ACTIONS OF
PARATHYROID HORMONE RELATED PEPTIDE
IN
MOUSE PARIETAL ENDODERM FORMATION

- SNAIL'S TRAIL TELLS A TALE -

ACTIES VAN HET BIJSCHILDKLIERHORMOON-GERELATEERD PEPTIDE IN DE VORMING VAN PARIËTAAL ENDODERM IN DE MUIS

- SNAIL VERLICHT -

met een samenvatting in het Nederlands

PROEFSCHIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen op
vrijdag 18 mei 2001 des middags te 12.45 uur

door

Johanna Maria Veltmaat
geboren op 7 januari 1969 te Heino

Promotor: Prof. Dr. S.W. de Laat

Co-promotor: Dr. L.H.K. Defize

Cover figure: False color phase contrast image of an isolated inner cell mass re-plated and grown out on gelatinized tissue culture surface
Layout: Jacqueline Veltmaat and Petra Veltmaat-van Hevele
Reproduction: FEBODRUK BV, Enschede, The Netherlands

ISBN: 90-393-2711-4

The work described in this thesis was carried out at the Hubrecht Laboratory, Netherlands Institute for Developmental Biology (NIOB) of the Royal Dutch Academy of Arts and Sciences (KNAW), within the Utrecht Graduate School for Developmental Biology (OOB). Part of the work was carried out at the Curie Institute in Paris, France, financed by a short-term fellowship of the Ter Meulen Fund of the KNAW to JMV.

INSPIRATIE

Hoe kreeg jij ooit een idee
Kan een gedachte ontstaan
Waar komt het helder ogenblik
Het inzicht toch vandaan
Vroeg jij je dat nooit eens af?
Dat komt door ieder; zin voor zin,
Gaven zij de woorden in.

Je noemde het inspiratie
'adem van de geest'
maar iedereen is het steeds geweest.

Zo krijgt een kind een idee
Zo vindt een mens wat hij zoekt
Zo wordt een sterveling een held
Zo wordt vooruitgang geboekt
Dat komt door iedereen hier
De mens bereikt op die manier
Het hogere niveau.

Noem het inspiratie
'adem van de geest'
inspiratie
maar iedereen is het steeds geweest.

gebaseerd op een songtekst van Ad en Koen van Dijk.

Voor mijn ouders

CONTENTS

ABBREVIATIONS

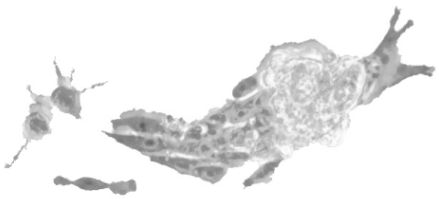
aa	amino acids
AC	adenylate cyclase
AFP	alpha-fetoprotein
AGA	18α-glycyrrhetinic acid
bp	base pairs
bHLH	basic helix-loop-helix
cAMP	cyclic adenosine-monophosphate
COLL-I or COLL-IV	type I collagen or type IV collagen
Cx43	connexin 43
dbcAMP	dibutyryl cAMP
DG	diacyl glycerol
DNA	deoxyribonucleic acid
E7.5 (or other number)	embryonic day 7.5 (other age)
EC cell	embryonal carcinoma cell
ECM	extracellular matrix
EMI	epithelio-mesenchymal interaction
EMT	epithelio-mesenchymal transition
ES cell	embryonic stem cell
FAK	focal adhesion kinase
FGF	fibroblast growth factor
FN	fibronectin
GFP	green fluorescent protein
GJIC	gap junctional intercellular communication
ICM	inner cell mass
IF	intermediate filament
IP_3	inositol 1,4,5 triphosphate
iso	isoproterenol
kDa	kilo Dalton
LN	laminin
MAP kinase	mitogen activated protein kinase
MET	mesenchyme-epithelial transition
MMP-9	matrix metallo proteinase 9
mRNA	messenger RNA
pc	*post coitum*
PE	parietal endoderm
PKA	protein kinase A
PLCβ	phospholipase Cβ
PKC	protein kinase C
PrE	primitive endoderm
PrEc	primitive ectoderm
PTH	parathyroid hormone
PTH(rP)-RI	type I receptor for PTH and PTHrP
PTHrP	PTH related peptide
PYS	parietal yolk sac
RA	(*all*-trans) retinoic acid
RER	rough endoplasmic reticulum

RNA	ribonucleic acid
sd	standard deviation
SEM	standard error of the means
Slu	slug
Sna	snail
SPARC	secreted protein acidic and rich in cysteines
SSEA-1	stem cell specific embryonic antigen I
TE	trophectoderm
TGF	transforming growth factor
TIS	transcription initiation site
TM	thrombomodulin
tPA	tissue plasminogen activator
uPA	urokinase plasminogen activator
UTR	untranslated region
VE	visceral endoderm
VYS	visceral yolk sac
ZO-1, ZO-2	zonula occludens 1, 2

CHAPTER 1

PARIETAL ENDODERM FORMATION IN THE MOUSE EMBRYO

- A GENERAL INTRODUCTION TO THIS THESIS -



Jacqueline M. Veltmaat

Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.

1

PARIETAL ENDODERM FORMATION IN THE MOUSE EMBRYO

- A GENERAL INTRODUCTION TO THIS THESIS –

INTRODUCTION

The development of eutherian mammalian embryos critically depends on the implantation of the embryo in the uterine wall of the mother. Consequently, the earliest differentiation processes in these embryos concern the formation of the extraembryonic tissues, which function as an early placenta. The first formed placental structure is the parietal yolk sac, in the mouse embryo consisting of trophectoderm (TE) and parietal endoderm (PE), with a thickening basement membrane in between. The importance of the establishment of the parietal yolk sac is often overlooked, but is underscored by the fact that the development of the definitive placental structures and the embryo proper, starting with gastrulation, does not begin until the materno-fetal contact has been firmly established by the parietal yolk sac.

Despite the fact that PE is recognized as an important layer of the parietal yolk sac, relatively little is understood about the molecular regulation of its formation. The research described in this thesis was aimed to gain more insight in this mechanism. This first chapter is an introduction to the subject of this research. It reviews the process of PE formation in the mouse embryo, and describes its resemblance to an epithelio-mesenchymal transition (EMT). Furthermore, it reviews the evidence for the involvement of cell-cell and cell-matrix interactions, and parathyroid hormone related peptide (PTHrP) in PE formation. This project led to the discovery of the transcription factor snail, an evolutionarily conserved key molecule in epithelial morphogenesis, as an important mediator of PTHrP induced PE formation. snail is therefore also introduced in this chapter. The subsequent chapters are based on experiments that were carried out to further investigate the role of PTHrP signaling through its type I receptor (chapter 2), and its downstream targets connexin 43 (chapter 3) and *snail* (chapters 4 and 5), in PE formation. In the final chapter the implications of these results are discussed.

THE ORIGIN AND DEVELOPMENT OF PARIETAL ENDODERM IN THE MOUSE EMBRYO

The first differentiation event of the embryo starts at about day 3 *post coitum* (pc), when the clump of cells that form the embryo (now called *morula*) compacts. At around that stage, the peripheral cells are committed to form the first extraembryonic tissue, the trophectoderm (TE). These cells segregate from the inner cell mass (ICM), except at one pole at about day 3.5 pc. The blastocoel cavity, that develops in between these cell populations fills with fluid and expands. At day 4-4.5 pc, shortly before the expanded blastocyst hatches from the surrounding *zona pellucida* and subsequently implants into the uterine wall, the approximately 20 ICM cells facing the blastocoel develop into the

primary or primitive endoderm (PrE) cells, also named hypoblast. These cells are the sole precursors of the endodermal extraembryonic tissues, while the approximately 20 cells underneath the PrE remain totipotent and are the only cells to constitute the embryo proper and extraembryonic mesoderm in a later stage (Gardner and Johnson, 1972; Gardner *et al.*, 1973; Nadijcka and Hillman, 1974; Papaioannou, 1982). The mural TE cells lining the blastocoel cavity cease to divide, but continue to replicate their DNA, resulting in the formation of aneuploid trophoblast giant cells in preparation for implantation (Barlow and Sherman, 1972).

Upon implantation of the blastocyst into the uterine wall, the interaction between the trophoblast giant cells and the uterine epithelium triggers the decidual response; a cascade of events that triggers the formation of the maternal part of the placenta over the next 5 days. Meanwhile, at day 4.5-5.5 pc, the PrE gives rise to two new extraembryonic tissues, the PE and the visceral endoderm (VE). First, at the late blastocyst stage, PrE cells delaminate at the marginal zone, i.e. the junction of the ICM with the TG, and form the first cells of the PE layer (figure 1, left). These cells assume a flat fibroblast-like form when they first appear in the late blastocyst (Enders *et al.*, 1978). Notably, the PrE is multi-layered at this stage, and the superficial cells have already adopted the flat fibroblast-like PE morphology before leaving the ICM (Enders *et al.*, 1978; Gardner, 1982). Based on studies with delayed blastocysts, it is believed that the formation of the PE layer does not occur before implantation during normal mouse embryonic development (Surani and Barton, 1977; Gardner *et al.*, 1988).

Later, at around day 5 pc, the extraembryonic ectoderm and the embryonic or primitive ectoderm (PrEc), derived from the polar TE and ICM respectively, form the egg cylinder, which invaginates the blastocoel. The PrE that invests the elongating egg cylinder differentiates into visceral endoderm (VE) (figure 1, right) (Gardner, 1982; 1984; 1985).

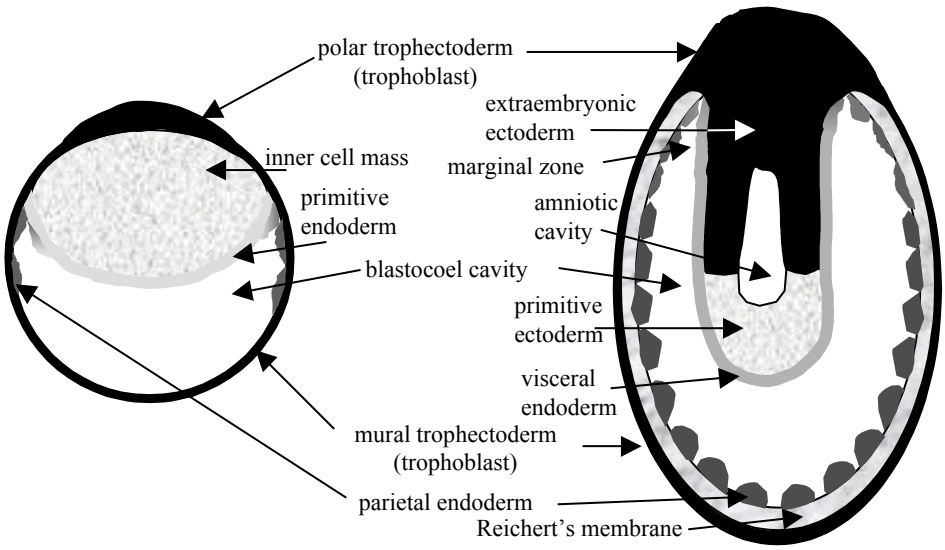


Figure 1: Schematic representation of PE formation in the early mouse embryo.
Left: Implanted blastocyst at around day 4.5 pc. **Right**: Egg-cylinder stage embryo at day 6.0 pc.

In turn, VE cells contribute to PE (Solter and Damjanov, 1973; Diwan and Stevens, 1976; Rossant *et al.*, 1978; Hogan and Tilly, 1981). In addition, they contribute to the visceral yolk sac, but not to the definitive endoderm or any other tissue of the embryo proper (Gardner, 1978; 1982; 1983; 1984; Rossant *et al.*, 1978; Gardner and Rossant, 1979).

Egg cylinder explant cultures, grafting experiments and blastocyst injections have demonstrated that the VE cells retain their potency to form PE until at least the 7^{th} day of gestation (Solter and Damjanov, 1973; Diwan and Stevens, 1976; Hogan and Tilly, 1981; Cockroft and Gardner, 1987). However, a similar capacity of VE of older embryos is suggested by the morphology of endodermal cells in the marginal zone of a day 8 pc embryo. The elongated morphology of these cells with branched filopodia is consistent with their detachment from the epithelial sheet and migration onto the basement membrane underlying the trophoblast cells (Hogan and Tilly, 1981; Hogan and Newman, 1984). Furthermore, the expansion of the parietal yolk sac at this and later stages of development occurs so fast, that the low proliferation rate measured in PE cells seems insufficient to solely account for the apparently constant cell-density of the tissue (Hogan *et al.*, 1983; Cockroft, 1987). This suggests that new PE cells must be recruited from the VE.

Although PE cells are initially flat and fibroblast-like, they acquire a more rounded morphology during later stages of development. However, temporal and regional differences persist as shown by scanning electron microscopy (Cockroft, 1986). While the cells in the proximal region predominantly have a blebby morphology, the distal cells change from a predominantly (60%) blebby to predominantly (90-100%) smoothly morphology in the course of the 9^{th} to 11^{th} day of gestation, concomitant with an about 2.5 lower motility measured in distal cells, compared to proximal cells. These distal cells become extremely rounded and smooth during the 12^{th} day, and develop variable numbers of short filopodia during the 13^{th} day, which persist during the 14^{th} day (the oldest embryos studied). Thus, the morphological differentiation of PE proceeds at least until day 13 pc, yet the functional significance of these ongoing modifications is not clear.

What is the function of PE cells? Their principal activity seems to be the secretion of extracellular matrix components (ECM), which constitute the Reichert's membrane between themselves and the TE cells. This membrane is first seen at day 6 pc (Adamson and Ayers, 1979) and it is relatively porous (Jollie, 1968). While it thickens well beyond day 11 pc (Clark *et al.*, 1975), it eventually becomes thinner and ruptures at around day 17 pc. The Reichert's membrane may serve primarily as a coarse filter, allowing free access of nutrients to the embryo (Gardner, 1983; Freeman, 1990), and it enhances the efficiency of nutrient uptake by the VE (Payne and Deuchar, 1972). In addition it excludes maternal cells from the embryo, since TE cells can not fulfill this latter function, as these do not remain present as a continuous cell layer. Thirdly, it may protect the embryo against mechanical damage. Thus the function of PE may reside in establishing and modifying the Reichert's membrane. The parietal yolk sac as a whole, later on together with the visceral yolk sac and other extra-embryonic structures, functionally comprises an 'early placenta'. Besides the functions fulfilled by the Reichert's

membrane, this early placenta forms an integral part of the life support system, essential for the maintenance of the fetus and for its nutrient and waste exchange between days 5 and 10 of gestation, until the definitive placenta is formed (Cross *et al.*, 1994; Bielinska *et al.*, 1999).

It is worth noting that VE and PE are defined by topography, visceral and parietal being derived from the Latin words *visceralis* and *paries*, freely translated as 'adjacent to the organ' and 'outer wall' respectively. However, the definition also serves to distinguish two cell populations that differ strikingly from each other in several aspects, as discussed below in more detail. Throughout this thesis, 'PE layer' is used to refer to PE topographically, while 'PE differentiation' is used to indicate the changes in morphology and gene expression that the endodermal cells undergo while migrating to or residing at the 'parietal' position, and 'PE formation' may include all aspects of the development of the tissue.

IN VITRO MODEL SYSTEMS TO STUDY PARIETAL ENDODERM FORMATION

At the time when PE formation starts, there are only about 20 PrE cells in the mouse embryo, which soon differentiate into VE. In addition, the embryo is extremely small, has just implanted, and is consequently hardly accessible for experimentation. All this severely hampers the study of the regulation of PE formation *in vivo*. Therefore, several approaches have been developed to enable this study *in vitro*. The first relies on the culturing of immunosurgically isolated embryonal tissue, such as ICMs from expanded blastocysts, or isolated egg cylinders. These isolates show PE outgrowth when cocultured with TE or cultured in TE-conditioned medium (Hogan and Tilly, 1981; Behrendtsen *et al.*, 1995). Furthermore, ICMs cultured on fibronectin (FN) coated substrates produce solitary cells, thus considered PE, of which the number increases when the ICMs are plated on various ECM components in TE-conditioned medium (Behrendtsen *et al.*, 1995). These experiments suggested an interaction between ECM components and a factor secreted by the TE in the regulation of PE formation.

The second model system relies on the use of cultured embryonic stem (ES) cells or embryonal carcinoma (EC) cells. ES cells differentiate to PE when cultured in monolayer in the absence of the leukemia inhibiting factor, which inhibits differentiation, and in the presence of retinoic acid (RA) and cyclic adenosine-monophosphate (cAMP) elevating factors, such as dibutyryl cAMP (dbcAMP) (Mummery *et al.*, 1990; van de Stolpe *et al.*, 1993). Most studies on PE formation are performed with teratocarcinoma cells, of which the most frequently used line is the F9 EC cell line. The F9 cell line was established in culture from a sub-line of the transplantable testicular teratocarcinoma OTT6050, itself derived from a 6-day old male embryo of a strain 129 mouse (Berstine *et al.*, 1973). This cell line has the advantage over ES cells that it can not differentiate into other lineages than the extraembryonic endoderm lineage, and it has the advantage over some other teratocarcinoma cells (such as PSA-1 and PYS-2 cells) that it can be induced to differentiate into all three forms of extraembryonic endoderm. When treated with RA in monolayer it irreversibly forms PrE, which upon concomitant or

subsequent treatment with e.g. dbcAMP differentiates into PE, as indicated by elevated production of a.o. tissue plasminogen activator (tPA), laminin (LN) and type IV collagen (COLL-IV) and thrombomodulin (Strickland and Mahdavi, 1978; Strickland *et al.*, 1980; Imada *et al.*, 1990a, b; van de Stolpe *et al.*, 1993; Weiler-Guettler *et al.*, 1996). Alternatively, when cultured in suspension in the presence of RA, these cells form aggregates with an outer layer of VE, as indicated by morphology and the production of alpha-fetoprotein (Dziadek, 1978; Dziadek and Adamson, 1978; Hogan *et al.*, 1981). These so-called embryoid bodies generate PE when treated with cAMP elevating agents, or when re-plated on FN (Grover and Adamson, 1986; Grabel and Watts, 1987), thus mimicking the behavior of isolated ICMs.

Although the role of RA as an endogenous inducer of PrE has not been established, F9 PrE is considered phenotypically similar to PrE, and F9 derived VE and PE faithfully mimic their *in vivo* counterparts, as reviewed (Hogan *et al.*, 1983), and described below. This cell line was therefore used in this thesis project.

It is worth mentioning that, whether studying PE formation *in vivo* or *in vitro*, the problem is to distinguish PrE, VE and PE cells unambiguously, since many of the gene products now used as markers are expressed at different levels in the three phenotypes rather than in an all-or-none way (e.g. laminin, tPA), or are part of a multigene family (e.g. cytokeratins). This finding has been partly overcome with the finding of alpha-fetoprotein as a marker exclusively expressed in VE among these three cell types (Dziadek, 1978; Dziadek and Adamson, 1978; Hogan *et al.*, 1981), and thrombomodulin in mouse PE (Imada *et al.*, 1987, 1990a, b; Weiler-Guettler *et al.*, 1996) and in F9 derived PE (figure 2).

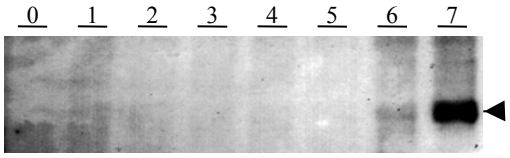


Figure 2: Western blot of thrombomodulin expression in differentiating F9 cells.
From left to right, F9 EC cells were left untreated (lane 0), or treated with RA alone for 1 to 5 days (lane 1-5), or with RA during 5 days of which the last 2 days in the presence of the PE inducers PTHrP (lane 6) or dbcAMP (lane 7). Thrombomodulin expression was detected with the monoclonal antibody 273-34A (Kennel *et al.*, 1987) as a band of approximately 85 kDa (arrowhead).

THE DIFFERENTIATION OF PARIETAL ENDODERM BEARS THE HALLMARKS OF AN EPITHELIO-MESENCHYMAL TRANSITION

The obvious change in cell shape of PrE and VE cells during their differentiation to PE cells (as demonstrated in figure 3) points to a reorganization in one or more of the following: (i) cytoskeletal and junctional elements, (ii) synthesis of matrix glycoproteins and (iii) mode of attachment to the substratum, as already suggested by others (Hogan *et al.*, 1983). In fact, the basis of this thesis lies in the postulation that these changes are indicative for an EMT. To support this postulation, the general tissue organization, junctional specialization and cytoskeletal arrangement in epithelial and mesenchymal tissues is described in more detail below, and later on compared to their organization in PrE, VE and PE.

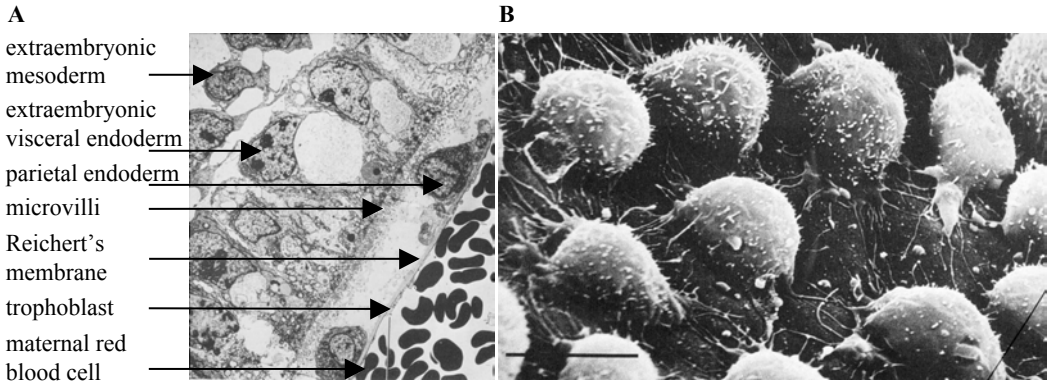


Figure 3: Electron micrographs of mouse extraembryonic endoderm.
A: Transmission electron micrograph of the extraembryonic layers of a day 7.5 pc mouse embryo, demonstrating the columnar morphology of extraembryonic VE cells and the rounded morphology of PE cells. **B**: Scanning electron micrograph of the PE cells on the Reichert's membrane of a day 13 pc mouse embryo, demonstrating the rounded morphology and filopodia of these cells (bar 10 µm). From: Hogan *et al*., 1994, Stephanie Ellington, Cambridge.

Epithelial and mesenchymal tissue organization.

Most of the cells in multicellular organisms are held together by a complex network of secreted extracellular macromolecules (a variety of versatile (glyco)proteins and polysaccharides) referred to as the extracellular matrix (ECM). The cells contribute to and organize their own matrix, and together with it, assemble a tissue. The two basic or extreme phenotypes which cells can assume are found in epithelial and mesenchymal tissues respectively (Alberts *et al.*, 1983). How can these two phenotypes be distinguished? The most important discriminator is the extent of specialized cell junctions. These occur at many points of cell-cell and cell-matrix contacts in all tissues, but are especially important and plentiful in epithelia. The cell-cell contacts seal the epithelial cells to sheets that form barriers to the movement of molecules and cells between different compartments of an organism. Epithelial cells have an apical-basal polarity. They are usually positioned on and anchored to a thin, continuous layer of ECM, the basal lamina, and as such line the surface of organs. In early life forms consisting of epithelial cells only, complexity was introduced by e.g. folding of the epithelium, such that a cavity was formed, of which the internal milieu was controlled by the epithelium. In later life forms, complexity became even greater with the conversion of epithelial cells into a new, mesenchymal cell type, via an epithelio-mesenchymal transition (EMT) (Hay, 1995; Thiery and Chopin, 1999).

Mesenchymal cells are no longer linked by cell-cell anchoring contacts, and display a front-to-back polarity. They have usually acquired migratory behavior, enabling them of occupying subepithelial spaces. There they can retain their mesenchymal phenotype or condensate and re-establish an epithelial tissue via the reverse process, a mesenchymal-epithelial transition (MET). The archetypal EMTs during mammalian development are the formation of mesoderm from the PrEc during gastrulation (Viebahn, 1995), and later the migration of neural crest cells from the neurectoderm (Duband *et al.*, 1995). Mesenchymal cells produce copious amounts of ECM components, which they organize into a lattice through which they can migrate, and with which they

establish specialized cell-matrix contacts. Since epithelial and mesenchymal cells experience different forms of mechanical stress, from the adjacent cells and the matrix respectively, both cell types have differently organized cytoskeletons, as described below.

Cell-junctional and cytoskeletal arrangements in epithelial and mesenchymal tissues.

Cell junctions can be classified into 3 functional groups: 1) anchoring junctions that mechanically attach cells and their cytoskeletons to their neighbors or to the ECM, thereby enabling groups of cells, such as those in an epithelium, to function as robust structural units; 2) the occluding junctions that seal cells together in an epithelial cell sheet in a way that prevents paracellular diffusion of water and even small molecules, and additionally confine membrane-bound proteins to the apical or basolateral side of the cells; and 3) communicating junctions, which mediate the passage of metabolic or electrical signals from one interacting cell to its partner, and thus function in coordinating the activities of groups of cells.

Table I

Functional group	Specified junctions	Molecular constituents
Anchoring junctions F-actin filament attachment	adherens junctions (cell-cell)	E-cadherin, α-catenin, β-catenin or γ-catenin (plakoglobin), vinculin, α-actinin
	focal contacts (cell-matrix)	integrins, FAK, vinculin, talin, α-actinin
intermediate filament attachment	desmosomes (cell-cell)	desmoplakin, desmoglein, desmocollin a, desmocollin b, plakoglobin, plakophilin
	hemi-desmosomes (cell-matrix)	integrins, desmoplakin-like proteins
Occluding junctions	tight junctions (cell-cell)	ZO-1, ZO-2, occludin, cingulin, Rab13
Communicating junctions	gap junctions	connexins

Functional classification of cell junctions in early embryos of vertebrate animals.
This table may incorrectly suggest a junction-specific localization of most junctional proteins. It is worth noting that some connexins have been found to associate with ZO-1 and to cadherin/catenin complexes. The physiological meaning of this interaction remains largely elusive (Fujimoto *et al.*, 1997; Giepmans and Moolenaar, 1998), but connexin43 may be stabilized in complex with ZO-1, and as such maintain involved in gap junctional intercellular communication (Toyofuku *et al.*, 2001). Additionally, ZO-1 and ZO-2 can link β-catenin at macular adherens junctions in non-epithelial cells to actin filaments (Rajasekaran *et al.*, 1996; Itoh *et al.*, 1997). Interestingly, some of these molecules are not exclusively junctional, but can translocate to the nucleus and regulate gene transcription, such as ZO-1 and β-catenin. Despite the promiscuity of some molecules among the various junctions, detailed structure analysis and immunohistochemistry in electron microscopy and confocal microscopy have provided insight in how the subcellular localization of the respective components correlates with their assembrly various junctions.

The variety of junctions and their molecular components in table I is limited to what is relevant for tissues of early vertebrate embryonic development

(Fawcett, 1981; Alberts *et al.*, 1983; Collins and Fleming, 1995). Epithelial cells have either one or both of the two types of cell-cell anchoring junctions: 1) the adherens junctions, organized in a circumferential belt-like structure, the *zonula adhaerens*, sometimes confusingly referred to as belt desmosome, and 2) the button-like (spot) desmosome (*macula adhaerens*). Adherens junctions are located apically, and are well-established in cells with a brush border. Desmosomes are located in parallel rows below the adherens junctions, if the latter are present. Adhesion is provided by a homophilic interaction between dimers of Ca^{2+}-dependent transmembrane linker proteins belonging to the family of cadherins on two adjacent cells (figure 4A). In adherens junctions of a pre-implantation mouse embryo, this is the prototype cadherin, E-cadherin. Its intracellular domain is linked to a dense structure on the plasma membrane, composed of a.o. catenins. In turn, this structure is linked to the cytoskeleton, predominantly actin, which interacts with myosin motor proteins. As such, the cytoskeleton forms a contractile belt along the *zonula adhaerens* (figure 7) which mediates epithelial morphogenesis, e.g. in tubule formation. In cells that do not fully express an epithelial phenotype, the adherens junctions exist as small punctate (macular) or streaklike attachments but fail to form a belt-like structure.

The cadherins used in desmosomes are desmoglein and desmocollin. Like E-cadherin, these cadherins link their cytoplasmic tail to a dense structure on the plasma membrane, i.c. composed of a.o. desmoplakin and plakoglobin (figure 4B). As illustrated in figure 7, desmosomes form a kind of rivets through which the intermediate filament cytoskeletons of two adjacent cells are interconnected. This cytoskeleton, mainly composed of type II intermediate filaments (IFs) or (cyto)keratins, has a scaffold-like structure criss-crossing the cytoplasma of each cell, which provides epithelial cells with tensile strength.

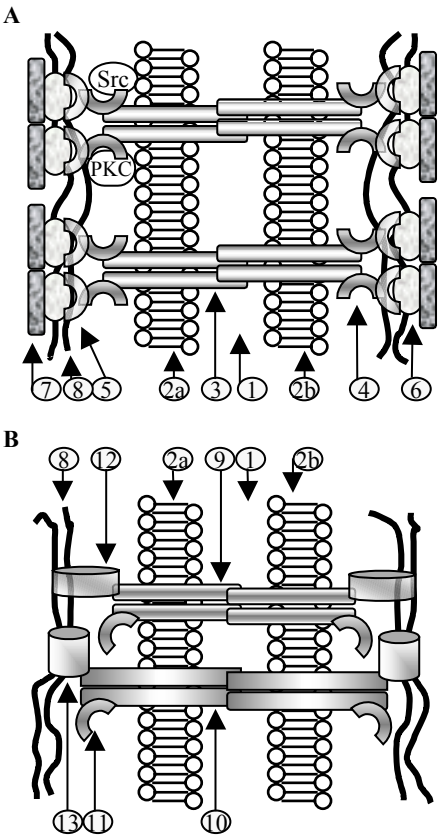


Figure 4: Schematic representation of cell-cell anchoring junctions.
A: Adherens junction, **B**: Desmosome. Only the major protein interactions are represented. 1: intercellular space, 2a, b, lipid bilayer of the adjacent cells 'a' and 'b', 3: E-cadherin, 4: β-catenin or plakoglobin, 5: α-catenin, 6: α-actinin, 7: vinculin, 8: F-actin, 9: desmocollin, 10: desmoglein, 11: plakoglobin, 12: desmoplakin, 13: plakophilin. The illustrated location of some signaling molecules (Src, PKC) is tentative.

Occluding or tight junctions (*zonulae occludentes*) exist in all epithelia, given some rare exceptions, and form the apical-most junctions. As depicted in figure 5A, they are composed of a network of anastosomizing strands (*maculae occludentes*) of 'intra'membrane particles, that completely encircles the apical end of each cell in the epithelial sheet. Im-

portantly, the ability of tight junctions to restrict the passage of ions through the spaces between cells increases logarithmically with increasing numbers of strands in the network, as if each strand acts as an independent barrier (Claude and Goodenough, 1973). Occludin and claudin are intramembrane components of tight junctions, and thought to be responsible for the close intercellular adhesion of the tight junction (Furuse *et al.*, 1993; Saitou *et al.*, 1998; Tsukita *et al.*, 1999). Both occludin and claudin can bind ZO-1 and ZO-2, which in turn are linked to actin filaments (Tsukita *et al.*, 1999) (figure 5B).

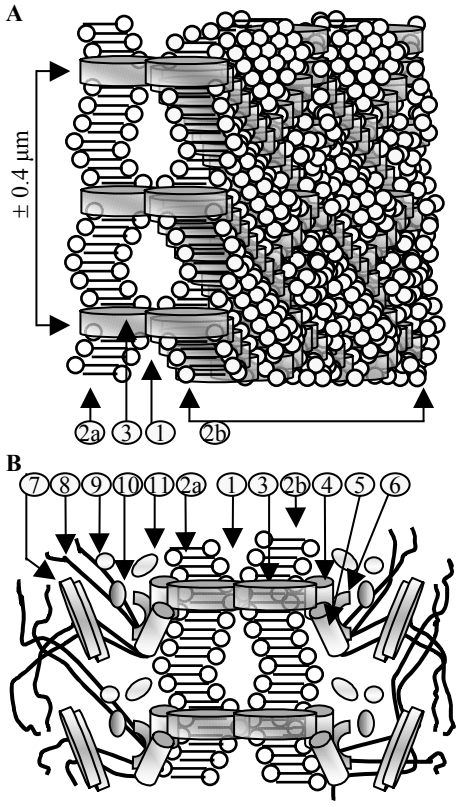


Figure 5: Schematic representation of tight junctions.

A: Tight junctions are composed of *maculae occludentes*, arranged into a *zonula occludens*. **B**: Protein interactions in the *maculae*.1: intercellular space, 2a, b: lipid bilayer of adjacent cells 'a' (frontal view on plane of section) and 'b' (viewed in perspective in A), 3: occludin or claudin, 4: ZO-1, 5: ZO-2, 6: p130, 7: cingulin, 8: F-actin, 9: p155 (7H6 antigen), 10: p192 (BCG9,1 antigen), 11: Rab13. The exact interaction is not yet known for each of the various components.

This may allow the modulation and periodically opening of the tight junctions to allow passage of molecules and even cells (Balda and Anderson, 1993). Another intracellular component, cingulin, has a structural organization similar to myosin, and thus may also regulate actinfilament anchorage to the tight junction.

Gap junctions (communicating junctions, nexuses) are perhaps the most intriguing cell junctions, since they represent one of the most widespread cell-cell junctions, and provide a way of communication. These junctions are usually located below adherens junctions, but in the absence of tight junctions, they may occasionally be the most apical junctions. They provide the passage of small molecules such as amino acids, sugars, cAMP and other nucleotides, but not proteins, nucleic acids, and other macromolecules, as the maximum size for passage is 1000 to 1200 daltons. A gap junction (figure 6) comprises an array of up to several hundreds of paired hemichannels of about 7 nm diameter, called connexons. In turn, connexons are hexamers of proteins, named connexins, around a central aqueous channel of approximately 1.5 nm in diameter. So far, 14 different connexin genes have been identified in the mouse genome (Simon and Goodenough, 1998).

Connexons can be composed of similar connexins (homomeric connexons) or different connexins (heteromeric connexons). Connexons of adjacent cells can be apposed to similar connexons, forming homotypic channels, or to different connexons, forming heterotypic channels. The diversity in connexins and connexons provides a variety of structurally and physiologically different channels each with their own distinct molecular permeability. A rise in intracellular calcium or a lowering of the

intracellular pH is associated with a change of gap junctions from the low resistance state (open) to a high resistance state (shut), resulting in uncoupling of the cell (Turin and Warner, 1977). These, together with the phosphorylation status of connexins (regulated by a.o. cAMP) may be physiological mechanisms of regulating coupling of neighboring cells. It is possible that the coupling of cells in embryos provides a pathway for long-range cell signaling within a developing epithelium. For example, Ca^{2+} or cAMP molecules, as second messengers of hormonal stimulation, can be transported from one cell to another by gap junctions, and as such, the signal may be propagated through a tissue, possibly via a smooth concentration gradient.

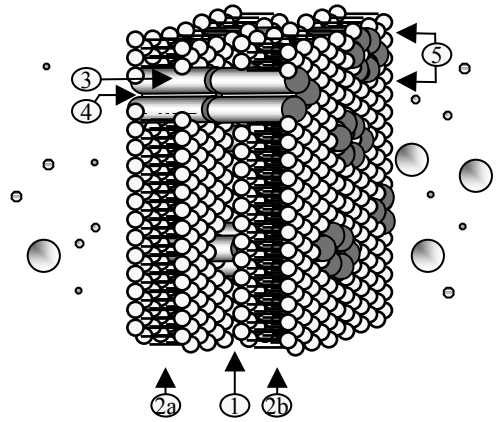


Figure 6: Schematic representation of the organization of gap junctions.
The hydrophilic pores of connexons permit the passage of ions and small molecules such as cAMP or fluorescein (small and medium-sized free-floating circles), but excludes macromolecules (large free-floating circles). 1: intercellular space, 2a, b: lipid bilayer of the two adjacentcells 'a' and 'b', viewed in perspective on the plane of section, 3: a connexin, 4: the hydrophobic pore, 5: six connexins assembled into a connexon.

Epithelio-mesenchymal transitions.

While mesenchymal cells are devoid of cell-cell anchoring junctions and tight junctions, they may still be connected by gap junctions, which allow them to function as a tissue instead of as individual cells. In addition, mesenchymal cells have many contacts with the ECM, by which they are surrounded. Epithelial and mesenchymal cells express hemi-desmosomes: sites where IFs terminate and attach to ECM via Ca^{2+}-dependent transmembrane cell-surface receptors named integrins, in complex with desmoplakin-like proteins.

The predominant IF in mesenchymal cells is vimentin. In addition to its association with hemi-desmosomes (Kartenbeck *et al.*, 1984), it can also associate with actin in focal contacts (Seifert *et al.*, 1992). Actin bundles terminate in focal contacts (focal adhesion plaques) in migrating cells, either epithelial or mesenchymal. Here the bundles are associated with the integrins, via a complex including e.g. talin, vinculin and focal adhesion kinase (FAK). However, in mesenchymal cells the actin cytoskeleton may have totally collapsed.

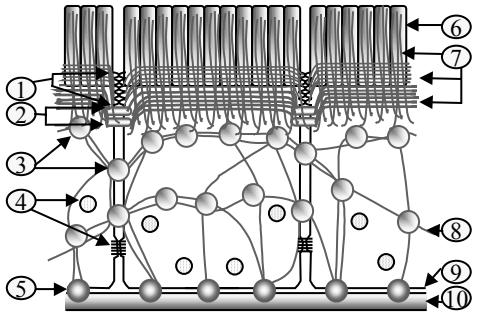


Figure 7: Schematic representation of cell junctions and cytoskeletal structures in an epithelium.
The tight junctions and adherens junctions are located apically and organized into a zonular junction by the subcortical actin belts. Spot-like desmosomes are interconnected by a scaffold-like cytokeratin cytoskeleton, which terminates in hemi-desmosomes located at the membrane, thus connecting the cells to the basal lamina. 1: tight junction, 2: adherens junction, 3: desmosome, 4: gap junction, 5: hemidesmosome, 6: microvillus, 7: actin filaments, 8: keratin filament, 9: cell membrane, 10: basal lamina.

The loss of the epithelial organization of cell-cell junctions and cytoskeletons, illustrated in figure 7, may be indicative for epithelial cells to transform into me-

senchymal cells. However, besides this 'de-epithelialization', epithelial cells need to acquire specific mesenchymal properties to undergo a complete EMT, such as elevated secretion of ECM molecules. Most importantly, cells feature (elevated) migration after the acquisition of a fibroblastic, mesenchymal morphology. Table II summarizes the differences between epithelial and mesenchymal tissues. It is important to bear in mind that none of the features listed in that table can serve as an exclusive marker for epithelium or mesenchyme, but the combination of characteristics is determinant, and may be helpful in determining whether a certain differentiation process occurs via an EMT.

Table II

Epithelium	Mesenchyme
Sheets of cells	Single, loose cells
Situated on basal lamina	Cells surrounded by superfluous ECM
Basal-apical polarity	Front-to-back polarity
Apical microvilli	Smooth surface, no microvilli
Linked by cell adhesion molecules (mainly E-cadherin and/or desmosomes)	No or low (E-)cadherin expression
Tight junctions	No tight junctions
Connexins, diverse forms	Connexins, including those expressed in epithelia
Cytokeratin intermediate filaments	Vimentin intermediate filaments
Subcortical actin fibers	Actin not subcortical, optionally in stress fibers
Non- or slightly motile and migratory	Highly motile and migratory
Non-invasive (attachment to basal lamina)	Invasive (migration through basal lamina)
	Secretion of copious amounts of ECM
	Production of proteases

Characteristics of epithelial and mesenchymal tissues.
(Based on: Hay, 1995; Davies and Garrod, 1997; Thiery and Chopin, 1999).

Tissue organization in PE and its precursors, *in vivo* and in model systems.

Now that we know the criteria for an EMT, we can demonstrate that the transition of PrE/VE to PE bears the hallmarks of an EMT. First of all, the ultrastructure of the tissue (figure 3) already gives a clue to the epithelial organization of VE and the lack of such organization of PE. Furthermore, early PE cells display a flat, fibroblast-like morphology, before they round up, suggesting a migratory capacity. Finally, the thickening of the Reichert's membrane is attributed to a secretory activity of PE cells. What is known about these aspects and the existence of cell-cell junctions in PrE, VE and PE will here be discussed in more detail.

During compaction of the morula, cells become electrically coupled to one another with Cx31 and Cx43 as the most prominent gap junctional components, and Cx30.3, Cx40, Cx45 are present in lower amounts (Ducibella *et al.*, 1975; Lo and Gilula, 1979; Dahl *et al.*, 1996). Immediately after implantation, Cx31 expression is restricted to trophectodermal lineages, while Cx43 is expressed in ICM-derived lineages including the PrE (Ruangvoravat and Lo, 1992; Yancey *et al.*, 1992). PrE cells also have macular junctional complexes, which are not

desmosomes (Enders *et al.*, 1978). The intercellular distance of 20 nm suggests that these junctions are adherens junctions, which have not yet been organized in zonular belts. Indeed, E-cadherin mediated adhesion between PrE cells has been suggested from studies with function-perturbing antibodies on preimplantation embryos and isolated ICMs (Damsky *et al.*, 1983; Vestweber and Kemler, 1984; Richa *et al.*, 1985; Collins and Fleming, 1995) or E-cadherin knock-out embryos (Ohsugi *et al.*, 1997). F-actin is uniformly distributed along all cell membranes in the morula and blastocyst (Lehtonen and Badley, 1980; Reima and Lehtonen, 1985; Lehtonen and Reima, 1986), thus supposedly also in the PrE, and this distribution is dependent on zygotic E-cadherin expression (Ohsugi *et al.*, 1997). The presence of cytokeratin material in some cells of the ICM at blastocyst stage could be an indicator of initial steps in IF assembly in PrE formation, as suggested before (Emerson, 1988), and as supported by the assembly of cytokeratin 8 and 18 filaments seen upon RA treatment of F9 cells (Grover *et al.*, 1983; Hogan *et al.*, 1983; Trevor and Oshima, 1985; Kurki *et al.*, 1989; Trevor and Steben, 1992). Preceding the appearance of keratin networks, there is a reorganization of vinculin in F9 cells treated with RA alone, leading to the formation of distinct plaques in the flattened cells (Lehtonen *et al.*, 1983).

The multilayered PrE has been referred to as an ill defined polarized epithelium (Nadijcka and Hillman, 1974). The cells have a rough appearance, suggesting the presence of microvilli (Gardner, 1982). Similarly, F9 PrE cells in culture are unable to fully express an epithelial morphology, with distinct basal and apical surface, and well-developed anchoring junctional complexes. Hence their 'epithelioid' instead of epthelial character (Hogan *et al.*, 1983).

Compared to the PrEc cells, the PrE cells exhibit a more extensive rough endoplasmic reticulum (RER). The RER is filled with secretory material, probably ECM components, since ultrastructural studies have revealed the presence of deposits of extracellular material such as FN, LN and COLL-IV between the PrEc and PrE in the late blastocyst (Nadijcka and Hillman, 1974). These deposits do not form a continuous layer until after egg cylinder formation. F9 PrE produces FN, as do F9 EC, but in contrast to the latter, the PrE cells are able to retain FN on their surface reviewed in (Gardner, 1983; Hogan *et al.*, 1983).

VE cells are underlaid by a thin basement membrane composed of LN, COLL-IV, and FN (Adamson and Ayers, 1979; Wartiovaara *et al.*, 1979; Leivo *et al.*, 1980) which they secrete themselves. Whereas the VE cells covering the embryonic part of the egg cylinder are squamous in form, the VE cells covering the extraembryonic part are columnar and have a very vacuolated cytoplasm and numerous apical microvilli indicative for a function in nutrient absorption (reviewed in Hogan and Tilly, 1981; Gardner, 1983; Bielinska *et al.*, 1999). Furthermore, the epithelial organization of VE is apparent as adjacent cells are linked together by E-cadherin mediated adhesion, desmosomes and tight junctions (Enders *et al.*, 1978; Hogan and Tilly, 1981; Vestweber and Kemler, 1984; Casanova and Grabel, 1988; Saitou *et al.*, 1998). Similarly, F9 derived VE cells possess desmosomes as well as tight junctions (Hogan and Tilly, 1981; Hogan *et al.*, 1983). Cx43 is expressed in the VE at early postimplantation stages (Ruang voravat and Lo, 1992; Yancey *et al.*, 1992), while Cx31 is restricted to tro-

phectodermal lineages, and these protein expression patterns correlate with the communication compartments within the embryo (Kalimi and Lo, 1988; Dahl *et al.*, 1996).

All VE cells show a distinct subapical distribution of cytokeratin filaments connected to intercellular desmosomes. Similar observations were made in F9 cells cultured in suspension in the presence of RA: epithelium formation is preceded by the expression of LN after 2 days, and starts with the expression of alpha-fetoprotein (AFP) and the intermediate filaments Endo A (keratin 8) and Endo B (Grover *et al.*, 1983), and disappearance of the stem cell marker SSEA-1 (Solter and Knowles, 1978) from the outer layer at around day 3 or 4. In the course of differentiation, these aggregates express increased amounts of COLL-IV, and this expression and that of LN and FN changes from a uniform pattern into a predominant localization under and around the outer layer of cells, indicating the start of the formation of the basement membrane (Grover *et al.*, 1983; Grover and Adamson, 1985). AFP is detectably secreted after at least 5 days, and immunohistochemical detection on sections is only possible after 7 days, and in the outer, endodermal cell layer only (Hogan *et al.*, 1981; Grover *et al.*, 1983).

In contrast to their precursors, PE cells, whether derived from the PrE or VE, establish few intercellular junctional complexes (Hogan and Tilly, 1981), and are only loosely associated with the surface of the mural TE on which they migrate (Enders *et al.*, 1978). Indeed, the PE does not seem to constitute a continuous cell layer at any stage of its existence (Jollie, 1968; Enders *et al.*, 1978). Antibodies to E-cadherin do not react with PE *in vivo*, or derived from F9 cells (Vestweber and Kemler, 1984). The loose association of PE cells is suggestive for the absence of tight junctions in PE. No desmosomes are found, and some controversy exists whether cytokeratin filaments are present in F9 PE, but if present, they are co-expressed with the mesenchymal IF, vimentin (Hogan *et al.*, 1983; Lane *et al.*, 1983; Lehtonen *et al.*, 1983; Trevor and Steben, 1992). While F9 cells express a basal level of vimentin, as often observed in cultured cells, the expression level is elevated in F9 PE cells.

PE cells have few microvilli, but an extensive RER filled with secretory material (Hogan and Tilly, 1981), such as the glycoprotein SPARC (Mason *et al.*, 1986) and the ECM components COLL-IV, FN, LN, entactin and heparan sulphate proteoglycans (Hogan *et al.*, 1980; Leivo *et al.*, 1980). The PE cells assemble these ECM in the continuous basal lamina underlying the TE (Nadijcka and Hillman, 1974) that such becomes the very conspicuous Reichert's membrane (Hogan and Tilly, 1981; Hogan *et al.*, 1982; Gardner, 1983). PE cells have been shown to be far more active in synthesis of COLL-IV and LN than VE cells. However, in contrast to VE cells, PE cells produce little, if any FN (Hogan *et al.*, 1980; Hogan, 1980; Howe and Solter, 1980; Smith and Strickland, 1981; Amenta *et al.*, 1983; Gardner, 1983). Studies with F9 cells have shown increased amounts of LN in PE intracellularly and associated in extracellular strands, while FN synthesis decreases in the course of PrE and VE derived PE formation (Wolfe *et al.*, 1979; Hogan *et al.*, 1983; Grover and Adamson, 1985). In addition, the PE cells also synthesize modulators of the ECM. Examples are the large amounts of tissue plasminogen activator (tPA) (Strickland *et al.*, 1976; Marotti *et al.*, 1982), as well as the matrix degrading

metalloprotease 9 (MMP-9, or collagenase), and its inhibitor TIMP-1 (Behrendtsen and Werb, 1997).

Table III summarizes the junctional specialization and cytoskeletal arrangement in PrE, VE and PE *in vivo* and *in vitro*. Taking into account that the first PE cells have already acquired a fibroblast-like appearance as they migrate through the marginal zone, an additional comparison of table III with table II supports the contention that PE-formation from either precursor bears the hallmarks of an EMT.

Table III

	PrE	F9 PrE	VE	F9 VE	PE	F9 PE
cell-cell contacts						
E-cadherin-based adherens junctions	+ (macular)	+	+	+	-	-
tight junctions			+	+	-	-
desmosomes	-	Subline dependent	+	+	-	-
gap junctions	+ (Cx43)?		+ (Cx43)			
cytoskeletal organization						
organized IF	keratins	keratins *	keratins	keratins*	vimentin	vimentin
subcortical actin	+		+	+	-	-
apical microvilli	+?	+?	+	+	-	-
matrix						
COLL-IV synthesis	+?	+?	+	+	++	++
LN synthesis	+	+?	+	+	++	++
FN synthesis	+	+	+	+	-	-
ECM modulators		little tPA	uPA		tPA, MMP-9	
basal lamina	spot-like		continuous and thin		continuous and thick (Reichert's membrane)	
tissue specific gene expression			alpha-fetoprotein	alpha-fetoprotein	thrombo-modulin	thrombo-modulin

Tissue organization of PrE, VE and definitive PE *in vivo* and *in vitro*.
For references, see text. When insufficient data were available for the indicated variables, positions in the table were left empty. * = F9 cells express low amounts of vimentin as an effect of culturing. Expression is highly increased in F9 PE cells. ? = indirect evidence.

PE-INDUCING FACTORS

Cell-cell and cell-matrix contacts.

How is the remarkable transition in cell phenotype during PE formation brought about? The earliest speculation about the induction of PE was that the contact of PrE/VE with the TE initiated the differentiation towards a PE phenotype. However, the PrE in the advanced blastocyst is multilayered, with the su

perficial cells having adopted a fibroblast-like PE morphology (Enders *et al.*, 1978; Gardner, 1982). This finding provided support for the hypothesis that the lack of contact with diploid ectoderm, rather than the contact with the mural TE, induces the differentiation towards a PE phenotype (Gardner, 1982). Further support for this hypothesis came from the fact that cells exhibiting the distinctive fibroblast-like appearance of early PE were often seen to migrate out from isolated ICMs explanted on a tissue culture surface (Gardner, 1983), and from the demonstration that PE cells will form on top of the VE layer in the absence of a TE layer to migrate on (Hogan and Tilly, 1981). However, these findings may also suggest that the lack of lateral contacts, instead of or combined with the lack of contact with the PrE, induces PE formation. Some support for this speculation comes from a vinculin deficient, thus adhesive-defective variant of the F9 cell line, that spontaneously differentiates to PE (Grover *et al.*, 1987; Samuels *et al.*, 1993), and the observation that plating a single-cell suspension of wild type F9 VE cells results in their loss of VE phenotype and acquisition of some PE-specific features, such as a swollen endoplasmic reticulum and enhanced tPA production (Grover and Adamson, 1986; Casanova and Grabel, 1988).

