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UNIVERSITY OF ALBERTA

CLONING AND IDENTIFICATION OF PL74: A NOVEL MEMBER OF TGF BETA SUPERFAMILY

By

HONGSHI LI ©

A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** in Experimental Medicine.

DEPARTMENT OF MEDICINE

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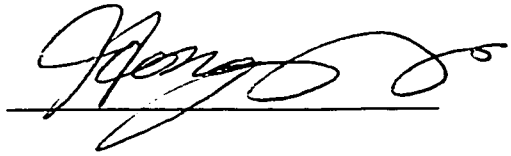

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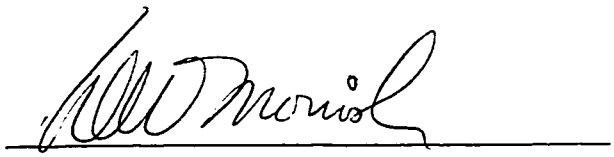

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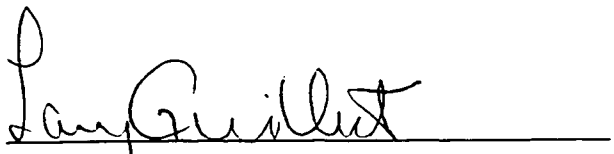

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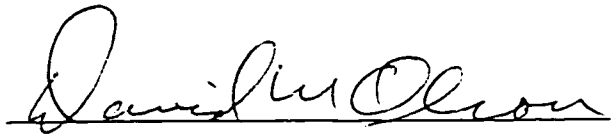

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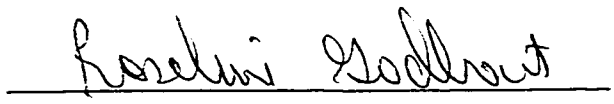

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ABSTRACT

To identify human gene regulators of placental differentiation, we prepared a subtractive cDNA library of *in vitro* differentiating human cytotrophoblast. Twenty clones were identified with increased expression during differentiation and six were novel. One of these novel clones, PL74, was fully sequenced and identified as a member of transforming growth factor beta (TGF-β) superfamily (GenBank accession number U51731). PL74 has a 1180 base pairs cDNA insert and contains an open reading frame of 308 amino acids with an estimated molecular mass of 34.2 kDa.

Amino acid sequence alignment of PL74 with representative members of all TGFβ subfamilies showed many conserved amino acids including the seven cysteines that are well-conserved in all TGF-β superfamily members. At the amino acid level, PL74 is 21-38% homologous with the members of TGF-β superfamily. It is most closely related to the bone morphogenetic protein (BMP) subfamily, sharing 33-38% similarities to BMP-8, BMP-7, BMP-6 and BMP-5. Northern analyses revealed that PL74 mRNA was expressed in human placenta at a much higher level than other human tissues. *In vitro*, PL74 mRNA level markedly increased during spontaneous cytotrophoblast differentiation. Overexpression of PL74 in human osteosarcoma MG-63 cells by transfection revealed that PL74 inhibits MG-63 cell proliferation by decreasing ^{3}H-thymidine uptake and cell number by 12% and 21%, respectively. TUNEL measurements showed that overexpression of PL74 increased the apoptotic frequency of MG-63 cells by six-fold. MG-63 cells stably transfected with PL74 revealed an increase of the cyclin-dependent kinase inhibitor p21 (WAF1/Cip1) mRNA expression, which indicates that

PL74 may act through the p21 (WAF1/Cip1) pathway. Human collagen 1 α and human osteocalcin, both of which are differentiation markers of MG-63 cells, showed increased mRNA expression in MG-63 cells stably transfected with PL74. Expression of PL74 mRNA in MG-63 cells was up-regulated by interferon γ, but not by EGF, GM-CSF, IGF-1,TGFβ1, and TNF-α.

Thus, we conclude that PL74 is a new TGF-β superfamily member and it that does not belong to any known subfamily of the superfamily. Based on studies in human osteosarcoma MG-63 cells, we propose that PL74 inhibits proliferation, induces apoptosis, and can also induce a differentiated phenotype.

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ABBREVIATIONS

A	Alanine
C	Cysteine
bp	Base pair
BMP	Bone morphogenetic protein
cDNA	Complementary deoxyribonucleic acid
CSF-1	Colony stimulating factor one
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EVT	Extravillous trophoblast
FSH	Follicle-stimulating hormone
GAPDH	Glyceraldehyde-3-phophate dehydrogenase
GM-CSF	Granulocyte-macrophage colony stimulating factor
hCG	Human chorionic gonadotrophin
hPL	Human placental lactogen
IFN-γ	Interferon gamma
IGF-1	Insulin-like growth factor one

IUGR	Intra-uterine growth restriction
kb	Kilobase
kDa	KiloDalton
M	Methionine
mRNA	Messenger ribonucleic acid
μg	Microgram
μl	Microlitre
N	Asparagine
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIH	Pregnancy-induced hypertension
R	Arginine
RNA	Ribonucleic acid
S	Serine
SDS	Sodium dodecyl sulfate
TdT	Terminal deoxynucleotidyl transferase
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TUNEL	TdT-mediated dUTP-biotin nick end labeling
U	Unit
VCAM-1	Vascular cell adhesion molecule 1

CHAPTER 1

INTRODUCTION

1 . 1 REGULATION OF HUMAN AND RODENT TROPHOBLAST DIFFERENTIATION

The placenta is an extraembryonic tissue that permits the embryo to develop within the female reproductive tract. The specialized epithelial cells of the placenta, termed trophoblasts, are the major cell components in first trimester placentas.

Trophoblast cell differentiation is crucial to the morphogenesis of the placenta and thus the establishment of pregnancy and development of the embryo / fetus. A search for the processes which govern trophoblast differentiation will therefore involve a functional analysis of growth factors, growth factor receptors and other gene products. Our work has described a new TGF-β family growth factor (PL74) highly expressed in the placenta. Since PL74 may regulate trophoblast and bone cell development, we will review the regulatory factors which affect human and rodent trophoblast differentiation, then discuss the TGF-β superfamily and the functions of its members in regulating the functions of trophoblasts, bone cells, and other cell types.

1.1.1 Human Trophoblast and Its Differentiation

In human placenta, two cell types are the main cellular components of the chorionic villi: the trophoblast and the mesenchymal cells. The trophoblast is discernible approximately 4 days after fertilization as the outermost layer of the blastocyst. From the early stage of gestation, it differentiates into two cell layers: the

outer differentiated syncytiotrophoblast and the inner proliferative cytotrophoblast. The syncytiotrophoblast and cytotrophoblasts form the primary villi. As pregnancy progresses, fusion of cytotrophoblastic cells with the syncytiotrophoblast leads to a largely single-layered epithelium of syncytial character. The remaining trophoblast, which is not used for villous formation, is the basic material used for the development of all other parts of the placenta: chorion laeve, marginal zone, chorionic plate and cell islands. It is named extravillous trophoblast, (Reviewed by Bernischke et al. 1995, Petraglia et al.1996). Villous cytotrophoblasts, extravillous cytotrophoblasts and syncytiotrophoblast thus comprise the trophoblast cells of the human placenta.

Development of the human placenta leads to two distinct trophoblast populations: the villous trophoblast primarily engaged in absorptive, exchange and endocrine function, and the extravillous trophoblast primarily engaged in remodeling the pregnant endometrium and its vasculature to achieve placental anchorage to the uterine wall and unhindered placental perfusion through low resistance uterine vessels. Both trophoblast populations arise by proliferation and differentiation of cytotrophoblast stem cells found in the proximal cytotrophoblast cell columns of the villus. The balance between proliferation and differentiation of trophoblast cells ultimately determines the structure and function of the developing placenta. The overall mechanism of human trophoblast differentiation was worked out by Tao & Hertig (1965), who incubated placental explants with ^{3}H-thymidine for different lengths of time. They found the syncytiotrophoblast does not replicate, in contrast to cytotrophoblasts. If the explants were maintained for 1-2 weeks, however, numerous

labeled nuclei were detected in the syncytial layer (Tao & Hertig, 1965). This suggested that the placental syncytium originated from the cytotrophoblast layer. The syncytiotrophoblast could form from mononuclear cytotrophoblasts by cell fusion. Kliman (1986) confirmed those conclusions by an *in vitro* differentiation model of term cytotrophoblast. The syncytiotrophoblastic layer is responsible for the exchange of gas, nutrients and waste products. Also it serves as a structural and immunological barrier between mother and fetus. A large number of steroid and peptide hormones are synthesized by the syncytiotrophoblast and secreted into the maternal blood. These factors play a role in the regulation of placenta development (Morrish et al. 1998).

1.1.2 Rodent Trophoblast and Its Differentiation

In mouse and rat, as in other mammalian species, the trophoblast lineage arises at the 64-cell blastocyst stage with the formation of an outer layer of cells, called the trophectoderm. The remaining cells of the embryo are the inner cell mass (ICM). Trophoblast differentiation is directed towards at least four recognizable phenotypes: (a) trophoblast giant cells; (b) spongiotrophoblast cells; (c) glycogen cells; and (d) syncytial trophoblast cells (Soares et al. 1993). Each of these cell types has unique functional and morphological attributes. Trophoblast giant cells and spongiotrophoblast cells represent endocrine cells of the rat placenta. The spongiotrophoblast forms the hormone-producing layer of placenta, producing proteins such as progesterone and products of the placental lactogen (PL) gene family (Faria et al. 1991, Lee et al. 1988). Glycogen cells are transitory cell-types that

accumulate glycogen and are surrounded by spongiotrophoblast cells. Syncytial cells arise by cell fusion and have a significant role in fetal-maternal exchange (Soares et al. 1993). Trophoblast giant cells, spongiotrophoblast cells, and glycogen cells situated at the decidual-placental interface comprise the junctional zone, while syncytial trophoblast cells and some trophoblast giant cells located between the junctional zone and the developing fetus constitute the labyrinth zone (Soares et al.1993).

1.1.3 Comparison Between Human and Rodent Trophoblast

The nature of placentation varies considerably across mammalian species (Steven et al. 1983). However, humans and rodents are similar in possessing an invasive hemochorial placenta. The trophoblast layers of human placenta arise from the trophectoderm of blastocyst as in mouse, but the final placental structure is somewhat different. Human trophoblast cells are concentrated into chorionic villi, in which villous cytotrophoblasts lie on the basement membrane in a layer under the multinucleated syncytiotrophoblast, which is the surface layer of the villus facing the maternal-fetal blood space. In rat and mouse, the destiny of differentiated trophoblast cells is to form four different types of cells: giant cells, spongiotrophoblast cells, glycogen cells and syncytial cells. There are a number of genes, such as chorionic gonadotrophin (CG) α and β (Jameson &Hollenberg, 1993), that are expressed in human placenta and not in mouse. The hCG α gene can be made to be expressed in the placenta of transgenic mice (Bokar et al.1989). This suggests that underlying transcriptional mechanisms of trophoblast gene regulation have been conserved

across mammalian evolution, despite the variation in gene expression in the placenta from species to species.

1.1.4 Molecular Indices of Trophoblast Differentiation

Some placental proteins such as chorionic gonadotrophin (CG), placental lactogen (PL), and pregnancy-specific β1 glycoprotein (SP1), are differentiation-linked proteins; that is, they are expressed nearly exclusively by the human syncytium. The presence of CG in the maternal circulation provides the earliest evidence of pregnancy. CG is a dimer of α and β subunits and is synthesized and secreted by trophoblast in increasing amounts during the first trimester of pregnancy. The synthesis and secretion of the α subunit continues to increase to term. CG α mRNA is detectable in villous syncytiotrophoblast, but also occurs in a subpopulation of villous cytotrophoblast (Hoshina et al. 1982). Immunohistochemical studies confirm that CG is produced mainly in the villous syncytiotrophoblast (Gosseye and Fox 1984). Recent studies demonstrated that cytotrophoblasts and syncytiotrophoblasts in human placenta contain hCG/LH receptor genes, with syncytiotrophoblasts expressing more than cytotrophoblasts (Reshef et al. 1990, Lei et al. 1992). The major role of hCG in human pregnancy is to stimulate estrodiol and progesterone from the corpus luteum in the first trimester. This is an essential function to maintain the pregnancy in the first trimester. After this time, hCG is not needed as the placenta has become self-sufficient in steroidogenesis. More recently, several roles for hCG have been described. For example, hCG can promote the differentiation of cytotrophoblasts into syncytiotrophoblasts, which was demonstrated by Shi et al.

(1993) using morphological, immunocytochemical, and molecular biological approaches.

PL is produced by the syncytiotrophoblast in steadily increasing amounts throughout pregnancy, and is also detected in an extravillous population (Gosseye and Fox, 1984). Many other glycoproteins detected in pregnancy plasma originate largely in the secretory syncytiotrophoblast. These include SP1 (Zheng et al. 1990) and plasminogen activator inhibitor α (PAI-2) (Feinberg et al. 1989).

The rat placenta synthesizes and secretes a number of polypeptides and steroid hormones. Among these are six members of the placental prolactin (PRL) family: placental lactogen-I (PL-I), PL-I variant (PL-Iv), PL-II, PRL-like protein A (PRL-A), PRL-B and PRL-C. These placental hormones are expressed in distinct cells and their expression can be used to monitor the state of differentiation (Soares et al. 1996).

1.1.5 The Regulation of Trophoblast Differentiation

In the developing placenta a search for key developmental events is likely to involve the identification of factors that control cell differentiation, proliferation and death. These factors include a number of hormonal factors, their receptors, and also involve some genes that might influence the complicated developing process.

Growth factors, cytokines, and other factors which affect trophoblast differentiation

Growth factors and their receptors play a role in the regulation of trophoblast growth and differentiation by stimulating or inhibiting placental growth from early to

late pregnancy or by influencing the differentiation process from cytotrophoblast to syncytial cells.

Epidermal growth factor (EGF) and transforming growth factor α (TGF-α)

EGF is a single chain polypeptide consisting of 53 amino acids which stimulates proliferation and differentiation in many kinds of cells and tissues (Carpenter & Cohen 1979). Morrish et al. (1987) and Barnea et al. (1990) have shown that EGF treatment gives rise to enhanced syncytium formation and increased production of CG and PL in vitro, indicating a function to enhance the differentiated phenotype. Trophoblast differentiation can be inhibited by incubating the trophoblast cells in the media which contains antibody against EGF (Ameniya et al. 1994). Several studies have demonstrated the presence of EGF receptor (EGFR) in human placenta (Rao et al. 1985, Hofmann et al. 1988). EGFR belongs to the family of receptors with tyrosine kinase activity. EGFR expression is modulated by trophoblast differentiation and by hormones or toxic substances. EGFR protein and mRNA expression increase with the in vitro differentiation of cytotrophoblast into syncytiotrophoblast (Alsat et al.1993). The same receptors may be occupied by TGFα, which is a product of decidua in humans and rats (Han et al. 1987). EGFR interacts with two ligands (EGF and TGFα) to stimulate cell differentiation. Since EGFR is expressed at high levels in differentiated trophoblasts in the mouse (Pollard et al. 1987) and EGF receptor is expressed in syncytiotrophoblasts of human placenta (Maruo et al. 1987), it is possible that the main biological role of EGF /EGFR autocrine and paracrine mechanism may be related to trophoblast differentiation.

In rodent, EGFR null mutant mice on a 129/Sv genetic background die at mid-gestation with a spongiotrophoblast cell defect (Sibilia and Wagner, 1995). This defect resembles the placental defect in Mash-2 null mutants (Threadgill, et al. 1995). The data suggest a potentially complex involvement of the EGFR in control of spongiotrophoblast cell development, possibly via interactions with Mash-2.

Transforming growth factor (TGF)β

TGF-β is a polypeptide growth factor, composed of two disulfide-linked monomers (MW 25,000) that are synthesized as large precursors. TGF-β1-2 are produced in human placenta, primarily in the syncytiotrophoblast (Dungy et al. 1991). TGF-βs block differentiation of villous cytotrophoblast into syncytiotrophoblast and thus reduces hormone (hCG, hPL) production most likely by interfering with the terminal events in the differentiation. Morrish et al. (1991) showed that exposure of monolayers of cultured cytotrophoblast to a range of TGF-β1 concentrations (0.001-10 ng/ml), resulted in decreased secretion of both hCG and hPL, as well as acting as a major inhibitor of trophoblast differentiation. Most recent studies indicate that early differentiation events such as the expression of α-hCG or pregnancy specific β1 glycoprotein genes remain unaffected by exposure to TGF-β, so that TGF-β possibly influences the terminal events in the villous trophoblast differentiation pathway (Morrish et al. 1996).

TGF-β also blocks villous cytotrophoblast stem cell differentiation into the extravillous trophoblast (EVT) cell pathway, and provides a key control for first

trimester EVT cell invasiveness (Graham and Lala, 1991), as well as proliferation (Graham et al. 1992). These controlling actions of TGF-β on EVT cells are mediated by multiple mechanisms such as an upregulation of PAI-I (Graham 1996), a stimulation of multinucleate cell formation (Graham et al. 1992) and an inhibition of trophoblast migratory ability (Irving and Lala, 1995). Thus, TGF-β family members have a significant role in regulation of trophoblast growth, invasion and differentiation. Because PL74 is a new member of TGF-β superfamily, with a highly expression in placenta, it may have an important role in trophoblast development.

GM-CSF and CSF-1

Trophoblast syncytialization is enhanced by the presence of GM-CSF and CSF-1. Both of these cytokines enhance production of human placental lactogen and human chorionic gonadotropin in placental cell preparations *in vitro* (Garcia-Lloret et al. 1994; reviewed by Wegmann and Guilbert 1992). Human choriocarcinoma cell lines JEG, JAR and BeWo spontaneously produce GM-CSF and CSF-1. Their proliferation can be inhibited by antibodies that react with GM-CSF and also antibodies that react with the receptor for CSF-1. Studying the morphological and functional differentiation of human placenta in vitro, Garcia-Lloret et al. (1994) found that both GM-CSF and CSF-1 stimulated cytotrophoblast aggregation into large multinucleated structures composed of extensive patches of syncytium interspersed with mononuclear cells. Concomitant with this morphological differentiation was upregulation of the production of the hPL and hCG (Garcia-Lloret

et al. 1994). In general, it is clear that GM-CSF and CSF-1 stimulate cytotrophoblast differentiation and hormone secretion.

Interleukin-1 (IL-1) and IL-6

Both IL-1 and IL-6 are present in human placenta, both in syncytiotrophoblast cells and bone marrow-derived cells (Kameda et al. 1990; Paulesu et al. 1991). Recent studies have shown that both IL-1 and IL-6 stimulate the release of hCG and hPL from cultured cells (Yagel et al. 1989; Nishino et al. 1990). Trophoblast derived IL-6 and IL-1 play a paracrine/autocrine role in the regulation of placental hormone secretion (Stephanou et al. 1995). These studies suggest a role for cytokines in the regulation of placental hormone secretion. There are no recent data indicating that IL-6 or IL-1 alter morphologic differentiation.

Leukemia Inhibitory Factor (LIF)

LIF is a glycoprotein that has multiple effects on different organ systems. It regulates the growth and differentiation of embryonic stem cells (Williams et al. 1988; Smith et al. 1988). Also it strongly associates with normal implantation in the mouse. A recent study showed that LIF in purified cultures of human cytotrophoblasts affects several morphologic and biochemical markers of trophoblastic differentiation (Margaret et al. 1995). LIF markedly decreased trophoblast production of hCG protein at 72 and 96 h, as well as expression of hCG β mRNA. LIF also significantly increased the expression of oncofetal fibronectin mRNA and secretion of the protein. These biochemical changes are characteristic of

cytotrophoblast differentiation toward an anchoring extravillous phenotype. LIF gene knockout experiments in mouse (Stewart et al 1992) showed that transient expression of LIF in mice is essential for implantation. Females lacking a functional LIF gene are fertile, but their blastocysts fail to implant and do not develop. However, the blastocysts are viable and, when transferred to wild type pseudopregnant recipients, they can implant and develop to term (Stewart et al 1992). Thus, LIF appears to be important for implantation.

Activin

Activin is produced in human placenta (Petraglia et al.1992; 1993). The presence of activin receptors on cytotrophoblast cells suggests that activin may have paracrine effects (Peng et al. 1993). Inhibin, a hormone related to activin, can antagonize the effects of activin on hCG secretion (Petraglia et al. 1989). The expression of activin as well as activin receptors by cytotrophoblast cells suggest that this factor may be a local regulator of placental development, including effects on promoting the differentiation of cytotrophoblast cells (Cross et al. 1996).

Recent data have shown that activin-A stimulates outgrowth of EVT cells from first trimester explants. The EVT growth was accompanied by the up-regulation of gelatinase, fibronectin and HLA-G expression, indicating that phenotypic differentiation was occurring (Caniggia et al. 1997). Also activin can stimulate EVT invasion, and this action can be blocked by follistatin. A physicochemical factor, hypoxia, also inhibits differentiation of EVT (Genbacev et al. 1996).

α_4 Integrins

Integrins comprise a family of cell surface receptors that mediate cell extracellular matrix (ECM) and cell-cell adhesions by interacting with ECM proteins such as fibronectin and vascular cell adhesion molecule 1 (VCAM-1) (Hynes et al. 1992). Integrin-mediated cell adhesion has been shown to play an important role in development. At the cellular level, integrins are involved in a number of cellular processes such as cell proliferation and differentiation (Hynes & Lander 1992; Adams & Watt 1993). By using targeted gene disruption, Yang et al. (1995) showed that a mutation, in which the α_4 integrin subunit has been disrupted, is recessive embryonic lethal as the result of two defects. The first defect is failure of fusion of the allantois with the chorion during placentation. The second is in the development of epicardium and coronary vessels leading to cardiac hemorrhage (Yang et al.1995). Thus α_4 integrins have essential functions during embryogenesis and to ensure normal placentation.

Other Factors Involved in Trophoblast Differentiation

There is evidence that retinoic acid (Kato and Braustein 1991) and its receptor (Stephanou et al.1994) may stimulate placental hormone secretion of the trophoblast, thus affecting its differentiation. Polyamines (Moore et al. 1988), inhibin (Petraglia et al. 1989), and interferons (Sekiya et al 1986) may also have a role in regulating trophoblast differentiation. 8-bromo-cAMP increased hCG secretion by up to 200 fold within 48h of incubation with cultured cytotrophoblast (Feinman 1986, Rodway 1990); hPL production was unaffected. It is possible that 8-bromo-cAMP acts as a

up-regulating factor of trophoblast differentiation. Differentiation can also be induced by matrix elements or cell attachment to plastic (Adams & Watt, 1993). Attachment to fibronectin, collagen I and other matrix elements can enhance in vitro trophoblast differentiation (Morrish et al 1996). As well, maternal cigarette smoking inhibits the trophoblast differentiation pathway that leads to column formation and uterine invasion (Genbacev et al. 1995). This effect, which is due at least in part to the effects of nicotine, may contribute to the developmental retardation of placenta (Genbacev et al. 1995).

Genes Involved in Regulating Human and Rodent Trophoblast Differentiation

Genes responsible for the normal growth and differentiation of trophoblast cells are just beginning to be identified. Identification of these important trophoblast-regulatory genes will provide a better understanding of trophoblast development and a road into the etiology of early pregnancy loss and various disorders of the placenta. At present, no trophoblast specific genes determining commitment to a trophoblast lineage have been identified. However, a specific genetic locus for controlling formation of the placenta has been demonstrated (Cross et al 1994).

Basic Helix-loop-Helix (bHLH) Gene - Hxt

A bHLH transcription factor gene, termed Hxt, has been implicated in the commitment of cells to differentiate along the trophoblast lineage in rodent (Cross et al 1994). Overexpression of Hxt in mouse blastomeres directed their development into trophoblast cells in blastocysts. In addition, overexpression of Hxt induced the

differentiation of rat trophoblast (Rcho-1) stem cells, measured by changes in cell adhesion and activation of the PL-I gene promoter, a trophoblast giant cell specific gene (Cross et al 1995). In contrast, the negative HLH regulator, Id-1, inhibited Rcho-1 differentiation and PL-I transcription. Hxt mRNA was also detected in trophoblast cells from human and rats (choriocarcinoma cell lines). Whether Hxt is an essential trophoblast determinant awaits further experiments. However, it is clear that overexpression of Hxt in rat trophoblast stem cells reduces their proliferation and promotes differentiation (Cross et al, 1994). These data demonstrate a role for HLH factors in regulating rodent trophoblast development and indicate a positive role for Hxt gene in promoting the formation of trophoblast giant cells in mouse (Cross et al. 1995). To date, a human homologue of Hxt has not been identified.

