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Signals governing extraembryonic endoderm formation in the mouse: involvement of the type 1 parathyroid hormone-related peptide (PTHrP) receptor, p21Ras and cell adhesion molecules

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ABSTRACT The formation of parietal endoderm (PE) from primitive endoderm (PrE) immediately after implantation of the early mouse embryo can be seen as the earliest example of an epithelio-mesenchyme transition (EMT) in murine development. Since EMT and EMI (epithelium-mesenchyme interactions) are at the very heart of morphogenesis, identifying molecular mechanisms governing these processes is of utmost importance. An excellent *in vitro* model system to study PE formation, i.e. F9 embryonal carcinoma cells, is available to this end. In the present paper we review our own recent results and those of others using these cells, and present our current view on the molecular mechanisms involved in PE formation.

KEY WORDS: *PTHrP, parietal endoderm, adhesion, p21ras, epithelium-to-mesenchyme transition*

Introduction

Extraembryonic endoderm formation

Embryonic development in mammals requires implantation in the uterine wall and placenta formation to physically connect the embryo to its mother. Since the establishment of this connection is one of the embryo's first priorities, the first differentiation events are concerned with establishing the necessary extraembryonic tissues, while organization of the foetus proper is postponed until somewhat later stages. The extraembryonic tissues are precursors of the placenta and are specialized in different functions: they are required for invasion of the uterine epithelium, they mediate the interaction between the maternal and foetal environment and they serve to protect and nourish the embryo.

While the trophoblast cells (the outer cells of the preimplantation embryo) are invading the uterine wall, the embryonic or inner cell mass (ICM) develops an epithelial structure distally (i.e. at the side facing the blastocoelic cavity), which has been named variously the extraembryonic, primary or primitive endoderm (PrE), or hypoblast (Fig. 1). This structure subsequently gives rise to two new cell populations: the parietal endoderm (PE), which forms the parietal yolk sac, a structure critical for both the absorption of nutrients during early gestation, and for protecting the embryo from mechanical damage (Gardner, 1983; Cross *et al.*, 1994), and the visceral endoderm (VE) which at its basal side remains in contact with the embryonic ectoderm or epiblast and later (together with extraembryonic mesoderm) gives rise to the visceral yolk sac. The three cell types are distinguishable not only topographically, but also by their morphology and expression of marker proteins (reviewed in Gardner, 1983; Hogan *et al.*, 1983). PrE is a small population of cells with extensive rough endoplasmic reticulum containing secretory material, which synthesizes and secretes tissue plasminogen activator. At its border the cells already have a PE-like morphology, in that they are elongated and loosely associated (Strickland *et al.*, 1976; Enders *et al.*, 1978). VE cells are characterized by their association in a polarized epithelium with apical tight junctions and numerous microvilli. They synthesize and secrete alpha-fetoprotein and the urokinase plasminogen activator (uPA) (Dziadek and Adamson, 1978; Enders *et al.*, 1978; Marotti *et al.*, 1982). PE cells have a flattened elongated fibroblast-like

Abbreviations used in this paper: PTHrP, parathyroid hormone-related peptide; PTH, parathyroid hormone; ICM, inner cell mass; PrE, primitive endoderm; PE, parietal endoderm; VE, visceral endoderm; EMT, eptihelio-mesenchyme transition; EMI, epithelium-mesenchyme interaction; ECM, extracellular matrix; EC, embryonal carcinoma; ES, embryonal stem; RA, retinoic acid; CAMP, cyclic adenosine monophosphate; CRE, cyclic amp responsive element; CREB, CRE-binding protein; MAP-kinase, mitogen activated protein-kinase.

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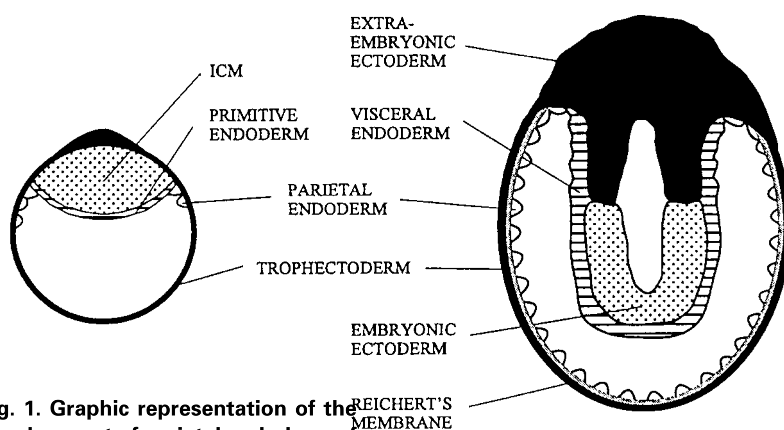

Fig. 1. Graphic representation of the development of parietal endoderm of the mouse embryo. **(Left)** *Implanted blastocyst around day 4.5 p.c.* **(Right)** *Post-implantation embryo at day 6.5 p.c.*

form when they first appear, but assume a rounded morphology at later stages. They are loosely associated, both with each other and with the basal lamina of the trophectoderm on which they migrate. In murine embryos they synthesize and secrete large amounts of extracellular matrix components, such as laminin, collagen IV, entactin, heparan sulphate proteoglycans and SPARC, which become assembled in the Reichert's membrane, an extremely thick layer of extracellular matrix formed between the PE cells and the basal lamina of the trophectoderm (Hogan *et al.*, 1980, 1982; Smith and Strickland, 1981; Kurkinen *et al.*, 1983; Mason *et al.*, 1986). The PE cells remain scattered individually over the surface of the Reichert's membrane and do not form a continuous epithelial layer at any stage (Enders *et al.*, 1978). Furthermore, these cells are characterized by the expression of high amounts of tissue plasminogen activator (tPA) and by the expression of the anti-coagulant thrombomodulin (Imada *et al.*, 1987; Weiler-Guettler *et al.,* 1996).