Also extracellular matrix components have been shown to influence several parameters of PE formation. F9 cells defective in post-translational modification of COLL-IV show reduced expression and secretion of LN and show delayed differentiation in response to RA and cAMP (Wang *et al.*, 1989), suggesting that COLL-IV is important for the complete expression of the differentiation program in response to RA and cAMP. FN promotes the haptotactic and chemotactic migration of F9 PE cells (Carnegie, 1994). COLL-I, via the binding of FN, stimulates the outgrowth of PE cells from F9 embryoid bodies (Grabel and Casanova, 1986; Grabel and Watts, 1987). In addition, COLL-I, COLL-IV and FN stimulate the migration of more solitary (thus considered PE) cells further away from isolated (rat) ICMs compared to LN (Carnegie and Cabaca, 1993). This may be partly due to the fact that more proliferating outgrown cells were detected on FN and COLL-IV than on LN. Although LN is less inductive for PE formation than FN, it is important to note that expression of the LAMC-1 gene and the assembly of its product, the laminin γ chain, in the basal lamina is required for PE migration (Smyth *et al.*, 1999). Behrendtsen and co-workers demonstrated an outgrowth promoting effect of FN on plated mouse ICMs (Behrendtsen *et al.*, 1995). Moreover, they showed that the addition of the collagenase MMP-9 inhibits outgrowth of cells with PE morphology from isolated ICMs on FN, while the addition of the inhibitor of MMP-9, TIMP-1, had a stimulatory effect on the number of outgrown cells and their migrated distance (Behrendtsen and Werb, 1997). In contrast, PE outgrowth from F9 embryoid bodies is reduced when tPA activity is inhibited. Therefore, tPA may facilitate the migratory behavior of PE cells by mediating the degradation of ECM components such as FN (Behrendtsen and Werb, 1997; Cheng and Grabel, 1997). Interestingly, if the adhesion to FN is altered due to loss of the β1-integrin, the most important receptor for LN and FN in F9 cells and early embryogenesis, migration of F9 PE on FN *in vitro* or ES-derived PE on the Reichert's membrane *in vivo* is reduced without apparent loss of differentiation markers (Stephens *et al.*, 1993; Stephens

et al., 1995).

It remains speculative to what extent FN contributes to PE formation *in vivo*, since PE cells have been shown to be far more active in synthesis of COLL-IV and LN than VE cells and moreover, in contrast to VE cells, PE cells produce little, if any FN (Hogan *et al.*, 1980; Hogan, 1980; Gardner, 1983; Howe and Solter, 1980; Smith and Strickland, 1981; Amenta *et al.*, 1983). Thus, the origin of the very low amounts of FN in the Reichert's membrane remains ambiguous. Since it is found in the outermost layer adjacent to the TE (Semoff *et al.*, 1982), it may be derived from e.g. the TE (Hogan *et al.*, 1983) or is a soluble form from the maternal serum (Gardner, 1983). Alternatively, it may be deposited by the earliest PrE/VE cells migrating over the TE (Semoff *et al.*, 1982) and play a role in the first stages of PE formation only. A major contribution of TE cells in FN deposition is not expected, since they play a role in degradation rather than synthesis of the Reichert's membrane (Minor *et al.*, 1976a, b). It was suggested by Grover and Adamson (1985), that FN in differentiating embryoid bodies is needed for aggregation and basement membrane formation, while LN is required for differentiation. LN is at least essential for the formation of VE as an initial step of PE formation, as the culture of aggregates in presence of antibodies to LN prevented formation of an outer epithelium, and no AFP staining was found (Grover *et al.*, 1983).

Parathyroid hormone related peptide (PTHrP) and the type I PTH/PTHrP-receptor.

Expression pattern and biological activity of PTHrP and its type I receptor.

Are cell-cell and cell-matrix contacts the only PE inducing factors? The differentiation of F9 cells to PE upon experimental elevation of intracellular cAMP levels suggested that hormonal activity, via cAMP as a second messenger, could also play a role in PE formation (Smith and Strickland, 1981; Grover and Adamson, 1986). Evidence from our group and others pointed to parathyroid hormone related peptide (PTHrP) as a likely candidate. First, F9 PE cells were shown to express parathyroid hormone (PTH) responsive adenylate cyclase activity (Evain *et al.*, 1981), but it was unclear whether this hormone and its receptor were expressed during early mouse embryogenesis. Later, a PTH-like protein (PLP or PTHrP) and an adenylate cyclase coupled PTHrP receptor were found to be expressed in F9 PrE cells. Addition of PTHrP to these cells leads to both PE formation, and elevated PTHrP receptor mRNA levels (Chan *et al.*, 1990; van de Stolpe *et al.*, 1993). Indeed, PTHrP generates a cAMP response in ES- and F9 derived PrE cells (van de Stolpe *et al.*, 1993). Coculture of isolated ICMs with TE, or the culture of isolated ICMs in TE-conditioned medium stimulated the outgrowth of solitary PE cells, suggesting that the TE secretes a PE-inducing factor (Behrendtsen *et al.*, 1995). Messenger RNA expression of PTHrP in TE, and of its receptor (now referred to as the classical, type I PTH/PTHrP-receptor or PTH(rP)-RI throughout this thesis) in PrE and its derivatives during early mouse embryogenesis support the contention that PTHrP may be such an *in vivo* PE-inducing factor secreted by the TE (van de Stolpe *et al.*, 1993; Karperien *et al.*, 1994, 1996). This contention was further supported by the inhibition of PE formation from isolated ICMs cultured in the presence of a function perturbing antibody to PTHrP, and the stimulation of PE outgrowth from isolated ICMs in

the presence of PTHrP on various ECM components (Behrendtsen *et al.*, 1995).

Surprisingly, no abnormalities in PE formation were found in mouse embryos in which the gene for either PTHrP or PTH(rP)-RI had been ablated (Karaplis *et al.*, 1994; Lanske *et al.*, 1996). However, dependent on their genetic background, 60% of PTH(rP)-RI -/- embryos die between day 9.5 and day 12.5 pc with no apparent abnormalities but a proportionally smaller body size from day 8.5 pc onwards (Lanske *et al.*, 1996; Verheijen *et al.*, 1999). While in PTHrP -/- embryos the maternal protein, abundantly present in the decidua, may rescue the embryos from a PE phenotype or early death, a similar mechanism cannot operate in the PTH(rP)-RI -/- embryos and account for an apparently normal PE phenotype. Importantly, the PTH(rP)-RI protein is only expressed in the developing extraembryonic endoderm layers of the early postimplantation embryo until day 8.5 pc, and exclusively in PE at day 9.5 pc (Verheijen *et al.*, 1999). Moreover, PTHrP signaling via a redundant receptor during PE formation is highly unlikely, since PTH(rP)-RI -/- ES cells do not form PE upon addition of PTHrP *in vitro* (Verheijen *et al.*, 1999). Therefore, the smaller body size and early death are likely due to a malfunctioning PE. This hypothesis waits further testing.

PTHrP induced signal transduction in PE formation.

PTHrP was first identified in tumor cells associated with the syndrome of hypercalcaemia of malignancy. It was given its name based on its physiological actions similar to PTH, including elevated blood calcium levels and increased adenylate cyclase activity leading to increased cAMP excretion, as well as its structural resemblance to PTH. The full-length mouse PTHrP protein consists of 139 amino acids (aa), but is posttranscriptionally processed to yield a family of PTHrP peptides before it is secreted. Most of the PTH-like activity of PTHrP resides in its N-terminal fragment of 34 aa, which shares 8 of the 13 N-terminal-most residues with PTH, as well as considerable three-dimensional homology in the region of aa 13-34, accounting for the fact that both peptides are able to act as ligands with equal affinity for the PTH(rP)-RI (Jüppner *et al.*, 1991; Mallette, 1991; Abou-Samra *et al.*, 1992; Orloff *et al.*, 1994; Wan *et al.*, 1996; Chorev and Rosenblatt, 1996; Goltzman and Henderson, 1996). Other receptors that bind this or other PTHrP fragments exist, but they seem not involved in PE differentiation (Verheijen *et al.*, 1999) or at least, if expressed (which remains to be demonstrated) are not capable of inducing PE in the absence of PTH(rP)-RI signaling.

The PTH(rP)-RI receptor is a 7-pass transmembrane G-protein coupled receptor, belonging to a family of receptors which can activate the Gαs and at least one other G-protein to transduce the signal (Segre and Goldring, 1993). Upon ligand binding, for which specific domains in the N-terminal extracellular region, the third extracellular loop, and the second and seventh transmembrane domain are necessary (Gardella *et al.*, 1993; Lee *et al.*, 1994; Turner *et al.*, 1996), the receptor presumably undergoes a conformational change, resulting in the activation of these G-proteins.

As illustrated in figure 8, binding of the N-terminal 34 aa fragment of PTH or PTHrP is sufficient to activate phospholipase Cβ (PLCβ) and elevate adenylate cyclase (AC) activity via respectively a Gαq and Gαs activation in several cell types, among which F9 PrE cells. In turn, PLCβ catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate to

produce two second messengers, diacylglycerol (DG) and inositol 1,4,5, triphosphate (IP_3), which activate protein kinase C (PKC) and release of calcium from intracellular stores respectively. Adenylate cyclase converts adenosine triphosphate to cAMP, which subsequently induces activation of protein kinase A (PKA). The activation of PLCγ requires high Gq expression and higher ligand concentrations and higher receptor densities and on the cell membrane than activation of AC in LLC-PK1 cells. Nonetheless, the addition of 100 nM $PTHrP_{1-34}$ to F9 PrE, normally used to induce the formation of PE, is sufficient to induce a calcium release in these cells (Verheijen, 1997).

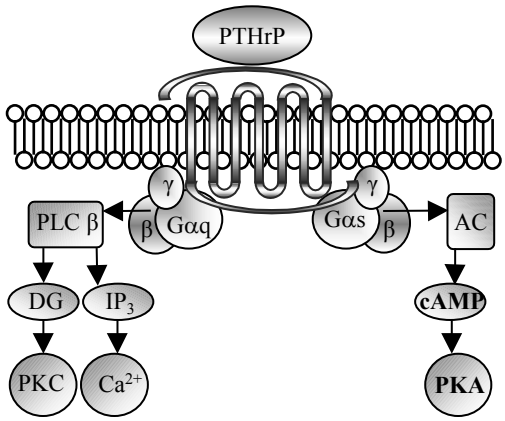


Figure 8: Schematic representation of the main signaling pathways activated by the type I PTH/PTH(rP) receptor.
The PTH(rP)-RI is a G-coupled receptor, that passes the lipid bilayer 7 times. Orientation: the extracellular space is above the lipid bilayer, the intracellular space with the two known signaling cascades are below the lipid bilayer. For details, see text.

Since the substitution of $PTHrP_{1-34}$ by dbcAMP or even active PKA is sufficient to induce PE formation in these cells, but does not produce a calcium transient (Verheijen, 1997; Verheijen *et al.*, 1999), the importance of PLCβ activation resulting in a calcium transient in PE formation remains unclear. Moreover, the contribution of various isoforms of the other PLCβ target, PKC, to the differentiation of F9 EC to PrE and subsequently to PE is probably negligible (Kindregan *et al.*, 1994; Khuri *et al.*, 1996).

Work in our group has indicated that cAMP inhibits Ras activity itself in F9 PrE cells, which is in contrast to models proposed by others in which cAMP inhibits the Ras/MAP kinase pathway downstream of Ras, (Verheijen and Defize, 1997). This Ras inhibition occurs via a yet unclear mechanism, but at the level of or downstream of PKA. Subsequent work elucidated a dual role for Ras signaling in extraembryonic endoderm formation (Verheijen *et al.*, 1999): Ras is rapidly activated by RA treatment during the formation of PrE in F9 EC cells. Furthermore, overexpression of constitutively active Ras is sufficient to induce F9 PrE. Support for a possible necessity of Ras activity during endoderm formation came from Grb2 knockouts. Grb2 is an adapter molecule that links various plasma membrane receptors to the activation of Ras. Grb2 -/- ES cells fail to form endoderm when stimulated to do so, and this failure is restored upon expression of an active form of Ras (Cheng *et al.*, 1998). Conversely, upon PTHrP treatment of F9 PrE cells Ras is slowly downregulated, and the use of constitutively active Ras elucidated that downregulation of Ras activity is required for PE formation (Verheijen and Defize, 1997). In parallel, Ras is expressed at extremely low levels in PE of rat embryos (Brewer and Brown, 1992), suggesting that low activity is probably important for *in vivo* PE differentiation as well.

MAP kinase activity is high in F9 PrE, and reduced upon treatment with PTHrP or cAMP. This strong correlation between p42 MAP kinase activity and Ras activity during the subsequent

phases of endoderm formation suggests that the cAMP-induced MAP kinase inhibition is a consequence of the inhibition of Ras activity. Experimental inhibition of MAP kinase activity in F9 EC cells does not prevent RA-induced PrE differentiation, nor is inhibition of MAP kinase activity in F9 PrE cells sufficient to induce PE. However, the inhibitory effect of constitutively active Ras on PKA-induced PE differentiation is completely abolished by blocking MAP kinase activity with PD98059, suggesting that downregulation of MAP kinase activity is necessary for PE differentiation (Verheijen *et al.*, 1999). What are the downstream targets of Ras and MAP kinase in the subsequent steps of PE differentiation remains speculative. Given the obvious morphological changes during differentiation, other small GTPases of the Ras family are interesting candidates for study, since they are known to modulate cytoskeleton assembly and cell membrane remodeling.

SNAIL ZINC FINGER TRANSCRIPTION FACTORS IN EPITHELIO-MESENCHYMAL TRANSITIONS

In a strategy to identify new targets of PTHrP signaling in PE formation, we screened F9 PrE and F9 PE for differentially expressed genes and identified upregulation of the gene encoding the zinc finger transcription factor snail (Sna) in PE (chapter 4 of this thesis). This gene was first identified in *Drosophila* on the basis that loss of function embryos exhibit defects in the invagination of the presumptive mesoderm and retraction of the germ band, leading to highly twisted, snail-like embryos (Grau *et al.*, 1984; Nusslein-Volhard *et al.*, 1984; Hemavathy *et al.*, 2000). The gene is a member of the larger family of *snail* zinc finger transcription factors, which have an evolutionarily conserved molecular structure and expression pattern. In *Drosophila*, the *snail* family includes three other genes, *escargot*, *worniu*, and *scratch* (Whiteley *et al.*, 1992; Roark *et al.*, 1995; Ashraf *et al.*, 1999). In vertebrates, each species studied usually possesses at least one *snail* gene and the closely related *slug*. This family of transcription factors is particularly interesting, because they are involved in various aspects of development and differentiation, such as cell fate determination, cell migration, cell cycle regulation, apoptosis, and cancer. Most strikingly, family members are expressed in mesenchymal cells, or cells undergoing an EMT. Illustrative for this are the expression of snail family members in (presumptive) mesoderm during gastrulation, neural crest migration, and metastasizing tumors, as reviewed (Hemavathy *et al.*, 2000). In some of these cases, *snail* or *slug* transcription is downstream of e.g. dorsal and twist (*Drosophila*) or mesoderm inducing factors (vertebrates), such as members of the families of transforming or fibroblast growth factors (TGFs, FGFs). However, it is not clear whether this activation is direct or indirect.

How do snail proteins regulate transcription? The most conserved feature of the snail family of proteins is the C-terminal zinc finger domain, consisting of 4 to 6 C_2H_2-type zinc fingers (Boulay *et al.*, 1987; Ashraf *et al.*, 1999), with a minimum of four fingers required for functioning (Fuse *et al.*, 1994; Grimes *et al.*, 1996; Nakayama *et al.*, 1998). These fingers are implied in DNA binding, although their use in protein-protein interactions, as shown for some other zinc

finger proteins (Mackay and Crossley, 1998), is not excluded. Outside the zinc-finger domain, there is much less conservation among all the members of the family. Nonetheless, one noticeable feature conserved among the *Drosophila* family members is a small, N-terminal segment, referred to as the NT box (Hemavathy *et al.*, 2000). No known function has been determined for this box, but one may postulate that it is involved in nuclear localization based on the presence of many basic residues. Interestingly, all the vertebrate members contain a different conserved motif at their extreme N-termini, which was first identified in the unrelated Gfi-1 oncoprotein, and thus called SNAG (snail/-gfi-1) domain. This motif, MPRSFLVK, is necessary but probably not sufficient for transcriptional repression (Grimes *et al.*, 1996; Nakayama *et al.*, 1998; Hemavathy *et al.*, 2000; Kataoka *et al.*, 2000). Residual repressor function may be located in the region in between the SNAG domain and zinc-finger domain (Nakayama *et al.*, 1998). All the targets of snail activity in *Drosophila*, *rhomboid*, *single-minded* and *lethal-of-scute* (Ip *et al.*, 1992; Kasai *et al.*, 1992) and studies with vertebrate snails show that snail orthologues act indeed as transcriptional repressors. However, they may function as activators under some circumstances, as the human SLUG and *Drosophila snail* each confer transcriptional activation when assayed by transfection (Mauhin *et al.*, 1993; Nakayama *et al.*, 1998; Hemavathy *et al.*, 2000).

How does snail confer its activity in transcriptional regulation? The evolutionarily conserved recognition consensus for DNA binding of the zinc finger region is 5'-CANNTG, with a preference for NN being GG or CC, but variants of this core may bind the proteins as well (Mauhin *et al.*, 1993; Fuse *et al.*, 1994; Inukai *et al.*, 1999). This core sequence resembles the E_2-box, the consensus of bHLH protein targets. Under some circumstances, the snail family proteins may compete directly with bHLH proteins for the same binding sequences. Interestingly, the E_2-box has also been identified in the promoter of the human *E-cadherin* gene, while repression via bHLH factors could not be demonstrated (Giroldi *et al.*, 1997). Later *Drosophila* snail was shown to repress *DE-cadherin* (Oda *et al.*, 1998). Since the loss of E-cadherin expression by epithelial cells is correlated with the acquisition of mesenchymal characteristics, which in turn is often correlated with snail expression, it was well conceivable that snail represses *E-cadherin* expression also in vertebrates, and is thus a key factor in EMT. The resemblance of the PrE or VE to PE transition to an EMT suggested a similar key role for snail in PE formation, which we investigated in chapters 4 and 5.

SCOPE OF THIS THESIS

For most mammalian embryos, the implantation of the embryo in the uterine wall is essential for its further development and survival. Therefore, the first differentiation events in such embryos are aimed at the establishment of placental structures, to establish the contact and nutrient and waste exchange between embryo and uterus. In the mouse embryo, the trophectoderm and the adjacent parietal endoderm (PE) form the first placental structure, the parietal yolk sac. Despite the importance of PE for further embryonic development, relatively little is known about the regulation of its formation and function. However,

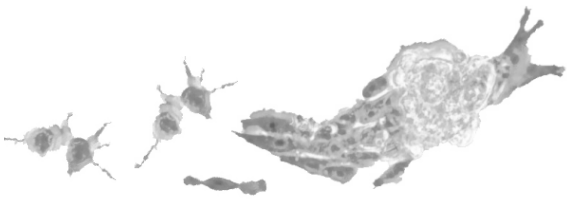
it was speculated already about twenty years ago that PE formation is induced by cell contacts and a cAMP elevating hormone, probably secreted by the trophectoderm. After the discovery of the peptide hormone PTHrP as an inducer of PE formation *in vitro* (Chan *et al.*, 1990; van de Stolpe *et al.*, 1993), our group has investigated whether and how PTHrP plays a similar role in the formation of PE in the mouse embryo. The work described in the thesis of M. Karperien demonstrated that the spatio-temporal expression of PTHrP and the type I PTH/PTHrP receptor (PTH(rP)-RI) strongly supports a role for this hormone/receptor system in PE formation in the mouse embryo. Subsequent *in vitro* studies, described in the thesis of M. Verheijen, demonstrated that the PTH(rP)-RI is the only receptor through which PTHrP induces PE formation. It additionally showed that the activation of PKA, a downstream effector of PTH(rP)-RI signaling, is sufficient for the induction of PE differentiation, and suggested that downregulation of Ras and MAP kinase activity are required for this process, but by themselves are not inductive.

Meanwhile, the genes encoding the PTHrP or the PTH(rP)-RI protein had been respectively ablated (Karaplis *et al.*, 1994; Lanske *et al.*, 1996) in mice, but surprisingly, no abnormalities in PE formation had been detected in embryos missing either gene. However, PTH(rP)-RI embryos are proportionally smaller and start dying at around the age when in the wild type situation, the receptor is only expressed in the PE. Therefore, it was tempting to investigate whether the growth retardation and early death of the knockout embryos were due to a thus far undiscovered PE phenotype in these embryos. Consequently, the project described in this thesis was aimed first at elucidating the cell-biological significance of PTHrP signaling via the PTH(rP)-RI in PE formation. The second aim was to identify new molecules, propagating the PTHrP induced signaling involved in PE formation. In addition to the analysis of PE layers in wild type and PTH(rP)-RI knockout embryos, we also made use of F9 embryonal carcinoma cells for our studies. These cells provide an excellent system to study PE formation *in vitro* (Hogan *et al*., 1983), and are thus a very helpful tool, as the onset of PE formation in the embryo occurs at a stage when the embryo is hardly accessible for experimentation.

As explained in this chapter, we postulate that PE formation is an epithelio-mesenchymal transition. We demonstrate in this thesis that PTHrP signaling via PTH(rP)-RI is required for this EMT, which in turn is required for proper PE differentiation. We identified the regulation of connexin 43 and *snail* by this signaling and studied their function in PE formation. Our results led to new insights in the molecular regulation of different aspects of PE formation. Furthermore, the findings described in this thesis open avenues to explain the growth retardation and death of the PTH(rP)-RI knockout embryos, as well as the role of PTHrP in tumor progression.

CHAPTER 2

PARATHYROID HORMONE RELATED PEPTIDE INDUCES AN EPITHELIO-MESENCHYMAL TRANSITION THAT IS REQUIRED FOR PARIETAL ENDODERM DIFFERENTIATION



Jacqueline M. Veltmaat*, Jeanine A. A. Hendriks*[1], Beate Lanske[2],Jean-Paul Thiery[3], Siegfried W. de Laat, and Libert H.K. Defize

*both authors contributed equally

Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands. [1]Current address: Isotis B.V., P.O. box 98, 3720 AB Bilthoven, The Netherlands. [2]Molecular Endocrinology, Max-Planck Institute for Biochemistry, Am Klopferspitz 18a, 82152 Martinsried, Germany. [3]UMR 144 Morphogenèse Cellulaire et Progression Tumorale, Institut Curie, Section de Recherche, 26 Rue d'Ulm, 75248 Paris Cedex 05, France.

PARATHYROID HORMONE RELATED PEPTIDE INDUCES AN EPITHELIO-MESENCHYMAL TRANSITION THAT IS REQUIRED FOR PARIETAL ENDODERM DIFFERENTIATION

ABSTRACT

Primitive endoderm (PrE) and visceral endoderm (VE) cells migrate over the trophectoderm and as such contribute to parietal endoderm (PE), the inner layer of the parietal yolk sac in the mouse embryo. Based on the loss of epithelial morphology of PrE and VE cells during their migration over the trophectoderm, and the mesenchymal morphology of PE cells, we consider PE formation an epithelio-mesenchymal transition (EMT). Here we studied the significance of Parathyroid hormone related peptide (PTHrP) signaling via the type I PTH/PTHrP receptor (PTH(rP)-RI) for the migration and morphological transformation of endoderm cells, two aspects of EMT, as well as for PE-specific gene expression, during *in vitro* and *in vivo* PE formation. We demonstrate that addition of PTHrP to F9 derived PrE and VE cells induces the loss of typical epithelial cell-cell junctions, concomitant with the induction of a mesenchymal morphology and PE-specific enhanced thrombomodulin expression. PTHrP enhances migration during the formation of F9 PE. However, analysis of the parietal yolk sac from mutant mouse embryos lacking the PTH(rP)-RI reveals that receptor activation is not required for migration, as endodermal cells are present. Importantly, these cells display the morphology and gene expression typical for epithelia, and lack PE-specific enhanced thrombomodulin expression. We conclude that signaling via the PTHrP hormone/receptor complex is required for PrE/VE cells to properly differentiate to mesenchymal PE, but not for migration. The failure of mutant PE cells to complete the EMT may cause the growth retardation and mortality that has been previously described for these mutant embryos.

INTRODUCTION

The two extreme tissue phenotypes are the epithelial and mesenchymal phenotype. Epithelia line surfaces of organs and body cavities, and can be defined as continuous sheets of cells with basal-apical polarity, situated on a basal membrane, with the cells tightly connected to each other by specific junctions. These junctions fulfill an anchoring function (adherens junctions and desmosomes) and form a permeability barrier across cell sheets (tight junctions), or they facilitate intercellular passage of molecules and with that allow communication between cells (gap junctions) (Alberts *et al.*, 1983; Hay, 1995; Birchmeier *et al.*, 1996; Davies and Garrod, 1997). Most of these junctions are interconnected through cytoskeletal networks: actin microfilaments organize tight junctions and adherens junctions into continuous belts along the lateral membranes of the cells (Collins and Fleming, 1995; Kusumi *et al.*, 1999), while cytokeratin intermediate filaments crosslink desmosomes across the cytoplasma with each other and with hemi-desmosomes, giving the epithelium tensile strength (Kouklis *et al.*, 1994; Collins and Fleming, 1995; Birchmeier *et al.*, 1996; Boyer *et al.*,

1996; Davies and Garrod, 1997). Mesenchymal cells develop from epithelial cells by loss of these junctions and reorganization of cytoskeletal structures, and acquisition of front-to-back polarity, allowing (enhanced) migration. As a result of this so-called epithelio-mesenchymal transition (EMT), the cells become solitary or only very loosely connected (Hay, 1995; Thiery and Chopin, 1999). Usually mesenchymal cells secrete high levels of extracellular matrix molecules and possess invasive capacity.

The formation of mesoderm from ectoderm during early gastrulation is usually considered the first EMT in mouse embryonic development (Hay, 1995; Viebahn, 1995). However, we noted that PE formation may also be considered an EMT, occurring even before gastrulation, since (i) PrE and VE, the precursors of PE, are considered 'epithelioid' or epithelial respectively, based on their morphological appearance, localization on a basal lamina, and possession of apical microvilli (Hogan and Tilly, 1981; Hogan, 1981; Franke *et al.*, 1982; Hogan and Newman, 1984; Salamat *et al.*, 1995) which are typical epithelial characteristics (Hay, 1995), (ii) they acquire a flattened, elongated fibroblast-like morphology as they migrate onto the basal lamina underlying the trophectoderm while forming the PE layer (Solter and Damjanov, 1973; Enders *et al.*, 1978; Gardner, 1982; Cockroft and Gardner, 1987), (iii) by ongoing morphological modifications the PE cells become increasingly solitary, smooth cells, with a rounded cell body from which long filopodia-like protrusions extend (Hogan and Newman, 1984; Cockroft, 1986), (iv) the cells secrete large amounts of extracellular matrix factors that constitute a thickening basal lamina, now called Reichert's membrane (Hogan and Tilly, 1981; Hogan *et al.*, 1982; Hogan *et al.*, 1982)

Previously, we and others have shown that PTHrP induces PE formation *in vitro* (Chan *et al.*, 1990; van de Stolpe *et al.*, 1993) and *ex vivo* (Behrendtsen *et al.*, 1995), and does so exclusively via the type I PTH/PTHrP receptor (PTH(rP)-RI) (Verheijen *et al.*, 1999). The expression of PTHrP by TE and decidual cells, and expression of the receptor by the VE in the marginal zone and by PE strongly suggest a similar role *in vivo* (Karperien *et al.*, 1994, 1996; Verheijen *et al.*, 1999). Mouse embryos lacking the PTH(rP)-RI are growth retarded and the first die at the 9th day of gestation, when wild type embryos express the receptor in PE only (Lanske *et al.*, 1996; Verheijen *et al.*, 1999). Since PE cells constitute the inner layer of the parietal yolk sac, which surrounds and protects the embryo and importantly, serves as a nutrient filter between mother and embryo (Gardner, 1983; Freeman, 1990; Cross *et al.*, 1994), the mutant phenotype is presumably caused by a dysfunctional PE layer (Lanske *et al.*, 1996; Verheijen *et al.*, 1999). However, no abnormalities were observed in histological examination: PE cells seemed to normally occupy the Reichert's membrane and contribute to its composition by the secretion of laminin (Lanske *et al.*, 1996), leaving the role of PTHrP and its receptor in PE formation *in vivo* enigmatic.

To better understand the cell-biological significance of this hormone/receptor complex in PE formation, we studied its role in two aspects of EMT seen during PE formation, i.e. the changes in expression of markers that discriminate epithelium from mesenchyme, and migration, as well as in PE-specific thrombomodulin expression. The results of experiments on *in vitro* PE formation from F9 cells indicate that addition of

PTHrP induces the morphological transition of PrE and VE to a mesenchymal cell type. Additionally, it enhances but importantly, is not required for migration during PE formation. This suggests that PTH (rP)-RI activation is not required for migration. By microscopical and molecular analysis of the parietal yolk sac of PTH(rP)-RI null mutant embryos, we confirmed that during PE formation *in vivo*, PE precursors migrate in the absence of PTHrP signaling via this receptor. However, the PTH(rP)-RI -/- cells that are considered PE on the basis of topography, display an epithelial instead of mesenchymal morphology, and additionally, do not express the PE-marker thrombomodulin (Imada *et al.*, 1987; 1990a, b; Weiler-Guettler *et al.*, 1996). Thus PTH(rP)-RI activation is required for the differentiation of PE precursors to a mesenchymal cell type with PE specific gene expression. The incomplete EMT and lack of cell-type specific differentiation during PE formation in the PTH(rP)-RI -/- embryos strongly support our previous assumption that the growth retardation and early death of these mutants is caused by a dysfunctional parietal yolk sac.

MATERIALS AND METHODS

Cell culture and differentiation

F9 EC cells were obtained from ATTC (American Type Tissue Culture, Rockville, MD) and cultured on gelatinized tissue culture surfaces as described elsewhere (Veltmaat *et al.*, 2000). All tissue culture plastic was from Costar (Badhoevedorp, The Netherlands). To obtain PrE, cells in monolayer were treated for 3 days with 10^{-6} M *all trans* retinoic acid (RA) (Sigma, Bornem, Belgium). To obtain VE, cells were cultured in suspension in bacterial dishes in the presence of 5 x 10^{-8} M RA for 7 days, generating aggregates of various sizes, with a core of undifferentiated cells surrounded by VE (Hogan et al., 1981). These so-called embryoid bodies were re-plated on gelatinized surfaces. PrE and VE were differentiated to PE by subsequent treatment with $PTHrP_{1-34}$ (PTHrP) (Bachem, Bubendorf, Switzerland) for 1 day (embryoid body outgrowths) or 2 days (monolayer).

Mice, dissection of extraembryonic endoderm

PTH(rP)-RI heterozygous mice (Lanske *et al.*, 1996) in a C57BL/6 x CBA genetic background were intercrossed and embryos were isolated at E7.5, E8.5 or E9.5. Midday of the day of appearance of a vaginal plug was designated E0.5. The Reichert's membrane with the PE attached were isolated and carefully anchored to polyethylene coverslips (Sarstedt Inc., Newton, NC) as described (Hogan *et al.*, 1994) using a pair of tweezers, and analyzed by means of phase contrast microscopy. Membranes were fixated mildly (10-20 minutes) in a 4% paraformaldehyde solution in PBS, and used for immunohistochemistry as described for F9 cells below combined with epifluorescence microscopy. Some embryonic material was used for a genotyping PCR.

Genotyping PCR

DNA was purified from embryonic tissue derived from the crossings described above. PCRs were done to test for the presence of the intact PTH(rP)-RI allele and substitution by the neo-cassette. Primers were GCAGAGATTAGGAAGTCTTGGA and GCCGTCGTCCTTGGGAACTGT (forward and reverse for receptor), and for the neomycin cassette GGAGAGGCTATTCGGCTATGAC (forward), and CGCATTGCATCAGCCATGATGG (reverse). Both products were simultaneously amplified in a reaction volume of 25 µl, using 1 µl DNA, 1.5 mM MgCl2, 0.5 mM of each dNTP, 20 pmol of each primer, and 1 U Taq Polymerase in its reaction buffer (Eurogentec, Seraing, Belgium). Reactions were cycled in a BIOMETRA UNO II thermocycler (Biometra, Göttingen, Germany) through 5 min. 95°C, 40x(30 sec. 95°C, 30 sec. 61°C, 45 sec. 72°C), 7 min 72°C and soaked at 4°C, and analyzed by gel electrophoresis.

Electron microscopy

F9 EC cells were grown in monolayer on polyethylene coverslips (Sarstedt) in the presence of RA for 3 days to obtain PrE. They were fixated in 2% paraformaldehyde/2% OsO_4/50 mM cacodylic acid pH 7.2 during 4 hr. at room temperature, and postfixated in 1% OsO_4/1.5% $K_4(FeCN_6)$/50 mM sodium phosphate pH7.3 during 1.5 hr. on ice, followed by a 30 minute 1% uranyl acetate treatment at room temperature. After dehydration, cells were flat embedded in Epon. Ultrathin sections of 70 nm were cut, of which contrast was enhanced by uranyl acetate and lead citrate treatment. Sections were analyzed by transmission electron microscopy (Philips).

Immunohistochemistry

F9 cells that had been treated with RA during 3

days were trypsinized and re-plated in a density of 25000 cells/cm^2 with RA or PTHrP on polyethylene coverslips (Sarstedt). After 2 days, they were washed in PBS (phosphate buffered saline supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$) at 37°C cells and fixated mildly during 10-20 minutes in 2-4% paraformaldehyde in PBS at room temperature for most immunohistochemical applications. For detection of desmoplakin I and II, cells were fixated 10 minutes in methanol at –20°C. After fixation, cells were washed in PBS at room temperature. For detection of E-cadherin, β-catenin, connexin43, and thrombomodulin, fixation was quenched 30 minutes in PBS/20 mM NH_4Cl at 4°C, followed by washings with PBS at room temperature. Cells were placed in NETGEL (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.05% (v/v) NP-40, 0.25% (w/v) gelatin, 0.02% (w/v) sodium azide) supplemented with 10 mM $CaCl_2$ and 1 mM $MgCl_2$, for at least one hour. Cells were incubated with first and subsequently secondary antibody during 1 hr in supplemented NETGEL, and washed in PBS after each incubation. For detection of ZO-1, cytokeratins and actin, cells were permeabilized 5 minutes in PBS/0.2% Triton X-100 after fixation, washed in PBS. Antibody incubations were diluted in PBS according to manufacturer's recommendations. Cells were analyzed by confocal laser scanning microscopy. Ratαmouse-E-cadherin (DECMA) was from Sigma, mouse-β-catenin and connexin43 were from Transduction Laboratories, mouseαmouse desmoplakin I/II antibodies were from Boehringer Mannheim, rabbit-αbovine-pan-keratin was from DAKO, Texas-Red conjugated phalloidin was from Molecular Probes Inc. Cy-3 conjugated secondary antibodies and Texas-Red conjugated secondary antibodies were from Jackson Laboratories. Rabbitαmouse-ZO-1 antibodies were a kind gift of Dr. M. Arpin (Institut Curie, Paris, France) and diluted 1:200. Ratαmouse thrombomodulin antibodies 273-34A were a kind gift of Dr. S.J. Kennel and diluted 1: 250 (Oak Ridge National Laboratory, Tennessee, USA).

Analysis of cell migration

To analyze whether PTHrP affects migration of PE precursors, F9 embryoid bodies were re-plated in the absence or presence of PTHrP and photographed 1 day later. Migration was defined as the distance that cells had migrated away from the edge of the embryoid body, calculated as the average distance of the cell farthest away from the edge, determined in 8 directions with 45° intervals. The mean total distance was calculated for 16 embryoid bodies without and 25 with PTHrP. In all cases, a two-sided Student's t-test was performed to calculate the significance of the difference in means +/- standard error of the mean (SEM).

RESULTS

PTHrP induces a mesenchymal morphology in the epithelial precursors of PE *in vitro*.

To gain more insight in the significance of PTHrP in PE formation, we started analyzing *in vitro* in which aspects of the EMT as it occurs during *in vivo* PE formation PTHrP plays a role. VE of an E8.5 mouse embryo has an epithelial morphology (figure 1A), while PE cells, which are formed by differentiation of PrE and VE, are solitary, rounded off cells (figure 1B).

PE formation can be mimicked *in vitro* by differentiating F9 EC cells. When treated with retinoic acid (RA) for 3 days in monolayer, about 80% of the EC cells acquire a flat, polygonal morphology, and are associated in continuous sheets, as shown in figure 1C. These cells are considered PrE, based on their morphology, the loss of expression of SSEA-1, a marker for undifferentiated cells that is expressed by untreated F9 EC cells (Solter and Knowles, 1978), and gain of expression of the endodermal markers TROMA-1, (Kemler *et al.,* 1981; Duprey *et al*., 1985), laminin (Strickland *et al*., 1980) and tissue plasminogen activator (tPA) (Strickland and Mahdavi, 1978). However, they do not yet express the PE marker thrombomodulin at the membrane (figure 2 A, A') (Imada *et al*., 1987, 1990a, b; Verheijen *et al*., 1999). At this time they express the PTH(rP)-RI (van de Stolpe *et al*., 1993). Subsequent addition of PTHrP results in a refractory, rounded off shape with filopodia-like extensions and less than 20% contact with neighboring cells in 50-80% of the cells (fig. 1D). These cells are considered PE on the basis of their rounded morphology and reduced contact with other cells (Behrendtsen et al., 1995), elevated expression of lami-

nin and tPA, and high expression of thrombomodulin (figure 2B, B').

Alternatively, treating F9 cultures in suspension with RA results in aggregates with an outer layer of VE cells characterized by the expression of alpha-fetoprotein (Grabel and Casanova, 1986). Upon re-plating of these so-called embryoid bodies on gelatinized tissue culture plastic the endoderm cells grow out as continuous sheets (figure 1E) with very low levels of thrombomodulin expression at the membrane (figure 2C, C'). Incidentally, solitary cells are seen, of which the number is greatly increased and morphology is more rounded by addition of PTHrP upon re-plating. These cells are considered PE as indicated by their rounded morphology (figure 1F), and a high expression level of thrombomodulin (figure 2D, D').

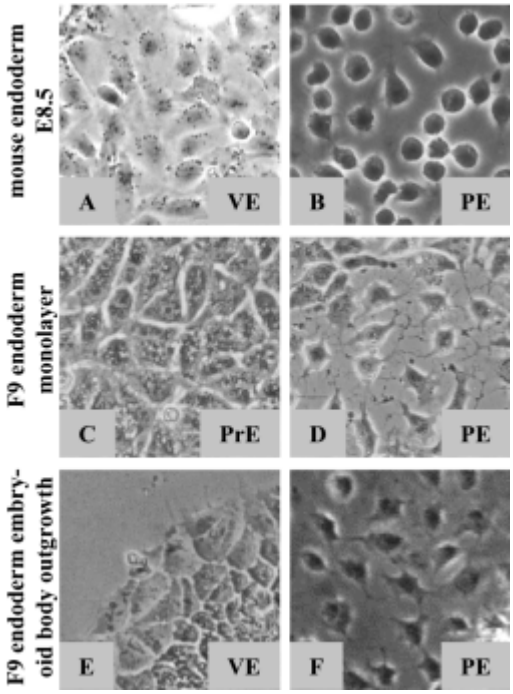


Figure 1: Morphology of extraembryonic endoderm (like) cells derived from mouse embryos and F9 cells.
Phase-contrast images of fresh *in vivo* isolates and F9 cultures. All photographs are displayed at the same magnification.

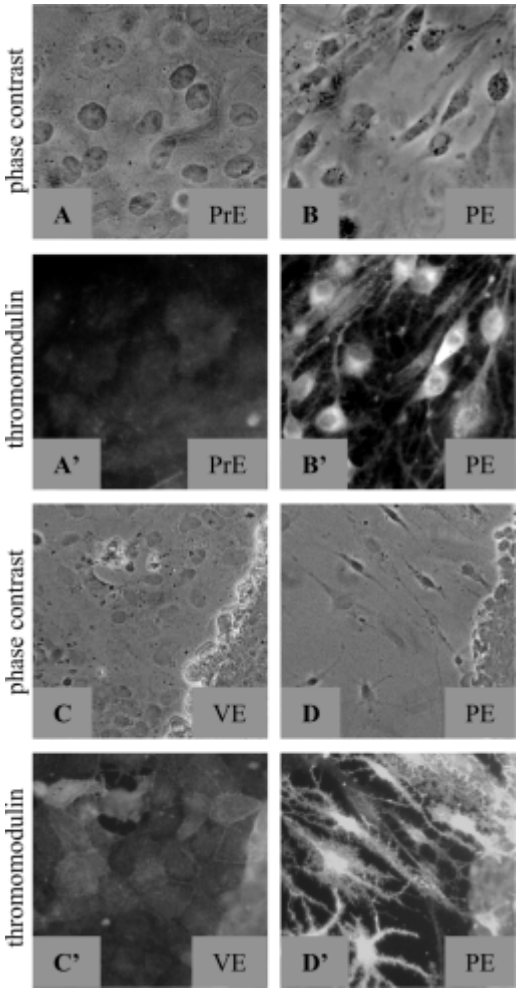


Figure 2: Expression of the PE marker thrombomodulin by F9 cells.
Phase-contrast (A-D) and g epifluorescence images of the corresponding areas (A'-D') showing immunoreactivity for the PE marker thrombomodulin in F9 endoderm after fixation. All photographs are displayed at the same magnification.

These data demonstrate that PTHrP induces a mesenchymal phenotype during PE *in vitro* formation, at least at the morphological level. We further investigated this at the molecular level, by analyzing the expression of molecular markers that distinguish epithelial cells from mesenchymal cells. Next we analyzed the role of PTHrP in migration, to shed light on the possible role of PTHrP in the EMT as it occurs during PE formation *in vivo*.

Epithelial junctions between PrE and VE cells are lost during PTHrP induced PE formation *in vitro*.

Epithelia can be discriminated from mesenchymal cells by their expression of specialized cell-cell junctions, e.g. zonular adherens junctions and/or desmosomes, and most often tight junctions (Davies and Garrod, 1997). We used the localized expression of proteins that constitute various junctions to further examine F9 PrE, VE and PTHrP induced PE cells for their epithelial or mesenchymal character. Figure 3A shows that PrE cells express zonula occludens 1 (ZO-1), an intracellular marker for tight junctions (Collins and Fleming, 1995), at the cell membrane, suggesting it is incorporated in intact junctions. E-cadherin (figure 3C) and β-catenin (figure 3E), the transmembrane and intracellular constituents respectively of adhe-

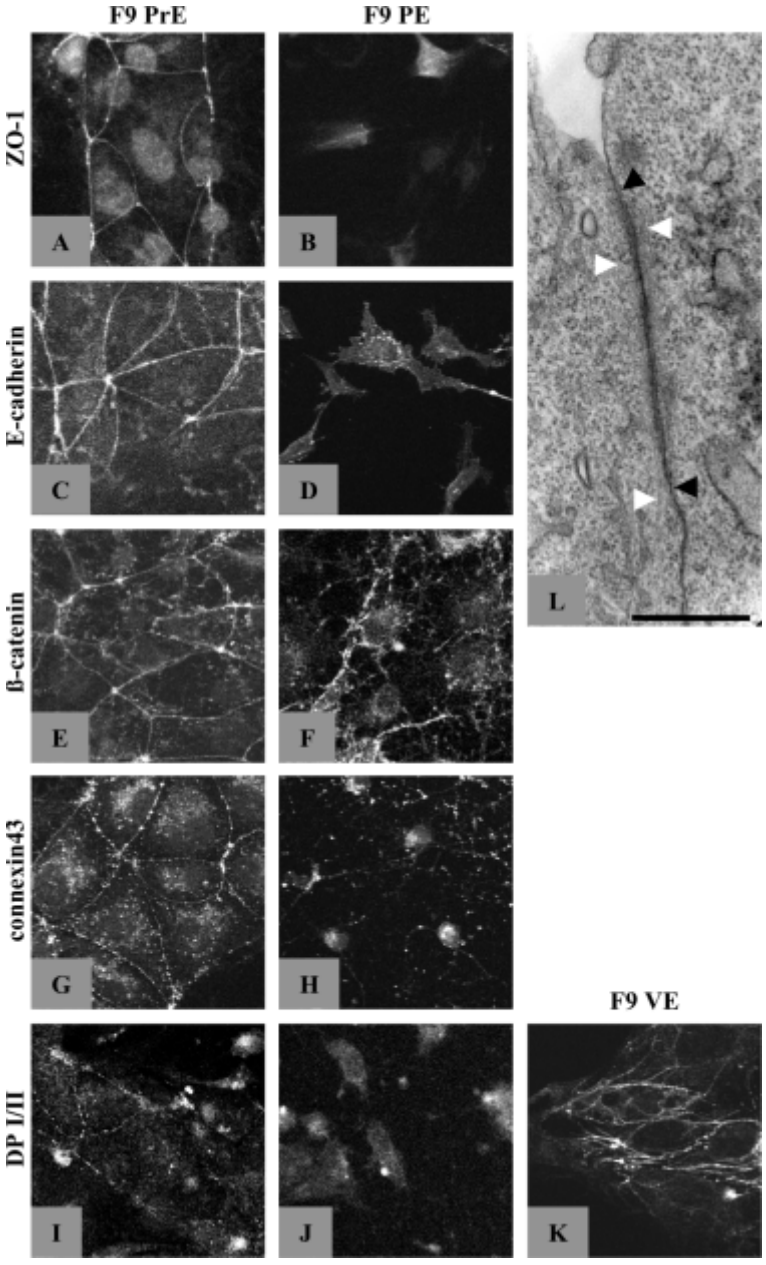


Figure 3: Analysis of cell-cell junctions in F9 endoderm.
Immunoreactivity of F9 endoderm as analyzed by confocal laser scanning microscopy on F9 PrE (left column, from a culture as displayed in figure 1C), F9 PrE derived PE (right column, from a culture as displayed in figure 1D) and F9 VE (K) as displayed in figure 1E). Shown are expression of ZO-1 as a marker for tight junctions (A, B), E-cadherin (C, D) and β-catenin (E, F) as markers for adherens junctions, connexin43 (G, H) as a marker for gap junctions, and desmoplakins (DP) I/II (I, J, K) as markers for desmosomes. The prevalence of positive cells is displayed in table 1. All photographs are displayed at the same magnification. Figure L is a transmission electron micrograph demonstrating the presence of a gap junction in F9 PrE between the black arrowheads. F-actin fibers located laterally are indicated by white arrowheads. Scale bar is 500 nm.

rens junctions (Boyer *et al*., 1996) are expressed at the membrane as well, indicative for the presence of adherens junctions, which we confirmed by transmission electron microscopy (figure 3L). The expression of connexin43 (Cx43), the most abundant component of gap junctions in F9 cells, which we have shown to be functional (van der Heyden *et al*., 2000), is shown in figure 3G. On the other hand, desmoplakin I/II (DP), the intracellular constituent of desmosomes (Boyer *et al*., 1996), are expressed at the membrane only in a few PrE cells (< 1%), of which an example is shown in figure 3I. In VE cells, expression patterns of ZO-1, E-cadherin and β-catenin were identical to those in PrE cells (not shown). While Cx43 was localized at the membrane as well as intracellularly in PrE, in VE cells it was mainly localized at the membrane (van der Heyden *et al*., 2000). Most strikingly, expression of DP was seen in more than 80% of VE cells (figure 3K), in contrast to the very low expression in PrE cells.

Upon treating PrE cells with PTHrP, membrane localized expression of E-cadherin (figure 3D) and β-catenin (figure 3F) was fragmented or completely abolished, and E-cadherin was often internalized. The majority of Cx43 was mainly localized intracellularly as well, with sparsely distributed spots remaining at the membrane (figure 3H), in accordance with a decreased, but still present, intercellular communication as we recently described (van der Heyden *et al*., 2000). Membrane localization of ZO-1 (figure 3B) and DP (figure 3J) was no longer detected. VE derived PE expressed all these molecules similar to PrE-derived PE (not shown).

Together, these data (as summarized in table I) show that F9 PrE and VE possess anchoring junctions, as well as tight and gap junctions, and thus can both be considered epithelia. They display qualitative differences in membrane localized expression of DP, which may reflect maturation of the epithelial state in VE, and in expression of Cx43. The partial or complete absence or relocation of all the junctional molecules and rounding of the cells upon treatment with PTHrP is indicative for a mesenchymal phenotype, induced by PTHrP. In addition to their loss of anchoring and tight junctions, the PE cells have less gap junctions, and therewith a reduced capacity for intercellular communication (van der Heyden *et al*., 2000).

Table I

	PrE	VE	PE
ZO-1 (tight junction)	+++	+++	-
E-cadherin (adherens junction)	+++	+++	-
β-catenin (adherens junction)	+++	+++	-
connexin43 (gap junction)	+++	+++	-
desmoplakin I/II (desmosome)	+/-	+++	-

Membrane-localized expression of junctional proteins in F9 endoderm.
The prevalence of membrane-localized expression of junctional proteins in F9 endoderm is indicated with – (absent in all cells), +/- (present in less than 1% of the cells) or +++ (present in 75-100% of the cells). Expression patterns are illustrated in figure 3.

F9 PrE cells have an epithelial cytoskeletal organization, which is lost upon PTHrP treatment.

Another hallmark, in which epithelial cells differ from non-epithelial cells, is the organization of cytoskeletal filaments (Alberts *et al*., 1983). In epithelial cells, the actin microfilaments are organized in a continuous subcortical belt at the lateral membranes, and cytokeratin intermediate filaments form an intricate network across the cytoplasm. In mesenchymal cells, cytokeratins do not form such a network, but may be linked to focal adhesion plaques instead, and their expression may be complemented or substituted by another intermediate filament, vimentin. In contrast, the subcortical actin fibers are fragmented, or may form stress fibers attached to focal adhesion plaques at the basal membrane, or alternatively, this cytoskeleton may completely collapse.

We tested F9 PrE cells and PTHrP induced PE cells for the expression and localization of actin microfilaments and keratin intermediate filaments. Actin microfilaments were mainly running along the lateral periphery of PrE cells as a thick subcortical belt, typical for epithelial cells (figure 4A). Occasionally these filaments formed stress fibers. In PTHrP induced PE cells, the thick subcortical belts were not detected at all (figure 4B). Instead, microfilaments were fragmented and in many cells perinuclear staining was observed.

In PrE cells, the epithelial cytokeratin filaments, although strongly variably abundant, were always organized in a network (figure 4C). In contrast, in the PTHrP induced PE cells, these keratins were no longer detected as a network, but they were still present perinuclearly (figure 4D). The intermediate filament that is typical for mesenchymal cells, vimentin, is also present to some extent in F9 PrE, but strongly upregulated in F9 PE cells (Trevor, 1990), indicative for the transition to a mesenchymal differentiation state. This is confirmed by the respective absence and presence of vimentin in VE and PE *in vivo* (Lane *et al*., 1983; Lehtonen *et al*., 1983).

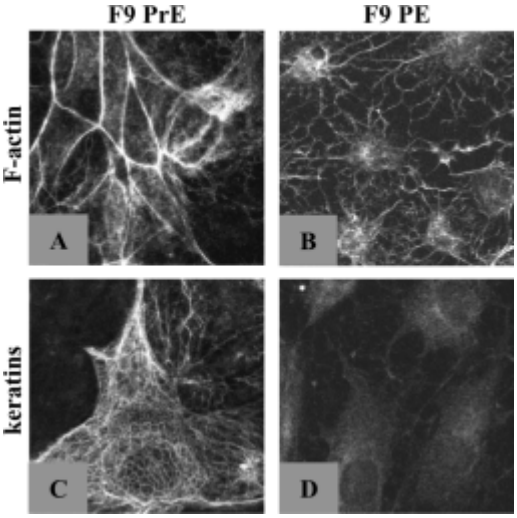


Figure 4: Cytoskeletal organization in F9 endoderm.
Immunoreactivity of F9 endoderm as analyzed by confocal laser scanning microscopy on F9 PrE (left column, from a culture as displayed in figure 1C), and F9 PrE derived PE (right column). Shown are expression of F-actin microfilaments (A, B) or keratin intermediate filaments (C, D). Photographs A and B are displayed at the same magnification. Photographs C and D are displayed at the same magnification, which is slightly higher than for A and B.