Mash-2 Gene

Mash-2 is a mammalian member of the *achaete-scute* family, which encodes basic-helix-loop-helix transcription factors and is strongly expressed in the extraembryonic trophoblast lineage. Recently the Mash-2 gene has been shown to play a role in the development of mouse placenta by using gene targeting to generate mice having no Mash-2 function (Guillemot et al. 1994). Mash-2 $^{-/-}$ embryos die from placental failure at 10 days postcoitum. In mutant placentas, spongiotrophoblast cells and their precursors are absent and chorionic ectoderm is reduced (Guillemot et al. 1994). Mash-2 expression is restricted to trophoblast cells (Guillemot et al. 1995). These data indicate that Mash-2 is the first transcription factor shown to play a critical part in the development of the mouse trophoblast lineage. A human

homologue, HASH-2, has been cloned (Oudejans et al. 1996), and is expressed in trophoblast cells. However, it is unknown if it can regulate trophoblast lineage commitment and development analogously to Mash-2.

Until now our knowledge about trophoblast differentiation is still limited. Multiple factors and genes influence trophoblast differentiation. These factors function probably like a network, in which different factors regulate specific steps in trophoblast differentiation. But in human placenta, no specific genes which regulate trophoblast differentiation have been found. To search for these factors is the motive of our work we are describing here.

1 . 2 THE GENERAL BIOLOGY OF THE TRANSFORMING GROWTH FACTOR-BETA (TGF-β) SUPERFAMILY

The TGF-β superfamily is named after the first member of the family to be isolated from human platelets as TGF-β1 (Assoian et al. 1983) and mediates many key events in normal growth and development. The TGF-β family of factors includes diverse activities, such as inhibiting proliferation, promoting apoptosis, inducing expression of various extracellular matrix proteins and modulating cell differentiation in a wide variety of cell types (reviewed by Massague 1990; Kingsly 1994).

1.2.1 Structure of the TGF-β superfamily

All members of the TGF-β superfamily are secreted as precursor molecules approximately four times larger than the mature form. The precursors usually contain a short amino-terminal signal sequence and a pro-domain of varying sizes. This precursor structure is shared by all known members of the superfamily, but the sequence of the precursor portions of the family members show poor sequence homology. This precursor protein is usually cleaved at a dibasic or RXXR site to release a mature carboxy-terminal segment of 104-140 amino acids. The active signaling molecule is made up of hetero- or homodimers of this carboxy-terminal segment (Massague 1990). The precessor part of the protein appears to be essential

for normal syntheses and secretion (Gray and Mason 1990; Hammonds et al. 1991; Thomsen and Melton 1993).

The conserved carboxy- terminal feature consists of a pattern of seven cysteine residues, C...CXGXC...CC...CXCX, while TGF-β itself and β chains of inhibins and activins display two additional cysteines C...CC...CXGXC...CC...CXCX. As a hallmark, all family members contain this structure, by which new family members are usually recognized (Fig.1 A). The crystal structures of two members of the TGF-β superfamily have now been solved: TGF-β2 (Daopin et al. 1992; Schlunegger and Grutter 1992) and BMP-7/ op-1 (Griffith et al. 1996). In both, the core of the monomer structure is this cysteine knot (McDonald and Hendrickson 1993) involving intramolecular bonding of six of the seven conserved cysteines; the seventh participates in intermolecular disulfide bonding. Extending from the monomer core in one direction are two finger-like projections made from antiparalled β strands and, on the opposite side, an α -helical loop (Fig. 1 B).

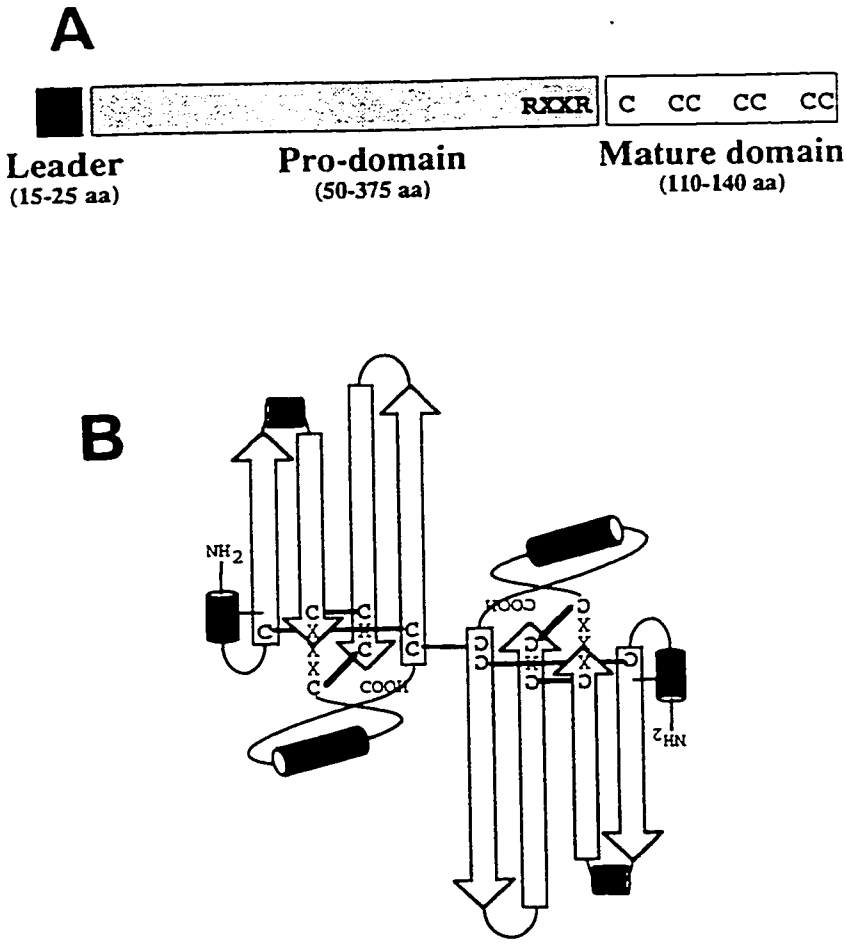


Figure 1. Structure of TGF-β superfamily members. (Copied from Kingsley, D. 1994).

A. TGF-β-related proteins are synthesized as large precursor proteins. An amino-terminal signal targets the precursor to the secretion pathway. A variable pro-domain may assist in folding, dimerization, and regulation of the factor activity. The actual signaling molecule is a homo-or heterodimer of a small carboxyl-terminal fragment. Seven characteristic cysteine residues in this region are almost unchanged in various members of the superfamily.

B. Crystal structure of the mature region of TGF-β2. Six of the characteristic cysteine forms a disulfide bond linking two monomers into a dimer. Much of the variation between members of the family are in the amino-terminal α-helix and the loops and helices that join the various β-sheet strands.

1.2.2 The members of TGF-β superfamily

The TGF-β superfamily includes almost 40 members from animals as diverse as *C. elegans, Drosophila, Xenopus* and humans. The superfamily is generally subdivided into three subfamilies by amino acid sequence similarity in the C-terminal domain of the family members. These are the TGF-β subfamily, the activin subfamily and the bone morphogenetic protein (BMP) subfamily (Massague 1990; Kingsley 1994). The genes encoding the Mullerian inhibiting substance (MIS) (Massague 1990), the glial-derived neurotropic growth factor (GDNF) (Lin, et al. 1993) and growth differentiation factor 9 (GDF-9) (McPherron and Lee 1993) are more divergent and thus have not been included in any of these subfamilies. The degree of amino acid sequence identity ranges from 25 to 90% between different family members. Kingsley (1994) gave an overview of the family members shown in Table 1.

The TGF-β subfamily

There are five members in this subfamily, TGF-β1 (Roberts et al. 1981), TGF-β2 (Holley et al. 1980; Segedin et al. 1985; Wrann at al. 1987), TGF-β3 (ten Dijke et al. 1988; Derynck et al. 1988), TGF-β4 (Jakowlew et al.1988) and TGF-β5 (Kondaiah et al. 1990). Different from other superfamily members, nine cysteines are conserved in all TGF-β superfamily members. The degree of identity between the five mature TGF-β sequences range from 64% (TGF-β1 vs. TGF-β4) to 82% (TGF-β2 vs. TGF-β4) (Kondaiah et al.1990). Individual TGF-βs are extremely well conserved, with as

high as 97% identity in TGF-βs of various mammalian and avian species (Derynck et al.1987; Jakowlew et al.1988; Madisen et al. 1988).

Activin subfamily

Activins are homo-or heterodimers of two subunits, A or B. Activins stimulate FSH production by pituitary cell cultures, steroidgeneses in granulosa cells and production of gonadotropin-releasing hormone and progesterone in cultured human placenta cells (Vale et al. 1986; Ling et al. 1986; Petraglia et al. 1989). Homodimeric β A activin also induces differentiation of erythroleukemia cells (Eto et al. 1987).

Bone Morphogenetic Protein (BMP) subfamily

Bone morphogenetic activity was first described by Urist (1965). The name BMP was first given to three proteins purified from a demineralized bovine bone preparation that induced ectopic cartilage and endochondral bone when implanted in experimental animals (Wozhey et al. 1988). One of these proteins (BMP1) was a putative protease of the astacin family, whereas the other two (BMP2 and BMP3) were related to human TGF-β. Now over twenty members have been identified in this family ranging from the sea urchin to mammals (Fig. 2). There is strong genetic and experimental evidence that these molecules regulate biological processes as diverse as cell proliferation, apoptosis, differentiation, cell-fate determination and morphogenesis. Also, the vertebrate BMPs are involved in the development of nearly all organs and tissues as well as in critical steps in the establishment of the basic embryonic body plan (Hogan, 1996).

In the BMP subfamily, the decapentaplegic (DPP) gene encodes important functions in embryonic *Drosophila* pattern formation. Mutation in various regions of the DPP gene complex result in failed dorsal-ventral patterning during early embryogenesis and defective patterning of the larval imaginal disks (Spencer, et al. 1982). The protein of the DPP gene has the predicted structure of a TGF-β-related molecule, with a C-terminal sequence that is 36% identical to the mature TGF-β1 sequence (Padgett et al. 1987). Another member of this subfamily, Vg1, is involved in embryonic development in *Xenopus* (Weeks and Melton 1987). The Vg1 product is 38 and 50% identical, respectively, to TGF-β1 and DPP. This subfamily also contains another *Drosophila* gene. It has been named 60A after its chromosomal map location, but its normal function is unknown (Wharton et al. 1991; Doctor et al. 1992).

BMP-2 and BMP-4 (Wozney et al. 1988) are 90% identical to each other and 75% to DPP in the C-terminal domain. Human BMP4 can rescue the dorsoventral axis defects caused by DPP mutation in *Drosophila* (Padgett et al. 1993). Conversely, purified preparations of the DPP protein can induce bone and cartilage in mammals (Sampath et al. 1993). Thus the similarity between the fly and mammalian genes extends to the functional level. They are also called the dpp group (Kingsley, 1994).

BMP 5 (Celeste et al. 1990), BMP6 or Vgr1 (Lyons et al. 1989; Celeste et al. 1990), BMP7 or osteogenic protein 1 (op-1) (Celeste et al 1990; Ozkaynak et al. 1990), and BMP-8 or osteogenic protein 2 (op-2) (Ozkaynak et al 1992) are ~75% to

90% identical to each other and 63~75% to 60A. They are also called the 60A group (Kingsley 1994).

The other members in this subfamily include BMP-3 (Woznney et al. 1988), growth differentiation factor1 (GDF-1) (Lee et al. 1991), GDF-3/Vgr-2 (Jones et al. 1992; McPherron and Lee 1993), dorsalin (Basler et al. 1993) and nodal (Zhou et al. 1993). These members cannot be assigned to any BMP subgroup because of lower C-terminal similarity. BMP-3 or osteogenin is another osteoinductive factor in mammals. GDF-1 is expressed primarily in the nervous system. GDF-3/Vgr-2 has highest similarity with Vg-1, BMP-2, BMP6 and GDF-1. The GDF-3/Vgr-2 protein product is unique in the TGF-β superfamily because it lacks one of the seven cysteine residues normally found in the mature region. The missing cysteine residue normally forms the interchain disulfide bond that links two monomer subunits into a dimer (Daopin et al.1992; Schlunegger and Grutter 1992). The GDF-3/Vgr-2 gene is expressed in ossifying skeletal tissue during embryonic development (Jones et al. 1992) and in thymus, spleen, and bone marrow in adult mice (McPherron and Lee 1993). Dorsalin is expressed in the developing chick nervous system. This molecule has two in vitro activities. It promotes the outgrowth of neural crest cells, and it inhibits the formation of motor neuron cells. This suggests that dorsalin may play an important role in chick neural patterning along the dorsovental axis (Basler et al. 1993). The nodal gene was identified at the site of a retroviral insertion that cause an early embryonic lethal phenotype in mice (Zhou et al. 1993). The product of this gene is almost equally similar to the BMP and the activin subfamilies.

Divergent genes

MIS

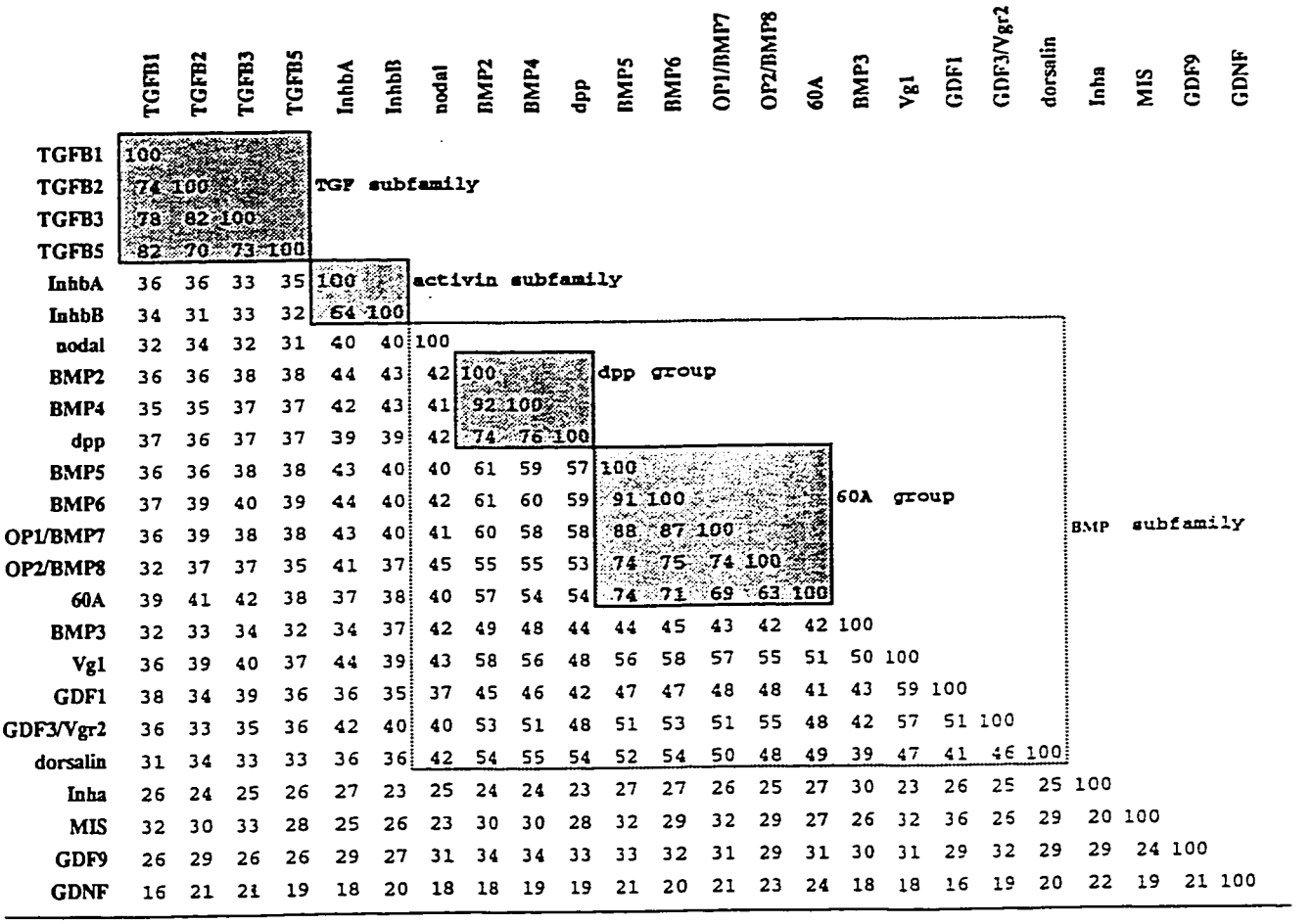
Mullerian inhibiting substance (MIS) is distantly related to the other members of the TGF-β superfamily (Massague, 1990). It was identified and later purified based on its ability to induce regression of the primordium of female genitalia, the mullerian duct, in mammalian male embryos (Blanchard & Josso 1974). The sequence of the MIS C-terminal domain is ~ 25% identical to that of other TGF-β family members. In contrast to other TGF-β - related factors, MIS contains the glycosylated N-terminal extension uncleaved from the C-terminal domain (Cate et al. 1986). MIS can be a regulator of gonadal morphogenesis and act on ovarian endocrine differentiation (Vigier et al. 1989).

GDF-9

The GDF-9 gene was isolated in a PCR screen for additional TGF-β superfamily members (McPherron and Lee 1993). Like GDF-3, its protein lacks the seventh cysteine residue. GDF-9 is expressed only in ovaries of adult animals. Its C-terminal sequence is ~26% identical with the other members of the TGF-β superfamily.

GDNF

Glial-derived neurotropic growth factor (GDNF) was isolated based on its ability to promote the survival and differentiation of dopaminergic neurons from the midbrain (Lin et al 1993). The factor shares the pattern of seven cysteines found in other members of the family, but the amino acid identity only has < 25% homology with other members.



	TGFB1	TGFB2	TGFB3	TGFB5	InhbA	InhbB	nodal	BMP2	BMP4	dpp	BMP5	BMP6	OP1/BMP7	OP2/BMP8	60A	BMP3	Vg1	GDF1	GDF3/Vgr2	dorsalin	Inha	MIS	GDF9	GDNF
TGFB1	100																							
TGFB2	74	100																						
TGFB3	78	82	100																					
TGFB5	82	70	73	100																				
InhbA	36	36	33	35	100																			
InhbB	34	31	33	32	64	100																		
nodal	32	34	32	31	40	40	100																	
BMP2	36	36	38	38	44	43	42	100																
BMP4	35	35	37	37	42	43	41	92	100															
dpp	37	36	37	37	39	39	42	74	76	100														
BMP5	36	36	38	38	43	40	40	61	59	57	100													
BMP6	37	39	40	39	44	40	42	61	60	59	91	100												
OP1/BMP7	36	39	38	38	43	40	41	60	58	58	88	87	100											
OP2/BMP8	32	37	37	35	41	37	45	55	55	53	74	75	74	100										
60A	39	41	42	38	37	38	40	57	54	54	74	71	69	63	100									
BMP3	32	33	34	32	34	37	42	49	48	44	44	45	43	42	42	100								
Vg1	36	39	40	37	44	39	43	58	56	48	56	58	57	55	51	50	100							
GDF1	38	34	39	36	36	35	37	45	46	42	47	47	48	48	41	43	59	100						
GDF3/Vgr2	36	33	35	36	42	40	40	53	51	48	51	53	51	55	48	42	57	51	100					
dorsalin	31	34	33	33	36	36	42	54	55	54	52	54	50	48	49	39	47	41	46	100				
Inha	26	24	25	26	27	23	25	24	24	23	27	27	26	25	27	30	23	26	25	25	100			
MIS	32	30	33	28	25	26	23	30	30	28	32	29	32	29	27	26	32	36	26	29	20	100		
GDF9	26	29	26	26	29	27	31	34	34	33	33	32	31	29	31	30	31	29	32	29	29	24	100	
GDNF	16	21	21	19	18	20	18	18	19	19	21	20	21	23	24	18	18	16	19	20	22	19	21	100

Table 1. Sequence relationships between members of the TGF-β superfamily. The different TGF-β family members were aligned beginning with the first invariant cysteine residue in the mature region of the molecules. Percent amino acid identities are shown for all pairwise combinations (Copied from Kingsley 1994).

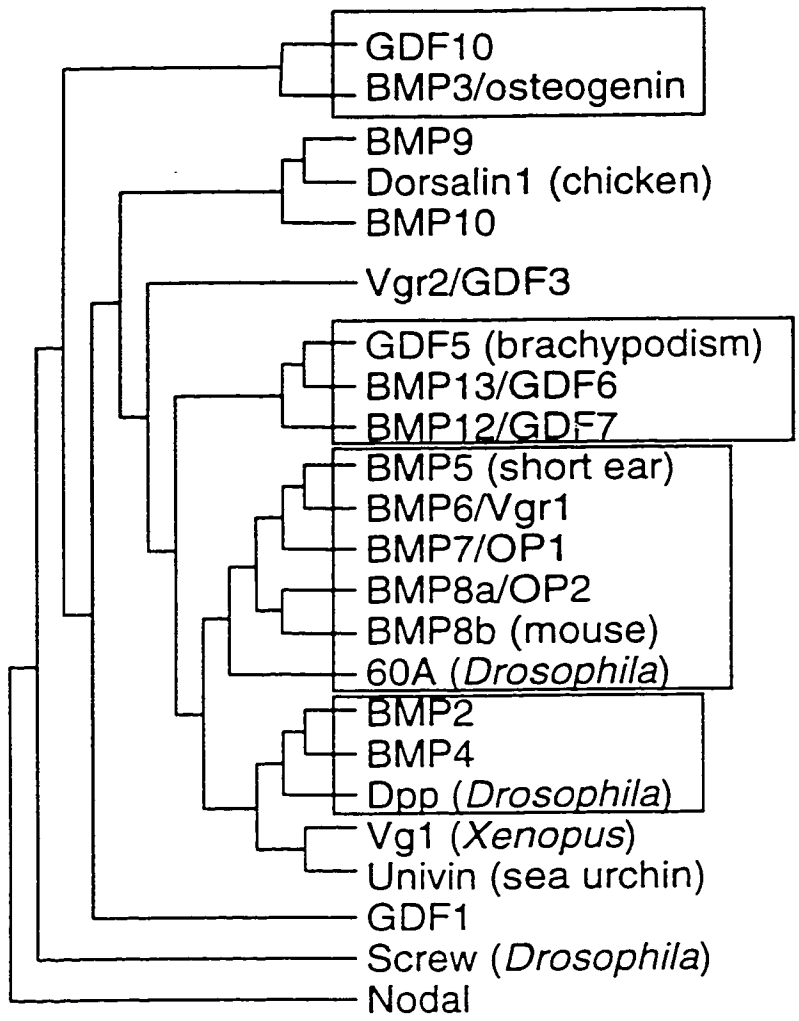


Figure 2. The BMP subfamily.

Relationships between members are shown, determined on the basis of sequence comparisons of the carboxy-terminal mature region. (Copied from Hogan 1996).

1.2.3 Receptors and Other Binding Proteins of the TGF-β Superfamily

Like other polypeptide growth factors, TGF- β ligands are believed to act on target cells by binding specifically to integral membrane proteins that are coupled to cytoplasmic signal transducers. Over the past several years, a group of the TGF-β receptors and binding proteins have been identified and molecularly characterized (Massague 1992; Massague et al. 1994; Yingling et al. 1995). Those studies have demonstrated that the TGF-βs signal through a heterometic receptor complex consisting of Type I and Type II transmembrane serine/threonine kinases. Also those studies have revealed that TGF-β signaling receptors belong to a larger family of transmembrane ser/thr kinases, all of which likely serve as receptors for the members of TGF-β superfamily (Massague 1996).

Members and Structure of TGF-β superfamily receptors

A classical crosslinking assay was used to identify cell surface TGF-β binding proteins that were widely expressed and thus likely to transmit TGF-β-binding signals across the plasma membrane. The main TGF-β-binding components detected in this assay are membrane proteins of 53 kDa, 70-85 kDa and 200-400 kDa, called receptors type I, II and III, respectively (Massague et al. 1985; 1986; Ohta et al. 1987; Cheifetz et al. 1988). Now a group of receptors of the TGF-β superfamily have been identified by gene cloning. They all belong to a conserved family of transmembrane serine/threonine kinase receptors (Fig.3). All members of this receptor family have a characteristic structure that includes a short, cysteine rich extracellular domain, a single

transmembrane domain and an intracellular serine/threonine kinase domain (Attisano & Wrana 1996; Massague & Weis-Garcia 1996; Miyazono et al. 1994).