While the PE is generated from the PrE immediately after implantation, somewhat later the VE contributes to PE formation as well. VE cells have PrE-like characteristics and can differentiate into PE until day 7.0 p.c. (Solter and Damjanov, 1973; Cockroft and Gardner, 1987). Scanning electron microscopy suggested that early VE cells differentiate into PE at the so called marginal zone, a junction between the two tissues were VE cells detach from the epithelial layer. Subsequently, they migrate on to the Reichert's membrane and differentiate into PE (Hogan and Newman, 1984).

The formation of PE from PrE and later VE can be considered to be one of the earliest examples of an epithelio-mesenchyme transition (EMT) in mouse development (see Table 1). Other examples of EMT occurring early in embryogenesis are: 1) at the onset of gastrulation the detachment from the embryonic ectoderm (an epithelium) of mesenchymal cells that give rise to the mesoderm and definitive endoderm and 2) the detachment from the neurectoderm of the mesenchymal neural crest cells. EMT and also EMI's (epithelium-mesenchyme interactions) are at the very heart of morphogenesis. Nevertheless, the signals that govern EMT and EMI remain largely enigmatic. Since mechanisms governing cell growth and differentiation are highly conserved, studying the PrE-PE transition will yield clues on the initiation and regulation of EMT in general. As will be outlined in the next section, the study of this process is highly facilitated by the availability of excellent *in vitro* model systems.

Models to study PE formation in vitro

Since the study of development at peri-implantation stages *in vivo* is difficult in view of the extremely small size and inaccessibility of the embryo, two approaches have been used to circumvent these problems. The first involves culturing immunosurgically isolated tissues from either pre-implantation embryos, or from postimplantation embryos stages that are relatively easy to isolate and dissect, i.e. after embryonic day 6.5.

The second approach relies on the use of cultured embryonal carcinoma (EC) and embryonic stem (ES) cells as *in vitro* models (see Mummery and Vandeneijndenvanraaij, this issue). ES cells, isolated directly from the ICM, can be induced to differentiate *in vitro* to form derivatives of all three germ layers depending on the culture conditions. The differentiation is predominantly directed toward PrE when the cells are cultured in monolayer in the presence of retinoic acid (RA) and leukemia inhibiting factor (LIF), while concomitant treatment with cAMP-elevating agents, such as dbcAMP, strongly increases PE differentiation (Mummery *et al.*, 1990; van de Stolpe *et al.*, 1993). However, most studies on the processes involved in PE differentiation have been carried out using F9 EC cells. These cells are derived from a testicular teratocarcinoma, they show very little spontaneous differentiation and can be induced to differentiate exclusively into extraembryonic endoderm. Differentiation of these cells to PE-like cells mimics the two-step differentiation of PE *in vivo*, and can be initiated either in monolayer culture or by aggregation of the cells into embryoid bodies. Treatment with RA of F9 EC cells in monolayer induces differentiation to PrE-like cells; these can be induced to further differentiate to PE-like cells by subsequent treatment with cAMP-elevating agents (Strickland and Mahdavi, 1978; Strickland *et al.*, 1980). Treatment of F9 EC cell embryoid bodies with RA results in the formation of an outer layer of VE-like cells, which can further differentiate into PE either again by treatment with cAMP elevating agents or by plating of the aggregates on fibronectin (Hogan and Tilly, 1981; Grover and Adamson, 1986; Grabel and Watts, 1987).

TABLE 1

SOME GENERAL CHARACTERISTICS OF EPITHELIAL VERSUS MESENCHYMAL CELLS, AND THEIR DISPLAY IN PRE, VE AND PE

Epithelium	*PrE and VE*	*Mesenchyme*	*PE*
Sheets of cells	✔	Single, loose cells	✔
High (E-)Cadherin expression	✔	No or low (E-) Cadherin expression	✔
Not motile	✔	Highly motile	✔
Basal-apical polarity	✔	Front-back polarity	✔
Apical microvilli	✔	No microvilli	✔
		Secretion of copious ECM	✔
		Production of proteases	✔
Attachment to basal lamina	✔	Migration through basal lamina	

Using cultured mouse embryos, it was shown that isolated extraembryonic VE indeed could differentiate into PE, but only when cocultured with trophectoderm (Hogan and Tilly, 1981). Furthermore, when isolated ICMs were plated on fibronectin, outgrowth of PrE-like cells that migrated and differentiated into PE-like cells was observed, a process that was strongly enhanced by trophectoderm-conditioned medium (Behrendtsen *et al.*, 1995). These data indicate that PE differentiation is probably regulated by interactions with ECM components and by (a) factor(s) secreted by the trophectoderm.

The similarities in PE formation between cells growing out from ES and EC cell-derived embryoid bodies and those growing out from isolated ICM's suggests that similar signal transduction mechanisms are at work. The observation that *in vitro*, cAMP-elevating agents strongly enhance differentiation and outgrowth of PE, suggested that, *in vivo,* PE differentiation may also be enhanced by (a) cAMP-elevating factor(s), possibly secreted by the trophectoderm. This concept had been supported by a substantial number of reports but the identity of the endogenous PE-inducing signal(s) remained unclear until the early 90's.

Around that time, we and others identified parathyroid hormone related peptide (PTHrP) as a molecule capable of inducing F9 derived PrE-like cells to differentiate into PE cells, showing that receptors for this hormone were present on the surface of the cells (Chan *et al.*, 1990; van de Stolpe *et al.*, 1993). This paper summarizes the subsequent research in our group on the relevance of this finding, the intracellular signal transduction pathways involved, as well as recent insights into other physiological roles of PTHrP and their possible involvement in the PrE-PE transition and EMT in general.