Thus, the cytoskeletal rearrangements that we observed upon PTHrP treatment confirm that this peptide induces a mesenchymal phenotype during the formation of PE.

Cell migration during the formation of PE *in vitro* occurs independently of exogenous PTHrP.

Cells undergoing an EMT usually acquire an increased migratory speed (Boyer *et al*., 1996). To assess the role for PTHrP in this aspect of PE formation, we mimicked the *in vivo* situation (where endoderm has to grow away from the inner cell mass) more closely by gro-

wing F9 cells in suspension in the presence of RA. This allowed the formation of aggregates with a core of undifferentiated cells, surrounded by VE cells as indicated by expression of alpha-fetoprotein (Dziadek and Adamson, 1978; Hogan *et al*., 1981). These so-called embryoid bodies were re-plated on gelatinized tissue culture plastic, and photographed the next day. Migration was determined by measuring the distance between the edge of the embryoid body and the farthest outgrown cell as measured in 8 pre-determined directions. As shown in table II and figure 5A, cells grow out in the absence of exogenous PTHrP, while retaining their epithelial character as indicated by morphology and membrane localized E-cadherin and β-catenin expression (figure 5C', C''). However, the addition of PTHrP increased the average distance significantly. Since the total number of outgrown cells in the absence or presence of PTHrP did not differ significantly (not shown), this larger distance was attributable to enhanced migration of individual cells in the presence of PTHrP. In the absence of PTHrP less than 10% of the outgrown cells had lost contact with neighboring cells, and these had only incidentally assumed a fibroblastic or more rounded, mesenchymal morphology (figure 5A, arrow). Upon addition of PTHrP, the percentage of solitary cells with such typical PE morphology and loss of adherens junctions was strongly increased (70%±24%, figure 5B arrow, D', D''), as was thrombomodulin expression (figure 2D, D').

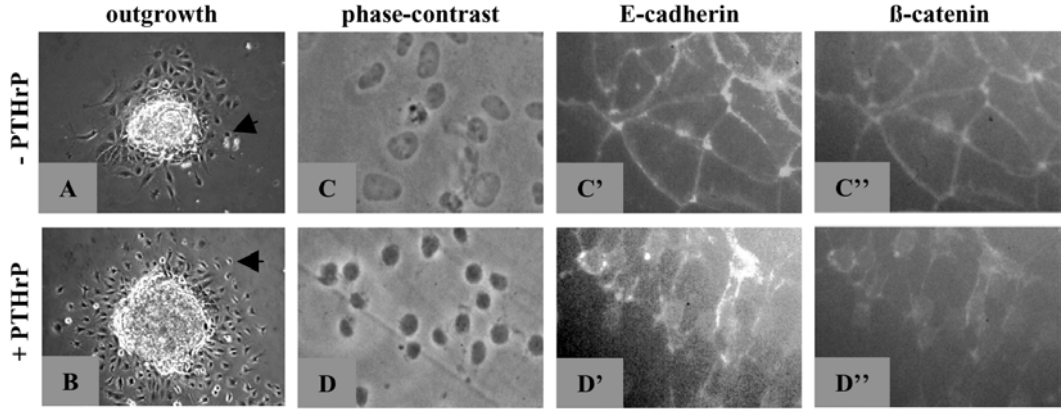


Figure 5: F9-embryoid body outgrowth in absence and presence of PTHrP.
F9 EC cells were cultured in suspension with RA during 7 days, generating embryoid bodies with a core of undifferentiated cells, surrounded by VE. Upon subsequent re-plating and 1 day culture in the absence (A) or presence (B) of PTHrP, outgrowths of cells with a predominantly epithelial or mesenchymal morphology were seen respectively. In the absence of PTHrP, most outgrown cells (of which the nuclei are shown in C at a larger magnification) have membrane localized expression of E-cadherin (C') and β-catenin (C''), while in the presence of PTHrP, more solitary cells are seen (B), often with a rounded morphology (as estimated from light-refraction), typical for PE (arrow in A, B), with a lack of membrane localized expression of E-cadherin (D') and β-catenin (D'') in outgrown cells (nuclei shown in D).

Table II

	- PTHrP (n = 16)	+ PTHrP (n = 25)
Distance from edge	5.4 ± 0.6	8.4 ± 0.7

Outgrowth of F9 embryoid bodies.
The migration of cells growing out from F9 embryoid bodies in the absence or presence of PTHrP was measured as end-point values at 1 day after re-plating on gelatinized surfaces. Values represent the mean distance (in arbitrary units, a.u.) of the cell farthest away of the edge of the aggregate, as measured in 8 directions per aggregate, with the S.E.M. over the number of aggregates (n) re-plated. The effect of PTHrP on outgrown distance is significant ($p < 0.01$, two-sided Student's t-test). 1 a.u is approximately equal to 4 VE-cell diameters.

These data indicate that VE cells, the epithelial precursors of PE, possess a capacity to migrate, which is independent of exogenous PTHrP. We can not exclude a role for endogenous PTHrP in this basal migration *in vitro*, as PTHrP and the PTH(rP)-RI are expressed upon RA treatment of F9 cells and may provide autocrine or paracrine stimulation (Chan *et al*., 1990; van de Stolpe *et al*., 1993). Importantly however, the addition of PTHrP significantly enhances the migration of outgrowing cells and their acquisition of a mesenchymal morphology, and as such PTHrP is sufficient to *in vitro* induce the EMT as seen during PE formation *in vivo*.

PTH(rP)-RI null mutant embryos form PE cells that have not acquired the mesenchymal morphology.

To determine whether PTHrP signaling is also required for these aspects, we analyzed PE formation in PTH(rP)-RI knockout mouse embryos. Signaling of PTHrP during PE formation through another receptor can most likely be excluded, since ES cells derived from such embryos do not form PE upon addition of PTHrP *in vitro* (Verheijen *et al*., 1999).

We isolated the Reichert's membrane from embryos at E7.5-E9.5 derived from crosses between PTH(rP)-RI heterozygous mice in a C57BL/6 genetic background, and investigated them by phase contrast microscopy. In accordance with our previous findings (Lanske *et al*., 1996), we found that cells were present on these membranes at all stages, indicating that PTHrP signaling is not necessary for migration. The cells constituting the PE layer of E7.5 embryos were difficult to analyze, but seemed to predominantly have an epithelial appearance, regardless of genotype. We found that cells of the PE layer in E8.5 heterozygous and wild type embryos mostly displayed a rounded, mesenchymal morphology (figure 6A, D), and were sometimes mingled with patches of epithelial cells, while in E9.5 embryos these patches were seldomly observed (table III). In contrast, in null mutants of E8.5 and E9.5, the PE layer consisted predominantly of cells with an epithelial morphology resembling VE (figure 1A, 6B, E, table III).

Table III

	+/- or +/+		-/-	
	E8.5	**E 9.5**	**E8.5**	**E 9.5**
epithelial	2	2	13	2
predominantly mesenchymal, with epithelial patches	17	0	0	1
mesenchymal	55	22	1	0
TOTAL	**74**	**24**	**14**	**3**

Morphology of PE of embryos from crosses between C57BL/6 PTH(rP)-R1 +/- mice.

Table III displays the number PE isolates dissected from 12 litters at E8.5 and 6 at E9.5 of the indicated PTH(rP)-R1 genotype with an epithelial versus mesenchymal morphology of PE cells on the Reichert's membrane, as observed by phase contrast microscopy at the time of isolation. 54 Embryos (24 at E8.5 and 30 at E9.5) of these litters were not available for analysis due to unresolved genotype or failed PE isolation.

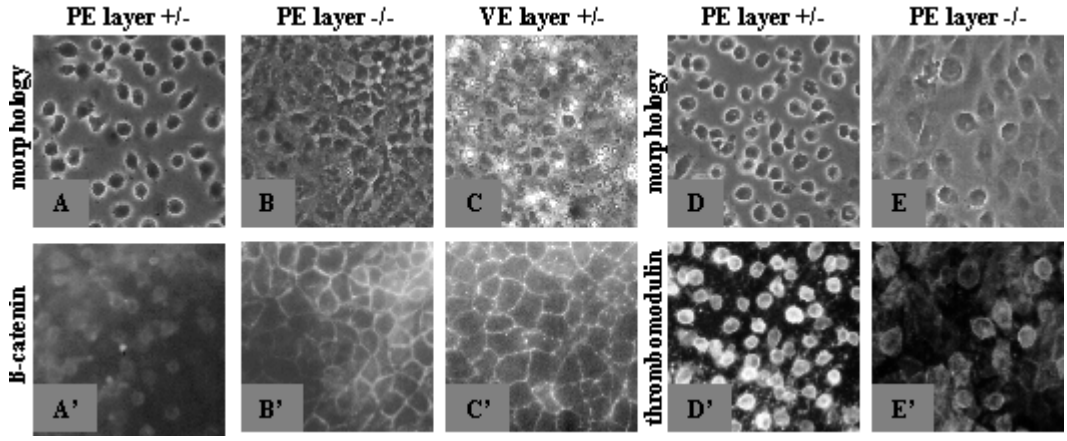


Figure 6: Morphology and protein expression in PE cells with different genotypes for the PTH(rP)-RI.

Phase contrast and epifluorescence images of the corresponding areas of cells occupying the Reichert's membrane in PTH(rP)-RI heterozygous mouse embryos (A, D) and their PTH(rP)-RI null mutant litter mates (B, E), and of heterozygous VE (C) at E8.5 (A-C) or E9.5 (D, E) in a C57BL/6 genetic background. Expression and localization of β-catenin (A'-C') and thrombomodulin (D', E') was analyzed by epifluorescence microscopy. Expression of these proteins in wild-type embryos resembled that of heterozygous littermates. The low expression of thrombomodulin in VE is masked by expression in vasculature, therefore not shown. All images are displayed at the same magnification.

To test if these morphologically epithelial cells were also linked by cell-cell junctions, we analyzed the cells of 9 wild type and heterozygous, and of 6 homozygous null mutant embryos at E8.5 for membrane-localized expression of β-catenin. We could not find membrane labeling of β-catenin in PE of any of the wild type and heterozygous embryos (figure 6A'), while it was localized at the membrane in PE of all null mutant littermates (figure 6B'), suggesting the presence of adherens junctions. Thus, both in morphology and expression of β-catenin, mutant PE cells resemble VE cells, which thus seem to fail to complete their EMT.

Additionally, we examined E9.5 embryos for expression of the PE marker thrombomodulin, and found that the mesenchymal morphology of PE of wild type and heterozygous embryos (n=21) always correlated with high levels of thrombomodulin expression (figure 6D, D'). In contrast, the epithelial morphology of PE in their null mutant littermates (n=3) always correlated with low levels of thrombomodulin expression at the membrane (figure 6E, E'). Whether these mutant PE cells resembled VE cells with respect to their expression of thrombomodulin was difficult to determine, since expression of thrombomodulin in VE was masked by expression of this protein in the vasculature *in vivo*. However, it closely resembled the low expression levels observed in F9 VE (figure 2C'), suggesting that the mutant PE cells are VE cells that fail to differentiate.

Taken together, our results indicate that PTHrP signaling via the PTH(rP)-RI is sufficient and required to induce the differentiation to a mesenchymal cell phenotype with PE specific gene expression during PE formation *in vitro* and *in vivo*. It enhances migration *in vitro*, and is therefore a true inducer of the EMT as seen during PE formation. However, our *in vivo* results demonstrate that signaling via this hormone/receptor system is not required for migration. Therefore, PE

precursors in PTH(rP)-RI null mutant embryos contribute to a PE layer albeit without acquisition of a mesenchymal, PE-specific differentiation state. The lack of differentiation that we found in cells of the PE layer of PTH(rP)-RI mutant embryos at the age at which they are first growth retarded and start dying, strongly supports our previous assumption that their phenotype is caused by a dysfunctional parietal yolk sac.

DISCUSSION

We propose to consider the differentiation of PrE and VE to PE an epithelio-mesenchymal transition (EMT). We provide evidence that PTHrP signaling via the PTH(rP)-RI is required for the epithelial precursor cells of PE to undergo the transition to a mesenchymal cell type with PE specific morphology and marker expression, but not for their migration over the Reichert's membrane, away from the embryo proper. We provide support for our previous assumption that PTH(rP)-RI null mutant embryos are growth retarded due to a malfunctioning PE.

PTHrP is sufficient for induction of a mesenchymal phenotype during PE formation.

PrE and VE have been recognized as epithelia before by others, mainly based on positional and structural features of the cells (Franke *et al.*, 1982; Hogan and Newman, 1984). PE cells *in vitro* and in early post-implantation mouse embryos are scattered over the surface as solitary cells with a fibroblastic morphology, and can thus be considered mesenchymal on a structural basis. Others and we have shown before that PTHrP is an inducer of PE formation *in vitro* (Chan *et al.*, 1990; van de Stolpe *et al.*, 1993) and *ex vivo* (Behrendtsen *et al.*, 1995).

We investigated the presence of cell-cell junctions in F9 derived PE and its precursors *in vitro* to test the hypothesis that PE formation by PTHrP occurs via an EMT. Adherens type junctions are the most important junctions for epithelial cells. Our immunohistochemical data for E-cadherin and β-catenin expression strongly suggested the presence of adherens junctions, which we confirmed to exist by performing electron microscopy. The existence of another adherens type junction, the desmosome, was suggested by the punctate membrane bound expression of DP in a low percentage of PrE cells, and in the majority of VE cells. The membrane localization of ZO-I in F9 PrE has not been reported before, and its presence in these cells and in F9 VE is indicative for existence of tight junctions. This may reflect maturation of these epithelia to functioning as a selective permeability barrier, similar as in trophoblast differentiation (Collins and Fleming, 1995). Moreover, the high abundance of DP at the membrane of VE cells suggests that this epithelium is maturer than PrE. The qualitative difference between PrE and VE was also seen in the membrane localized expression of Cx43 in a punctate pattern, which corresponds well with the existence of gap junctional intercellular communication, as we described elsewhere (van der Heyden *et al.*, 2000). Together, these data indicate that PE precursors are epithelial cells, intimately connected by anchoring junctions, tight junctions, and functional gap junctions.

We found no membrane localized DP, nor ZO-I, E-cadherin and β-catenin, in PE cells that had emerged after treating PrE or VE cells with PTHrP, indicating that anchoring junctions and tight junctions are disintegrated by the action of PTHrP. In addition to the loss of these typical epithelial junctions,

PTHrP leads to a reduction of membrane bound expression of Cx43, concomitant with reduced gap junctional communication, as we reported (van der Heyden *et al.*, 2000). The partial or complete loss of the various junctions by PrE and VE after treatment with PTHrP and the absence of these junctions in PE *in vivo* are indicative for mesenchymal cells.

We further characterized the process as EMT by analyzing the cytoskeletal organization in F9 endoderm cells. We found that actin and cytokeratins form subcortical bundles and trans-cytoplasmic networks respectively in PrE, similar to the findings of others (Tienari, 1989; Trevor and Steben, 1992), and indicative for epithelial cells. Alternatively, the loss of actin bundles in PE cells strongly suggests a mesenchymal character of these cells, as does the loss of organization of cytokeratins in PE cells. Moreover, the mesenchymal phenotype of PE cells *in vitro* and *in vivo* is confirmed by the substitution of cytokeratins by vimentin (Lane *et al.*, 1983; Lehtonen *et al.*, 1983; Trevor, 1990).

Also with respect to secretory behavior, PE cells *in vitro*, *ex vivo* and *in vivo* have typically mesenchymal features. They express and secrete high levels of a number of matrix components, such as laminin, collagen IV, entactin, fibronectin, heparan sulphate proteoglycans and SPARC, which become assembled in the Reichert's membrane (Hogan *et al.*, 1980, 1982; Smith and Strickland, 1981; Kurkinen *et al.*, 1983; Mason *et al.*, 1986). Additionally, PE cells secrete modulators of the extracellular matrix, such as tPA (Marotti *et al.*, 1982), gelatinase B/MMP9 and TIMPs (Behrendtsen and Werb, 1997). Such molecules are generally expressed by mesenchymal cells while not, or to a lesser extent, by epithelial cells (Hay, 1995).

Thus we have provided evidence that PTHrP induces a mesenchymal phenotype, by inducing the loss of cell-cell contacts and typical epithelial cytoskeletal organization and inducing the rounded morphology typical for PE cells. These PE cells secrete high amounts of extracellular matrix molecules and their modulators, typical for mesenchymal cells. Interestingly, as far as peptide growth factors have been shown to be involved in EMTs, they are usually signaling via tyrosine kinase receptors (Birchmeier *et al.*, 1996; Boyer *et al.*, 1996; Thery and Stern, 1996; Davies and Garrod, 1997; Thiery and Chopin, 1999), while PTHrP induces the EMT involved in PE formation via binding to the PTH(rP)-RI, a G-protein coupled 7-pass transmembrane receptor.

Signaling of PTHrP via the PTH(rP)-RI is required for a mesenchymal phenotype of PE *in vivo*.

Our analysis of C57BL/6 PTH(rP)-RI null mutants revealed that the cells residing on the inner side of the Reichert's membrane had retained the epithelial morphology of PE precursor cells, and had not acquired a mesenchymal morphology typical for PE cells. Although some matrix factors, e.g. fibronectin, induce a mesenchymal morphology of PE cells *in vitro* and *ex vivo* (Grabel and Watts, 1987; Behrendtsen *et al.*, 1995), our results indicate that the composition of the Reichert's membrane alone is not sufficient to support this morphological transition *in vivo*. In addition, we found that the cells were still connected by adherens junctions, as suggested by the membrane-localized expression of β-catenin. This clearly indicates that signaling via the PTH(rP)-RI is needed to complete the EMT during PE formation in mouse embryos in the C57BL/6 genetic background.

PTHrP signaling is not required for migration during PE formation.

We observed a basal migration of VE cells when we re-plated F9 embryoid bodies. In the absence of PTHrP, most outgrown cells had an endodermal phenotype, and proliferation was seldomly observed, which indicates that new cells must derive from migratory endoderm cells in the embryoid body itself. In these *in vitro* assays, signaling via endogenously produced PTHrP and the PTH(rP)-RI may still occur, and even be necessary for the migration that we found. However, the necessity of their signaling for migration *in vivo* was excluded by analysis of the Reichert's membrane of mouse embryos that lack the PTH(rP)-RI. Their Reichert's membrane appeared to be covered by outgrowth from PrE and VE. Since it is highly unlikely that PTHrP may signal via another receptor in these tissues, as we reported previously (Verheijen *et al.*, 1999), these results indicate that PTHrP signaling is not needed for migration. However, addition of PTHrP significantly enhanced the migration of PE precursors *in vitro* (this report) and is even required when embryoid bodies are re-plated on non- or low-permissive substrates, such as laminin (J. Veltmaat, unpublished results). Similarly, an additive effect of PTHrP in increasing the number of outgrowing cells from isolated inner cell masses on fibronectin, laminin, vitronectin and collagen IV has been reported (Behrendtsen *et al.*, 1995).

The observed migration of cells *in vivo* is not necessarily a default effect of proliferation of the VE, as daughter cells might also form an extra layer on top of the existing VE, or undergo apoptosis. Their migration onto the basal lamina may be facilitated by extracellular matrix factors, e.g. fibronectin, which has been shown to enhance outgrowth of cells from plated embryoid bodies or inner cell masses (Grabel and Casanova, 1986; Grabel and Watts, 1987; Carnegie and Cabaca, 1993; Behrendtsen *et al.*, 1995). Alternatively, it may be facilitated by tyrosine kinase receptor signaling activated by e.g. FGF-4, which is produced in the blastocyst and has been shown to stimulate migration of endoderm cells from isolated inner cell masses (Rappolee *et al.*, 1994). FGF-4 signaling increases the expression of Gelatinase B and tPA (Rappolee *et al.*, 1994) which may allow cells to modulate the substrate such, that it facilitates their migration (Behrendtsen and Werb, 1997; Cheng and Grabel, 1997).

Towards understanding the phenotype of PTH(rP)-RI null mutant embryos.

Our analysis of PE cells in null mutant embryos revealed that they had not acquired a mesenchymal morphology, and were still connected through adherens junctions. In addition, using thrombomodulin as a marker to analyze the differentiation of PE (Imada *et al.*, 1987, 1990a, b; Weiler-Guettler *et al.*, 1996; Verheijen *et al.*, 1999), we found that it was not expressed as abundantly as in wild type PE. Thus, although these cells can be considered PE on a topographical basis, they are not true PE cells since they failed to differentiate in the absence of PTHrP signaling.

PTH(rP)-RI null mutant embryos are normal at E7.5, but significantly smaller at E8.5 and E9.5, while 50% of them die between E9.5 and E12.5 (Lanske *et al.*, 1996; Verheijen *et al.*, 1999), and almost 100% has died at E18.5 (Lanske *et al.*, 1996). Since at E8.5 and E9.5 the only site of receptor expression is the parietal endoderm (Karperien *et al.*, 1994, 1996; Verheijen *et al.*, 1999), it was assumed that this tissue was not properly formed due to absence of

PTHrP signaling. Surprisingly, histological analysis indicated that PE cells were formed and secreted laminin (Lanske *et al.*, 1996). However, such analysis of paraffin embedded tissue does not allow detailed morphological analysis, and the deposition of laminin does not necessarily indicate that the cells are well differentiated parietal endoderm, since precursors cells of PE also produce laminin, albeit to a lesser extent. Our observation that the mutant PE cells have not lost their adherens junctions (figure 6), make it conceivable that they have retained their VE character, and thus are also connected by tight junctions. These may prevent the diffusion of lipids and membrane proteins between the apical and basolateral domains of the cells, and in addition, form an impermeable seal that prevents the passive passage of nutrients and waste products between the mother and the embryo. Additionally, the mutant PE cells may not function as PE, e.g. in depositing matrix factors in the Reichert's membrane, giving the Reichert's membrane its appropriate characteristics to support embryonic growth and life. This may explain the smaller body size and possibly the death of PTH(rP)-RI -/- embryos around mid-gestation.

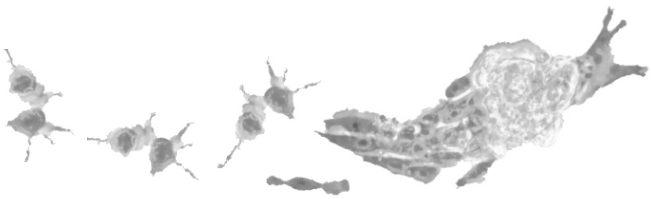
By demonstrating an epithelial phenotype and the lack of PE-specific enhanced thrombomodulin expression of the PE layer in PTH(rP)-RI -/- embryos, we have demonstrated that this tissue is aberrantly formed. This aberrant phenotype may explain the growth retardation and early death of these mutants.

ACKNOWLEDGMENTS

This work was carried out at the Hubrecht Laboratory within the Graduate School for Developmental Biology (Utrecht, The Netherlands) and at Institut Curie (Paris, France) financed by the Ter Meulen Fund from the Royal Netherlands Academy of Arts and Sciences to JMV. We thank Dr. M. Arpin (Institut Curie) and Dr. S. Kennel (Oak Ridge National Laboratories, Tennessee, USA) for kindly providing anti ZO-1 antibodies and anti thrombomodulin antibodies 273-34A respectively. We are also grateful to Drs. J. Salamero and C. Antony (Institut Curie), W. Hage (Hubrecht Laboratory), and Dr. H. Schwarz (Max Planck Institut für Entwicklungsbiologie, Tübingen, Germany) for the excellent assistance with microscopy techniques and to F. Vervoordeldonk and J. Heinen (Hubrecht Laboratory) for photographic reproduction.

CHAPTER 3

DYNAMIC CONNEXIN43 EXPRESSION AND GAP-JUNCTIONAL COMMUNICATION DURING ENDODERM DIFFERENTIATION OF F9 EMBRYONAL CARCINOMA CELLS



Marcel A.G. van der Heyden*[1], Jacqueline M. Veltmaat*, Jeanine A.A. Hendriks[2], Olivier H.J. Destrée and Libert. H.K. Defize

*both authors contributed equally

Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands. [1]Current address: University Medical Center, Department of Medical Physiology, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands. [2]Current address: Isotis B.V., P.O. box 98, 3720 AB Bilthoven, The Netherlands.

The European Journal of Cell Biology 79:272-282, (2000).

3

DYNAMIC CONNEXIN43 EXPRESSION AND GAP-JUNCTIONAL COMMUNICATION DURING ENDODERM DIFFERENTIATION OF F9 EMBRYONAL CARCINOMA CELLS

ABSTRACT

Gap junctional communication permits the direct intercellular exchange of small molecules and ions. In vertebrates, gap-junctions are formed by the conjunction of two connexons, each consisting of a hexamer of connexin proteins, and are either established or degraded depending on the nature of the tissue formed. Gap junction function has been implicated in both directing developmental cell fate decisions and in tissue homeostasis/metabolite exchange. In mouse development, formation of the extraembryonic parietal endoderm from visceral endoderm is the first epithelial-mesenchyme transition to occur. This transition can be mimicked *in vitro* by F9 embryonal carcinoma (EC) cells treated with retinoic acid, to form (epithelial) primitive or visceral endoderm, and then with parathyroid hormone related peptide (PTHrP) to induce the transition to (mesenchymal) parietal endoderm. Here, we demonstrate that connexin43 mRNA and protein expression levels, protein phosphorylation and subcellular localization are dynamically regulated during F9 EC cell differentiation. Dye injection showed that this complex regulation of connexin43 is correlated with functional gap junctional communication. Similar patterns of connexin43 expression, localization and communication were found in visceral and parietal endoderm isolated *ex vivo* from mouse embryos at day 8.5 of gestation. However, in F9 cells this tightly regulated gap junctional communication does not appear to be required for the differentiation process as such.

INTRODUCTION

Gap-junctions permit the direct exchange of small molecules and ions, including second messengers such as cAMP, IP_3, calcium, and metabolites, between the cytoplasm of adjacent cells in developing and differentiated tissues (for reviews see (Bruzzone *et al.*, 1996; Goodenough *et al.*, 1996). Exchange of these compounds may function in tissue homeostasis, electrical synchronization and transmission or sharing of signaling molecules between particular cell clusters. Impaired gap-junctional intercellular communication (GJIC), often resulting from mutations in the constituent proteins, has been linked to several human diseases including deafness, demyelinating neuropathies, lens cataractogenesis (reviewed by Simon and Goodenough (1998), White and Paul (1999)) and in the skin disease erythrokeratodermia variabilis (Richard *et al.*, 1998).

Gap-junctions are formed through the direct interaction of two connexons on neighboring cells. Each connexon consists of a hexamer of one or more types of connexins, oriented circularly around an aqueous pore of 1.2 nm diameter (Unger *et al.*, 1999) (for review see Yeager (1998)). More than 15 different types of mammalian connexins have been cloned and categorized by molecular weight; each has their own, more or less restricted, expression pattern and

permeability characteristics. The organization of connexin molecules into functional channels is very complex. For some, expression and function is regulated at, at least, four different levels: transcription, RNA turnover, phosphorylation-dependent subcellular localization and post-translational modification dependent gating of the channel that eventually forms. Connexin 43 (Cx43), for example, can be regulated at the transcriptional level by various hormones and ligands (estrogen, cAMP and Wnt (Yu *et al.*, 1994; van der Heyden *et al.*, 1998), at the level of RNA stability (by RA (Clairmont *et al.*, 1996)), by (in)direct phosphorylation (via PKC, MAP-kinase and c-Src (Postma *et al.*, 1998; Hossain *et al.*, 1998; Warn-Cramer *et al.*, 1998)) and gating (by pH and cAMP (Morley *et al.*, 1997; Bevans and Harris, 1999)).

Just after implantation in the uterine wall, cells derived from the inner cell mass of mouse blastocyst stage embryos express Cx43. These cells of the egg-cylinder are subdivided in several different communication compartments (Kalimi and Lo, 1989). The most extensive GJIC and prominent Cx43 expression is found in the extraembryonic visceral endoderm (VE) (Kalimi and Lo, 1989; Ruangvoravat and Lo, 1992; Dahl *et al.*, 1996) although to date, no specific function for this extensive coupling in the VE has been identified. This VE can transdifferentiate to parietal endoderm (PE) in the marginal zone of the conceptus where it comes into contact with trophectoderm. We consider this VE to PE conversion of the extraembryonic tissue, from an endocytotic cell providing nutrients to a migratory cell depositing extracellular matrix (Gardner, 1983; Freeman, 1990; Bielinska *et al.*, 1999) the earliest epithelial-mesenchyme transition (EMT) in mouse development. Current evidence suggests that it is largely controlled by parathyroid related peptide (PTHrP) dependent signaling (Chan *et al.*, 1990; van de Stolpe *et al.*, 1993; Karperien *et al.*, 1994, 1996; Behrendtsen *et al.*, 1995; Verheijen *et al.*, 1999).

In vitro, the VE to PE transition can be mimicked by F9 embryonal carcinoma (EC) cells. Treatment of these cells in monolayer or as aggregates in suspension culture ('embryoid bodies') with retinoic acid (RA) results in differentiation to primitive endoderm (PrE) or VE respectively (Strickland and Mahdavi, 1978; Hogan *et al.*, 1981). Subsequently, PrE/VE cells can be induced to transdifferentiate into PE by addition of cyclic AMP (cAMP) elevating agents such as PTHrP or by plasma membrane permeable forms of cAMP itself (Strickland *et al.*, 1980). Previous studies on Cx43 expression in F9 EC cells have suggested regulation by RA and cAMP at the RNA level (Nishi *et al.*, 1991; Clairmont *et al.*, 1996; Clairmont and Sies, 1997). However, a comprehensive study of Cx43 mRNA and protein expression and protein (sub)cellular localization in combination with a functional analysis of gap-junctional communication during differentiation has not been described so far. Furthermore, the effects of interference with gap junctional communication on F9 EC differentiation are unknown.

Here, we have examined in detail the regulation of Cx43 during differentiation in the PrE/VE to PE transition of F9 EC cells at the four different levels described and where feasible verified these results with regulation in VE and PE isolated *ex vivo* from the mouse conceptus We have correlated the complex Cx43 regulation with gap-junctional communication. Finally, we provide evidence that GJIC is not essential for F9

endoderm differentiation *in vitro* and propose that the presence of extensive GJIC in embryonic VE serves a role in metabolite exchange between mother and the early embryo.

MATERIALS AND METHODS

Cell culture

F9EC cells were obtained from the American Type Cell Culture, Rockville, MD), and maintained essentially as described before (Veltmaat *et al*., 2000). For experiments in monolayer, cells were seeded on gelatinized tissue culture dishes (Costar) or polyethylene tissue culture coverslips (Sarstedt) at a density of 500 cells/cm^2 and grown for 5 days in growth medium (EC cells), or seeded at a density of 1000 cells/cm^2 in presence of 10^{-6} M all-*trans* RA (Sigma, Zwijndrecht, The Netherlands) (PrE), for 5 days, with or without 10^{-7} M $PTHrP_{(1-34)}$ (here referred to as PTHrP) (Bachem, Bubendorf, Switzerland) or 1 mM dibutyryl cyclic AMP (Aldrich, Zwijndrecht, The Netherlands) (dbcAMP) for the last two days (PE). For shorter exposures to RA, $PTHrP_{1-34}$ (here referred to as PTHrP), or dbcAMP, the agents were added for the indicated time for the last stages of the 5 day differentiation period.

To obtain VE-like cells, EC cells were aggregated in bacterial dishes in medium containing $5x10^{-8}$ M RA, as described before (Veltmaat *et al.*, 2000), generating 'embryoid bodies'. Control aggregates did not receive RA and remained undifferentiated. Medium was refreshed on the 3rd or 4th day, and the embryoid bodies were re-plated (approx. 10 aggregates per 16 mm diameter dish) on gelatinized culture surfaces (tissue culture plastic or polyethylene coverslip) on the 7th day. Control aggregates were re-plated in growth medium while embryoid bodies were re-plated in presence of $5x10^{-8}$ M RA, 10^{-7} M PTHrP or 1 mM dbcAMP for one more day to obtain outgrowths with, respectively, EC, VE-like (RA), PE-like (PTHrP, dbcAMP) characteristics.

To block gap junctional intracellular communication, 75 µM 18α-glycyrrhetinic acid (AGA) (Sigma) was added to the culture after cells or aggregates had attached. A DMSO solvent control was included in all experiments.

VE/PE isolation from mice

F1 embryos were obtained from C57BL/6 females mated to CBA males. At day 8.5 of gestation (E8.5), embryos were collected in PBS/1% BSA. Visceral yolk sac (VYS) endoderm and parietal endoderm was carefully isolated and attached to tissue culture coverslips (Costar) using a pair of tweezers. Explants were either fixed for indirect immunofluorescence staining using the protocol below or kept in chemically-defined culture medium (bicarbonate buffered mixture of Iscove's modified Dulbecco's Medium and Ham's F12 Medium (Gibco), supplemented with 1% Glutamax (Gibco) 15 mg/l Transferrin (Sigma), 7 mg/l Insulin (Sigma), 0.05% (w/v) BSA (Sigma), 40 µl/l Monothioglycerol (Sigma), 1% Chemically Defined Lipids (Gibco) and 1 U/ml LIF) for 1-4 hours until dye spreading assays were performed.

Northern blotting and hybridization

Cells were washed twice with ice cold PBS and lysed in 0.1 M NaCl, 200 mM Tris/HCl pH 7.4, 10 mM EDTA, 500 µg/ml proteinase K, 0.5% SDS. NaCl was added to a final concentration of 0.5 M in 5 ml lysate (10^7 10^9 cells) and incubated for 2 hours at room temperature with 250 mg oligo dT cellulose (Pharmacia, Roosendaal, The Netherlands) suspended in 5 ml 0.4 M NaCl, 20 mM Tris pH 7.4, 10 mM EDTA, 0.2% SDS. This was loaded onto a polyprep chromatography column (BioRad, Veenendaal, The Netherlands) and washed once with suspension buffer and three times with 0.1 M NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 0.2% SDS. Subsequently, poly A^+ RNA was eluted with 4 ml 1 mM Tris pH 8, 1 mM EDTA, precipitated, and resuspended in 10 mM Tris pH 7.4. 2 µg of poly A^+ RNA was separated on a 1-% (w/v) agarose/6% (w/v) formaldehyde gel and blotted onto Hybond-N filter. Hybridization with 50-100 ng probe was performed in 0.5 M NaH_2PO_4, 7% (w/v) SDS, 1 mM EDTA, 0.1 mg/ml salmon sperm DNA at 65°C overnight. A 1.0 kb rat Cx43 probe and a 1.4 kb GAPDH probe were labeled using a multiprime labeling set (Rediprime, Amersham, Den Bosch, The Netherlands) and 1[α-^{32}P]dCTP (Amersham) resulting in specific activities around $4x10^8$ dpm/µg DNA. Signal was visualized and quantified using a PhosphorImager (Molecular Dynamics) and ImageQuaNT 4.2 Software.

Dye injections

Cells were cultured and induced to differentiate in 30 mm tissue culture dishes as described above. Prior to the experiments, the culture medium was replaced by serum-free HEPES buffered DF medium. Micro electrodes (tip diameter < 1µm) were backfilled with 4% Lucifer Yellow in 150 mM LiCl + 10 mM HEPES (pH 7.4). Dye containing microelectrodes were inserted in selected cells, which were located in clusters of at least fifty. The dye was allowed to diffuse out of the pipette into the impaled cell and its adjacent cells for 2 minutes. The electrode was then retracted, the culture was viewed with epifluorescent illumination (excitation 420 - 490 nm, emission > 520 nm) and the number of fluorescent cells, excluding the one injected, was counted.

Immunofluorescence

Cells and isolated mouse VE/PE on coverslips were prepared as described above, washed twice with phosphate buffered saline (PBS) and fixated in 3.5% formaldehyde in PBS for 30 minutes. Samples were then permeabilized with 0.3% Triton X-100 in PBS for 5 minutes and quenched with 50 mM glycine in PBS twice for 10 minutes. Non-specific binding was blocked by incubation in NET-gel (150 mM NaCl, 5 mM EDTA, 50 mM Tris/HCl pH 7.4, 0.05% NP40, 0.25% gelatin, 0.02% NaN_3) twice for 10 minutes. Cx43 antibody (Transduction labs, Lexington, KY, USA) was diluted 1:500 in NET-gel and cover slips were incubated for one hour at room temperature. Primary antibody was detected with CY3 conjugated sheep anti-mouse IgG (Jackson Immuno Research, West Grove, PA, USA) for one hour. Non-bound antibodies were removed by 6 washes with NET-gel and cells were embedded in Mowiol. Cells were viewed with a confocal laser-scanning microscope (Leica).

Immunoprecipitation and Western blot detection

Immunoprecipitation and Western blot detection was performed as described before (Van der Heyden *et al.,* 1998). In short, cells, cultured and differentiated in 95 mm dishes, were washed twice with PBS at 4EC and lysed in 1 ml cold radio-immunoprecipitation assay (RIPA) buffer (20 mM Tris/HCl pH 7.4, 150 mM NaCl, 10 mM Na_2HPO_4, 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1mM PMSF, 10µg/ml aprotinin). Lysates were clarified by centrifugation at 14,000 x g. 0.5 µg Cx43 antibody (Transduction labs, Lexington, KY) was added to the lysate containing 1 mg protein, and mixed for 4 hours at 4°C. Protein A-sepharose was added for 16 hours to precipitate the immune complexes. The precipitate was washed four times with RIPA, the proteins then dissolved in Laemmli sample buffer, resolved by 12.5% SDS-PAGE and subsequently electroblotted on Immobilon-P membrane (Amersham). For Western blot detection, Cx43 antibody (see above) or thrombomodulin antibody (273-201B; a gift from Dr. S.J. Kennel, Oak Ridge National Laboratory, Oak Ridge, TN) were used according to standard procedures.

RESULTS

Cx43 mRNA levels are enhanced in PrE and decreased in PE F9 cells.

We first examined the kinetics of Cx43 expression during F9 EC cell differentiation. F9 EC cells in monolayer culture were treated with RA for 5 days, with or without PTHrP or dbcAMP during the last two days (figure 1).

F9 EC cell differentiation in monolayer

day 1-3	day 4-5	cell state
-	-	EC
RA	RA	PrE
RA	RA+PTHrP	PE
RA	RA+dbcAMP	PE

F9 EC cell differentiation in embryoid bodies

day 1-7 hanging drops	day 8 plated	cell state
-	-	EC
RA	RA	VE
RA	PTHrP	PE
RA	dbcAMP	PE

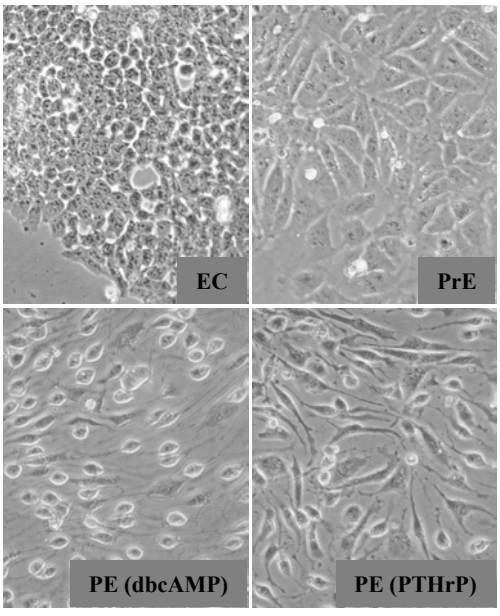


Figure 1: Schematic representation of F9 differentiation protocols.

F9 EC cells were differentiated in monolayer for 5 days with RA without or with PTHrP or dbcAMP added at day 4, for the indicated time. The morphology of the cultures is shown in the microphotographs shown in the lower panel. F9 EC were differentiated to embryoid bodies by aggregating cells in hanging drops in the presence of RA during 7 days and subsequently re-plating with RA or PTHrP or dbcAMP on a gelatinized culture surface, and culture for 1 day.

Messenger RNA was isolated, blotted and hybridized with rat Cx43 and mouse GAPDH probes. As shown in figure 2, Cx43 mRNA is present in undifferentiated F9 EC cells. RA treatment results in an increase in Cx43 mRNA levels, first evident at day 4 and continuing at least until day 5. Differentiated PrE (5 days RA) exhibits a 2.2 fold increase over undifferentiated cells, consistent with previous studies (Nishi *et al.*, 1991; Clairmont *et al.*, 1996). Subsequent differentiation into PE induced by either the natural ligand PTHrP, or by dbcAMP results in a decrease in Cx43 RNA levels. As a negative control for the latter differentiation step we used isoproterenol as a stimulus. Isoproterenol treatment, like PTHrP or dbcAMP treatment, results in elevated intracellular cAMP levels but does not lead to PE differentiation (M. Verheijen and L.H.K. Defize, unpublished observations). As shown in figure 2, isoproterenol does not affect Cx43 mRNA levels, suggesting that Cx43 expression is a differentiation related, and not an intracellular cAMP related, event.

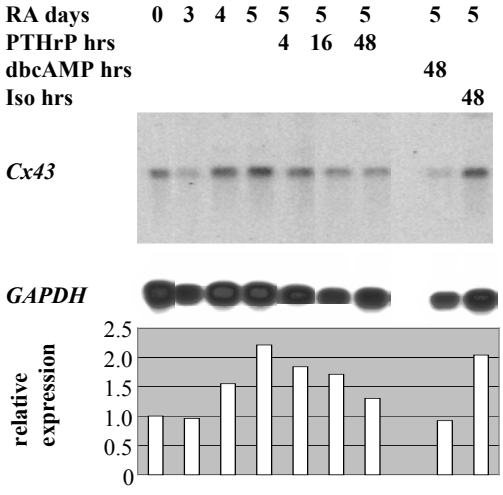


Figure 2: *Cx43* mRNA levels are regulated during F9 cell differentiation.
Northern blot of poly A^+ RNA, hybridized with a *Cx43* probe, stripped and rehybridized with a *GAPDH* probe. The Cx43/GAPDH ratio is depicted graphically.

RA treatment of F9 EC cells results in elevated Cx43 protein level and Cx43 phosphorylation.

To test whether the regulation at the RNA level was followed by an increase in Cx43 protein in RA treated F9 cells, we followed the kinetics by immunoprecipitation and Western blotting (figure 3a). F9 EC cells display predominantly two forms of Cx43, a major band with an apparent molecular weight of 42 kDa, representing non-phosphorylated Cx43 (NP), and a minor band at 46 kDa representing phosphorylated Cx43 (P2). This is in agreement with findings in other cell types (Musil *et al.*, 1990). The P2 form is correlated with plasma membrane localized and coupling competent Cx43 (Musil *et al.*, 1990; Musil and Goodenough, 1991). After three days of RA treatment this pattern starts to change and both NP and P2 bands become denser. Furthermore, a third band, representing another phosphoform of Cx43 (P1) becomes more apparent between NP and P2. The three bands are even more pronounced after 5 days of RA treatment and the P2/NP ratio is increased. Finally, three smaller bands of ~30, ~23 and ~19 kDa are observed, probably representing Cx43 degradation products (Guan and Ruch, 1996). The appearance of degradation products in combination with an increase in the intact protein probably results from an increased Cx43 turnover rate in response to RA treatment.

We next analyzed the protein pattern upon transition from PrE to PE by either a 2-day stimulation with PTHrP or dbcAMP. As shown in figure 3b, RA alone for five days again results in increased levels of Cx43 and in phosphorylation/degradation forms, with a clear increase in the P2/NP ratio. Co-stimulation of RA and PTHrP or dbcAMP during the last two days of the five-day

differentiation protocol, results in a slight decline in protein levels and degradation forms, while the P2/NP ratio clearly decreases. In general dbcAMP is a more potent inducer of PE than PTHrP as reflected by the lower level of Cx43 in cells treated with dbcAMP compared with PTHrP treated cells.

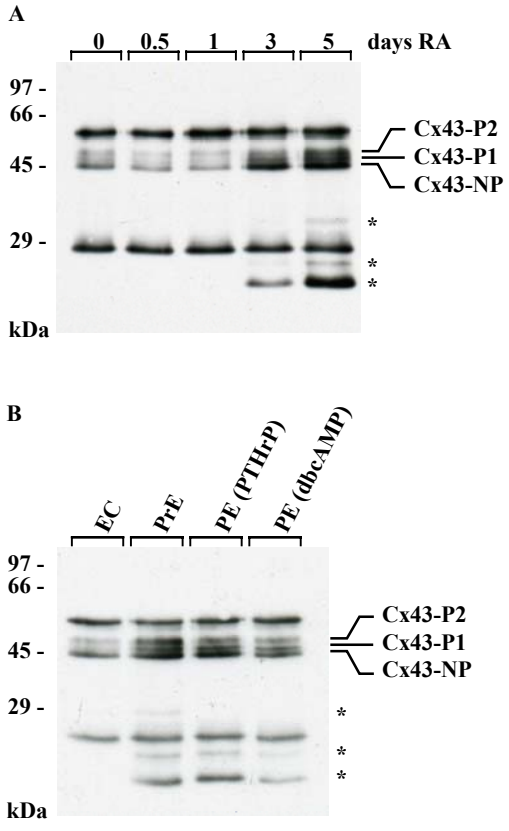


Figure 3: Cx43 protein levels and Cx phophorylated forms are regulated during F9 cell differentiation.

A: Cx 43 was immunoprecipitated from 1 mg of total cell lysate from F9 EC cells stimulated with 10^{-6}M RA for the indicated time points. Precipitated Cx43 was subsequently detected by Western blotting and Cx43 antibody detection. **B**: F9 EC cells were kept undifferentiated or were differentiated to PrE cells with 10^{-6}M RA for five days, or differentiated to PE cells with either RA+PTHrP or RA+dbcAMP. Subsequently, immunoprecipitation was performed as in (A). Positions of non-phosphorylated Cx43 (Cx43-NP) and phosphorylated Cx43 (Cx43-P1, Cx43-P2), and degradation products (*) are indicated at the right, molecular mass markers are indicated at the left. Bands at ~50 and ~25 kDa result respectively from heavy and light chain of the immunoprecipitating antibody.

Endoderm differentiation alters subcellular Cx43 distribution.

We next analyzed the subcellular localization of Cx43 protein in the light of the changes in expression level and phosphorylation described in the previous section. After Cx43 has been produced in the endoplasmic reticulum, its organization into connexons begins in the Golgi apparatus (Musil and Goodenough, 1993). From there intact connexons are transported to the plasma membrane and eventually form gap-junctions with opposing connexons on adjacent cells. After a relatively short period (2-4 hr) the connexins are degraded by proteasomal and lysosomal pathways (Laing and Beyer, 1995; Laing *et al.*, 1997). The distribution of Cx43 in F9 EC, PrE and PE cells in monolayer was examined by CSLM and data were subsequently transformed into pseudo-3D images to distinguish plasma membrane from intracellularly localized protein. As depicted in figure 4, Cx43 is detected in large clusters on and beneath the apical plasma membrane in undifferentiated EC cells. In PrE cells, much more Cx43 is found at the plasma membrane, most often between adjacent cells where it adopts the typical distribution of functional gap-junctions (figure 4). This has been shown previously to correlate with the P2 phosphorylation state of Cx43 in NRK cells (Musil *et al.*, 1990). In PE, either induced by PTHrP or dbcAMP, Cx43 is still present on the plasma membrane, however most of it appears to be internal, possibly already in the degradation pathway (figure 4). Furthermore, the altered morphology of the PE cells is clearly evident, and functional connections between cells are apparent from the typical gap-junctional distribution of Cx43 along the extensions.

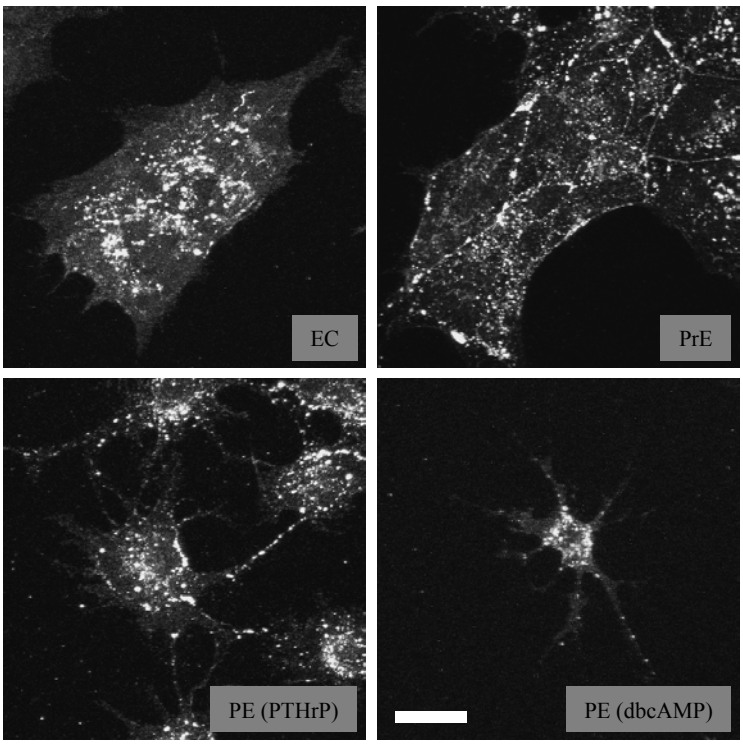


Figure 4: Subcellular Cx43 localization during F9 cell differentiation in monolayer.
Cells were grown on gelatinized polyethylene coverslips in monolayer and differentiated as depicted in figure 1. Cx43 was detected by indirect-immunofluorescence CLSM on EC, PrE and PE differentiated by PTHrP or dbcAMP. Pseudo-3D images are generated to facilitate discrimination between plasma membrane associated and intracellular localized Cx43 and should be viewed with red/green glasses. Bar, 20 μM.

color figure can be seen on page 85.

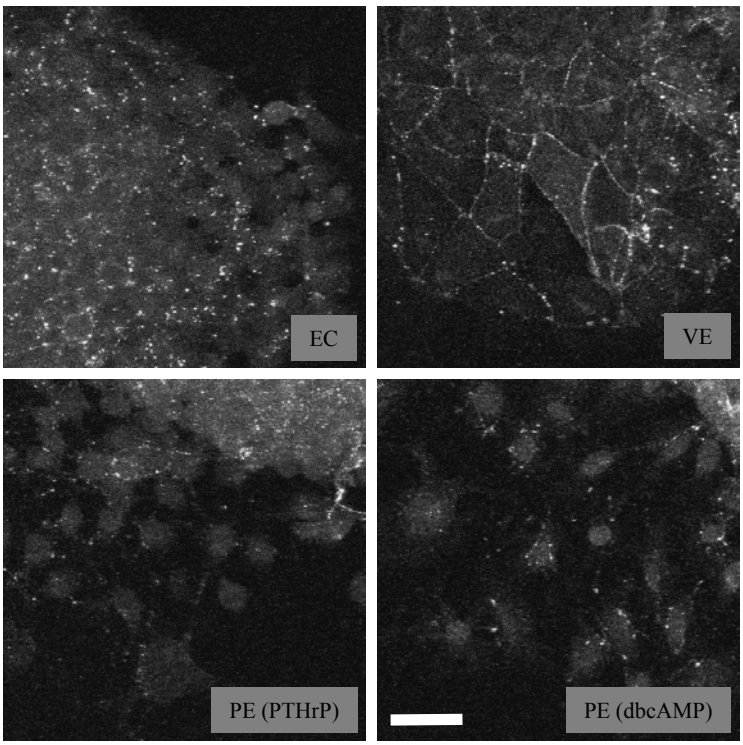


Figure 5: Subcellular localization of Cx43 during F9 cell differentiation in embryoid bodies.
Cells were differentiated as depicted in figure 1. Cx43 was detected and visualized as described in the legend of figure 4. Bar, 40 μM.

color figure can be seen on page 85.

