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**The Effects of Growth Factors on
Laminin Secretion by F9-Derived
Primitive Endoderm-Like Cells**

by Angela Marie Tonary, B.Sc.

University of Ottawa, 1994

Thesis submitted to the School of Graduate Studies
of the University of Ottawa as partial fulfillment of
the requirements for the degree of Master of Science,
Department of Physiology, Faculty of Medicine.



Angela Marie Tonary, Ottawa, Canada, 1994



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ABSTRACT

Reichert's membrane (RM) is a basement membrane that is deposited on the inner surface of the trophectoderm (TE) just prior to implantation in mice and rats. Components of RM include laminin, type IV collagen, fibronectin and heparan sulfate proteoglycan. The parietal endoderm (PE) cells have been shown to synthesize laminin, type IV collagen and heparan sulfate proteoglycan present in RM, while it appears that the fibronectin in RM is contributed by the TE cells. The PE, RM and TE together form the parietal yolk sac, which nourishes the developing fetus.

The PE cells arise from the first inner cell mass (ICM)-derived cell lineage, the primitive endoderm (PrE) cells. The PrE cells move away from the ICM and migrate along the TE, differentiating to PE cells at some point in this migration. Primitive endoderm cells also secrete laminin and type IV collagen, and it is the contention of this author that secretion of these extracellular matrix (ECM) glycoproteins by the PrE cells may provide the substratum for their migration by contributing to the initial formation of RM.

Very little is known about factors that may *regulate* the secretion of ECM proteins found in RM. Therefore, this research project studied the possible growth factor regulation of laminin glycoprotein secretion by PrE-like cells. F9 embryonal carcinoma (EC) cells were used as an *in vitro* model endoderm system, and the effects of transforming growth factors- β (TGF- β 1 and - β 2), platelet-derived growth factor (PDGF) and transforming growth factor- α (TGF- α) on laminin secretion by F9-derived PrE-like

cells were assessed. Thus, F9 EC cells were treated with retinoic acid to induce differentiation to a PrE-like cell type. The PrE-like cells were subsequently cultured with various concentrations of the four growth factors, and the levels of secreted laminin were measured using a specific enzyme-linked immunosorbent assay (ELISA).

The results indicated that TGF- β 1 decreased the amount of laminin secreted by PrE-like cells after 72 h. This inhibition of laminin secretion by TGF- β 1 was a specific effect of this growth factor since the levels of total cellular protein in untreated and TGF- β 1-treated PrE-like cells were the same. Additionally, the decreased laminin secretion did not reflect an increase in deposition of laminin by TGF- β 1-treated PrE-like cells. TGF- β 2 was not functionally equivalent to TGF- β 1 in this system, as it had no effect on the secretion of laminin. Although TGF- α appeared to stimulate increased secretion of laminin by PrE-like cells, it was not a statistically significant result. PDGF did stimulate a small, but significant, increase in laminin secretion by PrE-like cells. A model is presented to account for growth factor-induced changes in ECM composition in the early developing embryo.

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LIST OF ABBREVIATIONS AND CHEMICAL FORMULAE

ABTS	2,2'-azino-di[3-ethyl-benzathiazolinsulfonate] diammonium salts
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BSA	bovine serum albumin
$^{\circ}\text{C}$	degrees Celcius
Ca^{++}	calcium ion
cAMP	cyclic adenosine monophosphate
CO_2	carbon dioxide
CPSR-2	Controlled Process Serum Replacement-2
CRABP	cellular retinoic acid-binding protein
D-MEM	Dulbecco's Modified Eagle's Medium
dbcAMP	dibutyl cyclic adenosine monophosphate
dH_2O	distilled water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EC	embryonal carcinoma
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
fmol	femtamole
g	gram
G	guanine nucleotide-binding protein
G_i	inhibitory G protein
G_s	stimulatory G protein
GDP	guanine diphosphate
GTP	guanine triphosphate
h	hour
^3H	tritium
H_2O_2	hydrogen peroxide
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HRP	horseradish peroxidase
HSPG	heparan sulfate proteoglycan
ICM	inner cell mass
IgG	immunoglobulin
kDa	kilodalton
K_d	dissociation constant
l	litre

M	molar
MEM	Minimal Essential Medium
Mg ⁺⁺	magnesium ion
mg	milligram
min	minute
MIX	3-isobutyl-1-methylxanthine
ml	millilitre
mm	millimetre
mM	millimolar
mRNA	messenger RNA
NaCO ₃	sodium carbonate
NAD ⁺	nicotinamide adenosine diphosphate
ng	nanogram
NaHCO ₃	sodium bicarbonate
NaOH	sodium hydroxide
ND	nondetectable
ng	nanogram
nM	nanomolar
nm	nanometre
O ₂	oxygen
P _i	inorganic phosphate
PA	plasminogen activator
PAI	plasminogen activator inhibitor
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PE	parietal endoderm
PrE	primitive endoderm
RA	retinoic acid
RAR	retinoic acid receptor
RARE	retinoic acid response element
RBP	retinol binding protein
RGD	arginine-glycine-aspartic acid
RM	Reichert's membrane
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription and polymerase chain reaction
SEM	standard error of the mean
TE	trophectoderm
TGF- α	transforming growth factor-alpha
TGF- β	transforming growth factor-beta
U	unit
μ g	microgram
μ l	microlitre
UND	undifferentiated
VE	visceral endoderm

INTRODUCTION

1. Basement Membranes: Composition and Functions

Basement membranes are specialized extracellular matrix (ECM) structures. ECMs serve a variety of functions: acting as sheets to separate planes of cells and to filter molecules; giving tensile strength to, and accounting for, the elasticity of tissues; and maintaining the bulk of tissues (Mosher *et al.*, 1992). Basement membranes are also dynamic structures that regulate the morphology, proliferation, migration, differentiation and gene expression of cells (Timpl and Dziadek, 1986). Indeed, Schmidhauser and collaborators (1990) have demonstrated the existence of matrix-dependent elements that regulate transcription of the murine mammary β -casein gene.

The morphology of basement membranes varies, depending on both their location within the body and the cell type with which they are associated (Fisher *et al.*, 1985), and may reflect, in part, the physiological function they perform (Clark *et al.*, 1975a). However, all basement membranes are composed of basement membrane-specific macromolecules, which can include glycoproteins such as type IV collagen, laminin, entactin, and fibronectin, as well as heparan sulfate-rich proteoglycans (Semoff *et al.*, 1982; Amenta *et al.*, 1983; Hogan *et al.*, 1984; Grant *et al.*, 1985).

Studies documenting the pathophysiology caused by alterations in basement membranes emphasize the importance of these structures for normal cell function. For example, changes in basement membrane collagen underlie the Alport syndrome of renal failure (Zhou *et al.*, 1992) and lead to glomerular nephropathy in the diabetic renal

glomerulus (Cohen, 1978). Furthermore, aberrations in the normal interactions between epithelial cells and basement membranes can lead to tumor invasion and metastasis (Gehlsen *et al.*, 1988; Plantefaber and Hynes, 1989; Aznavoorian *et al.*, 1990).

Reichert's membrane (RM) is a basement membrane that is deposited on the inner surface of the trophectoderm (TE; Figure 1) during the preimplantation stage of mouse and rat embryo development, and it persists throughout most of gestation (Jollie, 1968; see section 2.3). This research project examined the regulation of secretion of laminin, a glycoprotein present in RM, by cultured endoderm-like cells.

2. Summary of Murine Early Embryo Development

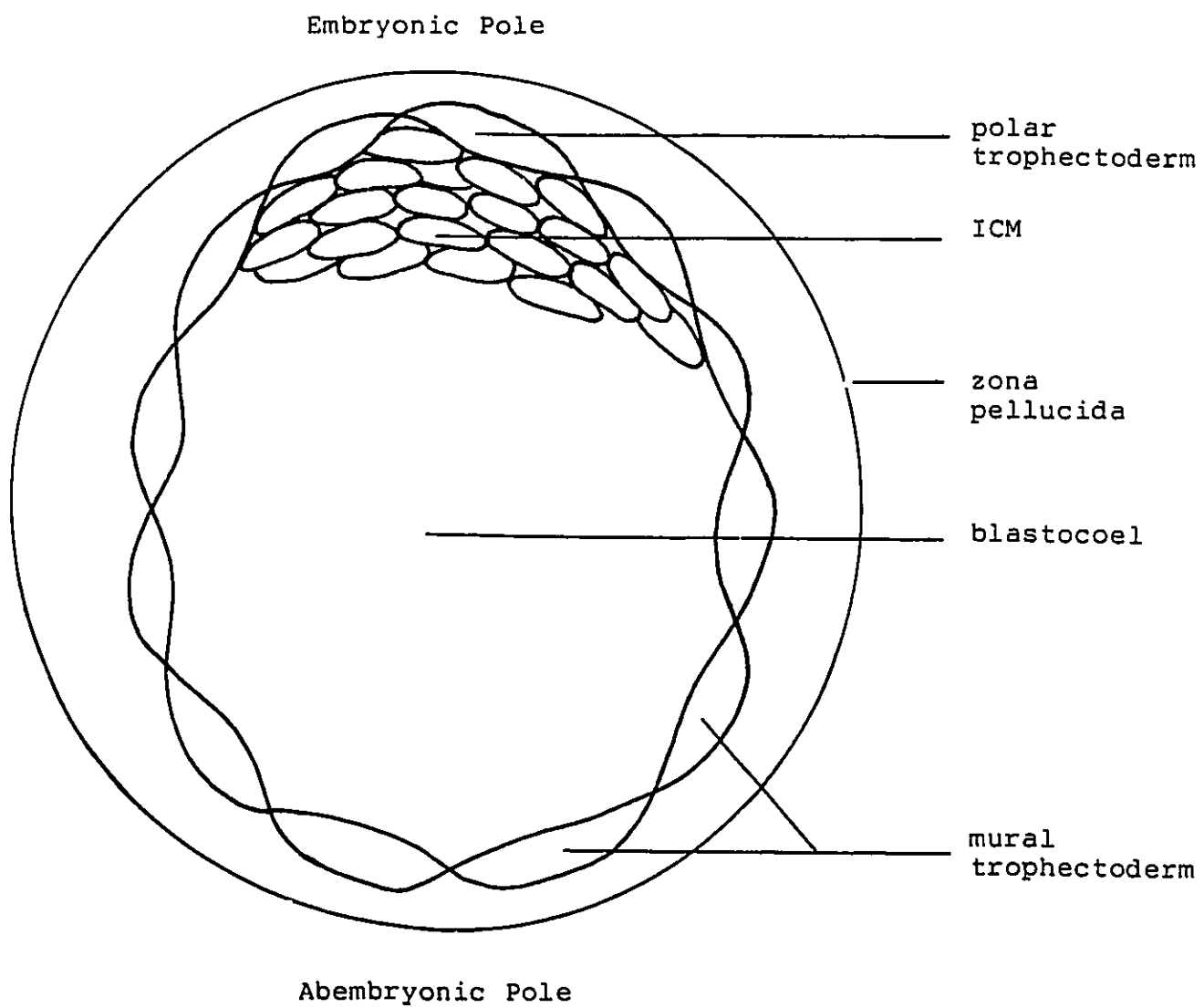
2.1 Zygote to Implanted Blastocyst

The typical gestation time for mice (and rats) is 19-21 days, depending on the strain. A fertilized egg cleaves three times to form an eight-cell uncompact embryo. Up to the mid-two-cell stage, the embryo relies largely on protein and RNA synthesized during oogenesis (Hogan *et al.*, 1986). By the mid-two-cell stage, many embryonic genes are switched on, while much of the maternally-inherited mRNA is rapidly degraded (Schultz, 1986; Telford *et al.*, 1990). Individual blastomeres from two- and four-cell embryos can each give rise to a mouse. Cleavage to the sixteen-cell stage is followed by a process known as compaction, which generates two distinct cell lineages, the TE and the inner cell mass (ICM; Hogan *et al.*, 1986). During compaction, the blastomeres increase their contact and communication with one another and polarization of the outer blastomeres occurs: the polar blastomeres develop into the TE while non-polar

blastomeres become the ICM and remain pluripotent (Johnson and Maro, 1986; Wiley *et al.*, 1990). The process of compaction is dependent on cell-cell adhesion molecules (Shirayoshi *et al.*, 1983; Richa *et al.*, 1985; Johnson *et al.*, 1986).

The embryo next undergoes cavitation, whereby a fluid-filled cavity (termed the blastocoel) is formed within the blastocyst. A fully expanded blastocyst consists of a trophectoderm vesicle containing a small group of anteriorly-located ICM cells and the blastocoel (Hogan *et al.*, 1986; Figure 1). During cavitation, the pluripotency of the ICM cells (to generate TE) is restricted by thin, TE-derived cytoplasmic processes that effectively enclose the blastocoelic surface of the ICM, separating it from the cavity (Fleming *et al.*, 1984). These processes are subsequently withdrawn, providing the opportunity for those ICM cells facing the blastocoel to differentiate into primitive endoderm (PrE; Wiley *et al.*, 1990). The fully expanded blastocyst contains about sixty-four cells, approximately twenty of which are in the ICM (Hogan *et al.*, 1983).

Figure 1. Drawing representative of a longitudinal section through a mouse blastocyst, approximately Day 3.5-4 of gestation. Drawing is about 1670X actual size.



Up to this developmental stage, the embryo is enclosed in an ECM coating called the zona pellucida (Figure 1). Studies using mouse embryos have shown this coat to be composed of three main proteins, termed ZP1, ZP2 and ZP3. The zona pellucida is important during fertilization of the oocyte (binds the sperm and induces the acrosome reaction) and also protects the cleaving embryo as it moves down the oviduct into the uterus (Wassarman and Mortillo, 1991). During the fifth day of development, the blastocyst hatches from the zona pellucida, attaches to the uterine epithelium and implants in the uterine endometrium (Hogan *et al.*, 1986). Immediately prior to implantation, the PrE cell layer differentiates on the blastocoelic surface of the ICM (4.0-4.5 days post-coitum; Hogan *et al.*, 1986; Figure 2). These PrE cells subsequently give rise to two morphologically and biochemically distinct subpopulations: the parietal endoderm (PE) and visceral endoderm (VE) cell layers in the yolk sacs surrounding the fetus (Hogan *et al.*, 1986; Figure 3). The remaining ICM cells become organized into the primitive ectoderm layer, which gives rise to the ectodermal, mesodermal and endodermal tissues of the fetus, to the germ cells, and to the mesodermal components of the extraembryonic membranes and placenta (Hogan *et al.*, 1986).

Figure 2. Drawing representative of a longitudinal section through a preimplantation stage mouse blastocyst, approximately Day 4.5 of gestation. The primitive endoderm (PrE) cell layer has differentiated on the blastocoelic surface of the inner cell mass (ICM) and PrE cells are beginning to migrate along the trophectoderm. Drawing is about 1670X actual size.

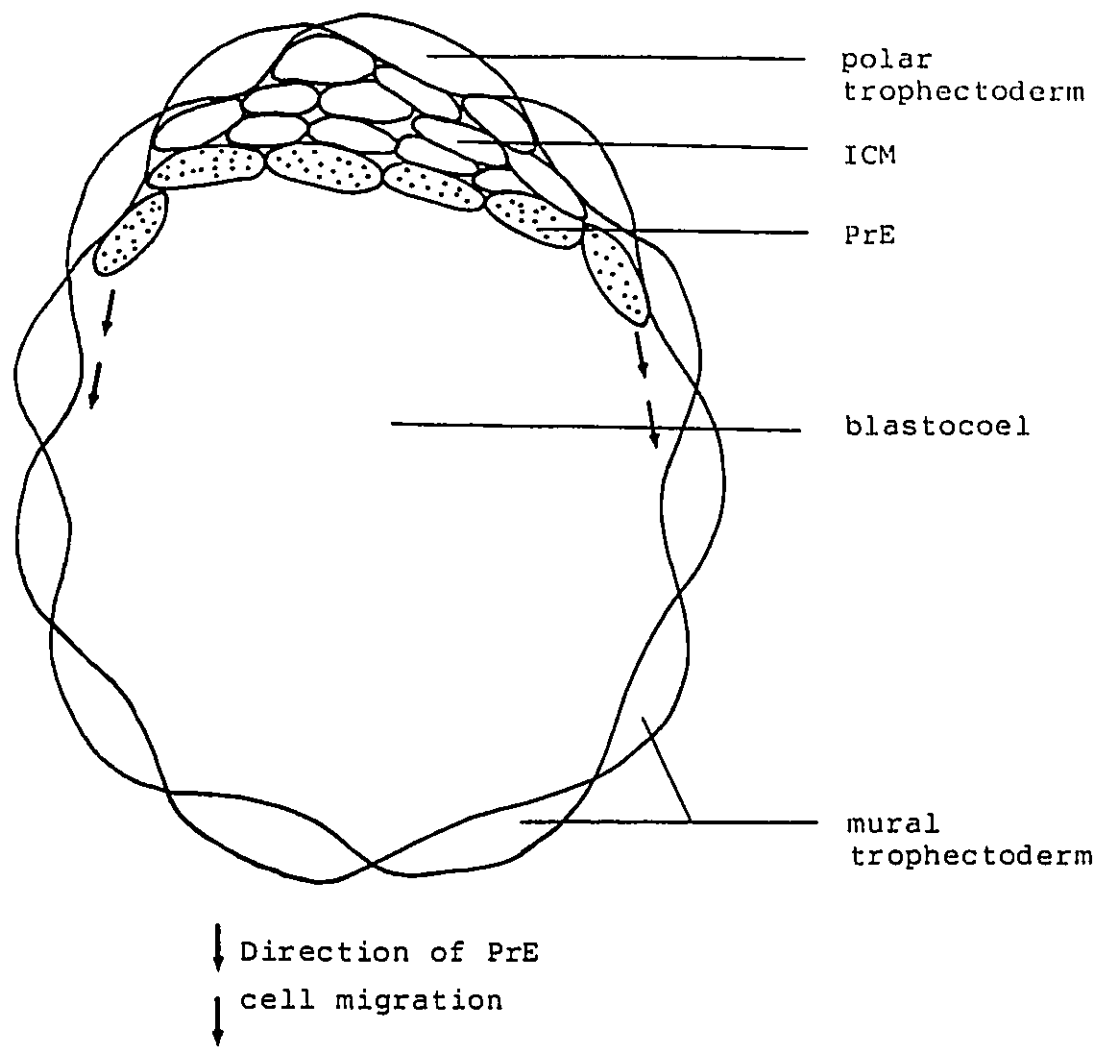
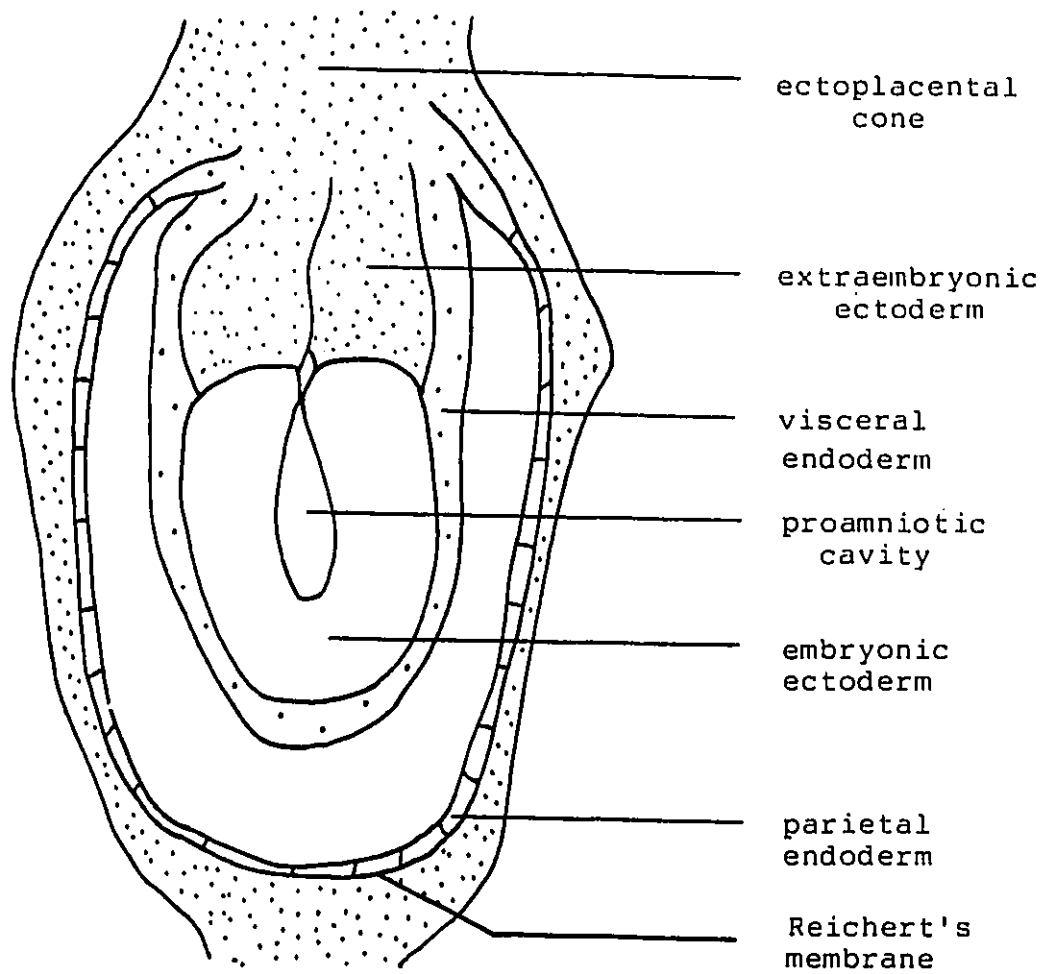


Figure 3. Egg cylinder-stage (approximately Day 6.5) of normal mouse embryo development. (Re-drawn from Slack, 1991)



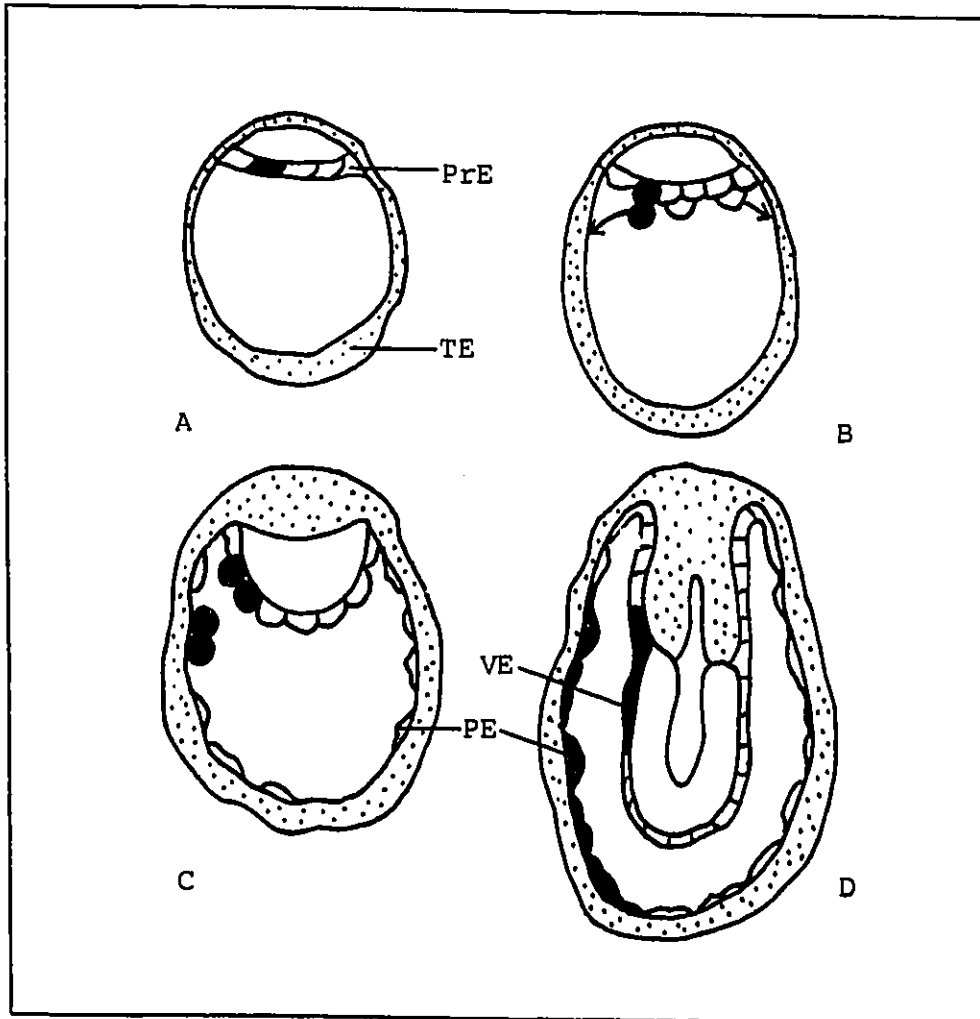
2.2 Primitive and Parietal Endoderm Cells

PrE cells can be distinguished from primitive ectoderm cells, using electron microscopy, because they contain a more extensive rough endoplasmic reticulum which is swollen with secretory material (Nadijcka and Hillman, 1974; Enders *et al.*, 1978). Both laminin and type IV collagen are seen intracellularly in the PrE (Adamson and Ayers, 1979; Leivo *et al.*, 1980) and, along with fibronectin, these glycoproteins are markers for the PrE cell type (Hogan *et al.*, 1983).