PTHrP and PTH/PTHrP receptors

Parathyroid hormone (PTH) plays an important role in the regulation of calcium homeostasis in the adult animal by acting on PTH/PTHrP receptors expressed in the target tissues kidney and bone (reviewed by Mallette, 1991). Soon after PTH is secreted by the parathyroid glands as a molecule consisting of 84 amino acids, it undergoes rapid inactivating proteolysis in the liver resulting in multiple fragments (Bringhurst *et al.*, 1982). Most of the calcium regulatory functions are mediated by the N-terminal portion of PTH, PTH (1-34). The function, if any, of midregion and C-terminal portions of PTH are unknown. Within PTH (1-34), amino acids 25-34 are involved in receptor binding. The N-terminal amino acids 1 and 2 are necessary for stimulation of adenylate cyclase, while region 28-34 is responsible for activation of the IP_3/calcium/PKC pathway (Nussbaum *et al.*, 1980; Jouishomme *et al.*, 1992; Gagnon *et al.*, 1993; Gardella *et al.*, 1993).

PTHrP, despite its name, is not very homologous to PTH. Only at its N-terminus 8 out of a stretch of 13 amino acids are identical to PTH. PTHrP was initially identified in tumors associated with humoral hypercalcemia of malignancy. Subsequently, PTHrP was found to be produced by a wide variety of normal adult and foetal tissues, in which it appears to exert primarily paracrine or autocrine effects. Mice in which the PTHrP gene was ablated died around birth, probably from asphyxia, and exhibited widespread abnormalities of endochondral bone formation (Karaplis *et al.*, 1994). Later studies have suggested that PTHrP and its receptor play a crucial role in the regulation of chondrocyte differentiation in the formation of long bones (Lanske *et al.,* 1996; Vortkamp *et al.*, 1996).

While full length mouse PTHrP consists of 139 amino acids, this molecule is posttranscriptionally processed to yield a group of mature daughter peptides before it is secreted (reviewed in Mallette, 1991; Orloff *et al.,* 1994; Wu *et al.*, 1996).

The amino-terminal fragments (1-34) of both PTH and PTHrP mediate their effects on bone and kidney via the 'classical' PTH/PTHrP receptor (type I) (Abou-Samra *et al.*, 1992). This receptor belongs to a family of G protein-coupled receptors (GPCRs), which are characterized by a structure consisting of seven hydrophobic domains that span the plasma membrane, connected by hydrophilic extracellular and intracellular loops (Fig. 2). The type I PTH/PTHrP receptor is a member of a growing family of GPCR that includes receptors for secretin, calcitonin, vasoactive intestinal peptide, growth hormone-releasing hormone, glucagon and glucagon-like peptide 1. These receptors have the following features in common: a relatively long extracellular N-terminus including a signal peptide, an amino acid homology of 31% to 55%, they bind small peptide ligands and have the unique property to activate G_s and at least one other G protein to transduce the signal (reviewed in Segre and Goldring, 1993). Not all of the actions of the N-terminal PTH and PTHrP fragments involve the type I PTH/PTHrP receptor. There are strong indications for a putative non-classical PTH/PTHrP receptor (type II) in keratinocytes which, upon activation by PTH (1-34) or PTHrP (1-34), elevates intracellular calcium but not intracellular cAMP levels (Orloff *et al.*, 1995). Furthermore, Usdin *et al.* (1995) cloned the so called PTH2 receptor from brain, which has 70% amino acid identity with the classical type I receptor and can only be activated by PTH(1-34) and not PTHrP(1-34), while work of Yamamoto *et al.* (1997) suggests the existence of a receptor in the rat supraoptic nucleus which can only be activated by PTHrP(1-34) and not by PTH(1-34). The biological activities of the mid-region and C-terminal parts of PTH and PTHrP suggest the existence of multiple receptors as well. For instance, the mid-region PTHrP (67-86) has been demonstrated to regulate fetal-placental calcium transport independent of the type I PTH/PTHrP receptor (Kovacs *et al.*, 1996). In addition, the C-terminal fragment PTHrP(107-139), which does not bind or activate the type I PTH/PTHrP receptor, was demonstrated to signal in osteoclasts and neurons (Fenton *et al.*, 1991; Fukayama *et al.*, 1995).

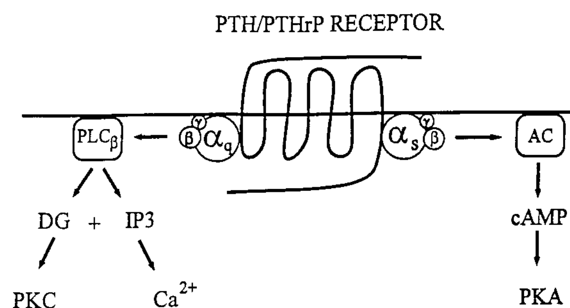

Fig. 2. Cartoon of the PTH/PTHrP receptor, its downstream effectors and the major signal transduction pathways involved.

Involvement of PTHrP in PE differentiation

As suggested above, PE differentiation is regulated by a cooperation of signals derived from cell-cell and cell-matrix contacts and presumably (a) cAMP-elevating factor(s) secreted by the trophectoderm. Several lines of evidence point to PTHrP as a cAMP-elevating factor involved in the formation of PE in the early mouse embryo. Firstly, F9 EC cells and ES cells, treated with RA to induce PrE differentiation, can be induced to differentiate towards a PE-like phenotype by subsequent addition of PTHrP (Chan *et al.*, 1990; van de Stolpe *et al.*, 1993). Secondly, function-perturbing antibodies against PTHrP inhibit the differentiation and outgrowth of PE from isolated ICM, while addition of PTHrP greatly increases the number of PE cells (Behrendtsen *et al.*, 1995). Thirdly, we showed that PTHrP mRNA and protein is expressed in the trophectoderm during early gestation, and that PE cells *in vitro* as well as *in vivo* express type I receptor mRNA (van de Stolpe *et al.*, 1993; Karperien *et al.*, 1994, 1996) and protein (Verheijen *et al.*, 1999a) (Fig. 3).