Many of these receptors have now been identified in a wide variety of vertebrates and invertebrates including flies, frogs, mouse and humans. Functional characterization and comparison of their primary amino acid sequence indicates that these receptors can be divided into two classes, the type I and type II receptors. The type I receptor-class is distinguished by a highly conserved sequence known as the GS domain, which contains a repetitive glycine-serine motif and is located between the transmembrane and kinase domains. In addition to these two types of receptors, TGF-β binds to two other transmembrane proteins, betaglycan (or type III receptor) and endoglin (Lin et al. 1995; Lopez-Casillas et al. 1993) . It is now clear that TGF-β receptor type I and type II are involved in signaling, whereas the type III receptors regulate access of TGF-β to the signaling receptors (Laiho et al. 1991). The role of endoglin in TGF-β signaling is less clear.

Receptor Kinase Signaling

Studies in TGF-β and activin signaling have provided a mechanistic understanding of how the type II and type I kinase domains interact to initiate signaling (Attisano et al. 1996; Chen et al. 1995; Chen and Weinberg 1995). The type II receptor is a constitutive receptor kinase that associates with type I receptor on binding to TGF-β. Upon association, the GS domain of the type I receptor kinase is phosphorylated by the active type II receptor (Wrana et al. 1994). Phosphorylation of the GS domain

subsequently activates the type I receptor kinase, which targets downstream signaling components of the pathway. Other TGF-β superfamily members also signal through a type II and type I receptor complex. Studies in *Drosophila* show that signaling by DPP requires both receptor II (PUNT) and receptor I (TKV) (Brummel et al. 1994; Letsou et al. 1995), and in mammalian cells, BMPs signaling occur through a complex of type II and type I receptors (Hoodless et al. 1996; Liu et al. 1995).

Biochemical and genetic data have also shown the signaling by TGF-β-like factors occur through this heteromeric complex. For instance, in mink lung epithelial cells lacking either component of TGF-β heteromeric receptor complex, signaling is abolished (Carcamo et al. 1994, Wrana et al. 1992). Thus a heteromeric complex of type II and type I receptors appear to be a general requirement for signaling by the TGF-β superfamily. Although some ser/thr kinase receptors appear to bind a limited subset of ligands, others can bind multiple members of TGF-β superfamily. TβR II and TβR I appear to be quite specific, interacting with only TGF-β1, 2 and 3 (Wrana et al. 1992). In contrast, the type II receptor ActR II and ActR II B appear to be capable of binding activin, BMP2, BMP7, GDF5 and likely many other members of the superfamily.

Smads: Effectors for TGF-β s receptors

In *Drosophila*, dpp is a TGF-β-like ligand (Padgett et al 1987). The receptors fot it are punt (homologous to typeII TGF-β receptor) and TKV (homologous to type I TGF-β receptor). In a genetic screen to determine dominant enhancers of a weak dpp allele, 'mother against dpp' (MAD) was isolated (Sekelsky et al. 1995). Loss-of-function

mutations in MAD result in organisms that phenotypically resemble those in null alleles of dpp. Moreover, MAD is required for dpp function especially in cells which respond to dpp, indicating that MAD is a component of the TGF-β-like signaling pathway (Newfeld et al. 1996). Three related homologues of MAD, sma-2, sma-3 and sma-4 have been identified in *C. elegans* (Savage et al. 1996). Mutation in these three genes yields a phenotype similar to that observed in mutants of type II receptor gene daf-4, suggesting that these genes are components of DAF-4 signaling pathway. These homologues of the SMA and MAD genes have been called Smads. Smads are critical downstream substrates of type I receptor kinase. Like Smad-2 and Smad-3, they directly interact with type I receptor and are phosphorylated on the last two serines of a conserved C-terminal SSxS motif.

The Smad family of proteins has been identified as mediators of the TGF-β signal pathway. So far seven Smad genes have been cloned, of these, Smad-2 and Smad-3 mediate the TGF-β and activin signals (Baker & Harland 1997; Yingling et al. 1996), whereas Smad-1 and Smad-5 mediate the BMB signal (Hoodless et al. 1996; Liu et al. 1996). Smad-4 appears to be a general partner for other Smads by bringing the cytoplasmic Smads into the nucleus (Hahn 1996). The first human homologue, DPC-4 (or Smad-4) was identified as a candidate tumor suppressor gene involved in pancreatic cancer (Hahn et al. 1996). DPC-4 and Smad-2 are closely linked on chromosome 18q21.1, a region often deleted in human cancers (Nakao et al. 1997), suggesting that cells that lack some of the Smads may escape from the TGF-β-mediated growth inhibition and promote cancer progression (Wrana, 1998).

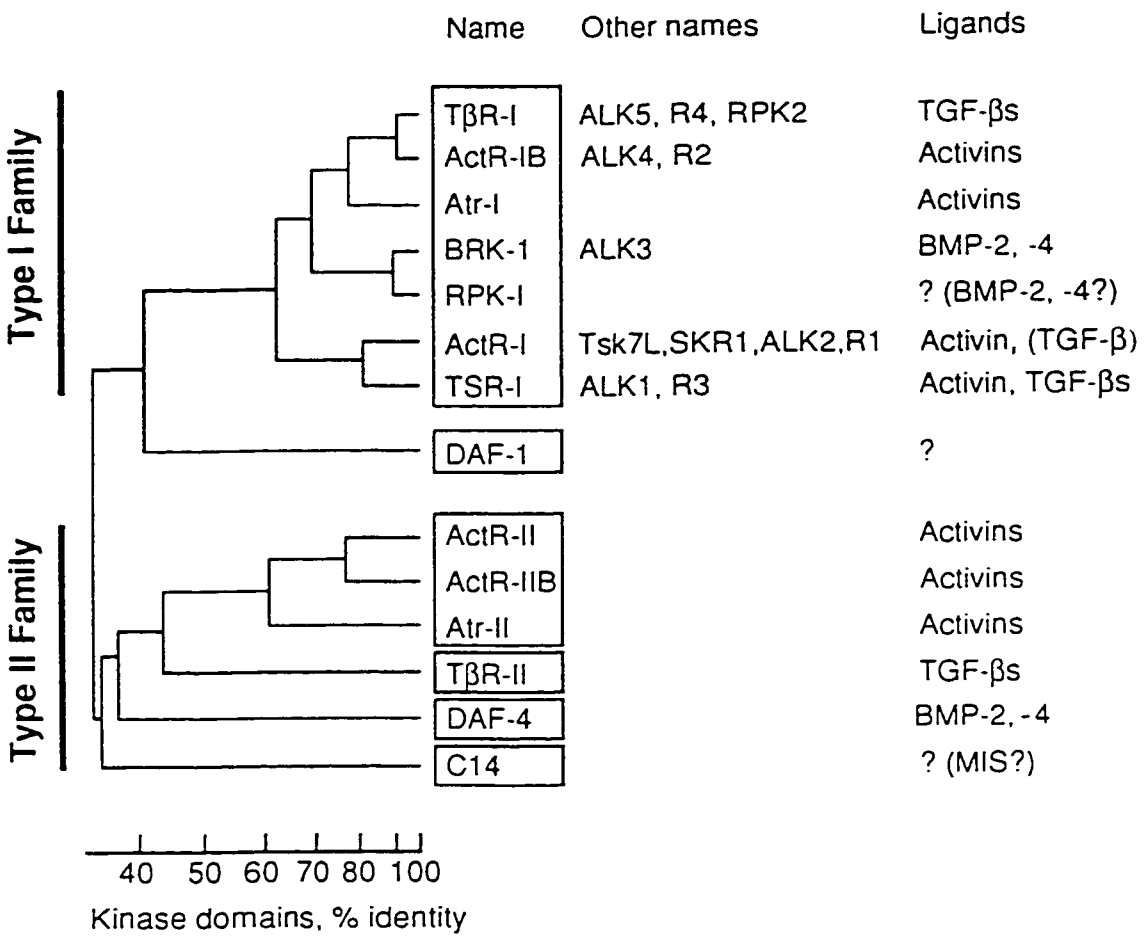


Figure 3. The TGF-β receptor family.

The alternative names and the known ligands of the type I and type II receptor subfamilies are shown. Boxes enclose receptors with similar extracellular cysteine arrangement (Copied from Massague et al. 1994).

1.2.4 Biological activities of TGF-β superfamily members

The TGF-β superfamily of growth factors has many diverse effects on cell biology depending on the type of cell, its environment and its state of differentiation. These actions include cell proliferation, cell cycle inhibition, expression of extracellular matrix protein, morphogenetic movements, apoptosis and induction of differentiation. Most of the effects of TGF-βs have been studied with in vitro systems. But many of the advances in genetic effects of TGF-βs have come from studies in transgenic mice. Here we focus on the roles of these factors in early mammalian development and bone growth.

TGF-β superfamily members and bone growth

TGF-βs are members of a large family of genes including the activins, BMPs and growth and differentiation factors (GDFs) that regulate growth and differentiation of many cell types including the osteoblast (Bonewald and Dallas 1994). TGF-β1 and TGF-β2 are produced by osteoblasts and incorporated into mineralized bone matrix. TGF-β1-2 are expressed at a high level in mature osteoblasts on bone surfaces during bone development and growth (Dodds et al.1994; D'souza 1993), and in healing fracture callus (Sandberg et al. 1993). TGF-β1 has been observed to both inhibit and stimulate osteoblastic cell proliferation in vitro, in part depending on TGF-β concentration, cell density, species and the stage of osteoblast differentiation (Centrella et al. 1994). In most studies, TGF-β increased expression of markers of osteoblast differentiation, such as alkaline phosphatase, type I collagen and osteonectin (Wergedad et al. 1992; Ingram et

al. 1994). In contrast, TGF-β inhibited mineralized bone nodule formation and osteoblast differentiation in rat osteoblast cultures (Harris et al. 1994).

BMPs were discovered for their ability to induce cartilage and bone formation from non-skeletal mesodermal cells and are now of considerable interest as therapeutic agents for healing of fractures and of periodental bone defects, and for inducing bone growth around implants and prostheses (Reddi et al. 1993; Wozney 1992). Besides their osteoinductive activities, BMPs have wide-reaching actions on other aspects of morphogenesis. For example, BMPs localized in the ventral cytoplasm of amphibian oocytes induce development of endodermal tissues, and BMPs expressed in the apical ectodermal ridge of the developing vertebrate limb bud are important in skeletal pattern formation. GDF-5 regulator closely related to BMPs, is important in pattern formation and skeletal morphogenesis in vertebrate limb development (Storm et al.1994).

Members of the TGF-β superfamily appear to act sequentially in regulating specific aspects of endochondral bone development (Centrella et al. 1996). BMP-2, TGF-β and BMP-6 are thought to act in a stepwise manner during osteoblast differentiation. BMP-2 promotes osteoblast differentiation in undifferentiated mesenchymal cells (Centrella et al. 1995) and alters TGF-β receptor expression, permitting increased osteoblast function in response to TGF-β (Centrella et al. 1995). BMP-6 has been shown to enhance osteoblast differentiation of ROB-C26 pluripotent mesenchymal cell line (Yamaquchi et al. 1991; Gitelman et al. 1995). Extracellular matrix from BMP-6 overexpressing cells demonstrated in vivo osteoinductive activity. This activity was blocked by BMP-6 antibodies (Gitelman et al. 1995). Members of the TGF-β superfamily may also act sequentially to regulate chondrocyte differentiation. TGF-β1, TGF-β2 and BMP-4 act

synergistically to promote chondrogenesis in chick limb bud mesenchymal cells (Chen et al. 1991).

Recently, exciting progress has been made in understanding the role of BMPs and TGF-βs in the formation of digits. As the limb extends and cells move away from the progress zone, they either undergo chondrogenic differentiation to form the cartilaginous skeleton of the digits or the cells remain undifferentiated in the interdigital regions. Eventually the cells in the interdigital regions undergo massive cell death by apoptosis. BMP 2, BMP 4 and BMP 7 display expression patterns which correlate with the interdigital spaces, suggesting that BMPs may regulate apoptosis in this region (Zou and Niswander 1996).

Recent studies of mouse mutants with skeletal defects have suggested important roles for BMPs and GDFs in skeletal development. *Short ear* mutations in mice, which express various defects in embryonic development of cartilage and bone, are associated with deletions and rearrangements of the BMP-5 gene (Kingsley et al. 1992). In addition to BMPs, growth and differentiation factor 5 (GDF-5) is also implicated in proper limb development, since the gene is mutated in the natural mutation brachypodism (bp) (Storm et al. 1994). Bp null mutants have shortened limb bones and alterations in segmentation of the digits which are detectable as early as day 12 of embryogenesis, thereby suggesting that GDF-5 is involved in regulating condensation of mesoderm in the limb. Thus, TGF-βs play important roles in osteoblast cell differentiation and proliferation, and regulate the early skeletal development and bone growth.

TGF-βs and embryogenesis

Some TGF-β superfamily members are expressed throughout embryogenic development, and their receptors are ubiquitously distributed. This, and the abilities of TGF-β family genes to control DNA replication, cell differentiation, cell adhesion, and extracellular matrix layout suggest a broad role for TGF-β family genes in generation and modification the morphogenic events of embryogenesis.

Homologous recombination was used to generate a null mutation of activin B in mice. These animals were viable, although defects in eyelid development were present (Vassalli et al. 1994). Activin A deficient mice die within 24 hours of birth with abnormalities in mouth formation (Matzuk et al. 1995). Taken together, these mutants suggest that activins are important regulators in mammalian development. In mammals, the BMPs appear to be important for mesoderm induction and gastrulation. Embryos deficient in BMP2/4 receptor, Alk3, exhibit a complete absence of mesoderm (Mishina et al. 1995). In chicken, dorsalin is expressed in the dorsal side of the developing neural tube where it stimulates neural crest cell outgrowth while restricting motor neuron formation to the ventral side (Basler et al. 1995).

TGF-βs are expressed in many embryonic tissues during morphogenetic processes. Interestingly TGF-β1 "knock-out" mice show no gross developmental abnormalities but die due to a massive infiltration of many organs by inflammatory cells at weaning (Shull et al. 1992, Kulkarni et al. 1993). The immunosuppressive function of TGF-β may result in part form the growth inhibitory effect of TGF-β on lymphoid cells. Growth inhibition is one of the most consistent activities of TGF-β and its study may uncover important

mechanisms of cell cycle arrest (Koff et al. 1993). The significance of this activity has been illustrated by introduction of TGF-β1 directly into the mammary tissue of mice (Siberstein and Daniel 1987). In these animals, TGF-β-1 inhibits the development and secretory function of mammary tissue during sexual maturation and pregnancy.

CHAPTER 2

HYPOTHESIS, RATIONALE, AND EXPERIMENTAL APPROACH

As mentioned in the introduction, human trophoblast differentiation is a complex process and so far no specific gene regulator has been found in controling this process. Also it is known that placental differentiation is abnormal in diseases of pregnancy, such as intrauterine growth restriction (IUGR), pregnancy induced hypertension (PIH), and diabetes. We have therefore postulated that there exist abnormalities in gene regulator of differentiation in human placenta. Thus our approach has been to identify normal genes regulating placental differentiation, then determine if they are abnormal in diseases of pregnancy. To reach this goal, we constructed a subtractive cDNA library between differentiating and undifferentiating trophoblast cells and found six novel genes. One of these was PL74.

We cloned and fully sequenced the PL74 cDNA. After sequence analysis of PL74, we determined that PL74 was a novel member of TGF-β superfamily and had both TGF-β-like sequence similarities (21-23%) and BMP-like sequence similarities (33-38%). Therefore we chose to study its function in both trophoblast and primitive osteoblast-like MG-63 cells. Based on these sequence similarities to both TGFβ and BMP peptides and the known functions of the peptides, we have formulated the following hypotheses:

(1) PL74 has TGF-β-like functions including inhibition of proliferation, induction of apoptosis and stimulation of matrix secretion.

(2) PL74 has BMP-like functions including the inhibition of proliferatiion and induction of differentiation.

Since primary trophoblast cell cultures do not replicate, and first trimester cell lines (HTR-8, ED27) do not differentiate or differentiate poorly, we have chosen to

perform most studies in the primitive osteoblastic cell line MG-63, which can proliferate but also show expression of a differentiated phenotype after appropriate stimuli. They are thus an appropriate model to test the hypotheses. Limited studies have also been performed in term primary cytotrophoblast cells.

CHAPTER 3

MATERIALS AND METHODS

3.1 CELLS AND CELL CULTURE

3.1.1 Human Trophoblast Cells

Human term placenta was cut up and the vessels and fibrous bits were carefully removed. Nine sequential 10 minute digestions in 0.25% trypsin-10 U/ml DNase I were carried out at 37°C. Digestions 5-9 were collected and centrifuged at 200Xg for 7 minutes. Cell pellets were combined in 10% FCS-DMEM, then resuspended. A 50 µl aliquot of well-mixed cells was mixed with 450 µl of 0.1% nigrosine and counted with a hemocytometer. Cells were plated in Corning 100 ml petri dishes at 12 x 10^6 cells/dish in 6 ml 10% FCS-DMEM medium. After 2 hours, the dishes were washed twice with DPBS, and 6 ml serum-free DMEM was added to each dish. Cytokines were added as indicated.

3.1.2 Human Osteosarcoma MG-63 cells

Biology of MG-63 cells

MG-63 was derived from an osteosarcoma by Heremans in 1978. In their first passages MG-63 cells could be distinguished from most of the primary cultures grown from tumor tissues in that cell growth was more profuse. In later passages the MG-63 cultures consisted of a morphological uniform population of fusiform cells. As soon as these cultures reached confluency, cells started to pile up into aggregates, scattered over the culture surface. The poorly differentiated cells contained a relatively large nucleus with multiple invaginations of the membrane. Electron microscopic examination of the pelleted culture cells showed rounded cells with numerous short microvilli and a few small blebs at the cell surface (Heremans et al.1978). MG-63 cells multiplied rapidly

without loss of contact inhibition. The saturation density of the confluent monolayer was high: $10^{5.3}$ cells/cm^2 compared to $10^{4.9}$ or $10^{5.0}$ for normal human fibroblasts and $10^{5.6}$ for HT-1080. Its generation time during exponential growth was 38 hours (Heremans et al. 1978).

MG-63 Cell culture

Human osteosarcoma MG-63 cells were obtained through the American Type Culture Collection (ATCC CRL-1427, Rockville MD, U.S.A.). MG-63 cells were grown in Dulbecco's modified Eagle's medium supplement with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin. The cells were cultured in an incubator with 5% CO_2 at 37^{0}C. The medium was changed twice a week.

3.2 SCREENING THE cDNA LIBRARY

3.2.1 Construction of the subtractive cDNA Library

Highly purified normal term cytotrophoblast cells prepared by trypsin-DNase I digestion were cultured in serum-free Dulbecco's modified Eagle's medium (DMEM) (Morrish et al.1987). mRNA from undifferentiated cytotrophoblast were used as the '-' pool in library construction. Equal amounts of mRNA from cytotrophoblasts stimulated by 10 ng/ml EGF for 24, 48, and 96 hours were pooled and used as the '+' pool. cDNA from both population of cells was generated from oligo d(T) cellulose prepared poly(A) + mRNA. Subtraction was then performed by the clonal deletion method of Lamar and Palmer (Lamar and Palmer, 1984) using a 50-fold excess of the '-' pool cDNA (Morrish, et al. 1996).

3.2.2 Screening the library

After differential screening, 20 genes were identified as having increased mRNA expression during differentiation by Northern analysis. Six were novel clones by homology searching in GenBank. One of these, PL74, which contained a 330bp piece of DNA, was used as a radiolabeled probe for extensive screening of the human placenta library to isolate the full-length cDNA. Human Placenta 5'-Stretch cDNA Library inλDR2 vector was obtained from Clontech (CAT.#: HL1144x). The screening of this library was carried out by following the company's protocol.

3.3 CLONING AND SEQUENCING

The full length cDNA of PL74 was subcloned into the pBluescript vector. The complete nucleotide sequence was determined by primer walking method using Applied Biosystem Automated Sequencer according to manufacturer's instruction.

3.4 CELL TRANSFECTION

3.4.1 Plasmids

Eukaryotic expression vector pcDNA3 was obtained from Invitrogen Inc. The PL74 cDNA was subcloned in 5'$\rightarrow$ 3' orientation at the 3' end of cytomegalovirus (CMV) promoter/enhancer of plasmid pcDNA3 to create sense PL74. pcDNA3 contains a G418-resistance gene for selection.

3.4.2 Transfection

MG-63 cells were transfected by LIPOFECTIN Reagent (GIBCO BRL). MG-63 cells were seeded at 1-2 x 10^5 cells per well in a 24 well plate and cultured until the cells were 40-60% confluent. 6 μg of purified plasmid DNA and 15 μl Lipofectin reagent were added in different tubes containing 100 μl serum-free medium, respectively. After 45 minutes, the two solutions were combined and incubated at room temperature for 15 minutes. 1.3 ml serum-free medium was added to the tube which contained the LIPOFECTIN Reagent -DNA complexes, and 250 μl of the complex was overlayed onto each well. After 24 hours, the DNA-containing medium was replaced with 500 μl MEM containing 10% FCS and cells incubated at 37°C in a CO_2 incubator for 48 hours. Stable clones expressing PL74 were selected by ability to grow in the presence of the antibiotic G418 (0.5 mg/ml). The selection took 3 weeks until the stable transfectants were selected.

3.5 ^{3}H-THYMIDINE UPTAKE

^{3}H-thymidine (Amersham) was added to the medium to a final concentration of 20 μCi/ml. 50 μl medium containing ^{3}H-thymidine was added to each well for 30 minutes. The cells were washed three times with DPBS buffer. Cells were detached with trypsin-EDTA (Gibco) and centrifuged at 1000 rpm for 3 minutes for collection. The cells were placed in scintillation tubes containing 5 ml scintillation solution and counted in scintillation counter.

3.6 NORTHERN BLOT

cDNA probes were prepared using the Wizard Minipreps Purification System (Promega Inc.). Inserts were run in a 1% agarose gel, excised, purified with Geneclean II (BIO 101 Inc.) and labeled with ^{32}P-dCTP using Random DNA Labeling System (BRL). Total RNA from trophoblast and MG-63 cells was prepared using RNeasy kits(QIAGEN Inc.).RNA was run on 1.2 % agarose formaldehyde denaturing gels, transferred to Nytran membranes (Schleicher and Schuell). Hybridization was performed at 42° C for 20 hours in a buffer containing 5 x PIPES, 50% formamide, 5 x Denhardt's solution, 1% SDS, 0.1% sodium pyrophosphate and 0.1% salmon sperm DNA. The blots were washed with 1x SSC-0.4% SDS for 15 minutes at room temperature, 0.5 x SSC-0.4% SDS for 15 minutes at room temperature and 0.1x SSC-0.4% SDS for 45 minutes at 65°C. Equality of lane loading was determined using either the 18 S ribosomal RNA or G6PDH as a probe.

3.7 TUNEL ASSAY

The *in situ* Cell Death Detection Kit was purchased from Boehringer Mannheim (Cat. No.1684809). Terminal deoxynucleotidyl transferase (TdT) has been used in this kit for the incorporation of labeled nucleotides to DNA strand breaks *in situ*. The tailing reaction using TdT, is called TUNEL (TdT-mediated dUTP nick end labeling) technique, and has been used to detect individual apoptotic cells. Air dried cell samples were fixed in cold 1:1 methanol and acetone solution for 10 minutes at room temperature, washed 3 times with PBS, and incubated in 50 µl TUNEL reaction mixture. The cells were incubated for 60 minutes at 37°C, followed by 3 washes with PBS. 50 µl Converter-AP was added to

each well and the cells incubated for 30 minutes at 37°C. Then 50 µl substrate solution (X-phosphate/BCIP) was added to the cells. After 5-10 mintues at room temperature, reaction product had developed and the cells were analyzed under the light microscope. Photographs of cell cultures were used to count apoptotic cells.

3.8 STATISTICS

3.8.1 ^{3}H-thymidine uptake

Six separate transfections were performed and in each there were 3 control and 3 PL74 transfected wells in a 24-well plate. Comparison between control and PL74 was by 2-tailed student's t-test.

3.8.2 Cell number

Four separate experiments were done with triplicate dishes for each of control and PL74 transfected cells. Comparison between control and PL74 was by 2-tailed student's t-test.

3.8.3 TUNEL assay

Three separate transfections were performed. Analysis of each experiment was by photographing representative microscope fields. Three to four microscope fields were randomly chosen and PL74-transfected cultures and TUNEL-labeled and unlabeled (background) cells counted. Comparison of labeled and unlabeled cell numbers was by 2-tailed student's t-test.