PE cells first appear at the time of implantation (Enders *et al.*, 1978), when PrE cells migrate onto the inner surface of the TE. Several studies have shown that the PrE cells give rise to the parietal (and visceral) extraembryonic endoderm. For example, both the PE and VE of conceptuses developing from blastocysts that had been reconstituted from genetically dissimilar TE and ICM expressed the marker of the ICM donor blastocyst (Gardner *et al.*, 1973; Papaionnou, 1982). Also, injection of single Day 5 PrE cells into host blastocysts resulted in those cells contributing to the PE and VE of the host embryos (Gardner, 1982).

It has been proposed that mitosis of PrE cells releases some daughter cells from contact with the primitive ectoderm, thereby enabling these cells to migrate peripherally and, subsequently, to differentiate into PE cells (Gardner, 1982; Figure 4). Consequently, the entire surface of the former blastocoelic cavity is lined with endoderm in the early post-implantation embryo (Gardner, 1983).

Figure 4. Diagram illustrating a proposed scheme for the initial steps in extraembryonic endoderm differentiation. (A) Represents a mouse blastocyst at the end of the fourth day of development showing the recently differentiated primitive endoderm (PrE) cell layer. The developmental fate of one PrE cell, depicted in black, is followed in B-D. When the PrE cell divides in the later blastocyst (B), only one daughter cell retains the position of the parent cell. The other daughter is released and is free to migrate laterally and contribute its progeny to the parietal endoderm (PE; C). Division of the daughter cell remaining adjacent to the ectoderm may be oriented in one of two directions, depending on whether it occurs prior to or during expansion of the ectodermal surface. In the former situation, it will repeat the pattern of the parent cell (B); in the latter, both daughters may remain adjacent to the ectoderm (C). Thus, individual PrE cells would contribute mitotic descendants to the PE and visceral endoderm (VE) of the post-implantation embryo (D). (Re-drawn from Gardner, 1982)



Cells exhibiting the characteristic appearance of PE cells are often seen to migrate out from isolated ICMs explanted on a tissue culture surface (Gardner, 1983; Carnegie and Cabaca, 1991; Carnegie and Cabaca, 1993). That these migratory cells were endoderm cells was confirmed when they colonized the PE following injection into genetically dissimilar host blastocysts (Gardner, 1983); furthermore, they stained positively for laminin and type IV collagen (Carnegie and Cabaca, 1991; Carnegie and Cabaca, 1993).

The most significant feature of PE cells is their grossly enlarged endoplasmic reticulum filled with secretory material, including the components of RM (Hogan *et al.*, 1986; see section 2.4). RM components may be secreted through the coated pits seen on the undersurface of PE cells (Hogan *et al.*, 1984). PE cells lie scattered over the surface of RM and have been shown to be motile (Hogan *et al.*, 1984; Grover and Adamson, 1986). Indeed, they establish only focal contacts with each other and with the surface of the mural TE on which they migrate (Enders *et al.*, 1978).

It is currently unknown when the differentiation of PrE cells to PE cells actually occurs. PrE cells migrate out from the ICM and move along the TE. At some point in this continuum, the migrating PrE cells differentiate into PE cells that secrete abundant quantities of laminin and type IV collagen, as well as entactin and heparan sulfate proteoglycan. However, PrE cells also secrete significant quantities of laminin and type IV collagen (Hogan *et al.*, 1983), albeit in reduced quantities compared to PE cells. It is the contention of this author that the ECM glycoproteins secreted by PrE cells may contribute to the matrix for their migration by contributing to the initial formation of

RM. Thus, PrE-like cells were used in this research work to elucidate a role for growth factors in the regulation of laminin secretion and PrE to PE differentiation in the early mouse embryo.

2.3 Reichert's Membrane: Structure and Proposed Function

RM is a basement membrane that is laid down between the extraembryonic PE layer and the TE beginning early in mouse and rat embryo development (Jollie, 1968); it is first visible in the mouse embryo 6-8 days after fertilization (Pierce, 1966). The PE, RM and TE layers together form the parietal yolk sac (Jollie, 1968). The parietal yolk sac, along with the placenta, the visceral yolk sac and the amnion, are responsible for the nourishment, protection and maintenance of the fetus inside the uterus (Hogan *et al.*, 1986). Although a human embryo does not have a RM, cells which may be analogous to PE cells do migrate from the ICM and, rather than depositing a thick basement membrane like RM, instead form a meshwork of cells with basement membrane material around each cell (Luckett, 1978).

RM has been proposed to regulate the passage of material between maternal blood and the fetal yolk sac cavity, since neither the trophoblastic layer nor the PE layer are continuous over the membrane (Jollie, 1968). Reichert's membrane has been shown to selectively regulate the passage of materials since ferritin, trypan blue and lithium carmine were found to cross RM, while thorotrast, iron ammonium citrate and lampblack did not (Jollie, 1968).

The size of RM increases linearly throughout much of gestation, achieving a maximum thickness of 11 μm , with a more than 10-fold increase in the surface area of RM occurring between 11.5 and 17.5 days of development in the rat (Clark *et al.*, 1975a). This is due to continued active secretion of RM components. Indeed, except for the lens capsule, RM is much thicker than other basement membranes which have been studied (Clark *et al.*, 1975a).

RM ruptures along the parietal wall and retracts to the peripheral margins of the chorioallantoic placenta on the eighteenth day of gestation in the rat (Clark *et al.*, 1975a). However, a residual portion of RM remains intact over the fetal surface of the placenta, so that maternal blood continues to be separated from the uterine yolk sac cavity (Jollie, 1968). Since the relative amount of collagen in RM decreases with increasing gestational age (Clark *et al.*, 1975a), it has been postulated that the rupture of RM occurs when the tensile strength of RM is no longer sufficient to contain the rapidly growing fetus and the increasing pressure of the extraembryonic fluids within the yolk sac (Clark *et al.*, 1975a).

2.4 Components of Reichert's Membrane

RM is composed of laminin, type IV collagen, entactin, fibronectin and heparan sulfate proteoglycan (Wartiovaara *et al.*, 1979; Leivo *et al.*, 1980; Smith and Strickland, 1981; Semoff *et al.*, 1982; Amenta *et al.*, 1983; Wu *et al.*, 1983). The composition of mouse and rat RM is remarkably similar (Smith and Strickland, 1981). The PE cells have been shown to synthesize laminin, type IV collagen, entactin and heparan sulfate proteoglycan (Clark *et al.*, 1975a,b; Hogan *et al.*, 1980; Leivo *et al.*, 1980), but secrete

little, if any, of the fibronectin found in the membrane (Jetten *et al.*, 1979; Hogan *et al.*, 1980; Smith and Strickland, 1981). The fibronectin associated with RM has been localized by immunoelectron microscopy towards the trophoblastic margin and not directly beneath the PE cells (Semoff *et al.*, 1982). Indeed, immunoreactive fibronectin has been localized to the cytoplasm of TE cells in rat blastocysts, therefore supporting a role for these cells in the synthesis of the fibronectin present in RM (Carnegie, 1991).

Laminin is a 900 kDa glycoprotein composed of three polypeptide chains (A, B1 and B2; Hogan *et al.*, 1984) interconnected to form a cruciform shape, with each chain contributing one short arm and all three chains forming the long arm (Akiyama *et al.*, 1990; Beck *et al.*, 1990). Laminin is the major non-collagenous protein found in virtually all basement membranes of mature tissues (Kleinman and Weeks, 1989; Beck *et al.*, 1990). This glycoprotein has binding domains for type IV collagen, heparin, cell surface receptors and itself (Ruoslahti *et al.*, 1981; Rao *et al.*, 1983), and it forms a stable complex with entactin (Paulsson *et al.*, 1987). Laminin promotes cellular attachment and is mitogenic for several cell types (Panayotou *et al.*, 1989; Beck *et al.*, 1990); indeed, the A, B1 and B2 chains all possess EGF-like repeats (Kleinman and Weeks, 1989).

Type IV collagen is a 500 kDa glycoprotein composed of two α -1 chains and one α -2 chain (Timpl and Dziadek, 1986) and it polymerizes via the formation of both end-to-end and lateral associations (Timpl *et al.*, 1981; Tsilibary *et al.*, 1988), thereby forming a network with which other basement membrane components can associate (Tsilibary *et al.*, 1988). Indeed, type IV collagen fulfills a key role as the main

supportive framework of basement membranes (Martinez-Hernandez and Amenta, 1983).

Fibronectin is a dimer of two 220 kDa glycoprotein chains connected by disulfide bridges. It exists as plasma (soluble) fibronectin and cellular (insoluble) fibronectin, both of which have similar molecular properties (Hynes and Yamada, 1982; Ruoslahti, 1988). Within the cellular fibronectin molecule are binding domains for collagen, hyaluronic acid, heparin, cell surface receptors and other fibronectin molecules (Hynes and Yamada, 1982; Ruoslahti, 1988), suggesting a key role for fibronectin in both the organization of ECM components and their interactions with cells. The smallest fibronectin fragment that retains full cell-binding activity is a 75 kDa fragment from the central one-third of the fibronectin polypeptide (Hayashi and Yamada, 1983; Akiyama *et al.*, 1985), which contains a key binding site of three amino acids, known as the RGD (arginine-glycine-aspartic acid) sequence (Ruoslahti and Pierschbacher, 1986). When present as a substratum for cultured endodermal (PFHR-9) cells, fibronectin promoted assembly of a basement membrane-type matrix containing laminin, type IV collagen and heparan sulfate proteoglycan; this was due to the interaction of the cells with the RGD sequence (Austria and Couchman, 1991).

Entactin (or nidogen) is a 158 kDa sulfated glycoprotein which was first isolated from a mouse embryonic endodermal cell line, but has since been found in basement membranes from a variety of rodent tissues including placenta, kidney, lung and smooth muscle (Carlin *et al.*, 1981). Entactin is a dumbbell-shaped molecule composed of a single polypeptide chain (Timpl and Dziadek, 1986). The extraction of laminin from Engelbreth-Holm-Swarm tumour-derived ECMs at physiological salt concentrations yields

laminin-entactin as a complex (Paulsson *et al.*, 1987). Similarly, antibodies against laminin specifically precipitated the laminin-entactin complex from eight mouse embryo-derived PE-like cell lines (Fowler *et al.*, 1990). It has been proposed that entactin mediates laminin binding to type IV collagen (Aumailley *et al.*, 1989).

Heparan sulfate is produced by a variety of cells and is incorporated into high molecular weight proteoglycans that are deposited on cell surfaces or in basement membranes (Iozzo, 1985). A prototype of a cell surface-associated heparan sulfate proteoglycan (HSPG) that is synthesized by human colon carcinoma cells has a molecular weight of approximately 300 kDa (Iozzo, 1985). HSPG can bind to fibronectin, laminin, type IV collagen and itself, and it appears to influence cell-fibronectin and fibronectin-collagen interactions (Iozzo, 1985; Ruoslahti, 1988). Rat parietal yolk sacs have also been shown to secrete chondroitin and dermatan sulfate proteoglycans during culture (Clark and Iozzo, 1984), suggesting that more than one type of proteoglycan may be involved in cell-ECM interactions during early embryogenesis.

3. Growth Factors and Early Embryonic Development

The formation of RM is a very important physiological process in early mouse and rat development. However, very little is known about factors that may regulate the production of ECM proteins for incorporation into RM. Therefore, this research project investigated the effects of transforming growth factor-beta 1 (TGF- β 1), transforming growth factor-beta 2 (TGF- β 2), platelet-derived growth factor (PDGF) and transforming growth factor-alpha (TGF- α) on laminin secretion by early embryo-like cells.

3.1 Transforming Growth Factors- β

TGFs- β are dimeric polypeptides composed of two 12 kDa chains which influence a wide range of developmental processes (Ignotz and Massagué, 1987; Bassols and Massagué, 1988). The mammalian TGF- β family consists of three related proteins: TGF- β 1, TGF- β 2 and TGF- β 3 (Nilsen-Hamilton, 1990); all forms are homo- or heterodimers of closely related subunits (Mummery *et al.*, 1990). TGF- β 1 appears to be the predominant TGF- β in various tissues, and sequences of TGF- β 1 from human, simian, bovine and porcine sources are identical and differ by only one amino acid residue from murine TGF- β 1 (Nilsen-Hamilton, 1990). TGF- β 2 is a homodimer of subunits sharing 71% amino acid homology with TGF- β 1 (Mummery *et al.*, 1990), while TGF- β 3 exhibits 77% and 79% amino acid homology compared with TGF- β 1 and TGF- β 2, respectively (ten Dijke *et al.*, 1988). TGF- β 1 and TGF- β 2 are generally equipotent in inducing TGF- β effects on cells (Massagué, 1987).

Although up to nine different proteins have been shown to bind TGF- β , to date, only three have been identified as high affinity TGF- β receptors: types I (53 kDa), II (73-95 kDa) and III (also called betaglycan, 300 kDa; Massagué, 1992). Types I and II bind TGF- β 1 and - β 3 more potently (10-20X) than TGF- β 2, while the type III receptor binds the three TGF- β s equally (Cheifetz *et al.*, 1987; Segarini *et al.*, 1987). The type III receptor has been correlated with many of the TGF- β effects on ECM, including increased synthesis of fibronectin, collagen, chondroitin/dermatan sulfate proteoglycans and cell adhesion receptors (Cheifetz *et al.*, 1987; Ignotz and Massagué, 1987; Bassols and Massagué, 1988). However, based on its structure, it appears unlikely that the type

III receptor is involved in signal transduction. Instead, it has been proposed that TGF- β binding to the type II receptor might be needed for binding to the type I receptor, and that both receptors then cooperate to initiate a productive signal (Laiho *et al.*, 1991; Massagué, 1992). Thus, betaglycan may function as a receptor accessory molecule in the TGF- β system (Massagué, 1992).

Using reverse transcription and polymerase chain reaction (RT-PCR), Rappolee and colleagues (1988) detected TGF- β 1 mRNA as early as the 4- to 8-cell stage of mouse preimplantation development; the mRNA level increased continuously thereafter. Additionally, TGF- β 1 antigens were identified by immunocytochemical localization in 70-90% of the cells of permeabilized blastocysts (Rappolee *et al.*, 1988). Paria and Dey (1990) demonstrated that the inferior development of singly cultured mouse embryos to blastocysts was markedly improved by TGF- β 1.

TGF- β 2 transcripts have been detected as early as the morula stage of murine embryonic development and continued to be expressed in preimplantation blastocysts (Kelly *et al.*, 1990). Positive staining of 4-cell mouse embryos with antisera to TGF- β 2 has been obtained (Slager *et al.*, 1991), while mid-blastocyst stage embryos showed anti-TGF- β 2 reactivity in the TE cells but not the ICM (Mummery *et al.*, 1990; Slager *et al.*, 1991). Additionally, blastocysts cultured on feeder layers to encourage TE outgrowth and endoderm differentiation showed the emerging PrE cells to be labelled above background for the presence of TGF- β 2 (Mummery *et al.*, 1990).

There is a significant body of evidence to support a key role for TGFs- β (β 1 and/or β 2) in inducing matrix accumulation via dual effects on the synthesis of ECM

components and inhibition of ECM degradation. Table 1 provides a comprehensive, but by no means exhaustive, compilation of these studies.

Table 1. Effects of TGF- β on ECM in various cell culture systems

REFERENCE	CELL CULTURE SYSTEM	EFFECT ON ECM
Centrella <i>et al.</i> , 1986	fetal rat calvariae in serum-free organ culture	increased synthesis of type I bone collagen and non-collagen proteins
Ignatz and Massagué, 1986	normal and transformed cells, incl. WI-38, NRK, Balb/c3T3, Mv1Lu	increased fibronectin & collagen synthesis and their incorporation into a matrix
Laiho <i>et al.</i> , 1986	HEL-299 and WI-38 human embryonic lung fibroblasts	decreased PA activity due to increased secretion of PAI
Roberts <i>et al.</i> , 1986	subcutaneous injection of newborn mice	rapid activation of fibroblasts to produce collagen
Saksela <i>et al.</i> , 1987	bovine capillary endothelial cells	decreased PA activity: decreased production of PA and increased secretion of PAI
Bassols and Massagué, 1988	NRK-49F rat kidney fibroblasts	increased synthesis of fibronectin, type I procollagen and chondroitin/dermatan sulfate proteoglycans
Keski-Oja <i>et al.</i> , 1988	A549 lung adenocarcinoma cells & WI-38 human lung fibroblasts	induction of fibronectin & type I procollagen synthesis; increased PAI mRNA levels
Penttinen <i>et al.</i> , 1988	3T3 mouse fibroblasts	increased mRNAs for $\alpha 1$ (I) collagen and fibronectin
Roberts <i>et al.</i> , 1988	IMR-90 human fetal lung fibroblasts	stimulated expression of fibronectin and fibronectin receptor complex
Wikner <i>et al.</i> , 1988	human keratinocytes in serum-free defined medium	increased fibronectin and PAI secretion
Helino <i>et al.</i> , 1989	WI-38 lung fibroblasts	elevated expression of $\alpha\beta_1$ integrins mRNAs and proteins
Carrington and Reddi, 1990	chick limb bud mesoderm cells	increased proteoglycan synthesis
Ichijo <i>et al.</i> , 1990	human oral squamous cell carcinoma cell line HSC-4	over 300-fold increase in secreted levels of fibronectin
Westergren-Thorsson <i>et al.</i> , 1990	human lung and skin fibroblasts	concentration-dependent stimulation of proteoglycan synthesis
Kahari <i>et al.</i> , 1991	normal human skin and gingival fibroblasts	increased expression of type I procollagen
Matrisian <i>et al.</i> , 1992	Rat-1, -2 fibroblasts	repression of transcription of metalloproteinase stromelysin

In summary, TGFs- β influence ECMs by (1) increasing the synthesis of ECM components, including glycoproteins and proteoglycans, (2) regulating the expression of integrins, (3) decreasing the expression of matrix-degrading enzymes, and (4) increasing the expression of inhibitors of matrix-degrading enzymes. These effects on ECM and their presence in the preimplantation embryo argue for a possible role of TGF- β s in the regulation of production of RM components.

3.2 Platelet-Derived Growth Factor

PDGF is a potent mitogen in serum which also induces fibroblast migration (Seppa *et al.*, 1982; Rizzino and Bowen-Pope, 1985). PDGF has two polypeptide chains: A and B. PDGF-AA and PDGF-BB homodimers as well as a PDGF-AB heterodimer have been identified and purified (Helden and Westermarck, 1989). The three dimeric forms of PDGF interact selectively with at least two forms of PDGF receptors (Hart *et al.*, 1988; Helden and Westermarck, 1989). The A-type receptor binds all three isoforms, whereas the B-type receptor binds PDGF-BB with high affinity, PDGF-AB with lower affinity, and PDGF-AA not at all (Kazlauskas *et al.*, 1988).

PDGF-A chain mRNA has been detected in early cavitation mouse blastocysts, and PDGF-A antigen was concentrated in punctate structures in the perinuclear area of all the cells of the blastocyst (Rappolee *et al.*, 1988; Kelly *et al.*, 1990; Mercola *et al.*, 1990). Preimplantation mouse blastocysts also expressed mRNA for the PDGF type A receptor (Mercola *et al.*, 1990). Furthermore, PDGF-BB has been measured in medium conditioned by human blastocysts (Svalander *et al.*, 1991).