We recently demonstrated that the induction of PE by PTHrP is mediated exclusively by the type I receptor, at least *in vitro* (Verheijen *et al.*, 1999a). This suggests that other receptors for PTH and/or PTHrP, if present, cannot compensate for a lack of the type I receptor. However, mice in which either the PTHrP or the type I receptor gene were ablated did not show obvious abnormalities in PE formation as judged by histological analyses (Karaplis *et al.*, 1994; Lanske *et al.*, 1996). PTHrP (-/-) mice seem to have a normally developed PE in early gestation, but die at birth (Karaplis *et al.,* 1994). The lack of fetal PTHrP in early gestation of PTHrP (-/-) embryos is, however, likely to be compensated by maternally derived PTHrP. Indeed, large amounts of PTHrP mRNA and protein have been found in the decidua and trophoblast giant cells of mice respectively (Karperien *et al.*, 1994; Verheijen *et al.,* 1999a). Also in the rat, PTHrP is strongly expressed in the uterus at sites of implantation (Beck *et al.*, 1993).

A more severe phenotype is observed in type I PTH/PTHrP receptor(-/-) mice (Lanske *et al.*, 1996). Depending on the genetic background, these mice are smaller than normal from at least day 8.5 p.c. (Verheijen *et al.*, 1999a), while 60% die before day 12.5 p.c. Since the only site of type I receptor mRNA expression before day 9.5. p.c. is the (developing) PE (Karperien *et al.*, 1994), this strongly suggests that the lack of the type I receptor leads to improper differentiation and/or functioning of the PE, causing the smaller size and the early death of these embryos. Dysfunction of components of the parietal yolk sac (trophoblast, Reichert's membrane, or PE) may be the indirect cause of the observed effect on embryonic growth and development.

A survey of the expression of mRNA encoding PTHrP and the type I receptor during later embryogenesis which we carried out using *in situ* hybridization analysis, showed that these molecules are present at various sites where EMI occurs, e.g. in the developing lung, the intestine, in endochondral bone formation and in hair follicles (Karperien *et al.*, 1994, 1996). Based upon the complementary expression of the two molecules in either the epithelium or the mesenchyme, we suggested that they probably played an important role in EMI. Two recent publications provide compelling evidence for such a role in the formation of the murine mammary glands. PTHrP is expressed in the mammary epithelial cells, while the receptor is expressed in the mesenchyme surrounding the mammary bud. In mice devoid of either molecule, morphogenesis was completely disturbed (Dunbar *et al.*, 1998; Wysolmerski *et al.*, 1998). The parallel between the expression patterns in the developing mammary gland with those in the peri-implantation embryo are striking in that the trophoblast (epithelial) expresses high levels of PTHrP and the PE (mesenchymal) expresses the receptor.

The growth of the embryo requires constant remodeling of the Reichert's membrane, which is probably regulated by PE cells, since they secrete large amounts of ECM components as well as proteases. Indeed, addition of PTHrP or dbcAMP to RA-treated ES or F9EC cells strongly induces the secretion of these factors (Strickland *et al.*, 1980; Adler *et al.*, 1990; van de Stolpe 1993). It can therefore be speculated that the phenotype of type I receptor (-/-) embryos is a consequence of a dysfunctioning Reichert's membrane. Indeed, injection of antibodies against preparations of the Reichert's membrane into pregnant rats of day 9.0 p.c. causes growth retardation and resorption of the embryos (Leung, 1977). Although the identity of the antigens is unknown, the phenotype observed is similar to that of type I PTH/PTHrP receptor (-/-) mice.

PTHrP strongly increases expression of the PE marker thrombomodulin in RA-treated F9EC and ES cells. Strikingly, thrombomodulin (-/-) embryos show a phenotype comparable to that of type I receptor (-/-) embryos. At day 8.0 p.c. these mice are smaller than normal, while the embryos fail to survive beyond day 9.5 p.c. Since no obvious pathological abnormalities were observed, and the only site of expression before day 9.5 p.c. was the parietal yolk sac, it was suggested that the failure of thrombomodulin (-/-) embryos to survive was a consequence of dysfunction in maternal-embryonic interaction (Healy *et al.*, 1995). It is possible that thrombomodulin is expressed in the PE to suppress the production of thrombin, thereby preventing coagulation at sites in close proximity to the maternal sinuses and the parietal yolk sac. Taken together, it seems likely that the expression of e.g. thrombomodulin, extracellular matrix components and/or proteases in PE is aberrant in type I PTH/PTHrP receptor (-/-) embryos.

Since there is formation of PE in type I receptor (-/-) embryos, this receptor is apparently not essential for (initial) PE formation. This is supported by our observation that type I PTH/PTHrP (-/-) ES cells can differentiate to some extent to PE after treatment with RA alone. Thus, other signals besides those triggered by PTHrP can also induce PE differentiation. In line with this, Behrendtsen *et al.* (1995) showed that differentiation and outgrowth of PE from isolated ICMs is stimulated more efficiently by (PTHrP-containing) trophectoderm-conditioned medium, than by pure PTHrP alone.

Signal transduction by the type I PTH/PTHrP receptor

Upon binding of PTH(rP) to the type I PTH/PTHrP receptor, the latter is thought to undergo a conformational change, resulting in the activation of G proteins (Fig. 2). Subsequently, these heterotrimeric proteins can activate different effectors either through their α or through their $\beta\gamma$ subunit. The activated type I PTH/PTHrP receptor can activate both adenylate cyclase (AC) and phospholipase Cβ (PLCβ) in several cell types. Adenylate cyclase converts ATP to cyclic AMP (cAMP), which subsequently induces activation of protein kinase A (PKA). PLCβ catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate to produce two second messengers, diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP3), which activate protein kinase C (PKC) and release of calcium from

intracellular stores respectively. Type I PTH/PTHrP receptor-mediated activation of adenylate cyclase is mediated by the G_s protein, while regulation of PLCβ-isoforms occurs through the α-subunits of the G_q-family (Maeda *et al*., 1996; Offermans *et al*., 1996; Iida-Klein *et al*., 1997).

