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Original Article

Snail is an immediate early target gene of parathyroid hormone related peptide signaling in parietal endoderm formation

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ABSTRACT In mouse development, parietal endoderm (PE) is formed from both primitive endoderm (PrE) and visceral endoderm (VE). This process can be mimicked *in vitro* by using F9 embryonal carcinoma cells (EC) cells, differentiated to PrE or VE cells, and treating these with Parathyroid Hormone related Peptide (PTHrP). By means of differential display RT-PCR, we identified *Snail (Sna)* as a gene upregulated during the differentiation from F9 PrE to PE. We show that *Sna* is an immediate early target gene of PTHrP action in the formation of F9 PE cells. Using RT-PCR, we detected *Sna* transcripts in pre-implantation mouse embryos from the zygote-stage onwards. *Sna* was strongly upregulated in parallel with *type 1 PTH/PTHrP Receptor (PTH(rP)-R1)* mRNA in mouse blastocysts plated in culture, concomitant with detection of the PE-marker *Follistatin* and appearance of PE cells. By radioactive *in situ* hybridization on sections of mouse embryos, we found *Sna* expression in the earliest PE cells at E5.5. *Sna* remained expressed until at least E7.5. At this stage, we also observed clear expression in endoderm cells delaminating from the epithelial sheet of VE cells in the marginal zone. We conclude that *PTH(rP)-R1* and *Sna* are expressed in endodermal cells that change from an epithelial to a mesenchymal phenotype. Since *Sna* expression has been described at other sites where epithelio-mesenchymal transitions (EMT) occur, such as the primitive streak at gastrulation and in pre-migratory neural crest cells, we hypothesize that Sna is instrumental in the action of PTHrP inducing PE formation, which we propose to be the first EMT in mouse development.

KEY WORDS: *Sna, PTHrP, parietal endoderm, F9 cells, mouse*

Introduction

Parathyroid Hormone related Peptide (PTHrP) induces parietal endoderm (PE) differentiation *in vitro* in F9 embryonal carcinoma (EC) cells that have been first treated with *all-trans* retinoic acid (RA) to form F9 primitive endoderm (PrE) (Chan *et al.*, 1990; van de Stolpe *et al.*, 1993). The only receptor involved in PE-formation *in vitro* is the "classical" or type 1 PTH/PTHrP-receptor (here referred to as PTH(rP)-R1) (Verheijen *et al.*, 1999). The complementary distribution of mRNA and protein of both *PTHrP* and *PTH(rP)-R1* during mouse development suggests that this hormone-receptor complex plays a similar role *in vivo* (Karperien *et al.*, 1994, 1996; Verheijen *et al.*, 1999). This is supported by data showing that function-perturbing antibodies against PTHrP inhibit outgrowth and differentiation of PE cells from isolated inner cell masses (Behrendtsen *et al.*, 1995). Binding of PTHrP or $PTHrP_{1-34}$ to PTH(rP)-R1 can activate two distinct G-protein coupled signaling cascades, leading to intracellular Ca^{2+} release and an increase in intracellular cyclic AMP respectively (Abou-Samra *et al.*,1992; Bringhurst *et al.*, 1993). Both pathways can be activated in F9 PrE cells, when treated with $PTHrP_{1-34}$ (Verheijen and Defize, unpublished results). However, elevation of intracellular cyclic AMP levels alone, by using the cell permeable cyclic AMP analog dibutyryl cyclic AMP (dbcAMP), is sufficient to induce complete transition to PE-like cells (Strickland *et al.*, 1980).

Abbreviations used in this paper: dbcAMP, dibutyryl cyclic AMP; DD-RT-PCR, differential display RT-PCR; EC, embryonal carcinoma; EMT, epithelio-mesenchymal transition; FGF, fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICM, inner cell mass; PE, parietal endoderm; PrE, primitive endoderm; VE, visceral endoderm; PrEc, primitive ectoderm; PTH, Parathyroid Hormone; PTHrP, PTH related Peptide; PTH(rP)-R1, type 1 PTH/PTHrP Receptor; RA, *all-trans* retinoic acid; Slu, Slug; Sna, Snail; TE, trophectoderm; TM, Thrombomodulin.

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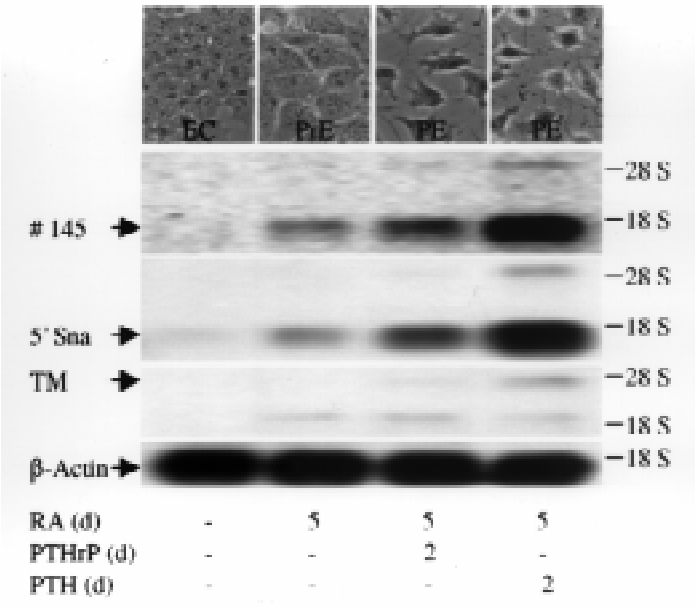

Fig. 1. Differential expression of *Sna* during F9 differentiation. *The top panel shows microphotographs of F9 cells in their various differentiation stages as a result of the treatment indicated at the bottom. Below the microphotographs are Northern blot results of poly-A$^+$ RNAs of differentiating F9 cells hybridized with differential display clone #145, a probe of the 5' region of Sna, Thrombomodulin (TM), and β-Actin. 28S and 18S ribosomal RNA bands as seen in control lanes with total RNA are indicated at the right. Abbreviations: EC, embryonal carcinoma; PrE, primitive endoderm; PE, parietal endoderm; d, days of treatment; RA, 10^{-6} M all-trans retinoic acid; PTH, 10^{-7} M Parathyroid Hormone; PHTrP, 10^{-7} M PTH related Peptide.*

In order to identify target genes for PTHrP in the formation of PE, we carried out a differential display RT-PCR on cDNA from F9 PrE and F9 PE cells, and found the *Snail* gene *(Sna)* to be differentially expressed. *Sna* belongs to the evolutionarily conserved *snail* family of the Cys_2His_2 class of zinc finger transcription factors. Their relationship is mainly based on strong conservation of the C-terminal zinc finger domain in all species. *Sna* was originally identified in *Drosophila* (Simpson, 1983; Grau *et al.*, 1984; Boulay *et al.*, 1987), that also expresses the closely related family members *escargot* (Whiteley *et al.*, 1992), *worniu* (Ashraf *et al.*, 1999) and the somewhat less related pan-neural gene *scratch* (Emery and Bier, 1995; Roark *et al.*, 1995). Most vertebrate species have two *snail* genes, *Snail (Sna)* and *Slug (Slu* or *Slugh)*, of which *Sna* molecularly and, in mouse, also functionally most closely resembles *Drosophila sna* and *escargot*.