Interestingly, the mitogenic effects of TGF- β on smooth muscle cells, fibroblasts and AKR-2B cells have all been suggested to be mediated via the induced expression of PDGF (Leof *et al.*, 1986; Majack *et al.*, 1990). Normal human mammary epithelial cells grown in serum-free medium in the presence of TGF- β had a rapid (within 1 h) 20- to 40-fold stimulation of PDGF-B chain mRNA expression, and secreted PDGF activity was increased 2- to 3-fold (Bronzert *et al.*, 1990). At present, no evidence exists for direct influences of PDGF on ECM composition. However, since proliferation in response to TGF- β can be mediated by PDGF, it was of interest to compare the effect of TGFs- β and PDGF, administered separately, on ECM protein secretion to determine if the anticipated stimulatory effect of TGF- β could be mimicked by PDGF.

3.3 Transforming Growth Factor- α

TGF- α activity was first detected in culture supernatants of rodent fibroblasts transformed with Moloney or Kirsten murine sarcoma viruses (reviewed by Derynck, 1986). Rat TGF- α is a 50 amino acid peptide, derived from the proteolytic cleavage of a 160 amino acid precursor; human TGF- α differs from rat TGF- α by only 4 amino acid residues (Derynck, 1986). TGF- α is related to epidermal growth factor (EGF) and binds to the EGF receptor. EGF and TGF- α exhibit a remarkably similar mode of interaction with the EGF receptor: both compete for binding with the same potency (Massagué, 1983). In fact, the interaction of either EGF or TGF- α with this receptor results in the activation of the receptor-associated tyrosine kinase activity (Pike *et al.*, 1982; Massagué, 1983; Paria *et al.*, 1991). EGF receptors have been detected on 8-cell/morula and

blastocyst-stage mouse embryos (Paria and Dey, 1990). TGF- α and EGF can exert similar or very different effects, depending on the biological system being studied; it is currently unclear how these ligands trigger divergent responses.

Along with PDGF, TGF- α mRNA is expressed in early cavitation mouse blastocysts, and TGF- α antigen is found in all cells of the blastocyst (Rappolee *et al.*, 1988; Kelly *et al.*, 1990). Tamada and collaborators (1991) demonstrated the co-localization of TGF- α (by immunohistochemistry) and its mRNA (by *in situ* hybridization) in mouse uterine luminal and glandular epithelial cells on Days 1-4 of pregnancy, and in uterine stromal cells on Days 3 and 4. Paria and Dey (1990) also showed that the mouse uterus produced TGF- α during the preimplantation period. Indeed, like TGF- β 1, TGF- α markedly improved the development of singly cultured mouse embryos to blastocysts (Paria and Dey, 1990). Additionally, Dardik and Schultz (1991) reported that TGF- α stimulated the rate of blastocoel expansion in early cavitating mouse blastocysts.

TGF- α (or EGF) often antagonizes TGF- β -induced effects on ECM composition (Laiho *et al.*, 1986; Roberts *et al.*, 1986; Matrisian *et al.*, 1992). Therefore, it was of interest to determine whether TGF- α would have an effect opposite to the anticipated stimulatory effect of TGF- β on ECM protein secretion.

4. Regulation of Extracellular Matrix Secretion by Embryonic Cells

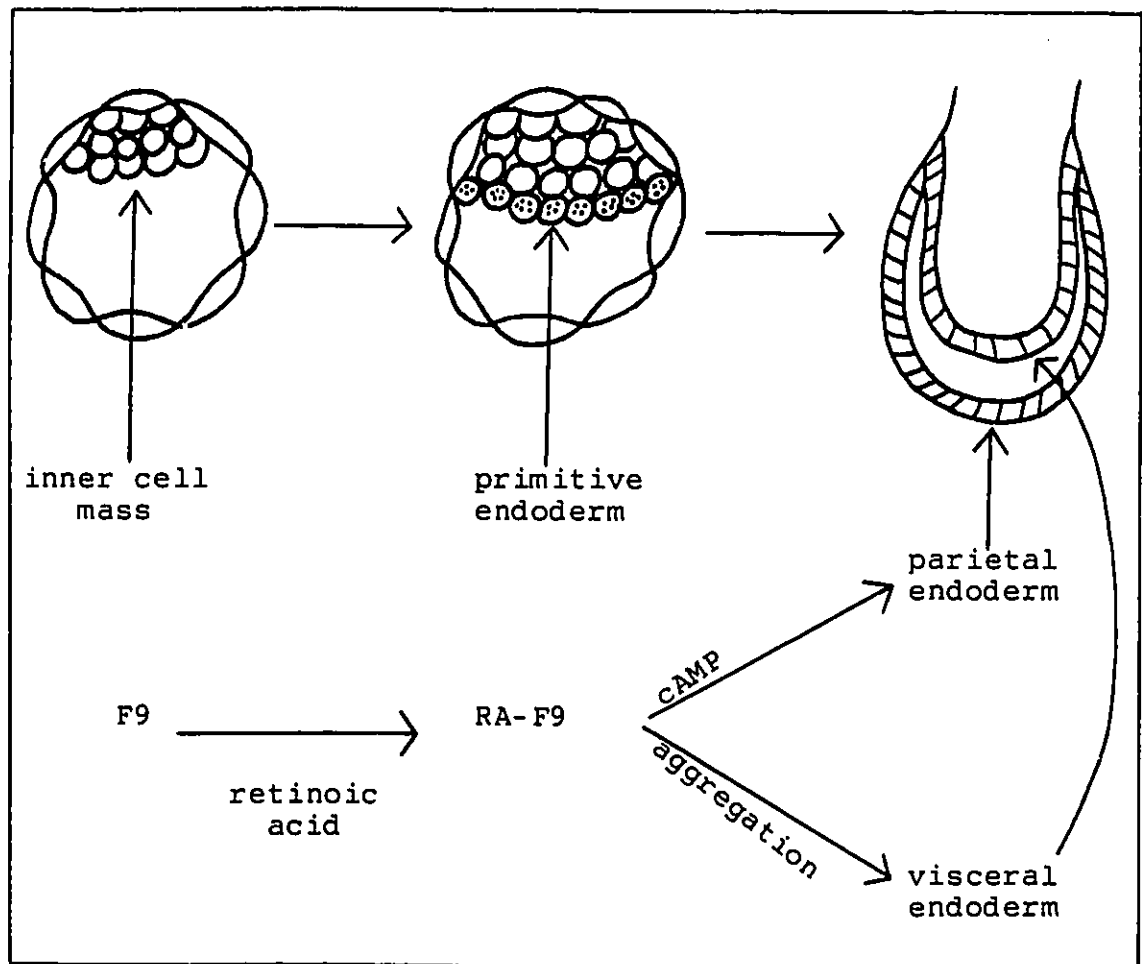
4.1 Development of a Model *in vitro* Endoderm System

In order to study growth factor regulation of laminin secretion during early

embryo development, the F9 mouse embryonal carcinoma (EC) cell line was selected to model endoderm formation in the early embryo. EC cells closely resemble the stem cells of the early mouse embryo and can differentiate *in vitro* into cells similar to definitive embryonic cells (Strickland, 1981). The F9 cell line was established in culture from a subline of the transplantable tumour OTT6050, which was derived from a Day 6 male embryo transplanted into the testis (Bernstine *et al.*, 1973).

In 1978, Strickland and Mahdavi showed that F9 cells which were treated with retinoic acid (RA) differentiated into cells resembling the PrE cells of the early embryo. Furthermore, monolayers of RA-treated F9 cells that were then exposed to dibutyryl cyclic adenosine monophosphate (dbcAMP) differentiated into cells that resembled PE. On the other hand, RA-differentiated F9 cells that were cultured as cell aggregates formed VE-like cells (Figure 5). In their study, F9 cell differentiation was assessed morphologically as well as biochemically by the secretion of plasminogen activator (PA), since embryonic PrE and PE cells have been shown to secrete PA (Strickland *et al.*, 1976). The addition of RA (10^{-7} M) to F9 cell cultures increased the secretion of PA more than 10-fold during three days of culture; at all concentrations of RA tested, dbcAMP (10^{-3} M) stimulated additional PA secretion by these cells (Strickland and Mahdavi, 1978).

Figure 5. Schematic comparison of the differentiation of F9 EC cells to normal mouse embryo development. Treatment of F9 EC cells with retinoic acid (RA) converts the stem cells, which are equivalent to inner cell mass cells, to a primitive endoderm-like (PrE) cell type. Depending on external influences, the PrE cells can form parietal (after cAMP treatment) endoderm or visceral (after aggregation) endoderm. (Re-drawn from Strickland, 1981)



The results of several studies have demonstrated similarities between differentiated F9 cells and native endodermal cells. Solter and coworkers (1979) showed that F9 cells treated with RA expressed decreased amounts of stage-specific embryonic antigen-1 (SSEA-1; an antigenic determinant characteristic of mouse embryos and teratocarcinoma stem cells), concomitant with increased PA production. Using immunofluorescence, Strickland and collaborators (1980) showed that the percentage of these cells expressing the SSEA-1 antigen was 8.5% (compared to 75.5% for control cultures) after RA treatment and 0% after RA + dbcAMP treatment.

RA stimulates F9 cells to secrete laminin and type IV collagen but reduces their secretion of fibronectin (Strickland *et al.*, 1980). Undifferentiated F9 cells synthesize little or no type IV collagen (Strickland *et al.*, 1980), and approximately 0.02% of their total protein is laminin (Hogan *et al.*, 1983). RA + dbcAMP treatment of F9 cells resulted in dramatically increased amounts of secreted laminin and type IV collagen (Strickland *et al.*, 1980; Carlin *et al.*, 1983; Grover and Adamson, 1986) as well as measurable secretion of entactin (not produced by undifferentiated F9 cells; Carlin *et al.*, 1983). The RA + dbcAMP-treated cells also synthesized less fibronectin (Strickland *et al.*, 1980; Carlin *et al.*, 1983; Grover and Adamson, 1986). RA is required for F9 cell responsiveness to dbcAMP; F9 cells treated with dbcAMP alone do not exhibit protein secretion patterns that differ from untreated F9 cells (Strickland and Mahdavi, 1978; Strickland *et al.*, 1980; Galvin-Parton *et al.*, 1990).

4.2 Retinoic Acid (RA): Mechanism of Action

RA is a naturally occurring metabolite (retinoid) of vitamin A (retinol). It is widely believed that RA acts similarly to the steroid family of hormones (Evans, 1988): RA binds to a cellular retinoic acid binding protein (CRABP) in the cytoplasm of the cell; the RA:CRABP complex then migrates to the nucleus, where it binds to a retinoic acid receptor (RAR); the RARs act like transcription factors and bind to specific DNA sequences, known as retinoic acid response elements (RAREs), to activate certain genes. Furthermore, evidence that high affinity binding of the RAR- α receptor to its RARE is dependent on interactions with additional nuclear proteins suggests that specific combinations of RARs and cell-type specific coregulatory proteins may function to integrate the effects of RA on patterns of gene expression during development (Glass *et al.*, 1990).

Much of the experimental work to elucidate the mechanism of action of RA has been carried out using F9 cells. In these cells, it has been shown that the association of RA with a specific intracellular CRABP is critical for retinoid action, and that this CRABP facilitates the delivery of RA to the nucleus (Ong and Chytil, 1979; Liao *et al.*, 1981; Omori and Chytil, 1982). F9 cytosol exhibited saturable, high affinity binding for ^3H -RA, binding 643 ± 105 fmol/mg protein with an apparent dissociation constant of 9.2 ± 1.1 nM (Grippo and Gudas, 1987); additionally, RARs have been detected in F9 EC cells (Zelent *et al.*, 1989; Rochette-Egly *et al.*, 1991).

RA has been found to be involved in the regulation of several genes. For example, Chiocca and coworkers (1989) showed that RA treatment of murine peritoneal

macrophages increased the expression of transglutaminase mRNA by stimulating the transcription of this gene. In contrast, alkaline phosphatase mRNA levels in ROS 17/2.8 osteosarcoma cells treated with RA were significantly decreased (Imai *et al.*, 1988). Murphy and colleagues (1988) observed dramatic increases in the level of *Hox-1.3* transcripts within 4 h after the addition of RA, attributable to a change in the rate of transcription of this gene. Transcription of the type IV collagen and laminin B gene were increased in F9 cells upon the addition of RA and dbcAMP (Levine *et al.*, 1984). Indeed, Vasios and coworkers (1989) have determined that a RARE is present between -477 to -432 bp of the 5' flanking region of the mouse laminin B1 gene.

It is possible that RA induces differentiation *in vivo* during early embryonic development. Several investigators have found components of the retinoid pathway in embryos. Using *in situ* hybridization, retinol binding protein (RBP; functionally equivalent to CRABP) was detected in the ICM and trophoblast of preimplantation-stage pig conceptuses (Harney *et al.*, 1990; Trout *et al.*, 1991), and sheep placental RBP was immunolocalized in the trophectoderm of 13 day old blastocysts (Liu *et al.*, 1992). RBP has also been detected via Western blot analysis in culture medium conditioned by conceptuses of pig, cattle and sheep (Trout *et al.*, 1991). Although there is little evidence at this time for the presence of components of the retinoid pathway in pre- or peri-implantation mouse embryos, there is a significant amount of data from F9 cells to suggest that they may be present in the early mouse embryo.

4.3 Dibutyl cyclic AMP (dbcAMP): Mechanism of Action

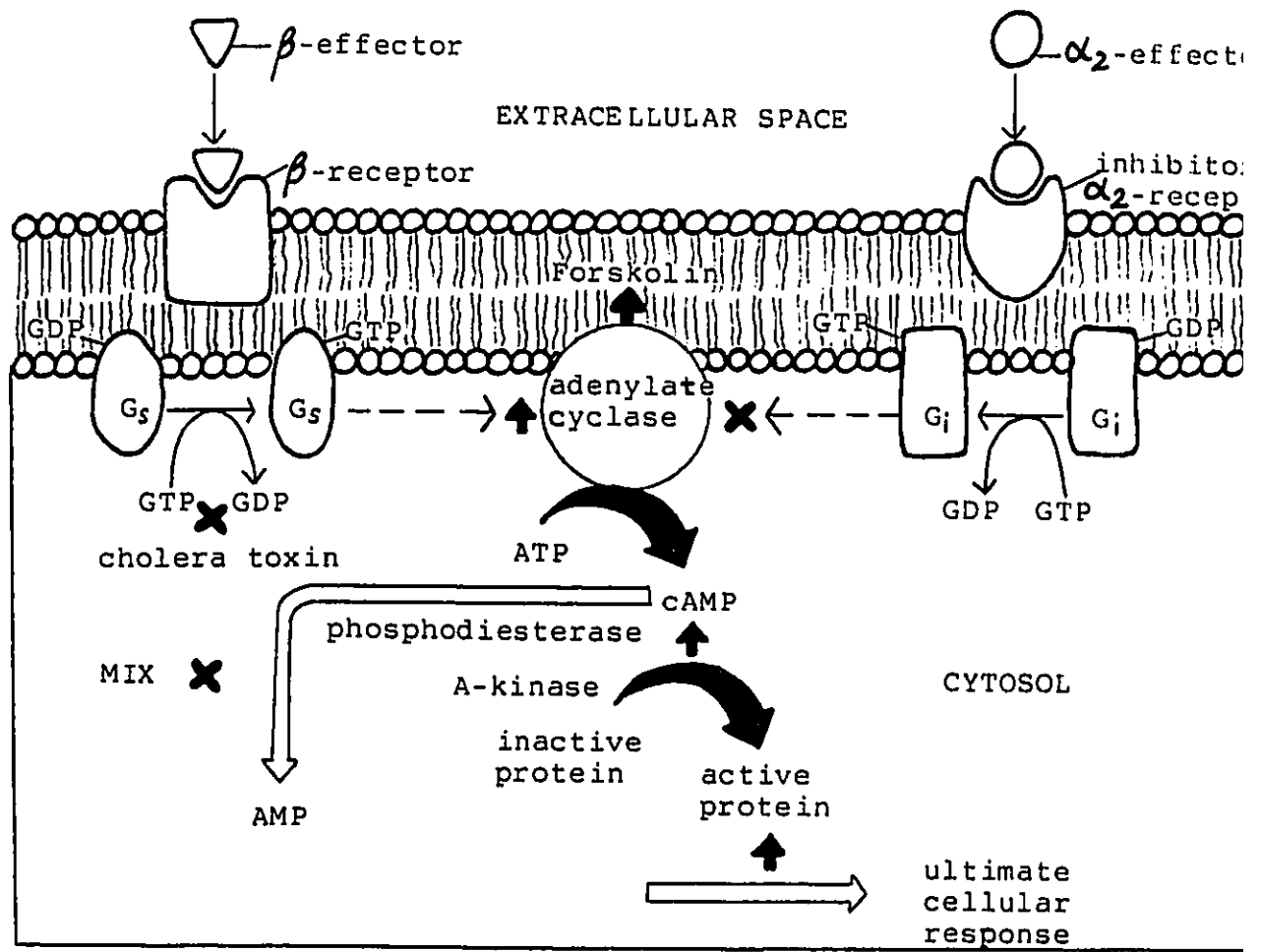
It is believed that RA treatment of F9 cells results in their differentiation to a stable phenotype whose cells are then able to respond to dbcAMP (Strickland and Mahdavi, 1978; Solter *et al.*, 1979); the continued presence of RA is not necessary to maintain this phenotype (Strickland and Mahdavi, 1978; Dong *et al.*, 1980; Strickland and Sawey, 1980). Similarly, the cell type produced by RA + dbcAMP treatment of F9 cells is also stable (Strickland *et al.*, 1980).

Signal transduction mechanisms involving cAMP consist of three separate proteins: a hormone receptor, adenylate cyclase and a G protein (Birnbaumer, 1990; Mathews and van Holde, 1990; Figure 6). Activation of adenylate cyclase results in the accumulation of cAMP. Forskolin is a product derived from *Coleus forskohlii* which has been shown to directly activate the catalytic subunit of adenylate cyclase, thereby stimulating cAMP production (Seamon *et al.*, 1981). DbcAMP is a membrane-permeable cAMP analogue; the action of dbcAMP in the F9 cell system is due to its cAMP activity. Indeed, the addition of cholera toxin (10^{-9} M) or 1-methyl-3-isobutylxanthine (MIX; 10^{-4} M) to RA-treated F9 cells caused similar effects to those seen with dbcAMP, specifically, increased expression of collagen, laminin and PA (Strickland and Mahdavi, 1978; Strickland *et al.*, 1980; Grippo and Gudas, 1987). Cholera toxin is an enzyme from *Vibrio cholerae* that cleaves NAD^+ and transfers the ADP-ribose moiety to a specific site in the α subunit of G_s . This modification of G_s inhibits its inherent GTPase activity, thus converting α to an irreversible activator of adenylate cyclase (Bennett *et al.*, 1975; Cassel and Selinger, 1977; Cassel and Pfeuffer,

1978; Gill and Meren, 1978). MIX potentiates cAMP accumulation by inhibiting the phosphodiesterase activity (Mathews and van Holde, 1990).

Chan and collaborators (1990) found that RA induced G_{α} gene transcription and protein expression in F9 cells, and that this expression preceded the induction of $\alpha 1$ (type IV) collagen. However, it is unknown whether a RARE is present in the mouse G_{α} gene. Galvin-Parton and coworkers (1990) found that F9 cells treated with RA had decreased $G_{\alpha 2}$ levels. Furthermore, RA treatment of F9 cells caused an increase in cytoplasmic and plasma membrane-associated cAMP protein kinase (Plet *et al.*, 1987). Thus, it would appear that treatment of F9 cells with RA results in altered gene expression leading to facilitation of cAMP-regulated processes. It is currently unknown, however, what the *in vivo* ligand might be for stimulating cAMP production in the early embryo. Since retinoids occur naturally in mammals, it is conceivable that local concentrations of RA and intracellular levels of cAMP may play a role in endoderm differentiation *in vivo* in the early mouse embryo.

Figure 6. Signal transduction pathways involving G proteins and adenylate cyclase. G_s is a family of G proteins involved in the stimulation of adenylate cyclase, while G_i proteins inhibit adenylate cyclase. When an extracellular hormone or agonist binds to a receptor and causes a conformational change in the receptor so that it interacts with G_s , this stimulates the release of GDP from G_s and its replacement with GTP. G_s with bound GTP stimulates adenylate cyclase to produce cAMP from ATP. This cAMP then activates a cAMP-dependent protein kinase, which phosphorylates target protein(s) to elicit a cellular response(s). The activity of the adenylate cyclase is controlled by an inherent GTPase activity of the G protein which converts the GTP to GDP + P_i , thereby terminating adenylate cyclase activation. G_i functions similarly, but the binding of GTP provokes an interaction with adenylate cyclase which inhibits the enzyme and decreases cAMP levels. The amount of cAMP is modulated by a phosphodiesterase which hydrolyzes cAMP to inactive AMP. (Re-drawn from Mathews and van Holde, 1990)



4.4 F9 Cell Expression of, and Responsiveness to, Growth Factors

F9-differentiated cells release both TGF- β 1 and TGF- β 2; however, these growth factors are in the non-active or latent form and require acidification for biological activity (Kelly *et al.*, 1990). It has not yet been shown that RA can fulfill this requirement. Using RT-PCR and primers specific for TGF- β 1 and TGF- β 2, both of these transcripts have been detected in F9-differentiated cells (Kelly *et al.*, 1990). Of these, only TGF- β 1 has been detected in undifferentiated F9 cells (Kelly *et al.*, 1990; Mummery *et al.*, 1990). F9 EC cells bound little, if any, TGF- β 1 or TGF- β 2 and seemed to lack receptors for these growth factors (Rizzino, 1987; Mummery *et al.*, 1990). However, RA-induced differentiation of F9 cells lead to the appearance of receptors for these growth factors, and the RA-treated F9 cells bound TGF- β 1 and TGF- β 2 with subsequent growth inhibition (Rizzino, 1987; Mummery *et al.*, 1990).

Undifferentiated F9 cells did not respond to PDGF and appeared to lack receptors for this growth factor; however, they did secrete a factor that was similar to PDGF (possibly an embryonic form of PDGF; Rizzino and Bowen-Pope, 1985). RA-differentiated F9 cells bound PDGF, exhibiting 8.3×10^3 PDGF receptors per cell ($K_d = 30$ pM) after 6 days of RA treatment (Rizzino and Bowen-Pope, 1985), and demonstrated increased proliferation in response to this growth factor (Rizzino and Bowen-Pope, 1984, 1985).

Undifferentiated F9 cells also did not respond to, and appeared to lack receptors for, EGF (TGF- α). Again, differentiation of F9 cells in response to RA induced the expression of EGF receptors and the differentiated cells responded to exogenously added

EGF with increased cell proliferation (Rees *et al.*, 1979).