Differentiation of F9 as embryoid bodies (figure 1) more closely resembles differentiation of the ICM/epiblast *in vivo* (Hogan *et al.*, 1981). Although less suited to quantitative biochemical assays than F9 PrE cultures it is amenable to assays comparing single cells within a culture such as indirect immunofluorescence. Embryoid bodies with an outer layer of VE were plated on tissue culture plates in the presence of RA, and then with PTHrP or dbcAMP for one day to form PE (figure 1), were compared with undifferentiated aggregates for the distribution of Cx43. Analysis of fluorescence by CLSM (figure 5), showed that undifferentiated cells growing out the aggregate express Cx43 in small clusters on the plasma membrane with some clusters localized in between adjacent cells. Outgrown VE cells show a more flattened morphology, typical for this cell type in culture: Here Cx43 is localized mainly at the plasma membrane between neighboring cells, with very little Cx43 intracellularly. In PE cells, derived from the embryoid body, clusters of Cx43 are mainly observed in extensions between neighboring cells. Cx43 distribution in embryoid body outgrowths is similar as in PrE and PE derived from monolayer culture, but it appears relatively more confined to the cell-to-cell borders.

F9 PrE/VE and PE are believed to represent the visceral and parietal endoderm respectively of the early post-implantation mouse embryo. To investigate whether the Cx43 distribution in F9 EC cells and their derivatives reflects that of their counterparts *in vivo*, we isolated the visceral and parietal yolk sac (VYS, PYS) from E8.5 mouse embryos, to analyze the expression of Cx43 in VE and PE *in vivo*. As shown in figure 6, Cx43 is localized almost exclusively at the plasma membrane of the VYS endoderm (VE), comparable with the F9 VE. No expression of Cx43 was observed in PE isolated at this stage (data not shown) suggesting that it is not equivalent in this respect to F9 PE.

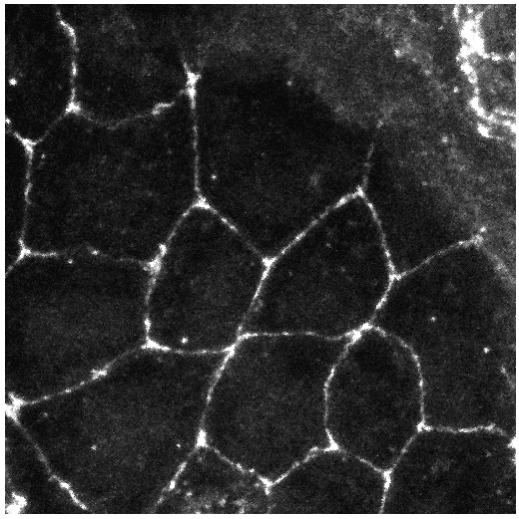


Figure 6: Cx43 localization in isolated mouse visceral yolk sac endoderm.
VYS endoderm was isolated from E8.5 mouse embryos and attached to cover slips. Cx43 was detected by indirect-immunofluorescence microscopy.

Gap-junctional intercellular communication correlates with Cx43 expression patterns.

To determine the extent to which Cx43 expression and subcellular localization relates to GJIC, we performed cellular injections with the gap junction permeable fluorescent dye Lucifer Yellow. In each differentiation state, cells within a cluster of surrounding, physically interacting cells were injected with the dye and spreading of the dye into neighboring cells was quantified after two minutes. As depicted in figure 7 and table I, EC cells in monolayer are well coupled. Coupling increased after the differentiation into PrE cells induced by RA as expected given the increase of Cx43 levels and localization in the plasma membrane at cell-cell contacts (figure 4, 5). Dye coupling

dropped below initial levels when cells were induced to differentiate to PE cells by either PTHrP or dbcAMP; this parallels the observed alterations in subcellular distribution of Cx43 (figure 4). Remarkably, PE cells physically only linked through small extensions to another are still capable of communicating, although the amount of dye flow into adjacent cells was substantially reduced. We found similar results in EC, VE and PE cells emerging from plated F9 embryoid bodies although the absolute number of coupled cells was lower for EC and VE (2- to 3-fold) than in monolayer cultures. Again, the extent of func-

Table I

Differentiation	Number of coupled cells	N
F9 monolayer		
EC	18.3 ± 2.3	17
PrE	32.8 ± 3.2	17
PE (PTHrP)	3.3 ± 0.6	16
PE (dbcAMP	5.4 ± 1.0	16
F9 embryoid body		
EC		
VE	7.1 ± 1.0	10
PE (PTHrP)	17.5 ± 1.8	12
PE (dbcAMP)	5.3 ± 0.6	10
	5.3 ± 0.8	14
mouse embryo		
VE		
PE	19.2 ± 1.6	5
	0.2 ± 0.2	6

Dye coupling in F9 cells and isolated mouse VYS endoderm (VE) and PE.
Chemical coupling in F9 cells is subsequently enhanced and decreased during EC to PrE and PrE to PE differentiation, which is similar to isolated mouse VYS and parietal endoderm. Junctional transfer was indexed by the number of cells to which the fluorescent dye Lucifer Yellow was transferred upon injection of a test cell (mean ± s.d.). The test cells were located in the middle of a cluster of at least 50 cells with similar cell density for all differentiation states. N determines the number of dye injections for each condition.

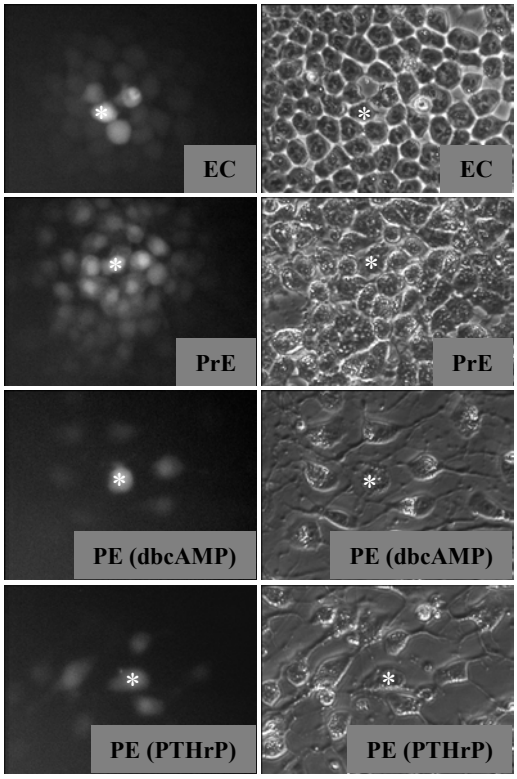


Figure 7: Dye coupling during F9 differentiation.
Cells were grown and differentiated in monolayer as described (fig. 1) resulting in EC, PrE and PE (dbcAMP or PTHrP). One cell (indicated with an asterisk), within a large cluster of cells, was injected with 4% Lucifer Yellow. The dye was allowed to spread for two minutes and was subsequently visualized by epifluorescent illumination (left). Subsequently, phase contrast pictures from the same area were taken (right). F9 differentiation is independent of functional coupling.

Table II

Treatment	Number of coupled cells	N
PrE control	40.8 ± 5.7	6
PrE AGA 1 day	0.0 ± 0.0	7
PrE AGA 2 days	0.0 ± 0.0	6

Dye coupling is blocked by 18α-glycyrrhetinic acid (AGA).
F9 EC cells were differentiated for 5 days with RA in monolayer, with or without 75 µM 18α-glycyrrhetinic acid (AGA) as indicated prior to examining. Control cells were treated with 0.1% DMSO during the last 2 days of the differentiation protocol. Junctional transfer was indexed as in Table I.

tional coupling correlates well with Cx43 localization (figure 5).

We also analyzed dye coupling in VYS endoderm and PE isolated *ex vivo*. VYS endoderm is well coupled (table I) as expected from the Cx43 localization (figure 6). No dye spreading was observed in isolated PE, which in combination with the lack of immunodetectable Cx43 again indicates a functional difference between PE from F9 cells and PE isolated from E8.5 mouse embryos.

In summary, the functional coupling of F9 EC cells, their differentiated derivatives and endoderm isolated from the mouse conceptus at E8.5 correlates remarkably well with Cx43 protein expression and subcellular localization for each cell and tissue, however, EC-derived PE does not reflect all the characteristics of PE *ex vivo*.

It has been reported that in another EC cell line, for instance P19 EC cells, Cx43 mediated coupling is required for proper neural cell differentiation (Bani-Yaghoub *et al.*, 1999), while in other systems, e.g. the lens, Cx43 mediated coupling is not essential for differentiation but is thought to regulate tissue homeostasis (Gao and Spray, 1998). To determine whether the alterations in Cx43 expression and gap-junctional communication were functionally involved in F9 cell differentiation to endoderm or were simply the result of differentiation, we induced F9 EC cells to differentiate in monolayer and as embryoid bodies in the presence and absence of 18α-glycyrrhetinic acid (AGA), a potent inhibitor of gap-junctional communication (Martin *et al.*, 1991). To confirm the inhibitory effect of AGA on GJIC in F9 cells, PrE cells were first generated in monolayer, with AGA present or absent for the last 1-2 days of the differentiation period. Dye spreading between neighboring cells was then measured. Control cells displayed a high level of communication (table II), similar to that found in earlier experiments (table I). In contrast, treatment of cells with AGA for one or two days completely abolished GJIC. However, by morphological criteria (i.e. cell flattening, formation of extensions, extent of contact, cell rounding), inhibition of GJIC by AGA does not alter differentiation of F9 cells into PrE/VE by RA or into PE by PTHrP or dbcAMP, whether the cells were treated in monolayer or as embryoid bodies (data not shown).

We confirmed the lack of effect of AGA on PE differentiation using the PE marker thrombomodulin (TM) (Imada *et al.*, 1987; 1990a, b). TM is only expressed at high levels in F9 EC cells treated with RA and cAMP or PTHrP, and not in F9 cells treated with RA or PTHrP/dbcAMP alone. Thus, PrE differentiation is a first requirement for TM induction in PE cells. F9 EC monolayers were treated with RA for three days, followed by RA, RA+PTHrP or RA+dbcAMP for two days, with or without AGA as indicated. Cell lysates were made and equal amounts of protein were separated by SDS-PAGE and blotted. TM was subsequently detected on Western blot with a thrombomodulin polyclonal antibody. As shown in figure 8, RA alone with or without AGA did not induce TM expression. Treatment with either PTHrP or dbcAMP resulted in TM expression with the strongest response observed with dbcAMP. Inhibition of GJIC during the whole differentiation period or only during the last two days of (PTHrP/dbcAMP) treatment only, did not prevent nor enhance TM upregulation, demonstrating that both PrE (required for subsequent PE formation) and PE differentiation are not blocked by inhibition of GJIC. However, a slight decrease in TM expression was observed

when differentiated with dbcAMP and treated with AGA.

In conclusion, GJIC does not seem to be essential for *in vitro* F9 differentiation into PrE/VE or PE, nor does blockage of GJIC induce or enhance F9 differentiation into either PrE/VE or PE. Therefore, alterations in Cx43 expression, localization and functioning seem rather a consequence of differentiation processes and probably reflect specific, but yet unidentified, aspects of tissue homeostasis of the visceral and parietal endoderm in mouse embryogenesis.

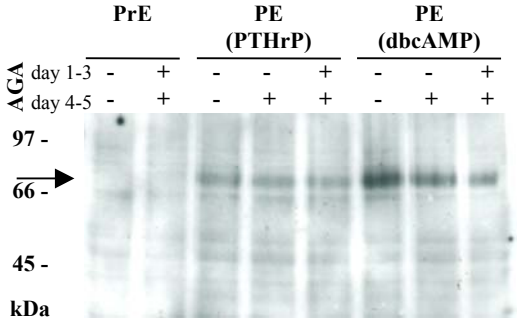


Figure 8: Thrombomodulin expression in F9 PrE and PE cells with or without inhibition of GJIC.

F9 EC cells were differentiated during 5 days in monolayer by RA, PTHrP and dbcAMP as indicated in the absence (-) of presence (+) of 75 µM 18α-glycyrrhetinic acid (AGA) during the whole period or only the last two days. Equal amounts of total cell lysate were separated by 10% SDS-PAGE and subsequently blotted onto Immobilon filter. Thrombomodulin was detected by antibody incubations and enhanced chemiluminisence (ECL) and is indicated by the arrow at the left. Molecular mass markers are also indicated at the left.

DISCUSSION

In this study we have performed a functional analysis of Cx43 during endoderm differentiation in F9 cells. F9 EC cells also express Cx26 and Cx37, while PrE and PE cells express Cx26, Cx32 and Cx37, although at relatively much lower levels than Cx43 (Nishi *et al.*, 1991; Willecke *et al.*, 1991); we could not detect Cx26 protein by immunofluorescence microscopy in either undifferentiated or differentiated F9 cells (data not shown). Therefore, Cx43 appears to be the most abundant and relevant connexin in F9 cells with respect to communication. This assumption is confirmed by our findings that dye spreading correlates well with Cx43 expression and subcellular localization. Since AGA is a general gap-junction blocker, the existence of other connexin types than Cx43 in F9 cells and their potential contribution to cell-cell coupling, does not affect the conclusions on the absence of a role for GJIC in F9 differentiation.

The effect of RA on GJIC and connexin expression is highly dependent upon the cell type. RA treatment of P19 EC cells, syrian hamster embryo cells and human NTera2 cells results in decreased gap-junctional communication, correlated with an altered subcellular localization and/or decreases in Cx43 protein and mRNA level (Rivedal *et al.*, 1994; Bani-Yaghoub *et al.*, 1997; Belliveau *et al.*, 1997). In contrast, Cx43 expression and gap-junctional communication is enhanced by RA in several other cell types, like rat liver epithelial cells, mouse C3H 10T1/2 cells and F9 EC cells by post-transcriptional regulation (Acevedo and Bertram, 1995; Bex *et al.*, 1995; Clairmont *et al.*, 1996). In F9 EC cells, it was found that the Cx43 message is stabilized by RA via elements located in the 3' UTR (Clairmont *et al.*, 1996; Clairmont and Sies, 1997).

Thus far, Cx43 regulation by PTHrP has not been reported. However, a functional analogue of PTHrP, i.e. PTH, has been studied for its role in Cx43 regulation in osteoblast cells. These studies reveal that PTH, and cAMP analogues, induce a rapid redistribution of Cx43 to the plasma membrane, and later in Cx43 gene expression probably via cAMP responsive promoter elements, resulting in a rapid and sustained increase in GJIC in

this cell type (Schiller *et al.*, 1992; Donahue *et al.*, 1995; Schiller *et al.*, 1997; Civitelli *et al.*, 1998). Furthermore, in many different cell types, for example C6 glioma, 3T3, 10T1/2, mouse mammary tumor cells and cardiac myocytes, cAMP analogues also increase GJIC and Cx43 expression (Mehta *et al.*, 1992; Atkinson *et al.*, 1995; Darrow *et al.*, 1996). In contrast, our results show that $PTHrP_{(1-34)}$ and dbcAMP decrease GJIC and Cx43 expression in F9 cells, which might be explained by the assumption that Cx43 regulation in F9 by PTHrP and dbcAMP is an indirect result of the differentiation process rather than a direct effect of the stimuli on Cx43 function. Alternatively, the rapid decrease in Cx43 mRNA upon PTHrP addition (figure 2) suggests a more direct regulation of GJIC by PTHrP.

We show that RA treatment of F9 EC cells results in the increase of Cx43 phosphoforms (figure 3). It has been shown previously that the P2 form of Cx43 is the plasma membrane localized, communication competent Cx43 type (Musil *et al.*, 1990; Musil and Goodenough, 1991). In F9 cells, we also see a strong correlation between P2 increase, Cx43 plasma membrane localization and GJIC consistent with earlier observations in other cell types. Remarkably, we observed the highest, relative increase in the P1 phosphoform upon RA addition. As was shown by Musil and Goodenough (1991), this phosphoform is modified into the P2 form.

We (this study) and others (Kalimi and Lo, 1989; Ruangvoravat and Lo, 1992; Dahl *et al.*, 1996) have shown that mouse VE express high levels of functional Cx43 and are very well coupled. These results are reflected in differentiated F9 cells, where we see the highest functional Cx43 expression in the PrE/VE cell state. We demonstrate that inhibition of GJIC in PrE/VE cells does not prevent or facilitate subsequent differentiation into PE however. From this we conclude that the significance of this high Cx43 expression and GJIC probably resides in a function in PrE/VE rather than in the PrE/VE to PE transition. The functions attributed to VE include nutrient uptake and transport, synthesis and secretion of proteins involved in nutrient transport, and it is the VE that coordinates blood cell differentiation and vessel formation of the adjacent mesoderm in the developing yolk sac (reviewed by Bielinska *et al.*, (1999)). A correlation between some secretion processes, like hormone secretion, and GJIC has been made in several systems. It has been shown for instance, that GJIC via Cx43 plays a critical role in the production and secretion of insulin (Vozzi *et al.*, 1995). GJIC modulates enzyme secretion by regulating calcium oscillations (Stauffer *et al.*, 1993). Furthermore, expression of Cx43 correlates with hormone production and secretion in human and baboon corpora lutea (Khan-Dawood *et al.*, 1996) and, luteinizing hormone-releasing hormone secretion in GT1-7 cells correlates with dye coupling (Matesic *et al.*, 1997). In the regulation of secretion, GJIC is thought to synchronize cell clusters to regulate and stimulate pulsative secretion of hormones and digestive enzymes in different tissues and organs. This, still unknown, mechanism could also provide means to regulate or optimize metabolite passage by VE.

Another system in which gap-junctions play an essential role in metabolite/nutrient exchange is found in the lens. Like the early embryo, the lens is devoid of a direct blood supply, and thereby totally relies on metabolite exchange to its numerous gap-junctions. The lens epithelial cells confine the

border between inner lens fiber cells and the extraocular space. These lens epithelial cells express relatively high amounts of Cx43 in various vertebrates. A Cx43 null mutation results, amongst many other defects, in cataract formation in the adult lens nucleus, probably due to osmotic imbalance, while embryonic lens differentiation is not affected (Gao and Spray, 1998). Thus, in analogy to the lens system, a role for GJIC in VE could be found in nutrient and metabolite exchange between the early embryo and uterus, rather than in governing differentiation as such.

We found a good correlation between our results obtained with the F9 model system and with isolated mouse VE and PE with respect to Cx43 localization and function. Although F9 PE already expresses less functional Cx43 than its precursors PrE and VE, these F9 PE cells are still dye coupled in contrast to PE derived from the mouse conceptus. It could be reasoned that, due to the absence of some factors in the culture medium, the *in vitro* derived PE is still not fully differentiated resulting in a residual Cx43 expression and communication. On the other hand, we cannot exclude that *in vivo* PE cells are still ionically coupled and cell-cell communication does play a role in PE function.

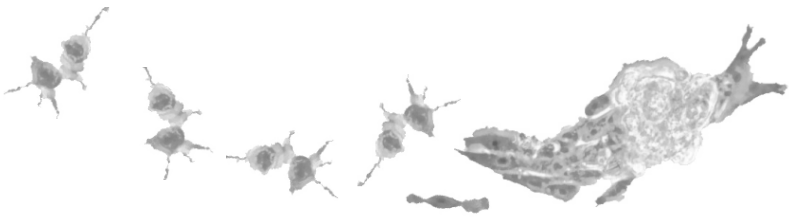
In conclusion, we demonstrate that GJIC in F9 EC cells can be differentially regulated, increased and decreased, dependent upon its differentiation state but has no obvious effect on the differentiation process itself. From this we hypothesize that GJIC in VE probably acts in metabolite and nutrient exchange between the early embryo and the endometrium. Furthermore we are the first to show that F9 differentiation to PE by its natural ligand PTHrP is accompanied by changes in GJIC and that this is correlated with differential expression of Cx43 mRNA and protein levels and subcellular localization. Finally, the observations made in the F9 model system are similar, but not identical, to isolated mouse VYS endoderm and PE.

ACKNOWLEDGMENTS

We thank M. Rook and H. Jongsma for using their dye injection equipment and advice and C. Mummery for careful reading of the manuscript. This research was supported by the Earth and Life Sciences Foundation (ALW), which is subsidized by the Netherlands Organization for Scientific Research (NWO).

CHAPTER 4

SNAIL *IS AN IMMEDIATE EARLY TARGET GENE OF PARATHYROID HORMONE RELATED PEPTIDE SIGNALING IN PARIETAL ENDODERM FORMATION*



Jacqueline M. Veltmaat, Claudia C. Orelio[1], Dorien Ward-van Oostwaard, Marga A. van Rooijen, Christine L. Mummery, Libert H.K. Defize

Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands. [1] Current address: Department of Cell Biology and Genetics, Erasmus University, PO Box 1738, 3000 DR, Rotterdam, The Netherlands.

The International Journal of Developmental Biology 44 (3): 297-307, (2000).

4

SNAIL *IS AN IMMEDIATE EARLY TARGET GENE OF PARATHYROID HORMONE RELATED PEPTIDE SIGNALING IN PARIETAL ENDODERM FORMATION*

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 I PTH/PTHrP Receptor (PTH(rP)-RI)* 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)-RI* 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.

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 I PTH/PTHrP-receptor (here referred to as PTH(rP)-RI) (Verheijen *et al.*, 1999). The complementary distribution of mRNA and protein of both *PTHrP* and *PTH(rP)-RI* 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)-RI 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, 1997). However elevation of

intracellular cyclic AMP (cAMP) levels alone, by using the cell permeable analogue dibutyryl cAMP (dbcAMP), is sufficient to induce complete transition to PE-like cells (Strickland *et al.*, 1980).

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 (i) it is expressed earlier and more widely in mouse development than *Slu,* (ii) *Slu* and *Sna* expression overlap in late development, (iii) 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 (iv) *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 trophec-

toderm (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 extracellular matrix which form the Reichert's membrane (Hogan *et al.*, 1980; Smith and Strickland, 1981; Hogan *et al.*, 1982; Kurkinen *et al.*, 1983; Mason *et al.*, 1986), are motile as indicated by their filopodia, and lack E-cadherin expression (Vestweber and Kemler, 1984). 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 I).

Table I

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	√

Characteristics of epithelial and mesenchymal cells and their display in PrE, VE and PE.
Abbreviations: PrE: primitive endoderm, VE: visceral endoderm, PE, parietal endoderm, ECM: extra-cellular matrix. (Modified from Verheijen and Defize, 1999).

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)-RI* 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* homologues 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.

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 1mM 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 minutes 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*., 1981). 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 petridish, 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) 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 20 bp long, with 50% GC content. The reaction was electrophoretically separated 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 γ[^{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 electrophoretically separated 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 EcoR1 insert from the PCR2.1-TOPO vector, or the EcoR1-BglII 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/:g DNA, using the Redivue kit and α[^{32}P]dCTP (both from Amersham). Hybridization with a 1.4 kb Pst1 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/6 x CBA 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 gelatinized 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 seeDNA (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 II. 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 :l) 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 µ:M 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)-RI* 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)-RI* and *follistatin* 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 (ISH)

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 Sac1-EcoR1 3' UTR fragment of clone 5-15 (described above), and high stringency washing at 65°C, as described (Feijen *et al*., 1994).

Table II

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)-RI	forward	GCAGAGATTAGGAAGTCTTGGA	
PTH(rP)-RI	reverse	AGCCGTCGTCCTTGGGAACTGT	280 with forward
follistatin	forward	atacggatccTTTTCTGTCCAGGCAGCTCCAC	
follistatin	nested reverse	ataggaattcACTTACTGTCCGGGCACAGCTCA	320 with forward
follistatin	reverse	ataggaattcGTCAACACTGAACATTGGTGG	545 with forward

Primers used in plateau-phase and semi-quantitative RT-PCRs.
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 (#).

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, figure 1). Sequence analysis revealed that this fragment of 300 bp was 98% homologous to part of the zinc finger domain of *Sna* (bp 536-836, 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 F9EC cells, but clearly visible in F9PrE and PE (figure 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* (I - mada *et al.*, 1990a, b), and a more profound rounded up morphology after 48 hr. treatment (figure 1, top panel). This is also reflected in *Sna* transcription.

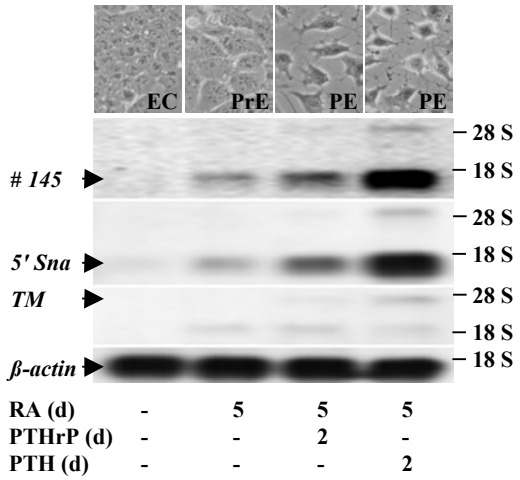


Figure 1: Differential expression of *Sna* during F9 differentiation.
The top panel shows microphotographs of F9 cells in their various differentiation stages as a result of the treatment indicated at the bottom. Below the microphotographs are Northern blot results of poly-A$^+$ RNAs of differentiating 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.

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 (figure 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 fig. 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 hours 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 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 hours 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.

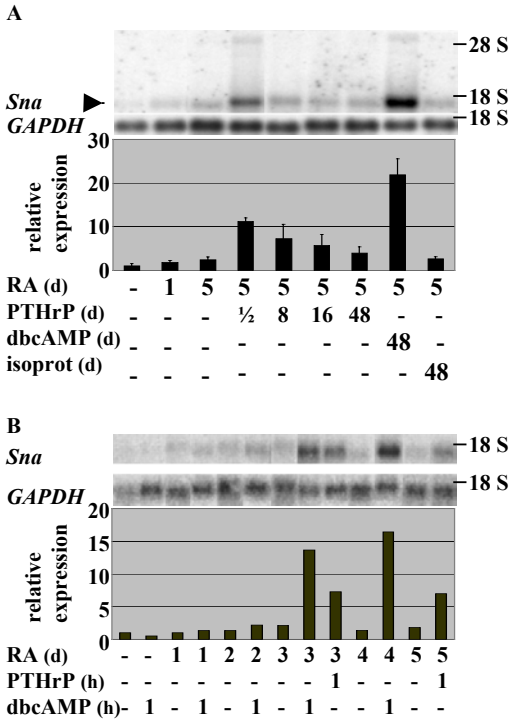


Figure 2: Rapid induction of *Sna* by PTHrP in F9 PrE cells, specific for differentiation to PE.

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 fig. 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 ½ to 48 hours 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 hr with PTHrP or dbcAMP.

In contrast, in F9 PrE cells treated for 48 hours with isoproterenol, *Sna* mRNA levels are comparable to those in cells treated with RA alone (figure 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 hour treatment with dbcAMP. Addition of dbcAMP for 1 hour 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)-RI* (van de Stolpe *et al.*, 1993). In these cells, PTHrP also effectively raises *Sna* levels (figure 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 (figure 2A, 3).

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

The capacity of PTHrP to induce *Sna* transcription within 30 minutes raised the question whether *Sna* is an immediate early target of PTH(rP)-RI sig-

naling and may thus be a key element in PE formation. To address this, we treated F9 PrE for 30 minutes or 2 hours with PTHrP in the absence or presence of cycloheximide or with cycloheximide alone. Cycloheximide was added 30 minutes prior to PTHrP addition, in order to block all protein synthesis. Northern blot analysis (figure 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)-RI signaling. Results are shown for 2 hours of PTHrP treatment only, and were similar in two other experiments in which cells were treated with PTHrP during 30 minutes.

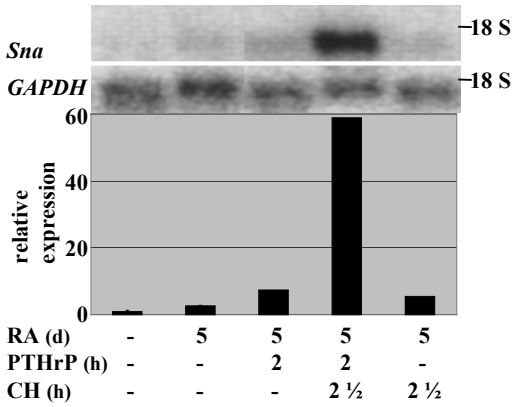


Figure 3: *Sna* is an immediate early target of PTHrP in F9 PrE cells.
Northern blot of poly-A$^+$ RNA of F9 EC, and PrE with or without induction with PTHrP for 2 hours, hybridized with the 5' *Sna* probe and *GAPDH* as a loading control. Abbreviations used are as in fig. 1. Cycloheximide (CX, 20 µg/ml) was added to the cells 30 minutes 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.

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 extracellular 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, diffe-

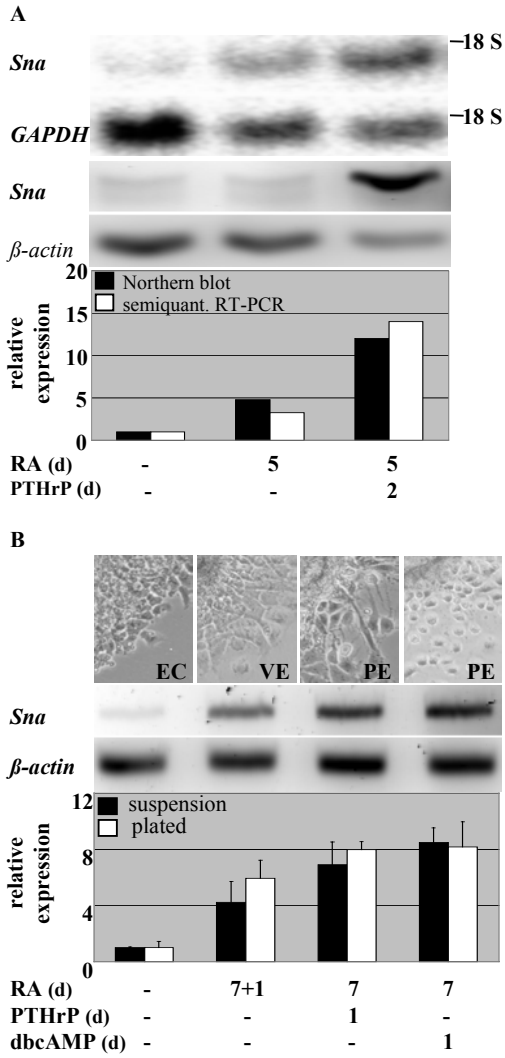


Fig. 4: *Sna* is upregulated by PTHrP in F9 embryoid bodies.

Abbreviations used are as in figs. 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 *ß-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 *ß-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, $p<0.05$).

rentiation 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 (figure 4B, white bars). However, this increase is 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)-RI 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 *ß-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-sided Student's t-test, $p<0.05$). Expression of *PTH(rP)-RI* and *follistatin* was also analyzed in the same samples. *PTH(rP)-RI* 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-sided 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)-RI*, suggestingthat *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 transcrip tion at earlier developmental stages. In an approximate equivalent of 1 embryo, *Sna* was detected in the zygote, at the 2-cell stage and beyond (figure 6A). The top row of figure 6a shows the results of a first round PCR 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 embryos at the 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 (figure 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 (figure 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.

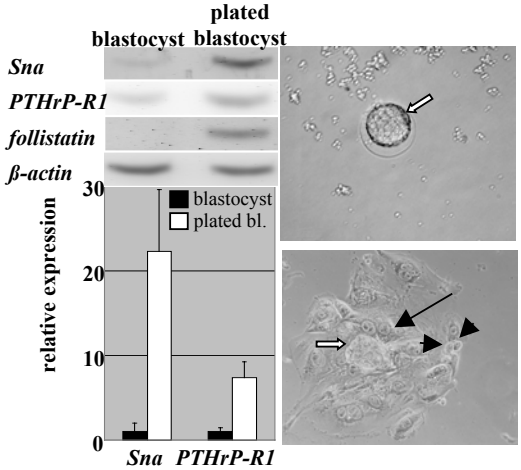


Figure 5: _Sna_ is upregulated in plated blastocysts.
RNA was extracted from blastocysts directly or several days after plating, and analyzed for *Sna*, *PTH(rP)-R1*, and *follistatin* mRNA expression relative to *β-actin* expression by semi-quantitative RT-PCR. Each PCR was repeated 3 times independently. 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.

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 (figure 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 (figure 6B, e, 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 (figure 6B, i, j, k, and 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: figure 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)-RI) 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)-RI 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).

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.

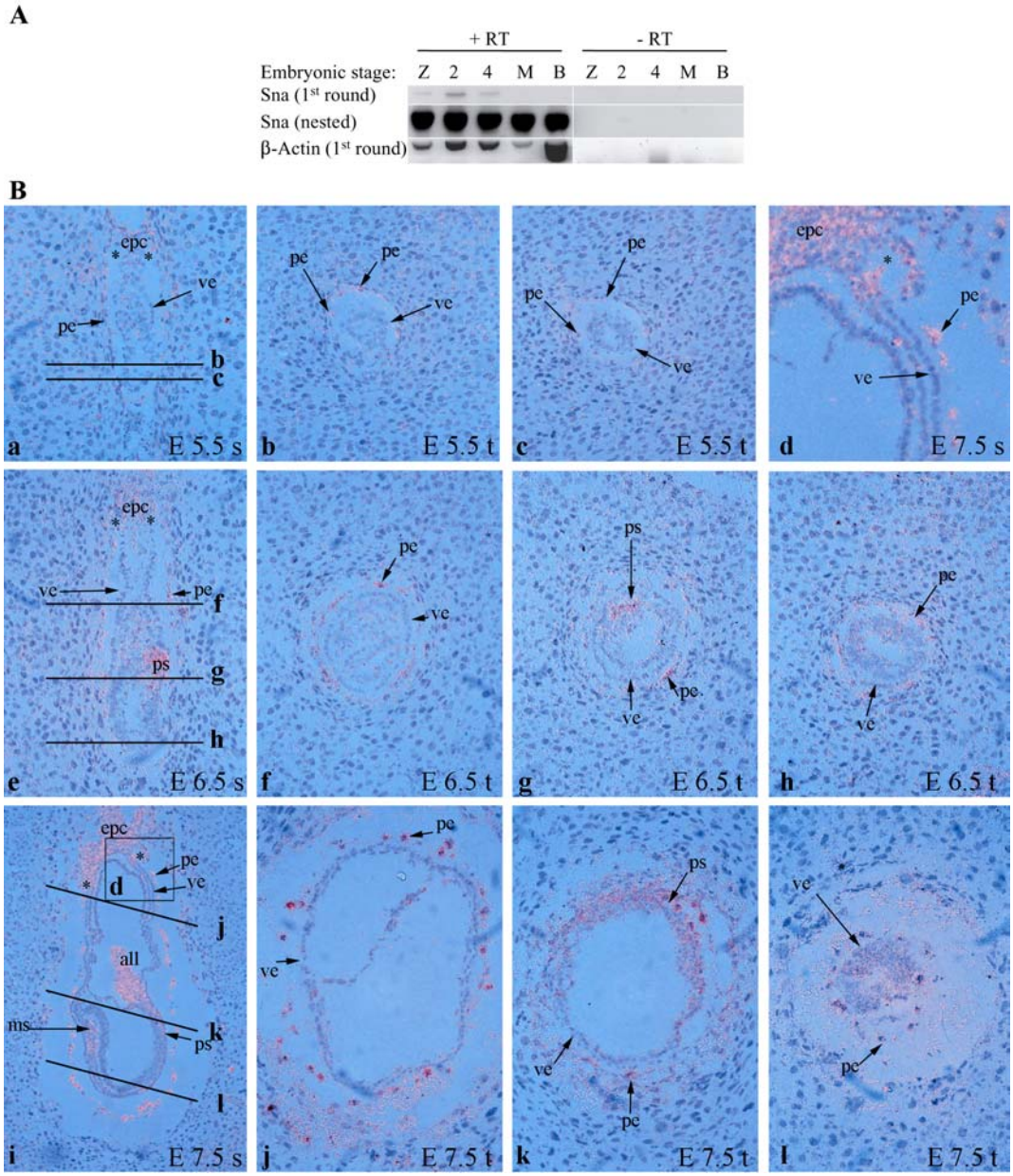


Figure 6: *Sna* expression during early mouse gestation.
A: Amplification products of a plateau-phase first round and nested PCR for *Sna* and a first round plateau phase PCR for *β-actin*. RNA was isolated from mouse embryos at zygotic (Z), 2 cell (2), 4 cell (4), morula (M) and blastocyst (B) stage. Three independent pools of 20 embryos were analysed 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).

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 gactor (bFGF) and the activin A homologue 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 hours, 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 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 hours. 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 seems 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)-RI 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 periments showed significant upregulation of *Sna* concomitant with the upregulation of *PTH(rP)-RI* 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; Fuse *et al.*, 1994, 1996; Hayashi, 1996) and for mouse *Sna* in trophoblast giant cells (Nakayama *et al.*, 1998). Alternatively, it might control programmed cell death, similar to the *C. elegans* snail family member *ces-1* (Metzstein and Horvitz, 1999) and human *SLUG* (Inukai *et al.*, 1999). 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)-RI* mRNA and protein at E5.5 and E6.5 is detected in PE and extraembryonic VE, most prominently in the marginal zone (Karperien *et al.*, 1994; Verheijen *et al.*, 1999). At E7.5, *PTH(rP)-RI* mRNA and protein expression is low in VE, but prominent throughout PE. From this stage, PTH(rP)-RI protein expression in the extraembryonic 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)-RI.

The way by which PTHrP, acting through the PTH(rP)-RI, 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.

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 (Cano *et al.*, 2000; Batlle *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.

ACKNOWLEDGMENTS

This study was carried out at the Graduate School for Developmental Biology, Utrecht, The Netherlands. The authors wish to thank Dr. Gridley for kindly providing the *Sna* cDNA clone 5-15, Dr. A. Caricasole, A. Feijen, E. de Groot, J. Hendriks, Dr. H. Kester, and Dr. B. Roelen for technical assistance and helpful discussions. We also thank T. Hoeijmakers for sequence analysis, J. Korving for sectioning the embryos, F. Vervoordeldonk for photographic reproduction, E. de Groot for sharing *follistatin* data before publication, and Drs. M. van der Heyden and A.J.M. van den Eijnden-van Raaij for critically reading the manuscript.

Color figures of page 59

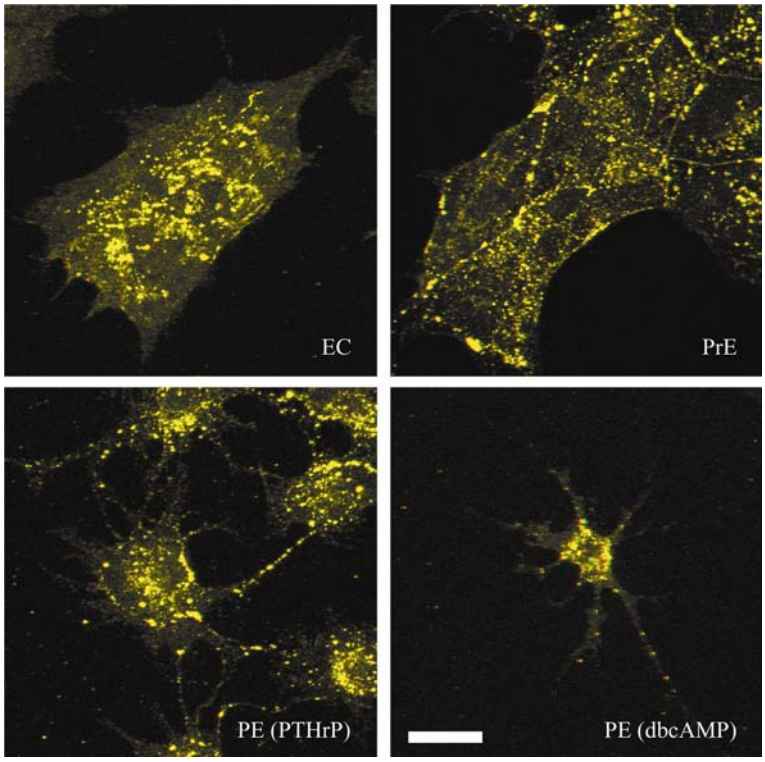


Figure 4: Subcellular Cx43 localization during F9 cell differentiation in monolayer.
Cells were grown on gelatinized polyethylene coverslips in monolayer and differentiated as depicted in figure 1. Cx43 was detected by indirect-immunofluorescence CLSM on EC, PrE and PE differentiated by PTHrP or dbcAMP. Pseudo-3D images are generated to facilitate discrimination between plasma membrane associated and intracellular localized Cx43 and should be viewed with red/green glasses. Bar, 20 µM.

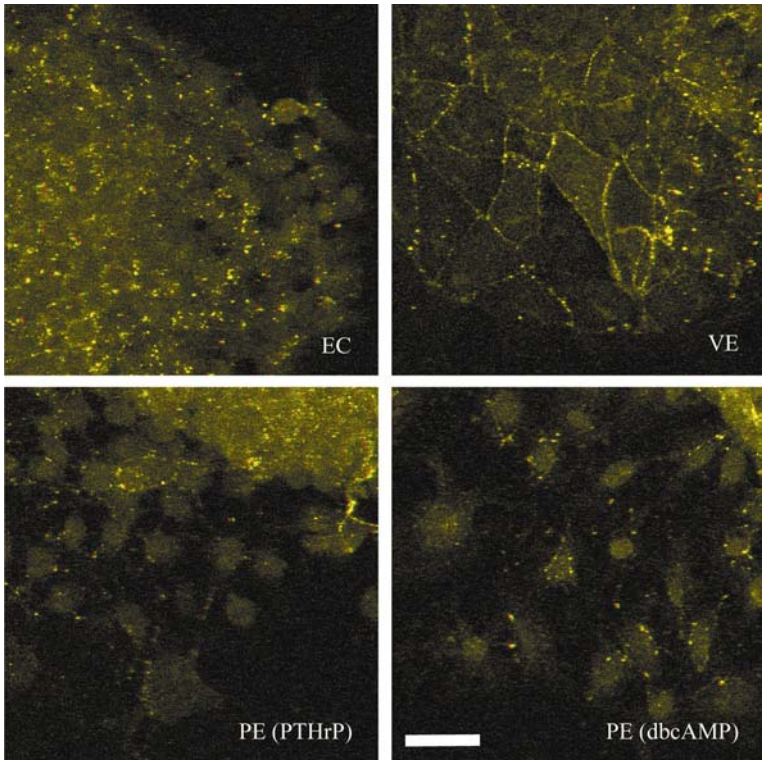


Figure 5: Subcellular localization of Cx43 during F9 cell differentiation in embryoid bodies.
Cells were differentiated as depicted in figure 1. Cx43 was detected and visualized as described in the legend of figure 4. Bar, 40 µM.

CHAPTER 5

SNAIL *INDUCES MESENCHYMAL CHARACTERISTICS DURING PARIETAL ENDODERM FORMATION*

Jacqueline M. Veltmaat, Alie Feijen, Beate Lanske[1], Jean-Paul Thiery[2], Libert H.K. Defize

Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8 3584 CT Utrecht, The Netherlands. [1]Molecular Endocrinology, Max-Planck Institute for Biochemistry, Am Klopferspitz 18a, 82152 Martinsried, Germany. [2]UMR 144 Morphogenèse Cellulaire et Progression Tumorale, Institut Curie, Section de Recherche, 26 Rue d'Ulm, 75248 Paris Cedex 05, France.

manuscript in preparation

SNAIL *INDUCES MESENCHYMAL CHARACTERISTICS DURING THE FORMATION OF PARIETAL ENDODERM*

ABSTRACT

Previously, we have demonstrated that the proper formation of parietal endoderm (PE) in the mouse occurs via an epithelio-mesenchymal transition (EMT), which requires the action of parathyroid hormone related peptide (PTHrP) via its type I PTH/PTHrP receptor. Using differentiating F9 embryonal carcinoma cells as an *in vitro* model for PE formation, we have also identified the transcription factor *snail (Sna*) is an immediate early target gene of PTHrP in this process. Again using the F9 model system, we here demonstrate that high levels of exogenous *Sna* expression can replace PTHrP in evoking the dissociation of adherens junctions, as well as the migration, dispersion, and rounding off of the cells that normally occur during PE formation. Additionally, we show that the distal PE cells in E7.5 PTH(rP)-RI knockout embryos, which fail to acquire the mesenchymal morphology and PE-specific differentiation, lack *Sna* expression, while the proximal cells still express *Sna*. We propose a pivotal role for *Sna* in the PTHrP induced EMT during PE formation.

INTRODUCTION

Epithelium and mesenchyme constitute the two extreme phenotypes of tissue organization. Epithelial cells can transform into mesenchymal cells by a so-called epithelio-mesenchymal transition (EMT), a process in which cells undergo drastic morphological changes. These involve the downregulation of e.g. adherens junctions and a re-organization of cytoskeletal structures. In addition, a switch in integrin expression, the assembly of focal adhesion plaques, and an increased production of matrix components occur. This leads to changes in adhesion to the extracellular matrix, and is usually accompanied by increased migration. As a result of EMT, cells individualize while dissociating from the epithelial sheet (Hay, 1995; Birchmeier *et al.*, 1996; Boyer *et al.*, 1996; Davies and Garrod, 1997). This process is recurrently used during embryonic development of many species to establish the complex three dimensional body plan, and it also underlies the invasive/metastatic behavior of epithelial tumors (Thiery and Chopin, 1999). As outlined previously (chapters 1 and 2) we consider the formation of the extra-embryonic tissue parietal endoderm (PE) from its precursors primitive endoderm (PrE) and visceral endoderm (VE) during mouse embryonic development an EMT, which requires signaling by parathyroid hormone related peptide (PTHrP) via its type I receptor (PTH(rP)-RI) (chapter 2 of this thesis).

In the last several years, expression of family members of the *snail* (*Sna*) zinc finger transcription factors has been found in various species in mesenchymal cells, and in cells undergoing an EMT (Nieto *et al.*, 1992; Smith *et al.*, 1992; Essex *et al.*, 1993; Hammerschmidt and Nusslein-Volhard, 1993; Thisse *et al.*, 1993; Mayor *et al.*, 1995; Thisse *et al.*, 1995; Jiang *et al.*, 1998b; Savagner *et al.*, 1998; Sefton *et al.*,

1998; Romano and Runyan, 1999). It is conceivable that they mediate a reduction of cell-cell contacts during EMT, since experimentally induced high expression levels of the orthologues *slug* and *Sna* lead to the dissociation of the desmosomes and adherens junctions respectively in cultured cells (Savagner *et al.*, 1997; Cano *et al.*, 2000; Batlle *et al.*, 2000). It has been shown that Sna exerts this effect by evolutionarily conserved binding to E-boxes (Kasai *et al.*, 1992; Mauhin *et al.*, 1993; Giroldi *et al.*, 1997) that are present in the promoter of the human and mouse *E-cadherin* gene (Cano *et al.*, 2000; Batlle *et al.*, 2000). Ectopic expression of *Sna* in epithelial (carcinoma) cells leads to repression of *E-cadherin* transcription. This is followed by the dissociation of adherens junctions and acquisition of a migratory, invasive phenotype, and increased tumorigenic potential of these cells when injected into nude mice (Cano *et al.*, 2000; Batlle *et al.*, 2000). Although slug, an orthologue of Sna, can bind to the same E-boxes in the mouse, *E-cadherin* transcription seems to be regulated specifically by Sna, given the mutually exclusive expression patterns of *Sna* and *E-cadherin* mRNA during tumorigenesis and embryonic development, while *slug* and *E-cadherin* partially overlap (Cano *et al.*, 2000; Batlle *et al.*, 2000). These findings suggested a conserved role for *Sna* in inducing EMTs during tumor progression and embryonic development.

We have previously shown that membrane-localized expression of E-cadherin is lost during the PTHrP-induced EMT that underlies PE differentiation (chapter 2 of this thesis). Moreover, we have demonstrated *in vitro* that *Sna* is an immediate early target of PTHrP in the formation of PE (Veltmaat *et al.*, 2000), and that it is upregulated early during PE formation *ex vivo* and *in vivo*. Therefore, we asked here whether elevated *Sna* expression is sufficient to induce aspects of this PTHrP-induced EMT. We studied this *in vitro* by using F9 embryonal carcinoma (EC) cells, differentiated to PrE or VE-like cells, the epithelial precursors of PE-like cells. We demonstrate that overexpression of *Sna* in these cells was sufficient for the loss of adherens junctions, as well as for cell migration, cell dispersion and the rounded morphology as also seen as a result of PTHrP treatment. In addition, we show that in the distal PE cells of E7.5 mouse PTH(rP)-RI -/- embryos, which fail to undergo differentiation to a mesenchymal cell type and lack the PE-specific expression of thrombomodulin, *Sna* expression is absent. We propose that *Sna* plays a pivotal role in mediating the EMT that is induced by PTHrP during PE formation.

MATERIALS AND METHODS

Cell culture and differentiation

F9 embryonal carcinoma (EC) cells (ATTC) were cultured and differentiated as described before (Veltmaat *et al.*, 2000). In short, to obtain primitive endoderm-like cells (PrE), cells in monolayer were treated for 3 days with 10^{-6} M *all trans* retinoic acid (RA). To obtain visceral endoderm-like cells (VE), cells were aggregated in suspension cultures in bacterial dishes in presence of $5x10^{-8}$ M RA for 7 days, giving rise to so-called embryoid bodies with a core of undifferentiated cells surrounded by VE. F9 PrE and VE were differentiated to parietal endoderm-like cells (PE) by subsequent treatment with 10^{-7} M $PTHrP_{1-34}$ (PTHrP) for 1 or 2 days.

Transient transfections

For transient transfections, F9 PrE cells were trypsinized and seeded in a density of 25000 cells/cm^2 on gelatinized polyethylene coverslips (Sarstedt) in 24 well clusters. The following day, they were transfected with 375 ng pREP7-*Sna* (a generous gift of Dr. J. Cross) or empty vector, and 125 ng pREP7-*Green fluorescent protein* (GFP), by lipofectamin (Life Technologies) according to the manufacturer's protocol. The second day after transfection, they were used for immunohistochemistry.

Immunohistochemistry on transient transfectants

At the second day after transfection, cells were washed in PBS (phosphate buffered saline supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$) at 37°C cells and fixated mildly in 2-4% paraformaldehyde in PBS at room temperature during 10 minutes. Quenching, permeabilization, antibody incubations and analysis by confocal laser scanning microscopy were carried out as described in chapter 2 of this thesis.