Activation of PLCβ via the PTH/PTHrP receptor requires higher ligand concentrations than receptor-mediated adenylate cyclase activation (Abou-Samra *et al*., 1992; Offermans *et al*., 1996). Furthermore, the activation of PLCβ by PTH is dependent on high receptor density and high G_q expression levels (Civitteli *et al*., 1992; Bringhurst *et al*., 1993; Guo *et al*., 1995; Maeda *et al*., 1996). It is therefore not surprising that most of the actions of PTH and PTHrP are mediated by adenylate cyclase. PLCβ activation was shown to be involved in only a few PTH responses, such as the inhibition of the apical Na^+/H^+ exchanger in renal proximal tubule cells (Azarani *et al*., 1995), and the activation of sodium-dependent phosphate transport in LLC-PK1 cells (Iida-Klein *et al*., 1997).

G protein-coupled receptors and the Ras-MAP kinase pathway

It is becoming increasingly clear that the signaling pathways activated by GPCRs can converge with signaling pathways activated by cell surface receptor tyrosine kinases (RTKs) (for a recent review, see Luttrell *et al*., 1999). They converge particularly on the small GTP binding protein Ras and on mitogen activated protein (MAP) kinase, a serine/threonine kinase occupying the last position in a cascade of kinases activated downstream of Ras, which has been shown to be important in the transduction of extracellular signals involved in cell proliferation and differentiation (Noda *et al*., 1985; Konieczny *et al*., 1989; Pagés *et al*., 1993; Marshall, 1995; Katz and McCormick, 1997). GPCRs regulate Ras and/or MAP kinase activity depending on the identity of the G protein, the receptor and the cell-type involved. G_i-coupled receptors, such as the M2 muscarine acetylcholine receptor, the α_2-adrenergic receptor and the receptors for lysophosphatidic acid (LPA) or thrombin, stimulate MAP kinase in a Ras-dependent manner (Alblas *et al.,* 1993; Howe and Marshall, 1993; van Corven *et al*., 1993; Hawes *et al*., 1995), which is mediated through the release of Gβγ subunits (Crespo *et al.,* 1994; Faure *et al*., 1994; Koch *et al*., 1994; Hawes *et al*., 1995).

Studies on the activation of MAP kinase via G_q have produced contradictory results. As an example, when the M1 acetylcholine receptor was transfected into COS-7 cells, it was reported to activate MAP kinase in a Gβγ subunit- and Ras-dependent fashion by one group (Crespo *et al*., 1994), but in a Ras independent fashion by another (Hawes *et al*., 1995). In contrast, when expressed in Rat-1a cells, the same receptor inhibited MAP kinase activation (Russell *et al*., 1994).

G_s-coupled receptors, such as the type I PTH/PTHrP receptor, the β-adrenergic receptor, or the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor, can either trigger or inhibit MAP kinase activation (Burgering *et al*., 1993; Frödin *et al*., 1994; Hordijk *et al*., 1994; Crespo *et al*., 1995; Verheijen and Defize, 1995, 1997; Vossler *et al*., 1997). This is cell-type dependent and can in most cases be mimicked by addition of cell-permeable cAMP analogs. With respect to the inhibition of MAP kinase activation, it was demonstrated that activation of PKA interfered with Ras-mediated activation of MAP kinase at the level of Raf-1, a serine-threonine kinase upstream of MAPK (reviewed in Burgering and Bos, 1995). Recently, we and others demonstrated that the stimulatory action of cAMP is Ras-independent and probably involves an indirect activation of MEK, a kinase located between raf-1 and MAPK (Faure and Bourne, 1995; Verheijen and Defize, 1997; Vossler *et al*., 1997).

Involvement of the Ras-MAP kinase pathway in PrE and PE differentiation

We recently showed that Ras is rapidly activated by RA treatment of F9EC cells (Verheijen *et al*., 1999b). Furthermore, Ras induces primitive endoderm differentiation of F9 EC cells when overexpressed in a mutated constitutively active form (Yamaguchi-Iwai *et al*.,1990; Verheijen *et al*., 1999b). A possible target of both RA and Ras involved in PrE differentiation is AP-1, a transcription factor family consisting of homo- or heterodimers of Jun, Fos or ATF proteins (Karin *et al*., 1997). Both RA as well as oncogenic Ras induce c-jun expression in EC cells (de Groot *et al*., 1990b; Yamaguchi-Iwai *et al*., 1990; Yang-Yen *et al*., 1990; Kitabayashi *et al*., 1992), while forced expression of c-fos or c-jun in EC cells is sufficient to induce differentiation (Müller and Wagner, 1984; de Groot *et al*., 1990a; Yamaguchi-Iwai *et al*., 1990; Yang-Yen *et al*., 1990). The kinetics of RA-induced Ras activation are comparable with the RA-induced expression of c-jun in F9 EC cells (Yang-Yen *et al*., 1990; Kitabayashi *et al*., 1992; Verheijen *et al*., 1999b). Taken together, this suggests that AP-1 plays an important role in RA- and Ras-induced PrE differentiation.

Additional experiments indicated that Ras has a dual role in the formation of extraembryonic endoderm: while elevation of its activity is tightly coupled to PrE formation, its activity needs to be downregulated in order for PE formation to occur. Thus active Ras probably serves to maintain the PrE phenotype. PTHrP-induced PE differentiation is accompanied by a decrease in Ras activity, and expression of constitutive active Ras prevents PTHrP-induced PE differentiation (Verheijen *et al*., 1999b). *In vivo*, in rats, Ras is expressed at extremely low levels in PE (Brewer and Brown, 1992), suggesting that low activity of Ras is probably important for PE differentiation *in vivo* as well.