In the chick embryo, expression patterns of *Sna* and *Slu* are largely inverted compared to mouse. However, their combined expression pattern is highly conserved in all vertebrate embryos. This, with the molecular similarities, led to the suggestion that the genes arose by duplication of an ancestor gene, and that they exert similar functions. At sites where they were originally co-expressed, their functional redundancy could lead to one gene being lost (Sefton *et al.*, 1998). Analysis of expression patterns and function, have clearly shown *cSlu* to be important for the epithelio-mesenchymal transition (EMT) that takes place in the presumptive mesoderm and neural crest (Nieto *et al.*, 1994), limb bud formation (Buxton *et al.*, 1997; Ros *et al.*, 1997), and in the developing heart (Romano and Runyan, 1999). A similar role for *Xenopus laevis Slu* has also been shown in the EMT that takes place in the neural crest (LaBonne and Bronner-Fraser, 1998; Carl *et al.*, 1999), and has been suggested for the murine *Slu* gene on the basis of expression pattern (Savagner *et al.*, 1998) and *in vitro* studies (Savagner *et al.*, 1997). However, the mouse null mutant for *Slu* (Jiang *et al.*, 1998b) showed no obvious EMT-defect in mesoderm and neural crest formation. The explanation for this may be that *Slu* is expressed late in mouse development (after E8.5), and relatively late in presumptive migratory neural crest (Jiang *et al.*, 1998b; Savagner *et al.*, 1998; Sefton *et al.*, 1998); another gene, which may be partially redundant later, is therefore likely to regulate earlier EMTs. *Sna* would be a good candidate for this, since (1) it is expressed earlier and more widely in mouse development than *Slu,* (2) *Slu* and *Sna* expression overlap in late development, (3) expression of *Sna* is high in parietal endoderm, presumptive mesoderm in the primitive streak and derivatives, in mesenchymal cells in limb bud, lung, kidney, and in pre-migratory as well as migratory neural crest cells (Nieto *et al.*, 1992; Smith *et al.*, 1992; Sefton *et al.*, 1998), all possible sites of EMT, and (4) *sna* has been shown to be functionally redundant to *escargot* and *worniu* in *Drosophila* (Ashraf *et al.*, 1999).

We have recently proposed that the differentiation of PrE or visceral endoderm (VE) to PE resembles an EMT (Verheijen and Defize, 1999). At the blastocyst stage, an epithelial layer of PrE cells covers the inner cell mass (ICM) of the mouse embryo. Where PrE cells are in contact with the trophectoderm (TE), however, they have a PE-like morphology, in that they are elongated and only loosely associated (Enders *et al.*, 1978). As implantation begins, PrE cells delaminate from the elongating egg-cylinder, and start forming PE. PrE cells remaining in contact with the epiblast develop into VE, a polarized epithelium with basal-apical polarity, apical tight junctions and numerous microvilli. At the junction between the epiblast and the trophectoderm, the so-called marginal zone, PE cells delaminate from this epithelial sheet of VE cells and assume an elongated, fibroblast-like morphology. Later, they acquire a rounded cell body from which long, filopodia-like protrusions extend (Enders *et al.*, 1978), providing a front-to-back polarity. PE cells have a smooth surface, produce and secrete copious amounts of extra-cellular matrix which form the Reichert's membrane (Hogan *et al.*, 1980, 1982; Smith and Strickland, 1981; Kurkinen *et al.*, 1983; Mason *et al.*, 1986), are motile as indicated by their filopodia, and lack E-Cadherin (Defize *et al.,* unpublished).

TABLE 1

CHARACTERISTICS OF EPITHELIAL AND MESENCHYMAL CELLS AND THEIR DISPLAY IN PrE, VE AND PE

EPITHELIUM	*PrE VE*	*MESENCHYME*	*PE*
Sheets of cells	√	Single, loose cells	√
Linked by cell adhesion molecules (mainly E-Cadherin)	√	No or low (E-)Cadherin expression	√
Non-motile	√	Highly motile	√
Basal-apical polarity	√	Front-to-back polarity	√
Apical microvilli	√	Smooth surface, no microvilli	√
Non-invasive (attachment to basal lamina)	√	Invasive (migration through basal lamina)	–
		Secretion of copious amounts of ECM	√
		Production of proteases	√

Abbreviations: PrE, primitive endoderm; VE, visceral endoderm; PE, parietal endoderm; ECM, extra-cellular matrix. (Modified from Verheijen and Defize, 1999).

These features of PE are all characteristic of mesenchymal cells (Hay, 1995), suggesting that PrE to PE and VE to PE transitions may be the first EMT in mouse development (see also Table 1).

PE expresses *Sna* at E6.5, and maintains expression until at least E8.5 (Nieto *et al.*, 1992; Smith *et al.*, 1992). However, the onset of PE formation occurs earlier, around the time of implantation. In the light of *Sna* upregulation in F9 cells undergoing a PrE to PE transition, we investigated the onset and regulation of its expression both *in vitro* in F9 cells and *in vivo*. We here demonstrate for the first time that in F9 PrE cells, *Sna* is an immediate early response gene to PTHrP and dbcAMP, and that this induction is correlated with the formation of PE. We furthermore document a link between the spatio-temporal expression of the *PTH(rP)-R1* and *Sna* in PE-formation *ex vivo* and *in vivo*. Thus, *Sna* is likely to be important both in inducing and in maintaining the changes in morphology and motility arising during PE formation. In the light of the expression of s*nail* homologs in EMTs in a variety of tissues, and the present results, we conclude that the formation of PE is the earliest EMT in mouse development.

Results

DD-RT-PCR reveals Sna upregulation in F9 parietal endoderm

Differential display RT-PCR with 12 arbitrary primers on cDNA derived from F9 PrE and PE cells yielded 25 fragments upregulated in F9 PE, and 20 of these fragments could be re-amplified (results not shown). These were cloned into PCR2.1-TOPO vector and of each cloned fragment, 8 colonies were screened to eliminate false positives as described under materials and methods. All but 4 clones, representing 4 different fragments, were eliminated, and ultimately tested in a Northern blot. Of these 4 fragments, we confirmed upregulation of only one during differentiation of F9 cells (#145, Fig. 1). Sequence analysis revealed that this fragment of 300bp was 98% homologous to part of the zinc finger domain of *Sna* [bp536-836, Nieto *et al.* (1992)]. Indeed, the fragment hybridized to a transcript of approximately 1.7 kb in Northern blot analysis, as previously reported for *Sna* (Nieto *et al.*, 1992; Smith *et al.*, 1992). This transcript was hardly detectable in F9-EC cells, but clearly visible in F9-PrE and PE (Fig. 1). In addition to this transcript, which is the only one reported in mouse (Nieto *et al.*, 1992; Smith *et al.*, 1992), we detected a transcript larger than 4.8 kb, the approximate genomic size of *Sna*, in cultures treated with PTHrP or PTH (right 2 lanes in Fig. 1). In our hands, PTH is more potent in inducing F9 PE than PTHrP is, as indicated by transcription of the PE-marker *Thrombomodulin* (Imada *et al.*, 1990) and a more profound rounded up morphology after 48 h treatment (Fig. 1, upper panel). This is also reflected in *Sna* transcription.

To rule out the possibility of cross hybridization with another (zinc finger) containing mRNA, parallel blots were hybridized with a probe spanning the 5' (non-zinc finger) region (Fig. 1) and a probe spanning the 3'-UTR (not shown), both generated from the *Sna* cDNA clone 5-15. These probes recognize the 1.7 kb transcript, as well as the larger one, strongly suggesting that both are *Sna* mRNAs. The 5' probe was used in subsequent Northern blot experiments.

Sna is a target for elevated intracellular cyclic AMP levels specific for PE-formation

Snail family members are mostly associated with the onset of EMT and are particularly expressed in mesenchyme. Since aspects of the differentiation of PE from PrE resemble such a transition to mesenchymal cells, we focused on this differentiation step, and determined the kinetics of *Sna* transcription following addition of PTHrP to F9 PrE cells by Northern blot hybridization. As can be seen in Figure 2A, RA alone already induces *Sna* to some extent within 1 day, and the level is maintained during prolonged treatment. However, within half an hour after subsequent PTHrP treatment of F9 PrE cells, *Sna* expression increases significantly. The upregulation is high initially, while *Sna* mRNA levels gradually decrease, but after 48 h they are still higher than in cells treated with RA alone. In three independent experiments, we observed a similar rapid increase and subsequent downregulation in *Sna* mRNA levels, although the extent and duration varied and seemed to positively correlate with the extent to which cells show the morphological changes (as visualized in microphotographs in Figure 1) associated with terminal PE differentiation (data not