Generation of semi-stable cell lines

For semi-stable *Sna* expression, F9 EC cells were first stably transfected with p-βAct-tTA-neo. This construct expresses the tetracycline repressible transactivator (tTA) under the control of human β-actin promoter sequences, and the neomycin resistance gene from an independent promoter. Clones resistant to 400 µg/ml G418 (GibcoBRL) were tested for expression of tTA, and the regulation of its activity by tetracycline, by transient transfection with pUHD16-3, expressing the *β-galactosidase* gene under the control of the minimal CMV promoter preceded by tet-operon sequences. Clone 621 stably expressing tTA was co-transfected with pTRE-HA-*Sna* (*Sna* fused to the haemagglutinin tag, a generous gift from Dr. Garcia de Herreros) or the mock construct, empty pUHC10-3 (Gossen and Bujard, 1992), which both contain the minimal CMV promoter preceded by tet-operon sequences. Either of these constructs was co-transfected with pPGK-*Hyg* (expressing the hygromycin resistance gene) and pPGK-*GFP* in a 2:1:2 molar ratio, using 10 µg DNA per 10 cm diameter dish with 50 µl Superfect (Qiagen) according to the manufacturer's protocol. Per transfection, 3 independent pools of hygromycin resistant clones were generated and used for RT-PCR and aggregation assays. From transfection onwards, pools were expanded in the presence of 1µg/ml tetracycline (Sigma) to suppress expression from pTRE-HA-*Sna,* and 400 µg/ml G418 (GibcoBRL) and 200 µg/ml hygromycin (Calbiochem).

Analysis of transfection efficiency and regulation of expression by semi-stable cell lines

After sufficient expansion of the pools, each culture was trypsinized and seeded simultaneously for aggregation assays (see below) and expression analysis by epifluorescence microscopy and semi-quantitative RT-PCR. For the latter two, cells were seeded in triplicate in monolayer in presence of RA to generate PrE-like cultures during 3 days. One culture of each line was analyzed by epifluorescence microscopy for GFP expression. GFP was found in each line in at least 50% of the cells, suggesting that also at least 50% of the cells contained the *snail* or mock construct. The corresponding cultures of each line were washed 3 times with PBS with or without tetracycline, and refreshed with medium with or without tetracycline for 6 hours, harvested and assayed for relative *Sna* expression levels by semi-quantitative RT-PCR analysis using the *β-actin* primerset and nested primerset for *Sna* as described before (Veltmaat *et al.,* 2000).

Aggregation assay of semi-stable cell lines

Cells were aggregated during 7 days in presence of RA, G418, hygromycin and tetracycline to generate embryoid bodies, and medium was replaced once during this period. These embryoid bodies were re-plated on gelatinized tissue culture plastic with fresh medium, in absence or presence of tetracycline and absence or presence of PTHrP and grown for 1 day. Photographs were taken and used to analyze migration (by measuring the average outgrown distance per aggregate as the distance of the cell farthest away from the aggregate in 8 directions with 45° intervals), cell dispersion (by determining the total number of outgrown cells, and the percentage of outgrown cells with less than 20% contact with neighboring cells, which is an indication for differentiation to PE (Behrendtsen *et al.*, 1995; Roelen *et al.*, 1998), and the morphology of cells, as parameters that are affected during PTHrP-induced PE formation. Only 1 mock cell line (M1) and all three sense Sna cell lines (S1, S2, and S3) were used for these experiments. By one-way ANOVA analysis, S1, S2, and S3 were found not to differ from each other for outgrown distance, nor number of outgrown cells, nor the percentage of mesenchymal cells in each culture condition (two-sided, $p>0.05$), except for a significant lower percentage of mesenchymal cells in S1 compared to S2 in the presence of tetracycline and absence of PTHrP. S1 showed a significant increase in the percentage of mesenchymal cells upon withdrawal of tetracycline (and absence of PTHrP), to the level of S2 (no S3 data available for this culture condition). We therefore pooled S1, S2 and S3 to S in all conditions except for the + tet/- PTHrP condition, and subsequently tested M1 and S pair-wise per culture condition by one-way ANOVA for differences in outgrown distance, total number of outgrown cells and the percentage of mesenchymal cells in the absence or presence of tetracycline or PTHrP (two-sided, $\alpha=0.05$).

In Situ Hybridization (ISH)

PTH(rP)-RI heterozygous mice (Lanske *et al.*, 1996) in a C57BL/6 x CBA genetic background were intercrossed. Midday of the day of appearance of a vaginal plug was designated E0.5, and embryos were dissected in decidua at E7.5 (Hogan *et al.*, 1994), embedded in paraffin, and sectioned sagittally. Per litter, 2 central sections of each embryo were collected on one slide to perform a genotyping *in situ* hybridization for expression of the PTH(rP)-RI,

while the remaining sections were used for i*n situ* hybridization for *Sna* expression. Probes and protocols were as described before (Karperien *et al.*, 1994; Veltmaat *et al.*, 2000).

RESULTS

Sna induces the dissociation of adherens junctions.

Previously, we have shown that PTHrP signaling is required for the EMT that occurs during the differentiation of PrE and VE to PE in mouse embryos (chapter 2 of this thesis). Using differentiating F9 EC cells as an *in vitro* model for PE formation, we have demonstrated that PTHrP is sufficient to induce the disintegration of adherens junctions during this EMT, as illustrated by the loss of membrane-localized expression of E-cadherin and β-catenin. We have also Identified the transcription factor *Sna* as an immediate early target gene of PTHrP action in this process (Veltmaat *et al.*, 2000). Since *Sna* has been shown to downregulate E-cadherin in epithelial carcinoma cells (Cano *et al.*, 2000; Batlle *et al.*, 2000), we now asked whether *Sna* can exert a similar function during PE formation from F9 PrE.

Therefore, we transiently transfected monolayer cultures of F9 PrE cells with pREP7-*Sna* (Nakayama *et al.*, 1998), or an empty vector (mock), together with pREP7-*green fluorescent protein* (GFP) to detect the transfected cells. We analyzed membrane-localized expression of E-cadherin and β-catenin two days after transfection. Non-transfected and mock transfected cells expressed E-cadherin (figure 1A) and β-catenin (figure 1B) mostly in a continuous line along the membrane (black area in left bar in figure 1E, F). Since the F9 PrE cultures were subconfluent at the time of analysis, we always found cells that were only partially surrounded by adjacent cells, or completely solitary. Even in the latter cells, membrane-localized expression of E-cadherin and β-catenin was still present, although mostly discontinu-

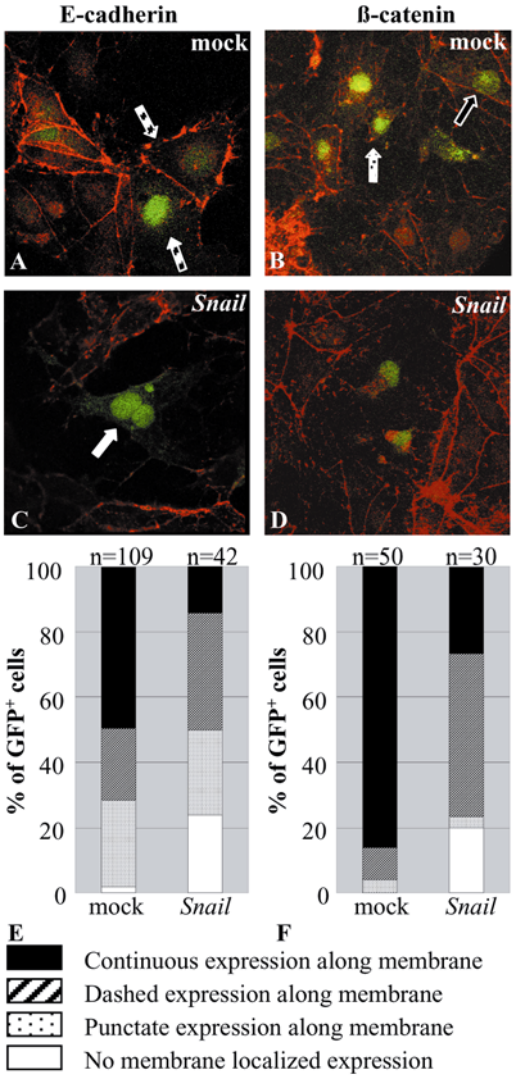


Figure 1: *Snail* downregulates the membrane-localized expression of E-cadherin and β-catenin.
The left column shows immunoreacitivity for E-cadherin in F9 PrE cells co-transfected with *green fluorescent protein* (GFP) and an empty vector (mock) (A), or a *Sna*-expressing vector (C). The right column shows immunoreactivity for β-catenin in similarly co-transfected cells: mock (B), or *Sna* (D). The bar diagrams represent the extent of membrane-localized expression of E-cadherin (E) or β-catenin (F) of GFP positive cells in mock or *Sna* co-transfected cultures. Membrane-localized expression was classified into 4 categories, being continuous (black bars and arrows), dashed (hatched bars and arrows), punctate (dotted bars and arrows) or absent (white bars and arrows). See also text.

ous,as a dashed or punctate line (hatched and dotted arrows and bar areas respectively in figure 1). There was a strong reduction of membrane-localized expression of E-cadherin and β-catenin in GFP-positive cells when they were co-transfected with pREP7-*Sna* (right bar in figure 1E, F). Most of these cells showed a dashed or punctate expression along the membrane, even when they were completely enclosed by adjacent cells along all lateral membranes. Almost all the solitary GFP-positive cells had completely lost their membrane-localized expression of E-cadherin and β-catenin (figures 1C and D respectively.

From these experiments, we conclude that elevation of *Sna* induces the dissociation of adherens junctions in F9 PrE, as indicated by the reduction or complete loss of membrane localized expression of E-cadherin and β-catenin. As such, *Sna* mimics the effect of PTHrP in the formation of PE *in vitro* and *in vivo* (chapter 2 of this thesis).

Sna induces migration, dispersion, and rounding of cells during embryoid body outgrowth.

Other features of PE formation *in vivo* are the migration of PrE or VE cells away from the embryo proper, while differentiating to PE cells, which are found as dispersed (solitary) cells on the Reichert's membrane, with rounded cell bodies. This process can be mimicked *in vitro* by re-plating of F9 embryoid bodies, which are aggregates with a core of undifferentiated cells surrounded by a layer of VE-like cells. These VE-like cells grow out as an epithelial sheet upon re-plating of the embryoid bodies on gelatinized culture surface in absence of exogenous PTHrP. When PTHrP is added at the time of re-plating, the cells migrate further and become solitary (dispersed) with a more rounded cell body (chapter 2 of this thesis). We have shown previously that *Sna* is upregulated during PTHrP-induced PE-formation from VE-like cells (Veltmaat *et al.,* 2000), and now wished to investigate whether *Sna* alone can induce the migration, dispersion and morphology of cells in this process. Given the low transfection efficiency of F9 cells, we decided to transfect F9 EC cells stably expressing a tetracycline repressible transactivator (tTA), with an empty vector (mock, M) or a vector expressing *Sna* (S) under a tTA-responsive promoter for these assays. We co-transfected a hygromycin expression vector, and used hygromycin to isolate stably transfected cells.

Of each transfection, we generated 3 independent pools of transfected clones (semi-stable cell lines). To test these pools for the levels of *Sna* transcription, we differentiated duplicate cultures of each to PrE in presence of tetracycline, and subsequently withdrew tetracycline from half of the cultures during the last 6 hours before RNA-extraction and semi-quantitative RT-PCR (figure 2).

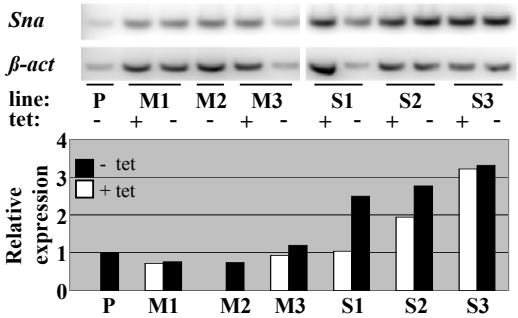


Figure 2: _Sna_ mRNA levels in F9 semi-stable cell lines.
F9 cells semi-stably transfected with a mock vector or a vector expressing *Sna* under the control of tetracycline were analyzed for *Sna* expression relative to *β-actin* expression by semi-quantitative RT-PCR (Veltmaat *et al*., 2000). RT-PCR products were loaded on an agarose gel containing Vistra-Green and analyzed by fluorimaging (top). Results shown here are preliminary. Ratios between *Sna* and *β-actin* are represented as relative expression over the ratio of the parental cell line in the lower panel. Abbreviations: P: parental cell line, M: mock transfected cell line, S: *Sna* transfected cell line, tet: tetracycline.

Our preliminary results shown here indicate that the levels of *Sna* mRNA in the PrE cultures of the three mock (M) cell lines in the presence or absence of tetracycline did not notably differ from each other or from the level in the parental cell line. While in S1, *Sna* expression in the presence of tetracycline seems equal to M, and elevated in the absence of tetracycline, *Sna* expression was elevated in the PrE cultures of S2 and S3 in the presence as well as the absence of tetracycline, indicating that in these lines, tetracycline could not completely suppress exogenous *Sna* expression. In the absence of tetracycline, *Sna* expression in S1, S2, and S3 is about three-fold elevated compared to M1, M2 and M3, which is comparable to the elevation that is seen at 30-60 minutes after PTHrP treatment of F9 PrE (Veltmaat *et al.*, 2000).

The semi-stable cell lines M1, S1, S2 and S3 were aggregated in the presence of tetracycline to generate embryoid bodies. One day after re-plating in absence or presence of tetracycline and PTHrP, they were photographed for analysis of embryoid body outgrowths. As can be seen in figure 3, the size of the embryoid bodies was variable, but this did not significantly affect the distance of outgrowth or the total number of outgrown cells (not shown). The photographs shown are representative for the morphological observations during culture, but not for the average number of outgrown cells (see below).

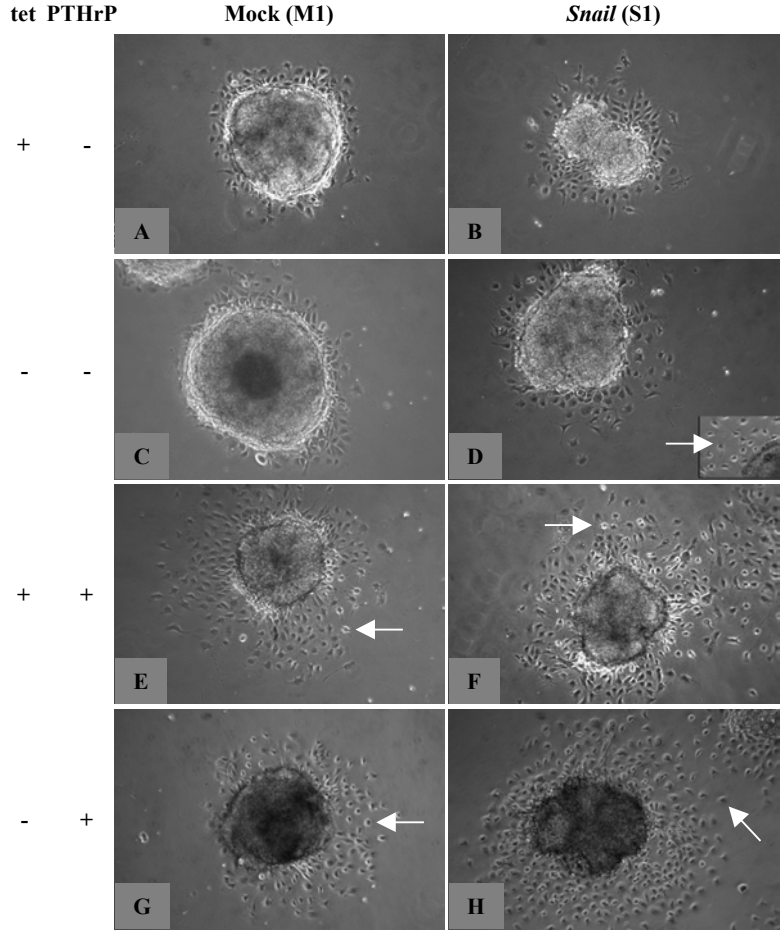


Figure 3: Morphology of outgrown cells of F9 semi-stable cell lines.
The photographs show outgrowths of embryoid bodies, representative for the morphology of F9 cell lines semi-stably transfected with a mock vector (left) or a *Sna* expressing vector (right). Photographs were taken at 1 day after re-plating in the absence (A-D) or presence of PTHrP (E-H) and the presence (A, B, E, F) or the absence (C, D, G, H) of tetracycline (tet). White arrows indicate cells with a rounded up morphology, as estimated from the light refraction. This is indicative for a more terminal differentiation of PE.

To analyze if overexpression of *Sna* affected the migration and dispersion of cells, we (i) measured the average outgrown distance (figure 4A), (ii) counted the total number of outgrown cells and (iii) the percentage of outgrown cells that had less than 20% contact with neighboring cells (figure 4B). These solitary, dispersed cells were considered mesenchymal, PE-like cells (Behrendtsen *et al.*, 1995; Roelen *et al.*, 1998). By one-way ANOVA analysis we first tested per culture condition and per parameter if S1, S2, and S3 differed among each other. As no significant differences were found (two-sided, $p>0.05$), except for the percentage of solitary cells in the +tet/-PTHrP condition (two-sided, $p<0.05$), we pooled all data from these cell lines in the other culture conditions per parameter, and tested it as a group S against M1, again by one-way ANOVA (two-sided, $\alpha=0.05$).

The outgrown distance in the presence of PTHrP was significantly larger than in the absence of PTHrP for M (figure 4A, white bars) and S (black bars), regardless of the presence or absence of tetracycline. The outgrown distance of S was significantly larger than that of M1 in all culture condition. While the number of outgrown cells was not affected by the absence or presence of tetracycline or PTHrP, nor the overexpression of *Sna* (not shown), these results indicate that cells migrate further away from the embryoid body in the presence of PTHrP, and that this effect is mimicked by *Sna*. Furthermore, the addition of PTHrP had an additive effect on the enhanced migration in S (figure 4A, right two black bars).

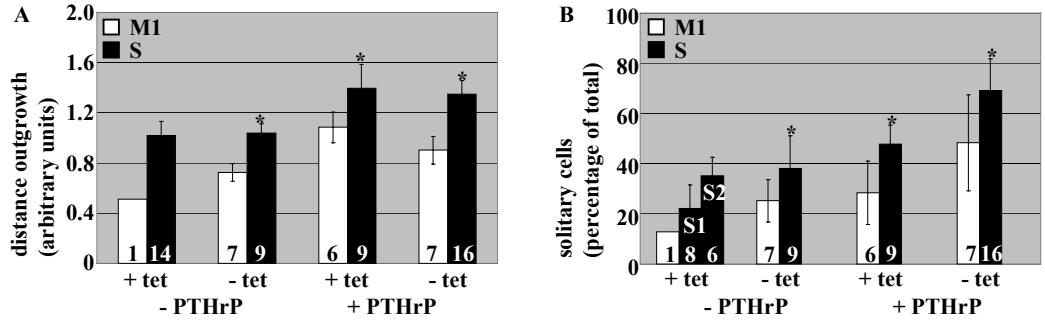


Figure 4: Quantitative analysis of embryoid body outgrowths of F9 semi-stable cell lines.

Photographs, obtained as described at figure 3, were used to measure the average outgrown distance per aggregate (in arbitrary units) (**A**), to count the total number of outgrown cells and the percentage of solitary cells (less than 20% contact with neighboring cells) (**B**) within the outgrowths of F9 semi-stably transfected cell lines. Bars represent the results as obtained with cells transfected with a mock vector (M, white bars) or pooled results for cells transfected with a *Sna* (S, black bars) expressing vector, with one exception (see material and methods). Error bars in figure A represent the standard error of the means over the number of embryoid bodies. Error bars in figure B represent the standard deviation over the number of embryoid bodies. Differences were tested for significance with a two-sided one-way ANOVA. The addition of PTHrP significantly increased the outgrown distance and the percentage of solitary cells for both M and S ($p<0.05$). A significant difference between S and M within the same culture condition is indicated with an asterisk for $p<0.05$. The outgrown distance and the percentage of solitary cells were not significantly different between S –PTHrP and M + PTHrP, in the absence of tetracycline. The numbers of embryoid bodies used are indicated in the bars.

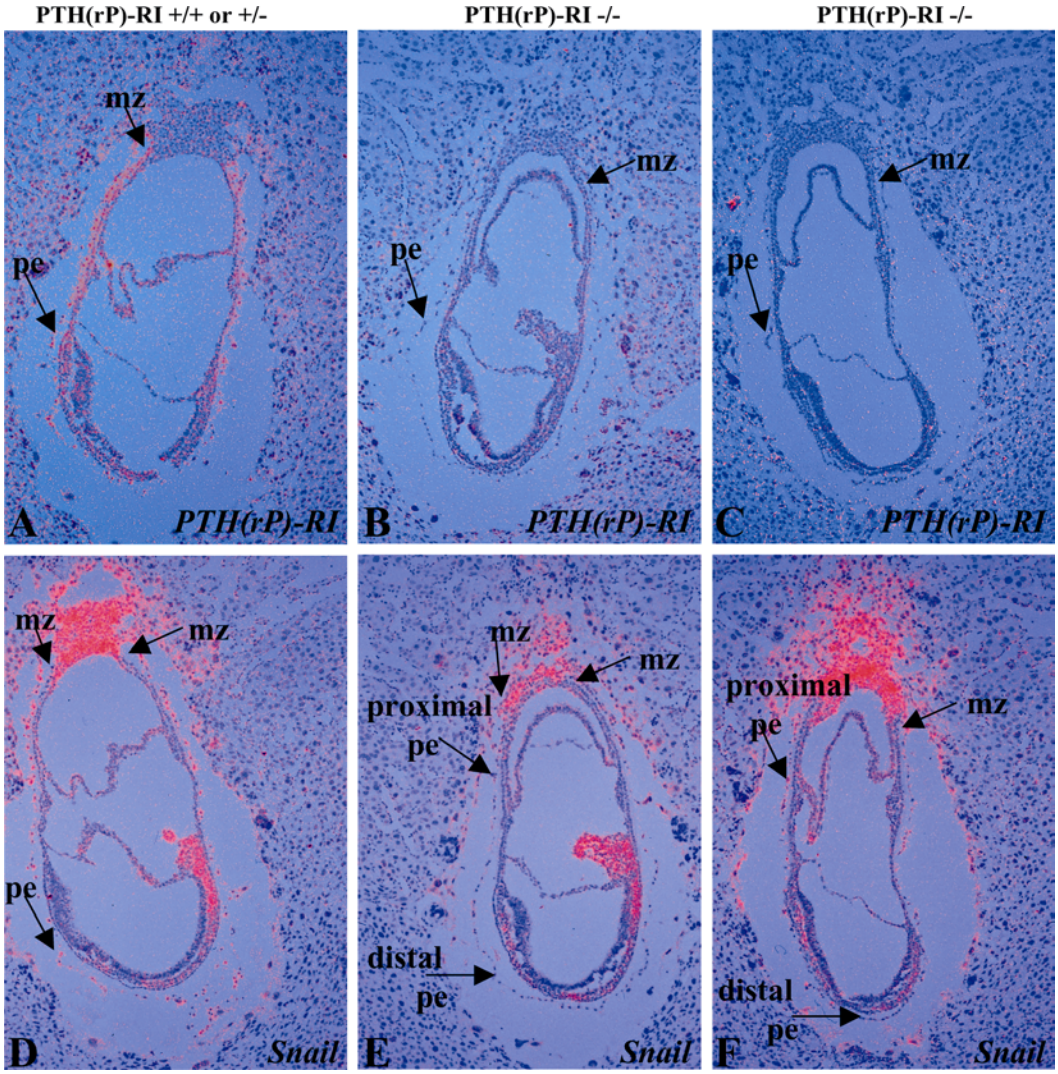


Figure 5: Expression of *Sna* mRNA in E7.5 wild type and PTH(rP)-RI -/- mouse embryos.

Mouse embryos derived from matings between PTH(rP)-RI +/- were dissected at E7.5 and processed for *in situ* hybridization on sagittal sections. Top panels show the hybridization results for the *PTH(rP)-RI* probe, while the lower panels show the hybridization results for the *Sna* probe on sections from the corresponding embryos. *Sna* expression in the distal PE is lost in the knockout embryos (B, C, E, F), where it is present in the wild type or heterozygous and wild type litter mates (A, D). mz: marginal zone, pe: parietal endoderm.

The percentage of solitary cells in the outgrowths of M and S in the presence of tetracycline was significantly increased by the addition of PTHrP. Similarly, addition of PTHrP in the absence of tetracycline yielded a significantly higher percentage of solitary cells in outgrowths of both M and S. The percentage of solitary cells in outgrowths of S was significantly higher than in the outgrowth of M in all culture conditions (figure 4B), with the exception of S1 in the presence of tetracycline and the absence of PTHrP, corresponding with the lower level of *Sna* expression in S1 (figure 2).

These results indicate that *Sna* mimics the effect of PTHrP in inducing cell

dispersion. Furthermore, the addition of PTHrP has an additive effect on the increased dispersion in S. Among these dispersed, solitary cells were extremely rounded off cells, indicative for a more terminal differentiation to PE-like cells (an example of which is indicated by the white arrow, insert fig. 3D). This was more often observed in PTHrP-treated outgrowths of both M and the three S cell lines (examples indicated by white arrows in figure 3E-H), suggesting that overexpression of *Sna* mimics the effect of PTHrP in inducing cell rounding, typical for PE.

From these experiments, we conclude that elevated expression levels of *Sna* enhance migration and induce cell dispersion, as well as rounding off of solitary cells in outgrowths of F9 embryoid bodies. As such, *Sna* mimics the effect of PTHrP. Additionally, we conclude from figure 4 that overexpression of *Sna* and the presence of PTHrP additively enhance cell dispersion and migration.

Sna expression is absent in distal PE of E7.5 PTH(rP)-RI -/- mouse embryos.

To investigate whether the epithelial phenotype of PE in mutant mice lacking PTH(rP)-RI expression could be due to a lack of (efficient) upregulation of *Sna* during PE formation *in vivo*, we dissected mouse E7.5 mouse embryos derived from 2 crosses between PTH(rP)-RI +/- matings. While in the extraembryonic endoderm of wild type and heterozygous embryos (n=10) *Sna* was expressed throughout the entire parietal layer, from marginal zone to the distal-most region (figure 5A, B), in all of the knockout embryos (n=5) *Sna* was expressed only in the marginal zone and proximal-most region, but not in the more distal region (figure 5C-F). This lack of *Sna* expression correlates well with the lack of a mesenchymal phenotype in these mutant PE cells observed at E8.5 and E9.5 (chapter 2 of this thesis), and strongly suggests that *Sna* expression is required for the mesenchymal, PE-specific morphology. While *Sna* was expressed in the endoderm in the marginal zone and proximal-most region of PE in these embryos, these data suggest that for the maintained expression of *Sna* in PE cells in the more distal region PTH(rP)-RI signaling in at least the distal region is required. Since we observed a mesenchymal morphology throughout the PE in wild type embryos, these results indicate that the residual *Sna* expression observed in the proximal PE in the PTH(rP)-RI -/- embryos is not sufficient to induce a complete EMT.

DISCUSSION

PE is formed first from PrE and later from VE migrating away from the embryo proper, initially as an epithelial sheet. Cells at the leading edge dissociate from this sheet and they round up while they differentiate to PE. We consider the formation of this tissue the first epithelio-mesenchymal transition during mouse embryogenesis. In this process, the disruption of the E-cadherin-mediated adhesion system (adherens junctions) is a major event, as it is in other EMTs later during development and during tumor progression. The action of PTHrP through its type I PTH/PTHrP receptor is required for the loss of the adherens junctions and the acquisition of the rounded morphology (chapter 2 of this thesis). We have previously shown that the zinc finger transcription factor *Sna* is an immediate early target gene of PTHrP in PE formation (Veltmaat *et al.*, 2000). Here, we demonstrate *in vitro* that *Sna* mimics the action of PTHrP in

the dissociation of adherens junctions, in enhancing the migration and dispersion of cells, and in establishing a rounded morphology of these dispersed, solitary cells.

Our data from *in vitro* studies on F9 PrE cells indicate that elevated levels of *Sna,* as normally observed early during PE formation *in vitro* and *in vivo*, are sufficient for the down-regulation of membrane-localized expression of E-cadherin and β-catenin, and therewith the dissociation of the adherens junctions during this process. Our results are supported by a similar downregulation observed in F9 cells stably transfected with avian *slug* (D. Newgreen, personal communication), which is assumed to have swapped its function with *Sna* in chick development (Sefton *et al.*, 1998). The downregulation of the cell-cell adhesion system seems to be a conserved function of Sna, since *Drosophila Sna* mutant embryos show a gastrulation-defective phenotype that has been associated with impaired downregulation of DE-cadherin (Oda *et al.*, 1998), and mouse *slug* has been shown to downregulate the desmosomal adhesion system as an early event in EMT in NBT-II cells (Savagner *et al.*, 1997). Recently, evidence for the downregulation of the E-cadherin based adhesion system during the EMT by the action of *Sna* in the late stages of tumor progression has been given (Cano *et al.*, 2000). Human and mouse Sna exert this action directly by binding to E-boxes in the promoter of the *E-cadherin* gene in the respective species (Batlle *et al.*, 2000). Sna may act similarly in the dissociation of the adherens junction in the differentiation of F9 PrE to PE, although interference at the translational or post-translational level is an additional possible mode of action.

We show that elevation of *Sna* leads to further outgrowth of embryoid bodies, mimicking the outgrowth of PE precursors *in vivo*. Since the number of outgrown cells is not affected, this outgrowth can be ascribed to enhanced miration of individual cells. This is in accordance with the implication of Sna and slug in cell migration during the EMTs in e.g. gastrulation, neural crest emigration, and tumor progression (Hemavathy *et al.*, 2000). Repression of their function results in reduced migration during e.g. gastrulation in *Drosophila* (Simpson, 1983; Grau *et al.*, 1984) and neural crest formation in *Xenopus* (LaBonne and Bronner-Fraser, 1998; Carl *et al.*, 1999), presumably by impaired downregulation of cell-cell adhesion.

Besides the downregulation of E-cadherin and promotion of migration, *Sna* also induces dispersion of cells, and morphological changes, two other aspects of PE formation, as we demonstrate here. Interestingly, *Sna* induces a fibroblastic morphology and cell dispersion during tumorigenesis (Cano *et al.*, 2000; Batlle *et al.*, 2000), while during PE formation in F9 cells, it induces the typical rounded instead of fibroblastic morphology. This suggests that *Sna* has a conserved general effect on modulation of cell contacts and cytoskeletal structures, while fine-tuning of the resulting morphological changes may be mediated by cell-type specific genes or processes.

By carrying out *in situ* hybridization on E7.5 embryos derived from crosses between C57BL/6 PTH(rP)-RI +/- mice, we demonstrate that *Sna* is not expressed in the PE cells outside the proximalmost region in knockout mice, while it is always expressed throughout all PE cells in heterozygous and wild type littermates. We have demonstrated before (Veltmaat *et al.*, 2000) that *Sna* is expressed in F9 PrE and in F9 embryoid

bodies in the absence of exogenous PTHrP, and that the level of expression is upregulated as an immediate early response to PTHrP during the formation of PE. The basal level of *Sna* expression in F9 PrE and embryoid bodies may reflect the *Sna* expression in the marginal zone and proximal PE of the knockout embryos. The expression of *Sna* in the endoderm in the marginal zone and the proximal-most PE cells in these mutants indicates that another factor besides PTHrP induces *Sna* expression during PE formation. This pathway may be essential in PE formation, e.g. for the migration of PE precursor cells that still occurs in PTH(rP)-RI knockout embryos, as well as in re-plated F9 embryoid bodies in the absence of exogenous PTHrP (chapter 2 of this thesis, and this report). However, such an essential role for *Sna* remains to be established. The absence of *Sna* expression in the distal PE of knockout embryos correlates well with the lack of mesenchymal morphology that we found in PE cells of knockout embryos at E8.5 and E9.5 (chapter 2 of this thesis). These data strongly suggest that *Sna* needs to be expressed at E7.5 of PE formation (and presumably maintained during later stages) to induce (and maintain) the mesenchymal morphology of true PE cells.

It is of interest to study whether *Sna* acts as a key molecule in PE, i.e. whether it induces more or all mesenchymal characteristics during PE formation, such as the upregulation of vimentin. This gene is known to be upregulated during PE formation, and shown to be upregulated in MDCK cells transfected with *Sna* (Cano *et al.*, 2000). Ultimately, the levels of expression of the PE marker thrombomodulin would provide insight in whether the EMT induced by *Sna* in PE precursors results in differentiation towards true PE. Sna -/- embryos have been generated, with a yet undefined PE phenotype (Jiang *et al.*, 1998a). Detailed analysis of PE formation in these embryos would reveal for which aspects of this process *Sna* is required.

Our results may also open avenues to better understand the role of PTHrP in tumor progression, since (i) this processes involves an EMT as well, (ii) elevated PTHrP expression is found in metastatic lesions of e.g. breast tumor cells (Southby *et al.*, 1990; Powell *et al.*, 1991; Vargas *et al.*, 1992), (iii) *Sna* is absent in epithelial (breast) carcinoma cells, but present in metastatic carcinoma cells, and inversely correlated with E-cadherin expression (Batlle *et al.*, 2000), (iv) overexpression of *Sna* induces metastatic behavior of epithelial tumor cells (Cano *et al.*, 2000).

In conclusion, we found that elevated expression of the zinc finger transcription factor *Sna* is sufficient to induce the loss of cell-cell contacts, enhanced cell migration and cell dispersion, as normally occurring as a result of PTH(rP)-RI signaling during PE formation. The absence of *Sna* in the PE cells outside the proximal-most region in PTH(rP)-RI -/- embryos strongly suggests that *Sna* is required for the PTHrP induced mesenchymal phenotype of PE.

ACKNOWLEDGMENTS

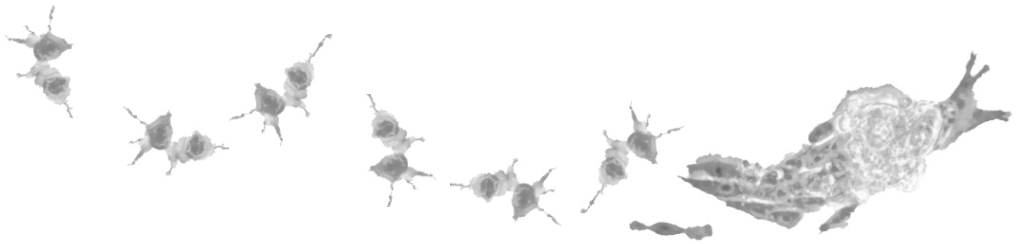
This work was carried out in the Graduate School of Developmental Biology in Utrecht, The Netherlands, and at the Institut Curie in Paris, France with the financial support of the Ter Meulen Fund of the Royal Netherlands Academy of Arts and Sciences to JV. The authors wish to thank Dr. J. Salamero, and W. Hage for help with fluorescence and confocal microscope techniques, J. Korving for embedding and sectioning of the embryos, A. Mailleux for valuable assistance with data-retrieval, and Dr. L. Tertoolen for help with statistical analysis of the data. Constructs were kindly provided by Dr. Cross at the Mount Sinai Hospital, Toronto, Canada (pREP7-*Sna*), Dr. den Hertog at the Hubrecht Labo-

ratory, Utrecht, The Netherlands (human *β-actin* promoter sequences), and Dr. García de Herreros at the Community Institute for Medical Research, Barcelona, Spain (pTRE-HA-*Sna*).

CHAPTER 6

NEW INSIGHTS IN THE MOLECULAR REGULATION OF CELLULAR EVENTS DURING PARIETAL ENDODERM FORMATION

- A GENERAL DISCUSSION OF THIS THESIS -



Jacqueline M. Veltmaat

Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.

6

NEW INSIGHTS IN THE MOLECULAR REGULATION OF CELLULAR EVENTS DURING PARIETAL ENDODERM

- A GENERAL DISCUSSION OF THIS THESIS -

INTRODUCTION

*I*t has become clear that the same or similar molecules and mechanisms govern embryonic development as well as tissue homeostasis in the adult. Much attention has been paid to e.g. growth factor activated kinase receptor signaling in e.g. epithelio-mesenchymal transitions (EMT) and interactions (EMI), two mechanisms that govern organogenesis. Much less attention has been paid to hormones activating G-protein coupled receptor signaling. One such example is parathyroid hormone related peptide (PTHrP) signaling via the type I PTH/PTHrP receptor (PTH(rP)-RI). The variety of functions exerted by this hormone-receptor system during embryonic and adult life and pathology suggests that it has important regulatory functions. The spatio-temporal pattern of expression of PTHrP and the receptors to which it can bind (Karperien *et al.*, 1996) are of critical importance for the embryonic development of e.g. bone, skin, mammary gland and lung as suggested by the malformation or malfunctioning of these organs in embryos or neonates in which the expression of PTHrP or the PTH(rP)-RI has been experimentally modified (Kong *et al.*, 1994; Karaplis *et al.*, 1994; Wysolmerski *et al.*, 1995; Lanske *et al.*, 1996; Foley *et al.*, 2001). In adult life, elevated PTHrP production has been associated with e.g. hypertension and more advanced stages of various neoplastic disorders, prompting researchers to investigate the role of PTHrP in these diseases (Goltzman and Henderson, 1996).

At the start of the work described in this thesis, it was clear that mouse embryos lacking the type I receptor for PTHrP (PTH(rP)-RI) die at a time when PTHrP signaling can only be involved in the formation of parietal endoderm (PE), an extraembryonic tissue (Karperien *et al.*, 1994, 1996; Lanske *et al.*, 1996; Verheijen *et al.*, 1999). The experiments described here address the role of PTHrP signaling via the PTH(rP)-RI in this process in the mouse embryo. For this, we made use of F9 embryonal carcinoma cells, which provide a good *in vitro* model system for PE formation, as well as of wild type and PTH(rP)-RI knockout mouse embryos. Our findings are discussed in this chapter and have lead to a model for PE formation and function.

PARIETAL ENDODERM FORMATION IS AN EPITHELIO-MESENCHYMAL TRANSITION

In chapter 1 of this thesis, the formation of PE is proposed to be an epithelio-mesenchymal transition (EMT). Many researchers have provided evidence that primitive endoderm (PrE) and visceral endoderm (VE) cells, the consecutive precursors of PE cells, are respectively 'epithelioid' and epithelial in character,

as described in chapter 1. PE cells have been referred to as fibroblast-like, or stellate cells, with filopodia that suggest a migratory property of these cells. However, the formation of PE has not been considered an EMT before, and gastrulation is thus generally considered to involve the first EMT during mouse embryogenesis (Hay, 1995; Viebahn, 1995).

The process of EMT as it occurs during e.g. gastrulation and neurulation in the mouse embryo should not be confused with the epithelial morphogenetic events that occur during gastrulation in the *Drosophila* or *Xenopus laevis* embryo. During these events, the epithelial precursors of mesoderm move to a new position either by apical constriction and stretching followed by invagination of the epithelium, or by cell intercalation followed by involution of the epithelium respectively (Keller *et al.*, 1992; Leptin, 1995). Real EMTs differ from the foregoing examples of epithelial plasticity, in that the epithelial cells lose their epithelial morphology, change their adhesion status and acquire a migratory phenotype as individual fibroblast-like cells, and as such move to a new position (Thiery and Chopin, 1999). The earliest PE precursors in the mouse embryo have already acquired a fibroblast shape as they migrate onto the basal membrane underlying the trophectoderm (Enders *et al.*, 1978; Gardner, 1982), while later precursors are elongated and have filopodia-like protrusions, and thus are also fibroblast-like as they migrate through the marginal zone, as reviewed in chapter 1. The loss of cell-cell adhesion, the cytokeratin cytoskeleton and subcortical F-actin belt, together with the elevated secretion of extracellular matrix components and proteases by PE cells are all hallmarks of a mesenchymal phenotype. Therefore, we propose to consider the process by which PE is formed a real EMT. However, PE cells differ from most other mesenchymal cells in that they line a cavity, generally an epithelial characteristic, and in that they do not invade through the matrix, but instead migrate over it. Furthermore, this particular EMT is induced by G-protein coupled receptor signaling (chapter 2 of this thesis), while other EMTs are usually induced by tyrosine or serine/threonine kinase receptor signaling or matrix factors (Duband *et al.*, 1995; Birchmeier *et al.*, 1996).

PTH(rP)-RI SIGNALING IS REQUIRED FOR THE MORPHOLOGICAL TRANSFORMATION AND DIFFERENTIATION DURING PE FORMATION

Work from our group and others has shown that PTHrP is a potent inducer of parietal endoderm *in vitro* in F9 cells, as reviewed in chapter 1. The spatio-temporal expression of PTHrP by the decidua and trophectoderm, and of the PTH(rP)-RI by the PrE, VE and PE strongly suggest that this hormone-receptor system plays an inductive role in PE formation *in vivo* as well (Karperien *et al.*, 1994, 1996). To specify the inductive action of PTHrP signaling in PE formation, we studied migration, morphological transformation and differentiation of the cells.

As we have shown in chapter 2 of this thesis, signaling via the PTH(rP)-RI leads to enhanced migration of endodermal cells growing out from plated F9 embryoid bodies. Whether this effect on migration is due to facilitated adhesion of the embryoid bodies to the culture substrate and/or enhanced migratory speed of outgrowing cells remains to be

determined. The VE cells can migrate in the absence of exogenous PTHrP, suggesting that PTHrP is not strictly needed for the migration during PE formation. Indeed in C57BL/6 embryos in which PTHrP signaling during PE formation is abrogated by homozygous deletion of the PTH(rP)-RI, endodermal cells migrate away from the egg cylinder and form a PE layer (Lanske *et al.*, 1996). However, as we show in chapter 2, the cells of this layer do not display the mesenchymal morphology, typical for PE. Instead, they display the epithelial character typical for the endodermal precursors of PE, indicated by membrane-localized expression of E-cadherin and β-catenin. Moreover, these cells do not express enhanced levels of thrombomodulin, which we consider a marker for differentiated PE cells (chapter 2 of this thesis) (Imada *et al.*, 1987, 1990a, b; Verheijen *et al.*, 1999). Although laminin is expressed by these cells (Lanske *et al.*, 1996), it is possible that this expression is not elevated to the level that is typical for PE cells.

In cultures of F9 PrE and VE cells, we found that the addition of PTHrP leads to the loss of membrane-localized expression of E-cadherin and β-catenin, thus adherens junctions, as well as the loss of markers for tight junctions and desmosomes, and a reduction of connexin 43 expression, the most abundant gap junctional protein in extraembryonic endoderm. Moreover we found a loss of the intermediate filament cytoskeleton and subcortical F-actin expression. In addition, PTHrP or its second messenger cAMP induces the rounding off of the cells (chapter 2), the expression of e.g. vimentin, tissue plasminogen activator, and elevated levels of laminin (Smith and Strickland, 1981; Marotti *et al.*, 1982; Lane *et al.*, 1983; Lehtonen *et al.*, 1983; Trevor, 1990; van de Stolpe *et al.*, 1993; Behrendtsen *et al.*, 1995), all indicative for a transition from an epithelial to mesenchymal phenotype.

As PTHrP signaling via the PTH(rP)-RI enhances migration during PE formation *in vitro*, and is sufficient and required for the morphological transition from an epithelial to a mesenchymal cell type *in vitro* and *in vivo*, we conclude that it controls the induction or at least the completion of the EMT during PE formation *in vivo*. In line with this, it is required for the terminal differentiation of PrE and VE cells to true PE cells *in vivo*.

PTHrP SIGNALING DURING PE FORMATION REDUCES GAP JUNCTIONAL COMMUNICATION

At the time when the project described in this thesis was started the significance of the extensive gap junctional coupling between VE cells had not been determined, nor had an effect of PTHrP on the regulation of connexin 43 (Cx43) been reported in these or other cells. Addition of PTHrP to F9 PrE and VE cultures reduces the expression of Cx43 (chapter 2 of this thesis), the most abundant component of gap junctions in extraembryonic endoderm *in vivo* (Kalimi and Lo, 1989; Ruangvoravat and Lo, 1992; Dahl *et al.*, 1996) and *in vitro* (Nishi *et al.*, 1991; Willecke *et al.*, 1991). Gap junctional intercellular communication (GJIC) provides a means to establish tissue homeostasis and electrical synchronization of clusters of cells, or to propagate hormonal stimulation by the transmission of signals, e.g. second messengers such as cAMP. We studied

the effect of PTHrP signaling on GJIC during PE formation, and its importance for PE formation. We demonstrate that the expression of phosphorylated (i.e. communication competent) Cx43 as well as its membrane localization and gap junctional coupling are high in F9 VE, as in VE isolated from day 8.5 pc mouse embryos. PTHrP treatment of F9 PrE or VE leads to a strong reduction in membrane-localization of Cx43 and dye coupling. The downregulation of *Cx43* mRNA expression occurs within 4 hours after PTHrP addition, suggesting a direct transcriptional regulation of *Cx43* by PTHrP, possibly by cAMP response elements in the promoter (Schiller *et al.*, 1992; Donahue *et al.*, 1995; Schiller *et al.*, 1997; Civitelli *et al.*, 1998). In this aspect, the regulation of Cx43 by PTHrP is opposite to the effect of PTH or cAMP analogues on Cx43 in other cell systems, in which a rapid upregulation, membrane localization and GJIC is seen (Mehta *et al.*, 1992; Atkinson *et al.*, 1995; Darrow *et al.*, 1996). Some residual Cx43 expression and GJIC remain present in F9 PE. However, PE cells of parietal yolk sacs isolated from day 8.5 pc mouse embryos completely lack detectable levels of Cx43 expression and dye coupling, suggesting that GJIC does not play a role in PE function. Nevertheless, GJIC may be important in the inductive phase of PE formation, and furthermore, it remains possible that PE cells *in vivo* are still ionically coupled.

Thus, to determine whether GJIC is necessary for PE formation, we cultured F9 cells in the presence of 18α-glycyrrhetinic acid (AGA), which blocks all gap junctions. Differentiation of F9 EC to either PrE or VE was not affected by the presence of AGA, nor was their subsequent differentiation to PE, as determined by morphology and thrombomodulin expression (chapter 3 of this thesis). Therefore we conclude that Cx43 function and GJIC are not needed for the formation and differentiation of PE *in vitro*. Instead, the high expression and membrane localization of Cx43 and the high extent of GJIC found in F9 PrE and VE, and in mouse VE, may play a role in exo- and endocytotic processes, e.g. nutrient and metabolite exchange as well as hormone and enzyme production and secretion (Bielinska *et al.*, 1999). Similarly, GJIC and Cx43 expression in other systems have been shown to correlate well or even be critical for hormone production and secretion, or in nutrient and metabolite exchange, e.g. in the pancreas and lens (Vozzi *et al.*, 1995; Khan-Dawood *et al.*, 1996; Matesic *et al.*, 1997; Gao and Spray, 1998). GJIC modulates enzyme secretion by regulating calcium oscillations (Stauffer *et al.*, 1993). Interestingly, calcium oscillations have been observed in ES cell-derived PrE and VE, and these oscillations are involved in the exo/endocytotic vesicle shuttle (Sauer *et al.*, 1998). This suggests that GJIC in VE has a function in the nutrient and metabolite exchange and protein secretion, rather than in differentiation to PE.

THE ZINC FINGER TRANSCRIPTION FACTOR *SNAIL* IS AN IMMEDIATE EARLY RESPONSE GENE OF PTH(RP)-RI SIGNALING IN PE FORMATION

In search for downstream targets of PTH(rP)-RI signaling with a function in PE formation, we screened F9 PrE before and after PTHrP treatment for differentially expressed genes, and found transcriptional upregulation of the zinc finger transcription factor *snail (Sna)* (chapter 4 of this thesis). Express-

sion of this gene had been found by others in PE in the day 7.5 and 8.5 pc mouse embryo (Nieto *et al.*, 1992; Smith *et al.*, 1992), We identified *Sna* as an immediate early response gene to PTHrP in F9 PrE. The gene is also upregulated in the transition from F9 VE to PE, while F9 PrE and VE cells already express basal levels of *Sna.* The expression in PrE and VE as presumptive mesenchymal cells is similar to the expression found in pre-migratory cells in mesoderm formation and neural crest migration. These *in vitro* results reflect the upregulation of *Sna* expression during PE formation in the embryo: *Sna* is expressed from zygote stage onwards with a significant increase of expression during parietal endoderm formation *ex vivo* in plated blastocysts. In accordance with this, *Sna* expression is detected in the earliest PE cells *in vivo* at day 5.5 pc, and its expression is continued to at least day 8.5 pc. By *in situ* hybridization we detected *Sna* at day 7.5 pc in the endodermal cells in the marginal zone, that are in their transition from VE to PE, as well as in the PE cells in more abembryonic regions (chapter 4 of this thesis). This expression coincides spatio-temporally with the expression of the PTH(rP)-RI (Karperien *et al.*, 1994; Verheijen *et al.*, 1999). Since PTHrP is expressed and secreted by the adjacent trophectoderm cells (Karperien *et al.*, 1996), this indicates that *Sna* could also be an early response gene to PTHrP in PE formation *in vivo*.

Sna orthologues are known to be induced by growth factors that signal via tyrosine and serine/threonine kinase receptors, such as family members of fibroblast and transforming growth factors (Sargent and Bennett, 1990; Hammerschmidt and Nusslein-Volhard, 1993; Thisse *et al.*, 1993; Thisse *et al.*, 1995; Buxton *et al.*, 1997; Savagner *et al.*, 1997; Romano and Runyan, 2000). However, a direct regulation of *Sna* expression by these growth factors has never been demonstrated. We are the first to identify a direct regulation of *Sna* by a cytokine, i.c. PTHrP, and in contrast to the aforementioned examples, this regulation is mediated by activation of a G-protein coupled receptor.

SNAIL MEDIATES THE PTHRP INDUCED EMT DURING PE FORMATION

The *Sna* gene is evolutionarily conserved, expressed at sites of epithelial plasticity, in particular EMT, and in mesenchymal tissues. Reduced function of *Drosophila* Sna and or its family members hampers the epithelial morphogenetic events during gastrulation and neurulation, indicating a role for Sna in these processes (Grau *et al.*, 1984; Nusslein-Volhard *et al.*, 1984; Ashraf *et al.*, 1999; Hemavathy *et al.*, 2000). Similarly, *Xenopus* slug is essential for neural crest migration (Carl *et al.*, 1999; LaBonne and Bronner-Fraser, 2000). This prompted us to study whether mouse Sna is involved in mediating the PTHrP induced EMT in PE formation.

