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Human Placental Trophoblast As A Model For Tumor Progression

Nelson K. Khoo

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ABSTRACT

The human placenta is a highly invasive structure. Extravillous trophoblast (EVT) cells of the placenta invade the uterus and its vasculature to establish a proper maternal-fetal exchange of key molecules. This laboratory has shown that mechanisms responsible for EVT cell invasiveness in vitro are similar to those of malignant tumor cells, but unlike tumor cells, their invasiveness is stringently regulated in situ by locally derived factors. Furthermore, they are not tumorigenic in nude mice, thus invasiveness alone is insufficient for malignancy. The long term goal of this laboratory is to transform normal EVT cells for a stepwise induction of premalignant, malignant and/or metastatic phenotypes in order to identify genetic changes relevant for tumor progression.

As a first step, SV40 T antigen (Tag) was successfully introduced into the mortal, invasive first trimester EVT cell line, HTR8, resulting in a long lived line, RSVT-2, and a postcrisis immortal line, RSVT2/C. The first objective of this study was to characterize these SV40 Tag transformed lines. Both lines exhibited EVT cell markers cytokeratin and cytoplasmic hPL, and are more proliferative and invasive than the parental HTR8 cells. The increased invasiveness could be explained by a reduced expression of tissue inhibitor of metalloprotease (TIMP)-1 mRNA in both cell lines and, in addition, a reduced expression of TIMP-2, as well
Human Placental Trophoblast as a Model for Tumor Progression

by

Nelson K. S. Khoo

Department of Anatomy & Cell Biology

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
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ABSTRACT

The human placenta is comprised of extravillous trophoblast (EVT) cells which invade the uterus and its vasculature establishing maternal-fetal exchange of key molecules. The mechanisms of in vitro EVT cell invasiveness are similar to those of malignant tumor cells but are regulated in situ by locally derived factors. This invasiveness is insufficient for malignancy. The goal of this laboratory is to transform normal EVT cells and induce premalignant, malignant and/or metastatic phenotypes to identify genetic changes relevant to tumor progression.

Two SV40 T antigen (Tag) transformed cell lines (long lived RSVT-2; immortal RSVT2/C) derived from the invasive first trimester EVT cell line, HTR8 were characterized. Both lines exhibited EVT cell markers cytokeratin and cytoplasmic hPL, and were more proliferative and invasive than the parental cells. Increased invasiveness can be explained by i) reduced expression of tissue inhibitor of metalloprotease (TIMP)-1 mRNA in both lines and ii) reduced expression of TIMP-2, and plasminogen activator inhibitor (PAI)-1 in RSVT2/C cells. RSVT-2 cells retained sensitivity to the anti-proliferative action of TGFβ. RSVT2/C cells were also resistant to its anti-invasive action. Resistance to the anti-invasive action of TGFβ may be explained by a lack of TGFβ-mediated upregulation of TIMP-1 and PAI-1 mRNA noted with normal HTR8 cells. These results, and the finding that neither cell line was tumorigenic in nude mice, suggest
that the SV40 Tag transformants acquired a "premalignant" phenotype, and RSVT2/C cells were possibly more advanced along the tumor progression pathway.

GJIC (dye coupling) and the expression of various connexin proteins and mRNA were also compared in HTR8 and SV40 Tag transformed cells. Only Cx43 was expressed by HTR8 cells. This expression was reduced in RSVT-2, and undetectable in RSVT2/C cells. GJIC was also reduced in RSVT-2 cells, and drastically reduced in RSVT2/C cells. TGFβ reduced Cx43 mRNA expression and GJIC in HTR8 cells, but not in the SV40 Tag transformants. These findings suggest that a down regulation of connexins and resultant impairment in GJIC are early events in tumor progression.

Gene(s) gained or lost during normal EVT cell transition to premalignancy achieved by SV40 Tag immortalization were also examined. Using differential display, a putatively novel gene was isolated. This gene was identified in RSVT2/C and JAR choriocarcinoma cells but not in the RSVT-2 or HTR8 cells. Sequencing revealed partial homology with Homo sapiens clone 135069 and EST 29905 cDNA. A complete sequence and the function of this gene remain undetermined.
ABSTRACT

The human placenta is a highly invasive structure. Extravillous trophoblast (EVT) cells of the placenta invade the uterus and its vasculature to establish a proper maternal-fetal exchange of key molecules. This laboratory has shown that mechanisms responsible for EVT cell invasiveness in vitro are similar to those of malignant tumor cells, but unlike tumor cells, their invasiveness is stringently regulated in situ by locally derived factors. Furthermore, they are not tumorigenic in nude mice, thus invasiveness alone is insufficient for malignancy. The long term goal of this laboratory is to transform normal EVT cells for a stepwise induction of premalignant, malignant and/or metastatic phenotypes in order to identify genetic changes relevant for tumor progression.

As a first step, SV40 T antigen (Tag) was successfully introduced into the normal, invasive first trimester EVT cell line, HTR8, resulting in a long lived line, RSVT-2, and a postcrosis immortal line, RSVT2/C. The first objective of this study was to characterize these SV40 Tag transformed lines. Both lines exhibited EVT cell markers cytokeratin and cytoplasmic hPL, and are more proliferative and invasive than the parental HTR8 cells. The increased invasiveness could be explained by a reduced expression of tissue inhibitor of metalloprotease (TIMP)-1 mRNA in both cell lines and, in addition, a reduced expression of TIMP-2, as well
as plasminogen activator inhibitor (PAI)-1 in RSVT2/C cells. RSVT-2 cells retained sensitivity to the anti-proliferative, but not the anti-invasive action of TGFβ, whereas RSVT2/C cells were resistant to both TGFβ actions. Their resistance to the anti-invasive action of TGFβ could be explained by a lack of TGFβ-mediated upregulation of TIMP-1 and PAI-1 mRNA, which was noted with normal HTR8 cells; TGFβ also upregulated 72 kDa type IV collagenase mRNA in RSVT-2 cells. These results, combined with the findings that neither cell line was tumorigenic in nude mice, suggested that the SV40 Tag transformants acquired a "premalignant" phenotype, and RSVT2/C cells were possibly more advanced along the tumor progression pathway.

Since an impairment of gap junctional intercellular communication (GJIC) has been reported in preneoplastic and neoplastic cells, the second part of this study compared GJIC and the expression of connexin proteins (immunostaining) and mRNA (northern analysis) in HTR8 and SV40 Tag transformed cells. Cx43, but not Cx32, Cx26, Cx40, or Cx33, was expressed by HTR8 cells. This expression was reduced in RSVT-2, and undetectable in RSVT2/C cells. GJIC, as measured by dye coupling, was also similarly reduced in RSVT-2 cells, and drastically reduced in RSVT2/C cells (HTR8 >> RSVT-2 >> RSVT2/C). TGFβ reduced Cx43 mRNA expression as well as GJIC in HTR8 cells, but not in the SV40 Tag transformants. These findings suggested that a down regulation of
connexins with the resultant impairment in GJIC is an early event in tumor progression.

The third objective of this study was to identify gene(s) gained or lost during the transition of normal EVT cells to a premalignant stage achieved by SV40 Tag immortalization. Using the differential display method, a putatively novel gene, 7-4, was initially isolated from HTR8/SVneo, an SV40 Tag immortalized derivative of parental HTR8 cells. This gene was identifiable in the immortal RSVT2/C as well as JAR choriocarcinoma cells but not in the long lived RSVT-2 or mortal HTR8 cells. Partial sequencing (357 nucleotides at the 5' end, 267 nucleotides at the 3' end) revealed some homology with Homo sapiens (clone 135069 and EST 29905) cDNA. A complete sequence as well as the function of this gene remain undetermined. It is speculated that this gene may be relevant to the acquisition of immortality.
Dedication

To my friends,

For their friendship, care and encouragement
Acknowledgements

I would like to thank my supervisor, Dr. Peeyush K. Lala, for his support, encouragement, and helping hand (even when I sometimes failed to recognize my need for help). I am extremely grateful for his tutelage, friendship (though at times I may not have deserved it) and for his care and words of wisdom throughout my studies. It is with respect and admiration for his drive and love of science (that he has passed onto to all his students) that I would like to say “Thank you”.

Throughout my years here, I have also been lucky to have so many great people that I have been able to call friends. I would like to thank all of them (Scot Hamilton, Nahla al-mutter, Trevor Shepherd, Amila Orucevic, Andrew Athanassiades, Ian Connelly, Jeff Lysiak, Walter Rushlow, Yuchun Zhang, and many more) for making my stay here enjoyable, for their friendship and for listening to my problems.

I would like to thank Dr. Francis Chan, for his advice and friendship during my stay here at Western. I will always try to remember his advice.

I am indebted to Dr. Shari L. Bond for her friendship, encouragement, and help in the lab and during my writing exile. I am extremely grateful for Shari and Ian for their generosity to allow persons of no fixed address to stay at their home for extended period of time. Thank you very much. I will try to follow their example. “Done yet?” Yes!

I am also indebted to Mr. John Bechberger for his friendship, unlimited patience for the "northern impaired”, and help in the lab. I am very appreciative of the generosity of John and Lynne for opening their home to the homeless for extended stays. Thank you very much. I will try to be as generous as they are.

I would like to thank Dr. Christian Naus for his generosity, advice, friendship, and many collaborations that he has allowed me to participate.

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I would like to thank everyone in the department and hope they will forgive me if I have not mentioned them... but I will remember them all.

