loop. We are currently directing our efforts towards identifying substances that modulate PTH/PTHrP-receptor or PTHrP expression in EC and ES cells.

Our data suggest an important role for the PTH/PTHrP-receptor/PTHrP-signalling system in early mouse development and as such this would provide the earliest example of an identified differentiation inducing mechanism in the early mouse embryo.

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