In chapter 5 we provide preliminary evidence that overexpression of *Sna* in F9 PrE or VE is sufficient to reduce or completely eliminate membrane localized expression of E-cadherin and β-catenin. Thus, Sna reduces cell-cell adhesion mediated by adherens junctions, which we have shown to occur in response to PTHrP signaling (chapter 2 of this thesis). These results are in accordance with the dissociation of E-cadherin-mediated adherens junctions by snail orthologues in *Drosophila*, human and mouse (Oda *et al.*, 1998; Cano *et al.*, 2000; Batlle *et al.*, 2000), and the iden-

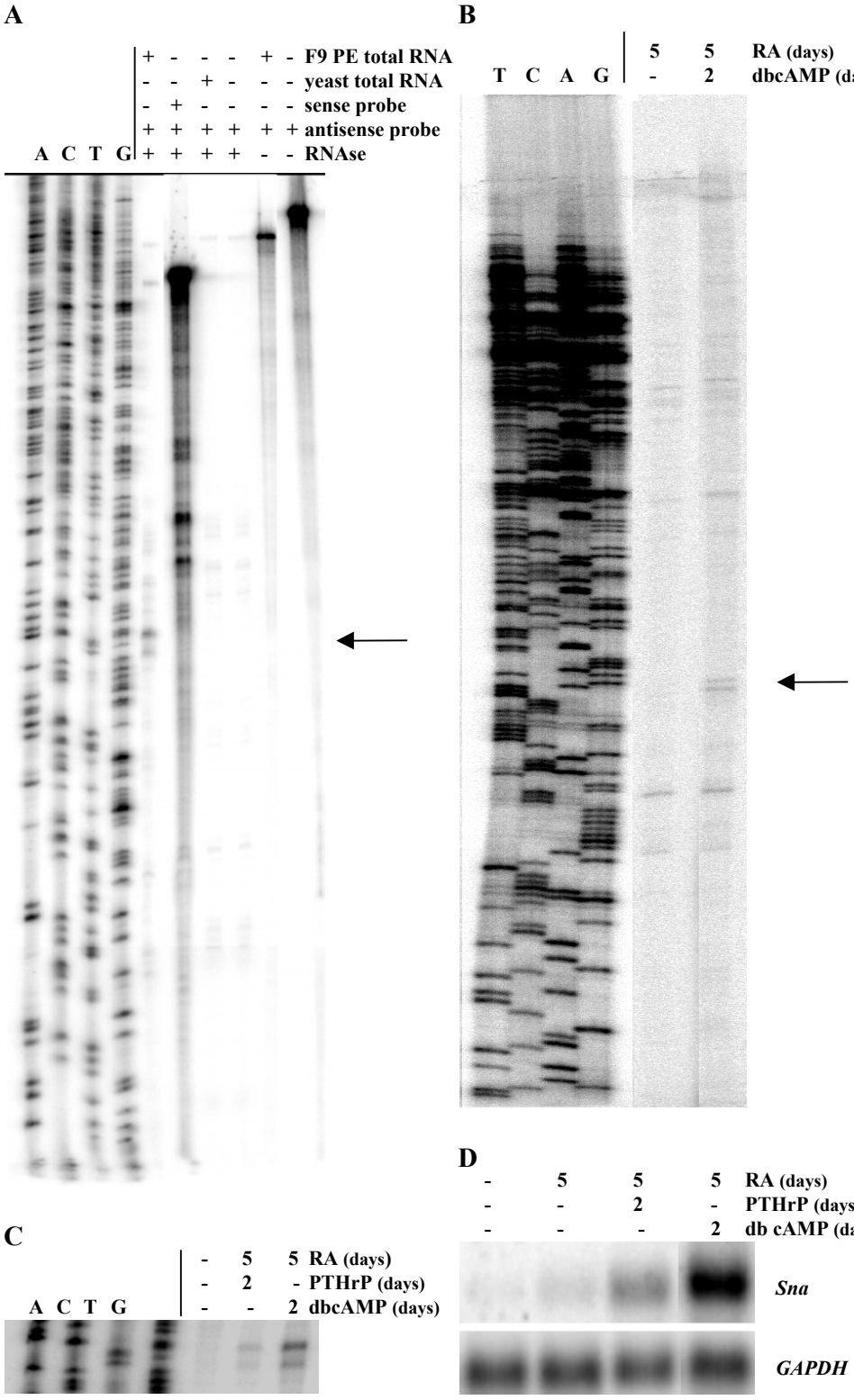
tification of the mouse and human *E-cadherin* gene as direct targets of Sna repressor activity. The loss of E-cadherin mediated cell-cell adhesion is considered an important step in increased migration and morphological transformation of cells during tumor progression and EMTs in general (Navarro *et al.*, 1993; Hay, 1995; Llorens *et al.*, 1998; Lozano and Cano, 1998; Cano *et al.*, 2000; Batlle *et al.*, 2000). We have shown that overexpression of *Sna* in F9 VE cells enhances their migration away from F9 embryoid bodies re-plated on gelatin, as well as their acquisition of a mesenchymal, rounded morphology typical for PE cells, obtained by e.g. PTHrP treatment (chapter 5). Our results indicate that elevated *Sna* expression is sufficient to enhance the migration and induce the morphological events, normally seen during PTHrP induced PE formation *in vitro*. Similarly, Sna orthologues have been shown to induce mesenchymal morphology and invasive properties when overexpressed in epithelial cells (Cano *et al.*, 2000).

Importantly, we have found a lack of *Sna* expression only in the distal PE cells in day 7.5 pc mouse embryos lacking the PTH(rP)-RI (chapter 5 of this thesis), while *Sna* was still expressed in the marginal zone and proximal PE cells. The lack of *Sna* expression at day 7.5 pc and the epithelial morphology of distal PE cells at day 8.5 and 9.5 pc support the contention that elevated *Sna* expression is not only sufficient, but also necessary for the complete and/or maintained mesenchymal phenotype.

PROMOTER ANALYSIS OF THE MOUSE *SNA* GENE

One of the questions we would like to answer is via which mechanism PTHrP signaling elevates *Sna* transcription. We isolated approximately 900 bp upstream of the start codon of the *Sna* gene of a C57BL/6xCBA mouse. This region has been designated promoter region by Jiang *et al*. (1997) based on consensus sequences for DNA binding sites of several transcription factors, but has not been further investigated.

Figure 1: Identification of the transcription initiation site in the *mSna* promoter.
A: RNase protection assay in F9 cells. The pCR2.1-TOPO vector (Invitrogen,Groningen, The Netherlands) spanning nucleotides 16-969 of the Genbank sequence for the *mSna* promoter region (accession number U95961, (Jiang *et al.*, 1997)) of which nucleotide 906-969 are coding sequence, was linearised with *SacI*, blunted with T4 DNA Polymerase (Amersham, Roosendaal, The Netherlands) and used as a template for transcribing an antisense riboprobe labelled with α[^{32}P]UTP (Amersham). The protection assay was carried out using the RPA II kit (Ambion) according to manufacturer's protocol with slight modifications. In short, 0.6 µg poly-A$^+$ RNA from F9 PE cells was hybridized overnight at 46°C with 100,000 cpm riboprobe in 20 µl hybridization buffer. The left four lanes show the T7 sequencing product of pCR2.1-TOPO-954. The arrow at the right indicates a protected doublet in F9 PE (5th lane), absent in the three negative control lanes (6th, 7th and 8th lane), co-migrating with 158 and 164 basepair (bp) sequencing products. The 3'-most 64 bp of the protected fragment are located in the coding sequence. When corrected for differences in mobility between RNA and DNA, the estimated transcription start site is located approximately 90 bp upstream of the translation start site. The right-most two lanes indicate the absence of RNAse contamination in the RNA and probe, and the length of protected full length probe. **B:** Primer extension in F9 cells. The Primer Extension Assay was carried out as described (Sambrook *et al.*, 1989), using a reverse primer spanning bases 45-64 of the coding sequence. In short, 50 µg total RNA (isolated as described (Chomczynski and Sacchi, 1987)) from F9 PrE and PE cells was used as a template in an extension reaction of the γ[^{32}P]ATP labelled reverse primer described above. The product of a manual sequencing reaction (T7 sequencing kit, Pharmacia Biotech) pBluescript KS1 was run alongside as a marker (left four lanes). The arrow at the right indicates the extension product (doublet) of 153 and 155 bp that is seen at low levels in F9 PrE (5th lane) and upregulated in F9 PE (6th lane). Subtracting the 64 bp in the coding region, this product indicated the location of the transcription initiation site at 90 bp upstream of the translation initiation site. The RNAse protection and primer extension products were separated on a 5% polyacrylamide sequencing gel. Gels were analysed using the Phosphorimager and Image QuaNT 4.2 software (both from Molecular Dynamics) **C**: Sequencing reaction of T7 (first four lanes), and RNase protection of pCR2.1-TOPO-954 linearized with SacI in poly-A+ RNA from F9 EC (5th lane), PrE (6th lane) and PE (7th lane) cells showing upregulation of the protected doublet of approximately 160 bp. **D**: Northern blot analysis of the same poly-A+ RNA samples used in C) of F9 EC (1st lane), PrE (2nd lane) and PE derived from PTHrP treatment (3rd lane) and dbcAMP treatment (4th lane) as a control for the observed upregulation of protected fragments in the RNase protection assay in C).


A
+ - - - + - F9 PE total RNA
- - + - - - yeast total RNA
- + - - - - sense probe
+ + + + + + antisense probe
A C T G + + + + - - RNAse
B
T C A G
5 5 RA (days)
- 2 dbcAMP (days)
C
A C T G
- 5 5 RA (days)
- 2 - PTHrP (days)
- - 2 dbcAMP (days)
D
- 5 5 5 RA (days)
- - 2 - PTHrP (days)
- - - 2 db cAMP (days)
Sna
GAPDH

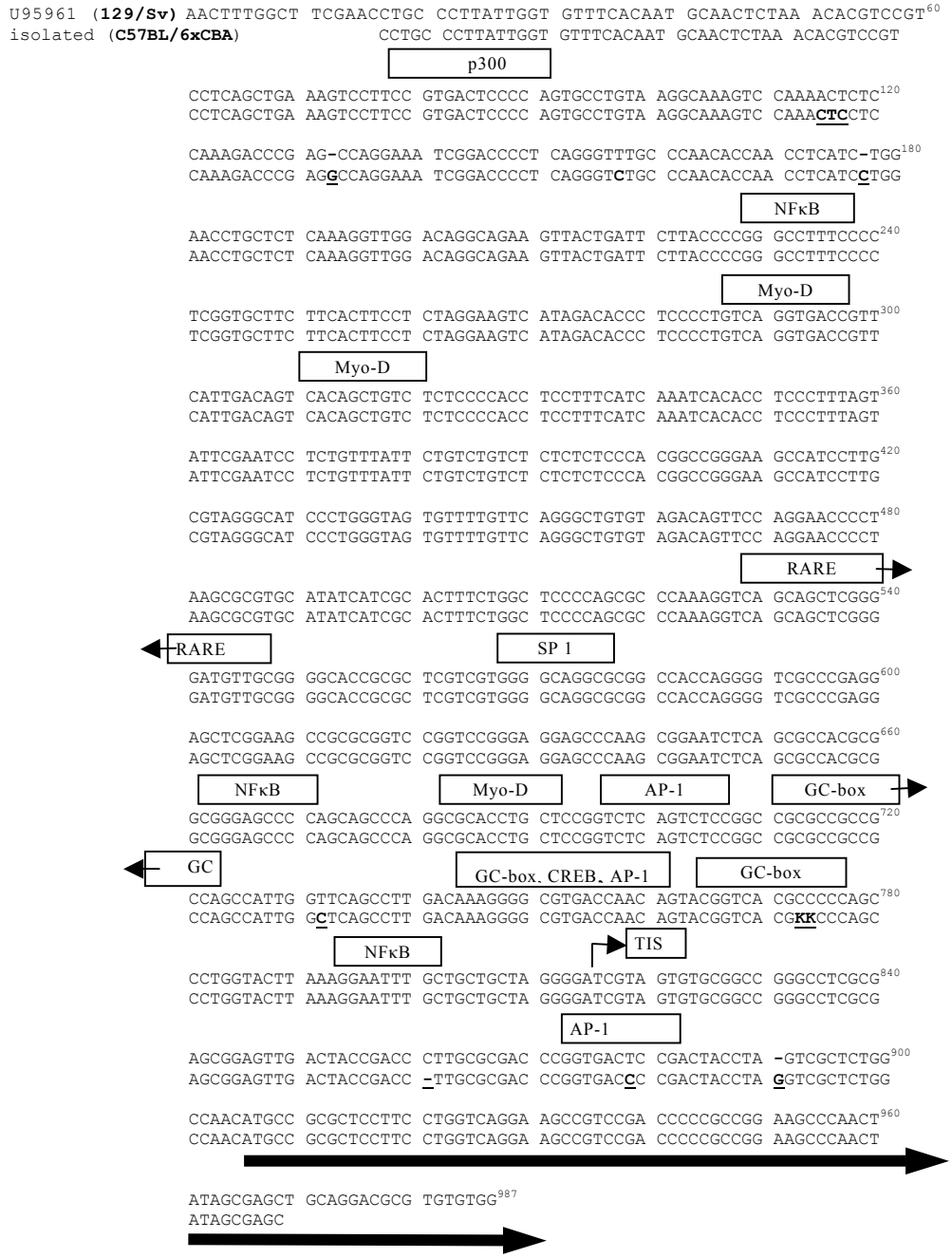


Figure 2: Sequence analysis of the murine *Sna* promoter region.
Two independent pCR2.1-TOPO-954 products were sequenced with the ABI Prism 377 DNA Sequencer (Perkin Elmer, Foster City, CA, USA). Their sequence (bottom line) is was compared to the sequence deposited in Genbank under accession nr. U95961 (top line). Differences in nucleotides are depicted in bold underlined and may be strain specific. DNA binding sites were identified using the MOTIF search database and PAPIA TFSearch database. TIS: transcription initiation site as found by primer extension and RNAse protection analysis, see figure 1.

By primer extension analysis and RNase protection analysis, we identified a transcriptional start site 90 bp upstream of the translation initiation site (figure 1).These techniques yielded products indicating putative other transcrip-

tion initiation sites downstream of the aforementioned site. However, none of these sites were found by both techniques. Moreover, given the existence of EST-sequences partially overlapping the *Sna* coding region and extending up to 74 bp upstream of the translation initiation site (BLAST mouse EST database, NCBI), we here propose the location of the transcription initiation site at 90 bp upstream of the translation initiation site. Thus, like the *Xenopus Sna* gene (Mayor *et al.*, 1993), the mouse *Sna* gene has a short 5' UTR.

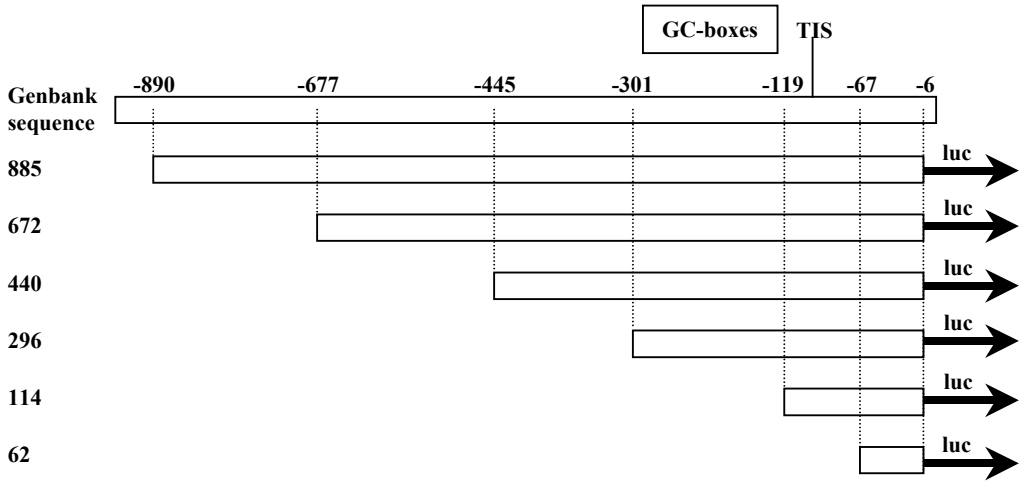


Figure 3: Promoter deletion constructs.
Deletion constructs of the promoter region were generated by fusing the promoter region at the *Msc*1 site (at 6 bp upstream of the translation initiation site) with designated upstream sequences to the *Sma*1 site in pGL2-basic-luc (Promega, Leiden, The Netherlands). As a control, the E1B-TATA box with a 5' flanking *Pst*1 restriction site (AGGGTATATAATG) was inserted between the *Sac*1 and *Xho*1 restriction sites of pGL2-basic-luc (pGL2-E1B). TIS:transcription initiation site.

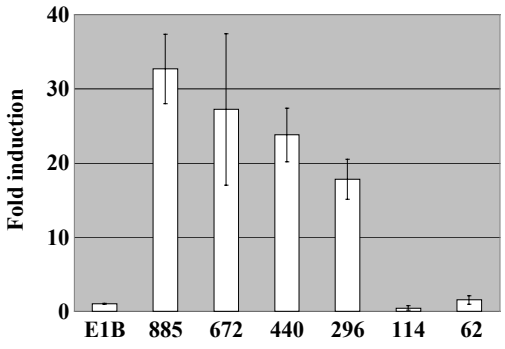


Figure 4: Activity of the promoter deletion constructs in F9 EC cells.
F9 EC cells were seeded 3000 cells/cm^2 in 3.8 cm^2 gelatinized tissue culture wells, and transiently transfected using lipofectamin (Gibco BRL) according to manufacturer's descriptions. For a typical lipofectamin transfection, 0.6 µg promoter construct and 0.4 µg PGK-LacZ were cotransfected with 4 µg lipofectamin in 500 µl medium. Cells were harvested after 2 days and assayed for luciferase expression, corrected for LacZ expression as described before (Wissink *et al.*, 1997). The graph is representative for 2 experiments.

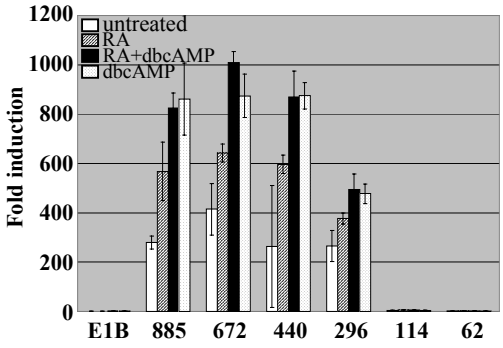


Figure 5: Activity of the promoter deletion constructs in F9 PrE cells.
F9 PrE were seeded 25000 cells/cm^2 and transfected as described under figure 4. After transfection, F9 PrE cells were not treated or received RA and/or dbcAMP to induce differentiation to PE. The graph is representative for 2 experiments.

Sequencing analysis revealed no TATA box in the promoter region, similar to the *Xenopus Sna* gene. However, in contrast to the *Xenopus Sna* gene, the region preceding the transcription initiation site is highly GC-rich, and displays putative binding sites for Sp1 and AP-1 near the transcription initiation site, which may provide the context for binding of the basal transcription machinery. Furthermore, as shown in figure 2, consensus sites for NFκB, as well as for cyclic AMP and retinoic acid response elements (CRE, RARE) are located upstream of this transcription initiation site. In addition we found consen sus sequences for several E-boxes, to which basic helix loop helix factors may bind. Additionally, Sna itself may compete in binding to these sequences, similar to *Drosophila* sna binding to the *Drosophila sna* promoter (Mauhin *et al.*, 1993).

To identify possible functional consensus sites, we generated deletion constructs of this promoter region and fused them to the *luciferase* gene (illustrated in figure 3). These were transfected in F9 EC cells, either untreated, or treated for 3 days with retinoic acid to generate PrE like cells before transfection. As shown in figure 4, the shortest 2 deletion constructs did not confer any luciferase activity in F9 EC cells, nor F9 PrE cells, while the longer constructs, containing at least the transcription initiation site and the GC-boxes did (figure 4). Preliminary data suggest that addition of cAMP to F9 PrE after transfection elevates transcription from the latter constructs (figure 5). This suggests that the CRE consensus sequence at approximately 60 bp upstream of the transcription initiation site may be functional. However, the extent of cAMP elevated luciferase expression is not as high as could be expected from the cAMP elevated *Sna* transcription in F9 PrE cells. Thus, additional or cis-acting elements may be upstream of the promoter sequence we describe here, or sequences that allow binding of RNA stabilizing factors may be located even in the 3'-UTR. Our transfection data await further testing and support from e.g. mutation analysis, footprinting and DNA electrophoretic mobility shift analyses, and will be complemented with analysis of more upstream sequences.

PARIETAL ENDODERM FORMATION; A MODEL

The sequence of events.

Based on the literature, the following model is presented for the sequence of events during PE formation (illustrated in figure 6): During the earliest phase of PE formation in a wild type embryo at the late blastocyst stage the PE precursors acquire a flat fibroblast-like appearance before they migrate onto the basal lamina (Enders *et al.*, 1978; Gardner, 1982). The loss of their cell-cell contacts allows cytoskeletal rearrangements leading to rounding of the cells, first seen at day 6.5 pc (Behrendtsen and Werb, 1997). Blastocyst injections with VE cells of early post-implantation embryos have demonstrated that the donor cells end up in the distal-most area of the PE layer of the host embryo (Cockroft and Gardner, 1987). This may suggest that during normal development, the earliest PE cells become the distal-most PE cells, as is also assumed by Behrendtsen and Werb (1997). These earliest cells don't need to migrate far since the embryo is still small. As such, while the embryo grows and the parietal

yolk sac expands, newly formed PE cells never need to migrate far since they occupy the more proximal regions. They may stop migrating once they have reached their destination, as suggested by the decrease in motility of PE cells from the proximal to more distal regions (Cockroft, 1986). While the yolk sac expands, PE persists until day 13.5 pc with an equal density of cells (Cockroft, 1987), partly due to newly recruited VE cells, and partly due to a residual capacity of PE cells to proliferate (Cockroft, 1986). Until at least around day 13 and 14 pc, the PE cells undergo ongoing morphological modifications by which they become more rounded and their surface smoothens, while their proliferative capacity decreases (Cockroft, 1986).

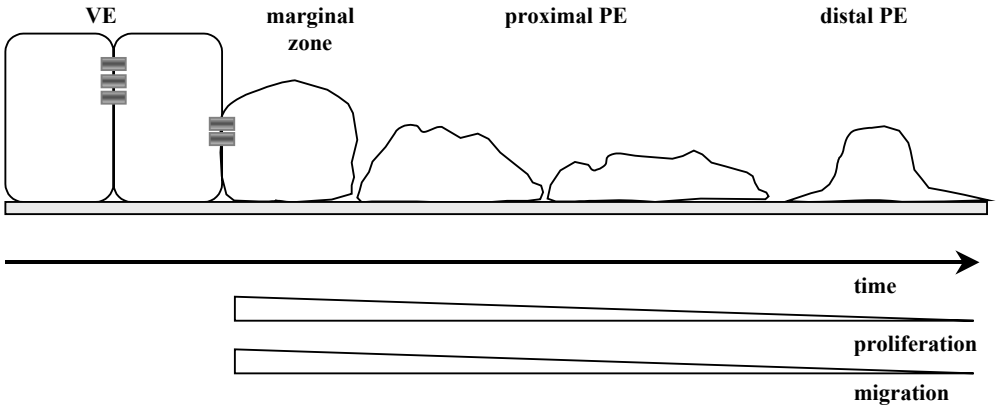


Figure 6: A model for PE formation, the sequence of events.
The morphological changes of endodermal cells occur in time. Since at the same time the embryo grows and new cells occupy the proximal region, the ongoing morphological changes in time can be seen from proximal to distal region. This is correlated to a reduction in motility and migration, as well as proliferation, as indicated by the conical shape of the bars. See also text.

Importantly, the rounding of the cells and their migration over the basal lamina underlying the trophectoderm are both required for proper PE formation *in vivo*, but are independent events, as (i) rounded, differentiated PE cells can form on the outer rim of floating embryoid bodies of ES and EC cells or on isolated egg cylinders in the absence of a lamina or substrate to migrate on (Hogan *et al.*, 1981; Hogan and Tilly, 1981), and (ii) the plating of isolated ICMs and the re-plating of F9 embryoid bodies in the absence of PE-inducing factors results in migration of endodermal cells over the substrate that do not display the PE-specific, rounded morphology, but remain epithelial instead (Behrendtsen *et al.*, 1995) (chapter 2, and time-lapse video data not shown).

The role of PTHrP signaling via the PTH(rP)-RI in PE formation.

Combining our *in vitro* and *in vivo* observations a dual role for PTH(rP)-RI signaling in migration and morphological transformation during PE formation can be proposed. Firstly, PTH(rP)-RI activation enhances migration of PE precursor cells *in vitro* and may do so similarly *in vivo*, but is not required for migration, as this still occurs in PTH(rP)-RI knockout embryos. Secondly, PTH(rP)-RI activation is sufficient and required for the mesenchymal phenotype of PE cells during PE formation.

As has been reviewed in chapter 1,

several studies have demonstrated that cell position (Hogan and Tilly, 1981; Gardner, 1982), cell-cell interactions and even the three-dimensional configuration of the substrate (Casanova and Grabel, 1988), are important in PE-formation. This has led to the hypothesis that the maintenance of the integrity of the epithelial junctions promote the VE phenotype by establishing polarity and/or anchoring the cytoskeleton, while the loss of these junctions would promote differentiation to PE (see also Casanova and Grabel, 1988)). The composition of and interaction with the extracellular matrix is also important for PE formation, e.g. in modulating migration of cells, as also reviewed in chapter 1. Importantly, the immunoreactivity of the outgrown cells from rat inner cell masses to various matrix components depends on the matrix components on which they are cultured (Carnegie and Cabaca, 1993). Further evidence that the interaction with the matrix affects the production of matrix components came from studies with ES cells lacking β1-integrin, which have a decreased laminin production, and show a disorganized basement membrane assembly (Sasaki *et al.*, 1998). Thus the initial interaction of endoderm cells with the matrix affects the secretion of matrix components and as such changes the cell-matrix interactions that a cell experiences. The cell-cell and cell-matrix interactions also affect the morphology of the cells, as observed in outgrowth experiments of rat inner cell masses (Carnegie and Cabaca, 1993), and with vinculin or β1-integrin deficient ES and F9 cells (Stephens *et al.*, 1993; Coll *et al.*, 1995). However, in the case of β1-integrin deficiency, the observed altered morphology and reduced migration did not interfere with PE-specific gene expression (Stephens *et al.*, 1993), indicating that migration and morphological differentiation are not necessarily linked to tissue-specific gene expression.

Evidence for a cooperative interaction between cell-matrix interactions and PTHrP during PE formation came from a study with isolated inner cell masses (Behrendtsen *et al.*, 1995). In that study, a function-perturbing antibody against PTHrP reduced the number of PE cells growing out on various substrates, while the addition of PTHrP made non-permissive concentrations of laminin and vitronectin permissive to outgrowth and PE differentiation. The question that now remains is at which level and for which aspects of PE formation crosstalk from signals derived from PTH(rP)-RI activation and from cell-cell and cell-matrix contacts occurs.

The simplest model for the action of PTHrP in PE would be the breaking of cell-cell contacts in the VE layer, after which the induction of a fibroblastic, later more rounded off cell type with enhanced migration may occur in collaboration with the matrix. PTHrP exerts this effect on cell-cell contacts by a rapid upregulation of *Sna*, which in turn downregulates the transcription of *E-cadherin* and leads to the disintegration of adherens junctions. However, some preliminary observations suggest that the action of PTHrP on cell-cell adhesion may be hampered by pre-existing cell-cell contacts: (i) Solitary F9 PrE cells overexpressing *Sna* have completely lost the expression of adherens junctions within 48 hr after transfection, while cells overexpressing *Sna* while completely or partially surrounded by other cells display a fractionation, but not a complete loss of the *zonula adherens*. Thus the complete loss of epithelial junctions by the action of Sna is less rapid in cells with well-established junctions. Moreover, rounded off cells are

never observed in the middle of epithelial sheets. Since the acquisition of a mesenchymal, i.c. rounded, morphology depends on the loss of cell-cell adhesion, the rapidity with which epithelial cells acquire a mesenchymal phenotype in response to elevation of *Sna* transcription thus seems delayed by the pre-existing cell-cell contacts. It is even possible that PTHrP does not efficiently upregulate *Sna* in cells in epithelial sheets, and would thus not induce dissociation of adherens junctions in these cells at all, (ii) Solitary cells in subconfluent F9 PrE cultures retain membrane localized expression of E-cadherin and β-catenin, components of adherens junctions, albeit it mostly streak-like or punctate instead of as an intact *zonula adherens* (chapter 5). This may allow them to quickly re-establish adherens junctions when they contact other solitary cells. Dissociated leading edge cells in F9 embryoid body outgrowths can reconnect to the trailing cells, as observed in time-lapse video-analyses (not shown), and this may occur *in vivo* as well, which in the presence of residual membrane localized E-cadherin and β-catenin could lead to a re-establishment of the epithelium.

These observations suggest that PTHrP, at least *in vitro*, probably does not actively break cell-cell contacts in the intact epithelium. Instead, PTHrP prevents the re-establishment of cell-cell adhesion once cells have already reduced cell-cell contacts, due to their position at the edge of the epithelium, or due to being already dissociated as a result of e.g. migratory forces, cytokinesis, or signaling from other factors such as the matrix. For example, cells can dissociate from isolated PE layers from PTH(rP)-RI -/- embryos when cultured on fibronectin, as shown in figure 8 in the next section. This indicates that PTHrP signaling via this receptor is not required for breaking of the cell-cell contacts in these cells. PTHrP prevents the re-establishment of cell-cell adhesion in dissociated cells by rapidly upregulating *Sna* transcription, which in turn leads to the downregulation of *E-cadherin* transcription and the dissociation of E-cadherin and β-catenin from the cell-membrane, preventing these cells from reconnecting to other cells. Thus PTHrP is required for the loss of the epithelial character. Given the fact that the F9 model system mimics the *in vivo* situation remarkably well, this mechanism may be applicable to the *in vivo* situation as well, and is therefore added to the model of PE formation illustrated in figure 6, resulting in a model illustrated in figure 7.

PTHrP signaling via the PTH(rP)-RI contributes to, but is not needed for the migration of PE precursors over the basal lamina, as a PE layer is formed in PTH(rP)-RI null mutant embryos. This migration may be induced by signaling derived from the matrix, e.g. fibronectin (reviewed in chapter 1), or from soluble factors, e.g. FGF-4, which is produced in the blastocyst and has been shown to stimulate migration of endoderm cells from isolated inner cell masses (Rappolee *et al.*, 1994). FGF-4 signaling increases the expression of gelatinase B and tPA (Rappolee *et al.*, 1994), which may allow cells to modulate the substrate such, that it facilitates their migration (Behrendtsen and Werb, 1997; Cheng and Grabel, 1997). In accordance with this, preliminary results from Boyden chamber experiments suggest that FGF-4 alone increases migration of F9 PrE cells, and interestingly, when used in combination with PTHrP, a synergistic effect of PTHrP was seen (L. Defize, unpublished results).

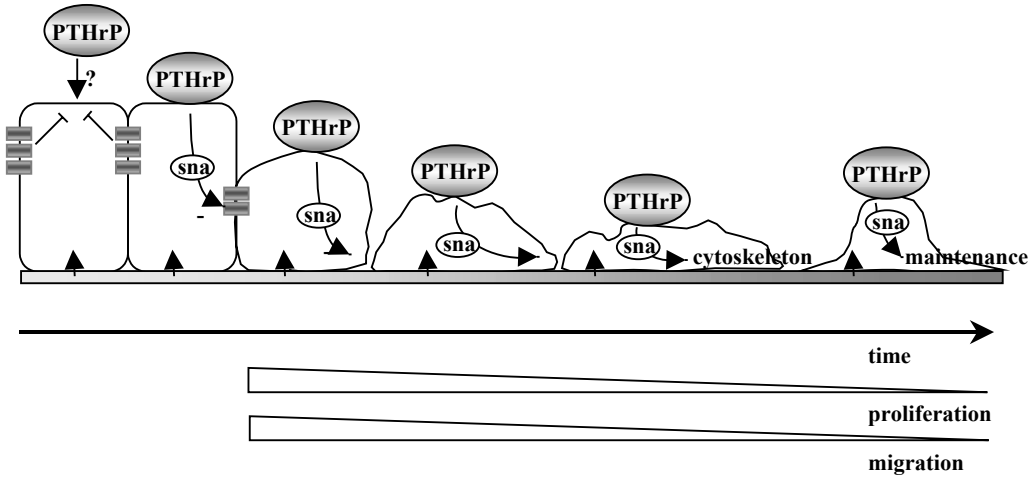


Figure 7: A model for the action of PTHrP in PE formation.
See also text. As is proposed in the model, PTHrP is inhibited by well-established cell-cell contacts. When endodermal cells are only loosely connected to neighboring cells, PTHrP prevents the re-establishment of adherens junctions by interfering with E-cadherin transcription and membrane-localization, and may increase migration. In subsequent phases, PTHrP induces rounding of the cells and a decrease in migration, concomitant with reduced proliferation.

In vitro, PTHrP induces rounding off of F9 PrE cells that are already solitary at the time of PTHrP addition to the culture within 1 hour (not shown). This indicates that PTHrP signaling has a rapid effect on cytoskeletal arrangement in cells without cell-cell adhesive contacts. However, as mentioned above, this effect may be modulated by the matrix, which itself may be modulated by the action of PTHrP. Since PTHrP and PTH(rP)-RI expression by the trophectoderm and PE cells respectively continues until at least day 11 pc (Karperien *et al.*, 1994; Karperien *et al.*, 1996), their action may account for the maintenance of the mesenchymal morphology of existing PE cells and their ongoing smoothening in the distal region (Cockroft, 1986). In addition, their action may be required for the induction of a mesenchymal character in new PE cells.

In vivo, the distal-most cells have the least proliferative capacity (Cockroft, 1987). Similarly, reduced proliferation has been observed upon treatment of F9 PrE with dbcAMP, as assessed by thymidine incorporation (Verheijen, 1997). Preliminary data from time-lapse video analyses showed that 20% of tracked cells residing at the edge of epithelial sheets in F9 PrE cultures at the start of the recording period proliferated during the first 16 hr. after PTHrP addition. In contrast, none of the cells that were solitary at the time of PTHrP addition proliferated during the same period (not shown), suggesting a reduced proliferation in solitary cells compared to cells at the edge of epithelial sheets in F9 PrE cultures treated with PTHrP. These observations support a role for PTHrP in reducing the proliferation in the distal PE cells *in vivo*.

Finally, PTH(rP)-RI activation is required for terminal PE differentiation, as no PE specific enhanced thrombomodulin expression is observed in the PE layer of PTH(rP)-RI knockout embryos. So far we have no evidence that enhanced thrombomodulin expression depends on the morphological transition. However, in the model PE differentiation by the action of PTHrP is only inhi-

bited by cell-cell contacts. Thus, like the morphological transition, high thrombomodulin expression would depend on the breaking of cell-cell contacts.

Is *snail* a master gene in PE formation?

The formation of a PE layer in PTH(rP)-RI knockout embryos demonstrates that PTHrP signaling is not needed for migration of endodermal cells *in vivo*. Interestingly, despite the absence of PTHrP signaling, *Sna* is expressed in the endoderm of the marginal zone and the proximal region of the PE layer. One can ask if this *Sna* expression plays a role in the onset of migration of those cells, as *Sna* expression has been observed by others in premigratory cells during gastrulation and neural crest migration in various species (Nieto *et al.*, 1992; Smith *et al.*, 1992; Hemavathy *et al.*, 2000). Similarly, we have observed a basal level of *Sna* expression in F9 PrE and VE (chapter 4 of this thesis), which are both migratory in the absence of exogenous PTHrP (chapter 2 and data not shown). The region where *Sna* is expressed in the mutant PE coincides with the region where the highest motility of PE cells is observed in the wild type situation, while still displaying a 'blebby' morphology (Cockroft, 1986). Additionally, others and we have shown that overexpression of *Sna* induces migration in epithelial cells (chapter 5) (Cano *et al.*, 2000), while ablation of the function of *Sna* orthologues inhibits migration (Carl *et al.*, 1999; Hemavathy *et al.*, 2000; LaBonne and Bronner-Fraser, 2000). Thus it is tempting to assume that this expression of *Sna* induces, and may even be necessary for, the migration of PE cells observed in mutant embryos. Moreover, the *Sna* expression in the proximal region of PE in the PTH(rP)-RI knockout indicates that there is another factor besides PTHrP inducing *Sna* during PE formation, which could thus be partially responsible for PE formation. For reasons mentioned earlier, it is likely that this factor could be fibronectin or FGF-4, which could be studied in F9 differentiation. Interestingly, besides having the appropriate spatio-temporal expression and the capacity to induce migration, FGF-4 has been demonstrated to enhance expression of the chick *Sna* orthologue in limb formation (Buxton *et al.*, 1997).

The epithelial character of the PE cells of PTH(rP)-RI deficient embryos at day 8.5 and 9.5 pc suggests that a transition to mesenchymal morphology does not occur in the absence of PTHrP signaling, e.g. because *Sna* expression is not sufficiently high. However, we can not exclude that a transient mesenchymal morphology is induced early in the formation of mutant PE, e.g. by the *Sna* expression observed at day 7.5 pc in the proximal region (chapter 5) and/or by signals derived from cell-matrix contacts (Behrendtsen *et al.*, 1995). Such signals may be induced by fibronectin, as we have observed that PE isolates from mutant embryos, when cultured on fibronectin, show outgrowth of solitary cells lacking membrane-localized β-catenin expression (J. Hendriks, unpublished results, figure 8).

Since fibronectin elevates cAMP in endothelial cells (Fong and Ingber, 1996) and downregulates N-cadherin activity in migrating neural crest cells (Monier-Gavelle and Duband, 1997), it may exert a similar action on cAMP and E-cadherin in PrE or VE, explaining its PE-inducing potential. This potential may still involve the action of Sna, since addition of dbcAMP to PrE elevates *Sna* transcription in these cells (chapter 4). Moreover, fibronectin induces PE diffe-

rentiation, as it enhances thrombomodulin expression upon re-plating of F9 embryoid bodies (not shown). Although it is still controversial whether fibronectin is available during later stages of *in vivo* PE formation, it is not unlikely that it contributes to morphology and differentiation early during PE formation, as mentioned in chapter 1. Nonetheless, the aberrant PE cells of mutant embryos at day 8.5 and 9.5 pc suggest that PTHrP signaling is needed for the maintenance and/or elevation of *Sna* expression in the distal PE cells, and indicate that PTHrP signaling is necessary for the maintenance of or progression to the mesenchymal phenotype and PE-specific differentiation of these cells.

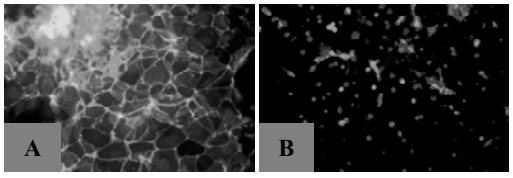


Figure 8: Fibronectin induces a mesenchymal phenotype in PE cells from PTH(rP)-RI -/- embryos.
PTH(rP)-RI -/- PE layers were isolated at day 8.5 pc and cultured on a substrate coated with 50 mg/ml fibronectin. They were used for standard immunodetection of β-catenin. While the cells in the original isolate form an intact epithelial sheet with membrane localized expression of β-catenin (**A**), fibronectin induces migration of cells out of this sheet, with the acquisition of a rounded cell body and lack of membrane localized expression of β-catenin (**B**). As such, these solitary cells resemble wild type PE cells.

We can now propose a model (illustrated in figure 9) in which *Sna* is induced by PTHrP signaling and another factor(s), during PE formation. While the *Sna* expression induced in the proximal PE cells by the unidentified factor(s), e.g. FGF-4 or fibronectin, may provide the migration and optional transient mesenchymal morphology of these cells, elevation and/or maintenance of *Sna* expression by PTHrP is necessary for the mesenchymal phenotype of the distal, definitive PE. If *Sna* expression would be sufficient and required for migration and morphological transformation, as well as PE-specific gene expression, e.g. thrombomodulin, it would act as a true master gene in PE formation. While we have demonstrated that *Sna* expression enhances migration and is sufficient to induce mesenchymal morphology, it is conceivable that it is sufficient and also required for both aspects, as (i) several reports have indicated that *Sna* or an orthologue is necessary for the migration and morphological changes during EMT, (ii) *Sna* is expressed in the migratory, proximal PE cells, even in the absence of PTHrP signaling, and (iii) *Sna* -/- embryos show defective PE formation, although the nature of this defect is not described (Jiang *et al.*, 1998a). Although the mor phological transition during EMT is generally considered to be dependent on the loss of cell-cell adhesion, it is not clear whether all the signals that induce the mesenchymal phenotype are downstream of E-cadherin. Since the transfection of antisense *E-cadherin* in keratinocytes is not sufficient to induce a full EMT in these cells (Llorens *et al.*, 1998), and moreover, transfection of *E-cadherin* is not sufficient to revert the fibroblastic phenotype of spindle carcinoma cells (Navarro *et al.*, 1993; Lozano and Cano, 1998), it is likely that some events are mediated by other tar gets of Sna. These may be genes involved in the control of adhesion and migration, such as α6-integrin, which contains E-boxes in its promoter region, and of which the α6B isoform is downregulated during PE formation (Jiang and Grabel, 1995; Morini *et al.*, 1997). *Sna* may also act as a transcriptional activator (Mauhin *et al.*, 1993; Nakayama *et al.*, 1998; Hemavathy *et al.*, 2000) and induce other genes required for the acquisition of the mesenchymal pheno

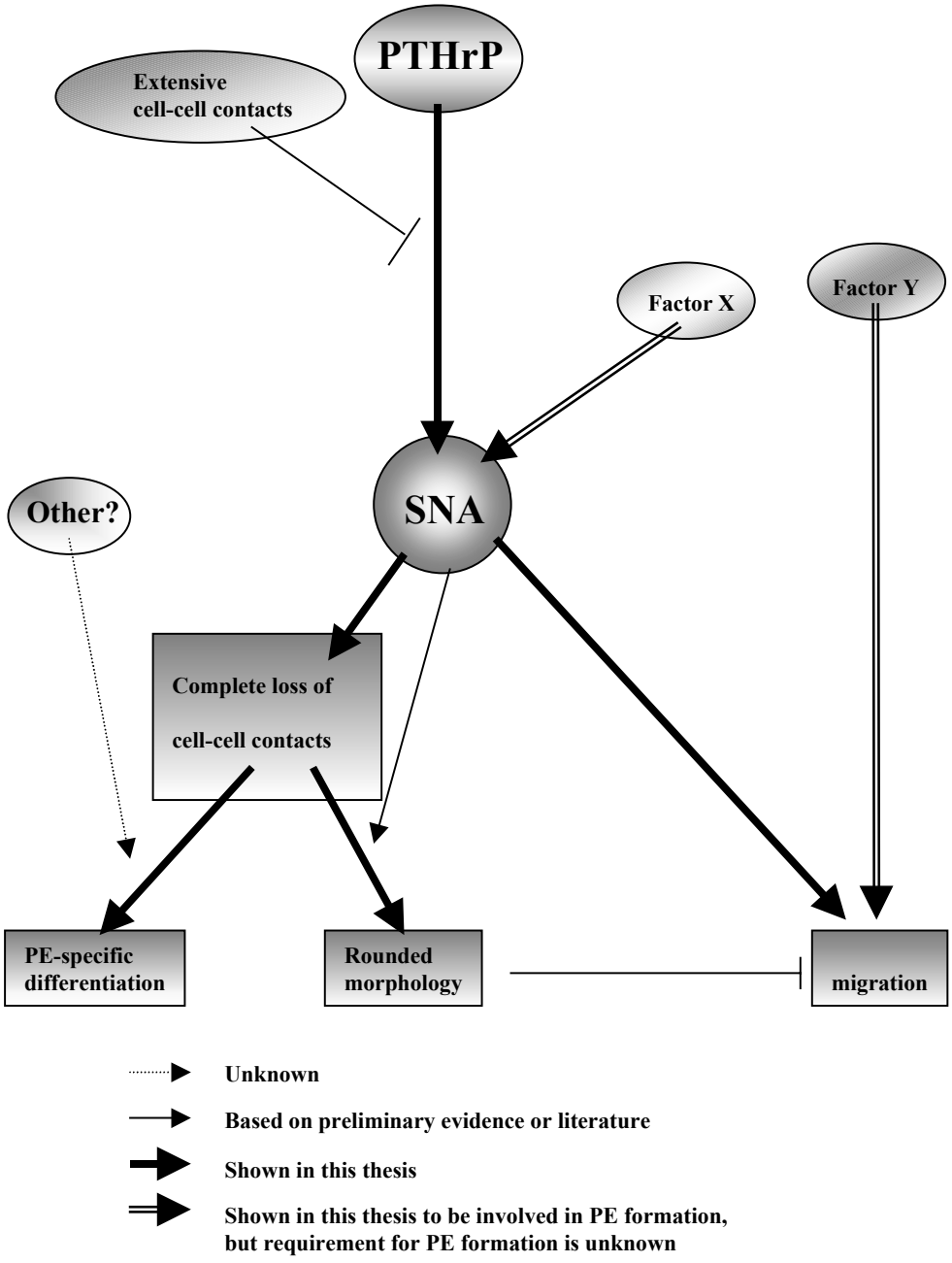


Figure 9: A model for the role of Sna in PE formation.
See also text. Factor X can be factor Y, e.g. fibronectin or FGF-4, and may have an inductive role in migration. Additionally, it can be the LNγ subunit, which needs to be incorporated in the basal membrane to allow migration (Smyth *et al.*, 1999).

type, such as vimentin, which is shown to be upregulated upon transfection of *Sna* in MDCK cells (Cano *et al.*, 2000).

Detailed analysis of PE formation in *Sna* -/- embryos and restoration of PE specific *Sna* expression in PTH(rP)-RI-/- ES cells or embryos (e.g. transgenic *Sna* under the control of the PTH(rP)-RI

promoter) would give clues as to whether *Sna* is indeed a master gene in PE formation. A more detailed and quantitative comparison of *Sna* expression during PE formation, as well as regional analysis of PE morphology in the PTH(rP)-RI mutant and wild type embryos in a larger time-window would be required to test this hypothesis.

TOWARDS UNDERSTANDING THE PHENOTYPE OF PTH(RP)-RI NULL MUTANT EMBRYOS

While the first C57BL/6 PTH(rP)-RI knockout embryos die between day 9.5 and 12.5 pc, and only 3% of the embryos that reach day 18.5 of gestation have the homozygous knockout genotype, this percentage is 12% among Black Swiss embryos (Lanske *et al.*, 1996). Despite the reduced lethality found among Black Swiss knockout embryos, they are still proportionally smaller (Lanske *et al.*, 1996), although it is unclear whether they display the same extent of growth retardation as C57BL/6 knockout embryos. This points to an essential role for PTH(rP)-RI signaling in growth of the early post-implantation mouse embryo. C57BL/6 knockout embryos are first growth retarded at day 8.5 pc, and until that stage the receptor in wild type embryos is only expressed in the PE and its precursors in wild type embryos. Therefore, the growth retardation may be an effect of absence of PTH(rP)-RI signaling in the VE in a yet unidentified function other than inducing PE formation. For example, if due to the lack of the PTH(rP)-RI VE cells would be affected in their function in metabolite transfer to the embryo and the regulation of the volume of the extraembryonic fluids, this could lead to growth retardation (Payne and Deuchar, 1972). Alternatively, the growth retardation could be an effect of absence of PTH(rP)-RI signaling in the proper formation or maintenance of PE, or in both VE and PE.

How could the absence of PTH(rP)-RI signaling in PE lead to growth retardation? Several mechanisms may either alone or in combination cause the retardation. Firstly, based on the lack of increased thrombomodulin expression in differentiated PTH(rP)-RI -/- ES cells *in vitro*, we hypothesized before that PTH(rP)-RI -/- embryos may be growth retarded because of a lack of thrombomodulin expression by their PE cells (Verheijen *et al.*, 1999). Support for this contention came from a study on thrombomodulin -/- embryos, which display a phenotype with striking similarity to PTH(rP)-RI -/- embryos: they are smaller at day 8.5 pc, and show lethality before day 10.5 pc (Healy *et al.*, 1995). In chapter 2 of this thesis, we demonstrate that the PE cells of PTH(rP)-RI -/- embryos indeed do not express elevated levels of thrombomodulin. Since thrombomodulin functions as an anticoagulant, it is conceivable that the lack or reduction of thrombomodulin expression in the parietal yolk sac leads to a dysfunctional maternal-embryonic interaction, possibly interfering with the permeability of parietal yolk sac by the deposition of fibrin, or the degradation of laminin, as discussed by Healy *et al.*, (1995), similar to fibrin deposition in lung by thrombomodulin -/- cells in thrombomodulin chimaeric mice (Healy *et al.*, 1998). Thus the lack of PE specific enhanced thrombomodulin expression in PTH(rP)-RI knockout embryos further supports the contention that the growth retardation of these embryos is the result of a dysfunctional PE.

Secondly, we hypothesized before, that the PE cells lacking the PTH(rP)-RI may produce insufficient matrix components to build the Reichert's membrane and give it the appropriate properties for nutrient and waste exchange (Verheijen, 1997). This was supported with results from a study in which day 9.0 pc rat embryos were injected with antibodies against the Reichert's membrane, leading to growth retardation or even death (Leung, 1977). Since we have demonstrated in chapter 2 that the PE cells in mutant embryos failed to differentiate to true PE cells, in terms of morphology and thrombomodulin expression, it is conceivable that they also do not function as true PE cells, e.g. in secreting matrix factors. Besides regulating the permeability of the Reichert's membrane, it is also possible that a lack of secretion of ECM molecules by the PE cells does not allow the Reichert's membrane to expand sufficiently to allow embryonic growth. Thus it is of interest to test the PTH(rP)-RI -/- PE cells for their secretion of various ECM molecules, and compare the composition and dimensions of the Reichert's membrane of knockout embryos with that of their wild type littermates.

Thirdly, the epithelial morphology of the PE layer in knockout embryos, as demonstrated in chapter 2 of this thesis, may prevent the exchange of nutrients and waste. The presence of tight junctions could, by preventing the movement of membrane lipids and proteins between the apical and basal compartments of the cell membrane, interfere with e.g. the availability of receptors for their ligands. Additionally, tight junctions could reduce the permeability of the parietal yolk sac. Therefore, it is of interest to study the existence of tight junctions and the permeability of the PE layer in knockout embryos. This could be done by performing electron microscopy and/or e.g. analyzing the penetration of tracer macromolecules, e.g. various sizes of dextran coupled to FITC (Kubota *et al.*, 2001) through the parietal yolk sac after incubating dissected embryos with an intact parietal yolk sac in a solution containing these molecules.

Fourthly, PTHrP and its receptor are often expressed at sites of epithelio-mesenchymal reciprocal interactions (EMI), e.g. in skin, teeth, and mammary gland development (Karperien *et al.*, 1994, 1996; Wysolmerski *et al.*, 1994, 1995, 1998; Philbrick *et al.*, 1998; Foley *et al.*, 2001). Since the trophectoderm is initially an epithelial tissue, which is replaced by secondary trophectoderm after de-epithelialization of the first, it is possible that PTHrP signaling has a function in an EMI between trophectoderm and PE. If so, the lack of PTHrP signaling in mutant PE may lead to aberrant development or functioning of the (secondary) trophectoderm layer, thus to an aberrant maternal-embryonic interaction.

The lethality observed in C57BL/6 PTH(rP)-RI -/- embryos may be a result of the growth retardation. Therefore, it remains an intriguing question what can explain the reduced lethality of Black Swiss compared to C57BL/6 knockout embryos (Lanske *et al.*, 1996). We have so far analyzed the PE layers of 36 Black Swiss embryos, dissected at day 8.5 and 9.5 pc and derived from 4 litters of crosses between PTH(rP)-RI +/- mice. The cells of all these isolates display a mesenchymal, PE-specific rounded morphology (J. Hendriks and L. Defize, data not shown). Till now we have genotyped only 1 litter of 6 embryos, and found one homozygous knockout. Based on Mendelian ratios, we could expect to find approximately 7

more knockout embryos, which would then all possess a PE layer with mesenchymal cells. Even though this morphologically apparently normally developed PE does not prevent growth retardation, these preliminary data suggest that the increased lethality among C57BL/6 knockout embryos be correlated with the epithelial PE morphology. The factor inducing the presumed mesenchymal PE morphology in Black Swiss knockout embryos, and the specific PTHrP response associated with embryonic growth, remains enigmatic.