Last but not least, I would like to say many thanks to Evelyn—my best friend, my strongest supporter, and my many hands to do the many things that I neglect (like packing for another life in another country). Thank you Evelyn for I could not have done so much without you.
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<tbody>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>Anti-TGFα</td>
<td>anti transforming growth factor alpha antibody</td>
</tr>
<tr>
<td>AR</td>
<td>amphiregulin</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>Cx</td>
<td>connexin</td>
</tr>
<tr>
<td>DD</td>
<td>differential display</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>dideoxynucleotide triphosphate, where N=cytosine</td>
</tr>
<tr>
<td>EACA</td>
<td>epsilon-aminocaproic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFr</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EVT</td>
<td>extravillous trophoblast</td>
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</table>
GJ      gap junction
GJIC    gap junctional intercellular communication
HLA     human leukocyte antigen
hPL     human placental lactogen
$^{3}$H-TdR tritiated thymidine
HTR8    human trophoblast cell line 8
IGF-n   insulin growth factor, where n=I, II
IGFBP-n insulin growth factor binding protein, where n=1, 2, 3, 4, 5, 6
LAP     latency-associated protein
LRP     lipoprotein receptor-like protein
MMP     matrix metalloproteases
mRNA    messenger ribonucleic acid
MT-MMP  membrane type matrix metalloprotease
NDOG    Nuffield Department of Obstetrics and Gynecology
MTS     (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxybrethoxyphenyl)) -2-(4-sulfophenyl)-2H-tetrazolium, inner salt
PAI-n   plasminogen activator inhibitor, where n= 1,2
PBS     phosphate buffered saline
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>PBS-EDTA</td>
<td>phosphate buffered saline-ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PMS</td>
<td>phenazine methosulfate</td>
</tr>
<tr>
<td>pRSVT</td>
<td>plasmid Rous sarcoma virus driven SV40 Tag oncogene</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine-glycine-aspartic acid tripeptide</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>STn</td>
<td>stromelysin, where n=1,2,3</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>SV40 Tag</td>
<td>Simian virus 40 large T antigen</td>
</tr>
<tr>
<td>TGFα</td>
<td>transforming growth factor α (alpha)</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor β (beta)</td>
</tr>
<tr>
<td>TIMP-n</td>
<td>tissue inhibitor of metalloproteases (where n=1,2,3)</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue-type plasminogen activator</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>uPAR</td>
<td>urokinase-type plasminogen activator receptor</td>
</tr>
</tbody>
</table>
Units of Measure

μCi  microCurie

μF  microFaraday

μl  microlitre

μm  micrometre

cm  centimetre

h  hour

kDa  kilodalton

M  molar

min  minute

ml  millilitre

mM  millimolar

mmol  millimoles

ng  nanogram

°C  degrees Celsius

sec  second

V  Volts

W  Watts
Chapter 1

1.1 Introduction

Invasion is a feature shared by all malignant tumors, and by the trophoblast-derived component of the hemochorial type of placenta in many species including human/primate, and rodent. Invasion, first recognizable at the hatched blastocyst stage of the embryo during implantation, allows trophoblast cells to breach the uterine epithelium and its basement membrane, and is dependent on the ability of the cells to secrete proteolytic enzymes (Denker, 1977). In contrast to tumor invasion of the host tissue, trophoblastic invasion of the uterus during placental development is stringently controlled, both spatially and temporally, so that in the human, it is limited to the endometrium-myometrium or endometrium-endothelial junctions and stops during mid-gestation. In addition, the human placenta grows exponentially and proliferates at a rapid rate during the first and early second trimesters of gestation. The rate of trophoblast-derived tissue growth and its invasive nature rivals that of the most aggressive tumors. Thus, these pseudomalignant features of the early to mid gestation placenta provide a unique model to study the mechanism of tumor progression.

Tumor progression is dependent upon a number of mechanisms which allow tumor cells to grow, invade, and migrate: (a) interaction with autocrine or
paracrine growth factors in the surrounding environment via specific receptors; (b) the cell's ability to invade the basement membrane and extracellular matrix using matrix degrading enzymes; and (c) interactions with the constituents of the extracellular matrix via integrins and other cell surface receptors, which may modulate invasion and migration. Some of these mechanisms are also shared by the first trimester extravillous trophoblast (EVT) cells of the human placenta, in particular those required for their invasive ability (Yagel et al. 1988). However, in contrast to the behavior of tumor cells, the proliferative, invasive and migratory behavior of trophoblast cells is tightly regulated in situ (Graham and Lala, 1992; Lala and Lysiak, 1995). Nevertheless, studies of the placental trophoblast have yielded many insights into both normal development and tumorigenesis.

Since invasive ability is shared by the invasive and/or metastatic tumor cells as well as the invasive, but nontumorigenic and nonmetastatic trophoblast cells, it is clear that this property is an essential, but not a sufficient, prerequisite for tumorigenesis or the metastatic process. Unfortunately, however, many investigations in tumor biology have failed to consider this important point. In many tumors, the invasive characteristic is acquired, thus leading, in many investigations, to the characterization of invasion-associated enzymes as the markers for tumor progression and metastasis. Given the many similarities in behavior between human first trimester EVT and malignant tumor cells, this
project was designed to exploit the invasive, but nonmalignant, nature of the EVT cells to investigate the genetic changes that occur early following SV40 large T antigen (SV40 Tag) transformation of these cells.

Since SV40 Tag transformation of the normal human EVT cells led to a premalignant (hyperproliferative and hyperinvasive, but nontumorigenic) phenotype, it was hoped that a comparison of the transformed cells with the parental cells would allow detection of genetic alterations responsible for the premalignant phenotype, distinct from invasion-associated enzymes. The purpose of this thesis was to examine the effects of SV40 Tag transformation on functional responses to certain growth factors which regulate normal trophoblast behavior, the expression of invasion-associated enzymes, and the repertoire of genes expressed. These studies will form the basis for future studies of the genetic mechanisms responsible for trophoblastic tumor progression, i.e., progression to choriocarcinoma.
1.2 Historical Review

1.2.1 The Human Placenta: Trophoblast Invasion In Situ

The human placenta is an invasive organ of fetal origin that allows for the proper development of the embryo by bringing the fetal blood circulation into proximity to the maternal blood with an intervening thin tissue barrier known as the placental membrane (Boyd and Hamilton, 1970). Placental invasion of the uterus also brings fetally-derived trophoblast cells into close proximity to maternally-derived decidual tissue, creating a feto-maternal tissue interface responsible for molecular interaction between the fetal and maternal cells, and is necessary for the growth, and survival of the embryo (Strickland and Richards, 1992).

Normal placental development in the human begins shortly after fertilization. The blastocyst implants into the posterior wall of the uterus after attaching itself to the endometrial epithelium (Pijnenborg, 1990; Aplin, 1991). Subsequently, trophoblast cells of the blastocyst transgress the epithelium, and begin to invade into the endometrial stroma of uterus. The uterine epithelium soon re-epithelializes and seals the blastocyst within the endometrial stroma. Thereafter, fetally-derived mononuclear cytotrophoblast and multinucleated syncytiotrophoblast cells are observed intermixed with the maternal decidual cells (Boyd and Hamilton, 1970).
Within the mononucleated cytotrophoblast population, cells undergo two morphologically and functionally distinct differentiation pathways to give rise to villous and intermediate or extravillous trophoblast (EVT) cells (Kurman et al. 1984). The EVT cells invading the uterine stroma also invade the endometrial spiral arterioles replacing their endothelial cell lining with trophoblast cells (called endovascular trophoblasts), thus establishing the human hemochorial placenta (Pijnenborg et al. 1980; Kurman et al. 1984; Pijnenborg, 1990). The blood flows into sinusoidal spaces surrounding primary villi, lined by an outer layer of noninvading, multinucleated syncytiotrophoblast cells which arise by proliferation and fusion of noninvading, mononucleated cytotrophoblast cells in the underlying layer attached to a basement membrane (Boyd and Hamilton, 1970; Kurman et al. 1984).

Secondary villi begin to form when the extraembryonic mesoderm penetrates the primary villus cores. Blood vessels linked to the fetal circulation begin to form inside the secondary villus cores, transforming them into tertiary villi. Tertiary villi grow rapidly to form floating villi, which are tree-like projections bathed in maternal blood. Some of these villus projections anchor to the uterine decidual tissue by columns of intermediate cytotrophoblast cells which perforate through the syncytiotrophoblast layer. These become sites of attachment for the developing placenta, and are then termed anchoring villi. At these sites of attachment, the
invasive EVT cells can migrate as a continuous layer along the decidual surface of the sinusoids to form cytotrophoblastic shell. Others remain dispersed in the decidua as the interstitial trophoblasts or fuse to form the placental bed multinucleated giant cells. Yet others are seen to remodel the spiral arteries as the endovascular trophoblast which replaces the endothelium, extending to the endometrium-myometrium junction (Boyd and Hamilton, 1970; Pijnenborg et al. 1980; Aplin, 1991).

It is evident that proliferation, migration, and invasion are three essential functions of the trophoblast required for the development of the hemochorial type of placenta. These functions are also hallmark properties of malignant tumor cells. However, unlike malignant tumors, the placenta does not destroy the uterus. What controls trophoblast invasion in situ to maintain utero-placental homeostasis? This question was addressed in the 1960's by Kirby (1960; 1963a; b; 1964) who designed elegant experiments in the murine model. He examined the in vivo invasiveness of murine trophoblast cells by transplanting the blastocyst and the ectoplacental cone orthotopically into the uterus or ectopically into the testes, or underneath the kidney capsule of recipient mice. Histologically, trophoblast invasion was observed to be greater in the nonpregnant uterus and extra-uterine sites than in the decidualized, pseudopregnant, or the pregnant uterus. Kirby concluded that the murine trophoblast cells were inherently invasive, and that
invasion is likely controlled by maternal decidual tissue. However, Kirby was unable to conclude whether the invasion controlling functions of the decidua were mediated by mechanical (barrier function) or chemical means. In the rabbit and the cat, Denker (1977) showed that the trophoblast cells attaching to the uterus in these animals were invasive. Degradation of the stroma was postulated to involve a number of metallo-, serine- and thiol-proteolytic enzymes (Denker, 1977). However, it remained unknown what controlled their invasive function.