In line with the idea that PE differentiation depends on the activation of CRE-regulated genes, Ras inhibits PTHrP-induced CRE activation (M. Verheijen, unpublished). In PC12 cells, a similar effect of Ras has been shown to be caused by binding of pp90rsk to CBP, a transcription factor which is an important cofactor for CREB in transcriptional activation (Nakajima *et al*., 1997). Interestingly, in F9 cells, targeting of CBP with adenovirus 12S E1A protein inhibits CRE activation and PE differentiation (Weigel *et al*., 1990; Lundblad *et al*., 1995; Nakajima *et al*., 1997). In analogy with PC12 cells, where overexpression of the pp90rsk-binding domain of CBP prevents Ras-induced CRE inactivation, it will be interesting to determine the effect of overexpression of this domain or full length CBP on the inhibition of PE differentiation by Ras. Another target of Ras could be molecules involved in cell morphology. PTHrP-induced cell retraction is probably mediated by PKA-induced breakdown of stressfibers (Miller *et al*., 1976; Aubin *et al*., 1983), and possibly involves inactivation of Rho (Lang *et al*., 1996), or myosin light chain kinase (Lamb *et al*., 1988). Since Ras-mediated activation of Rho can induce stress fiber formation (Ridley and Hall, 1992), this might prevent PTHrP-induced disrup-

tion of cell-cell contacts (see below), and thereby inhibit PE differentiation.

In contrast to models proposed by others in which cAMP inhibits the Ras-MAP kinase pathway downstream of Ras (see above), we found that cAMP inhibits Ras activity itself in PrE-like F9 cells. We observed a strong correlation between the reduction in Ras and p42 MAP kinase activity, suggesting that the cAMP-induced MAP kinase inhibition is a consequence of the inhibition of Ras activity (Verheijen *et al.,* 1999b). The mechanism involved in the inhibition of Ras activity by cAMP is unclear. cAMP might inhibit Ras activation via inhibition of Ras nucleotide exchange factors, as it has been reported to induce phosphorylation of one such factor, i.e. Sos (Burgering *et al.*, 1993). On the other hand, cAMP may inhibit Ras activity via activation of a RasGTP'ase activating protein (GAP), since PKA has been demonstrated to phosphorylate neurofibromin, a member of the GAP family (Izawa *et al.*, 1996). Also, in addition to its effect on Ras, RasGAP inhibits Rho activity through activation of Rho-GAP, resulting in breakdown of stress fibers and impaired cell adhesion (McGlade *et al.*, 1993). Since PTHrP inhibits Ras and induces disruption of cell-cell contacts and breakdown of stress fibers (L.H.K. Defize, unpublished results), one could speculate that these effects are mediated by cAMP-induced RasGAP activation.

Thus, the activity of Ras seems a crucial determinant in PE formation (Fig. 3), suggesting that it may be tightly regulated *in vivo*. Attractive candidates for *in vivo* regulators of Ras activity and PE differentiation are cell-cell and cell-matrix contacts, as will be discussed in the next paragraph. Other possible candidates could be soluble growth factors, such as PDGF, EGF and TGFβ, since these factors and their receptors are expressed by endoderm-like ES and F9 cells (reviewed in Mummery and van den Eijnden-van Raaij, 1990). Interestingly, several lines of research indicate that TGFβ is indeed involved in PE differentiation: (i) TGFβ inhibits retraction of PE-like PYS-2 cells (Kelly and Rizzino, 1989), (ii) TGFβ inhibits PE differentiation and proliferation of RA-treated F9 cells (M.H.G. Verheijen and L.H.K. Defize, unpublished results), (iii) TGFβ inhibits differentiation and outgrowth of PE from plated ICMs (Roelen *et al.*, 1998), and iv) treatment of ES cells with RA in the absence of LIF induces differentiation predominantly into a PE-like phenotype when the cells express a dominant negative TGFβ-II receptor (Goumans *et al.*, 1998). TGFβ has been demonstrated to activate the Ras-MAP kinase pathway in several cell-types (Mulder and Morris, 1992; Huwiler and Pfeilschifter, 1994; Hartsough and Mulder, 1995; Yamaguchi *et al.*, 1995). Furthermore, the mesoderm-inducing activity of TGFβ on animal caps from *Xenopus Laevis* embryos expressing the TGFβ-receptor II ectopically, is inhibited by dominant-negative Ras (Bhushan *et al.*, 1994). Whether TGFβ-induced inhibition of PE differentiation is mediated by activation of Ras-coupled signal transduction pathways remains to be determined.

We observed that the RA-induced PrE differentiation of F9 cells is not affected by inhibiting MAP kinase activity with a specific inhibitor (Verheijen *et al.*, 1999b). Conversely, while PTH inhibited MAP kinase activity in PrE like cells, inhibition of MAP kinase activity was not sufficient to induce PE differentiation. Therefore, we conclude that MAP kinase is not necessary for RA-induced PrE differentiation and that inhibition of MAP kinase in PrE-like cells is not sufficient to trigger PE differentiation. Other Ras-effectors, like phosphoinositide 3-kinase (PI3-kinase) or Ral guanine nucleotide exchange factor (Ral-GEF) could play an important role in mediating the effects of Ras on PrE and PE differentiation as well. For instance, PI3-kinase has recently been shown to be involved in cell transformation and reorganization of the cytoskeleton by Ras (Rodriguez-Viciana *et al.,* 1997), while Rlf, a member of the Ral-GEF family, mediates Ras-induced gene expression independent of MEK and MAP kinase (Wolthuis *et al.*, 1997). Experiments in which the effects of expressing constitutively active forms of the various Ras effectors on the induction of PrE and the inhibition of PE differentiation will hopefully provide insight in this complex issue.

Very recently, additional evidence for the crucial role of Ras signaling in extraembryonic endoderm formation was obtained by Cheng *et al.* (1999). These authors showed that ablation of the gene encoding Grb-2, an adaptor molecule linking various plasma membrane receptors to the activation of Ras, results in an early embryonic lethal phenotype. No homozygotic Grb-2$^{-/-}$ embryos could be recovered after embryonic day 7.5. Furthermore, these authors showed that Grb-2$^{-/-}$ ES cells are not growth inhibited, but completely fail to form endoderm *in vitro* when cultured under conditions that otherwise readily lead to endoderm formation. Importantly, the failure to form endoderm could be rescued completely by expressing a constitutively active form of ras in the cells. It will be of interest to test whether also the effect of RA on endoderm differentiation is attenuated in these cells.