PTHrP SIGNALING IN TUMOR PROGRESSION

As mentioned in the introduction of this chapter, PTHrP signaling is not only important for PE formation and organogenesis during embryonic development, but is also associated with a variety of diseases, such as bone malformation (e.g. Jansen's osteochondrodysplasia), cardiovascular disorders (e.g. hypertension) and malignant transformation in neoplastic disorders. Interestingly, PE formation and malignant transformation both occur via an EMT, and thus our findings concerning the role of PTHrP in PE formation may provide clues to the role of PTHrP in tumor progression. Of particular interest may be the role of PTHrP in breast tumors: Approximately 60% of primary breast neoplasms and more than 90% of metastatic foci of breast tumors in bone have been reported to constitutively express PTHrP, while only 20% of soft tissue (e.g. lung) metastases express PTHrP (Southby *et al.*, 1990; Powell *et al.*, 1991; Vargas *et al.*, 1992). High levels of PTHrP expression by these tumors and their metastasis to bone have been associated with a poor prognosis for the patient, and moreover, breast cancer is the main cause of death due to malignancy in west European women. The progression of epithelial breast tumors to a malignant phenotype is associated with a loss of E-cadherin, and high levels of *Sna* expression, while importantly, overexpression of *Sna* in epithelial breast tumor cell lines is sufficient to induce a metastatic phenotype (Battle et al. 2000; Cano et al., 2000). Human *SNAIL* is detectably expressed in all 7 soft tissue metastases of MDA-MB-231 breast tumor cells, and none of the 7 bone metastases examined (S. Bleuming, G. van der Pluijm and L. Defize, unpublished). Moreover *Sna* expression is elevated upon PTHrP treatment of MDA-MB-231 cells in culture. Given the role of PTHrP and *Sna* in PE formation, these data strongly suggest that PTHrP, via *Sna*, may play an inductive role in breast tumor progression. A similar mechanism may exist in some skin tumors, as PTHrP has been found to stimulate skin tumorigenesis in nude mice (Manenti *et al.*, 2000).

Interestingly, PTHrP contributes to prostate cancer progression by preventing apoptosis of the metastasizing cells (Dougherty *et al.*, 1999). Elevated PTHrP levels are associated with a variety of hematological malignancies (Goltzman and Henderson, 1996), while a human and *C. elegans Sna* orthologue prevent apoptosis, in human thus contributing to transformation of pro-B cells (Inukai *et al.*, 1999; Metzstein and Horvitz, 1999). Therefore, a different mechanism downstream of PTHrP, but possibly still via a *Sna* orthologue may act in such tumors.

SUMMARIZING REMARKS

The work described in this thesis demonstrates an inductive role of PTHrP and its type I receptor in cell migration, morphological transformation and differentiation, three distinct aspects of the EMT as it occurs during PE formation, as well as its requirement for the latter two aspects. The gene encoding the zinc finger transcription factor Sna has been identified as an immediate early target of PTH(rP)-RI signaling in PE formation, and its involvement in migration and morphological transformation during PE formation has been demonstrated. A model is presented in which migration and morphological transition during PE formation are two separable events, in which the action of PTH(rP)-RI signaling via *Sna* is inhibited by cell-cell contacts. We propose that the signaling mechanism of PTHrP via *Sna,* here identified in PE formation, acts similarly in (breast) tumor progression.

The here demonstrated lack of morphological transformation and differentiation of the PE cells in PTH(rP)-RI -/- embryos is strongly suggestive for a dysfunctional PE. This dysfunction may reside in reduced nutrient transfer from mother to embryo, and/or in aberrant formation of the Reichert's membrane. In turn, this may explain the growth retardation and early death of such embryos. Studies concerning the composition and permeability of the Reichert's membrane or parietal yolk sac as a whole may reveal more insight herein, and possibly also allow to explain the strain-dependent mortality observed in PTH(rP)-RI deficient embryos. A detailed analysis of PE formation in *Sna* -/- ES cells and embryos, and a rescue approach introducing a *Sna* transgene in PTH(rP)-RI -/- PE cells may answer the question whether *Sna* functions as a master gene in PE formation and differentiation.

ACKNOWLEDGMENTS

I would like to thank J. Hendriks, S. Bleuming, and Drs. G. van der Pluijm and L. Defize for allowing me to use their unpublished results. I would also like to express my gratitude to R. van Horssen, E. de Groot and T. Hoeijmakers for their valuable contribution to the analysis of the murine *snail* promoter.

REFERENCES

ABOU-SAMRA, A.B., JÜPPNER, H., FORCE, T., FREEMAN, M.W., KONG, X.F., SCHIPANI, E., URENA, P., RICHARDS, J., BONVENTRE, J.V. and POTTS, J.T.J. (1992). Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. *Proc. Natl. Acad. Sci. U. S. A. 89:* 2732-2736.

ACEVEDO, P. and BERTRAM, J.S. (1995). Liarozole potentiates the cancer chemopreventive activity of and the up-regulation of gap junctional communication and connexin43 expression by retinoic acid and beta-carotene in 10T1/2 cells. *Carcinogenesis 16:* 2215-2222.

ADAMSON, E.D. and AYERS, S.E. (1979). The localization and synthesis of some collagen types in developing mouse embryos. *Cell 16:* 953-965.

ALBERTS B., BRAY D., LEWIS J., RAFF M., ROBERTS K., and WATSON J.D. (Eds.) (1983). *Molecular biology of the cell*. Garland Publishing Inc., New York.

AMENTA, P.S., CLARK, C.C. and MARTINEZ-HERNANDEZ, A. (1983). Deposition of fibronectin and laminin in the basement membrane of the rat parietal yolk sac: immunohistochemical and biosynthetic studies. *J. Cell Biol. 96:* 104-111.

AOKI, F., WORRAD, D.M. and SCHULTZ, R.M. (1997). Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo. *Dev. Biol. 181:* 296-307.

ASHRAF, S.I., HU, X., ROOTE, J. and IP, Y.T. (1999). The mesoderm determinant Snail collaborates with related zinc-finger proteins to control Drosophila neurogenesis. *EMBO J. 18:* 6426-6438.

ATKINSON, M.M., LAMPE, P.D., LIN, H.H., KOLLANDER, R., LI, X.R. and KIANG, D.T. (1995). Cyclic AMP modifies the cellular distribution of connexin43 and induces a persistent increase in the junctional permeability of mouse mammary tumor cells. *J. Cell Sci. 108:* 3079-3090.

BALDA, M.S. and ANDERSON, J.M. (1993). Two classes of tight junctions are revealed by ZO-1 isoforms. *Am. J. Physiol. 264:* C918-C924.

BANI-YAGHOUB, M., BECHBERGER, J.F. and NAUS, C.C. (1997). Reduction of connexin43 expression and dye-coupling during neuronal differentiation of human NTera2/clone D1 cells. *J. Neurosci. Res. 49:* 19-31.

BANI-YAGHOUB, M., UNDERHILL, T.M. and NAUS, C.C. (1999). Gap junction blockage interferes with neuronal and astroglial differentiation of mouse P19 embryonal carcinoma cells. *Dev. Genet. 24:* 69-81.

BARLOW, P.W. and SHERMAN, M.I. (1972). The biochemistry of differentiation of mouse trophoblast: studies on polyploidy. *J. Embryol. Exp. Morphol. 27:* 447-465.

BATLLE, E., SANCHO, E., FRANCI, C., DOMINGUEZ, D., MONFAR, M., BAULIDA, J. and GARCIA, D.H. (2000). The transcription factor Snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat. Cell Biol. 2:* 84-89.

BEHRENDTSEN, O., ALEXANDER, C.M. and WERB, Z. (1995). Cooperative interactions between extracellular matrix, integrins and parathyroid hormone-related peptide regulate parietal endoderm differentiation in mouse embryos. *Development 121:* 4137-4148.

BEHRENDTSEN, O. and WERB, Z. (1997). Metalloproteinases regulate parietal endoderm differentiating and migrating in cultured mouse embryos. *Dev. Dyn. 208:* 255-265.

BELLIVEAU, D.J., BECHBERGER, J.F., ROGERS, K.A. and NAUS, C.C. (1997). Differential expression of gap junctions in neurons and astrocytes derived from P19 embryonal carcinoma cells. *Dev. Genet. 21:* 187-200.

BERSTINE, E.G., HOOPER, M.L., GRANDCHAMP, S. and EPHRUSSI, B. (1973). Alkaline phosphatase activity in mouse teratoma. *Proc. Natl. Acad. Sci. U. S. A. 70:* 3899-3903.

BEVANS, C.G. and HARRIS, A.L. (1999). Direct high affinity modulation of connexin channel activity by cyclic nucleotides. *J. Biol. Chem. 274:* 3720-3725.

BEX, V., MERCIER, T., CHAUMONTET, C., GAILLARD-SANCHEZ, I., FLECHON, B., MAZET, F., TRAUB, O. and MARTEL, P. (1995). Retinoic acid enhances connexin43 expression at the post-transcriptional level in rat liver epithelial cells. *Cell Biochem. Funct. 13:* 69-77.

BIELINSKA, M., NARITA, N. and WILSON, D.B. (1999). Distinct roles for visceral endoderm during embryonic mouse development. *Int. J. Dev. Biol. 43:* 183-205.

BIRCHMEIER, C., BIRCHMEIER, W. and BRAND-SABERI, B. (1996). Epithelial-mesenchymal transitions in cancer progression. *Acta Anat. (Basel.) 156:* 217-226.

BOULAY, J.L., DENNEFELD, C. and ALBERGA, A. (1987). The Drosophila developmental gene snail encodes a protein with nucleic acid binding fingers. *Nature 330:* 395-398.

BOYER, B., VALLES, A.M. and THIERY, J.P. (1996). Model systems of epithelium-mesenchyme transitions. *Acta Anat. (Basel.) 156:* 227-239.

BREWER, L.M. and BROWN, N.A. (1992). Distribution of p21ras in postimplantation rat embryos. *Anat. Rec. 234:* 443-451.

BRINGHURST, F.R., JUPPNER, H., GUO, J., URENA, P., POTTS, J.T.J., KRONENBERG, H.M., ABOU-SAMRA, A.B. and SEGRE, G.V. (1993). Cloned, stably expressed parathyroid hormone (PTH)/PTH-related peptide receptors activate multiple messenger signals and biological responses in LLC-PK1 kidney cells. *Endocrinology 132:* 2090-2098.

BROWN, S.J. and DENELL, R.E. (1996). Segmentation and dorsoventral patterning in Tribolium. *Cell & Developmental Biology 7:* 553-560.

BRUZZONE, R., WHITE, T.W. and PAUL, D.L. (1996). Connections with connexins: the molecular basis of direct intercellular signaling. *Eur. J. Biochem. 238:* 1-27.

BUXTON, P.G., KOSTAKOPOULOU, K., BRICKELL, P., THOROGOOD, P. and FERRETTI, P. (1997). Expression of the transcription factor slug correlates with growth of the limb bud and is regulated by FGF-4 and retinoic acid. *Int. J. Dev. Biol. 41:* 559-568.

CANO, A., PEREZ-MORENO, M.A., RODRIGO, I., LOCASCIO, A., BLANCO, M.J., DEL BARRIO, M.G., PORTILLO, F. and NIETO, M.A. (2000). The transcription factor Snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat. Cell Biol. 2:* 76-83.

CARL, T.F., DUFTON, C., HANKEN, J. and KLYMKOWSKY, M.W. (1999). Inhibition of neural crest migration in Xenopus using antisense slug RNA. *Dev. Biol. 213:* 101-115.

CARNEGIE, J.A. (1994). Influence of extracellular matrix gradients on the haptotactic migration of F9 embryocarcinoma-derived primitive and parietal endoderm-like cells. *Biol. Reprod. 50:* 413-420.

CARNEGIE, J.A. and CABACA, O. (1993). Extracellular matrix composition and resilience: two parameters that influence the in vitro migration and morphology of rat inner cell mass-derived cells. *Biol. Reprod. 48:* 287-299.

CASANOVA, J.E. and GRABEL, L.B. (1988). The role of cell interactions in the differentiation of teratocarcinoma-derived parietal and visceral endoderm. *Dev. Biol. 129:* 124-139.

CHAN, S.D., STREWLER, G.J., KING, K.L. and NISSENSON, R.A. (1990). Expression of a parathyroid hormone-like protein and its receptor during differentiation of embryonal carcinoma cells. *Mol. Endocrinol. 4:* 638-646.

CHENG, A.M., SAXTON, T.M., SAKAI, R., KULKARNI, S., MBAMALU, G., VOGEL, W., TORTORICE, C.G., CARDIFF, R.D., CROSS, J.C., MULLER, W.J. and PAWSON, T. (1998). Mammalian Grb2 regulates multiple steps in embryonic development and malignant transformation. *Cell 95:* 793-803.

CHENG, L. and GRABEL, L.B. (1997). The involvement of tissue-type plasminogen activator in parietal endoderm outgrowth. *Exp. Cell Res. 230:* 187-196.

CHOMCZYNSKI, P. and SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem. 162:* 156-159.

CHOREV, M. and ROSENBLATT, M. (1996). Parathyroid hormone: structure-function relations and analog design. In *Principles of bone biology*, (Eds. Bilezikian J.P., Raisz L.G.,

and Rodan G.A.) Academic Press Inc., San Diego, pp. 305-323.

CIVITELLI, R., ZIAMBARAS, K., WARLOW, P.M., LECANDA, F., NELSON, T., HARLEY, J., ATAL, N., BEYER, E.C. and STEINBERG, T.H. (1998). Regulation of connexin43 expression and function by prostaglandin E2 (PGE2) and parathyroid hormone (PTH) in osteoblastic cells. *J. Cell Biochem. 68:* 8-21.

CLAIRMONT, A. and SIES, H. (1997). Evidence for a posttranscriptional effect of retinoic acid on connexin43 gene expression via the 3'-untranslated region. *FEBS Lett. 419:* 268-270.

CLAIRMONT, A., TESSMANN, D. and SIES, H. (1996). Analysis of connexin43 gene expression induced by retinoic acid in F9 teratocarcinoma cells. *FEBS Lett. 397:* 22-24.

CLARK, C.C., MINOR, R.R., KOSZALKA, T.R., BRENT, R.L. and KEFALIDES, N.A. (1975). The embryonic rat parietal yolk sac. Changes in the morphology and composition of its basement membrane during development. *Dev. Biol. 46:* 243-261.

CLAUDE, P. and GOODENOUGH, D.A. (1973). Fracture faces of zonulae occludentes from "tight" and "leaky" epithelia. *J. Cell Biol. 58:* 390-400.

COCKROFT, D.L. (1986). Regional and temporal differences in the parietal endoderm of the midgestation mouse embryo. *J. Anat. 145:35-47:* 35-47.

COCKROFT, D.L. (1987). Growth and proliferation in mouse parietal yolk sac during whole embryo culture. *J. Reprod. Fertil. 81:* 575-581.

COCKROFT, D.L. and GARDNER, R.L. (1987). Clonal analysis of the developmental potential of 6th and 7th day visceral endoderm cells in the mouse. *Development 101:* 143-155.

COLL, J.L., BEN-ZE'EV, A., EZZELL, R.M., RODRIGUEZ, F.J., BARIBAULT, H., OSHIMA, R.G. and ADAMSON, E.D. (1995). Targeted disruption of vinculin genes in F9 and embryonic stem cells changes cell morphology, adhesion, and locomotion. *Proc. Natl. Acad. Sci. U. S. A. 92:* 9161-9165.

COLLINS, J.E. and FLEMING, T.P. (1995). Epithelial differentiation in the mouse preimplantation embryo: making adhesive cell contacts for the first time. *Trends. Biochem. Sci. 20:* 307-312.

CROSS, J.C., WERB, Z. and FISHER, S.J. (1994). Implantation and the placenta: key pieces of the development puzzle. *Science 266:* 1508-1518.

DAHL, E., WINTERHAGER, E., REUSS, B., TRAUB, O., BUTTERWECK, A. and WILLECKE, K. (1996). Expression of the gap junction proteins connexin31 and connexin43 correlates with communication compartments in extraembryonic tissues and in the gastrulating mouse embryo, respectively. *J. Cell Sci. 109:* 191-197.

DAMSKY, C.H., RICHA, J., SOLTER, D., KNUDSEN, K. and BUCK, C.A. (1983). Identification and purification of a cell surface glycoprotein mediating intercellular adhesion in embryonic and adult tissue. *Cell 34:* 455-466.

DARROW, B.J., FAST, V.G., KLEBER, A.G., BEYER, E.C. and SAFFITZ, J.E. (1996). Functional and structural assessment of intercellular communication. Increased conduction velocity and enhanced connexin expression in dibutyryl cAMP-treated cultured cardiac myocytes. *Circ. Res. 79:* 174-183.

DAVIES, J.A. and GARROD, D.R. (1997). Molecular aspects of the epithelial phenotype. *Bioessays 19:* 699-704.

DIWAN, S.B. and STEVENS, L.C. (1976). Development of teratomas from the ectoderm of mouse egg cylinders. *J. Natl. Cancer Inst. 57:* 937-942.

DONAHUE, H.J., MCLEOD, K.J., RUBIN, C.T., ANDERSEN, J., GRINE, E.A., HERTZBERG, E.L. and BRINK, P.R. (1995). Cell-to-cell communication in osteoblastic networks: cell line-dependent hormonal regulation of gap junction function. *J. Bone Miner. Res. 10:* 881-889.

DOUGHERTY, K.M., BLOMME, E.A., KOH, A.J., HENDERSON, J.E., PIENTA, K.J., ROSOL, T.J. and MCCAULEY, L.K. (1999). Parathyroid hormone-related protein as a growth regulator of prostate carcinoma. *Cancer Res. 59:* 6015-6022.

DUBAND, J.L., MONIER, F., DELANNET, M. and NEWGREEN, D. (1995). Epithelium-mesenchyme transition during neural crest development. *Acta Anat. (Basel.) 154:* 63-78.

DUCIBELLA, T., ALBERTINI, D.F., ANDERSON, E. and BIGGERS, J.D. (1975). The preimplantation mammalian embryo: characterization of intercellular junctions and their appearance during development. *Dev. Biol. 45:* 231-250.

DUPREY, P., MORELLO, D., VASSEUR, M., BABINET, C., CONDAMINE, H., BRULET, P. and JACOB, F. (1985). Expression of the cytokeratin endo A gene during early mouse embryogenesis. *Proc. Natl. Acad. Sci. U. S. A. 82:* 8535-8539.

DZIADEK, M. (1978). Modulation of alphafetoprotein synthesis in the early postimplantation mouse embryo. *J. Embryol. Exp. Morphol. 46:* 135-146.

DZIADEK, M. and ADAMSON, E. (1978). Localization and synthesis of alphafoetoprotein in post-implantation mouse embryos. *J. Embryol. Exp. Morphol. 43:* 289-313.

EMERSON, J.A. (1988). Disruption of the cytokeratin filament network in the preimplantation mouse embryo. *Development 104:* 219-234.

EMERY, J.F. and BIER, E. (1995). Specificity of CNS and PNS regulatory subelements comprising pan-neural enhancers of the deadpan and scratch genes is achieved by repression. *Development 121:* 3549-3560.

ENDERS, A.C. (1997). The role of hypoblast endoderm in early develoment of higher primates. In *Microscopy of reproduction and develoment: a dynamic approach*, (Ed. Motta P.M.) Antonio Delfino Editore, Rome, pp. 299-305.

ENDERS, A.C., GIVEN, R.L. and SCHLAFKE, S. (1978). Differentiation and migration of endoderm in the rat and mouse at implantation. *Anat. Rec. 190:* 65-77.

ERIVES, A., CORBO, J.C. and LEVINE, M. (1998). Lineage-specific regulation of the Ciona snail gene in the embryonic mesoderm and neuroectoderm. *Dev. Biol. 194:* 213-225.

ESSEX, L.J., MAYOR, R. and SARGENT, M.G. (1993). Expression of Xenopus snail in mesoderm and prospective neural fold ectoderm. *Dev. Dyn. 198:* 108-122.

EVAIN, D., BINET, E. and ANDERSON, W.B. (1981). Alterations in calcitonin and parathyroid hormone responsiveness of adenylate cyclase in F9 embryonal carcinoma cells treated with retinoic acid and dibutyryl cyclic AMP. *J. Cell Physiol. 109:* 453-459.

FAWCETT D.W. (Ed.) (1981). *The Cell*. W.B. Saunders, Philadelphia.

FEIJEN, A., GOUMANS, M.J. and VAN DEN EIJNDEN-VAN RAAIJ, A.J. (1994). Expression of activin subunits, activin receptors and follistatin in postimplantation mouse embryos suggests specific developmental functions for different actiVins. *Development 120:* 3621-3637.

FOLEY, J., DANN, P., HONG, J., COSGROVE, J., DREYER, B., RIMM, D., DUNBAR, M.E., PHILBRICK, W. and WYSOLMERSKI, J.J. (2001). Parathyroid hormone-related protein maintains mammary epithelial fate and triggers nipple skin differentiation during embryonic breast development. *Development 128:* 513-525.

FONG, J.H. and INGBER, D.E. (1996). Modulation of adhesion-dependent cAMP signaling by echistatin and alendronate. *Biochem. Biophys. Res. Commun. 221:* 19-24.

FRANKE, W.W., GRUND, C., KUHN, C., JACKSON, B.W. and ILLMENSEE, K. (1982). Formation of cytoskeletal elements during mouse embryogenesis. III. Primary mesenchymal cells and the first appearance of vimentin filaments. *Differentiation. 23:* 43-59.

FREEMAN, S.J. (1990). Functions of extraembryonic membranes. In *Postimplantation mammalian embryos, a practical approach*, (Eds. Copp A.J. and Cockroft D.L.) Oxford University Press, New York, pp. 249-265.

FREEMAN, W.M., WALKER, S.J. and VRANA, K.E. (1999). Quantitative RT-PCR: pitfalls and potential. *Biotechniques 26:* 112-115.

FUJIMOTO, K., NAGAFUCHI, A., TSUKITA, S., KURAOKA, A., OHOKUMA, A. and SHIBATA, Y. (1997). Dynamics of connexins, E-cadherin and alpha-catenin on cell membranes during gap junction formation. *J. Cell Sci. 110:* 311-322.

FURUSE, M., HIRASE, T., ITOH, M., NAGAFUCHI, A., YONEMURA, S. and TSUKITA, S. (1993). Occludin: a novel integral membrane protein localizing at tight junctions. *J. Cell Biol. 123:* 1777-1788.

FUSE, N., HIROSE, S. and HAYASHI, S. (1994). Diploidy of Drosophila imaginal cells is maintained by a transcriptional repressor encoded by escargot. *Genes Dev. 8:* 2270-2281.

FUSE, N., HIROSE, S. and HAYASHI, S. (1996). Determination of wing cell fate by the escargot and snail genes in Drosophila. *Development 122:* 1059-1067.

GAO, Y. and SPRAY, D.C. (1998). Structural changes in lenses of mice lacking the gap junction protein connexin43. *Invest. Ophthalmol. Vis. Sci. 39:* 1198-1209.

GARDELLA T.J., WILDON, A.K., KEUTMAN, H.T., OBERSTEIN, R., POTTS, J.R.T., KRONENBERG, H.M. and MUSSBAUM, S.R. (1993). Analysis of parathyroid hormone's principal receptor-binding region by site-directed mutagenesis and analog design. *Endocrinology 132:* 2024-2030.

GARDNER, R.L. (1978). The relationship between cell lineage and differentiation in the early mouse embryo. *Results. Probl. Cell Differ. 9:205-41:* 205-241.

GARDNER, R.L. (1982). Investigation of cell lineage and differentiation in the extraembryonic endoderm of the mouse embryo. *J. Embryol. Exp. Morphol. 68:175-98:* 175-198.

GARDNER, R.L. (1983). Origin and differentiation of extraembryonic tissues in the mouse. *Int. Rev. Exp. Pathol. 24:63-133:* 63-133.

GARDNER, R.L. (1984). An in situ cell marker for clonal analysis of development of the extraembryonic endoderm in the mouse. *J. Embryol. Exp. Morphol. 80:251-88:* 251-288.

GARDNER, R.L. (1985). Clonal analysis of early mammalian development. *Philos. Trans. R. Soc. Lond. B. Biol. Sci. 312:* 163-178.

GARDNER, R.L., DAVIES, T.J. and CAREY, M.S. (1988). Effect of delayed implantation on differentiation of the extra-embryonic endoderm in the mouse blastocyst. *Placenta. 9:* 343-359.

GARDNER, R.L. and JOHNSON, M.H. (1972). An investigation of inner cell mass and trophoblast tissues following their isolation from the mouse blastocyst. *J. Embryol. Exp. Morphol. 28:* 279-312.

GARDNER, R.L., PAPAIOANNOU, V.E. and BARTON, S.C. (1973). Origin of the ectoplacental cone and secondary giant cells in mouse blastocysts reconstituted from isolated trophoblast and inner cell mass. *J. Embryol. Exp. Morphol. 30:* 561-572.

GARDNER, R.L. and ROSSANT, J. (1979). Investigation of the fate of 4-5 day post-coitum mouse inner cell mass cells by blastocyst injection. *J. Embryol. Exp. Morphol. 52:* 141-152.

GIEPMANS, B.N. and MOOLENAAR, W.H. (1998). The gap junction protein connexin43 interacts with the second PDZ domain of the zona occludens-1 protein. *Curr. Biol. 8:* 931-934.

GIROLDI, L.A., BRINGUIER, P.P., DE WEIJERT, M., JANSEN, C., VAN BOKHOVEN, A. and SCHALKEN, J.A. (1997). Role of E boxes in the repression of E-cadherin expression. *Biochem. Biophys. Res. Commun. 241:* 453-458.

GOLTZMAN, D. and HENDERSON, J.E. (1996). Expressio of PTHrP in disease. In *Principles of bone biology*, (Eds. Bilezikian J.P., Raisz L.G., and Rodan G.A.) Academic Press Inc., San Diego, pp. 809-836.

GOODENOUGH, D.A., GOLIGER, J.A. and PAUL, D.L. (1996). Connexins, connexons, and intercellular communication. *Annu. Rev. Biochem. 65:* 475-502.

GOSSEN, M. and BUJARD, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. U. S. A. 89:* 5547-5551.

GRABEL, L.B. and CASANOVA, J.E. (1986). The outgrowth of parietal endoderm from mouse teratocarcinoma stem-cell embryoid bodies. *Differentiation. 32:* 67-73.

GRABEL, L.B. and WATTS, T.D. (1987). The role of extracellular matrix in the migration and differentiation of parietal endoderm from teratocarcinoma embryoid bodies. *J. Cell Biol. 105:* 441-448.

GRAU, Y., CARTERET, C. and SIMPSON, P. (1984). Mutations and chromosomal rearangements affecting the expression of snail, a gene involved in embryonic patterning in *Drosophila Melanogaster*. *Genetics 108:* 347-360.

GRIMES, H.L., CHAN, T.O., ZWEIDLER-MCKAY, P.A., TONG, B. and TSICHLIS, P.N. (1996). The Gfi-1 proto-oncoprotein contains a novel transcriptional repressor domain, SNAG, and inhibits G1 arrest induced by interleukin-2 withdrawal. *Mol. Cell Biol. 16:* 6263-6272.

GROVER, A. and ADAMSON, E.D. (1985). Roles of extracellular matrix components in differentiating teratocarcinoma cells. *J. Biol. Chem. 260:* 12252-12258.

GROVER, A. and ADAMSON, E.D. (1986). Conditions affecting the differentiation of F9 teratocarcinoma cells: potentiation of response by cyclic AMP. *In Vitro Cell Dev. Biol. 22:* 280-284.

GROVER, A. and ADAMSON, E.D. (1986). Evidence for the existence of an early common biochemical pathway in the differentiation of F9 cells into visceral or parietal endoderm: modulation by cyclic AMP. *Dev. Biol. 114:* 492-503.

GROVER, A., ANDREWS, G. and ADAMSON, E.D. (1983). Role of laminin in epithelium formation by F9 aggregates. *J. Cell Biol. 97:* 137-144.

GROVER, A., OSHIMA, R.G. and ADAMSON, E.D. (1983). Epithelial layer formation in differentiating aggregates of F9 embryonal carcinoma cells. *J. Cell Biol. 96:* 1690-1696.

GROVER, A., ROSENTRAUS, M.J., STERMAN, B., SNOOK, M.E. and ADAMSON, E.D. (1987). An adhesion-defective variant of F9 embryonal carcinoma cells fails to differentiate into visceral endoderm. *Dev. Biol. 120:* 1-11.

GUAN, X. and RUCH, R.J. (1996). Gap junction endocytosis and lysosomal degradation of connexin43-P2 in WB-F344 rat liver epithelial cells treated with DDT and lindane. *Carcinogenesis 17:* 1791-1798.

HAMMERSCHMIDT, M. and NUSSLEIN-VOLHARD, C. (1993). The expression of a zebrafish gene homologous to Drosophila snail suggests a conserved function in invertebrate and vertebrate gastrulation. *Development 119:* 1107-1118.

HARDIN, J. (1995). Target recognition by mesenchyme cells during sea urchin gastrulation. *American Zoology 35:* 358-371.

HAY, E.D. (1995). An overview of epithelio-mesenchymal transformation. *Acta Anat. (Basel.) 154:* 8-20.

HAYASHI, S. (1996). A Cdc2 dependent checkpoint maintains diploidy in Drosophila. *Development 122:* 1051-1058.

HAYASHI, S., HIROSE, S., METCALFE, T. and SHIRRAS, A.D. (1993). Control of imaginal cell development by the escargot gene of Drosophila. *Development 118:* 105-115.

HEALY, A.M., HANCOCK, W.W., CHRISTIE, P.D., RAYBURN, H.B. and ROSENBERG, R.D. (1998). Intravascular coagulation activation in a murine model of thrombomodulin deficiency: effects of lesion size, age, and hypoxia on fibrin deposition. *Blood 92:* 4188-4197.

HEALY, A.M., RAYBURN, H.B., ROSENBERG, R.D. and WEILER, H. (1995). Absence of the blood-clotting regulator thrombomodulin causes embryonic lethality in mice before development of a functional cardiovascular system. *Proc. Natl. Acad. Sci. U. S. A. 92:* 850-854.

HEMAVATHY, K., ASHRAF, S.I. and IP, Y.T. (2000). Snail/slug family of repressors: slowly going into the fast lane of development and cancer. *Gene 257:* 1-12.

HOGAN, B., BARLOW, D.P. and TILLY, R. (1983). F9 teratocarcinoma cells as a model for the differentiation of parietal and visceral endoderm in the mouse embryo. *Cancer Surv. 2:* 115-140.

HOGAN B., BEDDINGTON R., COSTANTINI F., and LACY E. (Eds.) (1994). *Manipulating the mouse embryo, a laboratory manual*. Cold Spring Harbor Laboratory Press, Plainview, NY.

HOGAN, B. (1981). Laminin and epithelial cell attachment. *Nature 290:* 737-738.

HOGAN, B.L. (1980). High molecular weight extracellular proteins synthesized by endoderm cells derived from mouse teratocarcinoma cells and normal extraembryonic membranes. *Dev. Biol. 76:* 275-285.

HOGAN, B.L., COOPER, A.R. and KURKINEN, M. (1980). Incorporation into Reichert's membrane of laminin-like extracellular proteins synthesized by parietal endoderm cells of the mouse embryo. *Dev. Biol. 80:* 289-300.

HOGAN, B.L. and NEWMAN, R. (1984). A scanning electron microscope study of the extraembryonic endoderm of

the 8th-day mouse embryo. *Differentiation. 26:* 138-143.

HOGAN, B.L., TAYLOR, A. and ADAMSON, E. (1981). Cell interactions modulate embryonal carcinoma cell differentiation into parietal or visceral endoderm. *Nature 291:* 235-237.

HOGAN, B.L., TAYLOR, A. and COOPER, A.R. (1982). Murine parietal endoderm cells synthesise heparan sulphate and 170K and 145K sulphated glycoproteins as components of Reichert's membrane. *Dev. Biol. 90:* 210-214.

HOGAN, B.L., TAYLOR, A., KURKINEN, M. and COUCHMAN, J.R. (1982). Synthesis and localization of two sulphated glycoproteins associated with basement membranes and the extracellular matrix. *J. Cell Biol. 95:* 197-204.

HOGAN, B.L. and TILLY, R. (1981). Cell interactions and endoderm differentiation in cultured mouse embryos. *J. Embryol. Exp. Morphol. 62:379-94:* 379-394.

HOSSAIN, M.Z., AO, P. and BOYNTON, A.L. (1998). Platelet-derived growth factor-induced disruption of gap junctional communication and phosphorylation of connexin43 involves protein kinase C and mitogen-activated protein kinase. *J. Cell Physiol. 176:* 332-341.

HOWE, C.C. and SOLTER, D. (1980). Identification of noncollagenous basement membrane glycopolypeptides synthesized by mouse parietal entoderm and an entodermal cell line. *Dev. Biol. 77:* 480-487.

IMADA, M., IMADA, S., IWASAKI, H., KUME, A., YAMAGUCHI, H. and MOORE, E.E. (1987). Fetomodulin: marker surface protein of fetal development which is modulatable by cyclic AMP. *Dev. Biol. 122:* 483-491.

IMADA, S., YAMAGUCHI, H. and IMADA, M. (1990b). Differential expression of fetomodulin and tissue plasminogen activator to characterize parietal endoderm differentiation of F9 embryonal carcinoma cells. *Dev. Biol. 141:* 426-430.

IMADA, S., YAMAGUCHI, H., NAGUMO, M., KATAYANAGI, S., IWASAKI, H. and IMADA, M. (1990a). Identification of fetomodulin, a surface marker protein of fetal development, as thrombomodulin by gene cloning and functional assays. *Dev. Biol. 140:* 113-122.

INUKAI, T., INOUE, A., KUROSAWA, H., GOI, K., SHINJYO, T., OZAWA, K., MAO, M., INABA, T. and LOOK, A.T. (1999). SLUG, a ces-1-related zinc finger transcription factor gene with antiapoptotic activity, is a downstream target of the E2A-HLF oncoprotein [In Process Citation]. *Mol. Cell 4:* 343-352.

IP, Y.T., PARK, R.E., KOSMAN, D., BIER, E. and LEVINE, M. (1992). The dorsal gradient morphogen regulates stripes of rhomboid expression in the presumptive neuroectoderm of the Drosophila embryo. *Genes Dev. 6:* 1728-1739.

ITOH, M., NAGAFUCHI, A., MOROI, S. and TSUKITA, S. (1997). Involvement of ZO-1 in cadherin-based cell adhesion through its direct binding to alpha catenin and actin filaments. *J. Cell Biol. 138:* 181-192.

JÜPPNER, H., ABOU-SAMRA, A.B., FREEMAN, M., KONG, X.F., SCHIPANI, E., RICHARDS, J., KOLAKOWSKI, L.F., HOCK, J., POTTS, J.T. and KRONENBERG, H.M. (1991). A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. *Science 254:* 1024-1026.

JIANG, R, LAN, Y, NORTON, C, and GRIDLEY, T (1998a). Defects in gastrulation and mesoderm migration in mice lacking the Sna gene. *Developmental Biology 198:* 224 (Abstr.).

JIANG, R., COPELAND, N.G., GILBERT, D.J., JENKINS, N.A. and GRIDLEY, T. (1997). Genomic organization and chromosomal localization of the mouse snail (Sna) gene. *Mamm. Genome 8:* 686-688.

JIANG, R. and GRABEL, L.B. (1995). Function and differential regulation of the alpha 6 integrin isoforms during parietal endoderm differentiation. *Exp. Cell Res. 217:* 195-204.

JIANG, R., LAN, Y., NORTON, C.R., SUNDBERG, J.P. and GRIDLEY, T. (1998b). The Slug gene is not essential for mesoderm or neural crest development in mice. *Dev. Biol. 198:* 277-285.

JOLLIE, W.P. (1968). Changes in the fine structure of the parietal yolk sac of the rat placenta with increasing gestational age. *Am. J. Anat. 122:* 513-531.

KALIMI, G.H. and LO, C.W. (1988). Communication compartments in the gastrulating mouse embryo. *J. Cell Biol. 107:* 241-255.

KALIMI, G.H. and LO, C.W. (1989). Gap junctional communication in the extraembryonic tissues of the gastrulating mouse embryo. *J. Cell Biol. 109:* 3015-3026.

KARAPLIS, A.C., LUZ, A., GLOWACKI, J., BRONSON, R.T., TYBULEWICZ, V.L., KRONENBERG, H.M. and MULLIGAN, R.C. (1994). Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes Dev. 8:* 277-289.

KARPERIEN, M., LANSER, P., DE LAAT, S.W., BOONSTRA, J. and DEFIZE, L.H. (1996). Parathyroid hormone related peptide mRNA expression during murine postimplantation development: evidence for involvement in multiple differentiation processes. *Int. J. Dev. Biol. 40:* 599-608.

KARPERIEN, M., VAN DIJK, T.B., HOEIJMAKERS, T., CREMERS, F., ABOU-SAMRA, A.B., BOONSTRA, J., DE LAAT, S.W. and DEFIZE, L.H. (1994). Expression pattern of parathyroid hormone/parathyroid hormone related peptide receptor mRNA in mouse postimplantation embryos indicates involvement in multiple developmental processes. *Mech. Dev. 47:* 29-42.

KARTENBECK, J., SCHWECHHEIMER, K., MOLL, R. and FRANKE, W.W. (1984). Attachment of vimentin filaments to desmosomal plaques in human meningiomal cells and arachnoidal tissue. *J. Cell Biol. 98:* 1072-1081.

KASAI, Y., NAMBU, J.R., LIEBERMAN, P.M. and CREWS, S.T. (1992). Dorsal-ventral patterning in Drosophila: DNA binding of snail protein to the single-minded gene. *Proc. Natl. Acad. Sci. U. S. A. 89:* 3414-3418.

KATAOKA, H., MURAYAMA, T., YOKODE, M., MORI, S., SANO, H., OZAKI, H., YOKOTA, Y., NISHIKAWA, S. and KITA, T. (2000). A novel snail-related transcription factor Smuc regulates basic helix-loop-helix transcription factor activities via specific E-box motifs. *Nucleic. Acids. Res. 28:* 626-633.

KAUFMAN M.H. (Ed.) (1992). *The atlas of mouse development*. Academic Press Limited, London, UK.

KELLER, G., KENNEDY, M., PAPAYANNOPOULOU, T. and WILES, M.V. (1993). Hematopoietic commitment during embryonic stem cell differentiation in culture. *Mol. Cell Biol. 13:* 473-486.

KELLER, R., SHIH, J. and SATER, A. (1992). The cellular basis of the convergence and extension of the Xenopus neural plate. *Dev. Dyn. 193:* 199-217.

KEMLER, R., BRULET, P., SCHNEBELEN, M.T., GAILLARD, J. and JACOB, F. (1981). Reactivity of monoclonal antibodies against intermediate filament proteins during embryonic development. *J. Embryol. Exp. Morphol. 64:45-60:* 45-60.

KENNEL, S.J., HOTCHKISS, J.A., RORVIK, M.C., ALLISON, D.P. and FOOTE, L.J. (1987). Rat monoclonal antibodies to mouse lung components for analysis of fibrosis. *Exp. Mol. Pathol. 47:* 110-124.

KESTER, H.A., VAN DER LEEDE, B.M., VAN DER SAAG, P.T. and VAN DER BURG, B. (1997). Novel progesterone target genes identified by an improved differential display technique suggest that progestin-induced growth inhibition of breast cancer cells coincides with enhancement of differentiation. *J. Biol. Chem. 272:* 16637-16643.

KHAN-DAWOOD, F.S., YANG, J. and DAWOOD, M.Y. (1996). Expression of gap junction protein connexin-43 in the human and baboon (Papio anubis) corpus luteum. *J. Clin. Endocrinol. Metab. 81:* 835-842.

KHURI, F.R., CHO, Y. and TALMAGE, D.A. (1996). Retinoic acid-induced transition from protein kinase C beta to protein kinase C alpha in differentiated F9 cells: correlation with altered regulation of proto-oncogene expression by phorbol esters. *Cell Growth Differ. 7:* 595-602.

KINDREGAN, H.C., ROSENBAUM, S.E., OHNO, S. and NILES, R.M. (1994). Characterization of conventional protein kinase C (PKC) isotype expression during F9 teratocarcinoma differentiation. Overexpression of PKC alpha alters the expression of some differentiation-dependent genes. *J. Biol. Chem. 269:* 27756-27761.

KONG, X.F., SCHIPANI, E., LANSKE, B., JOUN, H., KARPERIEN, M., DEFIZE, L.H., JUPPNER, H., POTTS, J.T.J., SEGRE, G.V. and KRONENBERG, H.M. (1994). The rat, mouse and human genes encoding the receptor for parathyroid hormone and parathyroid hormone-related peptide are highly homologous [published erratum appears in

Biochem Biophys Res Commun 1994 Jun 15;201(2):1058]. *Biochem. Biophys. Res. Commun. 200:* 1290-1299.

KOSMAN, D., IP, Y.T., LEVINE, M. and ARORA, K. (1991). Establishment of the mesoderm-neuroectoderm boundary in the Drosophila embryo. *Science 254:* 118-122.

KOUKLIS, P.D., HUTTON, E. and FUCHS, E. (1994). Making a connection: direct binding between keratin intermediate filaments and desmosomal proteins. *J. Cell Biol. 127:* 1049-1060.

KUBOTA, H., CHIBA, H., TAKAKUWA, Y., OSANAI, M., TOBIOKA, H., KOHAMA, G.I., MORI, M. and SAWADA, N. (2001). Retinoid X Receptor alpha and Retinoic Acid Receptor gamma Mediate Expression of Genes Encoding Tight-Junction Proteins and Barrier Function in F9 Cells during Visceral Endodermal Differentiation. *Exp. Cell Res. 263:* 163-172.

KURKI, P., LAASONEN, A., TAN, E.M. and LEHTONEN, E. (1989). Cell proliferation and expression of cytokeratin filaments in F9 embryonal carcinoma cells. *Development 106:* 635-640.

KURKINEN, M., BARLOW, D.P., JENKINS, J.R. and HOGAN, B.L. (1983). In vitro synthesis of laminin and entactin polypeptides. *J. Biol. Chem. 258:* 6543-6548.

KUSUMI, A., SUZUKI, K. and KOYASAKO, K. (1999). Mobility and cytoskeletal interactions of cell adhesion receptors. *Curr. Opin. Cell Biol. 11:* 582-590.

LABONNE, C. and BRONNER-FRASER, M. (1998). Neural crest induction in Xenopus: evidence for a two-signal model. *Development 125:* 2403-2414.

LABONNE, C. and BRONNER-FRASER, M. (2000). Snail-related transcriptional repressors are required in Xenopus for both the induction of the neural crest and its subsequent migration. *Dev. Biol. 221:* 195-205.

LAING, J.G. and BEYER, E.C. (1995). The gap junction protein connexin43 is degraded via the ubiquitin proteasome pathway. *J. Biol. Chem. 270:* 26399-26403.

LAING, J.G., TADROS, P.N., WESTPHALE, E.M. and BEYER, E.C. (1997). Degradation of connexin43 gap junctions involves both the proteasome and the lysosome. *Exp. Cell Res. 236:* 482-492.

LANE, E.B., HOGAN, B.L., KURKINEN, M. and GARRELS, J.I. (1983). Co-expression of vimentin and cytokeratins in parietal endoderm cells of early mouse embryo. *Nature 303:* 701-704.

LANGELAND, J.A., TOMSA, J.M., JACKMAN, W.R.J. and KIMMEL, C.B. (1998). An amphioxus snail gene: expression in paraxial mesoderm and neural plate suggests a conserved role in patterning the chordate embryo. *Dev. Genes Evol. 208:* 569-577.

LANSKE, B., KARAPLIS, A.C., LEE, K., LUZ, A., VORTKAMP, A., PIRRO, A., KARPERIEN, M., DEFIZE, L.H.K., HO, C., MULLIGAN, R.C., ABOU-SAMRA, A.B., JUPPNER, H., SEGRE, G.V. and KRONENBERG, H.M. (1996). PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science 273:* 663-666.

LEE, H.Y., CHUNG, H.Y., KIM, K.H., LEE, J.J. and KIM, K.W. (1994). Induction of differentiation in the cultured F9 teratocarcinoma stem cells by triterpene acids. *J. Cancer Res. Clin. Oncol. 120:* 513-518.

LEHTONEN, E. and BADLEY, R.A. (1980). Localization of cytoskeletal proteins in preimplantation mouse embryos. *J. Embryol. Exp. Morphol. 55:* 211-225.

LEHTONEN, E., LEHTO, V.P., BADLEY, R.A. and VIRTANEN, I. (1983). Formation of vinculin plaques precedes other cytoskeletal changes during retinoic acid-induced teratocarcinoma cell differentiation. *Exp. Cell Res. 144:* 191-197.

LEHTONEN, E., LEHTO, V.P., PAASIVUO, R. and VIRTANEN, I. (1983). Parietal and visceral endoderm differ in their expression of intermediate filaments. *EMBO J. 2:* 1023-1028.

LEHTONEN, E. and REIMA, I. (1986). Changes in the distribution of vinculin during preimplantation mouse development. *Differentiation. 32:* 125-134.

LEIVO, I., VAHERI, A., TIMPL, R. and WARTIOVAARA, J. (1980). Appearance and distribution of collagens and laminin in the early mouse embryo. *Dev. Biol. 76:* 100-114.

LEPTIN, M. (1995). Drosophila gastrulation: from pattern formation to morphogenesis. *Annu. Rev. Cell Dev. Biol. 11:189-212:* 189-212.

LEUNG, C.C. (1977). Embryotoxic effects of heterologous antisera against rat Reichert's membrane. *J. Exp. Zool. 200:* 295-301.

LLORENS, A., RODRIGO, I., LOPEZ-BARCONS, L., GONZALEZ-GARRIGUES, M., LOZANO, E., VINYALS, A., QUINTANILLA, M., CANO, A. and FABRA, A. (1998). Down-regulation of E-cadherin in mouse skin carcinoma cells enhances a migratory and invasive phenotype linked to matrix metalloproteinase-9 gelatinase expression. *Lab. Invest. 78:* 1131-1142.

LO, C.W. and GILULA, N.B. (1979). Gap junctional communication in the post-implantation mouse embryo. *Cell 18:* 411-422.

LOZANO, E. and CANO, A. (1998). Induction of mutual stabilization and retardation of tumor growth by coexpression of plakoglobin and E-cadherin in mouse skin spindle carcinoma cells. *Mol. Carcinog. 21:* 273-287.

MACKAY, J.P. and CROSSLEY, M. (1998). Zinc fingers are sticking together. *Trends. Biochem. Sci. 23:* 1-4.

MALLETTE, L.E. (1991). The parathyroid polyhormones: new concepts in the spectrum of peptide hormone action. *Endocr. Rev. 12:* 110-117.

MANENTI, G., PEISSEL, B., GARIBOLDI, M., FALVELLA, F.S., ZAFFARONI, D., ALLARIA, B., PAZZAGLIA, S., REBESSI, S., COVELLI, V., SARAN, A. and DRAGANI, T.A. (2000). A cancer modifier role for parathyroid hormone-related protein. *Oncogene 19:* 5324-5328.

MAROTTI, K.R., BELIN, D. and STRICKLAND, S. (1982). The production of distinct forms of plasminogen activator by mouse embryonic cells. *Dev. Biol. 90:* 154-159.

MARTIN, W., ZEMPEL, G., HULSER, D. and WILLECKE, K. (1991). Growth inhibition of oncogene-transformed rat fibroblasts by cocultured normal cells: relevance of metabolic cooperation mediated by gap junctions. *Cancer Res. 51:* 5348-5351.

MASON, I.J., TAYLOR, A., WILLIAMS, J.G., SAGE, H. and HOGAN, B.L. (1986). Evidence from molecular cloning that SPARC, a major product of mouse embryo parietal endoderm, is related to an endothelial cell 'culture shock' glycoprotein of Mr 43,000. *EMBO J. 5:* 1465-1472.

MATESIC, D., ATTARDI, B., DELLOVADE, T., PFAFF, D. and GERMAK, J. (1997). Differential LHRH secretion, dye coupling, and protein expression in two morphologically distinct cell types identified in GT1-7 cultures. *J. Neuroendocrinol. 9:* 467-478.

MAUHIN, V., LUTZ, Y., DENNEFELD, C. and ALBERGA, A. (1993). Definition of the DNA-binding site repertoire for the Drosophila transcription factor SNAIL. *Nucleic. Acids. Res. 21:* 3951-3957.

MAYOR, R., ESSEX, L.J., BENNETT, M.F. and SARGENT, M.G. (1993). Distinct elements of the xsna promoter are required for mesodermal and ectodermal expression. *Development 119:* 661-671.

MAYOR, R., MORGAN, R. and SARGENT, M.G. (1995). Induction of the prospective neural crest of Xenopus. *Development 121:* 767-777.

MEHTA, P.P., YAMAMOTO, M. and ROSE, B. (1992). Transcription of the gene for the gap junctional protein connexin43 and expression of functional cell-to-cell channels are regulated by cAMP. *Mol. Biol. Cell 3:* 839-850.

METZSTEIN, M.M. and HORVITZ, H.R. (1999). The C. elegans cell death specification gene ces-1 encodes a snail family zinc finger protein [In Process Citation]. *Mol. Cell 4:* 309-319.

MINOR, R.R., HOCH, P.S., KOSZALKA, T.R., BRENT, R.L. and KEFALIDES, N.A. (1976ba). Organ cultures of the embryonic rat parietal yolk sac. I. Morphologic and autoradiographic studies of the deposition of the collagen and noncollagen glycoprotein components of basement membrane. *Dev. Biol. 48:* 344-364.

MINOR, R.R., STRAUSE, E.L., KOSZALKA, T.R., BRENT, R.L. and KEFALIDES, N.A. (1976ab). Organ cultures of the embryonic rat parietal yolk sac. II. Synthesis, accumulation, and turnover of collagen and noncollagen basement membrane glycoproteins. *Dev. Biol. 48:* 365-376.

MONIER-GAVELLE, F. and DUBAND, J.L. (1997). Cross talk between adhesion molecules: control of N-cadherin activity by intracellular signals elicited by beta1 and beta3 integrins in migrating neural crest cells. *J. Cell Biol. 137:*

1663-1681.

MORINI, M., PICCINI, D., BARBIERI, O. and ASTIGIANO, S. (1997). Modulation of alpha 6/beta 1 integrin expression during differentiation of F9 murine embryonal carcinoma cells to parietal endoderm. *Exp. Cell Res. 232:* 304-312.

MORINI, M., PICCINI, D., DE SANTANNA, A., LEVI, G., BARBIERI, O. and ASTIGIANO, S. (1999). Localization and expression of integrin subunits in the embryoid bodies of F9 teratocarcinoma cells. *Exp. Cell Res. 247:* 114-122.

MORLEY, G.E., EK-VITORIN, J.F., TAFFET, S.M. and DELMAR, M. (1997). Structure of connexin43 and its regulation by pHi. *J. Cardiovasc. Electrophysiol. 8:* 939-951.

MUMMERY, C.L., FEIJEN, A., FREUND, E. and SHEN, S. (1990). Characteristics of embryonic stem cell differentiation: a comparison with two embryonal carcinoma cell lines. *Cell Differ. Dev. 30:* 195-206.

MUSIL, L.S., CUNNINGHAM, B.A., EDELMAN, G.M. and GOODENOUGH, D.A. (1990). Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. *J. Cell Biol. 111:* 2077-2088.

MUSIL, L.S. and GOODENOUGH, D.A. (1991). Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. *J. Cell Biol. 115:* 1357-1374.

MUSIL, L.S. and GOODENOUGH, D.A. (1993). Multisubunit assembly of an integral plasma membrane channel protein, gap junction connexin43, occurs after exit from the ER. *Cell 74:* 1065-1077.

NADIJCKA, M. and HILLMAN, N. (1974). Ultrastructural studies of the mouse blastocyst substages. *J. Embryol. Exp. Morphol. 32:* 675-695.