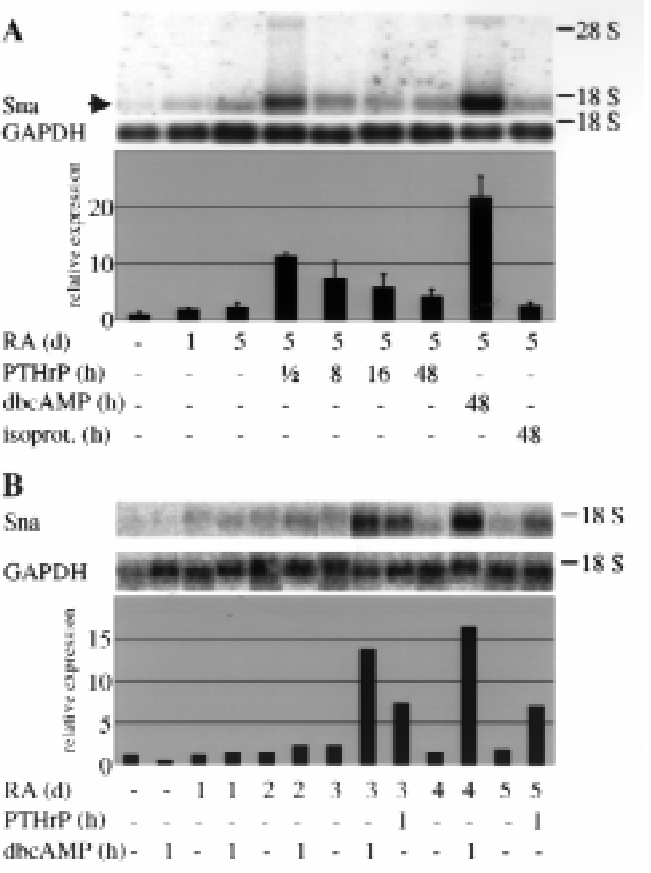

Fig. 2. Rapid induction of *Sna* by PTHrP in F9 PrE cells, specific for differentiation to PE. *Northern blots of poly-A⁺ RNA of F9 cells in various stages of differentiation, hybridized with the 5' Sna probe and GAPDH as a loading control. 28S and 18S ribosomal RNA bands as seen in control lanes loaded with total RNA are indicated at the right. Bars represent the ratio between Sna and GAPDH relative to EC cells. Abbreviations used are as in Figure 1 and dbcAMP, 1 mM dibutyryl cyclic AMP; isoprot, 10 μM isoproterenol; h, hours of treatment.* **(A)** *Cells were grown for 5 days in the absence or presence of RA to induce PrE, and treated for the last 1/2 to 48 h with PTHrP or dbcAMP to induce PE, or with isoproterenol as a control. Error bars indicate SEMs of three independent culturing experiments.* **(B)** *F9 cells were left untreated or treated for varying periods with RA and subsequently treated during 1 h with PTHrP or dbcAMP.*

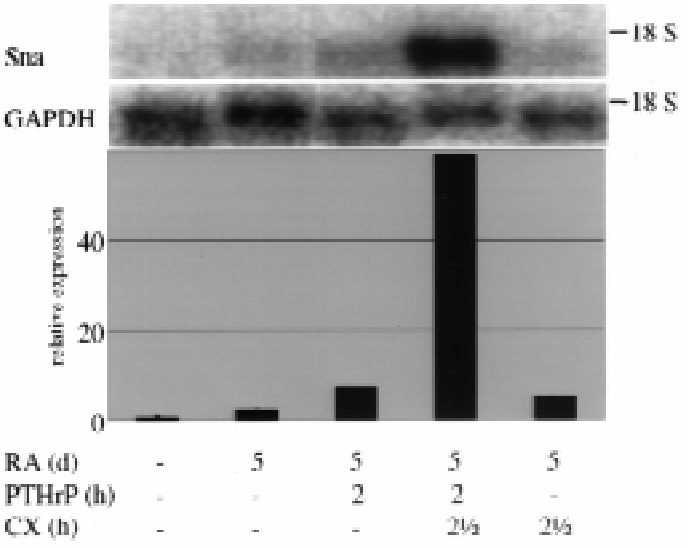

Fig. 3. *Sna* is an immediate early target of PTHrP in F9 PrE cells. *Northern blot of poly-A⁺ RNA of F9 EC, and PrE with or without induction with PTHrP for 2 h, hybridized with the 5' Sna probe and GAPDH as a loading control. Abbreviations used are as in Figure 1. Cycloheximide (CX, 20 μg/ml) was added to the cells 30 min prior to PTHrP addition, or as a control added without PTHrP. Bars represent the ratio between Sna and GAPDH relative to EC cells. This experiment was repeated twice with 30 min PTHrP treatment, and yielded similar results. The 18S ribosomal RNA band as seen in the control lane loaded with total RNA is indicated at the right.*

shown). When F9 PrE cells are treated with dbcAMP instead of PTHrP, *Sna* is also upregulated, suggesting the effect of PTHrP is mediated via the cyclic AMP/Protein kinase A pathway. After 48 h of treatment with dbcAMP *Sna* is higher than in cells treated with PTHrP and under these conditions more cells display a profoundly mesenchymal morphology than following PTHrP treatment, again suggesting that regulation of *Sna* expression correlates with the extent to which cells acquire the mesenchymal PE phenotype.

In contrast, in F9 PrE cells treated for 48 h with isoproterenol, *Sna* mRNA levels are comparable to those in cells treated with RA alone (Fig. 2A). Isoproterenol elevates intracellular cyclic AMP levels in these cells to a similar or higher extent as PTH and likewise is capable of activating reporter constructs via cyclic AMP response elements (Verheijen and Defize, unpublished results) but does not induce a differentiated PE morphology. The absence of elevated *Sna* mRNA levels upon prolonged isoproterenol treatment suggests that upregulation of *Sna* transcription via persistently high levels of cyclic AMP is correlated with differentiation, and not simply an effect of elevated intracellular cyclic AMP levels as such. This was further investigated by adding dbcAMP to cells that were previously either untreated or treated with RA for varying periods. As can be seen in Figure 2B, cultures untreated or treated with RA for 1 or 2 days do not show elevated *Sna* transcription after 1 h dbcAMP treatment. Addition of dbcAMP for 1 h results in elevated *Sna* mRNA levels only when cells are treated with RA for at least three days. This coincides with differentiation to PrE-like cells, as indicated by e.g. expression of the *PTH(rP)-R1* (van de Stolpe *et al.*, 1993). In these cells, PTHrP also effectively raises *Sna* levels (Fig. 2B). Longer treatment with RA does not induce higher *Sna* levels in response to PTHrP, and levels are comparable to those observed in other experiments in cells treated with RA during 5 days followed by a short treatment with PTHrP (Figs. 2A and 3).

Sna is an immediate early target gene of PTH(rP)-R1 signaling in the formation of PE

The capacity of PTHrP to induce *Sna* transcription within 30 min, raised the question whether *Sna* is an immediate early target of PTH(rP)-R1 signaling and may thus be a key element in PE formation. To address this, we treated F9 PrE for 30 min or 2 h with PTHrP in the absence or presence of cycloheximide or with cycloheximide alone. Cycloheximide was added 30 min prior to PTHrP addition, in order to block all protein synthesis. Northern blot analysis (Fig. 3) shows that *Sna* transcripts are still expressed, in fact at even higher levels in cultures treated with both PTHrP and cycloheximide, or cycloheximide alone. This indicates that *Sna* is indeed an immediate early target gene for PTH(rP)-R1 signaling. Results are shown for 2 h of PTHrP treatment only, and were similar in two other experiments in which cells were treated with PTHrP during 30 min.

Sna is upregulated in the formation of PE-like cells independent of the direct precursor

F9 EC cells treated in monolayer with RA and PTHrP differentiate non-synchronously, but specifically to PE. Their developmental potency is, however, increased by growth as aggregates in suspension culture. Exposure to RA under these conditions of "embryoid body formation" results in a core of undifferentiated cells, surrounded by an outer layer of VE-like cells that express the VE marker alpha-fetoprotein and are capable of trans-differentiation into PE upon re-plating on an appropriate extra-cellular matrix component. This process is enhanced by addition of dbcAMP (Grover *et al.*, 1983; Hogan *et al.*, 1983; Grabel and Watts, 1987) or PTHrP (our unpublished observations). We re-plated 5 aggregates per well to allow non-overlapping outgrowth. To determine the relative levels of *Sna* in such small cultures, we developed and validated a strategy for semi-quantitative RT-PCR (described under Materials and Methods). To further validate our strategy, a semi-quantitative RT-PCR on *Sna* and *β-Actin* was done with cDNA generated from RNA samples used for Northern blotting. Figure 4A shows that changes in mRNA levels determined by semi-quantitative RT-PCR reflect the changes as conventionally measured on a Northern blot.