4.5 Validity of the Model

In summary, use of the F9 EC cell line to model endoderm formation in the early embryo is a valid system to study the growth factor regulation of laminin secretion by PrE-like cells. The F9 cell line provides an abundant and readily available source of cells, compared to the limitations associated with using embryos. The cells are easily maintained in culture, growing as monolayers on gelatin-coated dishes (no feeder layers are required), and can be induced to differentiate in a reproducible manner. As has been presented in this literature survey, F9 cells have been used extensively to study the secretion of endodermal protein products and RA actions on cells. In addition, the growth factor ligand and receptor profiles for the four growth factors used in this study have been characterized for F9 cells. That is, PrE-like cells do possess the necessary receptors to respond to exogenously added TGF- β 1, TGF- β 2, PDGF and TGF- α .

5. Research Objectives

The purpose of this research work was to determine what, if any, effects TGF- β 1, TGF- β 2, PDGF and TGF- α would have on the secretion of laminin glycoprotein by F9-derived PrE-like cells. The objectives of this project were therefore:

- (1) To establish enzyme-linked immunosorbent assays (ELISAs) to quantify ECM proteins secreted into culture media;

- (2) To establish the F9 cell line in culture:
 - a) to assess the morphology of undifferentiated and differentiated F9 cells;
 - b) to quantify laminin and type IV collagen secretion by undifferentiated and differentiated F9 cells; and
 - c) to determine the localization of laminin in undifferentiated and differentiated F9 cells;
- (3) To measure secretion of laminin glycoprotein by PrE-like cells treated with TGF- β 1, TGF- β 2, PDGF and TGF- α using a laminin ELISA; and
- (4) To propose an *in vivo* model for observed growth factor effects on laminin glycoprotein secretion reported in this study.

In addressing these objectives, this research project contributed the following new information relating to F9 cells as a model for PrE and PE cells. First, all of the experimental cultures were performed in the absence of serum to allow any growth factor-induced changes in laminin secretion to be accurately assessed. Second, in contrast to the semi-quantitative methods of determining secreted protein levels used by other investigators (for example, immunoblots), quantitative ELISAs were developed to measure the amounts of laminin and type IV collagen secreted in response to growth factors and regulators of signal transduction pathways. Third, existing immunostaining protocols were modified and adapted to facilitate optimal localization of laminin in undifferentiated and differentiated F9 cells. Additionally, a unique method was developed to assess the amount of laminin *deposited* (as compared to secreted amounts

of this glycoprotein) on the culture substratum by these cells. Finally, for the first time, the effects of TGF- β 2 and TGF- α on laminin secretion by PrE-like cells were assessed.

MATERIALS AND METHODS

1. Maintenance of the F9 EC Cell Line

F9 EC cells were obtained from the American Type Culture Collection (Rockville, Maryland; ATCC No. CRL 1720). Unless otherwise specified, all culture media and reagents were obtained from GIBCO (Grand Island, NY). F9 cells were cultured in gelatin-coated petri dishes (60 x 15 mm, Becton-Dickinson, Bedford, MA) using Dulbecco's Modified Eagle's Medium (D-MEM) supplemented with sodium bicarbonate (NaHCO_3 ; 3.7 g/L), penicillin-streptomycin (25,000 U and 25,000 $\mu\text{g/L}$, respectively), fungizone (625 $\mu\text{g/L}$) and 15 % heat-inactivated fetal bovine serum (FBS). The dishes were gelatin-coated by application of a gelatin solution [0.1 % in sterile distilled water (dH_2O)] for 2 h at room temperature (RT), after which time the solution was removed, the dishes were rinsed three times with sterile dH_2O , and dried at RT prior to use. The cells were maintained at 37 °C in an incubator equilibrated with 5 % CO_2 /95 % O_2 .

For routine maintenance, the cells were plated at a maximum density of 2.5×10^4 cells/ml. Cell number was determined by haemocytometer counting. Cell viability was assessed by trypan blue (0.06 % final concentration; Eastman Kodak Company, Rochester, NY) exclusion and was always greater than 98 %. The cells were subcultured every 3-4 days using 0.25 % trypsin/ 5×10^{-4} M ethylenediaminetetraacetic acid (EDTA) in $\text{Ca}^{++}/\text{Mg}^{++}$ -free Dulbecco's phosphate-buffered saline (PBS).

For long-term storage of F9 cells in liquid nitrogen, a freezing medium consisting of D-MEM supplemented with 20% FBS and 15% dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO) was used. Cell pellets were cooled on ice for 20 min, resuspended at 4 °C in the freezing medium ($5\text{--}20 \times 10^6$ cells/ml), and cooled overnight at -80 °C before being put into liquid nitrogen. In order to minimize cell variability due to long-term culture, the cells were passaged no more than twenty-five times before a fresh aliquot was thawed and plated. A vial of cells was thawed by transferring it directly from liquid nitrogen to a 37 °C waterbath. The vial was gently agitated during thawing and the contents were immediately transferred into a cold 15 ml Falcon tube. While continuing to agitate, 2 ml FBS followed by 12 ml D-MEM (supplemented as for cell culture) were added to the thawed cells. The cells were centrifuged ($180 \times g$ for 10 min), resuspended in culture medium (1×10^6 cells/ml), and transferred to a 60 mm petri dish. After 24 h of culture, the medium was replaced to remove residual DMSO and dead cells.

2. Experimental Cell Cultures

2.1 Serum-Free Culture of F9 Cells

While serum was used for the routine propagation of F9 cells, no serum was added to the experimental cultures as this would interfere with the assessment of growth factor effects. Instead, a defined serum replacement was used: Controlled Process Serum Replacement-2 (CPSR-2; Sigma), which does not contain detectable levels of plasma-derived fibronectin ($< 6\text{ ng/ml}$; J.A. Carnegie, unpublished data). In addition, the

inclusion of "medium only" controls in some of the laminin and type IV collagen ELISAs revealed nondetectable levels of these glycoproteins in CPSR-2-supplemented medium. Cells were cultured in 0.1 % gelatin-coated multiwell tissue culture plates (6-well Falcon, Becton-Dickinson) at a plating density of 2.5×10^4 cells/ml in Opti-MEM I [a HEPES-buffered modification of Eagle's Minimal Essential Medium (MEM), designed to reduce the serum requirement of cells during culture] supplemented with NaHCO_3 (2.4 g/L), 0.1 % mercaptoethanol (facilitates attachment of cells to the substratum), penicillin-streptomycin (25,000 U and 25,000 $\mu\text{g/L}$, respectively), fungizone (625 $\mu\text{g/L}$), 1 % MEM non-essential amino acids solution, and 0.25 % bovine serum albumin (BSA; Sigma), adjusted to pH 7.2. Hereafter this medium will be referred to as the *base medium*.

2.2 Verification of the Model

2.2.1 F9 Differentiation Study

F9 cells were cultured in base medium (undifferentiated, UND) or base medium supplemented with RA (all-trans RA; Sigma; 10^{-7} M = 3×10^{-4} mg/ml) or dbcAMP (N^6 -2'-O-dibutyryl-adenosine 3':5'-cyclic monophosphate; Sigma; 10^{-3} M = 0.49 mg/ml) or RA + dbcAMP. RA stock solutions (10^{-3} M) were made in absolute ethanol and stored at -20°C for a maximum of 7 days. For addition to experimental cultures, the RA stock was first diluted 1/100 in serum-free medium and then further diluted 1/100 into the culture medium to yield 10^{-7} M (final concentration of ethanol was 0.01%). All RA manipulations were performed in subdued light. For all four treatment groups, the cells

were initially plated at a density of 2.5×10^4 cells/ml and were cultured $\pm$ the indicated test compounds for 72 h. All of the treatment groups were completed in triplicate and the experiment was repeated a minimum of three times. After the 72 h time period, medium was collected from each well (4-6 aliquots of 250 μ l) and stored at -20 °C pending measurement of ECM glycoproteins (laminin and type IV collagen) by ELISAs (see section 3.1). The mean number of cells per well was determined using a haemocytometer and the cells were then replated at a density of 2.5×10^4 cells/ml, under the same culture conditions, for an additional 72 h. Media aliquots were again collected and the cells were counted to determine the secreted levels of ECM glycoproteins for the second 72 h culture period.

2.2.2 RA Dose-Response Study

The experimental design was as described in section 2.2.1, with the undifferentiated cells cultured in base medium, while the test cells were exposed to the following concentrations of RA: 0, 10^{-9} , 10^{-8} , 10^{-7} and 10^{-6} M.

Upon completion of these preliminary experiments, a general experimental protocol was designed for the subsequent studies.

2.3 General Experimental Design

F9 cells were cultured in base medium (UND) or base medium with RA (10^{-7} M) for 48 h, after which time all treatment groups were given fresh base medium [no RA; previous studies had shown that a transient exposure to RA was sufficient to induce and

maintain F9 cell differentiation (Dong *et al.*, 1990)], either unsupplemented (UND and RA control groups), or containing various concentrations of the test substance (each described separately in subsequent sections), and the cultures were continued for a further 72 h. All treatment groups were completed in triplicate and, unless otherwise indicated, all experiments were repeated a minimum of three times. After the 72 h culture period, medium was collected from each well and stored at -20 °C pending measurement of ECM glycoproteins by ELISAs, and the mean number of cells per well was determined using a haemocytometer. Variations of this general design are described in section 2.4.

2.4 Specific Experimental Protocols

2.4.1 Analysis of dbcAMP Mechanism of Action: Activation of G_i Signal Transduction Pathway

(A) Forskolin Dose-Response Study

Four treatment groups were used in this study: UND; RA (10^{-7} M); RA + forskolin (CalBiochem Corporation, La Jolla, CA; 1-200 μ M=0.4-82 μ g/ml); and RA + dbcAMP (10^{-3} M). Forskolin was prepared as a 50 mM stock in absolute ethanol and was diluted directly into the culture medium to yield the final concentration. Control treatment groups (UND, RA) received an equivalent volume of absolute ethanol, which did not exceed 0.1 %. The cells were cultured $\pm$ RA (10^{-7} M) for 48 h prior to a 72 h exposure to forskolin or dbcAMP.

(B) Cholera Toxin Dose-Response Study

In this study, the treatment groups included: UND; RA (10^{-7} M); RA + MIX (10^{-5} M); and RA + MIX + cholera toxin (Sigma; 1 mg/ml stock in sterile dH₂O; 0.01-10 μ g/ml). An RA-differentiated dbcAMP (10^{-3} M) + MIX-treated group was also included for comparison. The cells were initially cultured in base medium (UND) or base medium with 10^{-7} M RA for 48 h. At this point, treatment with RA was discontinued and the cells were given fresh base medium $\pm$ the indicated concentrations of MIX, cholera toxin and dbcAMP. The cells were cultured for 4 h, after which time the UND and RA control cells received fresh base medium, while the MIX, dbcAMP and cholera toxin groups of cells received fresh base medium with 10^{-5} M MIX, and the cultures were continued for an additional 72 h.

2.4.2 Growth Factor Studies

Studies to assess the effects of growth factors on laminin glycoprotein secretion were carried out as described in section 2.3, using the following growth factors: human TGF- β 1 (Collaborative Research Inc., Bedford, MA; 0.01-10 ng/ml); porcine TGF- β 2 (R&D Systems, Minneapolis, MN; 0.01-10 ng/ml); human PDGF (Collaborative Research Inc.; 0.1-10 ng/ml); and human TGF- α (Collaborative Research Inc.; 0.001-10 ng/ml).

2.4.3 TGF- β 1 and dbcAMP: Time Course of F9 Cell Responsiveness

Additional experiments compared in more depth the influences of TGF- β 1 and

dbcAMP on laminin secretion by RA-differentiated F9 cells. Four treatment groups were used in this study: UND; RA (10^{-7} M); RA + dbcAMP (10^{-3} M); and RA + TGF- β 1 (3 ng/ml). Following routine pre-culture with RA for 48 h, the cells were given fresh base medium $\pm$ dbcAMP or TGF- β 1 according to the treatment group. Medium samples were collected and the cells were counted following 6, 12, 24, 48 and 72 h of culture (post-addition of dbcAMP or TGF- β 1).

3. Assays

3.1 ELISA: Description of the Procedure

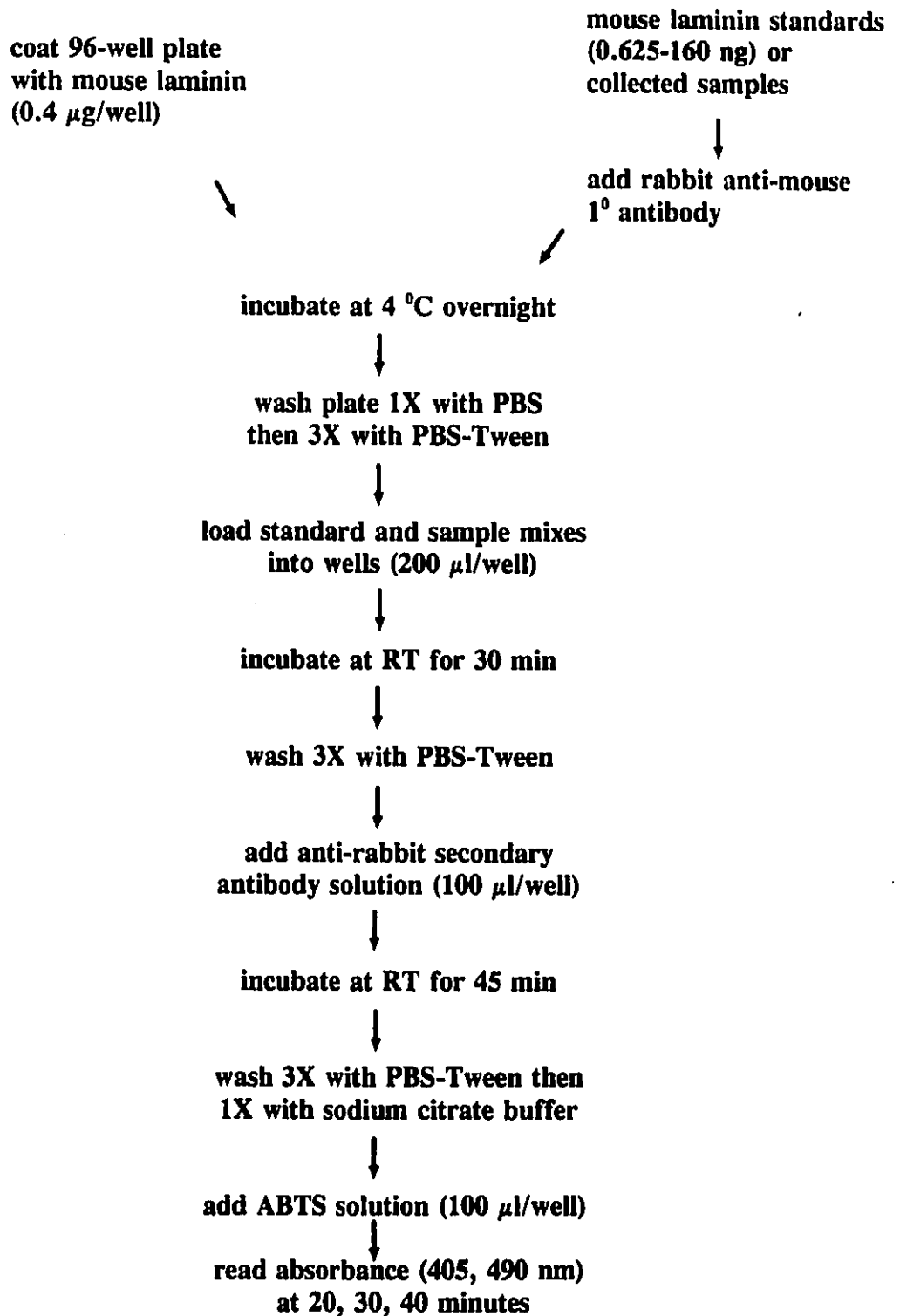
ELISAs were used to quantify the amount of laminin and type IV collagen secreted into the medium by cultured F9 cells. These ELISAs were all modifications of the fibronectin ELISA originally described by Skinner and collaborators (1985) and adapted by Carnegie (1990). The ELISA protocol (used to quantify laminin) is described below.

The ELISA was a two day procedure, as outlined in Figure 7. On Day 1, 96-well microtitre plates [Immulon 2 "U" Microelisa plates (Fisher) or Maxisorb U96 plates (GIBCO)] were coated with laminin [mouse laminin; Collaborative Research Inc.; 400 ng laminin/well in 100 μ l NaCO₃ buffer (20 mM; adjusted to pH 9.6)] at 4 °C overnight. Concurrently, standards of 0-160 ng laminin/100 μ l PBS-Tween (Tween 20, 0.05% in 10 mM sodium phosphate/0.85% sodium chloride, pH 7.4) were prepared (in triplicate) in polystyrene tubes. Anti-laminin antibody (rabbit anti-mouse laminin; Collaborative Research Inc.) was added (up to 1/6000 final antibody dilution, depending

on the antibody preparation) to both the standards and the samples. The antigen/antibody mixtures were then incubated at 4 °C overnight.

Figure 7. Overview of the protocol used for a laminin ELISA.

Laminin Enzyme-Linked Immunosorbent Assay (ELISA)



All of the Day 2 ELISA steps were performed at room temperature. First, each multiwell plate was washed once with PBS and then three times with PBS-Tween (last wash for 5 min). To each well was added 200 μ l of either the standard-primary antibody or sample-primary antibody mixture and incubation was for 30 min. Each plate was then washed three times with PBS-Tween (last wash for 5 min). Horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit IgG; Intermedico, Willowdale, ON; diluted 1/1,000 in PBS-Tween) was then added (100 μ l/well) and incubation was continued for a further 45 min. Each plate was then washed three times with PBS-Tween and once with sodium citrate buffer (38 mM sodium citrate, 8 mM sodium phosphate; adjusted to pH 4.25). The substrate was prepared just before use: 2 mg of 2,2'-azino-di [3-ethyl-benzathiazolinsulfonate] diammonium salts (ABTS; Boehringer-Mannheim, Dorval, PQ) in 5 ml sodium citrate buffer + 5 ml dH₂O + 5 μ l H₂O₂. The substrate was added to each well (100 μ l/well) and the plates were incubated in the dark for up to 40 min to allow the color reaction to develop. The absorbance at 405 and 490 nm was determined using a Dynatech Automated Microplate Reader (Model EL 310; Bio-Tek Instruments Inc., Winooski, VT). The 405 nm was the absorbance wavelength specific for the fluorochrome; the absorbance at 490 nm measured background absorbance (for example, due to light in the well) and this absorbance value was subtracted from the 405 nm value. Immediately prior to reading, each plate was gently swirled to ensure even distribution of the colored product. The standard and sample absorbance values were analysed using KinetiCalc software (Version 2.03, EIA Application Series; Bio-Tek Instruments Inc.). The concentration of laminin in each of the experimental samples was

expressed as ng protein/ 10^5 cells.

The ELISA to quantify type IV collagen followed the same protocol, with the following substitutions of antigen and antibody: mouse type IV collagen (Collaborative Research Inc.; 200 ng/well); and rabbit anti-mouse collagen IV primary antibody (Collaborative Research Inc.; 1/1,000 final dilution). The conjugated secondary antibody was the same as for the laminin ELISA.

3.2 Modified ELISA Application: Quantification of Laminin Deposition

F9 cells were cultured using the protocol described in section 2.3 with the following treatment groups: UND; RA; RA + dbcAMP; and RA + TGF- β 1 (3 ng/ml). The cells were cultured directly in 0.1% gelatin-coated Corning flat-bottomed 96-well plates, with nine wells per treatment group (experimental plate).

Twenty-four hours prior to completion of the cell culture, a gelatin-coated 96-well standard plate was prepared using serial dilutions (100-1.56 ng) of laminin in 100 μ l NaCO₃ buffer (100 μ l of each standard/well, done in triplicate) and stored at 4 °C overnight.

On the day of assay, the culture media was collected from the experimental plate and stored at -20 °C for subsequent determination of soluble laminin. Both the standard and experimental plates were washed once with PBS (all steps were performed at RT), and the cells were lifted from the experimental plate wells using either (1) PBS/EDTA (0.4% EDTA in PBS; 15 min) followed by 1% Triton X-100 (in PBS; 20 min) and

vigorous aspiration with a plastic pipette, or (2) a solubilization buffer (6 M urea, 1 mM phenylmethylsulfonylfluoride, 1 % Triton X-100 in PBS; 30 min; Scheidl *et al.*, 1992) and vigorous aspiration. The wells were then washed twice with PBS, and a 15 min PBS/BSA (2.4 mg/ml) incubation was done to reduce non-specific binding. After washing three times with PBS-Tween (last wash for 5 min), the two antibody incubations as well as the HRP substrate incubation were carried out as previously described for the laminin ELISA (section 3.1). The absorbance was again read at 405 and 490 nm and the relative amount of deposited laminin was calculated for each well.

3.3 Lowry Protein Assay

Total cellular protein levels were determined by the method of Lowry and coworkers (1951). Briefly, the cells were cultured with RA $\pm$ TGF- β 1 as outlined in section 2.3 and then harvested (3 wells/treatment group) using trypsin. The cells were centrifuged (180 x g, 10 min), rinsed once with PBS, and centrifuged again. The cell pellets were then suspended in 1 ml of 0.1 N NaOH and sonicated for 45 seconds at 30 % power (Fisher Sonic Dismembrator, Model 300 with microtip). A BSA standard curve was prepared using 0-100 μ g BSA in a total volume of 200 μ l of 0.1 N NaOH.

Standards and samples (diluted 1/4 and 1/8) were mixed with 1 ml of a 2 % sodium carbonate + 1 % copper sulfate/2 % sodium potassium tartate solution for 10 min. Next, 100 μ l of Folin-Ciocalteu's phenol reagent (BDH, Toronto, ON; diluted 1:2 with double dH₂O) was mixed with each standard and sample, and all were incubated in the dark for 30 min. The absorbance was read at 600 nm (Milton Roy Spectronic,

Model 1201) and protein levels in the samples were calculated using the BSA standard curve.

4. Microscopy

4.1 Cell Morphology

The cells were routinely observed using a Leitz Diavert inverted microscope. Representative cells were photographed onto Kodak Plus X film using the 10X objective.