A number of pregnancy associated diseases afflicting human females have been attributed to abnormal trophoblast invasion. For example, choriocarcinomas, ectopic pregnancy, and placenta accreta show high degrees of trophoblast invasion and a poor decidual response (Billington, 1971), while preeclampsia exhibits shallow invasion, resulting in inadequate remodeling of the spiral arteries (see review Cross et al. 1994). An understanding of the pathobiology of these diseases requires an understanding of the mechanisms underlying trophoblast invasiveness and its control in normal pregnancy.

1.2.1.1 In vitro studies on trophoblast invasion

In vitro studies of mechanisms responsible for trophoblast invasion and its control have been limited by the difficulty in isolating and propagating EVT cells which have retained their phenotype and functions e.g. proliferative, invasive and
migratory abilities. Some of the controversies in the literature can possibly be explained by the differences in the trophoblast subpopulations isolated by different techniques, and employed in those studies.

A number of laboratories have cultured chorionic villus explants on a gelatinous substrate e.g. reconstituted basement membrane or collagen gel to mimic trophoblast invasion in situ. Fisher and associates demonstrated that chorionic villus explants from first and second trimesters, but not villi from third trimester, were capable of adhering to and degrading a basement membrane-like matrix (Fisher et al. 1985; 1989a). Sprouting of EVT-like cells with transient invasive ability has been shown to occur in first trimester chorionic villus explants plated on Matrigel (Genbacev et al. 1992; 1993), rat tail collagen plus decidual cell extract (Vicovac et al. 1993) or decidual tissue fragments (Genbacev et al. 1992; 1993; Vicovac et al. 1995). Vicovac et al. (1995) found that EVT cells both invaded deep into the decidual fragments and differentiated to placental bed giant cells. These phenomena recapitulate the events previously reported in situ (Pijnenborg et al. 1980; Kurman et al. 1984).

Villous cytotrophoblast cells are traditionally isolated by enzymatic dispersion of chorionic villi followed by density gradient fractionation (Kliman et al. 1986). Using first, second, and third trimester human cytotrophoblast cells,
isolated by enzymatic dispersion, Fisher et al. (1989b) demonstrated that only first trimester cytotrophoblast cells were capable of matrix degradation in a Matrigel invasion assay, and concluded that trophoblast invasiveness is developmentally regulated during the course of human gestation. In contrast, Kliman and associates (1990) demonstrated that both first trimester and term human cytotrophoblast cells were capable of degrading Matrigel. Furthermore, Graham and associates (1993b) also showed that term trophoblast cells, isolated by the enzymatic dispersion method described by Kliman and associates (1986), were invasive in the human amnion invasion assay. Thus, these latter findings suggest that trophoblast invasion is likely controlled by the microenvironment, and is not pre-programmed.

Freshly isolated first trimester villous trophoblast cells have been used to enrich EVT-like cells by attachment to laminin-coated beads, possibly indicating their preferential adhesiveness to laminin (Loke et al. 1989). First trimester villous cytotrophoblast cells freed from leukocytes when plated on Matrigel have been reported to give rise to EVT-like cells in clusters and invade the matrix (Damsky et al. 1992; Bass et al. 1994b). However, unlike EVT cells in situ which exhibit proliferative ability (Bulmer et al. 1988), these cells fail to proliferate in culture. Our laboratory has used an explant culture method (Yagel et al. 1989a) which has been further refined (Graham et al. 1992; 1993b) to propagate EVT cells having a limited life span. In brief, mechanically derived fragments of first trimester
chorionic villi are allowed to attach on plastic petri dishes, and nonadherent villi are discarded. In about a third of the adherent villi, pure cytotrophoblastic outgrowth occurs with EVT cell phenotype. These cells can be further propagated in culture for up to 12-14 passages (Graham et al. 1992; Irving et al. 1995). Characterization of the phenotype of the migrant trophoblasts growing out of the primary explants and their progeny in longer term culture has shown that these cells express all the in situ markers of the EVT: cytokeratin, HLA class I framework antigen, NDOG-5 antigen, insulin-like growth factors (IGF II) peptide and mRNA, α1, α3, α5, αv and β1 integrin subunits and vitronectin receptor αvβ3/β5, but not α6/β4 integrin (an in situ marker for the villous cytotrophoblast) (Irving et al. 1995). As will be discussed later, these cells have the proliferative (Graham and Lala, 1992; Graham et al. 1992), invasive (Graham and Lala, 1991) and migratory (Irving and Lala, 1995) ability of the EVT cells in situ, making them an excellent tool for in vitro studies of EVT cell function (Graham and Lala, 1992; Lala and Lysiak, 1995).

1.2.2 Shared Mechanisms of Invasion of First Trimester Trophoblast and Metastatic Tumor Cells: Roles of Proteases and Their Natural Inhibitors

It has been established that tumor cells (Liotta, 1986; Yagel et al. 1988; Liotta, 1992; Stetler-Stevenson et al. 1993) and trophoblast cells (Liotta, 1986;
Yagel et al. 1988; Liotta, 1992; Stetler-Stevenson et al. 1993), share a common multistep process of invasion requiring: (a) attachment of cells to the extracellular matrix (ECM) components; (b) degradation of the ECM; and (c) migration through the ECM (Lala and Graham, 1990; Graham and Lala, 1992). Extracellular matrix degradation requires an array of proteolytic enzymes: matrix metalloproteases, serine proteases, and cathepsins (see reviews; Mignatti and Rifkin, 1993; Birkedal-Hansen et al. 1993). Specific activity of these three enzymes in situ depends on the balance between these enzymes and their natural inhibitors. In relation to the studies of invasion, the primary focus has been on two groups—(a) the matrix metalloproteases (MMPs) and tissue inhibitors of metalloproteases (TIMPs) (see reviews; Mignatti and Rifkin, 1993; Stetler-Stevenson et al. 1993; Ray and Stetler-Stevenson, 1994) and (b) the serine proteases, in particular the plasmin/plasminogen activator system and their specific inhibitors (PAI-1, PAI-2) (reviewed by Mignatti and Rifkin, 1993; Birkedal-Hansen et al. 1993).

1.2.2.1 Matrix metalloproteases and their inhibitors: Brief overview

Matrix metalloproteases (MMPs) are synthesized as latent proenzymes requiring activation. They are dependent on Zn$^{2+}$ and Ca$^{2+}$ cations for their activity (Birkedal-Hansen et al. 1993; Ray and Stetler-Stevenson, 1994), and are active at neutral pH (Brown et al. 1990; reviewed by Ray and Stetler-Stevenson, 1994). In addition, the regulation of many of these MMPs can be influenced by certain
growth factors (see section 1.2.3. below). Activation of these enzymes requires autoproteolytic removal of 80 amino acids from the NH₂-terminal (Mignatti and Rifkin, 1993; Birkedal-Hansen et al. 1993). MMPs can also be activated in vitro by treatment with organomercurial compounds, chaotropic agents or other proteases, such as plasmin (Birkedal-Hansen et al. 1993).

MMP activation is a sequential process in which the latent proenzyme is converted to the active proenzyme, and finally to the active enzyme. MMP enzymes share four similar domains: (a) signal peptide domains, (b) propeptide domain containing PRCGXPDV consensus sequence, (c) catalytic domain containing a thermolysin-type Zn²⁺-binding region, and (d) COOH-terminal hemopexin/vitronectin domain (Stetler-Stevenson et al. 1993). In addition, two of the MMPs (72 kDa type IV and 92 kDa type IV collagenases) have extra gelatin-binding domains inserted into the catalytic domain (Stetler-Stevenson et al. 1993).

MMPs are activated through a cysteine switch mechanism. The cysteine residue is coordinated with the Zn²⁺ bound to the catalytic site (Salowe et al. 1992; see reviews by Stetler-Stevenson et al. 1993; Ray and Stetler-Stevenson, 1994), which, when disrupted by physical or chemical means, activates the MMP. Proteolytic cleavage of the propeptide domain follows the disruption of the cysteine switch; thus the loss of the propeptide domain is indicative of the
activation of MMPs (see reviews; Birkedal-Hansen et al. 1993; Stetler-Stevenson et al. 1993). Synthetic peptides of the propeptide domain have been used to inhibit proteolytic activity, and to inhibit the in vitro invasion of human tumor cells, HT1080 and A2058 (Melchiori et al. 1992). Moreover, it has been shown that MMP activation in vivo may require plasmin (see review by Birkedal-Hansen et al. 1993) and the membrane-type MMP (MT-MMP; see review by Chen et al. 1994).

Two main pathways have been proposed for the biological activation of latent MMPs in vivo: (a) the serine protease/plasmin pathway for activation of interstitial collagenase, stromelysin-1 and 92 kDa type IV collagenase, and (b) the integral membrane protease pathway, for example, MT-MMP-mediated activation of 72 kDa type IV collagenase.