Involvement of cell-cell interactions in PE differentiation

Several lines of research suggest that the differentiation of PE is regulated by a crosstalk between signals derived from PTHrP and cell-cell/cell-matrix interactions. As already mentioned, PE differentiation has several characteristics of an epithelial-to-mesenchymal transition. In short, PrE and VE, which form an epithelial cell layer with tight junctions, contribute to PE by detaching from their neighboring cells, assuming an elongated morphology with extended filopodia, and migrating onto the Reichert's membrane, away from the epithelial layer from which they originated (Enders *et al.*, 1978; Hogan and Newman, 1984).

The importance of breaking cell-cell contacts for PE differentiation is demonstrated by several observations: 1) when cell-cell contacts of VE-like F9 cells are disrupted, by isolation of the cells and culture in monolayer, a transition from a VE-like to a PE-like phenotype is observed (Casanova and Grabel, 1988). 2) When an adhesion-defective variant of F9 EC cells is grown in suspension in the presence of RA, the cells do not from embryoid bodies and do not differentiate to VE, but instead acquire a PE-like phenotype (Grover *et al.*, 1987). 3) Addition of dbcAMP to RA-treated embryoid bodies induces cells in the outer layer of VE to differentiate into rounded, loosely packed PE-like cells expressing high level of tPA (Grover and Adamson, 1986). 4) Treatment of PrE-like F9 cells with dbcAMP or PTHrP induces a rapid and transient retraction of the cells prior to the stable PE-like morphology observed after 2 days of treatment. Thus, the breaking of cell-cell contacts might be a trigger for PE differentiation, or at least be permissive for PE differentiation to occur.

If so, what might be the nature of signals derived from (disruption of) cell-cell contacts that could be involved in PE differentiation? One possibility is that signals triggered by activated cell adhesion molecules are non-permissive for PE differentiation. An attractive candidate for such a signal is Ras, in the light of its inhibitory action

on PE differentiation of PrE-like F9 cells (Verheijen *et al.*, 1999b). Stimulation of cell-cell adhesion molecules has been demonstrated to induce Ras activation (Xu *et al.*, 1997), and PTH-induced differentiation of PrE-like F9 cells towards a PE-like phenotype is accompanied by breaking of cell-cell contacts and by a decrease in Ras activity (Verheijen *et al.*, 1999b). Whether cell adhesion-induced Ras activity is indeed manifest in PrE-like cells remains to be determined. If this hypothesis is correct, forced disruption of cell-cell contacts by using interfering peptides/antibodies, or by culturing of the cells at low density should decrease basal Ras activity.

A second possibility is that the breaking of cell-cell contacts triggers a PE-inducing signal. Recently, it became apparent that β-catenin is involved not only in cell adhesion processes by binding to cadherins (reviewed in Kemler, 1993), but also in signal transduction pathways by binding to members of the transcription factor family TCF/LEF (Molenaar *et al.*, 1996). Interestingly, treatment of PrE-like F9 or PYS-2 cells with PTHrP induces downregulation of cadherins and translocation of β-catenin to the nucleus (L.H.K. Defize, manuscript in preparation). An attractive hypothesis is that PTHrP-induced disruption of cell-cell contacts leads to release of β-catenin from cadherins, after which the free β-catenin molecules translocate to the nucleus and trigger the expression of genes involved in PE differentiation. It will be of interest to determine whether expression of wildtype or mutated forms of β-catenin and/or TCF affects PE differentiation.

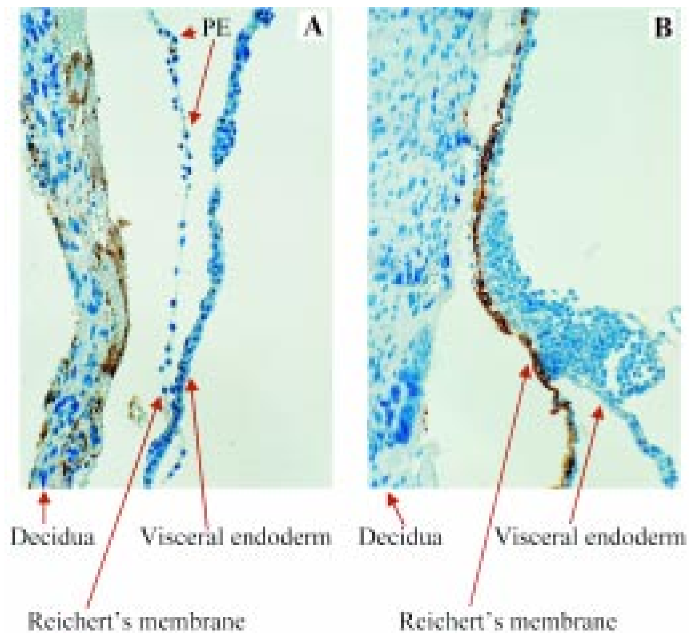

Fig. 3. Complementary immunohistochemical localization of PTHrP in the trophoblast giant cells and the decidua (panel A) and of the type I PTH(rP) receptor in the PE cells on the Reichert's mebrane (panel B).