4.2 Immunolocalization of Laminin in Cultured F9 Cells

Round coverslips (13 mm JBS #766; J.B. EM Services Inc., Dorval, PQ) were sterilized by soaking for 10 min in 70% ethanol at RT. The coverslips were placed upright in 6-well tissue culture dishes to allow the ethanol to evaporate, and then laid flat in the wells (3 per well). The wells + coverslips were gelatin-coated as previously described. F9 cells (1.0×10^5 cells/ml) were added to the wells (six coverslips/treatment group) in base medium alone (UND) or base medium supplemented with RA (10^{-7} M; PrE-like cells) or RA and dbcAMP (10^{-3} M; PE-like cells). After 48 h of culture, the cells were given fresh base medium $\pm$ RA $\pm$ dbcAMP as was appropriate for the treatment group and the culture was continued for a further 24 h. At the conclusion of the experiment, the coverslips with attached cells were removed from the wells and washed for 3 min in PBS (1X PBS diluted from a 10X stock, pH 7.0). All subsequent washes were carried out in 1X PBS for 3 min each and all manipulations were done at RT. The cells were fixed for 10 min in ice-cold 70% ethanol, washed twice, and

subsequently permeabilized in Nonidet P-40 (0.1% in 1X PBS) for 20 min. After being washed twice, coverslips from the control, PrE-like and PE-like treatment groups were incubated for 45 min with rabbit antibody to mouse laminin (same antibody as used for the ELISA, diluted 1:7 in 1X PBS), while some coverslips from the PE-like treatment group were incubated for the same length of time in PBS alone. Cells on the latter coverslips served as controls for the level of background fluorescence due to exposure to the labelled secondary antibody only. After exposure to the primary antibody, all of the coverslips were washed three times, and then they were exposed to fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG (Intermedico; diluted 1/10 in 1X PBS) for 45 min in the dark. The coverslips were then washed three times and each was mounted in a 10 μ l drop of anti-fade mounting medium (0.1% p-phenylenediamine in 50% glycerol, pH adjusted to 7.8). The p-phenylenediamine retards the bleaching of fluorescein during ultraviolet illumination (Johnson and Nogueira-Aranjo, 1981).

The cells were observed using a Zeiss Axiophot microscope (40X objective) equipped with epifluorescence optics. Representative cells were photographed onto Kodak Ektachrome Elite ASA 400 color slide film.

5. Statistical Analyses

Statistical comparisons of data in all studies were made by analysis of variance (ANOVA) using InStat software (Version 1.14, GraphPad Software, San Diego, CA; 1-way ANOVA) or SigmaStat software (Version 1.00, Jandel Corp., San Rafael, CA; 2-way ANOVA). When significant effects were observed, Duncan's Multiple Range Test

(Steel and Torrie, 1980) was used to determine post-hoc significance between specific treatment groups. Significance was inferred at $p < 0.05$.

In studies where the inter-experiment F9 cell responsiveness to RA was variable, all data were converted to percent of the RA-treated control value prior to statistical evaluation.

RESULTS

The possible growth factor regulation of laminin glycoprotein secretion by PrE-like cells was analyzed in this research project using F9 EC cells as an *in vitro* model for embryonic endoderm. The differentiation of F9 cells to PrE-like and PE-like cells was verified by both cell morphology and measurement of the secreted levels of laminin and type IV collagen using specific ELISAs. RA-differentiated F9 cells were then cultured with various concentrations of TGF- β 1, TGF- β 2, PDGF and TGF- α and the amount of laminin secreted by cells cultured under these conditions was measured using ELISA.

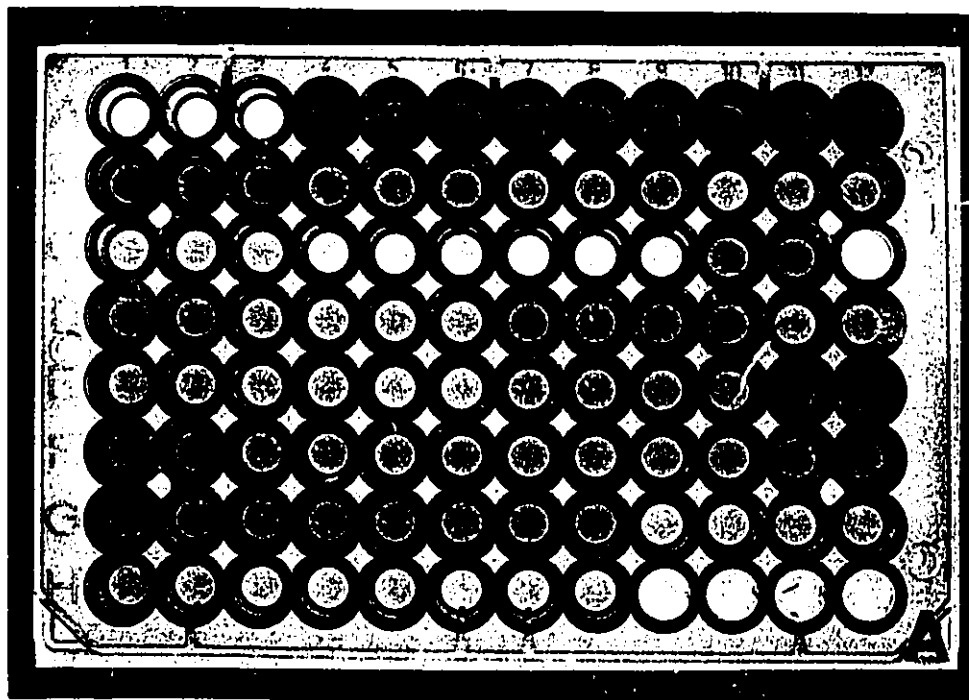
1. Verification of ELISAs for ECM Glycoproteins

Each ELISA included two conjugate blank microtitre wells plus two sets of internal standards (pools). The conjugate blank wells omitted exposure to the primary antibody; therefore, the absorbance values for these wells indicated the level of non-specific binding of the labelled secondary antibody. These wells were set as the absorbance blanks on the Microplate Reader: their mean value was subtracted from every other well. The pools were prepared by adding known concentrations of the antigens to Opti-MEM 1 + BSA. Pools were prepared in bulk and stored at -80 °C as 250 μ l aliquots. Each laminin ELISA included pools of 20 and 40 ng/100 μ l while those for the type IV collagen ELISA were 5 and 20 ng/100 μ l. For every assay, the pools were included as duplicate or triplicate wells, and they were treated identically to the samples on Day 1 of the assay. The pools monitored both the accuracy and the variability of the assays. The intraassay (within assays) coefficients of variation for these ELISAs were

7.2% for the low pools and 8.9% for the high pools, while the interassay (between assays) coefficients of variation were 11.8%, 13.5% and 12.2%, 13.1% for the laminin and type IV collagen (low, high) pools, respectively. Generally, both the laminin and type IV collagen ELISAs were linear from 2.5 ng to 80 ng (experimental sample values were thus read between these two values) and the sensitivity for each ELISA was 2.5 ng. A typical ELISA plate with the developed color reaction is shown in Figure 8A, and its corresponding table of standard values is given in Figure 8B.

Figure 8. Sample ELISA plate with corresponding standard curve values.

(A) 96-well microtitre laminin ELISA plate of culture media from two experiments, one in which the cells were immunostained for laminin, and a second in which the cells were assessed for total cellular protein levels. The plate was read 30 min after addition of substrate. See **MATERIALS AND METHODS** for a description of the ELISA protocol. Wells A1 through D6 are the standard curve: A1 and A2 conjugate blanks; A3 well not coated with laminin; A4-A6 primary antibody controls or "0 wells"; A7-A9 0.625 ng/100 μ l; A10-A12 1.25 ng/100 μ l; B1-B3 2.5 ng/100 μ l; B4-B6 5 ng/100 μ l; B7-B9 10 ng/100 μ l; B10-B12 20 ng/100 μ l; C1-C3 40 ng/100 μ l; C4-C6 80 ng/100 μ l; C7-C9 160 ng/100 μ l; C10 and C11 low pools; C12 empty well; D1 and D2 low pools; and D3-D6 high pools. Wells D7 through H8 are experimental culture media samples. The remaining wells were empty. Note the absence of color development in wells A1 to A3, showing minimal binding of labelled antibody in the absence of both antigen coating (A1, A2) and incubation with 1^o antibody (A3). (B) Standards values corresponding to the absorbance readings for the plate in (A), generated by KinetiCalc software.



STANDARD	LOCATION	ABSORBANCE	MEAN ABSORBANCE	COEFFICIENT OF VARIATION
0.625	A07	1.077	1.036	3.82%
0.625	A08	0.998		
0.625	A09	1.033		
1.25	A10	0.961	0.988	2.96%
1.25	A11	1.019		
1.25	A12	0.984		
2.5	B01	0.938	0.930	1.43%
2.5	B02	0.938		
2.5	B03	0.915		
5	B04	0.801	0.794	0.96%
5	B05	0.786		
5	B06	0.796		
10	B07	0.654	0.677	3.47%
10	B08	0.676		
10	B09	0.701		
20	B10	0.513	0.540	4.91%
20	B11	0.566		
20	B12	0.540		
40	C01	0.375	0.390	3.55%
40	C02	0.394		
40	C03	0.402		
80	C04	0.313	0.317	1.14%
80	C05	0.318		
80	C06	0.320		
160	C07	0.255	0.267	4.00%
160	C08	0.273		
160	C09	0.274		

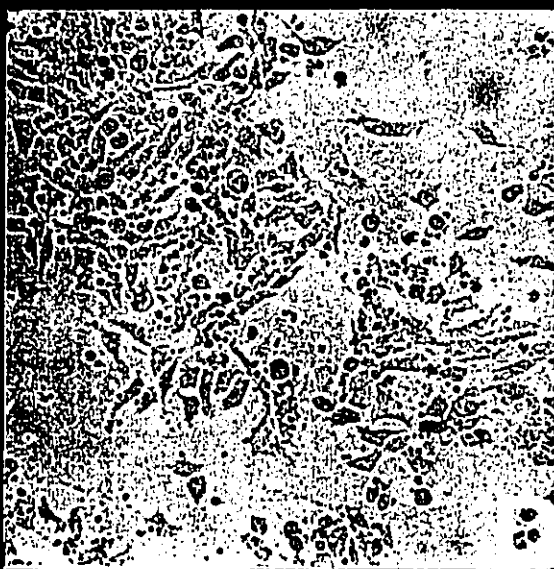
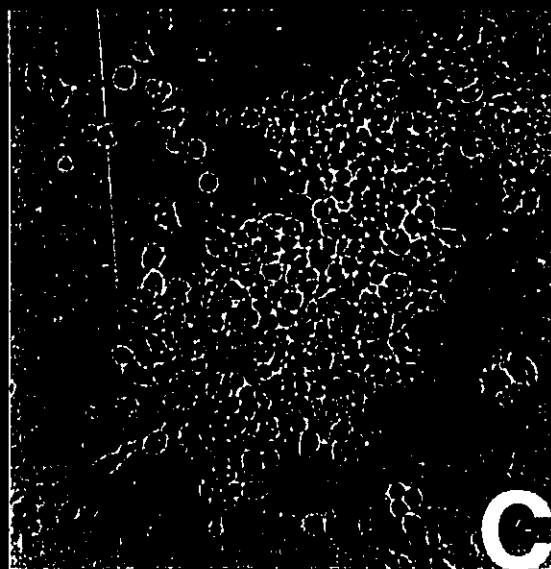
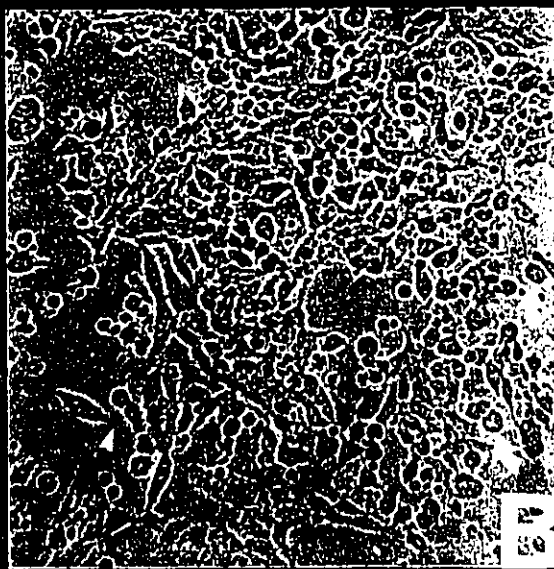
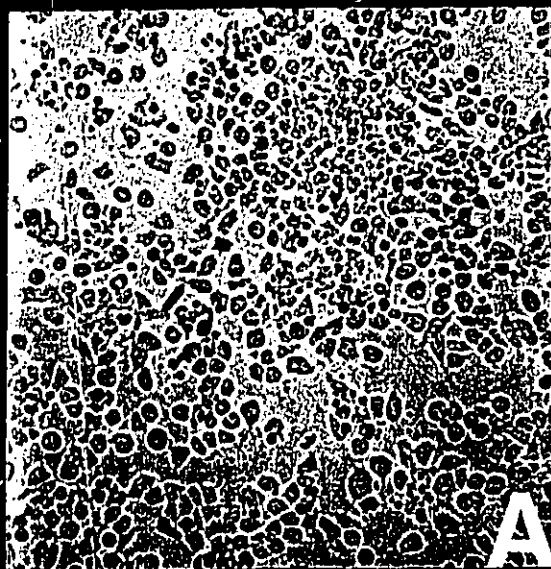
2. Establishing the F9 Cell Line as a Model for Embryonic Primitive and Parietal Endoderm

2.1 F9 Cell Morphology: Undifferentiated vs Differentiated Cells

F9 EC cells grew to confluent monolayers in culture. The maintenance cultures of F9 cells showed some degree of spreading due to the presence of serum in the medium; however, when cultured for experiments in serum-free base medium, these undifferentiated cells were round with minimal cytoplasm (Figure 9A). When F9 EC cells were treated with RA, the cells became elongated, with flattened cytoplasmic processes extending from their cell bodies (Figure 9B, arrowheads). However, even after six days of RA treatment, some round cells were still evident (Figure 9B, arrows). These likely represented cells preparing to undergo mitosis, since the cells did continue to proliferate during RA-induced differentiation. F9 cells treated with RA and dbcAMP had the same morphology as RA-treated cells, but they were less dense and, therefore, more spread than the PrE-like cells after six days of culture (Figure 9D). Cell cultures of F9 cells treated with only dbcAMP resembled the undifferentiated cell phenotype (Figure 9C). Concomitant with differentiation, there was a consistent decrease in cell proliferation (as determined at the conclusion of each experiment). RA-treated cell cultures were approximately 60% as dense as undifferentiated cell cultures, while RA + dbcAMP-treated cultures contained approximately 35% as many cells as the undifferentiated cultures.

Figure 9. Undifferentiated vs differentiated F9 cell morphology.

F9 cells were cultured for 144 h in base medium alone (A) or base medium supplemented with RA (10^{-7} M; B), dbcAMP (10^{-3} M; C) or RA + dbcAMP (D). The cells were given fresh culture medium ($\pm$ RA, dbcAMP) after the first 72 h of culture. Pictures shown are bright-field views of Day 6 unstained living cells. Magnification X200



2.2 Secretion of ECM Glycoproteins

Figure 10 summarizes the secretion of laminin and type IV collagen by undifferentiated and differentiated F9 cells. Undifferentiated F9 cells secreted a minimal amount of laminin: 6.5 ± 3.7 ng/ 10^5 cells. Similarly, cells treated with only dbcAMP secreted 8.1 ± 1.4 ng laminin/ 10^5 cells, an amount not significantly different from that released by undifferentiated cells ($p > 0.05$). Treatment of F9 cells with RA, however, caused a 19-fold increase ($p < 0.01$) in laminin secretion relative to undifferentiated controls, to 123 ± 22 ng/ 10^5 cells. RA-treated cells that were also exposed to dbcAMP demonstrated a further stimulation of laminin secretion, a 120-fold increase (780 ± 183 ng/ 10^5 cells; $p < 0.01$) compared to undifferentiated cells (Figure 10A).

In the same way but on a smaller scale, RA-treated F9 cells secreted 12 ± 4 ng type IV collagen/ 10^5 cells compared to nondetectable levels in undifferentiated cells. Treatment with dbcAMP alone also did not stimulate detectable levels of type IV collagen secretion. RA + dbcAMP-treated cells secreted 9.6-fold more type IV collagen compared to cells treated with just RA (Figure 10B).

Since subsequent experiments would utilize RA-differentiated cells, a RA dose-response study was performed. Figure 11 summarizes the influences of 10^{-9} M to 10^{-6} M RA on the secretion of laminin (Figure 11A) and type IV collagen (Figure 11B) by F9 cells during the second 72 h of a 144 h (6 day) culture period. Higher concentrations of RA were not tested because even 10^{-6} M RA appeared to have some toxic effect on the cells, as evidenced by a higher proportion of floating cells and cells that incorporated trypan blue (Tonary, unpublished data). RA at 10^{-9} M did not stimulate

laminin or type IV collagen secretion above that by undifferentiated F9 cells ($p > 0.05$). However, as the concentration of RA was increased up to 10^{-6} M, there was a corresponding increase in the secreted levels of laminin, with the highest levels measured at 10^{-6} M RA (395 ± 57 ng/ 10^5 cells). Type IV collagen secretion also increased with increasing concentrations of RA, to a maximum of 39 ± 2 ng/ 10^5 cells; however, the secreted levels measured in response to 10^{-8} M to 10^{-6} M RA were not significantly different ($p > 0.05$).

Only data from the second 72 h of the 144 h culture were shown in Figures 10 and 11, as these results more closely correlated with the five day cultures that were subsequently performed and analyzed. The laminin and type IV collagen secretion patterns for the first 72 h of culture were identical to those depicted in Figures 10 and 11, only smaller in magnitude.

Figure 10. ECM protein secretion by F9 EC cells and F9-differentiated cells.

F9 cells were cultured for two consecutive 72 h time periods in either unsupplemented base medium (UND group) or base medium supplemented with RA (10^{-7} M; RA group), dbcAMP (10^{-3} M; dbcAMP group) or RA and dbcAMP (RA + dbcAMP group). At the end of each 72 h, the culture media from the four treatment groups were collected and the levels of laminin (A) and type IV collagen (B) were determined using specific ELISAs. Data shown are the results from the second 72 h culture period. Bars represent the mean $\pm$ SEM of 3 experiments (3 replicates per experiment). ND means less than the 2.5 ng level of sensitivity of the ELISAs.

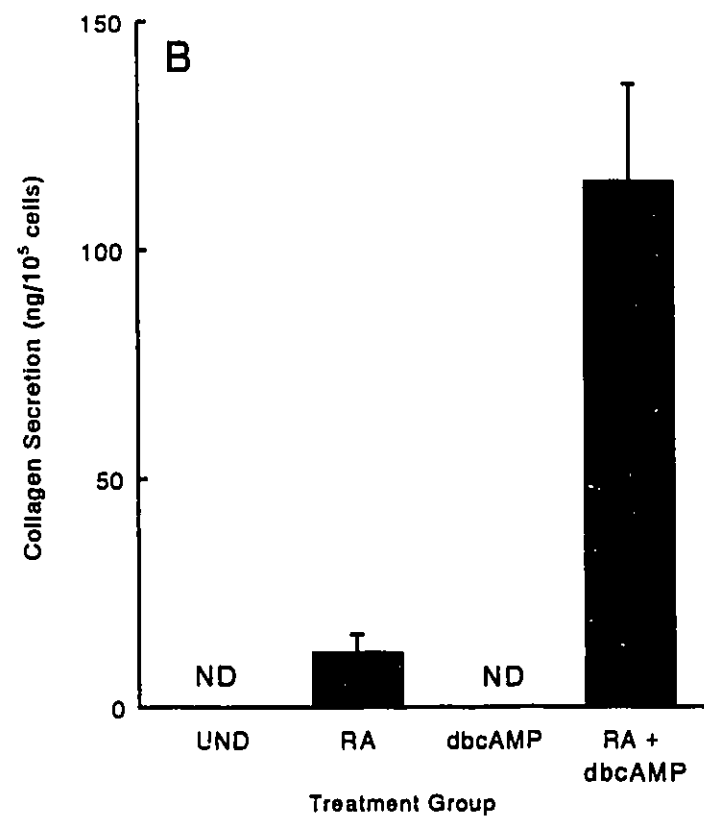
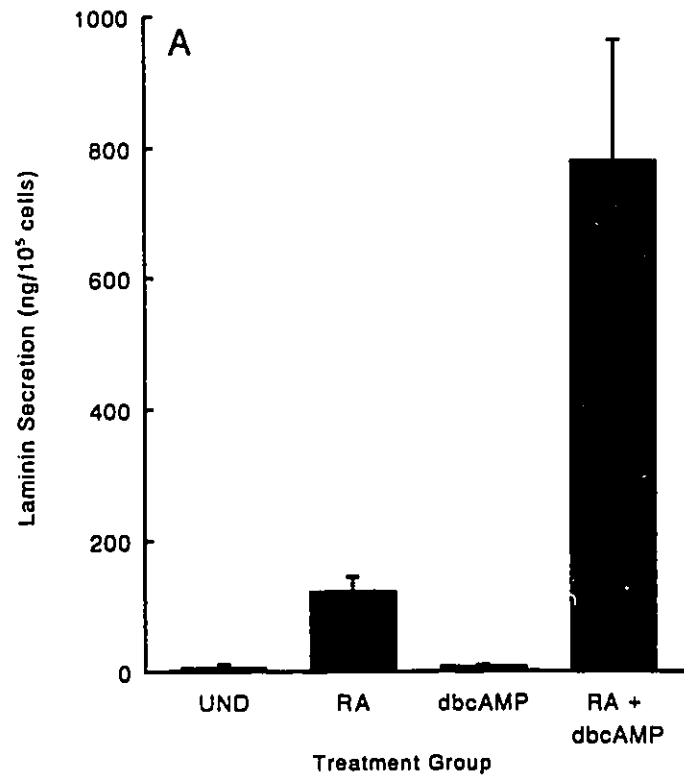
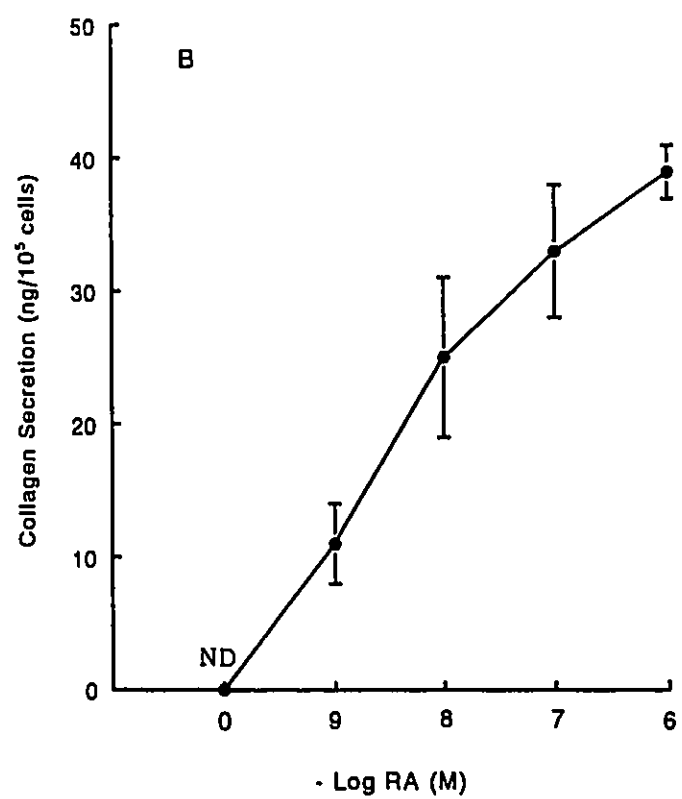
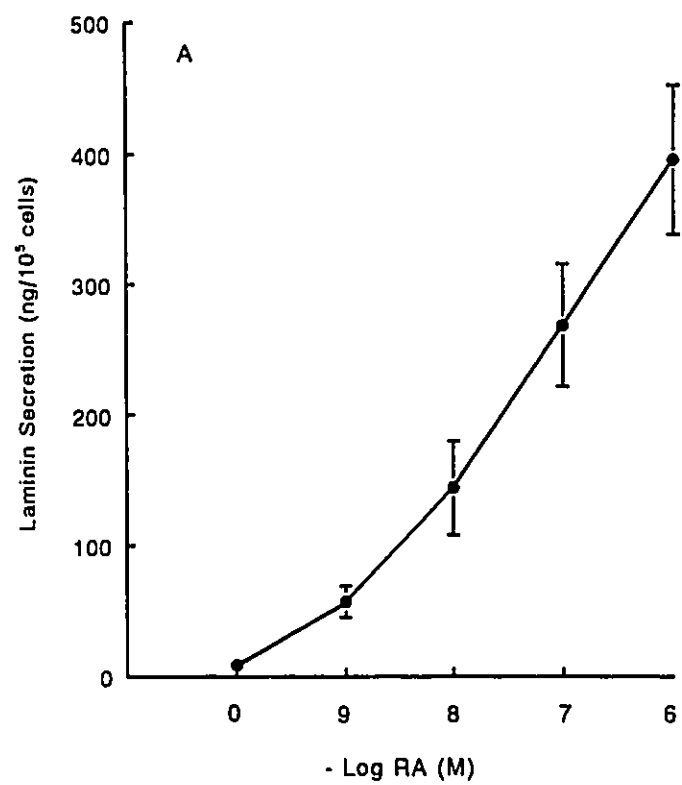