In contrast to 92 kDa type IV collagenase, stromelysins and interstitial collagenase which may be activated by a plasmin dependent pathway (reviewed by Mignatti and Rifkin, 1993; Birkedal-Hansen et al. 1993), 72 kDa type IV collagenase has been shown to be activated by the cell surface-associated MT-MMP (Strongin et al. 1994; Sato et al. 1994). Expression and activity of MT-MMP have been found in both normal (Azzam and Thompson, 1992; Yu et al. 1995; Nawrocki et al. 1995) and malignant cells (Azzam and Thompson, 1992; Chen et al. 1994; Nomura et al. 1995); it was observed that expression of MT-
MMP cDNA transfected into COS-1 cells increased specific activation of pro-72 kDa type IV collagenase and enhanced invasion in vitro (Sato et al. 1994).

Recent reports indicate that MT-MMP can be activated by plasmin, since it has an arg-arg-lys-arg motif upstream from the amino terminus that can serve as a substrate for plasmin or urokinase plasminogen activator (uPA) (Sato et al. 1994; Strongin et al. 1994). The activated MT-MMP subsequently binds TIMP-2, enabling it to activate pro-72 kDa type IV collagenase (Strongin et al. 1994; Sato et al. 1994). TIMP-2 plays an important role in the activation of pro-72 kDa type IV collagenase; it interacts with the carboxyl-end domain of 72 kDa type IV collagenase (Strongin et al. 1994), and is a required part of the cell surface activation complex. Furthermore, 72 kDa type IV collagenase is not activated if the interaction of the carboxyl end of 72 kDa type IV collagenase with TIMP-2 is abolished or if anti-TIMP-2 antibody is present (see review by Ray and Stetler-Stevenson, 1994; Strongin et al. 1994). In contrast to the events occurring on the cell surface, the 72 kDa type IV collagenase complexed with TIMP-2, when activated in vitro, has 20 fold less collagenolytic activity (Fridman et al. 1993). The mechanism by which MT-MMP removes the TIMP-2 from the complex on the cell membrane is currently unknown.
A key characteristic of all the MMPs illustrates a mechanism for regulation of their activity—all are inhibited by tissue inhibitor of metalloproteases (TIMPs) (Mignatti and Rifkin, 1993; Ray and Stetler-Stevenson, 1994). The balance between the levels of activated MMPs and TIMPs determines the net activity and the degree of extracellular matrix remodeling (Stetler-Stevenson et al. 1993). The complexities of the regulation and functions of MMPs and TIMPs have recently been reviewed (Birkedal-Hansen et al. 1993; Mignatti and Rifkin, 1993).

The role and importance of TIMPs in extracellular matrix remodeling has been clearly demonstrated both in culture and in vivo. Human TIMP-1, a glycoprotein with a molecular weight of 28.5 kDa, has been characterized (Stricklin and Welgus, 1983; Welgus et al. 1985; Docherty et al. 1985; Carmichael et al. 1986; Willard et al. 1989). TIMP-1 binds to activated type I collagenase, stromelysin-1, binds activated 72 kDa IV collagenase in vitro, and binds both the activated and the latent 92 kDa type IV collagenase in an equimolecular ratio. This binding inhibits metalloprotease activity (Goldberg et al. 1992; reviewed by Mignatti and Rifkin, 1993; Ray and Stetler-Stevenson, 1994). Most cells producing metalloproteases are also capable of producing TIMP-1; thus, the net collagenolytic activity for these cells is the result of the balance between activated metalloproteases and TIMP-1 levels (Ray and Stetler-Stevenson, 1994).
The second inhibitor, TIMP-2, is a nonglycosylated 21 kDa secreted protein that preferentially binds the latent proenzyme 72 kDa type IV collagenase as well as the activated form of the 72 kDa type IV collagenase enzyme at a 1:1 molar ratio, and also the activated 92 kDa type IV collagenase (Goldberg et al. 1989; Stetler-Stevenson et al. 1989). However, TIMP-2 binding to these two forms of the 72 kDa type IV collagenase enzyme is at two sites (Kleiner et al. 1993; see review, Stetler-Stevenson et al. 1993). It has a role as an inhibitor as well as a co-activation factor for 72 kDa type IV collagenase.

Human TIMP-3 (Apte et al. 1994b; Uria et al. 1994) and murine TIMP-3 (Leco et al. 1994) have recently been cloned and sequenced. Murine TIMP-3, like chicken ChIMP-3, has been found in the ECM, and also inhibits proteolytic degradation of the ECM (Leco et al. 1994). It has been observed in the murine uterus (Leco et al. 1994). Recently, TIMP-3 expression has been reported in the human trophoblast cells (Bass et al. 1994a), and in the spongiotrophoblast in the murine placenta (Apte et al. 1994a).

1.2.2.2 Serine proteases and inhibitors

Plasmin and plasminogen activators play an essential role in invasion and are required in the function of invasive cells, including human trophoblasts of the first trimester human placenta (see section 1.2.2.4.). There are two distinct plasminogen
activators, the tissue-type plasminogen activator (tPA) and the urokinase-type plasminogen activator (uPA). Both of these activators contribute to the conversion of the precursor plasminogen to plasmin (see reviews by Birkedal-Hansen et al. 1993; Mignatti and Rifkin, 1993). Activation of the plasminogen precursor occurs very rapidly when it is bound to its receptor on the cell surface (Plow and Miles, 1990), while free plasminogen is converted to its active form at a lower rate. Plasmin can degrade fibrin as well as cartilage proteoglycan, and although plasmin cannot degrade collagen, it can activate procollagenase (Werb et al. 1977). uPA is the best studied plasminogen activator, and has been found to play a major role in tumorigenesis (Plow and Miles, 1990; Ellis et al. 1993; Murphy et al. 1993).

Both the active and the proenzyme forms of uPA bind by their noncatalytic sites to a specific saturable receptor (uPAr) present on the cell surface (Vassalli et al. 1985; Blasi et al. 1990; Zini et al. 1992). The generation of plasmin requires uPA-uPAr interaction (Ellis et al. 1989; 1991; 1993). This represents a major regulatory mechanism: plasmin activity is restricted spatially to the cell surface, where its effect is exerted in a controlled manner; it is also temporally restricted, since it is rapidly inhibited when dissociated from the cell (Quax et al. 1991; Ossowski et al. 1991a; Ellis et al. 1993).
Matrix degradation by activated plasmin is controlled by PAI-1 and PAI-2, two powerful inhibitors of PAs (Vahe et al. 1992). PAI-1 has been shown to play a role in tumorigenesis (Sordat et al. 1990; Pedersen et al. 1994; Landau et al. 1994; see review by Nip and Brodt, 1995). PAI-1 inactivates uPA:uPAR forming a trimeric complex that is rapidly cleared from the cell surface following binding to the low density lipoprotein receptor-like protein (LRP)/α2-macroglobulin (α2-M) receptor (Orth et al. 1992; Nykjar et al. 1992; Bu et al. 1992). LRP is comprised of two noncovalently associated subunits: a 515 kDa extracellular subunit with a ligand binding region for PAI-1:uPA:uPAR complex (Orth et al. 1992; Nykjar et al. 1992; Bu et al. 1992) and the inhibitory receptor associated protein (RAP) (Herz et al. 1991; Moestrup and Gliemann, 1991; Williams et al. 1992), and a second 85 kDa subunit consisting of the endocytosis signaling sequence (Herz et al. 1990; 1992). The PAI-1:uPA:uPAR complex bound to LRP is internalized (Moestup and Gliemann, 1991; Orth et al. 1992; Nykjar et al. 1992; Bu et al. 1992; Herz et al. 1992), and both LRP and uPAR are recycled to the surface.

1.2.2.3 Matrix degradative enzymes, inhibitors, and cancer

Metastatic spread of malignant tumor cells requires degradation of the basement membrane barrier at both the primary and distant sites. A number of MMPs, as well as serine proteases, have been shown to be elevated during tumor progression (reviewed by Mignatti and Rifkin, 1993). Many of these enzymes are
expressed by the tumor cells themselves; however, in some cases MMPs can be
induced in host stromal cells depending on the type of tumors. The 72 kDa type IV
collagenase (gelatinase A, MMP-2), first characterized from H-ras oncogene
transformed human bronchial epithelial cells (Collier et al. 1988), is produced by
both normal human cell types (Kalebic et al. 1983; Salo et al. 1985; Garbisa et al.
1986), including trophoblast cells (Lala et al. 1989; Graham et al. 1993b; Polette et
al. 1994) and by several human tumor cell types (Hendrix et al. 1990; Monteogudo
et al. 1990) including choriocarcinomas (Graham et al. 1994). In addition, there is
a strong correlation between tumor cell invasiveness and expression of protease
production, e.g., 72 kDa type IV collagenase, during tumor progression (see
reviews by Ray and Stetler-Stevenson, 1994; Stetler-Stevenson et al. 1993).
Elevated 72 kDa type IV collagenase mRNA level was observed in the primary
tumor tissues of breast (Basset et al. 1990), prostate (Pajouh et al. 1991; Stearns
and Wang, 1993) and colon (Levy et al. 1991) suggesting that 72 kDa type IV
collagenase is likely a marker for tumor progression. The 92 kDa type IV
collagenase (gelatinase B and MMP-9; Wilhelm et al. 1989) is also expressed by a
number of normal human cell types (Murphy et al. 1982; Hibbs et al. 1985;
Mainardi et al. 1990), including cytotrophoblast cells (Fisher et al. 1989a; Librach
et al. 1991), and by a number of human tumors (Pyke et al. 1992; 1993; Canete-
Solar et al. 1994).
Interstitial collagenase (MMP-1) is produced by a variety of normal and malignant cells (Birkedal-Hansen et al. 1993), including cells of trophoblastic origin (Emonard et al. 1990). MMP-1 expression is elevated in a variety of human tumors in vivo (Polette et al. 1991; Muller et al. 1991; 1993) and in vitro (Templeton et al. 1990). Increased expression of MMP-1 has been positively correlated with histological differentiation in human colorectal tumors. Pulmonary tumors showed increased expression of MMP-1 in tumor cells but no elevation in the adjacent stromal cells. However, in colorectal cancer and squamous cell carcinomas of the head and neck, there was an observed elevation in MMP-1 transcripts in the stromal cells adjacent to the tumors but not in the tumor cells themselves (Polette et al. 1991; Muller et al. 1991; 1993).