CHAPTER 4

RESULTS

4.1 CLONING AND CHARACTERIZATION OF PL74

In an effort to isolate genetic regulators of human trophoblast differentiation, we constructed a subtractive cDNA library using undifferentiated cytotrophoblast and differentiating cytotrophoblast (Morrish et al. 1996). Six novel clones whose mRNA increased in expression during cytotrophoblast differentiation were isolated. One of those, termed PL74, had a 300 bp insert. In an attempt to get the full-length cDNA sequence of PL74, we screened a human placenta 5'- stretch cDNA library (Clontech, Inc.) using this 300 bp insert as a radiolabeled probe. Several clones were isolated and analyzed by restriction enzyme digestion. One of these which contained a bigger insert was sequenced. The 1180-bp sequence (GenBank accession number U51731) contained an open reading frame (ORF) of 308 amino acids with an estimated molecular mass of 34.2 kDa (Fig. 4).

Two potential ATG translation start sites were present in the PL74 RNA. Because the second, but not the first, methionine is immediately followed by a hydrophobic 19 amino acids long signal sequence, it suggests that the second methionine is more likely to correspond to the start site of translation. The 3' untranslated region contains four copies of the instability motif (ATTTA) that is present in many cytokine genes and is associated with post transcriptional regulation of the mRNA. The ORF also contains a potential N-linked glycosylation site at amino acid 70.

The open reading frame includes a prodomain of 186 amino acids and a mature domain of 112 amino acids. There is a consensus RXXRA/S cleavage signal at amino acids 193-196 used for the formation of the mature form of the protein. There are no

other RXXR sequences that are followed immediately by an alanine or serine. Processed TGF-β superfamily proteins all contain a highly conserved seven-cysteine domain spanning about 80 aa that forms the cysteine knot, a hallmark of this superfamily (Kingsley 1994). The mature carboxy terminal domain of PL74 contains all seven conserved cysteines and their spacing, like all other proteins of the family. This indicates that PL74 is a TGF-β superfamily member.

Sequence alignments of the seven-cysteine domain are used to classify proteins within the superfamily into subfamilies (Massague 1990), with the major subfamilies being TGF-β, BMP and activin (Table 1). PL74 is most closely related to the BMP subfamily, with 33-38% identity with different BMPs. In the BMP subfamily, PL74 shows most similarities with the 60A group, which includes BMP-8 (op-2), BMP-7 (op-1), BMP-6, BMP-5 and 60A. The similarities of those factors with PL74 are: BMP-8 38%, BMP-6 35%, BMP-7 34%, BMP-5 33%, 60A 35%, respectively. Also PL74 has 31% similarities with GDF-7 and GDF-1. However, the conserved carboxy terminal region of PL74 is 5 amino acids shorter than that of BMP members and lacks several conserved amino acids, suggesting that PL74 does not directly belong to the BMP subfamily.

Despite its overall similarity to BMP subfamily, PL74 has several features that are more consistent with the TGF-β 1-3 and inhibin βA and βB peptides. They all have two extra cysteines at the start of the mature protein. Significantly, there is a cysteine exactly 5 amino acids downstream of the RXXRA/S site that is conserved in both PL74 and TGF-β 1-3. This may play a role in the appropriate processing of the mature protein. PL74 also shared 21-23% identical residues with TGF-β 1-3.

As previously suggested (Massague 1990; Kingsley 1994), members of the same subfamily have identities of 40-95%. Members of different families bear 15-30% identity to each other. Overall PL74 shows 15-38% identity to the other TGF-β superfamily members, indicating that PL74 is a divergent member of this family and likely do not belong to any known group.

Figure 4. Nucleotide and amino acid sequence of PL74. The start codon (■), conserved cysteines (•) and stop codon (*) are indicated. The putative leading signal sequence (_ _ _), consensus N-linked glycosylation site(- - -) and the putative proteolytic cleavage site (_____) are underlined.

```
                                                    1 cgcggcacagcc
   13 atgcccgggcaagaactcaggacgctgaatggctctcagatgctc
      M  P  G  Q  E  L  R  T  L  N  G  S  Q  M  L
   58 ctggtgttgctggtgctctcgtggctgccgcatgggggcgccctg
      L  V  L  L  V  L  S  W  L  P  H  G  G  A  L
  103 tctctggccgaggcgagccgcgcaagtttcccgggaccctcagag
      S  L  A  E  A  S  R  A  S  F  P  G  P  S  E
  148 ttgcactccgaagactccagattccgagagttgcggaaacgctac
      L  H  S  E  D  S  R  F  R  E  L  R  K  R  Y
  193 gaggacctgctaaccaggctgcgggccaaccagagctgggaagat
      E  D  L  L  T  R  L  R  A  N  Q  S  W  E  D
  238 tcgaacaccgacctcgtcccggcccctgcagtccggatactcacg
      S  N  T  D  L  V  P  A  P  A  V  R  I  L  T
  283 ccagaagtgcggctgggatccggcggccacctgcacctgcgtatc
      P  E  V  R  L  G  S  G  G  H  L  H  L  R  I
  328 tctcgggccgcccttcctgaggggctccccgagaactcccgcctt
      S  R  A  A  L  P  E  G  L  P  E  N  S  R  L
  373 caccgggctctgttccggctgtccccgacggcgtcaaggtcgtgg
      H  R  A  L  F  R  L  S  P  T  A  S  R  S  W
  418 gacgtgacacgaccgctgcggcgtcagctcagccttgcaagaccc
      D  V  T  R  P  L  R  R  Q  L  S  L  A  R  P
  463 caggcgcccgcgctgcacctgcgactgtcgccgccgccgtcgcag
      Q  A  P  A  L  H  L  R  L  S  P  P  P  S  Q
  508 tcggaccaactgctggcagaatcttcgtccgcacggccccagctg
      S  D  Q  L  L  A  E  S  S  S  A  R  P  Q  L
  553 gagttgcacttgcggccgcaagccgccaggggggcgccgcagagcg
      E  L  H  L  R  P  Q  A  A  R  G  R  R  R  A
  598 cgtgcgcgcaacggggaccactgtccgctcgggcccgggcgttgc
      R  A  R  N  G  D  H  C  P  L  G  P  G  R  C
  643 tgccgtctgcacacggtccgcgcgtcgctggaagacctgggctgg
      C  R  L  H  T  V  R  A  S  L  E  D  L  G  W
  688 gccgattgggtgctgtcgccacgggaggtgcaagtgaccatgtgc
      A  D  W  V  L  S  P  R  E  V  Q  V  T  M  C
  733 atcggcgcgtgcccgagccagttccgggcggcaaacatgcacgcg
      I  G  A  C  P  S  Q  F  R  A  A  N  M  H  A
  778 cagatcaagacgagcctgcaccgcctgaagcccgacacggtgcca
      Q  I  K  T  S  L  H  R  L  K  P  D  T  V  P
  823 gcgccctgctgcgtgcccgccagctacaatcccatggtgctcatt
      A  P  C  C  V  P  A  S  Y  N  P  M  V  L  I
  868 caaaagaccgacaccggggtgtcgctccagacctatgatgacttg
      Q  K  T  D  T  G  V  S  L  Q  T  Y  D  D  L
  913 ttagccaaagactgccactgcatatgagcagtcctggtccttcca
      L  A  K  D  C  H  C  I  *
  958 ctgtgcacctgcgcgggggaggcgacctcagttgtcctgccctgt
 1003 ggaatgggctcaaggttcctgaaacacccgattcctgcccaaaca
 1048 gctgtatttatataagtctgttatttattattaatttattggggt
 1093 gaccttcttggggactcgggggctggtctgatggaactgtgtatt
 1138 tatttaaaactctggtgataaaaataaagctgtctgaactgtt
```

4.2 PL74 mRNA EXPRESSION AND BIODISTRIBUTION

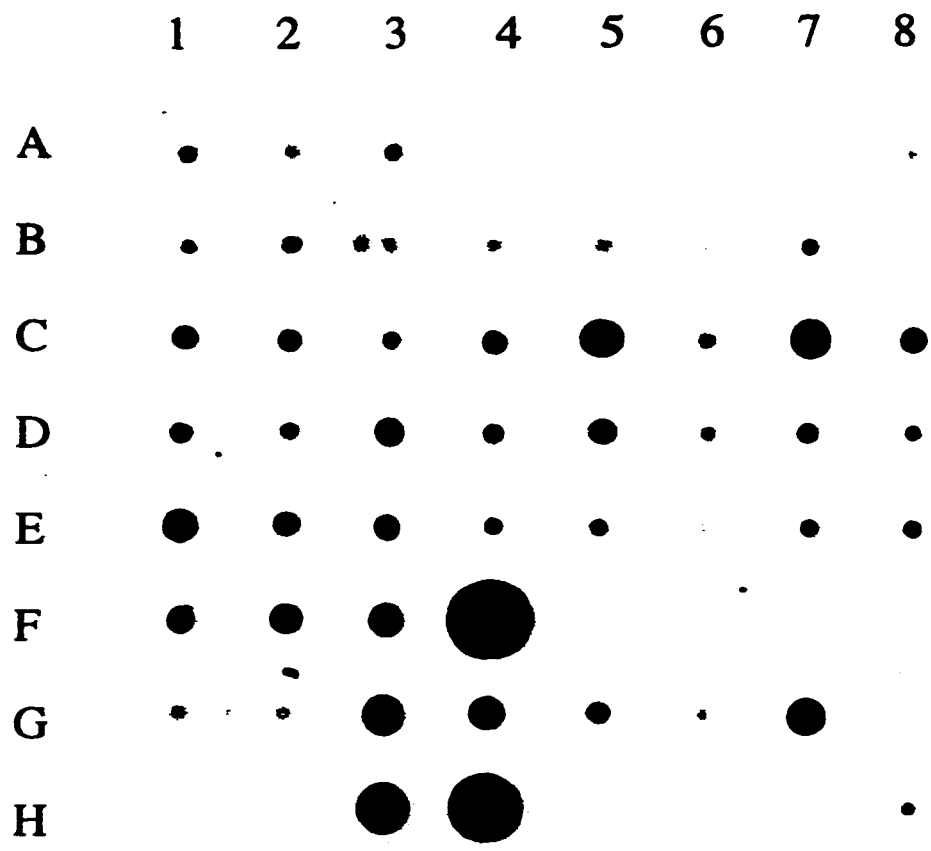
In order to determine the pattern of expression of the PL74 gene, a dot blot hybridization was performed with RNA derived from 43 human adult tissues and 7 fetal tissues (Clontech: RNA master Blot # 7770-1) (Fig. 5), and Northern blot analysis using 14 human cancer cell lines (a kind gift from Dr. R. Godbout) (Fig. 6). The signal decoded a transcript of 1.5 kb was detected by Northern blotting. The results obtained in these experiments revealed that PL74 was more highly expressed in placenta, the tissue from which it was cloned, than in any of the other human tissues surveyed. PL74 was also expressed at lower level in prostate, bladder, kidney, fetal lung, fetal kidney and fetal liver. The rest of human tissues had hardly detectable the PL74 mRNA by RNA dot blot. Apart from a nonspecific artifact, it is unclear why *E. Coli* rRNA and DNA showed hybridization with the PL74 probe. This suggests there were homologous sequences of PL74 in *E. Coli.*

In contrast to another trophoblast differentiation associated gene, PL48 (Dakour et al. 1997), PL74 mRNA was expressed in 10 out of 14 of the cancer cell lines surveyed. PL74 mRNA was expressed in first trimester extravillous cytotrophoblast (EVT) cell line HTR-8, and in the human choriocarcinoma cell lines BeWo, JAr and JEG-3, as well as in the human osteosarcoma MG-63 cells at low level (Fig. 7).

Thus, PL74 mRNA was expressed in human placenta to a much higher degree than other human tissues, and it is also expressed in some, but not all cancer cell lines.

Figure 5. Biodistribution of PL74 mRNA in normal human tissues. The diagram below shows the type and position of poly A^+ RNAs and controls dotted on the positively charged nylon membranes. Twelve housekeeping genes are used for normalization (CLONTECH, RNA Master Blots #7770-1). This experiment was carried out once.

	1	2	3	4	5	6	7	8
A	whole brain	amygdala	caudate nucleus	cere-bellum	cerebral cortex	frontal lobe	hippo-campus	medulla oblongata
B	occipital lobe	putamen	substantia nigra	temporal lobe	thalamus	sub-thalamic nucleus	spinal cord	
C	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
D	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
E	kidney	liver	small intestine	spleen	thymus	peripheral leukocyte	lymph node	bone marrow
F	appendix	lung	trachea	placenta				
G	fetal brain	fetal heart	fetal kidney	fetal liver	fetal spleen	fetal thymus	fetal lung	
H	yeast total RNA 100 ng	yeast tRNA 100 ng	*E. coli* rRNA 100 ng	*E. coli* DNA 100 ng	Poly r(A) 100 ng	human C_0t 1 DNA 100 ng	human DNA 100 ng	human DNA 500 ng


1 2 3 4 5 6 7 8
A
B
C
D
E
F
G
H

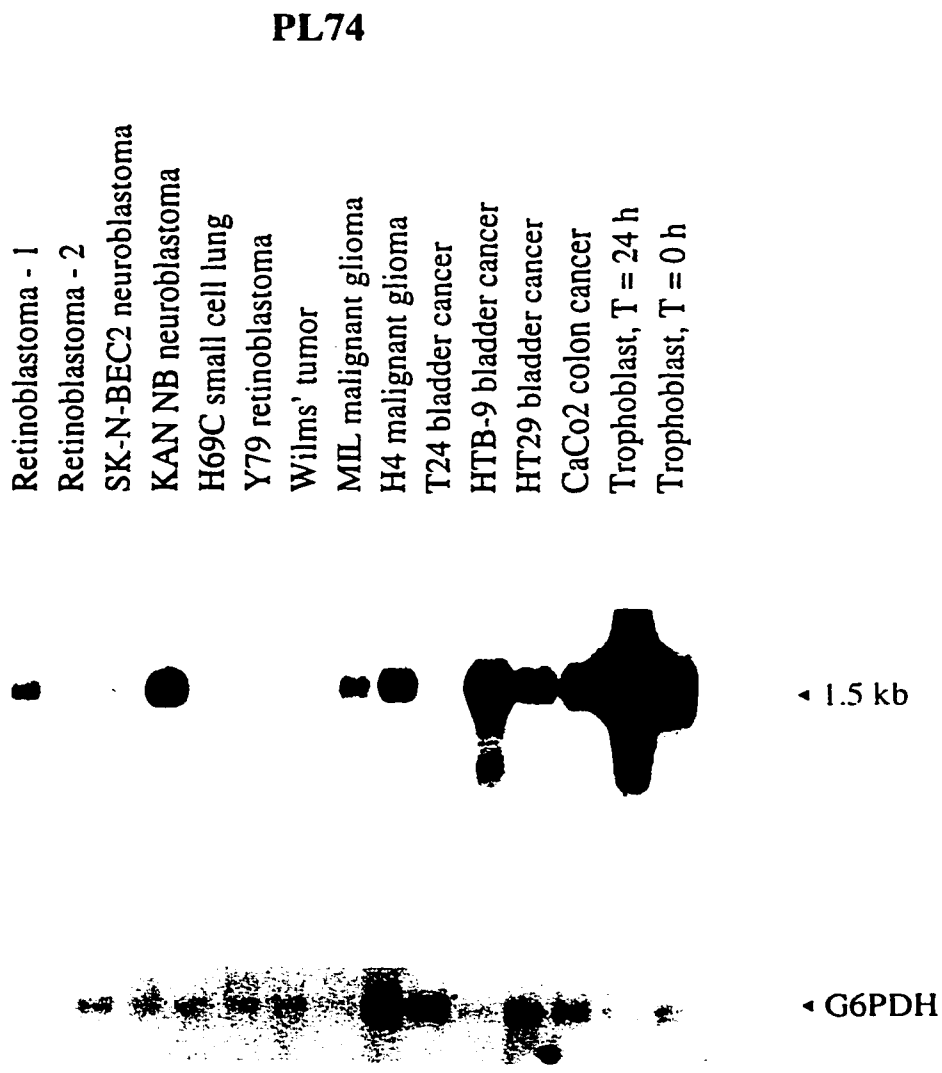


Figure 6. Expression of PL74 in cancer cell lines.

Northern blot analysis was performed using poly A^+ RNA (approximate 1 μg per line) and hybridized with ^{32}P labeled PL74 cDNA and G6PDH. This experiment was carried out once.

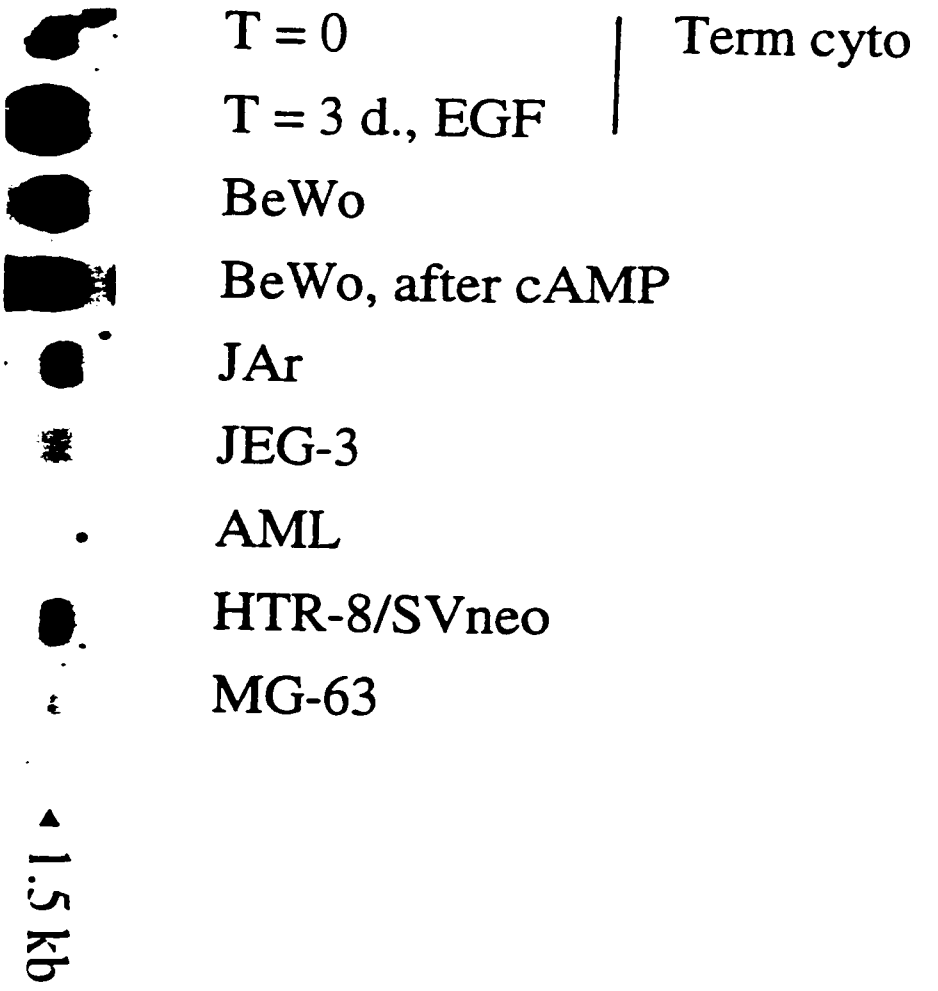


Figure 7. Expression of PL74 mRNA in human choriocarcinoma cell lines (BeWo, JAr and JEG-3), human EVT cells (HTR-8) and human osteosarcoma MG-63 cells.

10 μg total RNA per line was loaded. This experiment was carried out once.

4.3 PL74 ASSOCIATED WITH TROPHOBLAS DIFFERENTIATION

PL74 mRNA expression was markedly increased in the blot containing mRNA from cytotrophoblast cells undergoing spontaneous or EGF- induced differentiation. Time-course experiment showed that this increase was very early, as early as 2 hours from the time zero, suggesting that PL74 behaved as an early response gene (Fig. 8 A). Cells induced to differentiate by EGF had slightly greater PL74 mRNA expression than cells undergoing spontaneous differentiation, demonstrating that EGF may be an early or weak regulator of PL74. After 8 hours, the expression of PL74 mRNA reached a peak. The attached trophoblast cells showed no difference of PL74 expression compared with the suspension cells, indicating that signal transduction associated with attachment was not responsible for PL74 expression (Fig. 8 B).

To determine if PL74 was also expressed in other differentiating systems, we studied differentiating human keratinocytes (Fig. 9). PL74 mRNA expression was increased 2.5-fold (desitometry analysis) during keratinocyte differentiation, suggesting that PL74 may be more generally linked to the differentiation processes occurring in tissues other than the placenta.

Thus, PL74 expression increases during both trophoblast and keratinocyte differentiation.

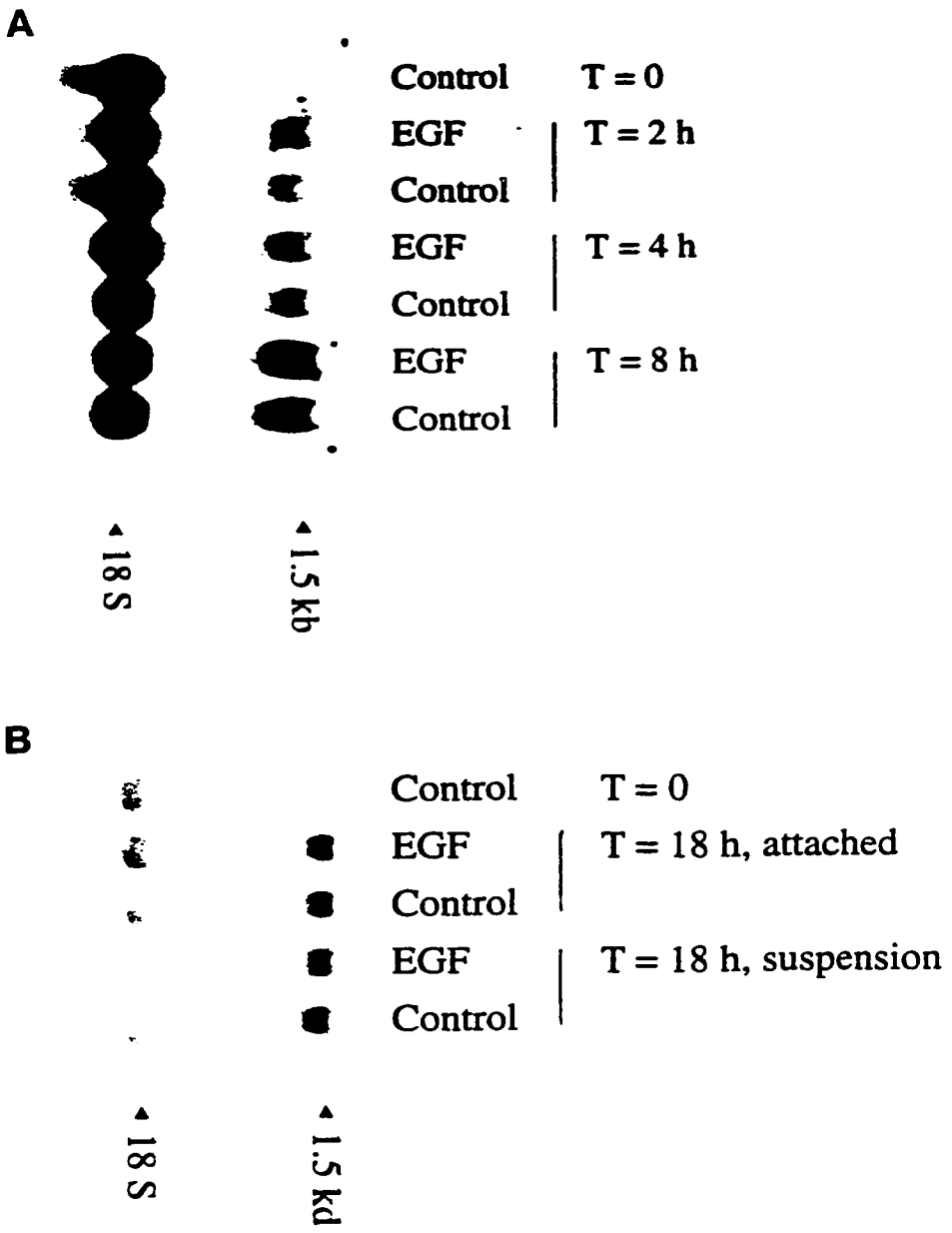


Figure 8. PL74 associated with trophoblast differentiation.

A. 8 hours time-course experiment.

B. Comparison between attached and suspension trophoblast cells.

Total RNA (10μg per lane) was used for Northern blot analysis. The blot was hybridized by radiolabeled PL74 cDNA and 18S rRNA oligonucleotide probes. These experiments were carried out three times with similar results.