Involvement of cell-matrix interactions in PE differentiation

Plating of F9 embryoid bodies or isolated ICMs on a fibronectin-coated substrate is sufficient to induce outgrowth and differentiation of PE (Grabel and Watts, 1987; Behrendtsen *et al.*, 1995). Furthermore, gradients of laminin or fibronectin promote the migration of primitive and PE-like F9 cells (Carnegie, 1994). It is unclear whether the interaction with ECM triggers PE differentiation or whether it is merely involved in migration of the cells. Deletion of β1-integrins in F9 EC cells prevents adhesion and migration on fibronectin, laminin and collagen, but does not reduce cAMP-induced PE differentiation, suggesting that matrix adhesion is not essential for PE differentiation, at least when induced by cAMP (Stephens *et al.*, 1993). Furthermore, plating RA-treated F9 cells from monolayer directly on an FN-coated surface does not induce differentiation, suggesting that the mere contact with FN is not sufficient (L.H.K. Defize, unpublished observations). Thus, migration induced by ECM contact could be instrumental in the breaking of cell-cell contacts in PrE, which in itself could trigger PE differentiation through mechanisms discussed in the previous paragraph. Interesting in this respect is that for neural crest cells migrating on fibronectin it has been demonstrated that the activity of the cell adhesion molecule N-cadherin is negatively regulated by intracellular signals elicited by integrins (Monier-Gavelle and Duband, 1997).

Adhesion to fibronectin has been demonstrated to elevate cAMP levels in endothelial cells (Fong and Ingber, 1996), and a similar action of fibronectin on primitive or VE cells might also explain its PE-inducing potential. Other ECM molecules such as laminin, collagen and vitronectin do not sustain PE outgrowth. Surprisingly, these substrates become permissive for PE outgrowth from ICM's when the cells are treated with PTHrP (Behrendtsen *et al.*, 1995). One explanation for the action of PTHrP can be that it affects the expression or functionality of integrins. Differentiation of PrE-like F9 cells into PE is accompanied by a switch in expression of the laminin integrin subunit α6B to α6A, while the localization of the laminin receptor is changed from cell-cell contacts to focal contacts (Jiang and Grabel, 1995; Morini *et al.*, 1997). Interestingly, integrin-matrix interactions modulate the Ras-MAP kinase cascade in several cell types and, furthermore, permit efficient activation of this pathway by growth factors (Clark and Hynes, 1996; Lin *et al.*, 1997; Mainiero *et al.*, 1997). Since Ras inhibits PE differentiation (Verheijen *et al.*, 1999b), it can be speculated that the switch in localization and expression of several integrins observed during PE differentiation (Hierck *et al.*, 1993; Burdsal *et al.*, 1994; Jiang and Grabel, 1995;

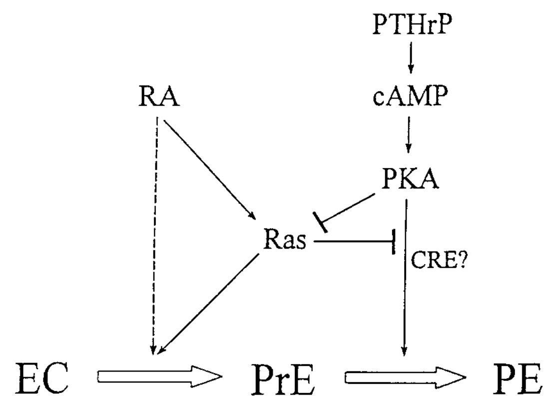

Fig. 4. A model for the regulation of PrE and PE differentiation by Ras and PKA.

Morini *et al.*, 1997), leads to downregulation of matrix-induced Ras activation, and thereby to permission of PE differentiation.

Concluding remarks

The type I PTH/PTHrP receptor is essential for the induction of PE differentiation by PTHrP *in vitro*, while the expression patterns of PTHrP and the type I receptor strongly support a role for this receptor-ligand system in PE formation *in vivo*. Taken together with the phenotype of type I receptor (-/-) embryos, and the potential of type I receptor (-/-) ES cells to differentiate into PE-like cells, we propose that this receptor, although not essential for the induction of a PE-like structure, is involved in proper differentiation and/or functioning of PE. We envision the action of PTHrP in PE differentiation *in vivo* as follows: upon secretion by the trophectoderm, PTHrP will activate type I PTH/PTHrP receptors expressed on PrE and VE cells. Especially the cells in the marginal zone express high levels of the type I receptor and will lose their cell-cell contacts upon stimulation with PTHrP. Subsequently, the loosely adherent cells will migrate out of the epithelial layer via interaction with the basal lamina of the trophectoderm at early stages, and with the Reichert's membrane at later stages. It is probable that the disruption of cell-cell contacts leads to the disappearance of a non-permissive signal derived from cell adhesion molecules which could involve Ras, and furthermore, that signals derived from activated integrins and the type I PTH/PTHrP receptor cooperate to induce differentiation into PE and migration over the trophectoderm. The continuous stimulation by PTHrP induces secretion of ECM proteins and proteases, leading to the formation and remodeling of the Reichert's membrane. Via this action and by its effects on thrombomodulin secretion, PTHrP will contribute to a properly functioning parietal yolk sac.

With respect to the signaling pathways involved in PTHrP-induced PE differentiation, the following can be concluded. Strong activation of PKA is sufficient to induce PE differentiation. This suggests that the action of PTHrP might be solely mediated by PKA. However, the involvement of G_q-mediated events, cannot at present be ruled out. PTHrP-induced inhibition of Ras in PrE-like cells seems crucial for PE differentiation, since high levels of Ras activity in PrE-like cells suppress differentiation towards PE. The mechanism of Ras inhibition by PTHrP is unclear, as is the suppression of PE differentiation by activated Ras. We are currently looking for genes that are expressed after PTHrP treatment of PrE-like F9EC cells. Analysis of their expression in the absence or presence of active Ras, followed by analysis of promoter elements involved might yield clues on how Ras suppresses PE formation. As mentioned, preliminary data suggest that indeed, active Ras inhibits PKA-induced transcriptional activation of a CRE-reporter construct. Taken together, we propose that PE differentiation in the early mouse embryo is tightly regulated by a cross-talk between signal transduction pathways involving Ras and PKA, respectively. While PTHrP can account for the activation of PKA, the factors involved in the activation of Ras remain to be identified, although cell adhesion molecules, ECM components and other growth factors such as TGFβ are attractive candidates.

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