Stromelysins-1 and -2 (ST-1, ST-2) are produced in cultures of normal (Murphy et al. 1989) and transformed cells (reviewed by Birkedal-Hansen et al. 1993). In head and neck tumors, the expression of ST-1 and ST-2 has been localized to the fibroblasts in the adjacent tumor stroma and their expression has been correlated with increased invasiveness (Polette et al. 1991). Stromelysin-3 (ST-3) has also been identified in breast carcinoma as well as the majority of invasive tumors, but its role in invasion is unclear (Basset et al. 1993). Basset et al. (1993) proposed that ST-3 is a stroma-derived factor necessary for the progression of epithelial malignancies and their metastasis, since it is correlated with
progressive disease (Muller et al. 1993). ST-3 protein as well as its mRNA were localized in situ to the stromal cells surrounding the malignant foci with the greatest expression found in stromal cells juxtaposed to tumor cells (Muller et al. 1993). At present, the substrate for ST-3 is not known; its significance in normal development and tissue remodeling remains to be elucidated.

TIMP-1 has been inversely linked to the tumorigenic ability of certain cells (reviewed by Khokha and Denhardt, 1989; Ray and Stetler-Stevenson, 1994). Gene targeted disruption of TIMP-1 increased invasiveness in embryonic stem cells. This increased invasion was reversed by exogenous addition of TIMP-1 (reviewed by Ray and Stetler-Stevenson, 1994). Expression of antisense murine TIMP-1 mRNA has also been reported to induce tumorigenic and metastatic ability in mouse 3T3 cells (Khokha et al. 1989), whereas TIMP-1 mRNA overexpression in B16F10 melanoma cells was demonstrated to reduce invasion (Khokha et al. 1992b) and tumorigenic ability of these cells (Khokha et al. 1992a). These results have supported the role of TIMP-1 as a metastasis suppressor gene (Stetler-Stevenson et al. 1993). TIMP-2 over-expression in the ras transformed rat embryonic fibroblast cell line was demonstrated to reduce growth and invasion of these cells in nude mice (DeClerck et al. 1992). Thus, down-regulation of TIMPs would increase invasiveness of tumor cells by altering the net balance towards higher degradative activity of MMPs.
The requirement of uPAR in the plasmin/PA proteolytic cascade was suggested by \textit{in situ} hybridization showing that uPA and uPAR expression were localized to the leading edge of the invasive tumor (Quigley \textit{et al.} 1990; reviewed by Ellis \textit{et al.} 1993). Thus, the active uPA bound to uPAR on the cell membrane may form a polarized invasion front (Plow \textit{et al.} 1986; Plow and Miles, 1990; Sordat \textit{et al.} 1990; Murphy \textit{et al.} 1993).

In some human tumors, for example in human colon carcinoma, uPA protein was seen in the stromal fibroblasts and uPAR was seen in the tumor cells (see review by Ellis \textit{et al.} 1993), suggesting a possible "cross-talk" between tumor cells and the surrounding stroma. Similar findings were reported for breast tumors (Bianchi \textit{et al.} 1994). uPA expression was elevated in human squamous carcinoma cells, and their invasiveness \textit{in vitro} was reduced in the presence of anti-uPA antibodies (Ossowski \textit{et al.} 1991b). Yu and Schultz (1990) showed that human uPA transfection into B16F1 cells increased their ability to form metastases while B16F1 controls were poorly metastatic. Conversely, transfection of anti-sense uPA into highly metastatic B16F10 resulted in a dramatic reduction of lung metastasis produced by the cell line compared to B16F10 controls. Similarly, Sordat \textit{et al.} (1990) showed that transfection of uPA cDNA in B16 melanoma increased invasion; in contrast, transfection of PAI-1 cDNA decreased their invasiveness. Several other studies have shown that PAI-1 plays a role in modulating tumor
invasion (Pedersen et al. 1994; Landau et al. 1994; Nip and Brodt. 1995). Taken together, these studies indicate that uPA may contribute to tumor invasion and metastasis.

1.2.2.4 Proteases and inhibitors in trophoblast invasion

Human trophoblasts, like tumor cells, produce a number of proteases and their inhibitors. These proteases include: 72 kDa type IV collagenase and 92 kDa type IV collagenase (Librach et al. 1991; Bischof et al. 1991; Fernandez et al. 1992; Graham et al. 1993B; 1994; Polette et al. 1994); interstitial collagenase (Emonard et al. 1990; Moll and Lane, 1990; Graham and Lala, 1991; Librach et al. 1991); MT-MMP (Nawrocki et al. 1995); uPA (Strickland et al. 1976; Queenan et al. 1987; Sappino et al. 1989); and its receptor (Multhaupt et al. 1994). Stromelysin-3 was also detected in the extravillous cytotrophoblasts of first and third trimester placentas (Maquoi et al. 1995). The inhibitors present include: TIMP-1 and TIMP-2 (Graham and Lala, 1991; Zini et al. 1992; Polette et al. 1994) and plasminogen activator inhibitors-1 and -2 (Astedt et al. 1986; Feinberg et al. 1989; Fazleabas et al. 1991; Zini et al. 1992). The broad panel of proteases and inhibitors expressed by the trophoblast cells is similar to those known to be expressed in many tumor cells.
In the Matrigel invasion assay, under serum-free conditions, Librach et al. (1991) reported that invasion by cytотrophoblast cells isolated from chorionic villi was blocked in the presence of an affinity purified anti-92 kDa type IV collagenase polyclonal antibody. They concluded that the 92 kDa type IV collagenase plays a critical role in trophoblast invasion. They also concluded that trophoblast cell invasion was plasmin-independent, since invasion was only partially blocked by the plasmin inhibitors, aprotinin and α2-antiplasmin. Alternatively, Graham and Lala (1992) suggested that the partial inhibition observed in their study may have been due to the inactivation of cell membrane associated plasmin only, since exogenous plasmin was not present in their serum-free incubation media.

As mentioned earlier, this laboratory has successfully propagated first trimester human EVT cells in vitro (Yagel et al. 1989a; Graham et al. 1992; 1993b). These cells were as invasive as metastatic tumor cells when tested with an in vitro amnion invasion assay. To study mechanisms responsible for their invasiveness, the invasion assays were conducted in the presence of metalloprotease inhibitors (1, 10-phenanthroline and recombinant TIMP-1), serine protease inhibitors (trasylol and EACA), anti-plasminogen and anti-uPA antibodies, and a metalloprotease activator (mersalyl) (Yagel et al. 1988). These studies revealed that inhibition of both proteases blocked invasion, whereas mersalyl enhanced invasion even in the presence of the serine protease inhibitor
trasylol. It was also observed that collagenase activity in trophoblast cultures was reduced in serum-free (plasminogen deficient) media and when incubated in the presence of trasylol, a serine protease inhibitor (Lala and Graham, 1990). These results suggested that metalloproteases are likely the key mediators of first trimester EVT cell invasiveness, and that plasmin on the cell membrane of trophoblast cells (Jensen et al. 1989) is required for metalloprotease activation.

There has been some controversy regarding the relative roles of 72 kDa and 92 kDa type IV collagenases in the mediation of trophoblast invasion possibly owing to the use of different subpopulations of trophoblast cells in these studies. For example, Librach et al. (1991) suggested that 92 kDa type IV collagenase is the principal mediator. On the other hand, Graham et al. (1993b) found that the 72 kDa type IV collagenase is the major metalloprotease expressed by first trimester invasive human EVT cells in culture. This collagenase species has been shown to be expressed by the first trimester human placenta (Fernandez et al. 1992) and selectively localized to the EVT cells in situ (Polette et al. 1994). It is likely that both type IV collagenase species are important for trophoblast invasion, since antisense oligonucleotides directed against specific domains of both collagenase species could block trophoblast invasion in vitro (Lala and Connelly, 1994).
A recent study demonstrated that MT-MMP is abundantly expressed by the EVT cells in situ, co-localized with 72 kDa type IV collagenase (Nawrocki et al. 1995). Thus, it is likely that MT-MMP activates 72 kDa type IV collagenase in first trimester EVT cells, similar to findings observed in tumor cells (Sato et al. 1994; Okada et al. 1995).