Next we analyzed *Sna* expression relative to *β-Actin* in RNA isolated from 7 day "hanging drop" aggregates which had subsequently been treated 1 day with fresh RA or PTHrP, while in suspension or re-plated. Black bars in Figure 4B show that *Sna* expression is detectable at a very low level in untreated EC aggregates in suspension and higher in aggregates exposed to RA. This is presumably attributable to the outer VE-like layer and its outgrowth, as elevation of *Sna* is also seen when cells are grown in monolayer in the presence of RA (Fig. 2A). When RA-exposed aggregates are treated with PTHrP or dbcAMP, differentiation to PE-like cells is induced and *Sna* levels show a further, significant increase (two-tailed Student's *t*-test, $p<0.05$). In our experiments, RA-treated aggregates that received fresh medium with RA and were simultaneously re-plated on gelatin, also show an increase in *Sna* mRNA levels (Fig. 4B, white bars). However, this increase is

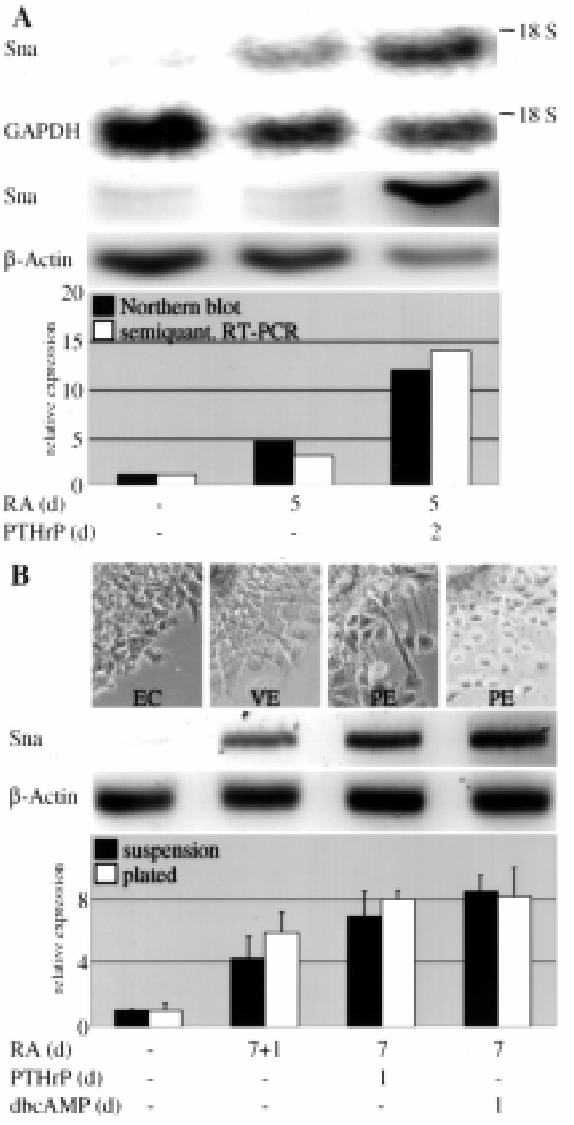

Fig. 4. *Sna* is upregulated by PTHrP in F9 embryoid bodies. *Abbreviations used are as in Figures 1 and 2, and VE: visceral endoderm.* **(A)** *RNA samples from monolayer cultures were analyzed by Northern blotting (top, with 18S ribosomal RNA band as seen in control lane indicated at the right) and semi-quantitative RT-PCR (middle) to validate the semi-quantitative RT-PCR strategy. The graph at the bottom shows results of one representative experiment. Bars represent the ratio between Sna and GAPDH (Northern blot, black bars) or Sna and β-Actin (semi-quantitative RT-PCR, white bars), relative to EC cells.* **(B)** *Semi-quantitative RT-PCR results of aggregation experiments. From left to right: undifferentiated aggregates were left untreated (EC), while aggregates differentiated with RA received fresh RA (VE), PTHrP or dbcAMP (both PE) while in suspension or re-plated. One day later, aggregates were used for semi-quantitative RT-PCR for Sna. Morphological changes associated with treatment while plated can be seen in the microphotographs at the top, and semi-quantitative RT-PCR results of plated aggregates below. The graph at the bottom shows the ratio between amplification products of Sna and β-Actin relative to untreated aggregates. Error bars represent standard deviations of three independent PCR results on 2 independent culture experiments. Differences between aggregates that had remained the last day in suspension (black bars) or that were plated (white bars) are not significant (two-tailed Student's t-test, p>0.10). RA-treated aggregates have significantly higher Sna levels than untreated aggregates, and PTHrP induced Sna levels differ significantly from RA induced levels (two-tailed Student's t-test, p<0.05).*

not significantly different from similar aggregates that had remained in suspension (two-tailed Student's *t*-test, p>0.10). In re-plated aggregates, the induction of *Sna* upon treatment with PTHrP is again significantly different from treatment with RA alone (two-tailed Student's *t*-test, p<0.05). From the experiments with F9 in monolayer and aggregate cultures, we conclude that *Sna* is induced by PTH(rP)-R1 signaling in the formation of PE *in vitro*, independent of whether its precursor is PrE or VE.

Sna is upregulated during the ex-vivo formation of PE.

We next tested whether there is a similar (rapid) upregulation of *Sna* as PE forms *ex vivo* from plated blastocysts. When plated on tissue culture substrates, blastocysts attach and trophectoderm cells grow out, liberating the inner cell mass. PrE cells thus migrate from the inner cell mass over the trophectoderm (TE) cells, where they are exposed to PTHrP secreted by these cells (Behrendtsen *et al.*, 1995). This results in their differentiation to PE cells during migration. In the experiments described here, blastocysts were plated for 3 to 5 days and then analyzed for expression of *Sna* relative to *β-Actin* by semi-quantitative RT-PCR. Figure 5 shows that *Sna* is already expressed in blastocysts at the time of plating, but that the level strongly increases upon plating (one-tailed Student's *t*-test, p<0.05). Expression of *PTH(rP)-R1* and *Follistatin* was also analyzed in the same samples. *PTH(rP)-R1* is expressed in endoderm migrating through the marginal zone of E5.5 mouse embryos (Karperien *et al.*, 1994). Figure 5 shows that it is also strongly upregulated in plated blastocysts (one-tailed Student's *t*-test, p<0.05). *Follistatin*, expressed in low levels in primitive ectoderm (PrEc) and very strongly in PE at E7.5 of gestation (Feijen *et al.*, 1994), was not detectable in blastocysts, even using a hemi-nested PCR (not shown), but is detectable in a single round of RT-PCR in plated blastocysts (Fig. 5). Upregulation of *Sna* in plated blastocysts is thus coincident with upregulation of *Follistatin* and *PTH(rP)-R1*, suggesting that *Sna* is most highly expressed in the emerging PE.

Sna is expressed in pre-implantation embryos and is specifically upregulated in the first PE cells of the mouse embryo

The presence of low levels of *Sna* RNA in mouse blastocysts prompted us to determine the onset of its transcription at earlier developmental stages. In an approximate equivalent of 1 embryo, *Sna* was detected in the zygote, at the 2-cell stage and beyond (Fig. 6A). The top row of Figure 6A shows the results of a first round PCR (Fig. 6A) for *Sna* on one series of pre-implantation embryos. In other series, first round PCRs for *Sna* showed variability in yield of an amplification product in 2-cell and later stages, probably due to variability in the RT-reaction. However, in a hemi-nested PCR, an amplification product for *Sna* was always detected in all stages of development (Fig 6A, middle row), indicating it is readily transcribed by the zygotic transcription machinery (Schultz, 1993; Aoki *et al.*, 1997).