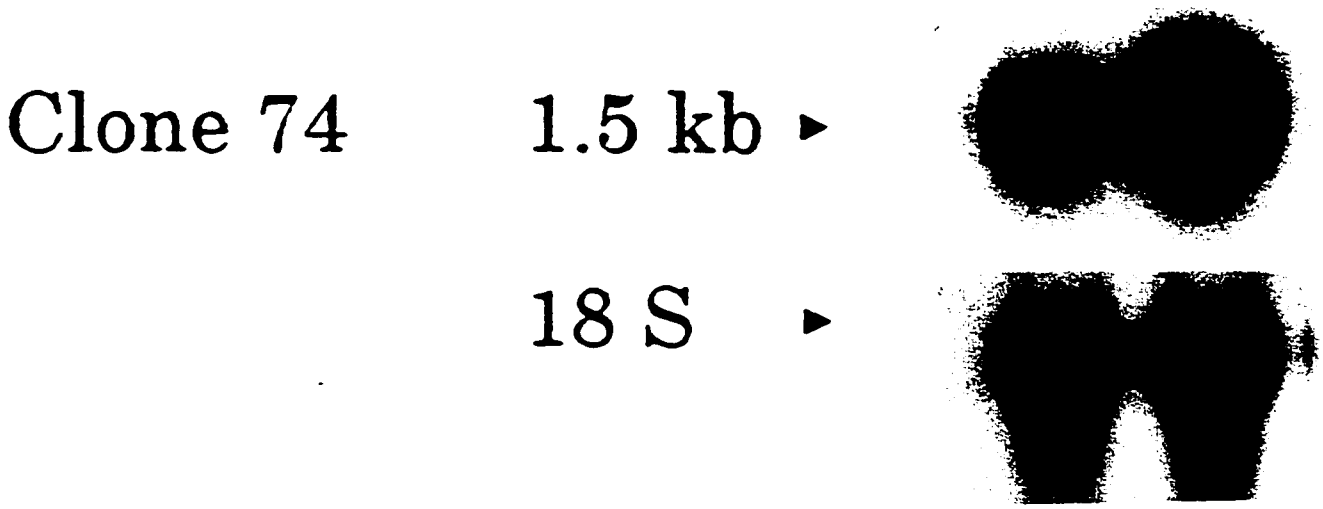


Figure 9. Expression of PL74 mRNA in undifferentiated and differentiated human keratinocyte cells.

15 μg of total RNA per line was loaded. This experiment was carried out twice with the same result.

4.4 PL74 INDUCED HUMAN OSTEOSARCOMA MG-63 CELLS DIFFERENTIATION

Since PL74 was most closely related to BMP family members, we chose human osteoblast-like MG-63 cells to study if PL74 could regulate bone cell function. Full-length cDNA of PL74 was subcloned into the expression vector pcDNA3 and transfected into MG-63 cells with Lipofectin. Northern analyses were used to measure the expression of PL74 mRNA. In stable transfectants of MG-63 cells, PL74 mRNA was overexpressed (Fig.10). In MG-63 cells stably transfected with PL74, human collagen I α and osteocalcin, both differentiation markers of MG-63 cells, showed increased mRNA expression (Fig.10). These results indicated that overexpression of PL74 mRNA induced MG-63 cells differentiation, suggesting that PL74 is a novel regulator of MG-63 cell differentiation. This result also are consistent with the previous results of PL74 we obtained from other differentiating cell systems like human cytotrophoblast and keratinocyte, although direct studies of function of PL74 on these cells have not been done yet.

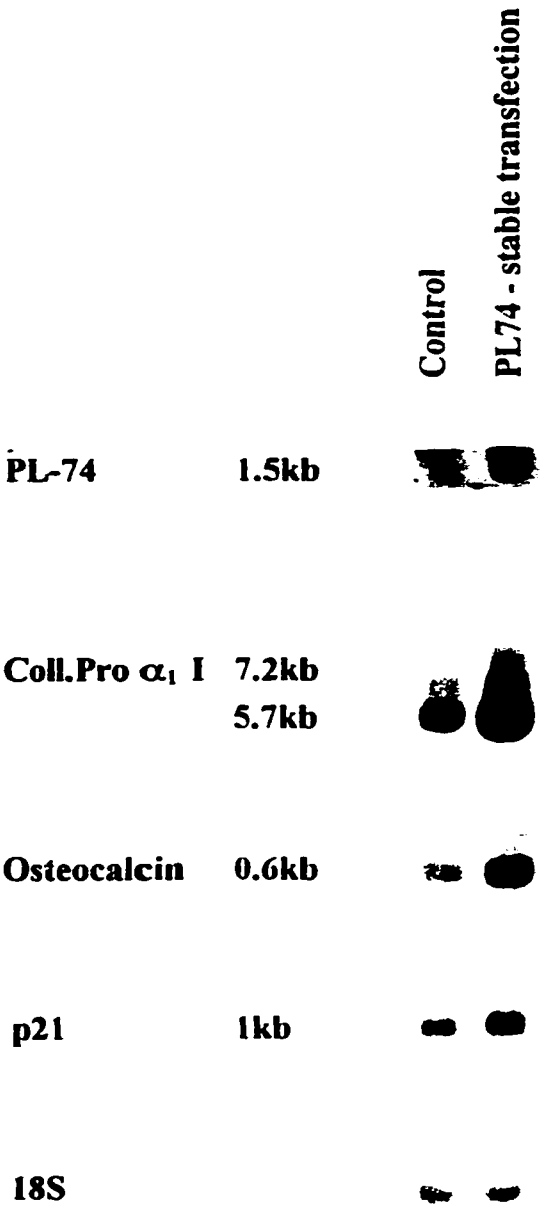


Figure 10. Northern analysis of stably transfected and untransfected MG-63 cells with different cDNA probes.

Five different cDNA probes were hybridized to the same blot containing mRNA from stably transfected MG-63 cell and untransfected cells (control). After hybridization, the signal was removed by boiling for 10 minutes in a solution containing 0.1% SDS, 2 mM EDTA and 1 mM Tris-HCl pH-8, and the blot was ready for reprobing. These experiments were carried out twice with the same results.

4.5 PL74 INHIBITED MG-63 CELL PROLIFERATION

Since PL74 is a TGF-β superfamily member, it is interesting to ask if PL74 has the growth inhibitory ability in MG-63 cells observed with other TGF-β family members. We therefore performed transient transfections of sense PL74 to determine if overexpression of PL74 would inhibit MG63 cell proliferation by measuring both ^{3}H-thymidine uptake and cell number. Six ^{3}H-thymidine uptake experiments were performed after 1 day transient transfection of pcDNA3-PL74 in MG63 cells, using transfection of pcDNA3 alone as control. The results are shown in Table 2 A. PL74 overexpression resulted in a 12% decrease in ^{3}H-thymidine uptake ($P<0.01$). Using the same approach, four experiments were performed to measure cell numbers by cell counting. The results are shown in Table 2 B. PL74 inhibited MG-63 cells proliferation by decreasing cell numbers 21% compared with control ($P<0.05$).

Also, Northern blot analysis of MG-63 cells stably transfected with PL74 showed an increase in cyclin D kinase inhibitor p21 (WAF1/Cip1) mRNA (Fig.10), indicating that the inhibition of PL74 may act through the p21 pathway.

Thus, PL74 appears to have TGFβ-like inhibitory activities on MG-63 cell proliferation, and also like TGF-β1(Datto et al.1995), appears to mediate this growth inhibition action through the p21 signal transduction pathway.

Cell Proliferation Indices
Transient Transfections of PL74 in MG-63 Cells

A. ^{3}H-Thymidine uptake (cpm ± SEM)

No.	Control	PL74	% Reduction
1	1166 ± 24	1024 ± 159	12.2
2	1093 ± 100	856 ± 99	21.7
3	8901 ± 402	8114 ± 315	8.8
4	8732 ± 670	8101 ± 567	7.2
5	8246 ± 251	7252 ± 237	12.1
6	8301 ± 506	7408 ± 266	10.8

Mean ± SEM: 12.1 ± 2.1
($P < 0.01$)

B. Cell Number

No.	Control	PL74	% Reduction
1	160.0 ± 4.3	120.0 ± 4.5	25.0
2	118.0 ± 10.6	98.7 ± 6.1	16.4
3	152.7 ± 17.5	114.3 ± 17.5	25.5
4	117.3 ± 1.8	96.7 ± 8.5	17.6

Mean ± SEM: 21.2 ± 2.4
($P<0.05$)

Table 2. Cell Proliferation Indices of Transient Transfection in MG-63 Cells.

A. ^{3}H-Thymidine uptake. Six expriments were performed and the data were analysed by t-test.

B. Cell Number. Four experiment were performed and the data were analysed by t-test

4.6 PL74 INDUCED MG-63 CELL APOPTOSIS

Because PL74 caused growth arrest in MG-63 cells, we wished tc determine if apoptosis was also induced. We therefore performed TUNEL (TdT-mediated nick end labeling) analysis to assess apoptosis in MG-63 cells transiently transfected with pcDNA3-PL74. Control transfections were with pcDNA3 vector alone. TUNEL measurements showed that transfection with PL74 significantly induced MG-63 cell apoptosis (Fig.11 a.b). Approximately six-fold more TUNEL positive cells were found in PL74 transfected MG-63 cells than the control ($p<0.05$-$p<0.0001$) (Table 3). Interestingly, the morphology of MG-63 cells stably transfected with PL74 showed rounding up of cells, and loss of cell processes. Also, these cells showed a reduction in cell number after 10 days of culture. At 15 days, all the PL74 stably transfected MG-63 cells had died but the control cells still had normal morphology and growth (Fig. 11 c.d). These data indicate that PL74 induced apoptosis by a gradual process and depended on PL74 expression over time.

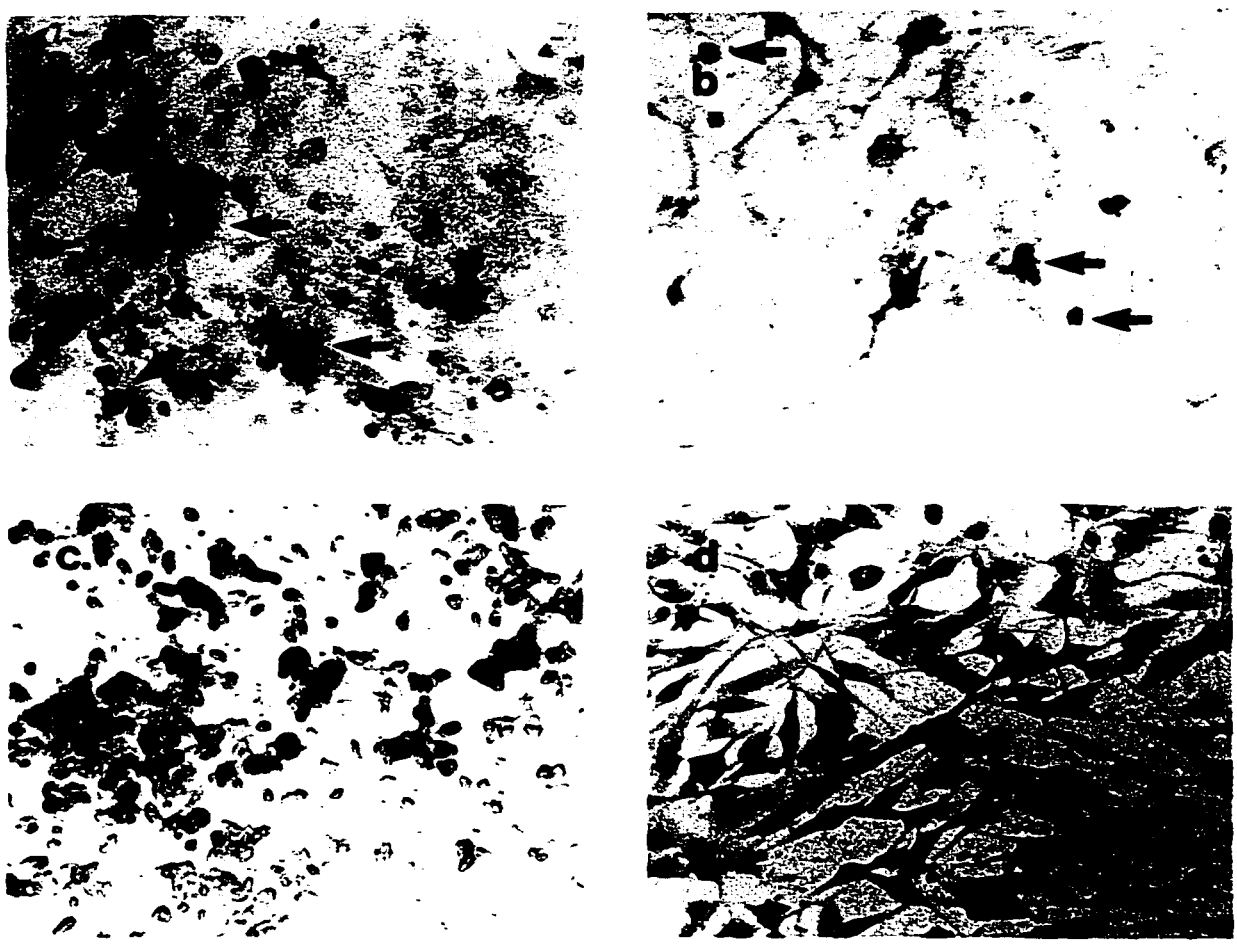


Figure 11. Overexpression of PL74 induced MG-63 cell apoptosis.

a. Transient transfection of pCDNA3-PL74 in MG-63 cells, 3 days after the transfection.

b. Transient transfection of pCDNA3 along in MG-63 cells as control, 3 days after the transfection.

c. Stably transfected MG-63 cells with pCDNA3-PL74, in 15 days of culture.

d. Untransfected MG-63 cells in 15 days of culture, as control.

These experiments were carried out three times with the similar results.

PL74: Induction of Apoptosis

Expt. No.	No. of Microscope Fields		No. Cells* Background	No. Cells Positive	% TUNEL Positive	p value
1	4	PL74	437±84	84±13	19.4±1.0	<0.0001
		Control	808±40	24±3	3.0±0.3	
2	3	PL74	602±75	80±34	19.0±4.3	<0.05
		Control	1027±50	19±4	1.8±0.4	
3	3	PL74	387±33	73±6	18.8±0.8	<0.005
		Control	590±76	21±3	3.6±0.8	

Table 3. TUNEL analysis of MG-63 cell apoptosis.

Apoptosis of MG-63 was induced by transient transfection of sense PL74.

Three experiments were carried out and the data was analysed by t-test.

4.7 CYTOKINE REGULATION OF PL74 EXPRESSION IN DIFFERENT CELL SYSTEMS

To determine whether cytokines were involved in regulating the expression of PL74, six cytokines (EGF, GMCSF, IGF-1, IFNγ, TGFβ1 and TNFα) were used to treat the cells of two different systems, human term placental trophoblast and human osteosarcoma MG-63. These cytokines were chosen because of their known proliferative or differentiative actions on trophoblast or MG-63 cells. The amount of cytokine used was 10 ng/ml of each of EGF, IGF-1, TGF-β, TNFα and 100 unit/ml of IFNγ and GMCSF.

In human trophoblast, prior studies have shown that the growth factors EGF, GM-CSF and CSF-1 induce, and TGFβ1 inhibits, human trophoblast differentiation (Morrish et al 1987, 1991; Garcia-Lloret et al. 1994). Work by Yui et al. (1994) and Garcia-Lloret et al. (1996) demonstrated that TNFα and IFNγ induce apoptosis of both mononucleus and multinucleus trophoblast in culture, and that EGF could block these effects. These data suggest that EGF, GMCSF, CSF-1 (among many other factors) are involved in inducing formation of the syncytium, counterbalanced by TGFβ1 acting as a negative feedback regulator (Morrish, 1998).

Our results (Fig. 12) showed that EGF and IFNγ induced a slightly increased expression of PL74 mRNA in cytotrophoblast at 3-6 hours of culture. TNFα and TGFβ1 did not show any induction of PL74 compared to the control. By 24 hours of culture, the cells had begun to differentiate spontaneously and this resulted in

maximal spontaneous expression of PL74. Thus, no cytokine effect could be determined at 24 hours.

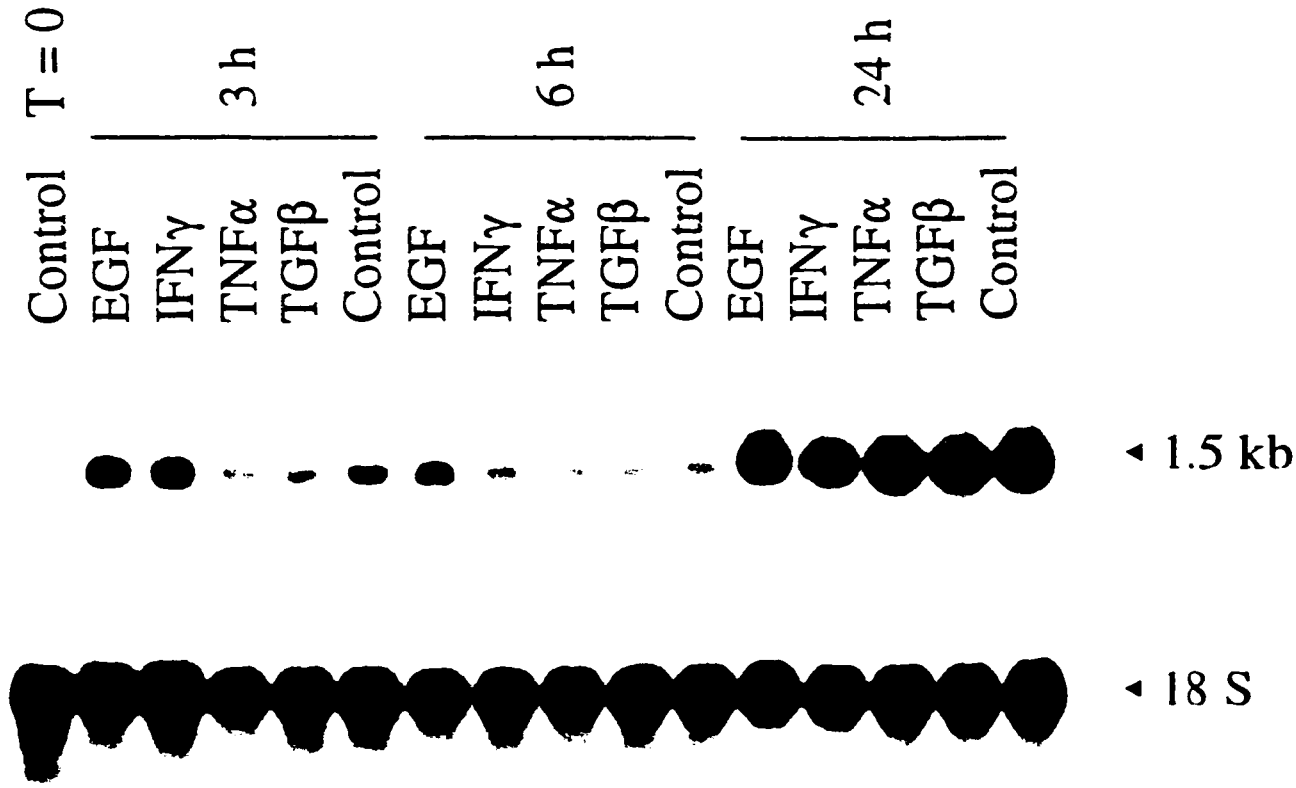
In contrast, by 3 days of culture, expression of PL74 mRNA in MG-63 cells was markedly upregulated by IFNγ and TGFβ1, but not by EGF, GM-CSF, IGF-1, or TNFα (Fig.13).

Figure 12. Cytokine regulation of PL74 expression in trophoblast cells.

A. Northern analysis. 10 μg of total RNA per line was loaded. The amount of cytokine used was 10 ng/ml of each of EGF, IGF-1, TGF-β, TNFα and 100 unit/ml of IFNγ and GMCSF.

B. Densitometry result (density of PL74 / density of 18S).

This experiment was carried out three times with similar results.


T = 0
3 h
6 h
24 h
Control
EGF
IFNγ
TNFα
TGFβ
Control
EGF
IFNγ
TNFα
TGFβ
Control
EGF
IFNγ
TNFα
TGFβ
Control
1.5 kb
18 S

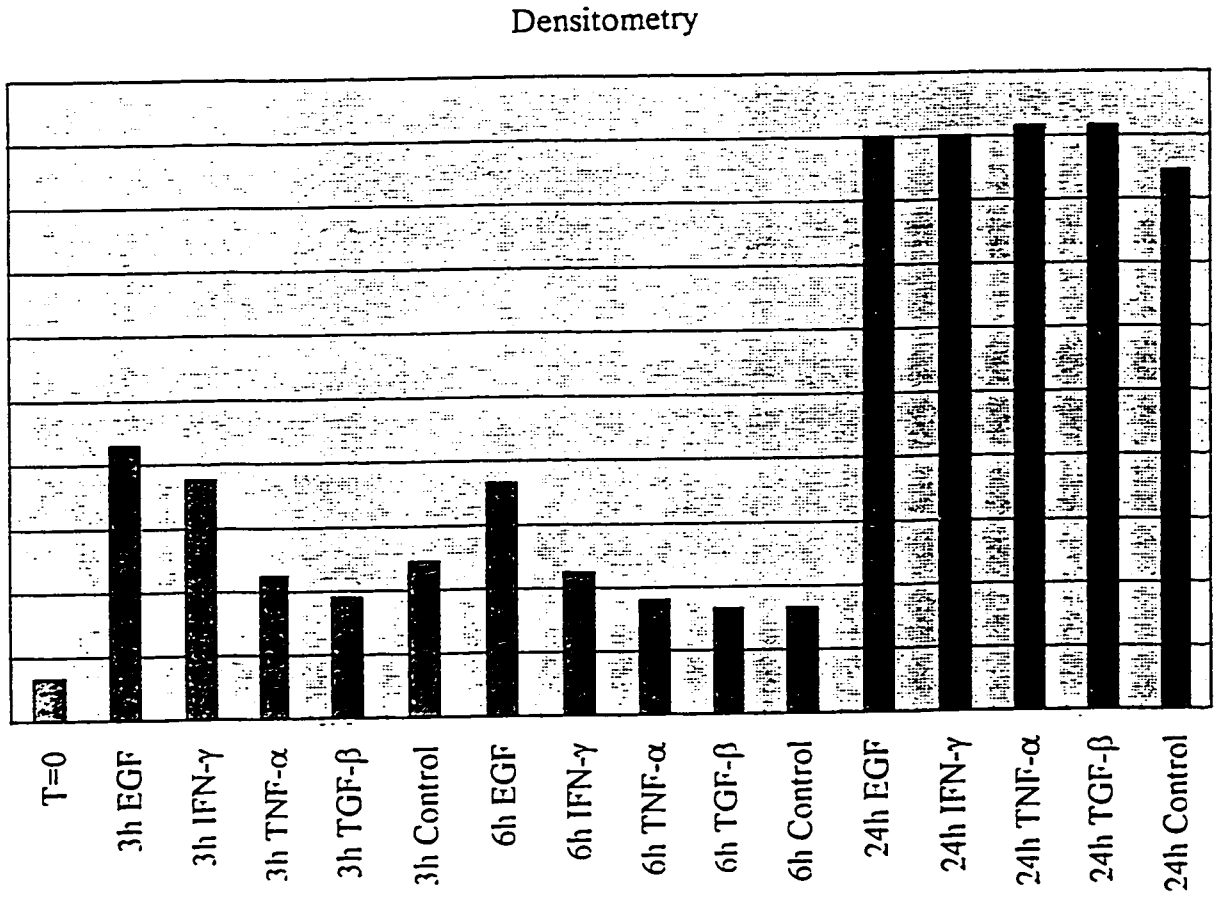
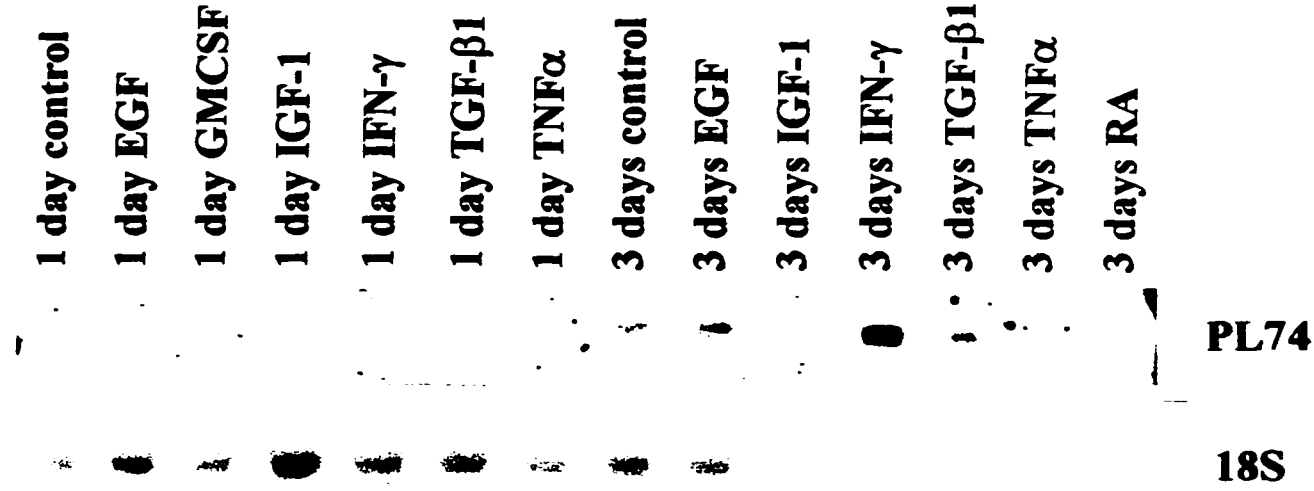

Densitometry
T=0
3h EGF
3h IFN-γ
3h TNF-α
3h TGF-β
3h Control
6h EGF
6h IFN-γ
6h TNF-α
6h TGF-β
6h Control
24h EGF
24h IFN-γ
24h TNF-α
24h TGF-β
24h Control

Figure 13. Cytokine regulation of PL74 expression in MG-63 cells.