First trimester (Yagel et al. 1989b) and term (Queenan et al. 1987) trophoblast cells produce uPA; they also express uPA receptors that remain saturated with endogenously produced uPA (Zini et al. 1992). A polarized expression of uPA receptors by the invasive front of first trimester EVT cells in situ is believed to determine the direction of trophoblast invasion of the uterus (Multhaupt et al. 1994). This has also been shown for certain tumors (Plow et al. 1986; Plow and Miles, 1990; Sordat et al. 1990; Murphy et al. 1993). PAI-1 and PAI-2 have been localized in both the human (Feinberg et al. 1989; Fazleabas et al. 1991) and the baboon (Fazleabas et al. 1991) placenta. During pregnancy, there is an increase in PAI concentrations (Astedt et al. 1986), and it is suggested that PAI-1 and -2 have different roles in maintaining homeostasis at the feto-maternal interface (Astedt et al. 1986; Fazleabas et al. 1991). PAI-1 may play a role in regulating trophoblast invasion, since it is observed in the invading EVT cell population closest to the maternal decidua (Feinberg et al. 1989; Fazleabas et al. 1991). PAI-1 expression has also been suggested to be a marker of differentiation.
for EVT cells (Yeh and Kurman, 1989), since it is not expressed in villous
cytotrophoblasts (Feinberg et al. 1989). In contrast, PAI-2 is present in the
syncytiotrophoblasts where it appears to be a regulator of the fibrinolytic pathway
(Fazleabas et al. 1991). These reports, along with the in vitro studies discussed
above, suggest that the uPA/plasmin pathway plays a prominent role in trophoblast
invasion. However, targeted gene disruption of uPA and tPA genes (Carmeliet et
al. 1994), as well as uPAR (Bugge et al. 1994) in mice did not result in the
expected impaired embryonic implantation, suggesting a redundancy of the system
in murine species. In contrast, targeted disruption of LRP/α2-macroglobulin
receptor gene blocked embryonic development at the implantation stage (Herz et
al. 1992). This is possibly because cell surface LRP provides a mechanism of
regenerating cell surface uPAR. LRP triggers endocytosis of uPA-PAI-1 complex
bound to uPAR. The trimeric complex is then degraded, and the UPA-R is recycled
back to the cell membrane, creating a functional invasion front. It is postulated that
the lack of uPA:PAI-1 clearance from the uPAR likely disrupted the plasmin
mediated invasive capability of trophoblasts in the transgenic mice (Herz et al.
1.2.3 Regulation of Trophoblast Invasiveness *In Situ*: Role of Growth Factors, Growth Factor Binding Proteins, and Proteoglycans

Growth factors are multifunctional soluble peptides which have been recognized to play key roles in cell-cell communication and in modulating cellular functions, including adhesion, proliferation, differentiation, locomotion, and invasion. Recent studies of tumor biology have shown that the constitutive production of growth factors in the tumor micro-environment, or malfunction of growth factor receptors in tumor cells, may contribute to the malignant phenotype (Weinberg, 1989a). Aberrant production of growth factors, or mutation/activation of growth factor receptors (Gill, 1989), may subvert normal growth regulatory mechanisms leading to partial or complete growth autonomy, a hallmark of tumorigenic cells.

Several laboratories, including ours, have examined the possible roles of some of these locally produced growth factors on trophoblast function (see review (Ohisson, 1989; Lala and Lysiak, 1994; 1995). These growth factors include transforming growth factor-β (TGF-β), insulin-like growth factor-II (IGF-II), and members of the epidermal growth factor (EGF) family which bind to EGF receptor: transforming growth factor-α (TGF-α), EGF and amphiregulin (AR). Some of these factors, e.g., TGFβ and IGFII have been shown to regulate invasion and/or
migration of the EVT cells, whereas EGFr ligands have been shown to stimulate EVT cell proliferation (Lala and Lysiak, 1995).

1.2.3.1 **EGF receptor and its ligands**

EGF receptor (EGFr) ligands share a common motif, CX₇CX₄₋₅CX₁₀₋₁₃CX₉₋₈C, comprised within forty amino acids (aa). They are all powerful epithelial cell mitogens which exert their signals through the tyrosine kinase domain of the receptor (Rall et al. 1985; Wong et al. 1989; Brachmann et al. 1989; Dobashi and Stern, 1991). EGF receptor has been localized on the villous cyto- and syncytiotrophoblast cells as well as EVT cells in the human placenta (Filla et al. 1993; Muhlhauser et al. 1993), suggesting a possible functional role of EGFr ligands on these cells. EGF and TGFα transcripts have been localized to the human endometrium as well as the placenta (Stromberg et al. 1982; Haining et al. 1991; Hofmann et al. 1991). It was proposed that these peptides may be required for post-menstruation epithelial regeneration (Haining et al. 1991) and could be mitogenic for the EGF receptor expressing trophoblasts *in vivo* (Lai and Guyda, 1984; Muhlhauser et al. 1993).

High levels of TGFα mRNA and protein are observed in all gestational ages in the human placenta, while EGF mRNA and peptide are present at lower levels (Bissonnette et al. 1992). This indicates a possible differential requirement of these
peptides for placental development and function. EGF and TGFα peptides are widely distributed and are present in the maternal decidual cells as well as the extravillous and the villous trophoblast cells in the human placenta (Hofmann et al. 1991; Filla et al. 1993; Lysiak et al. 1993). These in situ studies suggest that these EGFr ligands may have autocrine, paracrine or juxtacrine functions on the trophoblast.

The role of EGFr ligands in trophoblast function has been found to vary with the trophoblast cell subset. When added to freshly isolated villous cytotrophoblast cells, EGF was found to induce their differentiation into syncytiotrophoblasts with a concomitant increase in human chorionic gonadotrophin (hCG) production (Morrish et al. 1987). Both TGFα as well as EGF were found to stimulate proliferation of early (2-3) passage, first trimester EVT cells in culture, and this effect was blocked in the presence of an EGFr blocking antibody (Lysiak et al. 1993; 1994). It was also found that neither TGFα nor EGF stimulated invasion of EVT cells in vitro in the Matrigel invasion assay (Lysiak et al. 1994). However, both MMP and TIMP mRNA levels were equally increased, resulting in no net change in the balance between the protease and its inhibitor. In contrast, Bass et al. (1994b) reported that EGF stimulated Matrigel invasion by freshly isolated first trimester trophoblast cells. The differences in these results can be attributed to the use of different trophoblast subpopulations in each of the two studies.
TGF-α, like EGF, was found to be expressed in numerous tumor types in humans (Stromberg et al. 1987; Valverius et al. 1989a;b; Driman et al. 1992). Moreover, direct evidence from studies of TGF-α overexpressing transgenic mice demonstrated that it can influence growth and differentiation leading to neoplasms of the pancreas, mammary gland, and liver (Sandgren et al. 1990; Jhappen et al. 1990; Matsui et al. 1990). A significant role for TGFα in mammary carcinogenesis in murine models has been demonstrated (Sandgren et al. 1995; Matsui et al. 1990). This finding supports observations implicating TGFα in the progression of breast tumors in humans (Stromberg et al. 1987; Valverius et al. 1989a;b). In contrast, Lee and associates (1993) reported only minor abnormalities in hair and eye development in TGF-α null mutants, suggesting that other EGFr ligands compensate for TGF-α during development.

Amphiregulin (AR), a 84 aa glycoprotein is another member of the EGFr ligand family, recently found in human breast cancer cell culture medium (Shoyab et al. 1988; 1989). Characterization of AR indicates that it has a nuclear targeting domain (Shoyab et al. 1988). The expression has been immunolocalized in the nuclei of normal ovarian and colonic epithelium as well as their neoplastic counterparts. It is reported to function as both an inhibitor and a mitogen for proliferation depending on the cell type and concentration of the growth factor (Johnson et al. 1991; 1992). AR was immunolocalized to the nucleus and the
cytoplasm of terminally differentiated syncytiotrophoblast cells of only the early gestational (up to 18 weeks) chorionic villi (Lysiak et al. 1995b). Like other EGF ligands, TGFα and EGF, AR was also found to be growth stimulatory for EVT cells _in vitro_ (Lysiak et al. 1995b).

Recent murine studies, using EGF receptor null mutants, show that EGFr is needed for epithelial development as several abnormalities were observed in skin, liver, kidney brain, gastrointestinal tract. The effect on embryonic development depends on the strain of mouse and, while there was no defect in implantation, there was a reduction in placental size (Threadgill et al. 1995; Sibilia and Wagner, 1995; Miettinen et al. 1995). The observed reduction in placental sizes is in accord with the human studies showing growth stimulating role of EGFr ligands on the human trophoblast (Lysiak et al. 1993; 1994; 1995b).

### 1.2.3.2 Transforming growth factor β

Transforming growth factor β (TGF-β) is a 25 kDa homodimeric polypeptide growth factor family, acting by both autocrine and paracrine mechanisms (see Roberts and Sporn, 1991), which includes five TGFβ isoforms, TGFβ₁₋₅ (Delerco and Todaro, 1978; Roberts et al. 1980; Anzano et al. 1983; Tucker et al. 1984; ten Dijke et al. 1988; Derynck et al. 1988; Jakowlew et al. 1988a,b; Kondaiah et al. 1989; ten Dijke et al. 1990). Other related factors in the TGFβ family are:
activins/inhibins (Vale et al. 1991), Mullerian inhibiting substance (Cate et al. 1991) and bone morphogenetic protein (Wozney et al. 1988).