To determine the kinetics of *Sna* expression in endodermal cells, we carried out *in situ* hybridization on sections of early post-implantation mouse embryos. Just after implantation, at E5.5, the inner cell mass has grown out into an epiblast consisting of PrEc, covered by a layer of PrE, that differentiates into VE or PE. At this stage, the first few PE cells are evident on the basal surface of the TE (Kaufman, 1992), and *Sna* expression was detected in PE cells [Fig. 6B, a (sagittal section), b and c (transverse sections)], and in low levels in the ectoplacental cone, while neither their precursor,

PrE or VE, nor the PrEc shows expression. One day later, at E6.5, as gastrulation is initiated, *Sna* is again detected in PE cells, while expression remains undetectable in their precursor, VE [Fig. 6B, e (sagittal section), f, g and h (transverse sections)]. At E6.5, *Sna* can also be more clearly detected in the ectoplacental cone, and in addition, is highly expressed in the emerging primitive streak in the nascent mesoderm where an EMT takes place (Fig. 6B,e and g), as reported previously by Nieto *et al.* (1992) and Smith *et al.* (1992). At E7.5 expression at these sites was confirmed, and, additionally, found in the mesodermal wings and allantois (Fig. 6B,i,j,k,l). At this stage, endodermal cells delaminating from the epithelial sheet of VE cells in the marginal zone can be discriminated, expressing *Sna* (see magnification of sagittal section 6B,i; Fig. 6B,d).

Discussion

The formation of PE from PrE and later from VE has a number of characteristics of an EMT. The availability of the F9 EC cell system, in which PE differentiation can be studied *in vitro*, could thus also provide an excellent model system for studying molecular aspects of EMT. The data presented in this paper support this proposition.

In previous studies we have provided evidence that PTHrP and the type I PTH/PTHrP receptor (PTH(rP)-R1) play an important role in PE formation *in vivo* (van de Stolpe *et al.*, 1993; Karperien *et al.*, 1994, 1996; Verheijen *et al.*, 1999). While PTH(rP)-R1 is expressed by PE and its precursors, PTHrP is present in large amounts in the decidua and trophectoderm. Others have provided evidence in cultured blastocysts that PTHrP produced by trophectoderm indeed acts as an endogenous regulator of PE differentiation (Behrendtsen *et al.*, 1995). *In vitro*, PTHrP can also induce the formation of PE when added to F9 PrE cells (Chan *et al.*, 1990; van de Stolpe *et al.*, 1993).

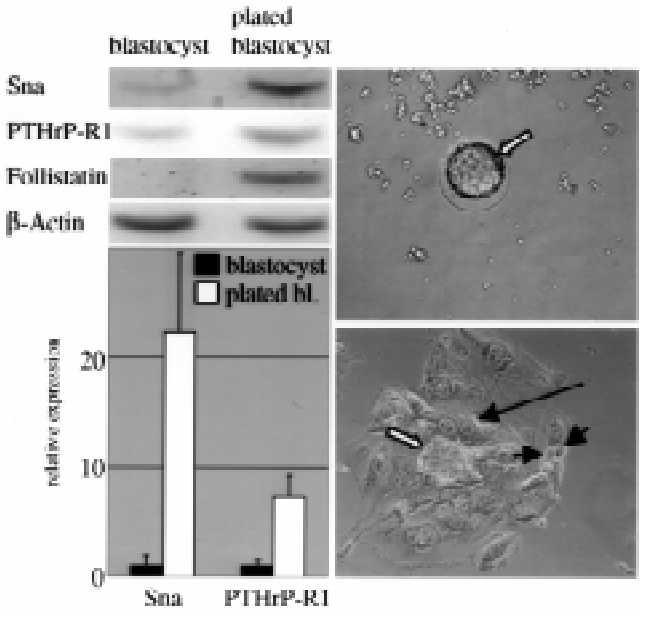

Fig. 5. *Sna* is upregulated in plated blastocysts. *RNA was extracted from blastocysts directly or several days after plating, and analyzed for Sna, PTH(rP)-R1, and Follistatin mRNA expression relative to β-Actin expression by semi-quantitative RT-PCR. Each PCR was repeated 3 times independently. Error bars represent SEMs of 6 (blastocyst, black bars), respectively 9 (plated blastocysts, white bars) independent pools of 20 blastocysts each. Sna and PTH(rP)-R1 are both significantly upregulated upon plating (one-tailed Student's t-test, p< 0.05). The right panel shows a blastocyst (top) and plated blastocyst (bottom) at the same magnification. White arrows indicate inner cell mass, short black arrows indicate individual PE cells. Large flat cells are trophectoderm cells, indicated by a long black arrow.*

Using differential display RT-PCR, we identified *Sna* as a target gene for PTHrP in F9 PrE cells. Interestingly, others have already described *Sna* expression in PE in mice (Nieto *et al.*, 1992; Smith *et al.*, 1992), and human *SNAIL* has recently been found in placenta (Twigg and Wilkie, 1999) to which PE cells also contribute (Enders, 1997). In several species, including the mouse, expression of either *Sna* or *Slu* has been detected in presumptive mesoderm and pre-migratory neural crest cells and maintained in mesenchymal derivatives (Nieto *et al.*, 1992; Smith *et al.*, 1992; Essex *et al.*, 1993; Hammerschmidt and Nusslein-Volhard, 1993; Thisse *et al.*, 1993, 1995; Mayor *et al.*, 1995; Jiang *et al.*, 1998b; Savagner *et al.*, 1998; Sefton *et al.*, 1998). Based upon these expression data, it was suggested that *Sna* or *Slu* could play a role in the formation and/or maintenance of mesenchymal cells in these processes. For murine *Slu*, such a role in EMT was indeed established at least *in vitro*: Savagner *et al.* (1997) showed that overexpression of the gene in NBT-II cells, a model system for EMT, resulted in desmosome breakdown an the appearance of a mesenchymal phenotype. Furthermore, a role for *cSlu* in EMT in the developing chicken heart was recently established as well (Romano and Runyan, 1999). It was suggested by the work of Sefton *et al.* (1998), that *cSlu* and mouse *Sna* play similar roles in their respective species in processes involving EMT.

In the present study, we demonstrate that *Sna* is strongly upregulated as a result of addition of the peptide hormone PTHrP to F9 PrE cells. *Sna* has been shown to be inducible by other hormones, such as Activin A in zebrafish animal cap assays for mesoderm induction (Hammerschmidt and Nusslein-Volhard, 1993), while injection of *Antivin* RNA (which inhibits Activin activity) in zebrafish downregulates *Sna* expression (Thisse and Thisse, 1999). Likewise, addition of basic Fibroblast Growth Factor (bFGF) and the Activin A homolog XTC-MIF to *Xenopus* animal caps (Sargent and Bennett, 1990) also upregulates *Sna*. By analogy, FGF-4 induces *Slu* in chick limb bud formation (Buxton *et al.*, 1997), as does FGF-1 when added to NBT-II cells (Savagner *et al.*, 1997). However, to date, an immediate early effect of any of these growth hormones has not been shown. Here, we show that the upregulation of *Sna* by PTHrP in F9 PrE cells still occurs in the presence of cycloheximide, providing evidence for the first time that *Sna* expression is an immediate early effect of this hormone in PrE cells.

The immediate rise in expression of *Sna* in response to PTHrP in F9 PrE cells is suggestive for a role of the gene in the onset of EMT. Supporting this, we find that *Sna* is already present at low levels in both epithelial precursors of F9 PE, i.e. F9 PrE and VE. This is in line with the reported expression of *Sna* in epithelial precursors of mesenchymal cells, such as the ectoderm cells in the primitive streak region which will become mesoderm, and in the pre-migratory cells in neural crest formation (Nieto *et al.*, 1992; Smith *et al.*, 1992; Sefton *et al.*, 1998).