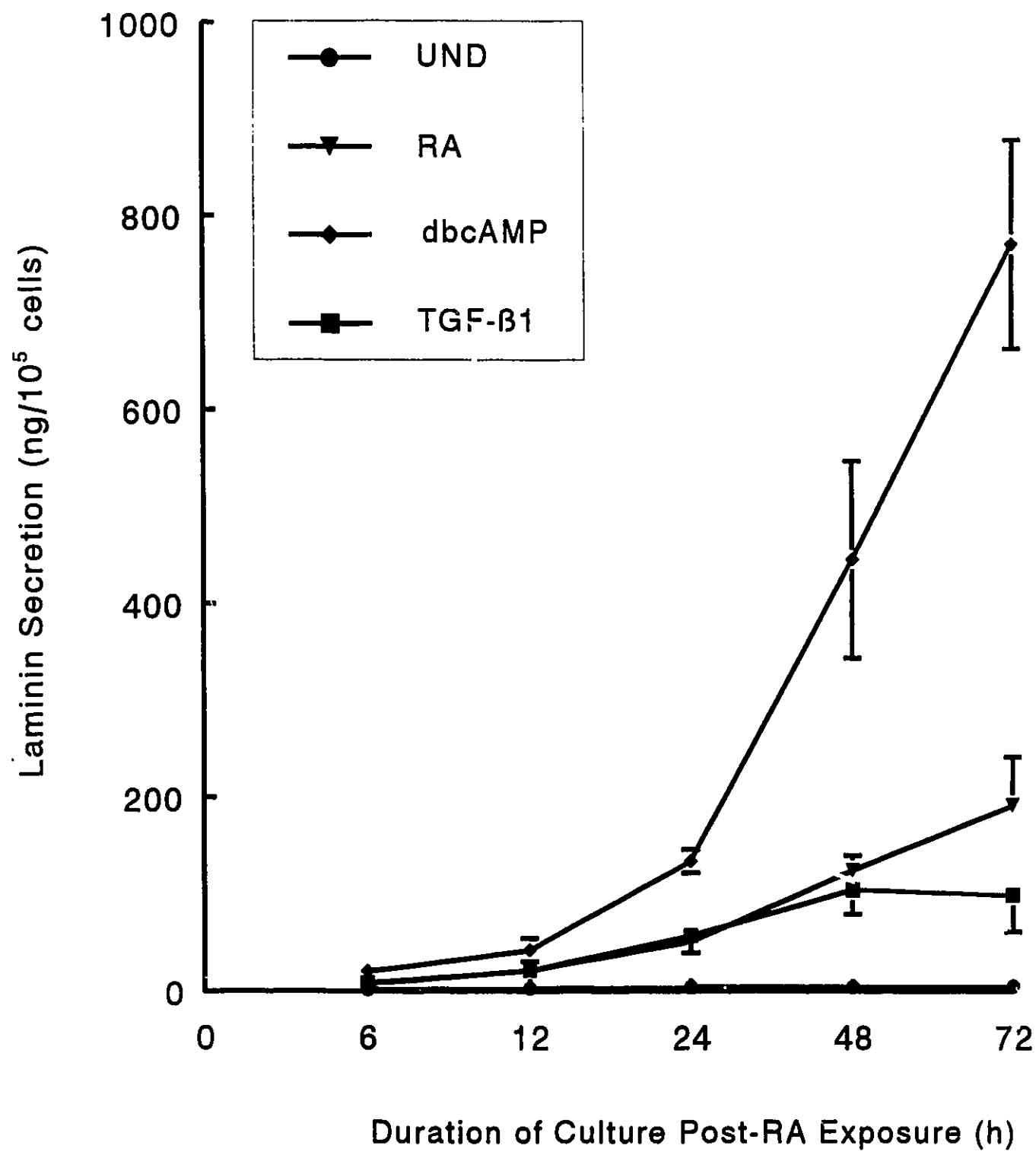
Figure 11. Influence of RA concentration on laminin and type IV collagen secretion by PrE-like cells. F9 cells were cultured for two consecutive 72 h time periods in base medium containing either no RA, or RA at concentrations of 10^{-9} M to 10^{-6} M. At the end of each 72 h, the culture media from the five treatment groups were collected and the levels of laminin (A) and type IV collagen (B) were determined using specific ELISAs. Data shown are the results from the second 72 h culture period. Points represent the mean $\pm$ SEM of 3 experiments (3 replicates per experiment). ND means less than the 2.5 ng level of sensitivity of the ELISAs.



2.3 Time Course of F9 Cell Responsiveness to RA & dbcAMP

Figure 12 shows laminin secretion by F9-differentiated cells treated $\pm$ RA for 48 h followed by culture $\pm$ RA and/or dbcAMP (see section 3.2.1 for mention of TGF- β 1 results) for 6, 12, 24, 48 and 72 h. Within 6 h, RA-treated cells were secreting 4-fold more laminin than the undifferentiated controls. At each subsequent time point, the amount of secreted laminin more than doubled the previous time point value, so that by 72 h post-RA, RA-treated cells secreted 35-fold more laminin than did undifferentiated cells. PrE-like cells treated with dbcAMP showed a 10-fold increase in laminin secretion compared to undifferentiated cells as early as 6 h post-RA treatment. By 24 h after RA treatment, the amount of laminin secreted in response to dbcAMP (134 ± 12 ng/ 10^5 cells) was six times that measured 6 h after RA treatment. Laminin secretion continued to increase in a linear manner and by 72 h the amount of laminin secreted by dbcAMP-treated PrE-like cells was 4X that by RA-treated cells and 141X that by undifferentiated cells.

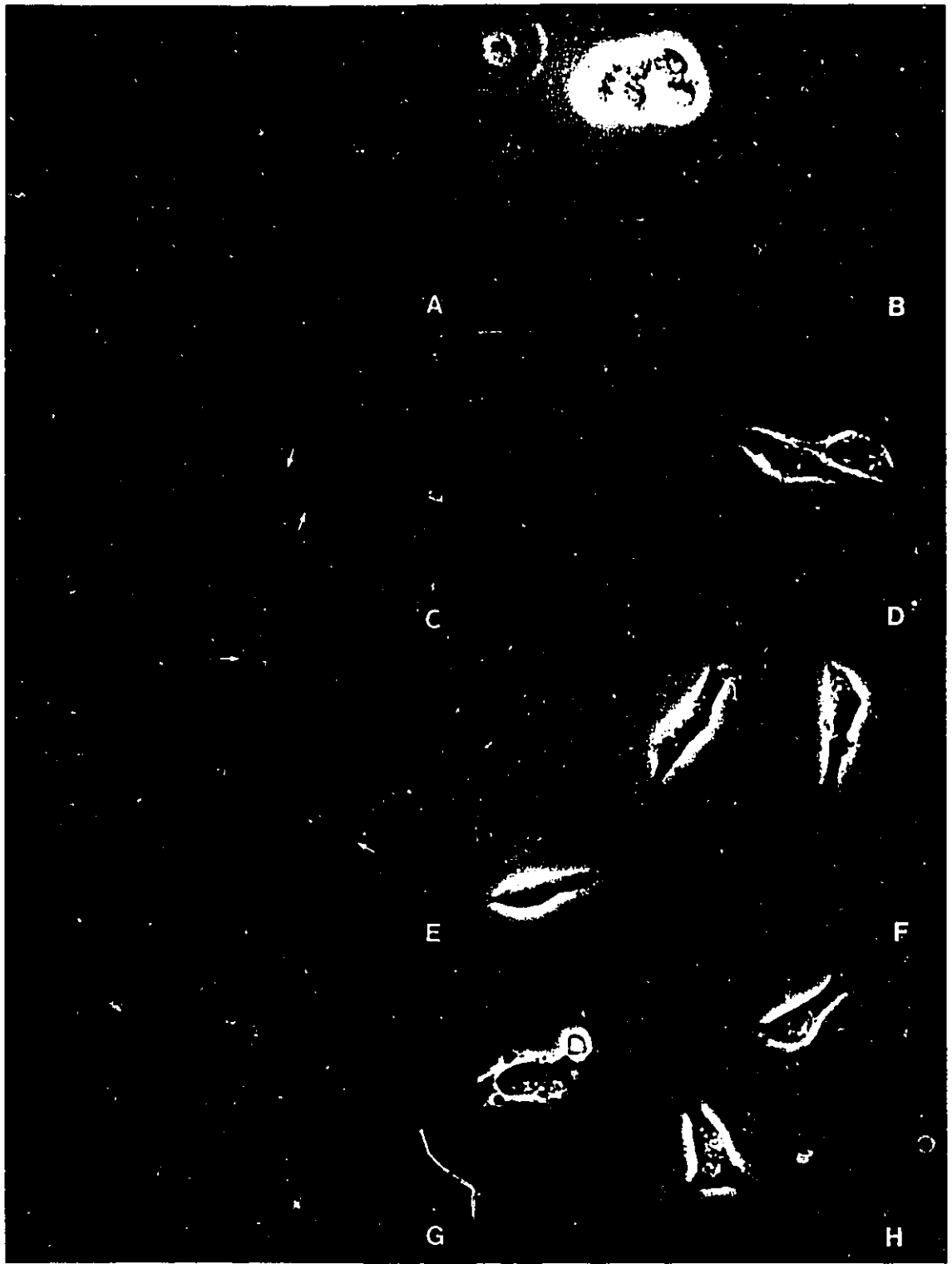
Figure 12. Time course of F9 cell responsiveness to RA, RA + dbcAMP and RA + TGF- β 1. F9 cells were cultured in base medium containing either no RA (UND) or RA (10^{-7} M) for 48 h. Subsequently, the cells were given fresh base medium (UND and RA) or base medium supplemented with dbcAMP (10^{-3} M; dbcAMP group) or TGF- β 1 (3 ng/ml; TGF- β 1 group). Medium samples from the four treatment groups were collected at 6, 12, 24, 48 and 72 h and assayed for laminin using a specific ELISA. Each point represents the mean $\pm$ SEM of 3 experiments (3 replicates per experiment).



2.4 Immunolocalization of Laminin in F9 EC Cells and F9-Differentiated Cells

Undifferentiated F9 cells (Figure 13A) showed a level of fluorescence slightly above the level of background staining (13G), but less than the degree of staining observed in the differentiated PrE-like and PE-like cells (13C,E). Undifferentiated F9 cells do synthesize a very small amount of laminin (see **DISCUSSION**), but the relatively homogeneous distribution of fluorescence was probably due to their being round and compact cells and, therefore, the top viewing represents a composite fluorescence of laminin in these cells. Immunostaining of differentiated cells (Figure 13C and E) showed a specific pattern of fluorescence. RA-treated cells (Figure 13C) had a punctate cytoplasmic distribution of laminin, sometimes with more intense fluorescence directly outside of the nucleus (arrows). The latter localization of laminin is suggestive of packaging of the protein for secretion. F9 cells treated with RA and dbcAMP also showed bright cytoplasmic staining for laminin; faint staining of cellular cytoplasmic processes was also evident (Figure 13E, arrows). PE-like cells taken through the laminin immunostaining procedure without a primary antibody incubation step showed a low level of non-specific staining (Figure 13G).

Figure 13. Immunolocalization of laminin in undifferentiated and differentiated F9 cells. F9 cells were cultured on gelatin-coated coverslips in a 6-well plate for 72 h, with a medium change after the first 48 h. Undifferentiated F9 cells (A, B) were cultured in base medium alone while PrE-like cells (C, D) and PE-like cells (E-H) were cultured in base medium supplemented with RA (10^{-7} M) and RA plus dbcAMP (10^{-3} M), respectively. The distribution of laminin within these cells was evaluated using immunofluorescence, as described in **MATERIALS AND METHODS**. Shown are representative cells from 1 of 3 experiments. Right-hand panels are phase-contrast views of the immunostained cells in the left-hand panels. Cells in A, C, E and G represent 8 second exposures. In G, the extent of non-specific binding of the FITC-labelled secondary antibody was assessed by omitting exposure to the primary antibody. Magnification X640



3. Investigations into the Regulation of ECM Secretion by Differentiating PrE-Like Cells

3.1 Dibutyl Cyclic AMP: Mechanism of Action

To determine if the action of dbcAMP on RA-treated cells could be mimicked by stimulating the G_i signal transduction pathway, RA-differentiated F9 cells were treated with forskolin and cholera toxin, as described below.

3.1.1 Forskolin

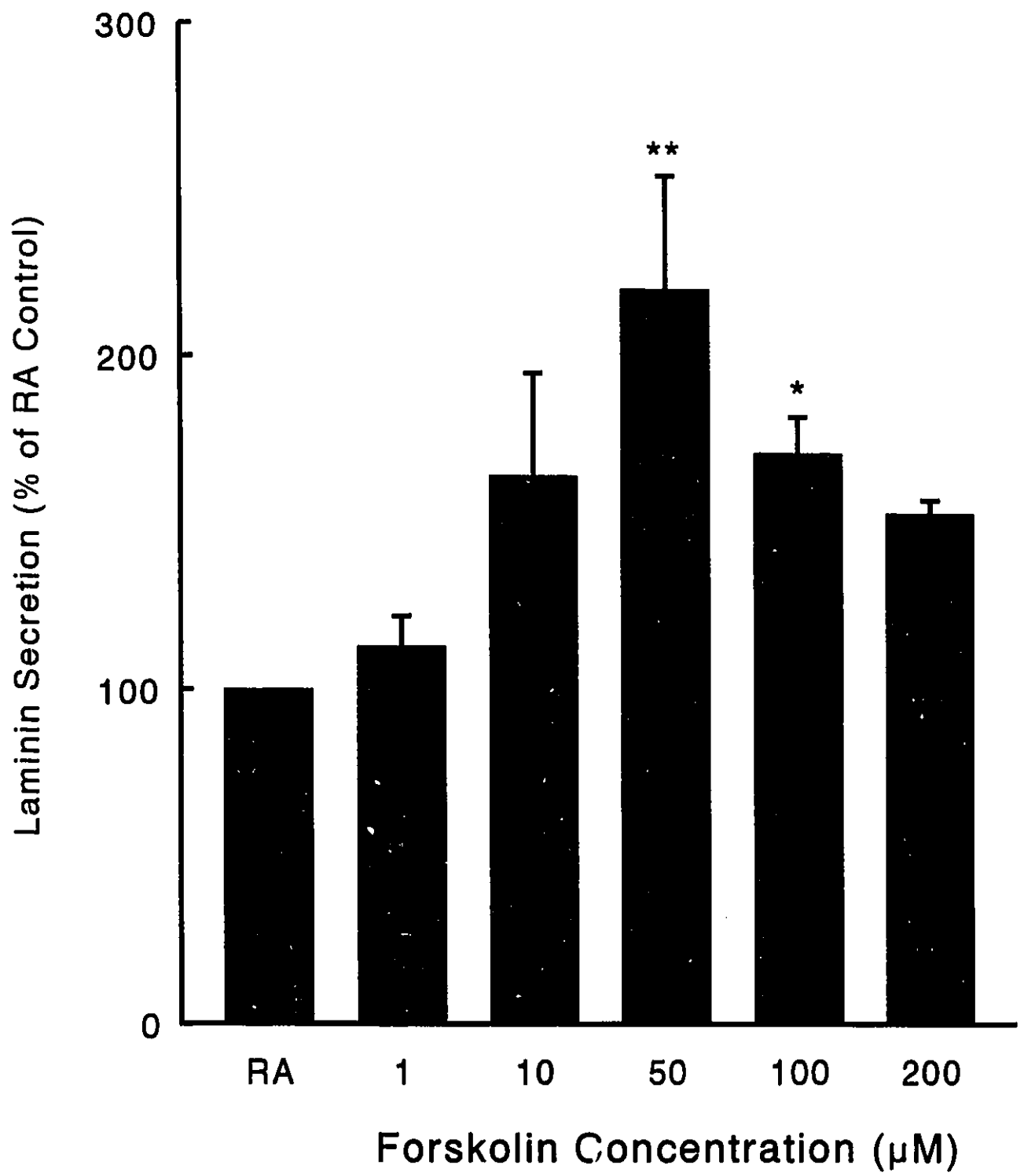
Figure 14 shows the influence of forskolin on laminin secretion by PrE-like cells. Laminin secretion by PrE-like cells treated with 1 μ M forskolin was not different from RA control cells ($p > 0.05$). Although the percent of laminin secreted by PrE-like cells treated with 10 μ M forskolin was 64% higher than that by RA control cells, inter-experiment variability in the extent of cell responsiveness to this concentration of forskolin produced an overall effect that was not significant ($p > 0.05$). Forskolin at 50 μ M, however, did significantly increase ($p < 0.01$) laminin secretion by 220%. Laminin secretion in response to 100 μ M forskolin was also a statistically significant increase ($p < 0.05$) compared to RA control cells; however, laminin secretion by PrE-like cells treated with 100 or 200 μ M forskolin did not significantly differ from the response elicited by 50 μ M forskolin ($p > 0.05$). The greatest response to forskolin (50 μ M; 220% of RA control) was less than the percent laminin secreted in response to dbcAMP (478% of RA control; data not shown).

Figure 14. Influence of forskolin on laminin secretion by PrE-like cells.

F9 EC cells were differentiated to PrE-like cells using RA (10^{-7} M) for 48 h, and were subsequently treated with forskolin (1-200 μ M) for 72 h. Laminin secreted into the culture medium during the 72 h was quantified using ELISA. Each bar represents the mean $\pm$ SEM from 3-5 experiments (3 replicates per experiment), expressed as a percent of the RA control (113 ± 15 ng/ 10^5 cells). Undifferentiated cells secreted only 4% of the laminin secreted by PrE-like cells.

* $p < 0.05$ compared to RA control

** $p < 0.01$ compared to RA control



3.1.2 Cholera Toxin

MIX was included in the cholera toxin experiments to allow the duration of cell exposure to this potentially toxic compound to be kept to a minimum. For these experiments, the cells were treated with cholera toxin for only 4 h, followed by a further 72 h culture period in the presence of MIX. Indeed, it was found that inclusion of MIX in the culture media *was required* to see a stimulatory effect of cholera toxin (data not shown). Treatment of PrE-like cells with cholera toxin (0.01-10 $\mu\text{g/ml}$) in the presence of MIX significantly increased laminin secretion by these cells at all concentrations tested ($p < 0.01$ at 0.01 and 0.1 $\mu\text{g/ml}$; $p < 0.05$ at 1 and 10 $\mu\text{g/ml}$; Table 2). Cholera toxin was quite potent in its effect on laminin secretion. Even the lowest cholera toxin concentration tested (0.01 $\mu\text{g/ml}$) caused a 3-fold stimulation of laminin secretion compared to a less than 2-fold stimulation with dbcAMP treatment of PrE-like cells ($180 \pm 8\%$ for 10^{-3} M dbcAMP in the presence of MIX; data not shown).

However, the concentrations of cholera toxin tested did not demonstrate a dose-response relationship with regard to laminin secretion: thus, there was no significant difference between the amount of laminin secreted by PrE-like cells treated with 0.01, 0.1, 1 or 10 $\mu\text{g/ml}$ cholera toxin ($p > 0.05$). Concentrations as low as 0.1 $\mu\text{g/ml}$ were evaluated and found to elicit similar responses as the 0.01-10 $\mu\text{g/ml}$ cholera toxin concentrations (data not shown).

Table 2.