TGFβ is produced by a variety of cell types (Assoian et al. 1983; Frolik et al. 1983; Roberts et al. 1983; Derynck et al. 1985; Miyazano et al. 1988; Rosen et al. 1990), and acts both as a stimulator and inhibitor of cell proliferation depending on the cell type (Roberts et al. 1985; Massague et al. 1994). It can also modulate cell differentiation, cellular migration, extracellular matrix production and angiogenesis (Roberts et al. 1986; Akhurst et al. 1990), which are all important processes for development, wound healing, and tumor progression. TGFβs are secreted from most cells in an inactive, latent form (Miyazano et al. 1988; 1990) composed of a mature processed peptide noncovalently associated with the latency associated protein (LAP) (Roberts et al. 1990). The latent protein is activated following cleavage by proteases such as plasmin or cathepsin D (Lyons et al. 1988) or changes in pH (below 3.5 or above 12.5) (Miyazono et al. 1990). Thus it appears that, although the peptide and its receptors are present on the cell, an autocrine action cannot be inferred, unless the activation of the latent TGFβ is observed (Wakefield et al. 1987). Mature TGFβ in the serum is found associated with α2-macroglobulin, which may represent a clearance mechanism for TGFβ (O'Connor-McCourt and Wakefield, 1987; Huang et al. 1988). TGFβ can be found sequestered in the ECM bound to decorin, a chondroitin-dermatan sulfate
proteoglycan, shown to moderate the biological activity of TGFβ (Yamaguchi et al. 1990). In addition, TGFβ binds to serum fibronectin, but unlike binding to α2-macroglobulin or decorin which inactivates TGFβ, the fibronectin-TGFβ complex remains active (Miyazano and Heldin, 1989).

Most cells, with few exceptions, have TGFβ binding receptors or proteins (see review by Massague et al. 1990). To date, nine membrane binding proteins have been observed to bind TGFβ (Massague, 1992; Massague et al. 1994). The most ubiquitous of these proteins are TGFβ receptors type I and II (Massague et al. 1990). Both of these glycoproteins are transmembrane serine/threonine kinase (Lin et al. 1992; Wrana et al. 1992; Attisano et al. 1993; see (Massague et al. 1994) that form heterodimers upon binding with TGFβ1 (Moustakas et al. 1993; Massague et al. 1994), and are interdependent components required for binding and signal transduction in response to TGFβ (Wrana et al. 1992; Attisano et al. 1993). Following the ligand dependent association of the type I with type II receptors, the kinase domain of type II phosphorylates the type I receptors leading to its activation for signal transduction (Wrana et al. 1992). Thus, the loss of type I or type II or both receptors has been correlated with loss of TGFβ response in many cancers, freeing these cells from inhibitory control (see review by Massague et al. 1990).
The third receptor, type III receptor, a betaglycan, is an integral membrane binding protein present on the majority of cells (Segarini and Seyedin, 1988; Massague et al. 1990; Lopez-Casillas et al. 1994). It does not transduce signals (Cheifetz et al. 1987; 1988; Ohta et al. 1987), but is involved in presenting bound peptides to the type I or II receptors (Ohta et al. 1987; Cheifetz et al. 1988; Moustakas et al. 1993), and can possibly act as a reservoir for TGFβ or modulate TGFβ binding to the TGFβ type II receptor (Moustakas et al. 1993). In addition, soluble betaglycan is a potent antagonist that inhibits TGFβ binding to the receptors blocking its action (Lopez-Casillas et al. 1994).

Types I and II as well as type III/betaglycan receptors for TGFβ have been demonstrated in freshly isolated trophoblast cells as well as first trimester trophoblast cells in culture (Mitchell et al. 1992), indicating that locally derived TGFβ may be important for trophoblast function.

Endoglin, a dimeric membrane glycoprotein, is a major TGFβ₁ binding protein in human endothelial cells, and is co-expressed with both TGFβ type I and II receptors (Cheifetz et al. 1988; 1992). In the placenta, endoglin expression was reported to be transiently upregulated in intermediate trophoblast cells coincident with the onset of migration and the appearance of α5β1 integrins within the columns (St.-Jacques et al. 1994). It has also been found in the syncytiotrophoblast
layer of human term placentas (Gougos et al. 1992). The role of endoglins in the syncytiotrophoblasts remains unclear (Gougos et al. 1992).

TGFβ has been immunolocalized to the syncytiotrophoblast layer of the chorionic villi (Dungy et al. 1991; Graham et al. 1992), the EVT of the cytотrophoblastic shell as well as the maternal decidua (Graham et al. 1992; Lysiak et al. 1995a). In the decidua, a stronger immunolocalization signal is seen in the ECM during the first trimester, whereas during the third trimester, it is localized mainly within the cytoplasm (Lysiak et al. 1995a). Cellular location of the peptide also corresponds to the location of the mRNA of TGFβ, indicating that these cells also make the peptide (Lysiak et al. 1995a). Since trophoblast cells have TGFβ receptors of all classes, and TGFβ is made by both the decidua and the trophoblast, TGFβ can potentially regulate trophoblast function in situ in a paracrine as well as autocrine manner.

TGFβ, derived from the decidua, is released as an inactive form; in contrast TGFβ from cultured trophoblast cells is an active peptide (Graham et al. 1992). Activation of TGFβ peptide in the placenta likely occurs by a plasmin-mediated cleavage pathway. In the placenta, TGFβ functions as an anti-invasive, anti-proliferative, as well as a differentiation inducing factor (Graham and Lala, 1992).
thus providing a possible regulatory mechanism to limit EVT cell invasion of the uterus. The manner by which TGFβ regulates EVT function is discussed below.

Human trophoblast cells are not genetically preprogrammed to stop invading after mid-gestation when invasion appears limited histologically. Isolated term trophoblast cells are also equipped with invasive ability (Graham et al. 1993b; Shiminovitz et al. 1994), indicating that the control of invasion in situ must be provided by the uterine micro-environment. Subsequently, it was discovered that soluble products of the decidua—a tissue layer of the uterus which forms during pregnancy and surrounds the placental trophoblast—can block trophoblast invasion in vitro in amnion (Graham and Lala, 1991) as well as in Matrigel (Graham and Lala, 1992) invasion assays. TGFβ and TIMP-1 are two key molecules that have been identified in the decidual cell conditioned medium (DCM) and are capable of blocking trophoblast invasion. Neutralizing antibodies to both of these molecules abrogated the invasion-blocking effects of DCM (Graham and Lala, 1991; 1992). Further experiments revealed that decidua-derived, and to a minor extent trophoblast-derived, TGF-β upregulated TIMP-1 message and its subsequent translation to TIMP-1 protein (Graham and Lala, 1991; 1992), leading to a drop in net natural collagenase activity. This occurs because collagenase is neutralized by TIMP-1 in a 1:1 stoichiometric ratio (reviewed by Liotta and Stetler-Stevenson, 1991). This laboratory also discovered that TGF-β
reduces trophoblast invasiveness by three other mechanisms: (a) reduced uPA production (Graham et al. 1993a), (b) reduced cell migration, most likely due to an upregulation of integrins making them more adhesive to the extracellular matrix (Irving and Lala, 1995), and (c) enhanced differentiation into nonproliferating, noninvasive multinucleated cells (Graham et al. 1992) the possible equivalents of placental bed giant cells. In addition to invasion and migration blocking effects, TGFβ has also been shown to have anti-proliferation effects on EVT cells in vitro (Graham et al. 1992). However, Lala and associates also found that choriocarcinomas were refractory to the anti-proliferative effects of TGFβ (Graham et al. 1994). JAR choriocarcinoma growth was stimulated by TGFβ, whereas JEG-3 was not. These cell lines have been shown to express receptors for the TGFβ ligand (Mitchell et al. 1992).

Homozygous TGFβ₁ null mutant mice showed no apparent developmental defects at birth (Kulkarni et al. 1993). However, by three weeks they were cachectic and showed severe inflammatory syndrome; the vital organs showed multifocal mononuclear cell infiltration leading to death of the mice. Thus, this study shows that other TGFβ peptides may compensate for the anti-invasive function of TGFβ₁ in the murine placenta, but not for the regulatory function on the immune system in the model studied.
1.2.3.3 Insulin-like growth factor-II

Insulin-like growth factor-II (IGF-II) is a 67 amino acid single chain polypeptide, which shares homology with IGF-I and insulin (see reviews Rechler and Nissley, 1991; LeRoith and Roberts, 1993). Regulation of IGF-II has recently been reviewed (LeRoith and Roberts, 1993). It is produced by normal cells (Rechler and Nissley, 1991) and in some tumors, it has been found to be aberrantly expressed (Scott et al. 1985; reviewed by Rechler and Nissley, 1991). By in situ hybridization, IGF-II mRNA levels were found to be 1 to 6 fold higher than IGF-I in many of the fetal tissues examined (Scott et al. 1985; Gray et al. 1987; Han et al. 1988). In many adult tissues, IGF-II mRNA is present at much lower levels than in the embryo (Rechler and Nissley, 1991), suggesting that it is likely a fetal growth factor (LeRoith and Roberts, 1993).

The biological activity of IGFs is mediated through the type I IGF receptor which possesses tyrosine kinase activity (Ullrich et al. 1986; Steele-Perkins et al. 1988a;b; Kato et al. 1993). The cellular and molecular biology of type I IGF receptor has been recently reviewed (Werner et al. 1991). Type I IGF receptor is a tetrameric membrane receptor structurally similar to insulin receptor, and is composed of two α and two β subunits linked by disulfide bonds which span the cell membrane (see reviews, Rechler and Nissley, 1991; LeRoith et al. 1993). IGF type I receptor, when bound to the ligand, activates the tyrosine kinase of the β
subunits, and phosphorylates insulin receptor substrate 1 (IRS-1), a common substrate for both IGF type I and insulin receptors (LeRoith et al. 1993). IGF-II can also bind to the IGF-II/mannose-6-phosphate (type 2) receptor which is responsible for directing lysosomal proteins. It is unclear what role type 2 receptor has on IGF-II bioactivity (Morgan et al. 1987; Baxter and Martin, 1989), but it is postulated that the receptor may mediate IGF action through G-protein coupled signaling pathway (Nishimoto et al. 1989).