The initial burst of *Sna* expression induced by PTHrP is transient, but after 48 h, when the cells have acquired a definitive PE morphology, *Sna* levels are still higher than in untreated F9 PrE cells. The transient nature of the expression could be due to the

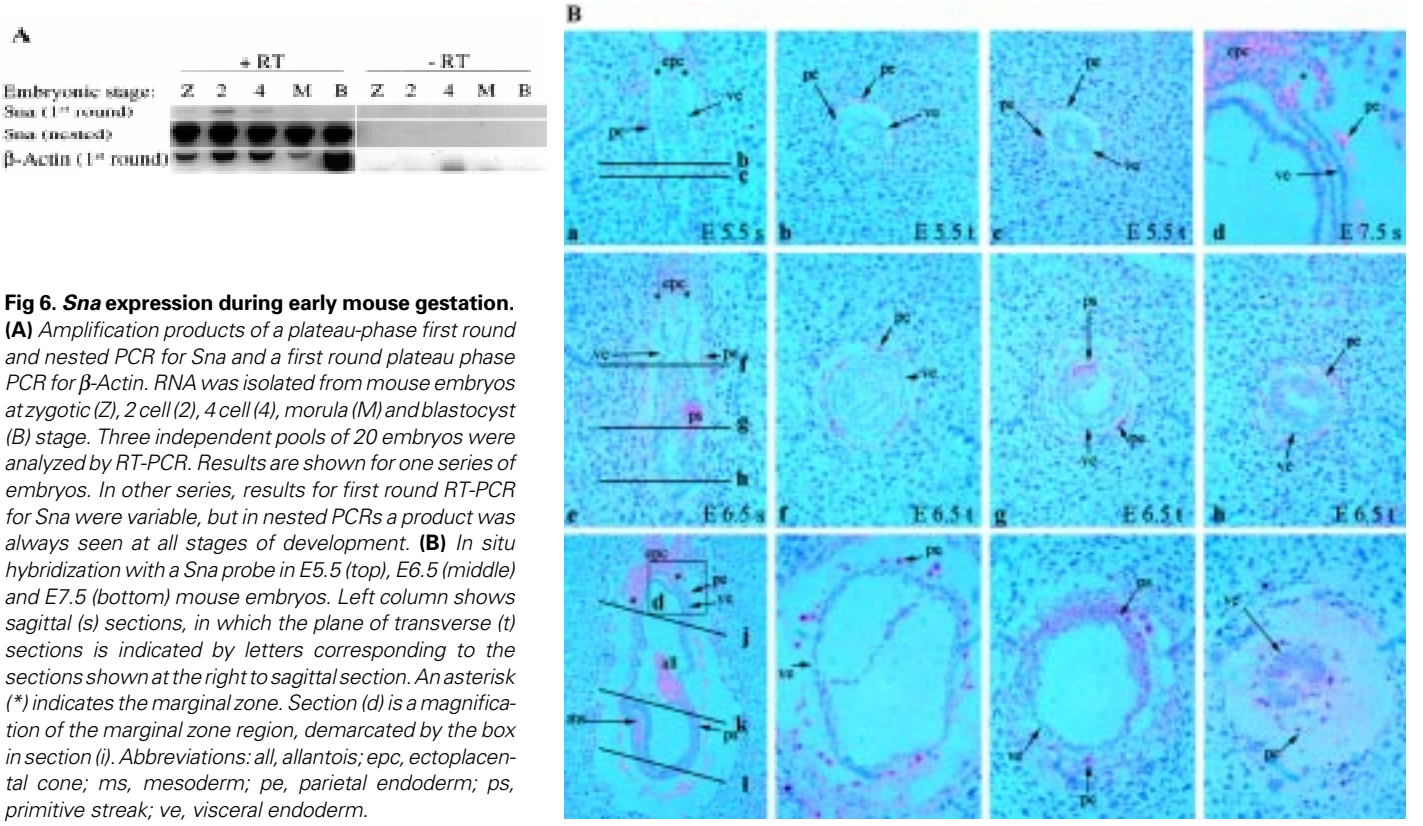

Fig 6. *Sna* expression during early mouse gestation. **(A)** *Amplification products of a plateau-phase first round and nested PCR for Sna and a first round plateau phase PCR for β-Actin. RNA was isolated from mouse embryos at zygotic (Z), 2 cell (2), 4 cell (4), morula (M) and blastocyst (B) stage. Three independent pools of 20 embryos were analyzed by RT-PCR. Results are shown for one series of embryos. In other series, results for first round RT-PCR for Sna were variable, but in nested PCRs a product was always seen at all stages of development.* **(B)** *In situ hybridization with a Sna probe in E5.5 (top), E6.5 (middle) and E7.5 (bottom) mouse embryos. Left column shows sagittal (s) sections, in which the plane of transverse (t) sections is indicated by letters corresponding to the sections shown at the right to sagittal section. An asterisk (*) indicates the marginal zone. Section (d) is a magnification of the marginal zone region, demarcated by the box in section (i). Abbreviations: all, allantois; epc, ectoplacental cone; ms, mesoderm; pe, parietal endoderm; ps, primitive streak; ve, visceral endoderm.*

instability of PTHrP under tissue culture conditions. In accordance with this idea, in F9 PrE and VE cells treated with PTH or dbcAMP instead of PTHrP, *Sna* levels remain high even after 24 or 48 h. This parallels more closely the situation *in vivo*, where high levels of *Sna* are detected in PE from E5.5 (our data) until at least E8.5 (Nieto *et al.*, 1992). The levels of *Sna* after prolonged treatment of F9 PrE or VE with PTHrP, PTH or dbcAMP seem related to the extent to which cultures acquire a rounded, mesenchymal, PE morphology. We therefore speculate that the early upregulation of *Sna* correlates with the initiation of the transition to a mesenchymal morphology, while the extent and/or maintenance of high *Sna* levels correlates with the extent and/or maintenance of the mesenchymal phenotype. *In vivo*, PTH(rP)-R1 could be continuously stimulated by PTHrP secreted by the TE cells, leading to constitutively high levels of *Sna* in PE.

The *ex vivo* blastocyst plating experiments showed significant upregulation of *Sna* concomitant with the upregulation of *PTH(rP)-R1* and *Follistatin*, as well as the appearance of PE cells. *In vivo*, *Follistatin* is expressed at E6.5 by primitive ectoderm (Feijen *et al.*, 1994), but here, in plated blastocysts, we use *Follistatin* as a marker for PE. Thus, these data strongly suggest that the emerging PE cells account for the increase of *Sna*.

We found *Sna* expression in the mouse embryo from zygote stage onward, well before the formation of PE, albeit in very low levels. This seems to be in contrast to expression in other species, where *Sna* is first detected at the onset of mesoderm formation (Sargent and Bennett, 1990; Kosman *et al.*, 1991; Nieto *et al.*, 1994; Hardin, 1995; Brown and Denell, 1996; Erives *et al.*, 1998; Langeland *et al.*, 1998). However, in zebrafish *Sna* has been found ubiquitously expressed before gastrulation as a maternal transcript, but it is strongly upregulated at the onset of zygotic transcription at the dome stage and becomes rapidly confined to the dorsal presumptive mesoderm at gastrulation (Hammerschmidt and Nusslein-Volhard, 1993; Thisse *et al.*, 1993). It is not clear whether there is a function for the *Sna* expressed at the early stages of mouse development. It could contribute to cell cycle control, as has been described for *Drosophila escargot* in imaginal wing disc cells (Hayashi *et al.*, 1993, Hayashi, 1996; Fuse *et al.*, 1994, 1996) and for mouse *Sna* in trophoblast giant cells (Nakayama *et al.*, 1998). Alternatively, as the *C. elegans* snail family member *ces-1* (Metzstein and Horvitz, 1999) and human *Slug* (Inukai *et al.*, 1999), it might control programmed cell death. It is also possible that these low expression levels are of no functional significance, which is supported by the report that *Sna* -/- mice survive until gastrulation. Interestingly, these mice show defects in parietal endoderm and mesoderm formation (Jiang *et al.*, 1998a). More detailed analysis of pre-implantation development of these *Sna* mutant embryos may reveal whether *Sna* is just present as a transcript or whether a functional protein is present at these stages.

By RT-PCR, we find relatively little *Sna* mRNA in blastocysts, which consists of inner cell mass and trophectoderm only, while *in situ* hybridization data show that expression is sharply upregulated at E5.5, when the embryo has just implanted in the uterine wall, specifically in the first PE cells. At E6.5, *Sna* is highly expressed in both PE and emerging mesoderm, and in lower levels in the ectoplacental cone. Unexpectedly, at these stages we could not detect expression in the PrE or VE cells, precursors of PE, in apparent contrast to the expression in F9 PrE and VE (this report),

and the expression in other epithelial pre-EMT cells, as shown by others. However, we cannot exclude the possibility that *Sna* is expressed below detection levels in PrE. Alternatively, *Sna* may be expressed in PrE only in the marginal zone; these cells are difficult to detect at early stages in conventional paraffin sections. At E7.5, *Sna* was clearly detected in PE and is present also in the endodermal cells that line the ectoplacental cone in the marginal zone. At this stage all PrE cells have differentiated into either PE or VE cells (Cockroft and Gardner, 1987), and it is until about this stage that the VE cells residing in the marginal zone retain their potential to contribute to PE (Hogan and Tilly, 1981; Cockroft and Gardner, 1987). *Sna* expression in endoderm at this stage is restricted to cells delaminating from the epithelial sheet of VE cells in the marginal zone and to PE more distally.