NAKAYAMA, H., SCOTT, I.C. and CROSS, J.C. (1998). The transition to endoreduplication in trophoblast giant cells is regulated by the mSNA zinc finger transcription factor. *Dev. Biol. 199:* 150-163.

NAVARRO, P., LOZANO, E. and CANO, A. (1993). Expression of E- or P-cadherin is not sufficient to modify the morphology and the tumorigenic behavior of murine spindle carcinoma cells. Possible involvement of plakoglobin. *J. Cell Sci. 105:* 923-934.

NIETO, M.A., BENNETT, M.F., SARGENT, M.G. and WILKINSON, D.G. (1992). Cloning and developmental expression of Sna, a murine homologue of the Drosophila snail gene. *Development 116:* 227-237.

NIETO, M.A., SARGENT, M.G., WILKINSON, D.G. and COOKE, J. (1994). Control of cell behavior during vertebrate development by Slug, a zinc finger gene. *Science 264:* 835-839.

NISHI, M., KUMAR, N.M. and GILULA, N.B. (1991). Developmental regulation of gap junction gene expression during mouse embryonic development. *Dev. Biol. 146:* 117-130.

NUSSLEIN-VOLHARD, C., WIESCHAUS, E. and KLUDING, H. (1984). Mutations affecting the pattern of the larval cuticle in Drosophila melanogaster. I. Zygotic loci on the second chromosome. *Wilhelm Roux's Arch. Dev. Biol. 193:* 267-282.

ODA, H., TSUKITA, S. and TAKEICHI, M. (1998). Dynamic behavior of the cadherin-based cell-cell adhesion system during Drosophila gastrulation. *Dev. Biol. 203:* 435-450.

OHSUGI, M., LARUE, L., SCHWARZ, H. and KEMLER, R. (1997). Cell-junctional and cytoskeletal organization in mouse blastocysts lacking E-cadherin. *Dev. Biol. 185:* 261-271.

ORLOFF, J.J., REDDY, D., DE PAPP, A.E., YANG, K.H., SOIFER, N.E. and STEWART, A.F. (1994). Parathyroid hormone-related protein as a prohormone: posttranslational processing and receptor interactions. *Endocr. Rev. 15:* 40-60.

PAPAIOANNOU, V.E. (1982). Lineage analysis of inner cell mass and trophectoderm using microsurgically reconstituted mouse blastocysts. *J. Embryol. Exp. Morphol. 68:* 199-209.

PAYNE, G.S. and DEUCHAR, E.M. (1972). An in vitro study of functions of embryonic membranes in the rat. *J. Embryol. Exp. Morphol. 27:* 533-542.

PHILBRICK, W.M., DREYER, B.E., NAKCHBANDI, I.A. and KARAPLIS, A.C. (1998). Parathyroid hormone-related protein is required for tooth eruption. *Proc. Natl. Acad. Sci. U. S. A. 95:* 11846-11851.

POSTMA, F.R., HENGEVELD, T., ALBLAS, J., GIEPMANS, B.N., ZONDAG, G.C., JALINK, K. and MOOLENAAR, W.H. (1998). Acute loss of cell-cell communication caused by G protein-coupled receptors: a critical role for c-Src. *J. Cell Biol. 140:* 1199-1209.

POWELL, G.J., SOUTHBY, J., DANKS, J.A., STILLWELL, R.G., HAYMAN, J.A., HENDERSON, M.A., BENNETT, R.C. and MARTIN, T.J. (1991). Localization of parathyroid hormone-related protein in breast cancer metastases: increased incidence in bone compared with other sites. *Cancer Res. 51:* 3059-3061.

RAJASEKARAN, A.K., HOJO, M., HUIMA, T. and RODRIGUEZ-BOULAN, E. (1996). Catenins and zonula occludens-1 form a complex during early stages in the assembly of tight junctions. *J. Cell Biol. 132:* 451-463.

RAPPOLEE, D.A., BASILICO, C., PATEL, Y. and WERB, Z. (1994). Expression and function of FGF-4 in peri-implantation development in mouse embryos. *Development 120:* 2259-2269.

REIMA, I. and LEHTONEN, E. (1985). Localization of nonerythroid spectrin and actin in mouse oocytes and preimplantation embryos. *Differentiation. 30:* 68-75.

RICHA, J., DAMSKY, C.H., BUCK, C.A., KNOWLES, B.B. and SOLTER, D. (1985). Cell surface glycoproteins mediate compaction, trophoblast attachment, and endoderm formation during early mouse development. *Dev. Biol. 108:* 513-521.

RICHARD, G., SMITH, L.E., BAILEY, R.A., ITIN, P., HOHL, D., EPSTEIN, E.H., DIGIOVANNA, J.J., COMPTON, J.G. and BALE, S.J. (1998). Mutations in the human connexin gene GJB3 cause erythrokeratodermia variabilis. *Nat. Genet. 20:* 366-369.

RIVEDAL, E., YAMASAKI, H. and SANNER, T. (1994). Inhibition of gap junctional intercellular communication in Syrian hamster embryo cells by TPA, retinoic acid and DDT. *Carcinogenesis 15:* 689-694.

ROARK, M., STURTEVANT, M.A., EMERY, J., VAESSIN, H., GRELL, E. and BIER, E. (1995). scratch, a pan-neural gene encoding a zinc finger protein related to snail, promotes neuronal development. *Genes Dev. 9:* 2384-2398.

ROELEN, B.A., GOUMANS, M.J., ZWIJSEN, A. and MUMMERY, C.L. (1998). Identification of two distinct functions for TGF-beta in early mouse development. *Differentiation. 64:* 19-31.

ROMANO, L.A. and RUNYAN, R.B. (1999). Slug is a mediator of epithelial-mesenchymal cell transformation in the developing chicken heart. *Dev. Biol. 212:* 243-254.

ROMANO, L.A. and RUNYAN, R.B. (2000). Slug is an essential target of TGFbeta2 signaling in the developing chicken heart. *Dev. Biol. 223:* 91-102.

ROS, M.A., SEFTON, M. and NIETO, M.A. (1997). Slug, a zinc finger gene previously implicated in the early patterning of the mesoderm and the neural crest, is also involved in chick limb development. *Development 124:* 1821-1829.

ROSSANT, J., GARDNER, R.L. and ALEXANDRE, H.L. (1978). Investigation of the potency of cells from the postimplantation mouse embryo by blastocyst injection: a preliminary report. *J. Embryol. Exp. Morphol. 48:239-47:* 239-247.

RUANGVORAVAT, C.P. and LO, C.W. (1992). Connexin 43 expression in the mouse embryo: localization of transcripts within developmentally significant domains. *Dev. Dyn. 194:* 261-281.

SAITOU, M., FUJIMOTO, K., DOI, Y., ITOH, M., FUJIMOTO, T., FURUSE, M., TAKANO, H., NODA, T. and TSUKITA, S. (1998). Occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions. *J. Cell Biol. 141:* 397-408.

SALAMAT, M., MIOSGE, N. and HERKEN, R. (1995). Development of Reichert's membrane in the early mouse embryo. *Anat. Embryol. (Berl.) 192:* 275-281.

SAMBROOK J., FRITSCH E.F., and MANIATIS T. (Eds.) (1989). *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Plainview, NY.

SAMUELS, M., EZZELL, R.M., CARDOZO, T.J., CRITCHLEY, D.R., COLL, J.L. and ADAMSON, E.D. (1993). Expression of chicken vinculin complements the adhesion-defective phenotype of a mutant mouse F9

embryonal carcinoma cell. *J. Cell Biol. 121:* 909-921.

SARGENT, M.G. and BENNETT, M.F. (1990). Identification in Xenopus of a structural homologue of the Drosophila gene snail. *Development 109:* 967-973.

SASAKI, T., FORSBERG, E., BLOCH, W., ADDICKS, K., FASSLER, R. and TIMPL, R. (1998). Deficiency of beta 1 integrins in teratoma interferes with basement membrane assembly and laminin-1 expression. *Exp. Cell Res. 238:* 70-81.

SAUER, H., HOFMANN, C., WARTENBERG, M., WOBUS, A.M. and HESCHELER, J. (1998). Spontaneous calcium oscillations in embryonic stem cell-derived primitive endodermal cells. *Exp. Cell Res. 238:* 13-22.

SAVAGNER, P., KARAVANOVA, I., PERANTONI, A., THIERY, J.P. and YAMADA, K.M. (1998). Slug mRNA is expressed by specific mesodermal derivatives during rodent organogenesis. *Dev. Dyn. 213:* 182-187.

SAVAGNER, P., YAMADA, K.M. and THIERY, J.P. (1997). The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial-mesenchymal transition. *J. Cell Biol. 137:* 1403-1419.

SCHILLER, P.C., MEHTA, P.P., ROOS, B.A. and HOWARD, G.A. (1992). Hormonal regulation of intercellular communication: parathyroid hormone increases connexin 43 gene expression and gap-junctional communication in osteoblastic cells. *Mol. Endocrinol. 6:* 1433-1440.

SCHILLER, P.C., ROOS, B.A. and HOWARD, G.A. (1997). Parathyroid hormone up-regulation of connexin 43 gene expression in osteoblasts depends on cell phenotype. *J. Bone Miner. Res. 12:* 2005-2013.

SCHULTZ, R.M. (1993). Regulation of zygotic gene activation in the mouse. *Bioessays 15:* 531-538.

SEFTON, M., SANCHEZ, S. and NIETO, M.A. (1998). Conserved and divergent roles for members of the Snail family of transcription factors in the chick and mouse embryo. *Development 125:* 3111-3121.

SEGRE, G.V. and GOLDRING, S.R. (1993). Receptors for secretin, calcitonin, parathyroid hormone (pth)/pth-related peptide, vasoactive intestinal peptide, glucagonlike peptide 1, growth hormone-releasing hormone, and glucagon belong to a newly discovered g-protein linked receptor family. *Trends in Endocrinology methods 4:* 309-314.

SEIFERT, G.J., LAWSON, D. and WICHE, G. (1992). Immunolocalization of the intermediate filament-associated protein plectin at focal contacts and actin stress fibers. *Eur. J. Cell Biol. 59:* 138-147.

SEMOFF, S., HOGAN, B.L. and HOPKINS, C.R. (1982). Localization of fibronectin, laminin-entactin, and entactin in Reichert's membrane by immunoelectron microscopy. *EMBO J. 1:* 1171-1175.

SIMON, A.M. and GOODENOUGH, D.A. (1998). Diverse functions of vertebrate gap junctions. *Trends. Cell Biol. 8:* 477-483.

SIMPSON, P. (1983). Maternal-zygotic gene interactions during formation of the dorsoventral pattern in drosophila embryos. *Genetics 105:* 615-632.

SMITH, D.E., FRANCO, D.A. and GRIDLEY, T. (1992). Isolation of Sna, a mouse gene homologous to the Drosophila genes snail and escargot: its expression pattern suggests multiple roles during postimplantation development. *Development 116:* 1033-1039.

SMITH, K.K. and STRICKLAND, S. (1981). Structural components and characteristics of Reichert's membrane, an extra-embryonic basement membrane. *J. Biol. Chem. 256:* 4654-4661.

SMYTH, N., VATANSEVER, H.S., MURRAY, P., MEYER, M., FRIE, C., PAULSSON, M. and EDGAR, D. (1999). Absence of basement membranes after targeting the LAMC1 gene results in embryonic lethality due to failure of endoderm differentiation. *J. Cell Biol. 144:* 151-160.

SOLTER, D. and DAMJANOV, I. (1973). Explantation of extraembryonic parts of 7-day-old mouse egg cylinders. *Experientia 29:* 701-702.

SOLTER, D. and KNOWLES, B.B. (1978). Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). *Proc. Natl. Acad. Sci. U. S. A. 75:* 5565-5569.

SOUTHBY, J., KISSIN, M.W., DANKS, J.A., HAYMAN, J.A., MOSELEY, J.M., HENDERSON, M.A., BENNETT, R.C. and MARTIN, T.J. (1990). Immunohistochemical localization of parathyroid hormone-related protein in human breast cancer. *Cancer Res. 50:* 7710-7716.

STAUFFER, P.L., ZHAO, H., LUBY-PHELPS, K., MOSS, R.L., STAR, R.A. and MUALLEM, S. (1993). Gap junction communication modulates [Ca2+]i oscillations and enzyme secretion in pancreatic acini. *J. Biol. Chem. 268:* 19769-19775.

STEPHENS, L.E., SONNE, J.E., FITZGERALD, M.L. and DAMSKY, C.H. (1993). Targeted deletion of beta 1 integrins in F9 embryonal carcinoma cells affects morphological differentiation but not tissue-specific gene expression. *J. Cell Biol. 123:* 1607-1620.

STEPHENS, L.E., SUTHERLAND, A.E., KLIMANSKAYA, I.V., ANDRIEUX, A., MENESES, J., PEDERSEN, R.A. and DAMSKY, C.H. (1995). Deletion of beta 1 integrins in mice results in inner cell mass failure and peri-implantation lethality. *Genes Dev. 9:* 1883-1895.

STRICKLAND, S. and MAHDAVI, V. (1978). The induction of differentiation in teratocarcinoma stem cells by retinoic acid. *Cell 15:* 393-403.

STRICKLAND, S., REICH, E. and SHERMAN, M.I. (1976). Plasminogen activator in early embryogenesis: enzyme production by trophoblast and parietal endoderm. *Cell 9:* 231-240.

STRICKLAND, S., SMITH, K.K. and MAROTTI, K.R. (1980). Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP. *Cell 21:* 347-355.

SURANI, M.A. and BARTON, S.C. (1977). Trophoblastic vesicles of preimplantation blastocysts can enter into quiescence in the absence of inner cell mass. *J. Embryol. Exp. Morphol. 39:* 273-277.

THERY, C. and STERN, C.D. (1996). Roles of kringle domain-containing serine proteases in epithelial-mesenchymal transitions during embryonic development. *Acta Anat. (Basel.) 156:* 162-172.

THIERY, J.P. and CHOPIN, D. (1999). Epithelial cell plasticity in development and tumor progression. *Cancer Metastasis Rev. 18:* 31-42.

THISSE, C. and THISSE, B. (1999). Antivin, a novel and divergent member of the TGFbeta superfamily, negatively regulates mesoderm induction. *Development 126:* 229-240.

THISSE, C., THISSE, B. and POSTLETHWAIT, J.H. (1995). Expression of snail2, a second member of the zebrafish snail family, in cephalic mesendoderm and presumptive neural crest of wild-type and spadetail mutant embryos. *Dev. Biol. 172:* 86-99.

THISSE, C., THISSE, B., SCHILLING, T.F. and POSTLETHWAIT, J.H. (1993). Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail mutant embryos. *Development 119:* 1203-1215.

TIENARI, J. (1989). Actin cytoskeleton of extraembryonic endoderm and teratocarcinoma-derived endoderm cells. *Exp. Cell Res. 184:* 388-395.

TOYOFUKU, T., AKAMATSU, Y., ZHANG, H., KUZUYA, T., TADA, M. and HORI, M. (2001). c-Src Regulates the Interaction between Connexin-43 and ZO-1 in Cardiac Myocytes. *J. Biol. Chem. 276:* 1780-1788.

TREVOR, K. and OSHIMA, R.G. (1985). Preimplantation mouse embryos and liver express the same type I keratin gene product. *J. Biol. Chem. 260:* 15885-15891.

TREVOR, K.T. (1990). Disruption of keratin filaments in embryonic epithelial cell types. *New Biol. 2:* 1004-1014.

TREVOR, K.T. and STEBEN, L.S. (1992). Distribution of desmosomal proteins in F9 embryonal carcinoma cells and epithelial cell derivatives. *J. Cell Sci. 103:* 69-80.

TSUKITA, S., FURUSE, M. and ITOH, M. (1999). Structural and signalling molecules come together at tight junctions. *Curr. Opin. Cell Biol. 11:* 628-633.

TURIN, L. and WARNER, A. (1977). Carbon dioxide reversibly abolishes ionic communication between cells of early amphibian embryo. *Nature 270:* 56-57.

TURNER, P.R., BAMBINO, T. and NISSENSON, R.A. (1996). Mutations of neighboring polar residues on the second transmembrane helix disrupt signaling by the parathyroid hormone receptor. *Mol. Endocrinol. 10:* 132-139.

TWIGG, S.R. and WILKIE, A.O. (1999). Characterisation of the human snail (SNAI1) gene and exclusion as a major disease gene in craniosynostosis. *Hum. Genet. 105:* 320-326.

UNGER, V.M., KUMAR, N.M., GILULA, N.B. and YEAGER, M. (1999). Three-dimensional structure of a recombinant gap junction membrane channel. *Science 283:* 1176-1180.

VAN DE STOLPE, A., KARPERIEN, M., LOWIK, C.W., JÜPPNER, H., SEGRE, G.V., ABOU-SAMRA, A.B., DE LAAT, S.W. and DEFIZE, L.H. (1993). Parathyroid hormone-related peptide as an endogenous inducer of parietal endoderm differentiation. *J. Cell Biol. 120:* 235-243.

VAN DER HEYDEN, M.A., ROOK, M.B., HERMANS, M.M., RIJKSEN, G., BOONSTRA, J., DEFIZE, L.H. and DESTREE, O.H. (1998). Identification of connexin43 as a functional target for Wnt signalling. *J. Cell Sci. 111:* 1741-1749.

VAN DER HEYDEN, M.A., VELTMAAT, J.M., HENDRIKS, J.A., DESTREE, O.H. and DEFIZE, L.H. (2000). Dynamic connexin43 expression and gap junctional communication during endoderm differentiation of F9 embryonal carcinoma cells. *Eur. J. Cell Biol. 79:* 272-282.

VARGAS, S.J., GILLESPIE, M.T., POWELL, G.J., SOUTHBY, J., DANKS, J.A., MOSELEY, J.M. and MARTIN, T.J. (1992). Localization of parathyroid hormone-related protein mRNA expression in breast cancer and metastatic lesions by in situ hybridization. *J. Bone Miner. Res. 7:* 971-979.

VELTMAAT, J.M., ORELIO, C.C., WARD-VAN OOSTWAARD, D., VAN ROOIJEN, M.A., MUMMERY, C.L. and DEFIZE, L.H. (2000). Snail is an immediate early target gene of parathyroid hormone related peptide signaling in parietal endoderm formation. *Int. J. Dev. Biol. 44:* 297-307.

VERHEIJEN, M.H. and DEFIZE, L.H. (1997). Parathyroid hormone activates mitogen-activated protein kinase via a cAMP-mediated pathway independent of Ras. *J. Biol. Chem. 272:* 3423-3429.

VERHEIJEN, M.H., KARPERIEN, M., CHUNG, U., VAN WIJNEN, M., HEYSTEK, H., HENDRIKS, J.A., VELTMAAT, J.M., LANSKE, B., LI, E., LOWIK, C.W., DE LAAT, S.W., KRONENBERG, H.M. and DEFIZE, L.H. (1999). Parathyroid hormone-related peptide (PTHrP) induces parietal endoderm formation exclusively via the type I PTH/PTHrP receptor. *Mech. Dev. 81:* 151-161.

VERHEIJEN, M. H. G. (1997). *Signal transduction by the PTH/PTHrP Receptor, its role in parietal endoderm formation*. Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Utrecht, The Netherlands. Thesis/Dissertation.

VERHEIJEN, M.H.G. and DEFIZE, L.H.K. (1999). Signals governing extraembryonic endoderm formation in the mouse: involvement of the type I parathyroid hormone-related peptide (PTHrP) receptor, p21Ras and cell adhesion molecules. *Int. J. Dev. Biol. 43:* 711-721.

VESTWEBER, D. and KEMLER, R. (1984). Rabbit antiserum against a purified surface glycoprotein decompacts mouse preimplantation embryos and reacts with specific adult tissues. *Exp. Cell Res. 152:* 169-178.

VESTWEBER, D. and KEMLER, R. (1984). Some structural and functional aspects of the cell adhesion molecule uvomorulin. *Cell Differ. 15:* 269-273.

VIEBAHN, C. (1995). Epithelio-mesenchymal transformation during formation of the mesoderm in the mammalian embryo. *Acta Anat. (Basel.) 154:* 79-97.

VOZZI, C., ULLRICH, S., CHAROLLAIS, A., PHILIPPE, J., ORCI, L. and MEDA, P. (1995). Adequate connexin-mediated coupling is required for proper insulin production. *J. Cell Biol. 131:* 1561-1572.

WAN, Y.J., WANG, L. and WU, T.C. (1996). Murine endodermal F9E cells, derived from the teratocarcinoma line F9, contain high basal levels of retinoic acid receptors (RARs and RXRs) but are not sensitive to the actions of retinoic acid. *Differentiation. 60:* 211-218.

WANG, S.Y., ROGUSKA, M.A. and GUDAS, L.J. (1989). Defective post-translational modification of collagen IV in a mutant F9 teratocarcinoma cell line is associated with delayed differentiation and growth arrest in response to retinoic acid. *J. Biol. Chem. 264:* 15556-15564.

WARN-CRAMER, B.J., COTTRELL, G.T., BURT, J.M. and LAU, A.F. (1998). Regulation of connexin-43 gap junctional intercellular communication by mitogen-activated protein kinase. *J. Biol. Chem. 273:* 9188-9196.

WARTIOVAARA, J., LEIVO, I. and VAHERI, A. (1979). Expression of the cell surface-associated glycoprotein, fibronectin, in the early mouse embryo. *Dev. Biol. 69:* 247-257.

WEILER-GUETTLER, H., AIRD, W.C., RAYBURN, H., HUSAIN, M. and ROSENBERG, R.D. (1996). Developmentally regulated gene expression of thrombomodulin in postimplantation mouse embryos. *Development 122:* 2271-2281.

WHITE, T.W. and PAUL, D.L. (1999). Genetic diseases and gene knockouts reveal diverse connexin functions. *Annu. Rev. Physiol. 61:* 283-310.

WHITELEY, M., NOGUCHI, P.D., SENSABAUGH, S.M., ODENWALD, W.F. and KASSIS, J.A. (1992). The Drosophila gene escargot encodes a zinc finger motif found in snail-related genes. *Mech. Dev. 36:* 117-127.

WILLECKE, K., HEYNKES, R., DAHL, E., STUTENKEMPER, R., HENNEMANN, H., JUNGBLUTH, S., SUCHYNA, T. and NICHOLSON, B.J. (1991). Mouse connexin37: cloning and functional expression of a gap junction gene highly expressed in lung. *J. Cell Biol. 114:* 1049-1057.

WISSINK, S., VAN HEERDE, E.C., SCHMITZ, M.L., KALKHOVEN, E., VAN DER BURG, B., BAEUERLE, P.A. and VAN DER SAAG, P.T. (1997). Distinct domains of the RelA NF-kappaB subunit are required for negative cross-talk and direct interaction with the glucocorticoid receptor. *J. Biol. Chem. 272:* 22278-22284.

WOLFE, J., MAUTNER, V., HOGAN, B. and TILLY, R. (1979). Synthesis and retention of fibronectin (LETS protein) by mouse teratocarcinoma cells. *Exp. Cell Res. 118:* 63-71.

WYSOLMERSKI, J.J., BROADUS, A.E., ZHOU, J., FUCHS, E., MILSTONE, L.M. and PHILBRICK, W.M. (1994). Overexpression of parathyroid hormone-related protein in the skin of transgenic mice interferes with hair follicle development. *Proc. Natl. Acad. Sci. U. S. A. 91:* 1133-1137.

WYSOLMERSKI, J.J., MCCAUGHERN-CARUCCI, J.F., DAIFOTIS, A.G., BROADUS, A.E. and PHILBRICK, W.M. (1995). Overexpression of parathyroid hormone-related protein or parathyroid hormone in transgenic mice impairs branching morphogenesis during mammary gland development. *Development 121:* 3539-3547.

WYSOLMERSKI, J.J., PHILBRICK, W.M., DUNBAR, M.E., LANSKE, B., KRONENBERG, H. and BROADUS, A.E. (1998). Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development. *Development 125:* 1285-1294.

YANCEY, S.B., BISWAL, S. and REVEL, J.P. (1992). Spatial and temporal patterns of distribution of the gap junction protein connexin43 during mouse gastrulation and organogenesis. *Development 114:* 203-212.

YEAGER, M. (1998). Structure of cardiac gap junction intercellular channels. *J. Struct. Biol. 121:* 231-245.

YU, W., DAHL, G. and WERNER, R. (1994). The connexin43 gene is responsive to oestrogen. *Proc. R. Soc. Lond. B. Biol. Sci. 255:* 125-132.

SUMMARY

Since about a decade, several reports have strongly suggested a role for parathyroid hormone related peptide (PTHrP) in the formation of parietal endoderm (PE) in the mouse embryo. This thesis is aimed first at elucidating the biological significance of parathyroid hormone related peptide (PTHrP) signaling via its type I receptor (PTH(rP)-RI) in PE formation, and secondly at finding new molecules downstream of this receptor that induce PE formation. In the first chapter a literature review about the formation of PE from primitive and visceral endoderm (PrE and VE) in the mouse embryo is given. In that chapter it is also illustrated that the changes in cell contacts, morphology and secretory properties, as well as the acquisition of migration of the cells during PE formation are indicative for an epithelio-mesenchymal transition (EMT). We therefore postulate that PE formation is the first EMT in mouse embryogenesis.

In chapter 2, we demonstrate that PTHrP signaling is sufficient to induce the transformation of an epithelial to mesenchymal cell type during PE formation in the F9 model system. In addition, we demonstrate that PTHrP enhances the migration of endoderm away from F9 embryoid bodies. A detailed analysis of the PE of receptor null mutants is described here, revealing that this tissue has an aberrant, epithelial instead of mesenchymal morphology, and lacks expression of the PE marker thrombomodulin in contrast to an apparently normal phenotype reported previously. These results indicate that PTHrP signaling, although not required for migration, is necessary for the proper differentiation of PE. In that chapter the downregulation of connexin43 (Cx43), the most abundant gap junctional molecule in F9 cells and the extraembryonic endoderm *in vivo*, is shown as a result of PTHrP signaling in PE formation. In chapter 3 we further investigated the importance of Cx43 and the function of gap junctions in the formation of PE, and found that their downregulation is a consequence of rather than a prerequisite for PE differentiation. We postulate that the high expression of Cx43 and high extent of gap junctional coupling in VE serves a function in metabolite exchange between the mother and the early embryo.

Chapter 4 describes the identification of the zinc finger transcription factor *snail* as a direct target of PTHrP signaling in F9 PrE signaling. It is furthermore shown there, that *snail* is also upregulated during the earliest formation of PE *ex vivo* and *in vivo*, clearly suggesting an important role in PE formation. This is further investigated in chapter 5, where it is described that high levels of expression of *snail* are sufficient to induce the disintegration of adherens junctions in F9 PrE, by loss of membrane localized expression of E-cadherin and β-catenin. Moreover, it is demonstrated that F9 VE cells overexpressing *snail* display enhanced migration, more cell dispersion and assume a rounded morphology, suggesting that elevated expression of *snail* is sufficient to at least induce the morphological transition of an epithelial (PrE or VE) to a mesenchymal cell with typical PE morphology. *In situ* hybridization results demonstrate a lack of *Sna* expression in the distal PE region in PTH(rP)-RI knockout embryos, strongly suggesting that it is due to a lack of Sna function that these cells have not acquired a mesenchymal phenotype. Finally, in chapter 6, preliminary data of a promoter analysis of the *snail* gene are shown, which are suggestive for a putative functional cyclic AMP responsive element in the promoter, which may confer the signal induced by PTHrP. In this chapter, all results are discussed and a model for a function of PTHrP in the downregulation of the cell-cell adhesion system in the earliest phase of PE formation is presented. Our finding that the PE of PTH(rP)-RI null mutant embryos displays an epithelial instead of mesenchymal morphology, and does not express PE-specific enhanced levels of thrombomodulin, suggests that this PE does not function properly. This may explain the growth retardation and mortality of the embryos. Given the similarities between PE formation and (breast) tumor progression with respect to the presence of PTHrP, expression of *Sna* and lack of E-cadherin expression at the cell membrane, our findings may also open avenues to better understand (breast) tumor progression.

SAMENVATTING

In de afgelopen jaren heeft het werk van verscheidene onderzoekers sterk de suggestie gewekt dat PTHrP (bijschildklier hormoon gerelateerd peptide) van belang is voor de vorming van het pariëtaal endoderm (PE) in het muizen-embryo. Dit proefschrift heeft als eerste doel de rol van PTHrP-signalering via zijn type I receptor (PTH(rP)-RI) in de vorming van PE te verhelderen. Het tweede doel is moleculen te identificeren die de signalen, afkomstig van PTH(rP)-RI-activatie, doorgeven. Ter introductie geeft hoofdstuk 1 een overzicht van de literatuur over de vorming van PE uit zijn voorlopers, primitief en visceraal endoderm (PrE, VE). Uit dat overzicht wordt duidelijk dat de veranderingen in cel-cel contacten, vorm en secretoire eigenschappen van de cellen die differentiëren naar PE, indicatief zijn voor een zogeheten epitheel-mesenchym transitie (EMT). Op basis daarvan beschouwen wij de vorming van PE de eerste EMT in de embryogenese van de muis.

In hoofdstuk 2 wordt getoond, dat de aanwezigheid van exogeen PTHrP voldoende is om de transformatie van een epitheliaal naar mesenchymaal celtype te induceren in het *in vitro* modelsysteem voor PE formatie, F9 embryocarcinoma cellen. Bovendien draagt PTHrP bij aan de migratie van endoderm cellen vanuit gedifferentieerde F9 aggregaten. In tegenstelling tot een eerder gerapporteerd ogenschijnlijk normaal PE in PTH(rP)-RI 'knockout' embryo's, is nu uit een meer gedetailleerde analyse van dit weefsel gebleken, dat het een afwijkende, namelijk epitheliale morfologie heeft, waarin de PE-specifiek verhoogde expressie van thrombomoduline ontbreekt. Dit betekent dat signaaltransductie geïnduceerd door PTHrP, hoewel niet vereist voor migratie, wel noodzakelijk is voor de juiste differentiatie van PE via een EMT. In hetzelfde hoofdstuk wordt getoond dat PTHrP de expressie van connexine 43 (Cx43), de meest geëxpresseerde component van 'gap junctions' in extra-embryonaal endoderm *in vitro* en *in vivo*, verlaagt. In hoofdstuk 3 is het belang van deze regulatie, alsmede van cel-cel communicatie via 'gap junctions' voor de vorming van PE verder bestudeerd. De verminderde expressie van Cx43 en de verminderde communicatie blijken een gevolg ván in plaats van voorwaarde vóór de differentiatie van PE te zijn. We postuleren dat de hoge expressie van Cx43 en de hoge mate van cel-cel communicatie van 'gap junctions' in VE van belang zijn voor de functie van VE in het uitwisselen van metabolieten tussen moeder en foetus.

In hoofdstuk 4 wordt de identificatie van de zinkvinger transcriptie factor *snail* als direct doelwit-gen van PTHrP-signalering in F9 PrE beschreven. Overeenkomstig wordt *snail* verhoogd tot expressie gebracht tijdens de vorming van de eerste PE cellen in het muizen-embryo *ex vivo* en *in vivo*. Dit suggereert in sterke mate een belangrijke rol voor snail in de vorming van PE. Dit is verder onderzocht in hoofdstuk 5, waar wordt beschreven dat experimenteel verhoogde expressie van (transgeen) *snail* voldoende is voor de desintegratie van 'adherens junctions' in F9 PrE, door het verlies van membraangebonden expressie van E-cadherine en β-catenine. Bovendien migreren endoderm cellen met verhoogde *snail* expressie verder vanuit gedifferentieerde F9 aggregaten, vertonen deze cellen meer dispersie en een meer afgeronde vorm. Dit suggereert dat verhoogde expressie van snail voldoende is om de transitie van een epitheliaal (PrE of VE) naar mesenchymaal

celtype met de voor PE karakteristieke afgeronde vorm te induceren. Door *in situ* hybridisatie werd duidelijk dat PTH(rP)-RI 'knockout' embryo's geen snail tot expressie brengen in het distale PE, wat sterk suggereert dat het epitheliaal zijn van dit PE toe te schrijven is aan het gebrek van snail activiteit. Tenslotte worden in hoofdstuk 6 preliminaire gegevens beschreven van de promoter analyse van het *snail* gen, die aanleiding geven te veronderstellen dat deze promoter een functioneel cyclisch AMP respons element bevat, via welke PTHrP de transcriptie van snail reguleert. In dit hoofdstuk worden alle resultaten bediscussieerd en wordt een model gepresenteerd voor een rol van PTHrP in de reductie van cel-cel adhesie in de vroegste fase van de vorming van PE. Onze bevinding dat het PE van PTH(rP)-RI 'knockout' embryo's epitheliaal is in plaats van mesenchymaal, en bovendien geen PE-specifieke expressie van thrombomoduline vertoont, suggereert dat dit mutant PE niet juist functioneert. Dit zou de groei-achterstand en sterfte van deze embryo's kunnen verklaren. Gezien de overeenkomsten tussen de vorming van PE en het metastaseren van (borst) tumoren, zoals de aanwezigheid van PTHrP, expressie van snail en afwezigheid van membraangebonden E-cadherine, is het aannemelijk dat onze resultaten ook kunnen leiden tot een beter begrip van (borst) tumor progressie.

SAMENVATTING VOOR DE NIET-BIOLOOG

De ontwikkeling van de meeste zoogdier-embryo's hangt volledig af van de innesteling van de embryo's in de baarmoederwand. Die innesteling kan niet plaatsvinden als het embryo niet eerst de dooierzak vormt, waarvan de buitenste cellaag aan de baarmoederwand 'plakt', en de binnenste cellaag veel bindweefsel-achtige stoffen produceert, die ervoor zorgen dat de dooierzak een stevige, ruime ballon is waarbinnen het embryo ruimte heeft om te groeien en tegelijkertijd beschermd is tegen schokken. Omdat de placenta en navelstreng pas later gevormd worden, veronderstelt men, dat deze dooierzak in eerste instantie de functie van de placenta en navelstreng waarneemt, namelijk de aanvoer van voedingsstoffen en de afvoer van afvalproducten. Het onderzoek dat in dit proefschrift staat beschreven is erop gericht om beter te begrijpen hoe deze dooierzak gevormd wordt. Het onderzoek heeft zich geconcentreerd rond de vorming van de binnenste cellaag van die dooierzak, het zogeheten pariëtaal endoderm (PE).

Er was al bekend dat een bepaald hormoon, PTHrP, door het embryo zelf en door de baarmoederwand geproduceerd wordt, juist op het moment dat ook het PE aangelegd moet worden. Daarom werd er verondersteld dat dit hormoon ook een belangrijke rol zou spelen in de aanleg van deze cellaag, maar of dat zo was, en hoe het die rol vervulde, was nog onbekend en daar heeft dit onderzoek zich op gericht. Om hun werking te kunnen uitoefenen moeten de meeste hormonen (zo ook PTHrP) binden aan een specifieke receptor die aan de buitenkant van een cel vastzit (zoals een sleutel specifiek op één slot past). Als die binding plaatsvindt, gaat er in de cel een heel systeem van eiwitten met elkaar 'praten' (zoals na het omdraaien van de sleutel in een ouderwetse klok een eerste tandwiel kan gaan draaien, waardoor vervolgens een heel systeem van tandwielen gaat draaien). Uiteindelijk krijgt de cel hierdoor het signaal dat hij zich anders moet gaan gedragen, en daarmee een andere specialistische functie krijgt (dit proces heet differentiatie, en is vergelijkbaar met het door de tandwielen in werking zetten van het slagmechanisme van de klok, die daardoor van functie verandert: van tijds-indicator naar 'wekker'). Normale embryos hebben ook de specifieke receptor voor PTHrP, wat het nog aannemelijker maakt dat PTHrP (via zijn receptor) een rol speelt in de vorming van PE.

Een groot gedeelte van dit promotie-onderzoek is verricht met behulp van celkweek, om een beter inzicht te krijgen op de effecten van PTHrP op uiterlijke eigenschappen en gedrag van cellen. Uiteindelijk is dit gecombineerd met onderzoek aan muizen-embryo's, om te bepalen of de waarnemingen die we met cellen hadden verkregen, relevant waren voor de vorming van het PE zoals die tijdens de ontwikkeling van het embryo zelf plaatsvindt. Door een vergelijking van het PE van normale muizen-embryo's en mutant embryo's (die geen receptor voor PTHrP hadden), werd duidelijk dat PTHrP van belang is voor de vorm van de cellen van het PE: In een normaal embryo zijn die cellen opgebold en liggen ze ver uit elkaar, terwijl in een mutant embryo de cellen strak aaneengesloten liggen. Mutant embryo's overlijden tijdens de zwangerschap. We veronderstellen nu dat die sterfte te wijten is aan die abnormale vorm van het PE, doordat bijvoorbeeld door het strak aaneenliggen van die abnormale cellen voedings- en afvalstoffen niet vrijelijk van moeder naar kind en omgekeerd kunnen gaan. Bovendien bleek dat die abnormale cellen, in tegenstelling tot normale PE cellen, geen eiwit maken dat mogelijk

betrokken is bij het tegengaan van bloedstolling. Het zou daarom bovendien zo kunnen zijn dat op de dooierzak bloed van de moeder stolt, waardoor de uitwisseling van voedings- en afvalstoffen (extra) wordt gehinderd. Het ontbreken van deze anti-bloedstollings-factor geeft aan dat de abnormale cellen niet helemaal gespecialiseerd, ofwel gedifferentieerd, zijn. Mogelijk scheiden ze ook minder bindweefselstoffen uit, waardoor de dooierzak niet groot of stevig genoeg is om groei en verder leven van het embryo mogelijk te maken.

Uit die afwijkende vorm van de cellen werd duidelijk dat PTHrP van belang is voor het verbreken van allerlei cel-cel contacten, waardoor de cellen kunnen opbollen en vrij komen te liggen van elkaar (zoals is weergegeven in hoofdstuk 6, figuur 7). In een poging om een aantal eiwitten (radertjes) te identificeren die het signaal van PTHrP-binding aan zijn receptor doorsturen naar die cel-cel contacten, stuitten we op een eiwit met de misleidende naam ‘snail’ (slak). De naam is in die zin misleidend, omdat het niet heel traag, maar juist razendsnel een signaal doorgeeft waardoor cel-cel contacten verbroken worden: Al binnen een half uur nadat PTHrP aan zijn receptor bindt, komt snail in actie, en uit de literatuur blijkt dat snail in staat is om zonder tussenkomst van andere eiwitten (radertjes) cel-cel contacten uiteen te laten vallen.

Samenvattend is uit dit onderzoek gebleken dat PTHrP een belangrijke rol speelt in de vorming van het PE, en is het mechanisme waarmee PTHrP deze rol vervult gedeeltelijk opgehelderd. PE kan weliswaar gevormd worden zonder dat PTHr ertoe bijdraagt (vanwege de afwezigheid van zijn receptor), maar de cellen van deze cellaag hebben een afwijkende vorm, en zijn niet volledig gedifferentieerd. Bovendien gaan muizen-embryo’s waarin PTHrP niet kan bijdragen tot de juiste vorming van het PE vroegtijdig dood. Dit maakt het aannemelijk dat het PE een belangrijke functie heeft in de overleving van het embryo, bijvoorbeeld door het tot stand brengen van het contact tussen moeder en kind, in de vorm van de uitwisseling van voedings- en afvalstoffen.

Hoewel de vorming van de dooierzak bij de mens iets anders verloopt dan bij de muis, en het niet helemaal duidelijk is of PTHrP daarin een vergelijkbare rol kan vervullen, zijn er opvallende overeenkomsten tussen de vormveranderingen die normale pariëtaal endoderm cellen in het muizen-embryo moeten ondergaan, en de vormveranderingen die tumorcellen ondergaan wanneer ze van goed- naar kwaadaardig omslaan. Opmerkelijke details hierbij zijn dat PTHrP in verhoogde mate aanwezig is bij een aantal van dit soort tumoren (bijvoorbeeld sommige kwaadaardige borsttumoren), evenals snail, terwijl de cel-cel contacten niet aanwezig zijn. Het is daarom aannemelijk dat PTHrP, via snail, een rol kan spelen in het verbreken van cel-cel contacten van goedaardige tumoren, die daardoor kwaadaardig worden. Om die reden kunnen de resultaten van dit onderzoek zeer relevant zijn voor het kankeronderzoek.

DANKWOORD

Dit proefschrift zou nooit geworden zijn tot wat het is zonder alle mensen om me heen, die me af en toe, op welke manier dan ook, een helpende hand toestaken. Met het risico enkele mensen te vergeten, wil ik er hier graag een aantal met name bedanken. Allereerst een woord van dank aan Bas, voor de dagelijkse begeleiding. Onze samenwerking was voorzien van vele discussies die, hoewel niet altijd van wetenschappelijk belang, hoe dan ook zeer leerzaam waren! Christine, met jouw onontbeerlijke hulp bleef ik in het zadel en kreeg ik voldoende inzicht om mijn einddoelen te definiëren en er doelgericht naartoe te werken. Siegfried, jouw betrokkenheid en nimmer aflatende, stimulerend kritische houding tijdens de laatste analyses en in de schrijffase is van onschatbare waarde geweest! De wijze lessen van jullie drieën zullen me in de nabije toekomst goed van pas komen!

De (ex-)leden van de groep wil ik niet alleen bedanken voor de gezelligheid en fijne samenwerking, maar Mark ook voor het duwtje in mijn rug en Jeanine voor de geestdriftige gezamenlijke lab-reorganisatie, brainstorms en zang, en niet te vergeten je bijdrage aan hoofdstuk 2, (zelfs in je vrije tijd naast je huidige baan!) en je vriendschap. Marcel vdH., bedankt voor het mij betrekken in jouw onderzoek en de stimulerende samenwerking, voor het werk- en knaagdieren-overleg, en je voortdurende belangstelling voor mijn eigen project, en Truus bedankt voor de grandioze inzet tijdens de eindsprint en het mij leren 'delegeren'. De studenten Claudia, Remco en Angela wil ik bedanken voor jullie enthousiaste inzet voor en bijdrage aan het project en omdat ik jullie (mede) mocht begeleiden. Het was voor mij een leerzame ervaring, die hopelijk wederzijds is. Studenten Bas en Sylvia, weliswaar niet 'mijn' studenten, we hebben helaas maar weinig, maar voor mij wel plezierige uurtjes samen op het lab doorgebracht. Veel succes allemaal in jullie verdere loopbanen elders!

Daarnaast wil ik Marga, Dorien, Egbert, Alie, Mark R., Marcel K. en Leon bedanken voor het mij leren isoleren van muizen-embryo's, het afstaan van embryonaal materiaal het uitvoeren van primer-extensie-analyse en de onmisbare *in situ's* of het helpen met statistiek. Een speciaal woordje van dank aan Dorien, en ook Jacqueline, voor jullie immer warme belangstelling. Jeroen K., bedankt voor het snijden van de vele embryo's, vooral die ene schitterende cover-sexy, en wie weet ligt er nog één in het verschiet! Andrea, thanks for the many fruitful and stimulating discussions, and taking the salsa class with me. Bernard, ik had toch maar mooi geluk dat ik een jaar de werkkamer met je mocht delen, bedankt voor die fijne tijd. Johannes Boonstra en José Bijveld, hartelijk dank voor jullie gastvrijheid tijdens de verbouwing van ons eigen lab. Beate und Leopold, ich möchte euch danken für eure Anstrengungen mir Embryonen zur Verfügung zu stellen wenn wir selber keiner hatten. Alle (ex-)AiO's/OiO's, en in het bijzonder Astrid, Egbert, Marie-José, Arjan, Christophe, Jaco, Lex en Marion, wil ik bedanken voor het van gedachten wisselen over (meta-) wetenschappelijke vragen en andere interessante dingen. Leden van de AIO-afvaardiging 1998, het was een plezier om met jullie (en met de fantastische hulp achter de schermen van Susann en Sabina!) de eerste AIO-retraite te organiseren.

Zonder administratieve en facilitaire voorzieningen zou het niet zo 'gemakkelijk' geweest zijn dit onderzoek te doen. Daarom wil ik ook alle 'niet-onderzoekende'

collega's van het Hubrecht bedanken. Zoals bijvoorbeeld José die midden in de nacht zijn bed uitkwam voor een vals alarm (mea culpa); Romke en Richard voor het op peil houden van het magazijn en het af en toe hanteren van flexibele openingstijden; Sander voor het voorzien in schoon water en glas en Patricia voor alles wat je erbij deed voor de cellen, bacteriën of voor de proefjes; Truus voor het sequencen; Obbe en consorten voor het verzorgen en kruisen van de muizen; Gerard, Rob en Henk voor 'h0merun' en overige hulp met bits en bytes; Jaap en Ferdinand voor jullie fantastische inzet bij fotografische reproducties van resultaten; Oeke voor het overzichtelijk en toegankelijk houden van de bieb en poëzie. Alle anderen die ik hier mogelijk vergeet te noemen, ik hoop dat ik op de betreffende momenten mijn dank heb laten blijken.

Jean Paul et Savério, merci beaucoup pour m'avoir accueillie chez vous l'année dernière. J'ai bien profité de votre expertise et facilités. Tout le monde dans l'équipe et à Curie, merci pour m'avoir aidée et passer du temps avec moi, aussi au dehors du labo. J'ai passé un temps merveilleux, et j'ai hâte de vous revoir. Anil, thanks for the many beers, interesting talks and squash games. A bientôt!

Petra, Remy, Helga, Bert, Michelle, Sylvie, Albertine, Jurjen, Marleen, Daan, Jeanine, Anita, Jack, Wilfred, Anneke, Ron, Josephine, Johanna, Ken, Marcel, Waltera, Hedwig, Petra, Brigitte, Gert, Rogier, Jasper, Nick, Julia, Joery, en Niels, bedankt voor jullie warme vriendschap en belangstelling, en voor het brengen van de broodnodige afleiding, afwisseling, ontspanning en relativering waarmee jullie hielpen de balans nog enigszins in evenwicht te houden! Petra, Marcel en Brigitte, veel dank voor jullie enthousiaste hulp bij de totstandkoming van dit boekje. Jeanine en Michelle, ik ben vereerd, dat jullie mijn paranimfen willen zijn.

Niet in de laatste plaats een woord van dank aan mijn ouders. Het stimuleren van doorzettingsvermogen en gedoseerd perfectionisme waren twee belangrijke pijlers in jullie opvoeding, zonder welke ik dit nooit zou hebben gered. Ook al waren mijn werk en levensstijl voor jullie vaak een 'ver van mijn bed show', jullie waren er altijd voor me met een luisterend oor, raad en daad, en onvoorwaardelijk vertrouwen. Oneindig veel dank daarvoor!!

CURRICULUM VITAE

Jacqueline Veltmaat werd geboren op 7 januari 1969 te Heino, alwaar zij het basisonderwijs genoot. Vervolgens doorliep zij succesvol het ongedeeld VWO aan het Florens Radewijns College te Raalte. In 1987 begon ze de studie Biologische Gezondheidskunde aan de Rijksuniversiteit Limburg te Maastricht, welk zij voltooide met het behalen van haar doctoraal diploma. Haar eerste stage en afstudeeronderzoek, op het gebied van (chemische) carcinogenese, verrichtte ze aan de vakgroepen Pathologie en Biologische Gezondheidkunde (nu Gezondheidsrisicoanalyse en Toxicologie), verbonden aan de Rijksuniversiteit Limburg en het Academisch Ziekenhuis te Maastricht, onder begeleiding van Dr. J. ten Kate en Ing. B. van Agen. Aansluitend vervolgde zij haar studie vanaf september 1991 met bijvak-onderwijs binnen de studierichting Moleculaire Wetenschappen aan de Landbouwuniversiteit Wageningen. Dit bijvak-onderwijs sloot zij af met een tweede afstudeer-onderzoek betreffende de meiose-kinetiek van muizen-spermatocyten met een ongebalanceerde reciproke chromosoom-translocatie, binnen de vakgroep Erfelijkheidsleer aan de Landbouwuniversiteit, onder de bezielende begeleiding van Ing. A. Peters en Dr. P. de Boer.

Jacqueline bekwaamde zich in een meer moleculair/biochemische benadering van onderzoek tijdens haar aanstelling als ‘Research Scholar’ binnen de vakgroep ‘Molecular Carcinogenesis’ aan de afdeling ‘Pathology Research and Laboratory Medicine’ aan het ‘Childrens Hospital of Los Angeles/University of Southern California’ te Los Angeles, Californië. Hier onderzocht ze van januari 1995 tot en met april 1996, onder supervisie van Dr. J. Groffen en Dr. N. Heisterkamp, de rol van een potentieel oncogen, de non-receptor tyrosine kinase Fer, in celcyclus en cel-adhesieve eigenschappen.

Van mei 1996 tot september 2000 was Jacqueline als Onderzoeker in Opleiding (OiO) in dienst van de Koninklijke Academie van Wetenschappen (KNAW), verbonden aan het Hubrecht Laboratorium/Nederlands Instituut voor Ontwikkelingsbiologie te Utrecht. Hier verrichtte zij het onderzoek beschreven in dit proefschrift, onder supervisie van Dr. L.H.K. Defize en Prof. Dr. S.W. de Laat. Een subsidie van het Ter Meulen Fonds van de KNAW stelde haar in staat om een gedeelte van dit onderzoek uit te voeren in de vakgroep ‘Morphogenèse Cellulaire et Progression Tumorale’ aan het ‘Institut Curie’ te Parijs, Frankrijk, onder supervisie van Dr. J.P. Thiery.

Ondersteund door een ‘Marie Curie Individual Fellowship’ van de Europese Commissie zal Jacqueline vanaf juni 2001 werkzaam zijn als post-doctoraal onderzoeker onder leiding van Dr. S. Bellusci en Dr. J.P. Thiery aan het ‘Institut Curie’ te Parijs, Frankrijk.

LIST OF PUBLICATIONS

Involvement of the tyrosine kinase Fer in cell adhesion (1998).
Roberto Rosato*, Jacqueline M. Veltmaat*, John Groffen and Nora Heisterkamp.
Molecular and Cellular Biology 18(10): 5762-5770.
*Both authors contributed equally

Parathyroid hormone-related peptide (PTHrP) induces parietal endo derm formation exclusively via the Type I PTH/PTHrP receptor (1999).
Mark H.G. Verheijen, Marcel Karperien, Ung-il Chung, Merel van Wijnen, Heleen Heystek, Jeanine A.A. Hendriks, Jacqueline M. Veltmaat, Beate Lanske, En Li, Clemens W.G.M. Löwik, Siegfried W. de Laat, Henry M. Kronenberg and Libert H.K. Defize.
Mechanisms of Development 81: 151-161.

Dynamic connexin43 expression and gap junctional communication du ring endoderm differentiation of F9 embryonal carcinoma cells (2000).
Marcel A.G. van der Heyden*, Jacqueline M. Veltmaat*, Jeanine A.A. Hendriks, Olivier H.J. Destrée and Libert H.K. Defize.
European Journal of Cell Biology 79: 272-282.
*Both authors contributed equally

Snail is an immediate early target gene of parathyroid hormone related peptide signaling in parietal endoderm formation (2000).
Jacqueline M. Veltmaat, Claudia C. Orelio, Dorien Ward-van Oostwaard, Marga A. van Rooijen, Christine L. Mummery and Libert H.K. Defize.
The International Journal of Developmental Biology 44(3): 297-307.

Cloning and analysis of the mouse follistatin promoter (2000).
Egbert de Groot, Jacqueline Veltmaat, Andrea Caricasole, Libert Defize and Adriana van den Eijnden-van Raaij.
Molecular Biology Reports 27: 129-139.