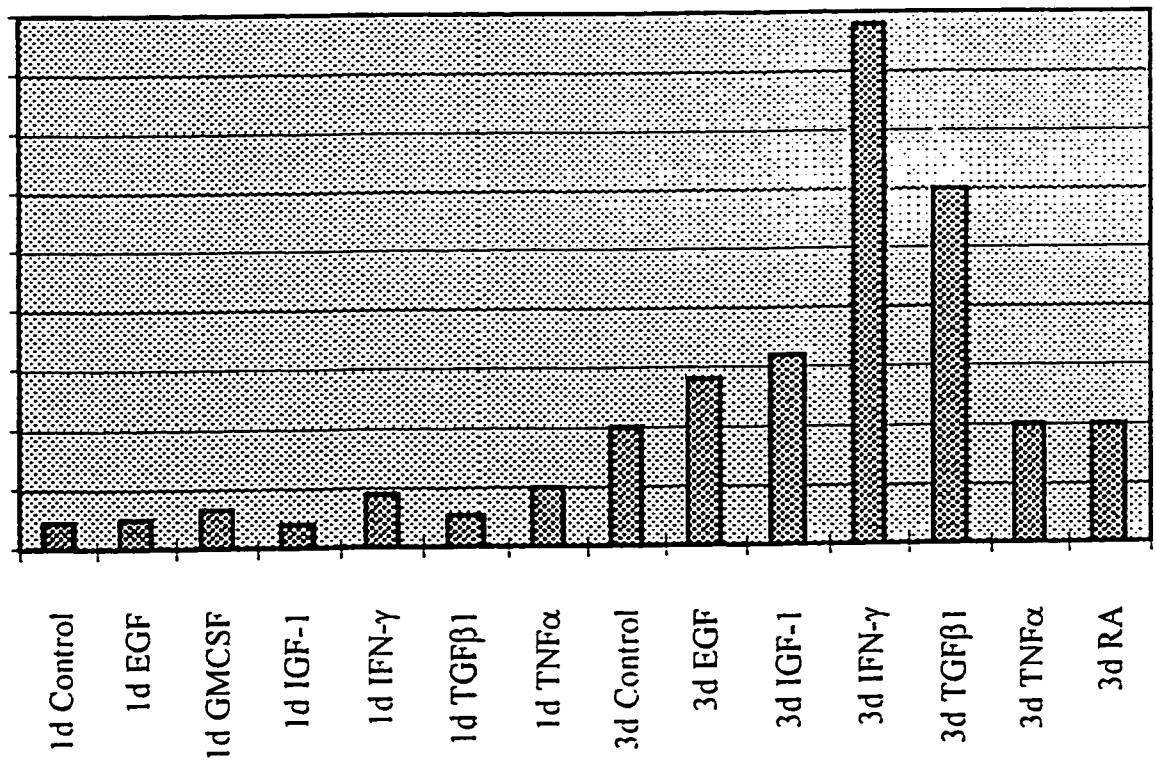
A. Northern analysis. 10 μg of total RNA per line was loaded.

B. Densitometry result (density of PL74 / density of 18s).

This experiment was carried out three times with similar results.



Densitometry



4.8 EXPRESSION OF PL74 IN PIH PLACENTA

Since some diseases of pregnancy are related to abnormal placental development, it is noteworthy to know if PL74 has abnormal expression in these abnormal placentas. Northern analyses were used to determine PL74 mRNA expression between normal and PIH patients term placentas. The average mRNA level of PL74 in 9 PIH placentas and 11 normal placentas showed no significant difference. At least in one case, PL74 mRNA expression significantly increased in PIH placenta samples comparing with the normal sample (Fig.15). This result indicated that the abnormal expression of PL74 in PIH placenta may be associated with abnormal trophoblast differentiation occurring in PIH placenta, and PL74 was associated with or even controlled this abnormal placental development.

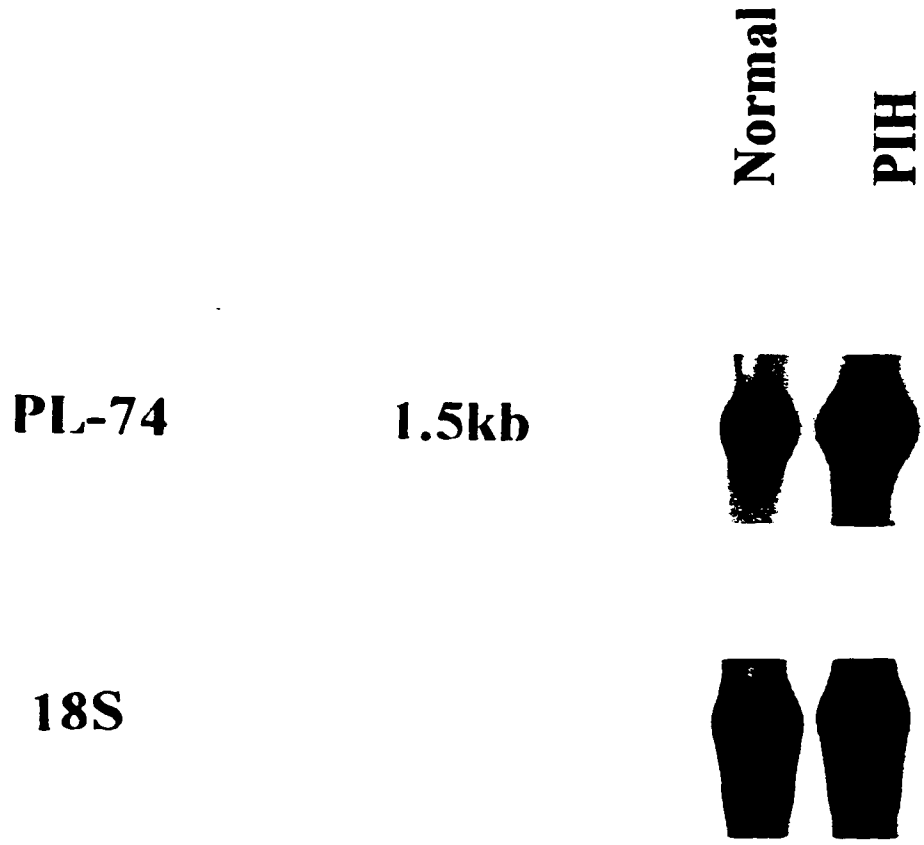


Figure 14. Expression of PL74 mRNA in normal and PIH placenta tissues.

10 μg of total RNA per line was performed. This experiment was carried out once.

CHAPTER 5

DISCUSSION

5.1 PL74 AND OTHER SIMILAR GENES PUBLISHED: STRUCTURE AND FUNCTION

In 1996, we first reported a partial sequence of PL74 (Morrish, et al. 1996). While the current work was in progress and the manuscript in preparation, five other groups cloned and published essentially the same gene (Bootcov et al. 1997; Hromas et al. 1997; Lawton et al.1997; Yokoyama-Kobayashi et al. 1997; Paralkar et al.1998). These other groups have named the gene PLAB, MIC-1, PDF, PTGFβ, or H00269. The GenBank Accession numbers are: AF019770, AF003934, AB000584, U88323, AF 008303. The sequences submitted were almost identical with the sequence that we independently determined for the PL74 mRNA. The following differences at the amino acid level were observed: (1) The 121th residue of PL74 is N, of which GenBank AF019770, AF003934, and AF008303 is A. (2) The 121th residue of PL74 is N, the 52th is S, of which GenBank AB00584 is A, and T. (3) The 121th residue of PL74 is N, the 13th is L, the 206th is H, the 273th is V, of which GenBank U88323 is A, V, D and E.

Only the 121th amino acid of PL74 is N in comparision with other sequences of which are all A. Therefore we sequenced the area three times and confirmed that our sequence is correct.

The five other groups who cloned the PL74 gene used different cell systems to investigate its functions. Bootcov et al. (1997) cloned MIC-1 from myelomonocytic cells. Expression of MCI-1 mRNA in monocytoid cells is up-regulated by a variety of stimuli associated with activation, including IL-1β, TNF-α, IL-2 and MCSF but not IFN-γ or

lipopolysaccharde (LPS). Its expression is also increased by TGF-β. Purified recombinant MCI-1 is able to inhibit LPS induced macrophage TNF-α production, suggesting that MCI-1 acts in macrophages as an autocrine regulatory molecule. Hromas et al. (1997) described that a novel BMP family member, termed PLAB, was cloned from placenta. Addition of recombinant protein inhibited the proliferation of primitive hematopoietic progenitors. Lawton et al. (1997) named this new gene placenta transforming growth factor beta (PTGFB). Beside the cDNA, they cloned the genomic sequence and found that the single copy of PTGFB to be composed of two exons of 309 bp and 891 bp, separated by a 2.9 kb intron. The gene was localized to chromosome 19p12-13.1 by fluorescence *in situ* hybridization. Yokoyama-Kobayashi et al. (1997) named the gene as H00269, and did not carry out functional studies. Finally Paralkar et al.(1998) reported this new gene, termed prostate-derived factor (PDF), with more functional studies, using active recombinant PDF protein and antibody. By *in situ* hybridization and immunohistochemistry, they found high expression of PDF in the placenta. In day 18 rat embryos, PDF protein expression was also seen in the skin and the cartilaginous tissue of developing skeleton, implicating PDF in bone formation. Interestingly, subcutaneous implantation of recombinant PDF induced cartilage formation and early stage of endochondral bone formation in 1-month-old male Long-Evans rats. This was the first direct support that this new member of TGF-β superfamily was functionally related to bone development. The work of Paralkar et al. (1998) suggests this new TGF-β superfamily gene has the ability to induce bone formation *in situ*. The results from the above researchers supported our results which showed an increase in differentiation markers (osteocalcin and collagen I) in MG-63 cells.

5.2 PL74 ACTS THROUGH THE p21 (WAF1/Cip1) PATHWAY

TGF-βs inhibit cell proliferation by causing growth arrest in the G1 phase of the cell cycle (Massague, 1990). The TGF-β induced G1 cell cycle arrest has been partially attributed to the regulatory effects of TGF-β on both levels and activities of G1 cyclins and Cdks. Recently, a family of low molecular weight cyclin-dependent kinase inhibitors (CkdIs) have been shown to play essential roles in arresting cell cycle procession. These CdkIs, which include p21, p16, p15 and p27, physically associate with the cyclin-Cdk complexes to inhibit their activities (Xiong et al 1993, Serrano et al 1993). p21 was first identified as a component of the quaternary complex composed of cyclin D, Cdk4 and the proliferating cell nuclear antigen (PCNA). p21 acts as an inhibitor of the multiple cyclin-Cdk complexes (Harper et al. 1993, Xiong et al. 1993). TGF-β1 can cause G1 cell cycle arrest by activated CdkIs such as p21, thereby inhibiting cyclin-CDK activity (Datto et al. 1995). Previous work has demonstrated that p21 was induced by DNA damage through p53 as a mediator (Dullic et al. 1994). Activation of TGF-β through a p53-independent mechanism can also cause a rapid transcriptional induction of p21 (Datto et al. 1995). TGF-β family members, such as BMP-4, can also induce p21 expression in isolated dental epithelia and stimulate apoptosis (Jernvall, et al 1998).

These facts prompted us to test if PL74, a novel TGF-β family member, could also regulate p21 transcription in MG-63 cells. Northern blot was used to measure p21 mRNA expression, comparing stably transfected MG-63 cells and control. The results revealed that p21 mRNA was markedly increased in the MG-63 cells stably transfected

with PL74 (Fig 11). Also the induction of p21 by PL74 was through a p53-independent pathway, because the cell line MG-63 contains only one allele of the p53 gene, which is rearranged (Chandar et al. 1992), and hence non functional. Our results showed that PL74 dependent inhibition of MG-63 cell growth is mediated through the p21 pathway. We do not know if differentiating activities of PL74 are upstream or downstream of p21.

5.3 TISSUE DISTRIBUTION

PL74 is widely distributed in nearly all normal human tissues, though often at a low level. This result indicates PL74 like TGF-β, may be important in the function of many tissues in the body. Since the highest expression is in the placenta, it would be reasonable to suppose PL74 has a particularly important role in placental function.

PL74 is expressed in varying amounts in many cancer cell lines. Future studies to determine a particular role in the regulation of tumor cell growth would be of interest. Our detailed studies of PL74 regulation of growth of the osteosarcoma cell line MG-63 suggest a potential role for PL74 in growth regulation at least in this tumor type. More detailed studies in clinical samples would be needed to establish if a significant *in vivo* funtion (or lack of growth control) by PL74 were important in the development or growth of osteosarcomas.

5.4 PL74 FUNCTIONS IN MG-63 CELLS

Based on structural similarities of PL74 to TGF-β and the BMP's, we postulated that PL74 had both TGF-β-like and BMP-like properties.

TGF-β-like properties are growth inhibition and induction of extracellular matrix secretion. TGF-βs are inhibitors of cellular growth for many cell types, including cells of epithelial, endothelial, neuronal, hematopoietic, osteoblast and lymphoid origins (Kingsley 1994). This ability of TGF-βs to inhibit growth is thought to play a critical role in their abilities to influence many aspects of cellular function. In addition, TGF-β-mediated growth inhibition may also play a regulatory role in complex physiological processes such as in immune response and development. Loss of cellular sensitivity to TGF-β-mediated growth inhibition may contribute directly to carcinogenesis or other human diseases (Hu et al. 1998).

Our data have shown that ^{3}H-thymidine uptake and cell numbers were significantly reduced in replicate, statistically significant experiments (Table 2). It is unclear why the reduction in ^{3}H-thymidine uptake was less than the reduction in cell number as the reverse, or equality, would be expected. The reason may be biological variation due to doing the experiments on different cell preparations that may be at different confluencies, which would change the results. In this regard, we have noted that as the cells achieve higher density, ^{3}H-thymidine uptake decreases (unpublished observation). We thus believe this is a valid result and confirms that, like TGF-β, PL74 is antiproliferative when overexpressed. However, although reduction in ^{3}H-thymidine

uptake and cell number suggest reduced proliferation, apoptosis alone may also cause this. Specific experiments to completely separate proliferation and apoptosis were not performed in this thesis.

Cells that cease proliferation do not necessarily die but may arrest in G1 or enter G0 phase of the cell cycle. Alternatively a cell may proceed to cell death by apoptosis or necrosis. Two distinct modes of cell death, apoptosis and necrosis, can be distinguished based on differences in the morphological, biochemical and molecular changes of dying cells. Programmed cell death or apoptosis is the most common form of eukaryotic cell death. It is a physiological suicide mechanism that preserves homeostasis, in which cell death naturally occurs during normal tissue turnover (Jacobson et al. 1997). In general, cells undergoing apoptosis display a characteristic pattern of structural changes in the nucleus and cytoplasm, including rapid blebbing of plasma membranes and nuclear disintegration. The nuclear collapse is associated with extensive damage to chromatin and DNA-cleavage into oligonucleosomal length DNA fragments after activation of an endonuclease. Apoptosis is essential in many physiological processes, including developmental and effective mechanisms of (i): the immune system, (ii): embryonic development of tissue, organs and limbs, (iii): development of nervous system and (iv): hormone-dependent tissue remodeling (Hoffman et al. 1994).

Our experiments have demonstrated that PL74, when overexpressed by transfection, induces MG-63 cell apoptosis. The overexpression of PL74 by stable transfection studies show that this effect is gradual over several days. Ultimately, by 2-3 weeks of culture, very few cells are left alive. The morphology also shows loss of normal cell structure and pre-terminal features, with loss of cell process and rounding up of

cells. At low levels of PL74 expression as observed in wild-type MG-63 cells, PL74 expression apparently has little effect as these cells grow rapidly in culture. This suggests a dose-response effect wherein high dose, as obtained by transfection, lead to cell death and apoptosis. These questions could be clarified by contructing dose-response courses with recombinant protein.

It should be noted that preparing stable tranfectants usually takes many weeks or months. However, since overexpression of PL74 is lethal, it was necessary to terminate clone selection after 2-3 weeks. Many separate small clones were pooled then reseeded together to circumvent the problem of lethality. The northern blot (Fig. 10) showed there was overexpression of PL74 mRNA. However, we cannot exclude that there may have been a mixed population including untransfected cells since the selection time was relatively short (only 2-3 weeks).

5.5 PL74 IS A REGULATOR OF CELL DIFFERENTIATION

In placenta, as in other complex organs, the development and maintenance of functional tissue structures depend on the balance between cellular proliferation, differentiation and death. So far, a number of studies have shown the regulation of expression of TGF-β family proteins during embryonic development, such as activins, TGF-β1 and TGF-β3.

PL74 has a somewhat restricted tissue distribution, in which its mRNA is highly expressed in human placenta in comparison with much lower expression in all other

normal tissues. This suggests it may play an important role in placental development and early embryogenisis. Interestingly, PL74 mRNA was expressed in many types of cancer cells, suggesting that its function may be more related to regulating cell differentiation rather than acting as a tumor suppressor gene. This type of function is in contrast with another trophoblast differentiation associated gene PL48 our laboratory has cloned, which was not expressed in the cancer cell lines we surveyed (Dakour et al. 1997).

Our results have shown that PL74 mRNA expression was associated with human trophoblast differentiation as well as human keratinocyte differentiation. PL74 also induced human osteosarcoma MG-63 cell differentiation. It is therefore clear that PL74 is involved in cell differentiation of several different cell types.

Differentiation induction is a second potential major function of PL74, implied by its similarity to the BMPs. PL74 clearly has this function as the data of Paralkar et al. (1998) show that in an *in vivo* system, PL74 (PDF in their terminology) could induce cartilaginous formation from osteoblast precursor cells in rats. Interestingly, in this system, the differentiative function was dominant and apoptosis was not. Since differentiative cells usually cease cell division, our results of antiproliferative activity were consistent with their data. Apoptosis may have arisen in our experiments and not theirs due to effects of dose or the particular conditions of the experiments. Our general interpretation would be that at moderate doses, PL74 induces cell cycle arrest and differentiation, but higher doses lead to apoptosis. Clearly this hypothesis would require testing as many other factors may be present to influence the commitment to apoptosis.

Our studies of increased osteocalcin and collagen I can be interpreted both as markers of differentiation (like BMPs), but also as a TGF-β-like action to increase extracellular matrix secretion.

5.6 CYTOKINE REGULATION IN MG-63 CELLS

In MG-63 cells, it is known that EGF stimulates cell proliferation (Mioh and Chen 1989), IGF-1 dose dependently stimulates cell proliferation and differentiation (Kawakami et al. 1998), and TGF-β inhibits cell growth (Mioh and Chen, 1989). In other bone cells, IFNγ inhibited collagen and DNA synthesis in cultured rat long bones and osteoblastic ROS 17/2.8 cells, therefore inhibiting bone formation (Nanes et al. 1989). Nanes et al. (1990) showed that IFNγ may inhibit bone formation by selective inhibition of osteoblast matrix protein, such as bone gla protein (BGP) production. Using cultured human osteoblast-like cells (hOB), Gowen et al. (1988) demonstrated that IFNγ inhibited proliferation and stimulated alkaline phosphatase activity of these cells. Our data indicate that IFNγ acts directly on bone-forming cells to affect both their proliferative and differentiated function.

We have not noted proliferation effects of IGF-1 and EGF in our cultures. We did find that IFN-γ induced PL74 significantly. Since IFN-γ arises from lymphocytes, it is possible that its effects are mediated on MG-63 cells through a paracrine/autocrine system (Fig. 15). Thus hematological cytokines may be postulated to play a role in *in vivo* bone growth as in this schema.

We also noted that TGF-β1 induces PL74, suggesting an additional regulation of bone cell development. TGF-β1 can induce both stimulation and inhibition of osteoblast proliferation *in vitro*, depending on the cell system and TGF-β concentrations. Our data suggest TGF-β action might be direct or possibly through autocrine induction of PL74. Experiments with PL74 blocking antibodies would be needed to explore this hypothesis.

These results suggest that PL74 may be involved in an intricate paracrine network with other cytokines such as TGF-β1 and IFN-γ in regulating osteoblast development and proliferation.

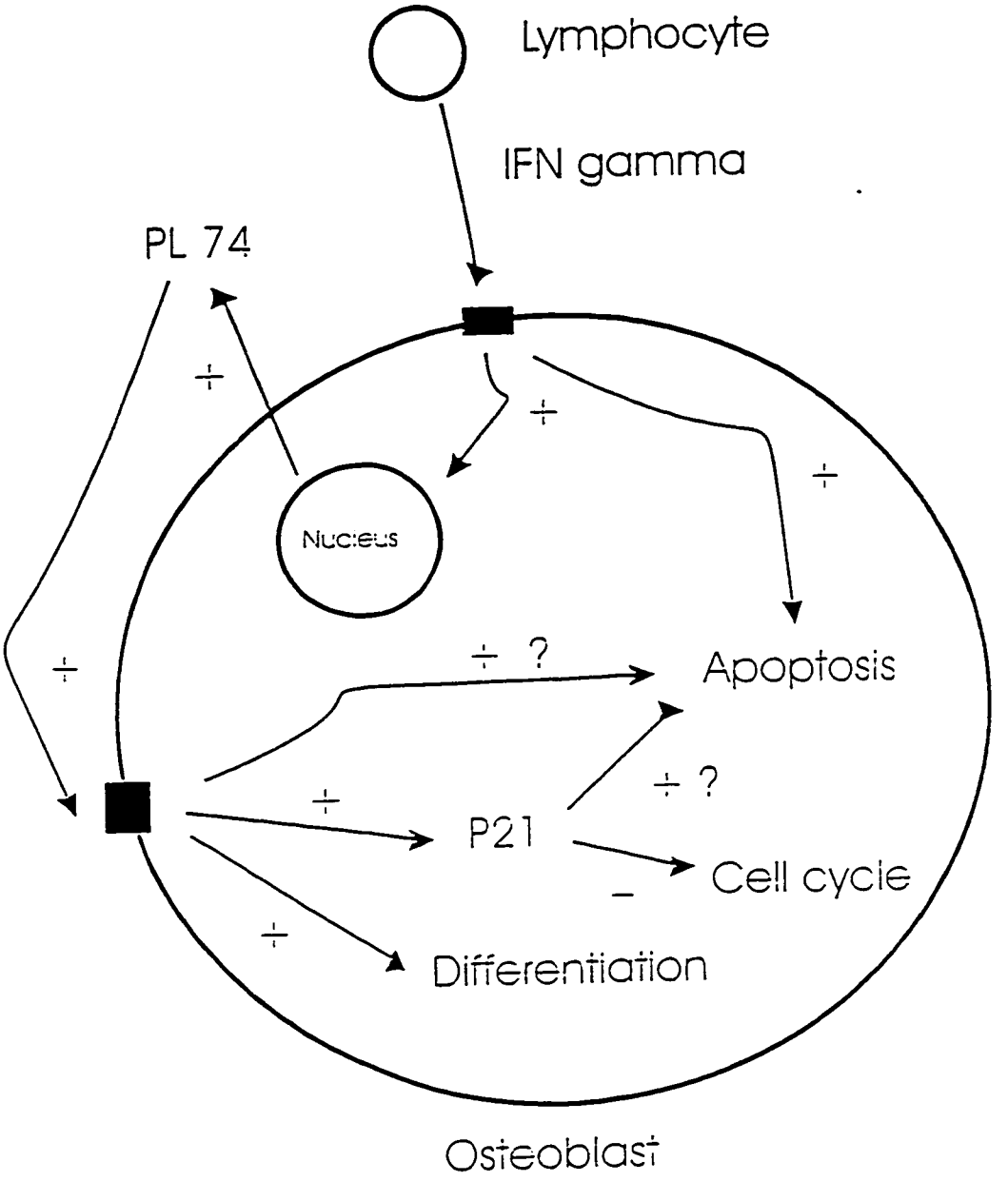


Figure 15. Possible PL74 Functional Actions in MG-63 cell by autocrine/paracrine mechanism.