Expression of *PTH(rP)-R1* mRNA and protein at E5.5 and E6.5 is detected in PE and extra-embryonic VE, most prominently in the marginal zone (Karperien *et al.*, 1994; Verheijen *et al.*, 1999). At E7.5, *PTH(rP)-R1* mRNA and protein expression are low in VE, but prominent throughout PE. From this stage, PTH(rP)-R1 protein expression in the extra-embryonic VE further decreases and is no longer detectable at E9.5 (Verheijen *et al.*, 1999). At all of these stages, *PTHrP* is expressed from the adjacent trophoblast and decidual cells (Karperien *et al.*, 1996), and the protein is detected in trophectoderm cells as early as the blastocyst stage (van de Stolpe *et al.*, 1993). These data suggest that the *Sna* expression observed at sites where PE differentiation is initiated and maintained, is indeed controlled by activation of the PTH(rP)-R1.

The way by which PTHrP, acting through the PTH(rP)-R1, regulates *Sna* expression is unclear. This could be direct, through enhancer sequences located in the promoter of the *Sna* gene or indirect, e.g. through rapid changes in cell-cell and cell-matrix interactions. The aggregate studies suggest that these latter changes could contribute to *Sna* expression as well, since we found some induction when RA-treated aggregates were plated on gelatin compared to aggregates that had remained in suspension. Probably, if aggregates were re-plated on fibronectin, which is a more potent inducer of PE than gelatin (Grabel and Watts, 1987; Behrendtsen *et al.*, 1995), and which contributes to the Reichert's membrane over which PE cells migrate *in vivo* (Wartiovaara *et al.*, 1979), induction would be stronger.

TABLE 2

PRIMERS USED IN PLATEAU-PHASE AND SEMI-QUANTITATIVE RT-PCRs

GENE	*PRIMER*	*SEQUENCE*	*PRODUCT SIZE (bp)*
β-Actin	Forward	CCTGAACCCTAAGGCCAACCG	
β-Actin	Reverse	GCTCATAGCTCTTCTCCAGGG	398 with forward
β-Actin	Nested reverse	TGTAGCCACGCTCGGTCAGGA	267 with forward
Sna	Forward	CTAGGTCGCTCTGGCCAAC	
Sna	Reverse	TCA(#)GCGAGGGCCTCCGGAGCAGCCAGACT	835 with forward
Sna	Nested forward	GGAAGCCCAACTATAGCGAGC	
Sna	Nested reverse	CAGTTGAAGATCTTCCGCGAC	424 with nested forward
PTH(rP)-R1	Forward	GCAGAGATTAGGAAGTCTTGGA	
PTH(rP)-R1	Reverse	AGCCGTCGTCCTTGGGAACTGT	280 with forward
Follistatin	Forward	atacggatccTTTTCTGTCCAGGCAGCTCCAC	
Follistatin	Nested reverse	ataggaattcACTTACTGTCCGGGCACAGCTCA	320 with forward
Follistatin	Reverse	ataggaattcGTCAACACTGAACATTGGTGG	545 with forward

Sequences of forward and reverse primers are denoted in 5' to 3' direction for each gene, with the size of their expected amplification product. Lower case is used to designate sequences used to insert a linker in primers for Follistatin. In the Sna reverse primer, a sequence encoding a HA-tag (AGGCTAGCGTAATCTGGAACATCGTA) is inserted, indicated by (#).

In summary, our data show that *Sna is* an immediate early response gene of PTHrP-R1 signaling in F9 endoderm-like cells, suggesting that *Sna* is instrumental in initiating and maintaining the PE phenotype. In the light of the proposed role of *Sna* and *Slu* in EMT, we suggest that the formation of PE is the first EMT in mouse development, a suggestion we have made previously on the basis of morphological and biochemical changes associated with this process.

Note: After submission of this manuscript, two reports were published in which the authors show that Sna represses *E-Cadherin* expression by binding to its promoter (Batlle *et al.*, 2000; Cano *et al.*, 2000). Accordingly, the expression patterns of *E-Cadherin* and *Sna* during mouse development are complementary (Cano *et al.*, 2000). Both during development and tumorigenesis, *Sna* expression and loss of E-Cadherin in epithelial cells are associated with the acquisition of a mesenchymal phenotype and tumorigenic and invasive properties. Thus, these two reports strongly support our hypothesis that the formation of PE is an EMT, and that it is induced and/or maintained by high levels of *Sna* expression.

Materials and Methods

Cell culture

F9 EC cells were obtained from ATCC (American Type Cell Culture, Rockville, MD), and maintained in monolayer culture on gelatinized tissue culture plastic in DMEM:Ham's F12 (1:1), buffered with 44 mM $NaHCO_3$ (DFbic), 7.5% FCS, supplemented with non-essential amino acids, 10 U/ml penicillin, and 10 μg/ml streptomycin (all from Gibco BRL, Breda, The Netherlands). EC cells were induced to differentiate to PrE with 10^{-6} M *all-trans* retinoic acid (RA) (Sigma, Bornem, Belgium) and subsequently to PE with 10^{-7} M $PTHrP_{1-34}$ (PTHrP) (Bachem, Bubendorf, Switzerland) or rat PTH_{1-34} (PTH) (Peninsula Laboratories, St. Helens, United Kingdom) or 1 mM dibutyryl cyclic AMP, (dbcAMP) (Aldrich, Zwijndrecht, The Netherlands). For experiments in monolayer culture, cells were seeded at a density of 500 cells/cm^2 and maintained as EC for 5 days, or seeded at a density of 1000 cells/cm^2 and grown for 5 days in the presence of RA, and with PTHrP, PTH or dbcAMP present during the last period as indicated in the figures. For inhibition of protein synthesis, 20 μg/ml cycloheximide was added 30 min prior to stimulation with PTHrP. Differentiation was monitored morphologically by photo-microscopy. On the 5th day, cells were lysed for RNA isolation.

To generate VE-like cells, F9 EC cells were suspended in a density of 10,000 cells/10 ml medium in the absence (for EC) or presence of $5x10^{-8}$ M RA (for VE) (Hogan *et al.*, 1994). We adapted the "hanging drop assay" (Mummery *et al.*, 1990) to obtain embryoid bodies of identical size and to control the ratio of "core"-to-VE cells: 20 μl drops containing 20 cells were placed on the lid of a petri dish, then inverted over the base filled with PBS. Aggregates received fresh medium at the 3rd or 4th day with medium without (for EC) or with RA (for VE). At the 7th day, EC-aggregates received fresh medium without RA, and RA-aggregated embryoid bodies received fresh medium containing $5x10^{-8}$ M RA, 10^{-7} M PTHrP or 1 mM dbcAMP, while in suspension or simultaneously re-plated to obtain outer layers or monolayer outgrowths of respectively, EC cells, VE- (RA), or PE-like (PTHrP, dbcAMP) cells. Re-plating at the 7th day was done on gelatinized tissue culture plastic (5 aggregates per well, 24 well dish). Outgrowth and morphological changes were monitored photo-microscopically. On the 8th day, cultures were lysed for RNA isolation.

Differential display RT-PCR (DD-RT-PCR)

F9 PrE (3 days RA) and F9 PE (3 days RA+2 days PTHrP or PTH) cells were harvested. Total RNA isolation, DNase treatment, cDNA synthesis by reverse transcription using Oligo(dT), purification, and differential display PCR were performed as described (Kester *et al.*, 1997). Before purified cDNA was used for differential display, its quality and recovery was verified

with a *β-Actin* PCR as described below. For DD-RT-PCR 2 μl cDNA (or H_2O as a control) was amplified using 200 ng of 1 arbitrary primer and 1 μCi [^{33}P]dATP in each of 12 parallel reactions. The primers used were on average 20bp long, with 50% GC content. The reaction was electrophoresed on a Sequagel XR (Biozym, Landgraaf, The Netherlands). Differential bands were excised from the gel, and re-amplified by PCR, using the same primer that had been used for the differential display reaction.