Cholera toxin increases laminin secretion by PrE-like cells in the presence of MIX

TREATMENT GROUP	LAMININ SECRETION (% of RA control)
RA + MIX control	100
0.01 $\mu\text{g/ml}$ CT	308 $\pm$ 62
0.1 $\mu\text{g/ml}$ CT	345 $\pm$ 83
1 $\mu\text{g/ml}$ CT	286 $\pm$ 8
10 $\mu\text{g/ml}$ CT	253 $\pm$ 61

F9 cells were differentiated to PrE-like cells by treatment with RA (10^{-7} M) for 48 h. The PrE-like cells were then cultured with cholera toxin (CT; 0.01-10 $\mu\text{g/ml}$) in the presence of MIX (10^{-5} M) for 4 h. After the 4 h, the CT-containing medium was removed from the wells and replaced with base medium containing MIX. The cells were cultured for a further 72 h, after which time the culture medium was collected and assayed for laminin using ELISA. Values represent the mean $\pm$ SEM from 3 experiments (3 replicates per experiment), expressed as a percent of the RA + MIX control (117 ± 49 ng/ 10^5 cells). Undifferentiated cells secreted 5.8 ± 0.7 ng laminin/ 10^5 cells during the 72 h culture period.

3.2 Growth Factors

3.2.1 Transforming Growth Factor- β 1

Preliminary experiments to determine a possible effect of TGF- β 1 on ECM protein secretion utilized TGF- β 1 at 3 ng/ml, a concentration commonly used by other investigators, and assessed secreted protein levels after 72 h and 120 h of TGF- β 1 treatment. The results are depicted in Figure 15. After 72 h of treatment with TGF- β 1, PrE-like cells secreted significantly less laminin (46% less; $p < 0.05$) than untreated PrE-like cells. Furthermore, PrE-like cells treated with TGF- β 1 for 120 h secreted 68% less laminin than untreated PrE-like cells ($p < 0.01$). However, there was no effect of *duration* of TGF- β 1 treatment at this concentration, since the measured decreases in laminin secretion recorded after 72 h and after 120 h were not significantly different ($p > 0.05$).

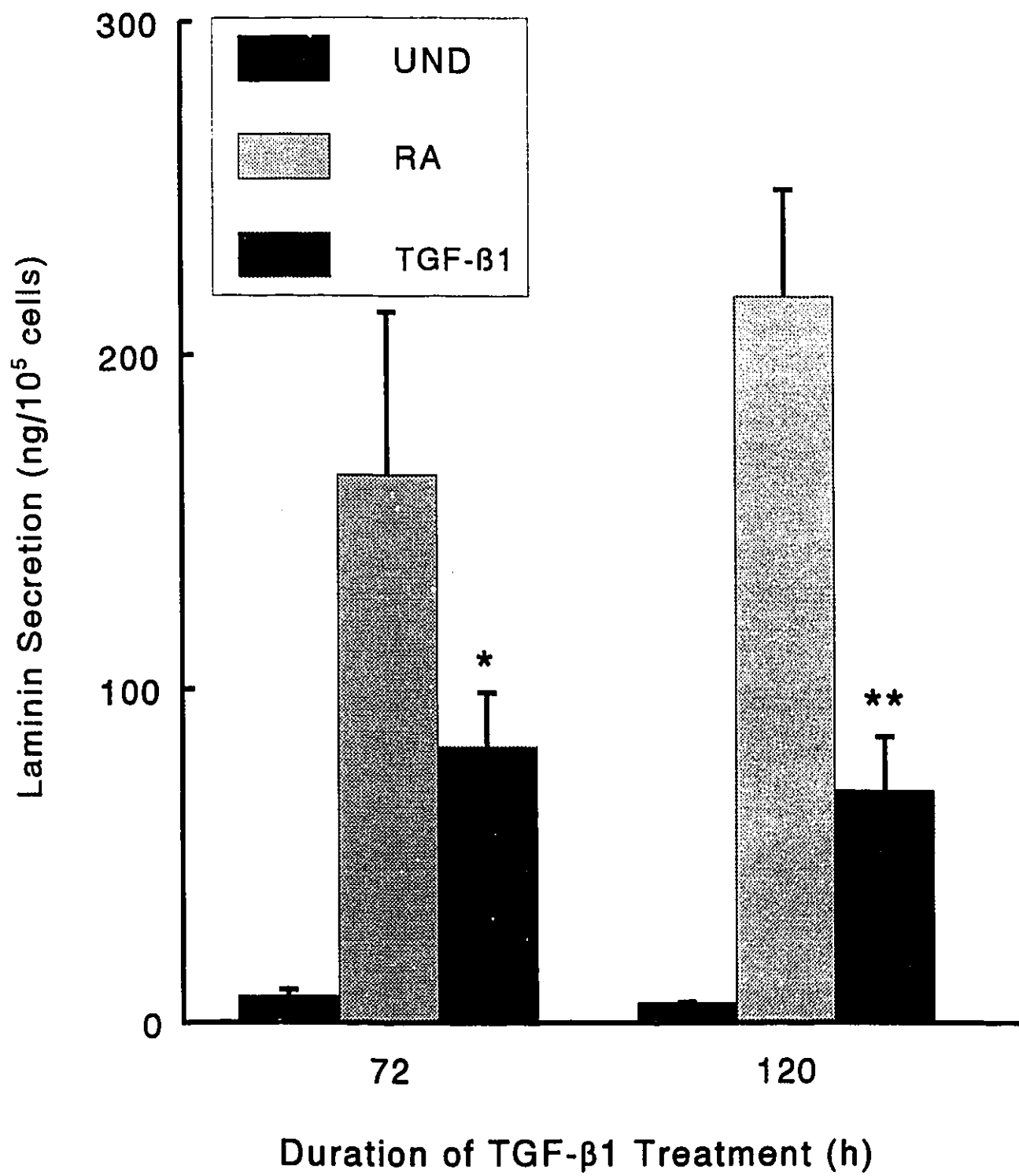
Interestingly, preliminary results on the effect of TGF- β 1 (3 ng/ml) on type IV collagen secretion by PrE-like cells indicated that TGF- β 1 did not affect type IV collagen secretion after only 72 h of treatment, but that the longer exposure to this growth factor resulted in a 43% reduction in secreted type IV collagen (result of one experiment, data not shown). However, this could not be confirmed by measuring type IV collagen secretion for the other two experiments in this study because this author became unable to obtain an antibody to type IV collagen which did not also show significant cross-reactivity with laminin present in the media samples. Numerous attempts were made to obtain an antibody specific for murine type IV collagen; none of the antibody preparations received were adequate for continuation of these studies.

Figure 15. TGF- β 1 inhibits laminin secretion by PrE-like cells.

F9 EC cells were differentiated to PrE-like cells using RA (10^{-7} M) for 48 h, and were subsequently treated with TGF- β 1 (3 ng/ml) for 72 h or 120 h. Laminin secreted into the culture medium by the cells during the TGF- β 1 treatment was quantified using ELISA. Bars represent the mean $\pm$ SEM of 3 experiments (3 replicates per experiment).

* $p < 0.05$ compared to 72 h RA control

** $p < 0.01$ compared to 120 h RA control



Regretably, due to time constraints, all further experimental work assessed only secreted laminin levels.

A dose-response study of the effect of TGF- β 1 on laminin secretion was subsequently attempted (Figure 16). Treatment of PrE-like cells with 0.01 ng/ml TGF- β 1 did not affect laminin secretion by these cells ($103 \pm 9\%$ of RA control). However, TGF- β 1 caused a significant decrease (approximately 40% after 72 h) in laminin secretion by PrE-like cells at concentrations of 0.1, 1 and 10 ng/ml ($p < 0.01$ at all concentrations). The concentrations tested did not reveal a dose-dependent effect of TGF- β 1 since there was no significant difference between the amounts of laminin secreted by cells treated with these inhibitory concentrations of TGF- β 1 ($p > 0.05$). Interestingly, the negative influence of TGF- β 1 on laminin secretion by PrE-like cells was not evident until 72 h of TGF- β 1 treatment (Figure 12).

Decreased laminin secretion by PrE-like cells treated with TGF- β 1 also appeared to be a specific effect of this growth factor, since determination of the total cellular protein levels for PrE-like control cells and those treated with TGF- β 1 revealed no significant difference between the two groups ($p > 0.05$; Table 3).

It is important to note that in both the initial TGF- β 1 (3 ng/ml) experiments and the TGF- β 1 dose-response study, the numbers of cells in the TGF- β 1-treated cultures were consistently the same as in the RA-treated control cultures, even after 72 h of TGF- β 1 treatment.

Figure 16. Influence of TGF- β 1 on laminin secretion by PrE-like cells.

F9 cells were sequentially cultured with RA (10^{-7} M; 48 h) and TGF- β 1 (0.01-10 ng/ml; 72 h). Laminin secreted into the culture medium during the 72 h TGF- β 1 treatment was quantified using ELISA. Bars represent the mean $\pm$ SEM of 3 experiments (3 replicates per experiment), expressed as a percent of the RA control value (mean level of laminin secretion by RA-treated cells was 157 ± 34 ng/ 10^5 cells). Undifferentiated F9 cells secreted 7.7 ± 0.6 ng laminin/ 10^5 cells during the 72 h of culture.

** $p < 0.01$ compared to RA control

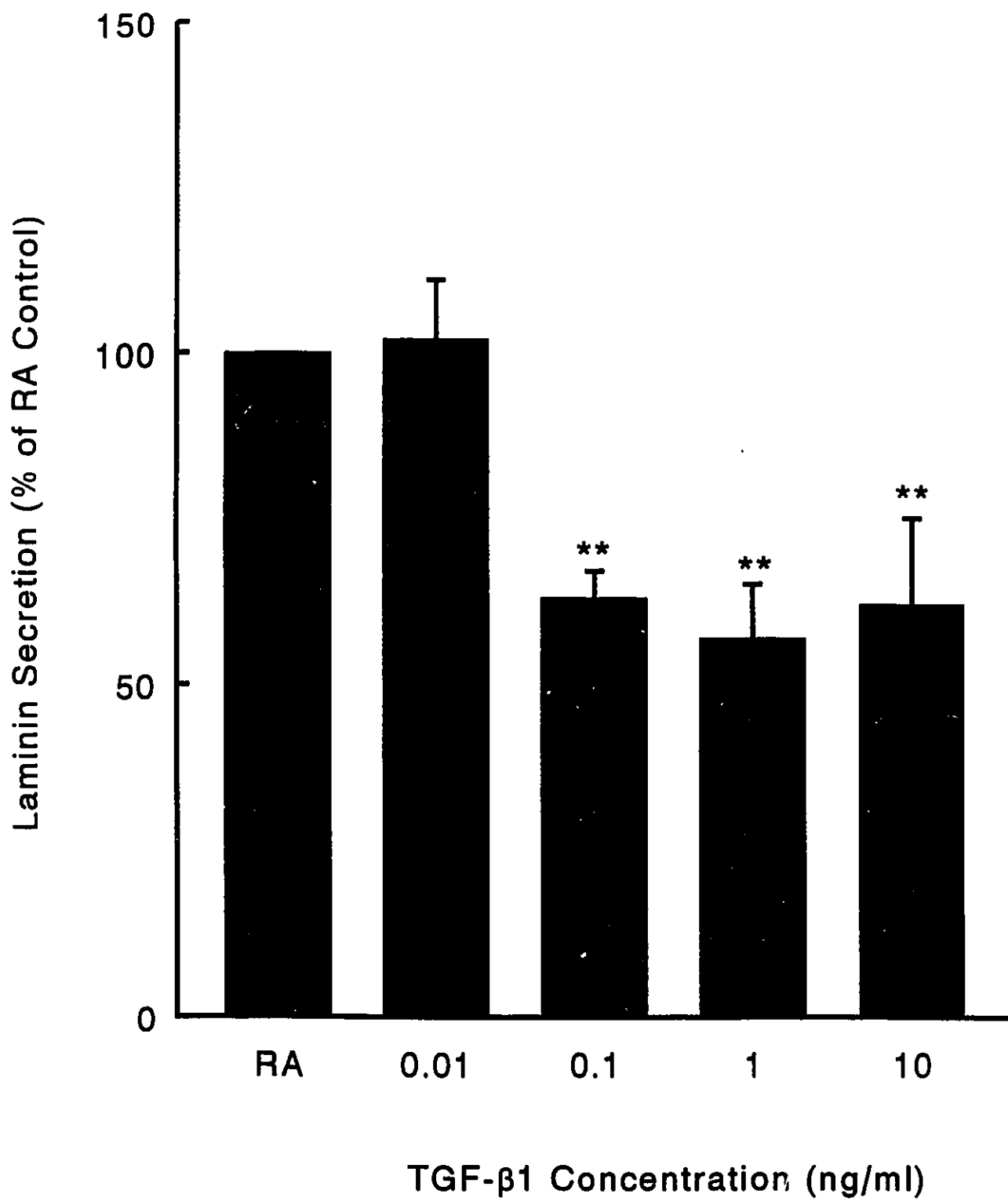


Table 3.

Total cellular protein in PrE-like cells vs TGF- β 1-treated PrE-like cells

TREATMENT GROUP	TOTAL PROTEIN (mg/ml cell extract)	LAMININ SECRETION (μg/dish)
RA control	44 $\pm$ 4	5.0 $\pm$ 0.4
TGF- β 1	36 $\pm$ 4	2.3 $\pm$ 0.1

F9 cells were differentiated to PrE-like cells by treatment with RA (10^{-7} M) for 48 h. The cells were then given fresh base medium, either unsupplemented (RA control) or supplemented with TGF- β 1 (3 ng/ml), and cultured for an additional 72 h. Total cellular protein levels were thereafter determined by the method of Lowry and coworkers (1951; see **MATERIALS AND METHODS**). Total protein values represent the mean $\pm$ SEM of two experiments (a total of 10 replicates), expressed as mg/ml cell extract (cell numbers for the RA control group and TGF- β 1 group were taken to be the same, see **DISCUSSION**). Shown also are the culture media laminin levels corresponding to one of the two experiments, expressed as μ g laminin/dish.

3.2.2 Transforming Growth Factor- β 2

In contrast to the inhibitory influence of TGF- β 1, there was no significant effect of TGF- β 2 (0.01-10 ng/ml) on the amount of laminin secreted by PrE-like cells ($p > 0.05$ compared to RA control; Table 4).

Table 4.

Secretion of laminin by PrE-like cells in the presence of TGF- β 2.

TREATMENT GROUP	LAMININ SECRETION (ng/10⁵ cells)
RA control	109 $\pm$ 16
0.01 ng/ml	144 $\pm$ 25
0.1 ng/ml	125 $\pm$ 20
1 ng/ml	124 $\pm$ 22
10 ng/ml	101 $\pm$ 6

F9 cells were differentiated to PrE-like cells using RA (10^{-7} M) for 48 h, and were subsequently treated with TGF- β 2 (0.01-10 ng/ml) for 72 h. Laminin secreted into the culture medium during the 72 h was quantified using ELISA. Values represent the mean $\pm$ SEM of three experiments (3 replicates per experiment). Undifferentiated cells secreted 6.2 ± 1.2 ng laminin/10⁵ cells during the 72 h culture period.

3.2.3 Platelet-Derived Growth Factor

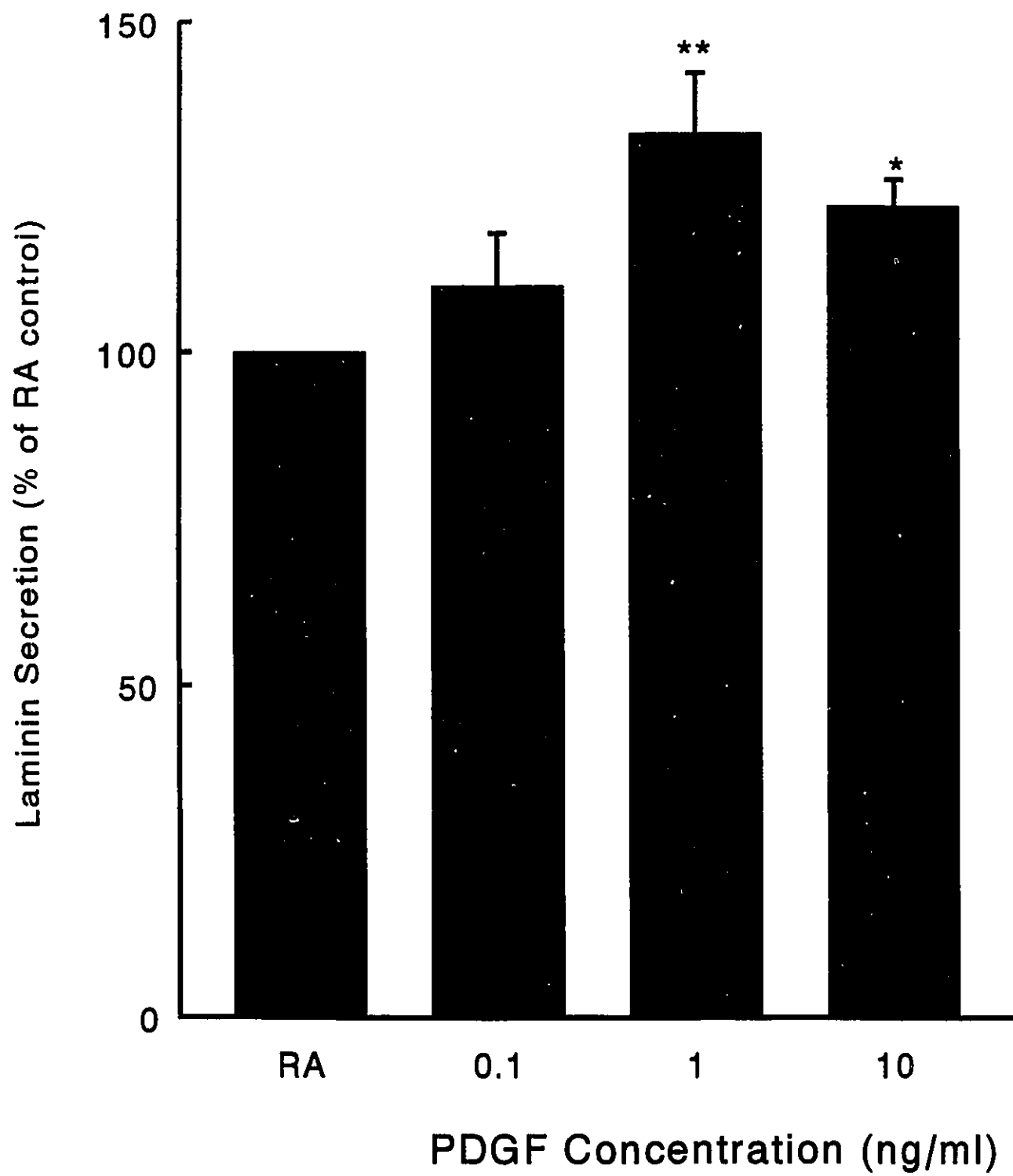
Treatment of PrE-like cells with PDGF at 0.1 ng/ml did not cause a significant change in the amount of laminin secreted by these cells ($p > 0.05$; Figure 17). However, PDGF concentrations of 1 and 10 ng/ml did stimulate small, but significant, increases in laminin secretion: 1.3-fold ($p < 0.01$) and 1.2-fold ($p < 0.05$), respectively. The amount of laminin secreted by PrE-like cells at these two concentrations was not significantly different ($p > 0.05$).

Figure 17. PDGF can stimulate laminin secretion by PrE-like cells.

F9 cells were differentiated to PrE-like cells using RA (10^{-7} M) for 48 h, and were subsequently treated with PDGF (0.1-10 ng/ml) for 72 h. Laminin secreted into the culture medium during the 72 h was quantified using ELISA. Values represent the mean $\pm$ SEM, expressed as a percent of the RA control, from 3 experiments (2-3 replicates per experiment). Undifferentiated F9 cells secreted 5.4 ± 0.8 ng laminin/ 10^5 cells during the 72 h culture period.

* $p < 0.05$ compared to RA control

** $p < 0.01$ compared to RA control



3.2.4 Transforming Growth Factor- α

While TGF- α appeared to increase the secretion of laminin by PrE-like cells, a dose-response relationship could not be discerned (Table 5). Furthermore, the extent of cell responsiveness to this growth factor was highly variable between cultures, resulting in mean effects which were not significant ($p > 0.05$) throughout the concentration range tested. Significance was not obtained by expressing the data either as a percentage of the RA control or transforming the data prior to statistical analysis.

Table 5.

TGF- α does not affect laminin secretion by PrE-like cells

TREATMENT GROUP	LAMININ SECRETION (ng/10⁵ cells)
RA control	115 $\pm$ 24
0.001 ng/ml	184 $\pm$ 59
0.01 ng/ml	195 $\pm$ 81
0.1 ng/ml	152 $\pm$ 28
1 ng/ml	186 $\pm$ 37
10 ng/ml	165 $\pm$ 26

F9 cells were differentiated to PrE-like cells using RA (10^{-7} M) for 48 h, and were subsequently treated with TGF- α (0.001-10 ng/ml) for 72 h. Laminin secreted into the culture medium during the 72 h was quantified using ELISA. Values represent the mean $\pm$ SEM of 4 experiments (3 replicates per experiment). Undifferentiated F9 cells secreted 7.1 ± 0.7 ng laminin/10⁵ cells during the 72 h culture period.

3.3 Laminin Deposition by F9 EC Cells and F9-Differentiated Cells Treated With dbcAMP or TGF- β 1

Undifferentiated F9 cells deposited a minimal amount of laminin (2.6 ng/well; Table 6). As might be expected from the laminin secretion profile of RA-treated and RA + dbcAMP-treated cells, the amount of laminin deposited in these wells was somewhat higher (17 ng/well and 48 ng/well, respectively) than in the wells occupied by undifferentiated cells. Of particular importance was the fact that TGF- β 1-treated PrE-like cells did not deposit more laminin than did RA control cells and, therefore, the negative regulation by TGF- β 1 of laminin secretion by PrE-like cells was not a result of increased deposition by these cells.

Table 6.

Laminin deposition by F9 undifferentiated and F9-differentiated cells treated with dbcAMP or TGF- β 1

TREATMENT GROUP	LAMININ DEPOSITION (ng laminin/well)	LAMININ SECRETION (ng laminin/well)
no cells control	ND ^a	ND
undifferentiated	2.6	9.7 $\pm$ 0.1
RA control	17	151 $\pm$ 13
RA + dbcAMP	48	424 $\pm$ 46
RA + 3 ng/ml TGF- β 1	4.4	60 $\pm$ 5

Cells were cultured and the levels of deposited laminin were measured directly in the 96-well microtitre plates used for ELISA. F9 cells were cultured $\pm$ RA (10^{-7} M) for 48 h. The cells were then given fresh base medium, either unsupplemented (no cells control, undifferentiated, and RA control) or supplemented with dbcAMP (10^{-3} M) or TGF- β 1 (3 ng/ml), and cultured for a further 72 h. The cells were lifted from the plate and the relative levels of deposited laminin were measured using ELISA (see **MATERIALS AND METHODS**). The values shown are a representative data set from one of three experiments (9 replicates per experiment). Shown also are the laminin levels in the corresponding culture media, expressed as ng laminin/well (the cell solubilization step in this method of determining deposited laminin did not allow for cell counts to be done; however, the proliferation rates of the cells under these conditions had been well established from previous experiments, see **DISCUSSION**).