5.7 PL74 IN NORMAL AND ABNORMAL PLACENTAL FUNCTION

We have demonstrated that PL74 increased markedly as the cytotrophoblast differentiated into syncytium. Due to the lack of normal human trophoblast cell lines that differentiated, we chose to do functional studies in MG-63 cells. Thus, we can only speculate on possible roles of PL74 in placenta at this time.

A large variety of cytokines and physicochemical factors can influence trophoblast proliferation and differentiation (Morrish et al. 1998). PL74 (PDF) is highly expressed in syncytium (Paralkar et al. 1998) and thus it is reasonable to suppose it has a significant function in regulating trophoblast function. Based on the functions in MG-63 cells, we speculate that PL74 would have one or more of the following functions:

(1) A syncytial product that acts as a negative feedback regulator to limit villus cytotrophoblast proliferation by causing cell cycle arrest or/and apoptosis.

(2) A syncytial product that regulates proliferation or invasive ability of extra villus trophoblast (EVT). In this regard, we note that decidual and/or syncytial TGF-β has significant regulatory effects to inhibit extravillus trophoblast invasion, promote differentiation of EVT to placental bed giant cells, and to stimulate extracellular matrix production from EVT (Lala et al. 1998).

(3) A syncytial product that induces cytotrophoblast differentiation into syncytium, as a positive feedback loop.

(4) PL74 may be a one of the mediators of IFN-γ action in the trophoblast. Yui et al. (1994) have shown that IFN-γ and TNF-α induce apoptosis in term human cytotrophoblast. We have shown an induction of PL74 by IFN-γ in very early

culture of human cytotrophoblast. At this stage, the cells are still cytotrophoblast in phenotype. Hence, IFN-γ may induce PL74 in cytotrophoblast. However, because the culture system used demonstrates rapid spontaneous differentiation into syncytium, it will be difficult to perform functional studies of this effect in this system. A solution of this problem would be to use cytotrophoblast cell lines (eg. HTR8/SVneo).

(5) PL74 may be a mediator of EGF action. Morrish et al. (1987) have shown that EGF induces cytotrophoblast differentiation. As well, EGF blocks TNF-α /IFN-γ induced apoptosis (Garcia-Lloret et al. 1996). It is possible that part of EGF action is to induce PL74 which in turn could promote differentiation and block apoptosis. Clearly, complex interactions are possible.

The above considerations are speculative. However, they represent testable hypotheses for future study of PL74 action in the placenta.

Abnormal placental development may be a primary causative factor in several human diseases of pregnancy including intrauterine growth restriction (IUGR), pregnancy induced hypertension (PIH), and diabetes. Multiple abnormalities in placental differentiation occur in diabetes (Stoz F. et al 1987; 1988; Boyd et al 1986) and poor implantation and impaired villous development also occurs in PIH (Zhou et al.1993; Roberts et al 1993). Taken together, these data showed a variety of cytotrophoblast differentiation abnormalities occur in diseases of pregnancy. However, the factors controlling placental growth and differentiation are complex and poorly understood. So far there are no specific human gene regulatory factors of trophoblast differentiation known. We are interested in understanding if PL74 is a potent gene regulator of

placental development, or if PL74 mRNA expression or regulation is abnormal in these diseases. Our results have shown that in one case, PL74 expression is markedly higher in a PIH placenta than the control. However, average mRNA level of PL74 in 9 PIH placentae and 11 normal placentae have not been significantly different in statistical analysis. Further work with PL74 needs to be done to determine if abnormal expression or regulation is involved in diseases of pregnancy.

Conclusion

The superfamily of TGF-β related proteins has recently emerged. It includes as many as 40 or more members such as TGF-β1-3, BMPs, activins and many other factors. Given the size and diversity of this superfamily, it is not surprising that studies on the biology of these factors continues to provide remarkable insights into how developmental and physiological processes are controlled. Our work has added a new member, PL74, to this superfamily.

We have cloned and characterized a novel TGF-β superfamily member, PL74. Sharing the same hallmark with all family members, PL74 is mostly similar to BMP subfamily, with 33-38% identity. PL74 is highly expressed in human placenta, suggesting that it may play an important role in placental development or embryogenesis. Our results have shown that PL74 is likely antiproliferative, and can induce apoptosis. As well it promotes differentiated function in MG-63 cells. Our data also indicate regulation by IFN-γ and TGF-β1 in osteoblast cells, and IFN-γ and EGF in cytotrophoblast. These results suggest new paracrine mechanisms for regulation of development and function of bone and placenta.

CHAPTER 6

BIBLIOGRAPHY

Adams, J.C. and Watt, F.M. 1993. Regulation of development and differentiation by the extracellular matrix. *Development* 117: 1183-1198.

Alsat, E., Haziza, J., Scippo, M.L., Frankenne, F., and Evain-Brion, D. 1993. Increase in epidermal growth factor receptor and its messenger ribonucleic acid level by parathyroid hormone (1-34) and parathyroid hormone-related protein during the differentiation of human trophoblast cells. *Journal of Cell Physiology* 154:122-132.

Amemiya, K., Kurachi, H., Adachi, H., Morishige, K.I., Adachi, K., Imai, T., and Miyake A. 1994. Involvement of EGF/EGFR autocrine and paracrine mechanism in human trophoblast cells: functional differentiation in vitro. *Journal of Endocrinology* 143:291-301.

Arceci, R.J., Shanahan, F., Stanley, E.R., and Pollard, J.W. 1989. Temporal expression and location of colony-stimulation factor 1(CSF-1) and its receptor in the female reproductive tract are consistent with CSF-1 regulated placental development. *Proc. Natl. Acad. Sci. USA* 86: 8818-8822.

Assoian, R.K., Komoriya, A., Meyers, C.A., Miller, D.M., and Sporn, M.B. 1983. Transforming growth factor-beta in human platelets. *Journal of Biological Chemistry* 258:7155-7160.

Attisano L. and Wrana T. L. 1996. Signal transduction by members of TGF-β superfamily. *Cytokine and Growth Factor Review* 7: 327-339.

Barnea, E.R., Feldman, D., Kaplan, M., and Morrish, D.W. 1990. The dual effect of EGF on hCG secretion by the first trimester placenta in vitro. *Journal of Clinical Endocrinology & Metabolism* 71: 923-928.

Basler, K., Edlund, T., Jessell, T.M., and Yamada, T. 1993. Control of cell pattern in the neural tube: Regulation of cell differentiation by dorsalin-1, a novel TGF-β family member. *Cell* 73: 687-702.

Bellido, T., Borba, V.Z., Roberson, P., and Manolagas, S.C. 1997. Activation of the Janus kinase/STAT signal transduction pathway by interleukin-6-Type cytokines promotes osteoblast differentiation. *Endocrinology*138 (9): 3666-3676.

Bernischke, K. 1995. Pathology of the Human Placenta. pp:154 New York edition.

Bistop, J. M. 1987. The molecular genetics of cancer. *Science* 235: 305-311.

Bokar, J.A., Keri, R.A., Farmerie, T.A., Fenstermaker, R.A., Andersen, B., Hamernik, D.L., Yun, J., Wagner, T., and Nilson, J.H. 1989. Expression of the glycoprotein hormone alpha-subunit gene in the placenta requires a functional cyclic AMP response element, whereas a different *cis*-acting element mediates pituitary-specific expression. *Molecular and Cellular Biology* 9:5113-5122.

Bonewald, L.F. and Dallas, S.L. 1994. Role of active and related transforming growth factor beta in bone formation. *Journal of Cell Biochemistry* 55:350-359.

Bootcov, M.R., Bauskin, A.R., Valenzuela, S.M., Moore, A.G., Bansal, M., He, X-Y., Zhang, H-P., Donnellan, M., Mahler, S., Pryor, K., and Breit, S.N. 1997.MCI-1, a novel macrophage inhibitory cytokine, is a divergent member of the TGF-β superfamily. *Pro. Natl. Acad. Sci. USA.* 94: 11514-11519.

Boyd, P.A., Scott, A., and Keeling, J.W. 1986.Quantitative structural studies on placentas from pregnancies complicated by diabetes mellitus. *Br. J. Obstet. Gynaecol.* 93:31-35.

Brummel, T.J., Twombly, V.. Marques, G., Wrana, J.L., Newfeld, S.J.,

Attisano, L., Massague, J., O'Connor, M.B., and Gelbart, W.M. 1994. Characterization and relationship of DPP receptors encoded by the saxophone and thick pein genes in *Drosophila. Cell* 78: 251-261.

Caniggia, I., Lye, S.J., and Cross, J.C. 1997. Activin is a local regulator of human cytotrophoblast cell differentiation. *Endocrinology* 138: 3976-3986.

Carcamo, J., Weis, F.M., Ventura, F., Wieser, R., Wrana, J.L., Attisano, L. and Massague, J. 1994. Type I receptors specify growth inhibitory and transcriptional responses to TGF-β and activin. *Molecular and Cellular Biology* 14: 3810-3821.

Carpenter, G., and Cohen, S. 1979. Epidermal growth factor. *Annual Review of Biochemitry* 48:193-216.

Cate, R.L., Mattaliano, R.J., Hession, C., Tizard, R., Farber, N.M., Cheung, A., Ninfa, E.G., Frey, A.Z., Gash, D.J., Chow, E.P., Fisher, R.A., Bertonis, J.M., Torres, G., Wallner, B.P., Ramachandran, K.L., Ragin, R.C., Manganaro, T.F., MacLaughlin, D.T., and Donahoe, P.K. 1986. Isolation of the bovine and human genes for mullerian inhibiting substance and expression of the human gene in animal cells. *Cell* 45:685-698.

Celeste, A.J., Iannazzi, J.A., Taylor, R.C., Hewick, R.M., Rosen, V., Wang, E.A., and Wozney, J.M. 1990. Identification of TGF-β family members present in bone-inductive protein purified from bovine bone. *Proc. Natl. Acad. Sci. USA* 87: 9843-9847.

Centrella, M. 1996. Transforming growth factor-β gene family members and bone. *Endocrine Review* 15:27-39.

Centrella, M., Casinghino, S., Kim, J., Pham, T., Rosen, V., Wozney, J., and

McCarthy, T.L. 1995. Independent changed in type I and type II receptors for TGF-β induced by bone morphogenetic protein-2 paralleled expression of the osteoblast phenotype. *Molecular and Cellular Biology* 15:3273-3281.

Chandar, N., Billig, B., McMaster, J., and Novak, J. 1992. Inactivation of p53 in human and murine osteosarcoma cells. *British Journal of Cancer* 65:208-214.

Cheifetz, S., Bassols, A., Stanley, K., Ohta, M., Greenberger, J., and Massague, J. 1988. Heterodimeric transforming growth factor-β: Biological properties and nteraction with three types of cell surface receptors. *Journal of Biological Chemistry* 263:10783-1078.

Chen, F., and Weinberg, R.A. 1995.Biochemical evidence of the autophosphorylation and transphosphorylation of TGF-β receptor kinase. *Proc. Natl. Acad. Sci. USA* 92: 1565-1569

Chen, P., Carrington, J.L., Hammonds, R.G., and Reddi, A.H. 1991.Stimulation of chondrogenesis in limb bud mesoderm cells by recombinant human bone morphogenetic protein 2B and modulation by transforming growth factor β1and β2. *Experimental Cell Research* 195:509-515.

Cross, J.C., Werb, Z., and Fisher, S.J. 1994. Implantation and placenta: key pieces of the development puzzle. *Science* 266:1508-1518.

Cross, J.C., Flannery, M.L., Blanar, M.A., and Werb, Z. 1995. Hxt encodes a basic helix-loop-helix transcription factor that regulates trophoblast cell development.*Development* 121: 2513-2523.

Dakour, J., Li, H., and Morrish, D.W. 1997. PL48, a novel gene associated with cytotrophoblast and lineage-specific HL-60 cell differentiation. *Gene* 185:153-157.

Daopin, S., Piez, K.A.,Ogawa, Y., and Davies, D.R. 1992. Crystal structure of transforming growth factor-β2: An unusual fold for the superfamily. *Science* 257: 369-373.

Datto, M.B., Li, Y., Panus, J.F., Howe, D.J., Xiong, Y., and Wang, X.F. 1995. Transforming growth factor β induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc.Natl. Acad. Sci. USA* 92:5545-5549.

Derynck, R., Jarrett, J.A., Chen, E.Y., and Goeddel, D.V. 1987. Intron-exon structure of human transforming growth factor-β precursor gene. *Nucleic Acids Research* 15:3188-3189.

Derynck, R., Lindquist, P.B., Lee, A., Wen, D., Tamm, J., Graycar, J., Rhee, L., Mason, A.J., Miller, D.A., Coffey, R.J., Moses, H.L., and Chen, E.Y. 1988. A new type of transforming growth factor-β, TGF-β3. *EMBO Journal* 7:3737-3743.

Doctor, J.S., Jackson, P.D., Rashka, K.E., Visalli, M., and Hoffmann, F.M. 1992. Sequence, biochemical characterization, and developmental expression of a new member of the TGF-β superfamily in Drosophila melanogaster. *Developmental Biology* 151: 491-505.

Dodds, R.A., Merry, K., Littlewood, A., and Gomen, M. 1994. Expression of mRNA for IL1β, IL6 and TGF-β, in developing human bone and cartilage. *Journal of Histochemistry & Cytochemistry* 42:733-744.

D'Souza, R.N., Niederreither, K., and de Crombrugghe, B. 1993.Osteoblast-specific expression of the α 2(I) collagen promoter in transgenic mice: correlation with the distribution of TGF-β 1. *J. Bone Miner. Res.* 8: 1127-1136.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., and Waterfield, M.D. 1984 Close similarity of EGF receptor and v-erb-B oncogene protein sequences. *Nature* 307: 521-527

Dungy, L.J., Sididiqi, T.A., and Khan, S. 1991. Transforming growth factor-β expression during placental development. *Am. J. Obstet. Gynecol.* 165:853-857

Eto, Y., Tsuji, T., Takezawa, M., Takano, S., Yokogawa, Y., and Shibai, H. 1987. Purification and characterization of erythroid differentiation factor isolated from human leukemia cell line THP-1. *Biochemical & Biophysical Research Communications* 142: 1095-1103.

Faria, TN., Ogren, L., Talamantes, F., Linzer, D.I., and Soares, M.J. 1991. Localization of placental lactogen-1 in trophoblast giant cells of the mouse placenta. *Biology of Reproduction* 44(2): 327-331.

Feinberg, R.F., Kao, L.C., Haimowitz, J.E., Queenan, J.T., Win, T.C., Strause, J.F., and Kliman, H.J. 1989. Plasminogen activator inhibitor type 1 and 2 in human trophoblast. *Labortory Investigation* 61:20-26.

Forage, R.G., Ring, J.M., Brown, R.W., McInerney, B.V., Cobon, G.S., Gregson, R.P., Robertson, D.M., Morgan, F.J., Hearn, M.T., and Findlay, J.K. 1986. Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. *Proc. Natl. Acad. Sci. USA* 83: 3091-3095.

Franceschi, R.T., Romano, P.R., and Park, K.Y.1988. Regulation of Type I collagen synthesis by 1, 25-Dihydroxyvitamin D_3 in human osteosarcoma cells. *The Journal of Biological Chemistry* 263(35): 18938-18945.

Garcia-Lloret, M.I., Morrish, D.W., Wegmann, T.G., Honore, L., Turner, A.R.,

And Guilbert, L.J. 1994. Demonstration of functional cytokine-placental interaction: CSF-1 and GM-CSF stimulate human cytotrophoblast differentiation and peptide hormone secretion. *Experimental Cell Research* 214: 46-54.

Garcia-Lloret, M.I., Yui, J., Winkler-Lowen, B., and Guilbert, L.J. 1996. Epidermal growth factor inhibits cytokine-induced apoptosis of primary human trophoblast. *Journal of Cell Physiology* 167: 324-332.

Genbacev O, Bass KE. Joslin RJ. Fisher SJ. 1995.Maternal smoking inhibits early human cytotrophoblast differentiation. *Reproductive Toxicology* 9(3): 245-255.

Genbacev, O., Joslin, R., Damsky, C.H., Polliotti, B.M., and Fisher, S.J. 1996. Hypoxia alters early gestation human cytotrophoblast differentiation / invasion in vitro and models the placental defects that occur in preeclampsia. *Journal of Clinical Investigation* 97:540-550.

Gitelman, S.E., Kirk, M., Ye, J.Q., Filvaroff, E.H., Kahn, A.J., and Derynck, R. 1995. Vgr-1/BMP-6 induces osteoblastic differentiation of pluripotential mesenchymal cells. *Cell Growth and Differentiation*n 6:827-836.

Gosseye, D. and Fox, H. 1984. An immunohistological comparison of the secretary capacity of villous and extravillous trophoblast in the human placenta. *Placenta* 5:329-348.

Gousin, A.S., Betsholtz, C., Persson, H., Rydnert, J., Bywater, M., Holmgren, G., Heldin, C., Westermark, B., and Ohlsson, R. 1985. Coexpression of the *sis* and *myc* Proto-oncogenes in developing human placenta suggests autocrine control of trophoblast growth. *Cell* 41:301-312.

Graham, A., Francis-West, P., Brickell, P., and Lumsden, A. 1994.The signaling

molecule BMP4 mediates apoptosis in rhombencephalie neural crest. *Nature* 372: 684-686.

Graham, C.H. and Lala, P.K. 1991. Mechanism of trophoblast invasion *in situ.* *Journal of Cell Physiology* 148:228-234.

Graham, C.H., Lysiak, J.J., McCrae, K.R., and Lala, P.K. 1992. Localization of TGF-β at the human fetal-maternal interface: Role in trophoblast growth and differentiation.*Biological Reproduction* 46:561-572.

Graham, C.H. 1996. Effect of TGF-β on plasminogen activator system in cultured first trimester human cytotrophoblasts. *Placenta* 17: A43.

Gray, AM. and A.J. Mason 1990. Requirement for activin A and transforming growth factor-beta pro-regions in homodimer assembly. *Science* 247:1328-1330.

Griffith, O. L., P.C. Keck, T.K., Sampath, D.C. Rueger, and W.D. Cardson 1996. Three-dimensional structure of recombinant human osteogenic protein 1: structural paradigm for the transforming growth factor superfamily. *Proc. Natl. Acad. Sci. USA* 93: 878-883.

Guillemot, F., Nagy, A., Auerbach,A., Rossant, J., and Joyner, A.L. 1994. Essential role of Mash-2 in extraembryonic development. *Nature* 371:333-336.

Hammonds, R.J., Schwall, R., Dudley, A., Berkemeier, L., Lai, C., Lee, J., Cunnigham, N., and Mason, A.J. 1991. Bone-inducing activity of mature BMP-2b produced form a hybrid BMP-2a/2b precursor. *Molecular Endorcrinology* 5:149-155.

Han, V.K., Hunter, E.S., Pratt, R.M., Zendegui, J.G., and Lee, D.C. 1987.

Expression of rat TGF α mRNA during development occurs predominantly in the maternal decidua. *Molecular and Cellular Biology* 7:2335-2343.

Harris, S.E., Bonewald, L.F., Harris, M.A., Sabatini, M., Dallas, S., Feng, J.Q., Ghosh-Choudhury, N., Wozney, J., and Mundy, G.R. 1994. Effects of TGF-β on bone nodule formation and expression of bone morphogenetic protein 2, osteocalcin, osteopontin, alkaline phosphatase and type I collagen mRNA in long-term cultures of fetal rat calvarial osteoblast. *Journal of Bone and Mineral research* 9:855-863.

Hoffman, B., and Liebermann, D.A. 1994. Molecular controls of apoptosis: differentiation/ growth arrest primary response genes, proto-oncogenes, and tumor suppressor genes as positive & negative modulators. *Oncogene* 9: 1807-1812.

Hofmann, G.E., Rao, C.V., Carman, F.R., and Jr. Siddiqi, T.A. 1988. ^{125}I-human epidermal growth factor specific binding to placentas and fetal membranes from various pregnancy states. *Acta Endocrinologica* 117:485-490.

Hogan, B.L. 1996. Bone morphogenetic proteins multifunctional regulators of vertebrate development. *Genes & Development* 10: 1580-1594.

Hoshina, M., Hussa, R., Pattillo, R., and Boime, I. 1982. Cytological localization of chorionic gonadotrophin α and placental lactogen mRNAs during development of the human placenta. *Journal of Cell Biology* 97:585-592

Holley, R.W., Bohlen, P., Fava, R., Baldwin, J.H., Kleeman, G., and Armour, R. 1980. Purification of kidney epithelial cell growth inhibitors. *Proc. Natl. Acad. Sci USA* 77(10): 5989-92.

Holte, H., Stokke, T., Smeland, E., Watt, R., Blomhoff, H.K., Kaalhus, O., and Ohlsson, R. 1989. Level of myc protein, as analyzed by flow cytometry, correlate with cell growth potential in malignant B-cell lymphomas. *International Journal of Cancer* 43:164-170.

Hromas, R., Hromas, R., Hufford, M., Sutton, J., Xu, D., Li, Y., and Lu, L. 1997. PLAB, a novel placenta bone morphogenetic protein. *Biochimica et Biophysica Acta*1354: 40-44.

Hu, P.P., Datto, M.B., and Wang, X.F. 1998. Molecular Mechanism of transforming growth factor-β signaling. *Endocrine Reviews* 19 (3): 349-363.

Hynes, R.O. 1992a. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69:11-25.

Hynes, R.O. and Lander, A.D. 1992b.Contact and adhesive specificities in the associations, migrations and targeting of cell and axons. *Cell* 68:303-322.

Ingram, R.T., Bonde, S.K., Riggs, B.L., and Fitzpatrick, L.A. 1994. Effects of TGF beta and 1, 25 dihydroxyvitamin D_3 on the function, cytochemistry and morphology of normal human osteoblast-like cells. *Differentiation* 55: 153-163.

Irving, J.A., and Lala, P.K. 1995. Functional role of cell surface integrins on human trophoblast cell migration: Regulation by TGFβ, IGF-II and IGFBP-1 . *Experimental Cell Research* 217:419-427

Jacobson, M.D. Weil, M., and Raff, M.C. 1997. Programmed Cell death in animal development. *Cell* 88:347-354.

Jakowlew SB. Dillard PJ. Sporn MB. Roberts AB. 1988a. Nucleotide sequence of

chicken transforming growth factor-β1 (TGF-β1). *Nucleic Acid Research* 1 6:8730-8733.

Jakowlew, S.B., Dillard, P.J., Sporn, M.B., and Roberts, A.B. 1988b.Complementary deoxy ribonucleic acid cloning of a novel transforming growth factor-β messenger ribonucleic acid from chick embryo chondrocytes. *Molecular Endocrinology* 2:747-755.

Jameson, J.L.and Hollenberg, A.N. 1993. Regulation of chorionic gonadotropin gene expression. *Endocrine Reviews* 14:203-221.

Jernvall, J., Aberg, T., Kettunen, P., Keranen, S., and Thesleff, I. 1998.The life history of an embryonic signaling center:BMP-4 induced p21 and is associated with apoptosis in the mouse tooth enamel knot. *Development* 125: 161-169.

Jones, C.M., Simon-Chazottes, D., Guenet, J.L., and Hogan, B.L. 1992. Isolation of Vgr-2, a novel member of the TGF-β related gene family. *Molecular Endocrinology* 6:1961-1968.

Kameda, T., Matsuzaki, N., Sawai, K., Okada, T., Saji, F., Matsuda, T., Hirano, T., Kishimoto, T., and Tanizawa, O. 1990. Production of interleukin 6 by normal tropholbast cells. *Placenta* 11: 205-213.

Kato, L. and Braunstein, G.D.1991. Retinoic acid stimulates placental protein hormone secretion by choriocarcinoma cell lines *in vitro*. *Endocrinology* 128:401-407.

Kelly, K., Cochran, B.H., Stiles, C.D., and Leder, P. 1983. Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet derived growth factor. *Cell* 35: 603-610.