Amplification products were cloned into the PCR2.1-TOPO vector (Invitrogen, Groningen, The Netherlands), and for each cloned product, 8 colonies were used for restriction analysis and screened for false positives as follows: per product, clones with different restriction patterns were again re-amplified with the primer used in the differential display. Per primer, amplification products were dot spotted on duplicate filters, which were hybridized with the α[^{32}P]dCTP labeled product of a DD-RT-PCR with the same primer on F9 PrE or PE, essentially as described (Kester *et al*., 1997). Products that showed differential hybridization were used as a probe for a conventional Northern blot and sequenced with the ABI Prism 377 DNA Sequencer (Perkin Elmer, Foster City, California).

PolyA$^+$ isolation and Northern blotting

Approximately 3 μg poly-adenylated RNA samples [isolated using Oligo(dT)-cellulose (type 7, Pharmacia Biotech, Roosendaal, The Netherlands)], were electrophoresed in formaldehyde/1% agarose gels (Sambrook *et al*., 1989), blotted and hybridized on Hybond N filters (Amersham, Roosendaal, The Netherlands) as described by the manufacturer. Probes comprised the differential display fragments as an *EcoR*1 insert from the PCR2.1-TOPO vector, or the *EcoR*1-*Bgl*II 5' Sna fragment from clone 5-15 (a kind gift of Dr. Gridley). All probes were labeled to a specific activity of approximately 10^8 cpm/μg DNA, using the Redivue kit and α[^{32}P]dCTP (both from Amersham). Hybridization with a 1.4 kb *Pst*1 fragment of the *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) cDNA or a *β-Actin* probe was used as a loading control.

Mouse embryos

For early pre-implantation stage embryos, wild-type C57bl/6xCBA F1 females were super-ovulated (Roelen *et al*., 1998) and mated with F1 males. Natural matings were used to obtain blastocysts and post-implantation embryos. Midday of the day of appearance of a vaginal plug was designated E0.5. Females were sacrificed by cervical dislocation at the required day of pregnancy. Collection of embryos in M2 medium and subsequent culture in M16 medium was as described (Hogan *et al*., 1994). Zygotes or 2-cell embryos from several crosses per stage were assigned randomly to pools of 20 embryos, and were either lysed directly, or cultured until 4-cell, morula or blastocyst stage and then lysed in Ultraspec (Biotecx, Veenendaal, The Netherlands) for later analysis by RT-PCR. For blastocyst outgrowths, E3.5 embryos were collected, distributed over 15 pools of 20 embryos, and grown for 1 day as described above. 6 Pools were then lysed in Ultraspec, and the other 9 pools were transferred to gelatinised tissue culture dishes containing DMEM, 20% FCS, 10 U/ml penicillin, 10 μg/ml streptomycin, 10^{-4}M β-mercapto-ethanol (Sigma) and grown for 3 to 5 days in a humidified incubator at 37°C, 7.5% CO_2 followed by lysis. Lysates were analyzed by semi-quantitative RT-PCR.

RT-mediated PCR and semi-quantitative RT-mediated PCR

Total RNA or polyA+ RNA was isolated from monolayer cultures of F9 cultured cells as described above. Total RNA from pools of 15 F9 embryoid bodies, or 20 mouse embryos were isolated from Ultraspec according to the manufacturer's protocol and precipitated in the presence of 1 μl see DNA (Amersham). All RNAs were DNase treated, using 1 U RQ1 RNase-free DNase (Promega, Leiden, The Netherlands) according to the manufacturer's protocol, then phenol-extracted and ethanol-precipitated. The pellet was resuspended in 15 or 20 μl H_2O, of which half was used for cDNA synthesis by reverse transcription (RT) using the Superscript kit (Gibco BRL) according to manufacturer's protocol. 4 U RNasin (Roche, Almere, The Netherlands) was added to a total reaction volume of 20 μl. cDNAs were used in PCRs without further purification, using an equivalent of the corresponding RNA as a negative control. The cDNAs and corresponding RNAs were routinely checked by a plateau-phase *β-Actin* RT-PCR to verify efficiency of cDNA-synthesis and test for contaminating DNA respectively.

RT-PCR was carried out on at least 3 independent samples, and run in a BIOMETRA UNOII Thermo cycler (Biometra, Göttingen, Germany). Primers are shown in Table 2. The following cDNA input was used: an equivalent of 50 ng total RNA or 2 ng polyA+ RNA from F9 monolayer cultures in 1 μl; an equivalent of 1/4 of an F9 embryoid body in 1 μl; an equivalent of 1 pre-implantation embryo or plated blastocyst in 2 μl. RT-PCR for *β-Actin* was performed with 165 ng (2.5 μl) of each primer, 0.2 mM dNTPs each, 1.5 mM $MgCl_2$, 0.625 U Goldstar polymerase (Eurogentec, Seraing, Belgium) in 1x Goldstar reaction buffer in a total of 50 μl. Amplification cycles were as follows: 3 min 94°C, 40x (15 sec. 94°C, 30 sec. 56°C, 45 sec. 72°C), 7 min 72°C, soak at 4°C. If no *β-Actin* product was seen after gel-electrophoresis, hemi-nested PCR was carried out with 2 μl of the first reaction under the same conditions. This was necessary for some pre-implantation embryo samples only. RT-PCR for *Sna* on pre-implantation embryos was performed with 0.8 μM of each primer, 0.2 mM dNTPs each, 10% DMSO, 2 mM $MgCl_2$, 1x Goldstar reaction buffer, 1 U Taq polymerase in a 25 μl reaction volume. Amplification cycles were as follows: 5 min 94°C, 40x (45 sec. 94°C, 45 sec. 58°C, 45 sec. 72°C), 7 min 72°C, soak at 4°C. If no amplification product was seen after gel-electrophoresis, nested PCR with 2 μl of the first reaction was carried out under the same conditions. RT-PCR for *PTH(rP)-R1* was performed as for *Sna*, but without DMSO and with 1.5 mM $MgCl_2$, and an annealing temperature of 61°C. RT-PCR for *Follistatin* was performed as for *β-Actin*, with the forward and nested reverse primer, and with an annealing temperature of 60°C. If no product was detected, a larger product was first amplified by using the forward and reverse primer, followed by a hemi-nested PCR on 2 μl of the first reaction with the forward and nested reverse primer.

To determine relative expression levels of *Sna* in F9 embryoid bodies, blastocysts and plated blastocysts, a semi-quantitative RT-PCR assay was developed. Accuracy and the range of linear DNA-quantification by the Fluorimager 595 (Molecular Dynamics) with ImageQuaNT 5.0 software were first determined by quantifying a serial dilution of DNA-fragments in the range of expected RT-PCR product sizes on a thin 2% agarose gel containing 1:13,000 VistraGreen (Amersham). We found that DNA is accurately quantifiable in a linear range (r= 0.99) when loaded within the 0.1 to 10 ng range on a thin gel, that has had minimal exposure to light. Since RT-reactions are a source of large variation in (semi-)quantitative PCR (Freeman *et al*., 1999), cDNA samples (and corresponding RNA samples) were titrated such that their *β-Actin* amplification products in the log-linear phase of the PCR varied less than four-fold among each other (Keller *et al*., 1993). These amounts of cDNA were subsequently used for semi-quantitative RT-PCRs. In these reactions, three subsequent samples were taken, loaded on gel within the range of linear quantification, and analyzed to verify that amplification occurred in the log-linear phase of each PCR (cycles 24, 27, 30 for *β-Actin;* cycles 30, 33, 36 for *Sna*; cycles 29, 31 and 33 for *PTHrP-R1*; and cycles 32, 34, 36 for *Follistatin*). *Follistatin* could only be measured accurately in the 36 cycle sample. If no product was seen, a sample from a 40 cycle PCR was analyzed on gel or used in a nested PCR to determine if any *Follistatin* transcript could be detected. Each RT-PCR was repeated 3 times to correct for variations in amplification efficiency. For each sample the amount of amplification products of *Sna, PTH(rP)-R1* and *Sna* relative to *β-Actin* were determined. The Student's *t*-test was used to determine significance of changes in relative expression levels.

In situ *hybridization*

Post-implantation embryos were dissected in decidua at E5.5-7.5 as described (Hogan *et al*., 1994), embedded in paraffin, and sectioned. *In situ* hybridization for *Sna* was performed with overnight hybridization at 55°C with a ^{35}S-UTP RNA probe spanning the *Sac*1-*EcoR*1 3' UTR fragment of clone 5-15 (described above), and high stringency washing at 65°C, as described (Feijen *et al*., 1994).

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