^a ND means less than the level of sensitivity of the ELISA, 3.12 ng and 1.25 ng for the deposition data and secretion data, respectively.

DISCUSSION

The constant, low level of laminin secretion by undifferentiated F9 cells measured in these studies was expected, given the fact that approximately 0.02% of their total protein is laminin (Hogan *et al.*, 1983). On the other hand, undifferentiated F9 cells did not secrete detectable levels of type IV collagen, as has been previously reported (Strickland *et al.*, 1980). The 19-fold increase in the amount of laminin secreted by RA-treated F9 cells reported in this research is in close agreement with the finding by Dziadek and Timpl (1985) that treatment of F9 cells with RA (100 μ M) stimulated a 20-fold increase in laminin secretion, as quantified by radioimmunoassay.

Several reports, including the present one, have shown a dramatic stimulation of laminin and type IV collagen secretion by RA + dbcAMP-treated F9 cells; however, there has been some variation in the extent of these responses. The approximately 100-fold stimulation of laminin and type IV collagen secretion by RA + dbcAMP-treated F9 cells compared to undifferentiated F9 cells reported here is contrasted with only a 30-fold increase in secreted levels of laminin reported by Dziadek and Timpl (1985), and a 15-fold increase in laminin in cytoplasmic extracts of RA + dbcAMP-treated F9 cells (Kurkinen *et al.*, 1983a,b). However, in all of these studies the concentration of RA (5×10^{-8} M; Kurkinen *et al.*, 1983a,b) and/or the concentration of dbcAMP (1×10^{-4} M; Kurkinen *et al.*, 1983a,b; Dziadek and Timpl, 1985) used to differentiate the F9 cells to PE-like cells were an order of magnitude lower than the concentrations used in the current study. Additionally, the RA+dbcAMP-treated F9 cell culture medium assayed

by Dziadek and Timpl (1985) represented only the last 24 h of a 7 day culture, while Kurkinen and colleagues (1983a,b) assessed laminin immunoprecipitates from F9 cells treated with RA + dbcAMP for only 56 h. The ability of PE-like cells to secrete large quantities of laminin and type IV collagen is supported by the finding that the concentration of translatable mRNAs for basement membrane proteins in RNA from F9-differentiated PE-like cells was at least 64-fold higher than in the RNA from undifferentiated cells (Kurkinen *et al.*, 1983b).

As has been previously reported for laminin, type IV collagen and PA, (Strickland and Mahdavi, 1978; Strickland *et al.*, 1980), treatment of F9 cells with dbcAMP alone did not generate an endoderm-like phenotype, as evidenced by levels of secreted laminin and type IV collagen that did not differ from undifferentiated cells.

The immunolocalization of laminin in F9-derived endoderm-like cells was comparable to previous findings (Solter *et al.*, 1979; Carlin *et al.*, 1983; Hogan *et al.*, 1983; Kurkinen *et al.*, 1983b), and served to demonstrate that RA and RA + dbcAMP treatment of F9 cells generated relatively homogeneous differentiated cell populations.

It is believed that the current study quantifying the secretion of laminin and type IV collagen by F9 cells treated with various concentrations of RA represents novel findings. Miki and Kitigawa (1988) provided a semi-quantitative assessment (densitometry of a fluorogram) of laminin A subunit secretion by F9 cells treated with RA at concentrations from 10^{-12} M to 10^{-5} M: the relative density of the laminin A subunit in Day 6 culture medium increased from 10^{-9} M to 10^{-6} M, and then decreased at 10^{-5} M. Type IV collagen was not evaluated in their study. The current research

work demonstrated that RA at concentrations from 10^{-8} M to 10^{-6} M caused a dose-dependent stimulation of laminin secretion, consistent with the findings of Miki and Kitigawa (1988). Type IV collagen secretion by F9 cells also increased in response to increasing concentrations of RA, but dose-related significance was not obtained.

A key observation of this research work was the difference in cellular proliferation rates between undifferentiated versus differentiated F9 cells. In all of the experiments, the number of cells in RA-treated cultures was less than in undifferentiated cultures, while RA + dbcAMP-treated cultures were even less dense than RA-treated cell cultures. These differences were noted after only six hours of culture. These results were also consistently recorded by J.A. Carnegie (personal communication), but are contrasted to the results of other investigators who recorded either no differences in cell numbers between undifferentiated and differentiated F9 cell cultures (Strickland and Mahdavi, 1978; Strickland *et al.*, 1980; Wang and Gudas, 1988), or higher cell numbers in differentiated cell cultures relative to the undifferentiated cultures (Kelly and Rizzino, 1989). Proliferation and differentiation are usually opposing cellular responses, and support for this notion regarding F9 cells was the reported increase in the generation time for differentiated versus undifferentiated F9 cells (Rosenstrauss *et al.*, 1982). Additionally, Minor and collaborators (1976) demonstrated that cultures of PE cells treated with [3 H]thymidine over 24 h showed only about 10% of the cells labelled. It is noteworthy that in the four reports cited above, in which reduced proliferation in response to RA-induced differentiation was *not* observed, the investigators used serum in their experimental manipulations and, therefore, the question must be posed as to the

influences of serum-derived factors.

The results of the current studies revealed that both forskolin and cholera toxin significantly stimulated laminin secretion by PrE-like cells. The highest level of laminin secreted in response to forskolin stimulation did not reach a level equivalent to that measured in response to dbcAMP-induced differentiation. However, cholera toxin, at all concentrations tested, was a more potent stimulator of laminin secretion by PrE-like cells than was dbcAMP (MIX was included in both the cholera toxin-treated and the dbcAMP-treated cell cultures).

Future studies should explore the possibility that a dose-dependent stimulation of laminin secretion by PrE-like cells treated with cholera toxin does exist by (a) looking at a time course of cholera toxin stimulation and (b) using a suboptimal concentration of MIX. It is possible that this author's reported levels of laminin secretion in response to a 4 h exposure to cholera toxin actually represent a plateau in the PrE-like cells' laminin secretory response, and therefore a time course of cholera toxin application from 0-4 h may be informative. Additionally, the 10^{-5} M concentration of MIX used in the current research work may have maximally sustained the levels of cAMP produced by the PrE-like cells in response to any of the concentrations of cholera toxin (i.e. absolutely no cAMP turnover). Therefore, a suboptimal concentration of MIX that still maintains sufficient levels of cAMP for PrE-like cell differentiation should be determined and used in conjunction with the time course study.

A previous report provided semi-quantitative results of fluorometric studies indicating that F9 cells treated with RA (10^{-7} M), cholera toxin (10^{-10} M or 0.008 $\mu\text{g/ml}$)

and MIX (10^{-5} M) for 72 h secreted about the same amount of laminin into the culture medium as cells treated with RA, dbcAMP (10^{-3} M) and MIX (Strickland *et al.*, 1980). Since the action of cholera toxin modifies G, so that it continually stimulates adenylate cyclase (Bennett *et al.*, 1975; Cassel and Selinger, 1977; Cassel and Pfeuffer, 1978; Gill and Meren, 1978), it is likely that the cAMP produced in response to cholera toxin would exceed the amount of cAMP available to the cells from the fixed concentration of exogenously-added dbcAMP. The presence of MIX in the culture medium would ensure that this higher level of cAMP would be maintained and, therefore, the F9-differentiated cells in cholera toxin-treated cultures would secrete more laminin. Since the concentration of cholera toxin used by Strickland and coworkers (1980) was within the range of cholera toxin concentrations employed in this author's experiments, it is unclear why those investigators did not observe a similar increased secretion of laminin by PrE-like cells in response to cholera toxin treatment, relative to that of dbcAMP-treated cells. However, once again it is possible that another factor(s) present in the serum used by those authors to supplement their cultures exerted some negative influence on laminin secretion in their system.

In this research work, concentrations of TGF- β 1 between 0.1 and 10 ng/ml were found to inhibit laminin secretion by PrE-like cells. The results of preliminary experiments also revealed a negative influence of this growth factor on the secretion of type IV collagen. In support of these findings is the data of Kelly and Rizzino (1989), who analyzed laminin immunoprecipitates from RA-differentiated F9 cells treated with TGF- β 1 (2 ng/ml; 24 h). Densitometry of the resultant fluorographs indicated that TGF-

$\beta 1$ decreased, by approximately 40%, the quantity of secreted laminin A and B subunits. However, their results were semi-quantitative in nature and, therefore, the data obtained from this author's experiments provide the first quantitation of the influence of TGF- $\beta 1$ on the secretion of laminin by F9-derived PrE-like cells. Furthermore, the cells used by Kelly and Rizzino (1989) were cultured in medium containing 10% serum. The use of this undefined medium supplement renders the analysis of the influences of a given growth factor on cellular processes inconclusive, due to an inability to separate the effect(s) of the exogenously-added growth factor from the influences of the numerous factors present in serum. The results of the current research project revealed that TGF- $\beta 1$ (0.1-10 ng/ml; 72 h) reduced, by approximately 40%, the amount of laminin protein secreted by PrE-like cells.

Although TGF- $\beta 1$'s effect on cells usually favors matrix accumulation, there are reported cases of inhibitory influences of this growth factor on the ECM. Salo and colleagues (1991) found that human mucosal and dermal keratinocytes cultured with TGF- $\beta 1$ in serum-free medium had increased expression of both 92- and 72-kDa type IV collagenases. The elevated synthesis of the type IV collagenases was associated with a marked increase in the levels of mRNA for these enzymes. Since type IV collagenases are able to degrade the main collagen component of basement membranes, the investigators postulated that TGF- $\beta 1$ -induced up-regulation of expression of these enzymes may explain the detachment of keratinocytes from basement membranes prior to their migration over the wound bed.

In another report, TGF- β 1 inhibited decorin (a small proteoglycan containing two and one chondroitin/dermatan sulfate glycosaminoglycan side chains, respectively, attached to a 45 kD protein core) mRNA expression by up to 70% in normal human skin and gingival fibroblasts. Quantitation by immunoprecipitation of both [35 S]sulfate and [3 H]leucine-labeled decorin in cell-conditioned media revealed a 50% reduction in decorin protein production by cells treated with TGF- β 1 (Kahari *et al.*, 1991).

In the current studies, TGF- β 1-treatment of PrE-like cells did not alter their cellular proliferation rate relative to that of control PrE-like cells. This finding is contrasted with the reports of other investigators showing that TGF- β 1 inhibited, by approximately 30%, the proliferation of RA-differentiated F9 cells cultured in medium with (Kelly and Rizzino, 1989) or without serum (Rizzino, 1987). Since the concentration of TGF- β 1, the duration of culture, and the method of detachment of cells employed by those investigators corresponded to this author's experimental conditions, an explanation for the lack of effect of TGF- β 1 on PrE-like cell proliferation in the current studies is not forthcoming. However, in support of the ability of TGF- β 1 to affect differentiation processes without influencing cell growth, Paria and Dey (1990) showed that TGF- β 1 markedly improved *in vitro* murine embryonic development to the blastocyst-stage, in serum-free medium, without increasing the cell number of the blastocysts.

To ensure that the decreased laminin secretion by PrE-like cells in response to TGF- β 1 treatment did not reflect increased deposition by these cells, a novel technique was designed to assess deposited laminin. Using this technique, this author reproducibly

demonstrated that PrE-like cells treated with TGF- β 1 did not deposit more laminin than did control PrE-like cells. Future experiments could measure the levels of laminin mRNA in TGF- β 1-treated PrE-like cells to determine if TGF- β 1 was acting to regulate transcription of the laminin gene in this system. In support of this possibility, it has been reported that repression of stromelysin induction by TGF- β in rat fibroblasts is mediated at the level of transcription by an element in the rat stromelysin promoter referred to as the TGF- β inhibitory element (TIE; Kerr *et al.*, 1990; Matrisian *et al.*, 1992).

Of interest in this research work was the fact that TGF- β 2 did not have any effect on laminin secretion by PrE-like cells. Since it has been shown that F9-differentiated cells bind TGF- β 2 with subsequent growth inhibition (Rizzino 1987; Mummery *et al.*, 1990), it would appear that TGF- β 2 does not function identically to TGF- β 1 in this system in terms of laminin secretion. Segarini and coworkers (1987) postulated that the sequence divergence between TGF- β 1 and TGF- β 2 gave reason to expect that functional differences might exist between the two proteins. Indeed, instances where TGF- β 2 was not functionally interchangeable with TGF- β 1 have been reported. Ohta and collaborators (1987) found that picomolar concentrations of TGF- β 1 potently inhibited hematopoietic progenitor cell proliferation, while TGF- β 2 at concentrations up to 1 nM did not affect colony formation. They determined that the progenitor cell lines examined were 100-fold more sensitive to TGF- β 1 than TGF- β 2, displaying type-I TGF- β receptors with a 15- to 20-fold higher affinity for TGF- β 1 compared to TGF- β 2. Rosa and coworkers (1988) studied mesoderm induction in *Xenopus laevis* blastula embryos by

exposing animal region explants to appropriate stimuli and assaying for the appearance of mesodermal products, such as α -actin mRNA. They found that TGF- β 2 at 3-200 ng/ml dose-dependently stimulated α -actin mRNA production. In contrast, TGF- β 1 (at the same concentrations) did not exhibit detectable mesoderm-inducing activity.

Since cAMP is clearly stimulatory for laminin and type IV collagen secretion by F9-differentiated cells, and TGF- β 1 has a definitive negative effect on the secretion of these glycoproteins, what might the signal transduction pathway be for TGF- β 1 in these cells? At least one of the TGF- β receptors (type II) is a serine/threonine kinase possessing autophosphorylation ability (Lin and Wang, 1992; Massagué *et al.*, 1992). Indeed, TGF- β -induced inhibition of the phosphorylation of the retinoblastoma susceptibility gene product can be alleviated by serine/threonine kinase inhibitors such as H7 (Massagué *et al.*, 1992). Therefore, it would be of interest to culture PrE-like cells in the presence of both TGF- β 1 and a serine/threonine kinase inhibitor and assess ECM glycoprotein secretion to determine if TGF- β 1 was signalling through this transduction pathway.

PDGF at 1 and 10 ng/ml was found to stimulate laminin secretion by PrE-like cells. In contrast, Kelly and Rizzino (1989) reported that the treatment of RA-differentiated F9 cells with PDGF (20 ng/ml; 24 h) did not affect the secretion of laminin A or B subunits. There are several possible explanations for this discrepancy between these two sets of data: (1) The cell cultures of Kelly and Rizzino (1989) contained 10% serum. As the increase in laminin secretion measured in the present research work was modest, perhaps any small stimulatory effect of PDGF in those authors' experiments was

masked by negative influences exerted by other factors present in the serum (for example, TGF- β 1). Alternatively, those authors may not have observed any stimulatory effect of exogenously-added PDGF since the serum most likely contained platelet-derived PDGF. (2) The use of PDGF at 20 ng/ml, a concentration two-fold higher than the highest PDGF concentration used in this research work (PDGF preparations obtained from the same source), may have caused a down-regulation of the PDGF receptors present on the PrE-like cells, rendering the cells incapable of responding to the available PDGF. (3) Finally, and perhaps most importantly, the shorter culture time used by those authors may indicate that PDGF requires greater than 24 h to stimulate laminin secretion by PrE-like cells.

TGF- β has been shown to effect a mitogenic response from several cell types via induction of PDGF expression (Leof *et al.*, 1986; Majack *et al.*, 1990). The fact that treatment of PrE-like cells with PDGF resulted in a modest stimulation of laminin secretion, in contrast to the decreased secretion measured after TGF- β 1-treatment of these cells, makes it unlikely that TGF- β 1's negative regulation of laminin secretion was via induction of PDGF.

TGF- α (or EGF) often antagonizes TGF- β -induced effects on ECM composition (Laiho *et al.*, 1986; Roberts *et al.*, 1986; Matrisian *et al.*, 1992). Thus, if TGF- α could stimulate increased laminin secretion by PrE-like cells, then this growth factor might be a candidate for an *in vivo* ligand stimulating PrE cell differentiation to PE. However, the results of the experiments assessing possible regulation of laminin secretion by TGF- α were inconclusive. Although there did appear to be some stimulation of laminin

secretion by PrE-like cells treated with TGF- α , these effects were found to be statistically insignificant. It would be of interest to extend this work with TGF- α to primary endoderm cell lines (for example, see Fowler *et al.*, 1990 and Mummery *et al.*, 1990) to determine whether those cells would respond to TGF- α with increased ECM protein secretion. Primary endoderm cells express ECM glycoproteins, including laminin, constitutively, thereby eliminating the variability of response associated with RA-induced differentiation.

It has been reported that EGF (6-100 ng/ml) potentiated cAMP accumulation in A-431 epidermoid carcinoma cells that were treated with forskolin, cholera toxin, isoproterenol or MIX in serum-free medium (Ball *et al.*, 1990). Since TGF- α signals through the EGF receptor (Pike *et al.*, 1982; Massagué, 1983; Paria *et al.*, 1991), this study raises the possibility that TGF- α could be involved in the differentiation of PrE to PE cells, for example, by acting synergistically with another ligand to stimulate cAMP production. Future experiments could, therefore, look at the effect of TGF- α on PrE-like cells that were concurrently exposed to forskolin or cholera toxin, and assess cAMP production as represented by secreted levels of ECM glycoproteins.

What might be the function of growth factor-induced changes in ECM glycoprotein secretion in the early embryo? PrE cells move away from the ICM to establish the PE layer (Enders *et al.*, 1978; Figure 2). The substratum for this migration is probably provided both by the trophectoderm and by the PrE cells themselves. A basal lamina has been localized on the trophoblastic side of RM (Jollie, 1968), and Carnegie and Cabaca (1991) found that the TE stained positively for the presence of

fibronectin and type IV collagen. This study as well as many others (Adamson and Ayers, 1979; Wartiovaara *et al.*, 1979; Leivo *et al.*, 1980; Strickland *et al.*, 1980; Dziadek and Timpl, 1985) have shown that PrE-like cells secrete ECM proteins. Cell adhesion to the ECM is necessary to initiate migration, but the cell-ECM contacts must also be broken for cells to move across the substratum. This could be accomplished by local dissolution of the underlying ECM, in conjunction with the action of local factors, such as TGF- β 1, to regulate the amount of matrix available for attachment.

One characteristic of F9-derived PrE-like and of murine ICM-derived PrE cells is secretion of PA (Strickland *et al.*, 1976; Strickland and Mahdavi, 1978). PA activity is associated with the oncogenic transformation of many cell types in culture (Ossowski *et al.*, 1973; Unkeless *et al.*, 1973). In addition, certain non-tumor cell types which are migratory, such as macrophages (Unkeless *et al.*, 1974), granulosa cells (Beers *et al.*, 1975) and trophoblastic cells (Sherman *et al.*, 1976), have also been shown to produce this enzyme. PA has been localized at cell-ECM adhesions (Pollanen *et al.*, 1987; Pollanen *et al.*, 1988) and plasminogen as well as PAs are able to bind to fibronectin and laminin (Salonen *et al.*, 1984; Salonen *et al.*, 1985; Vaheri *et al.*, 1990). It may be that the translocation of PrE cells is due, in part, to their production of PA, whose role is to permit localized digestion of cell-ECM contacts and, hence, cell movement. Indeed, in humans, migration of the trophoblastic cells is accompanied by focal dissolution of the matrix with which they are in contact (Fisher *et al.*, 1985).

Migration of PrE cells along their substratum may also be facilitated by local reductions in the amount of ECM material underneath the PrE cells, which would

decrease the likelihood of the cells remaining attached to the substratum and, therefore, allow the cells to detach from the matrix and translocate. Thus, the negative regulation of PrE-like cell secretion of laminin (and probably type IV collagen) by TGF- β 1 as determined in this research work may indicate that, in the early developing embryo, one function of TGF- β 1 is to regulate ECM glycoprotein secretion by these cells as they are migrating over the TE cell layer. Support for this theory is provided by a recent report from Lallier and colleagues (1994), who showed that maximal migration of neural crest cells occurred on intermediate concentrations of laminin substrata, while migration of these cells was decreased at high concentrations of laminin. They postulated that the self-assembly of laminin molecules at high concentrations might interfere with the ability of neural crest cells to access the migration-promoting domain of the laminin molecule.

The regulation of ECM glycoprotein secretion by PrE cells would likely be a finely-tuned process, since there must be sufficient matrix for attachment but not so much that the cells cannot translocate. Other growth factors, such as PDGF, may function to modestly stimulate secretion of ECM glycoproteins by PrE cells, as a counterbalance to the negative regulation by TGF- β 1, to ensure that there will be enough ECM for continued cell attachment.

It is interesting to speculate that TGF- β 1 may function as a morphogen for PrE cells in the early embryo and dose-dependently influence the secretion of ECM proteins by these cells, thereby creating gradients of ECM. Experiments in *Xenopus* have shown that embryonic cells are capable of interpreting growth factor concentrations (Green and Smith, 1991). Carnegie (1994) demonstrated that positive gradients of laminin and

fibronectin promoted the *in vitro* migration of F9-derived PrE-like cells, and postulated that the establishment of similar gradients from embryonic to abembryonic poles of the blastocyst may provide the means of promoting and guiding the outward migration of ICM-derived PrE cells. Future studies could use immunostaining in combination with confocal microscopy to determine if such gradients of ECM and/or TGF- β 1 exist in the peri-implantation embryo.

Thus, the presence of growth factors (of both maternal and embryonal origin) at the preimplantation-stage of murine development suggests a role for these factors in coordinating the growth and differentiation of the early embryo. Data presented here indicated that two growth factors, TGF- β 1 and PDGF, exerted significant effects on the *in vitro* secretion of laminin glycoprotein found in RM by F9-derived endoderm-like cells, thereby contributing towards the understanding of factors that may regulate the *in vivo* deposition of RM during early embryo development. Future studies detailing the the expression and physiological significance of growth factors, their receptors and their respective signalling pathways should provide a clearer picture of the multiple events occurring at this early stage of embryo development.

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