Kingsley, D.M., Bland, A.E., Grubber, J.M., Marker, P.C., Russell, L.B., Copeland, N.G., and Jenkins, N.A. 1992. The mouse *short ear* skeletal morphogenesis locus is associated with defects in a bone morphogenetic member of the TGF-β superfamily. *Cell* 71:399-410.

Kingsley, D.M. 1994.TGF-β superfamily: new members, new receptors and new genetics tests of function in different organisms. *Genes & Development* 8:133-146.

Kliman, H.L., Nestler, J.E., Sermasi, E., Sanger, J.M., and Strauss, J.F.I. 1986. Purification, Characterization, and *in Vitro* Differentiation of Cytotrophoblasts from Human Term Placentas. *Endocrinology* 118(4): 1567-1582.

Koff, A., Ohtsuki, M., Polyak, K., Roberts, J.M., and Massague, J. 1993. Negative regulation of G1 in mammalian cells: inhibition of cyclin E-dependent kinase by TGF-beta. *Science* 260:536-539.

Kondaiah, P., Sands, M.J., Smith, J.M., Fields, A., Roberts, A.B., Sporn, M.B., and Melton, D.A. 1990. Identification of a novel transforming growth factor-β (TGF-β5) mRNA in *Xenopus* laves. *Journal of Biological Chemistry* 265:1089-1093.

Kulkarni, A.B., Huh, C.G., Becker, D., Geiser, A., Lyght, M., Flander, K.C., Roberts, A.B., Sporn, M.B., Ward, J.M., and Karlson, S. 1993. Transforming growth factor beta 1 null mutation in mice cause excessive inflammatory response and early death. *Proc. Natl. Acad. Sci. USA* 90:770-774.

Laiho, M., Weis, M.B., and Massague, J. 1991. Responsiveness to transforming

growth factor-beta (TGF-beta) restored by genetic complementation between cells defective in TGF-beta receptors I and II. *Journal of Biological Chemistry* 266: 9108-9112.

Lawton, L.N., Bonaldo, M.F., Jelenc, P.C., Qiu, L., Baumes, S.A., Marcelino, R.A., de Jesus, G.M., Wellington, S., Knowles, J.A., Warburton, D., Brown, S., and Soares, M.B. 1997. Identification of a novel member of the TGF-β superfamily highly expressed in human placenta. *Gene*203: 17-26.

Lee, S.J. 1991. Expression of growth /differentiation factor 1 in the nervous system: Conservation of a bicistronic structure. *Proc. Natl. Acad. Sci.* 88: 4250-4254.

Lee, S.T. 1988. Trophoblast giant cells of mouse placenta as the site of proliferin synthesis. *Endocrinology* 122: 1761-1768.

Lei, Z.M., Rao, C.V., Ackerman, D.M., and Day T.G. 1992.The expression of human chorionic gonadotropin/human luteinizing hormone receptors in human gestational trophoblastic neoplasms. *Journal of Clinical Endocrinology & Metabolism* 74:1236-1241.

Letsou, A., Arora, K., Wrana, J., Simin, K., Twombly, V., Jamal, J., Staehling-Hampton, K., Hoffmann, F.M., Gelbart, W., Massague, J., and O'Connor, M. 1995. Dpp signaling in Drosophila is mediated by the punt gene product: a dual ligand blinding type II receptor of the TGF-β receptor family. *Cell* 80: 899-908.

Lin, H.Y., Moustakas, A., Knaus, P., Wells, R.G., Henis, Y.I., and Lodish, H.F.

1995. The soluble exoplamie domain of type II TGF-β receptor: a heterogeneously glycogylated protein with high affinity and selectively for TGF-β ligands. *Journal of Biological Chemistry* 270:2747-2754.

Lin, L.F., Doherty, D.H., Lile, J.D., Bektesh, R.A., and Collins, F. 1993. GDNF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* 260:1130-1132.

Ling, N., Ying, S.Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M., and Guillemin, R. 1986. Pituitary FSH is released by a heterodimer of the β-subunits from the two forms of inhibin. *Nature* 321: 779-782.

Lopez-Casillas, F., Wrana, J.L., and Massague, J. 1993. Betaglycan presents ligand to the TGF-β signaling receptor. *Cell* 73:1435-1444.

Lyons, K., Graycar, J.L., Lee, A., Hashmi, S., Lindquist, P.B., Chen, E,Y., Hogan, B.L., and Derynck, R. 1989. Vgr-1, a mammalian gene related to Xenopus Vg-1, is a member of TGF-β gene superfamily. *Proc. Natl. Acad. Sci. USA* 86: 4554-4558.

Maruo, T. and Mochizuki, M. 1987. Immunohistochemical localization of epidermal growth factor receptor and myc oncogene product in human placenta: implication for trophoblast proliferation and differentiation. *American Journal of Obstetrics & Gynecology*156: 172-177.

Mason, A.J., Hayflick, J.S., Ling, N., Esch, F., Ueno, N.,, Ying, S.Y., Guillemin, R., Niall, H., and Seeburg, P.H. 1985. Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-β. *Nature* 318: 659-663.

Massague, J. 1985. Subunit structureof a high-affinity receptor for type β-transforming growth factor. *Journal of Biological Chemistry* 260:7057-7066.

Massague, J. and Kelly, B. 1986. Internalization of transforming growth factor-β and its receptor in Balb/c 3T3 fibroblasts. *Journal of Cell Physiology* 128:216-222.

Massague, Joan 1990. The Transforming Growth Factor-β Family. *Annual Reviews of Cell Biology* 6:597-641.

Massague, J. 1992. Receptors for the TGF-β family. *Cell* 69:1067-1070.

Massague, J. 1994. The TGF-β family and its composite receptors. *Trends in Cell Biology* 4: 172-178.

Massague, J. 1996a. TGF-β signaling: receptors, transducers and MAD proteins. *Cell* 85: 947-950.

Massague, J. and Weis-Garcia, F. 1996b. Serine/Threonine kinase receptors: mediators of TGF-β family signals. *Cancer surveys cell signaling*. ICRF Press, London. Vol.27: 41-64.

Matzuk, M.M., Kumar, T., Vassalli, A., Bickenbach, J.R., Roop, D.R., and Bradley, A. 1995. Functional analysis of activins during mammalian development. *Nature* 374:354-356.

McPherron, A.C. and Lee, S.J. 1993. GDF-3 and GDF-9: Two new members of transforming growth factor-beta superfamily containing a novel pattern of cysteines. *Journal of Biological Chemisrty* 268: 3444-3449.

Mitrani E. Ziv T. Thomsen G. Shimoni Y. Melton DA. Bril A. 1990. Activin can induce the formation of axial structures and is expressed in the hypoblast of the chick. *Cell* 63:495-501.

Miyazno, K. 1994. Receptors for transforming growth factor-β. *Advanced Immunology* 55:181-220.

Moore, J.J, Lundgren, D.W., Moore, R.M., and Andersen, B. 1988. Polyamines control hCG production in JEG-3 choriocarcinoma cell. *Journal of Biological Chemistry* 263: 12765-12769.

Morrish, D.W., Bharwaj, D., Dabbagh, L.K., Marusyk, H., and Siy, O. 1987. Epidermal growth factor induces differentiation and secretion of human chorionic gonadotropin and placenta lactogen in normal human placenta. *Journal of Clinical Endocrinology and Metabolism* 65:1282-1290.

Morrish, D.W., Bhardwaj, D., and Paras, M.T. 1991.Transforming growth factor β1 inhibits placental differentiation and human chorionic gonadotropin and human placental lactogen secretion. *Endocrinolog* 129:22-26.

Morrish, D.W., Dakour, J., and Li, H. 1996a. TGFβ1 inhibits cytotrophoblast syncytial formation and hCG/hPL secretion in vitro but does not inhibit gene expression associated with differentiation. *Placenta* 17: A14.

Morrish, D.W., Bhardwaj, D., Berdan, R., and Grace, M. 1996b. Attachment to fibronectin confers qualitatively greater syncytium formation and induces preferential hPL secretion from cultured term human trophoblast. *Placenta*17, 31.

Morrish, D.W., Linetsky, E., Bhardwaj, D., Li, H., Dakour, J., Marsh, R.G., Paterson, M.C., and Godbout, R. 1996c. Identification by subtractive hybridization of a spectrum of novel and unexpected genes associated with in vitro differentiation of human cytotroblast cells. *Placenta* 18: 577-585.

Morrish, D.W., Dakour, J., Li, H., Xiao, J., Miller, R., Sherburne, R., Berdan, R.,

and Guilbert, L. 1997. In vitro cultured human term cytotrophoblast : a model for normal primary epithelial cells demonstrating a spontaneous differentiation program that requires EGF for extensive development of syncytium. *Placenta* 18: 577-585.

Morrish, Donald W, Dakour, Jamal, and Li, Hongshi 1998. Functional regulation of human trophoblast differentiation. *Journal of Reproductive Immunology* 39:179-185.

Nachtigall, M.J., Kliman, H.J., Feinberg, R.F., Olive, D.L., Engin, O., and Arici, A. 1995. The effect of leukemia inhibitory factor (LIF) on trophoblast differentiation: a potential role in human implantation. *Journal of Clinical Endocrinology and Metabolism* 81: 801-806.

Nishino, E., Matsuzaki, N., Masuhiro, K., Kameda, T., Taniguchi, T., Takagi, T., Saji, F., and Tanizawa, O. 1990.Trophoblast- derived interleukin-6 regulates hCG release through IL-6 receptor on human trophoblast. *Journal of Clinical Endocrinology & Metabolism* 71: 436-441.

Oudejans, C.B., Pannese, M., Simeone, A., Meijer, C.J., and Boncinelli E. 1990. The three most downstream genes of the Hox-3 cluster are expressed in human extraembryonic tissues including trophoblast of androgenetic origin. *Development.* 108(3): 471-477.

Ozkaynak, E., Rueger,D.C., Drier, E.A., Corbett, C., Ridge, R.J., Sampath, T.K., and Oppermann, H. 1990. Op-1 cDNA encodes an osteogenic protein in the TGF-β family. *EMBO Journal* 9:2085-2093.

Ozkaynak, E., Schnegelsberg, P.J., Jin, D.F., Clifford, G.M., Warren, F.D., Dried, E.A., and Oppermann, H. 1992. Osteogenic protein-2: A new member of TGF-β superfamily express early in embryogenesis. *Journal of Biological Chemistry* 267: 25220-25227.

Padgett, R.W., St. Johnston, R.D., and Gelbart, W.M. 1987. A transcript from a Drosophila pattern gene predicts a protein homologous to the transforming growth factor-β family. *Nature* 325: 81-84.

Padgett, R.W., Wozney, J.M., and Gelbart, W.M. 1993. Human BMP sequences can confer normal dorsal-ventral patterning in the Drosophila embryo. *Proc. Natl. Acad. Sci.* 90:2905-2909.

Paralkar, V.M., Vail, A.L., Grasser, W.A., Brown, T.A., Xu, H., Vukicevic, S., Ke, H-Z., Qi, H., Owen, T.A., and Thompson, D.D. 1998. Cloning and characterization of a novel member of the TGF-β/bone morphogenetic protein family. *Journal of Biochemical Biology* 273(22): 13760-13767.

Paulesu, L., King, A., Loke, Y.W., Cintorino, M., Bellizzi, E., and Boraschi, D. 1991. Immunohistochemical localization of IL-1α and IL-1β in normal human placenta. *Lymphokine and Cytokine Research* 10: 443-448.

Peng, C., Huang, T.H., Jeung, E.B., Donaldson, C.J., Vale, W.W., and Leung, P.C. 1993. Expression of the type II activin receptor gene in the human placenta. *Endocrinology* 133:3046-3049.

Petraglia, F., Vaughan, J., and Vale, W. 1989. Inhibin and activin modulate release of GnRH, hCG and progesterone from cultured human placental cells. *Proc. Natl. Acad. Sci. USA.* 86:5114-5117.

Petraglia, F., Woodruff, T.K., Botticelli, G., Botticelli, A., Genazzani, A.R., Mayo, K.E., and Vale, W. 1992. Gonadotropin-releasing hormone, inhibin, and activin in human placenta: evidence for a common cellular localization. *Journal of Clinical Endocrinology & Metabolism* 74:1184-1188.

Petraglia F, Anceschi MM. Calza L. Garuti GC. Fusaro P. Giardino L. Genazzani AR. Vale W. 1993 Inhibin and activin in human fetal membranes: evidence for a local effect on prostaglandin release. *Journal of Clinical Endocrinology & Metabolism* 77(2): 542-548.

Petraglia, F., Florio, P., Nappi, C., and Genazzani, A.R. 1996. Peptide Signaling in Human Placenta and Membranes: Autocrine, Paracrine, and Endocrine Mechanisms. *Endocrine Reviews* 117(2): 156-186.

Rao, C.V, Ramani, N., Chegini, N., Stadig, B.K., Carman, F.R, Jr.Woost, P.G., Schultz, G.S., and Cook, C.L. 1985. Topography of human placental receptors for epidermal growth factor. *Journal of Biological Chemistry* 260:1705-1710.

Reddi, A.H. and Cunningham, N.S. 1993. Initiation and promotion of bone differentiation by bone morphogenetic proteins. *J. Bone miner Res* 8 (Suppl.2) S499- S502.

Reshef, E., Lei, Z.M., Rao, C.V., Pridham, D.D., Chegini, N., and Luborsky, J.L. 1990. The presence of gonadotropin receptors in nonpregnant human uterus, human placenta, fetal membranes and decidua. *Journal of Clinical Endocrinology & Metabolism* 70: 421-430.

Roberts, A.B., Anzano, M.A., Lamb, L., Smith, J., and Sporn, M.B.1981. New

class of transforming growth factors potentate by epidermal growth factor. *Proc. Natl. Acad. Sci. USA* 78:5339-5343.

Roberts, J.M., and Redman, C.W.G. 1993. Pre-eclampsia: more than pregnancy-induced hypertension. *Lancet* 341:1447-1454.

Sampath, T.K., Rashka, K.E., Doctor, J.S., Tucker, R.F., and Hoffmann, F.M. 1993. Drosophila transforming growth factor β superfamily proteins induce endochondral bone formation in mammals. *Proc. Natl. Acad. Sci.* 90: 6004-6008.

Sandberg, M.M., Aro, H.T., and Vuorio, E.I. 1993. Gene expression during bone repair. *Clinical Orthopaedics & Related Research* 289:292-312.

Schlunegger, M.P. and Grutter, M. G. 1992. An unusual feature revealed by the crystal structure at 2.2 A resolution of human transforming growth factor-β2. *Nature* 358: 430-434.

Sekiya, S., Kuwata, T., Ishige, H., Tomita, Y., and Takamizawa, H. 1986. Effects of human interferons on human choriocarcinoma cells in vitro and in vivo. *Gynecologic Oncology* 25:115-124.

Serrano, M. Hannon GJ. Beach D. 1993. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 366: 704-707.

Seyedin, S.M., Thomas, T.C., Thompson, A.Y., Rosen, D.M., and Piez, K.A, 1985. Purification and characterization of two cartilage-inducing factors from bovine demineralized bone. *Proc. Natl. Acad. Sci.USA* 82:2267-2271.

Sherr, C.J, Rettenmier, C.W., Sacca, R., Roussel, M.F., Look, A.T., Stanley, E.R. 1985. The c-fms proto-oncogene product is related to the receptor for CSF-1. *Cell* 41: 665-676.

Shull, M., Ormsby, I., Kier, A.B., Pawlowski, S., Diebold, R.J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., Annunziata, N., and Doetschman, T. 1992. Targeted disruption of the mouse transforming growth factor beta 1 gene results in multifocal inflammatory disease. *Nature* 359: 693-699.

Shi,Q.J., Lei, Z.M., Rao, C.V., and Lin, J. 1993. Novel role of hCG in differentiation of human cytotrophoblasts. *Endocrinology* 132(3): 1387-1395.

Sibilia, M. and Wagner, E.F. 1995. Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science* 269:234-238.

Smith, A.G., Heath, J.K., Donaldson, D.D., Wong, G.G., Moreau, J., Stahl, M., and Rogers, D. 1988. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* 336:688-690.

Soares, M.J. 1993. Trophoblast cell differentiation: Expression of the placental prolactin family. *In Trophoblast Cells: Pathways for Maternal-Embroynic Communication* pp 45-67 Springer-verlag, New York

Soares, M.J., Chapman, B.M., Rasmussen, C.A., Dai, G., Kamei, T., and Orwig, K.E. 1996. Differentiation of trophoblast endocrine cells. *Placenta*17: 277-289.

Spencer, F.A., Hoffmann, F.M., and Gelbart, W.M. 1982. Decapentapleyic: A gene complex affecting morphogenesis in Drosophila melanogaster. *Cell* 28: 451-461.

Stephanou, A., Sarlis, N.J., Richards, R., and Handwerger, S. 1994. Expression of retinoic acid receptor subtypes and cellular retinoic acid binding protein-2 mRNA during differentiation of human trophoblast cells. *Biochemical and Biophysical Research Communication* 202 (2) : 772-780.

Stephanou, A., Myatt, L., Eis, A.L., Sarlis, N., Jikihara, H., and Handwerger, S. 1995. Ontogeny of the expression and regulation of IL-6 and IL-1 mRNAs by human trophoblast cells during differentiation in vitro. *Journal of Endocrinology* 147:487-496.

Steven, D.H. 1983 Interspecies differences in the structure and function of trophoblast. *Biology of Trophoblast* pp 111-137 Elsevier Press

Stewart, C.L,, . Kaspar, P., Brunet, L.J., Bhatt, H., Gadi, I., Kontgen, F., and Abbondanzo, S.J. 1992. Blastocyst implantation depends on maternal expression of leukemia inhibitory factor. *Nature* 359:76-79.

Storm, E.E., Huynh, T.V., Copeland, N.G., Jenkins, N.A., Kinsley, D.M., and Lee, S-J. 1994. Limb alterations in brachypodism mice due to mutations in a new member of TGF-β superfamily. *Nature* 368:639-643.

Stoz, F., Schuhmann, R.A., and Schmid, A. 1987. Morphometric investigations of terminal villi of diabetic placentas in relation to the White classification of diabetes mellitus. *J. Perinat. Med.* 15: 193-198.

Stoz, F., Schuhmann, R.A., and Schultz, R. 1988. Morphohistormetric investigations of placentas of diabetic patients in correlation to the metabolic adjustment of the disease. *J. Perinat. Med.*16: 211-216.

Tao, G. and Hertig, J. 1965.Viability and differentiation of human trophoblast in culture. *Am. J. Anat.* 116: 315-328.

ten Dijke, P., Hansen, P., Iwata, K.K., Pieler, C., and Foulkes, J.G.1988. Identification of another member of transforming growth factor type β gene family. *Proc. Natl. Acad. Sci. USA* 85: 4715-4719.

Thomsen, G.H., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W., and Melton, D.A. 1990. Activins are expressed early in Xenopus embryogenesis and can induce axial mesoderm and anterior structures. *Cell* 63: 485-493.

Thomsen, G.H. and Melton, D.A. 1993. Processed Vg1 protein is an axial mesoderm inducer in *Xenopus*. *Cell* 74:433-441.

Threadgill, D.W., Dlugosz, A.A., Hansen, L.A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R,C., et al. 1995. Targeted distribution of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science* 269:230-234.

Urist, M.R. 1965. Bone: formation by autoinduction. *Science*150: 893-899.

Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Karr, D., and Spiess, J. 1986. Purification and characterization of a FSH releasing protein from porcine ovarian follicular fluid. *Nature* 321: 776-779.

Vassalli, A., Matzuk, M.M., Gardner, H.A., Lee, K-F., and Jaenish, R. 1994. Activin/inhibin β B subunit gene disruption leads to defects in eyelid development and female reproduction. *Gene Development* 8: 414-417.

Vigier, B., Forest, M.G., Eychenne, B., Bezard, J., Garrigou, O., Robel, P., and Josso, N. 1989. Anti-mullerian hormone produces endocrine sex reversal of fetal ovaries. *Proc. Natl. Acad. Sci. USA* 86:3684-3688.

Weeks, D.L. and Melton, D.A. 1987. A maternal mRNA localized to the vegetal hemisphere in Xenopus eggs codes for growth factor related to TGF-β. *Cell* 51:861-867.

Wegmann, T. and Guilbert, L. 1992. Immune signaling at the maternal-fetal

Interface and trophoblast differentiation. *Developmental and comparative immunology* 16: 425-430.

Wergedal, J.E., Matsuyama, T., and Strong, D.D. 1992. Differentiation of normal human bone cells by transforming growth factor-β and $1,25(OH)_2$ Vitamin D_3. *Metabolism: Clinical & Experimental* 41:42-48.

Wharton, R.A., Ray, R.P., and Gelbart, W.M. 1993. An activity gradient of decapentaplegic is necessary for the specification of dorsal pattern elements in the Drosophila embryo. *Development* 117: 807-822.

Williams, R.L., Hilton, D.J., Pease, S., Willson, T.A., Stewart, C.L., Gearing, D.P., Wagner, E.F., Metcalf, D., Nicola, N.A., and Gough, N.M. 1988. Myeloid leukemia inhibitory factor maintain the developmental potential of embryonic stem cells. *Nature* 336: 684-687.

Wozney, J.M., Rosen, V., Celeste, A.J., Mitsock, L.M., Whiters, M.J., Kriz, R.W., Hewick, R.M., and Wang, E.A. 1988. Novel regulators of bone formation: molecular clones and activities. *Science* 242:1528-1534.

Wozney, J.M. 1992. The bone morphogenetic protein family and osteogenesis. *Molecular and Reproductive Development* 32:160-167.

Wrana, J.L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X-F., and Massague, J. 1992. TGF-β signals through a heteromeric protein kinase receptor complex. *Cell* 71:1003-1014.

Wrana, J.L., Attisano, L., Wieser, R., Ventura, F., and Massague, J. 1994. Mechanism of activation of the TGF-β receptor. *Nature* 370: 341-347.

Wrana, J.L. 1998. TGF-beta receptors and signalling mechanisms.

Current Opinion in Cell Biology 10(2):188-94.

Wrann, M., Bodmer, S., de Martin, R., Siepl, C., Hofer-Warbinek, R., Frei ,K., Hofer, E., and Fontana, A. 1987. T cell supressor factor from human glioblastomaa cells is a 12.5 kd protein closely related to transforming growth factor-β. *EMBO Journal* 6:1633-1636.

Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. 1993. p21 is a universal inhibitor of cyclin kinases. *Nature* 366:701-704.

Yagel, S., Lala, P.K., Powell, W.A., and Casper, R.F. 1989. Interleukin-1 stimulates hCG secretion by first trimester human trophoblast. *Journal of Clinical Endocrinology & Metabolism* 68: 992-995.

Yamaguchi, A., Katagiri, T., Ikeda, T., Wozney, J.M., Rosen, V., Wang, E.A., Kahn, A.J., Suda, T., and Yoshiki, S. 1991. Recombinant bone morphogenetic protein 2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro. *Journal of Cell Biology* 113:681-687.

Yang, J.T., Rayburn, H., and Hynes, R.O. 1995. Cell adhesion events mediated by α_4 integrins are essential in placenta and cardiac development. *Development* 121: 549-560.

Yingling, J.M., Wang, X-F., and Bassing, C.H. 1995. Signaling by the transforming growth factor-beta receptors. *Biochimestry & Biophysics Acta* 1242:115-136.

Yokoyama-Kobayashi, M., Saeki, M., Sekine, S., and Kato, S. 1997. Human cDNA encoding a novel TGF-β superfamily protein highly expressed in placenta. *Journal of Biochemistry* 122:622-626.

Zheng, Q-X., Tease, L.A., Shupert, W.L., and Chan, W.Y. 1990. Characterization

of cDNAs of the human pregnancy-specific β1 glycoprotein family, a new subfamily of the immunoglobin gene superfamily. *Biochemitry* 29: 2815-2852.

Zmuidzinas, A., Fisher, K.D., Lira, S.A., Forrester, L., Bryant, S., Bernstein, A., and Barbacid, M. 1995. The *vav* protooncogene is required early in embryogenesis but not for hematopoietic development in vitro. *EMBO Journal* 14:1-11.

Zou, H. and Niswander, L. 1996. Requirement for BMP signaling in the interdigital apoptosis and scale formation. *Science* 272: 738-741.

Zhou, X., Sasaki, H., Lowe, L., Hogan, B.L., and Kuehn, M.R. 1993. Nodal is a novel TGF-β like gene expressed in the mouse node during gastrulation. *Nature* 361: 543-547.

Zhou, Y., Damsky, C.H., Chiu, K., Roberts, J.M., and Fisher, S.J. 1993. Preeclampsia is associated with abnormal expression of adhension molecules by invasive cytotrophoblast. *Journal of Clinical Investigation* 91: 950-960.