In the serum and the extracellular fluid, IGFs are bound to a family of binding proteins, comprised of six members known to date, IGFBP-1 to IGFBP-6 (Shimasaki and Ling, 1991; Clemmons et al. 1993). Binding proteins regulate the biological actions of IGFs either enhancing or inhibiting their binding to the IGF receptors on the cells (DeMellow and Baxter, 1988; Clemmons, 1991). Interestingly, IGFBP-1 and IGFBP-2 have RGD (arginine-glycine-aspartic acid) sequences that can bind to RGD binding sites (Shimasaki and Ling, 1991), such as the fibronectin receptor (Jones et al. 1993; Irving and Lala, 1995), thereby potentiating the binding of IGFs to their receptors. Alterations in the RGD-tripeptide by site-directed mutagenesis, were found to block the migration stimulatory function of IGFBP-1 protein (Clemmons et al. 1993), showing that IGFBP-1 required the RGD peptide to be active.
Recently, it was reported that IGFII mRNA is selectively expressed in the invasive intermediate trophoblast in situ (Ohlsson, 1989), and that IGFBP-1 is produced by the decidual cells (Pekonen et al. 1988; Rutanen et al. 1988; Hill et al. 1993). Lala and associates (1995) showed that IGFII stimulated trophoblast invasion, independently as well as synergistically with IGFBP-1, but it did not stimulate proliferation. Subsequently, Irving and Lala (1995) showed that both IGFII as well as IGFBP-1 stimulated migration of first trimester EVT cells, which can, at least in part, explain their invasion stimulatory effects. The IGFBP-1 effect was dependent on access to cell surface α5β1 integrin (fibronectin receptor), since it could be abrogated by prior exposure of EVT cells to neutralizing antibodies to α5 or β1. It is likely that IGFBP-1 effects on migration occurred by its binding to the α5β1 integrin complex on the EVT cell surface via the RGD sequences in the IGFBP-1 molecule (Irving and Lala, 1995).

The IGFBP peptides may contribute to tumorigenesis both as a potentiator and a reservoir of IGFs (Reeve et al. 1993). In addition, proteolytic cleavage of binding proteins in situ has been reported to influence biological actions (see recent review by Clemmons et al. 1993). IGFBP-3 protease that degrades IGFBP-3 to a 31 kDa fragment has been found in decidual cells and placental epithelial cells (Guidice et al. 1990). This 31 kDa fragment was observed to increase IGF-I stimulation of DNA synthesis in rat osteoblast cells, whereas intact IGFBP-3
inhibited IGF-I response (Guidice et al. 1990). Clearly, in tumor cells where such proteolytic peptides are produced, this could result in the enhancement of IGF-mediated responses favorable for the tumor. Recently, Cohen et al. (1992) found that prostate specific antigen isolated from the seminal fluid is the IGFBP-3 protease. This finding implicates a possible function of IGFBP-3 protease in the progression of prostate hyperplasia and tumors.

1.2.4 Gap Junctions (GJs)

Gap junctions are specialized, gated intercellular channels between opposing cell membranes that form an aqueous conduit allowing molecules of less than 1000 daltons to pass between communicating cells (Flagg-Newton and Loewenstein, 1979; Sneyd et al. 1994). They may also play a role in development and differentiation of a number of organs within the host, by creating homeostatic micro-compartments. It has been shown that GJ alterations can interrupt the metabolic integration of the affected tissue. Moreover, gap junctional intercellular communication (GJIC) is usually reduced during carcinogenesis, for example following oncogene transformation or by tumor promoters (see reviews, Mesnil and Yamasaki, 1993; Ruch, 1994). As a tumor progresses, GJs are quantitatively reduced leading to faulty GJIC to the surrounding normal cells; subsequently, the foci become less regulated and more isolated from the host tissue (Mesnil and Yamasaki, 1993; Ruch, 1994).
1.2.4.1 Gap junction: structure and function

Each GJ channel is composed of two connexons or hexameric arrays of individual connexin (Cx) proteins with a central aqueous channel. Connexons within the cellular membrane float in the plane of the membrane until they connect to apposing connexons in the adjacent cell membrane (Peracchia, 1980; Caveney, 1985; Revel et al. 1985). Electron microscopic studies (Revel and Karnovsky, 1967; Peracchia and Peracchia, 1978) show that gap junctions are arranged as large plaques in the cell membrane and are made up of connexons numbering in the tens to the thousands (Caspar et al. 1977). Individual connexins show complex expression patterns, differing in their spatial distribution and temporal kinetics during development and differentiation (Goodenough, 1990; Lee et al. 1991).

Connexins are named based on their predicted size derived from the nucleotide sequence (see review by Bennett et al. 1995). For example, Cx32 nucleotide sequence predicted a 32 kDa protein (Henderson et al. 1979; Hertzberg and Gilula, 1979). When the Cx32 cDNA was isolated (Paul, 1986), and subsequently transfected into cell lines, functional coupling was observed (Dahl et al. 1987). The human Cx32 cDNA was cloned following hybridization with the rat cDNA (Kumar and Gilula, 1986). The Cx43 cDNA sequence was subsequently cloned from a rat heart cDNA library (Beyer et al. 1987), following isolation of Cx43 protein (Kensler and Goodenough, 1980; Manjunath et al. 1987). Since these
two prototypical proteins were first found, several other connexins have been isolated in this highly conserved gene family (reviewed by Bennett et al. 1995). Today, connexins are subdivided, based on their similarities to the two prototypical connexins, Cx 32 and Cx43 subfamilies. There are at least 12 different known members (Willecke and Traub, 1990; Beyer, 1993; Saez et al. 1993; Bennett et al. 1995). A number of the human Cx gene family members have been isolated and localized to their chromosomes (Willecke et al. 1990; Lee et al. 1992; Reed et al. 1993; Sorscher et al. 1993).

All Cx proteins share common structural features consisting of four hydrophobic and five hydrophilic domains that traverse the cell membrane four times yielding a highly conserved NH-terminal and variable COOH-terminal as well as two extracellular loops and one intracellular loop (Kumar and Gilula, 1986; Evans and Rahman, 1989; Zhang and Nicholson, 1989). The extracellular loops are highly conserved and serve to bind their respective counterpart on the apposing membrane. Some heterotypic couplings function well but others do not (Dahl et al. 1992). Phosphorylation of the connexins 32 and 43 has been demonstrated to alter GJIC (Gimlich et al. 1988; Saez et al. 1990; Holder et al. 1993), and it is postulated that other connexins can be similarly regulated.
GJs are cell-cell conduits that allow for metabolic coupling, *i.e.* the passage of essential metabolites, including cAMP and IP3, (Pitts and Finbow, 1986; Saez *et al.* 1989; Boitano *et al.* 1992; Charles *et al.* 1992) and ions (Charles *et al.* 1992; Sanderson *et al.* 1994; Sneyd *et al.* 1994), as well as electrical coupling *i.e.* electrical conductance between cells (Spray *et al.* 1985; Moreno *et al.* 1994; Waltzman and Spray, 1995). By gating (opening or closing of the aqueous pore) of these channels, cells can control the passage of various molecules, for example, by restricting the unwanted passage of molecules (Unwin and Ennis, 1984; Unwin, 1987). GJs are believed to attenuate cellular responses, and form functional regional units to: (a) buffer harmful xenotoxins by dispersing the xenotoxin to adjacent cells; (b) provide nourishment to an injured cell, allowing recovery; (c) allow rapid passage of electrical and essential metabolic signals, and their regulators; and (d) facilitate removal of waste byproducts from a cell distant from the vasculature (Holder *et al.* 1993).

### 1.2.4.2 Gap junctions in the placental trophoblast

The expression of various connexins has been reported in the placental trophoblast cells in the rat (Rişek and Gilula, 1991; Grummer *et al.* 1995), the mouse (Pauken and Lo, 1995; Dahl *et al.* 1995), and the human (Hellmann *et al.* 1995; Cronier *et al.* 1995a;b). Connexins in the three species differ in their spatial distribution. In the rat, Cx43 was found expressed in the spongiotrophoblasts and
the trophoblast giant cells, and Cx26 was present in the trophoblast cells of the labyrinthine zone (Risek and Gilula, 1991; Grummer et al. 1995). In addition, Cx31 was detected in the invasive trophoblast of the ectoplacental cone (Grummer et al. 1995; Reuss et al. 1995). In the mouse, Cx43 was not detected in the spongiotrophoblast cells in contact with the decidua cap, whereas Cx26 was found in the labyrinthine trophoblast (ectoplacental cone derivative) zone (Pauken and Lo, 1995; Dahl et al. 1995). Cx43 was detected in isolated human term trophoblast cells in vitro (Cronier et al. 1995a,b). *In situ* expression of Cx43, as well as Cx40, was reported in the human EVT cells (Hellmann et al. 1995). Furthermore, Hellmann et al. (1995) showed that Cx40 was also expressed in BeWo and JAR cells, two choriocarcinoma cell lines of trophoblastic origin; but there was no expression of Cx26 or Cx43 in these cells. Transfection of JAR with connexin43 resulted in the decrease of cell growth in vitro. The pattern and ontogeny of expression of connexins *in situ* in the human placenta remains to be explored further.

**1.2.4.3 Gap junction and malignancy**

Evidence to date suggests that carcinogenesis is a multistep and sequential process (Farber, 1984; Nowell, 1986; Bishop, 1991; Anderson et al. 1992) which may involve alterations in GJ function, including the involvement of connexins. The role of gap junctions in cancer was postulated by Loewenstein (1979) who
hypothesized that negative growth control factors are shared by intercommunicating cells through the GJs. Today, a number of studies of cancer models have shown that tumorigenic cells have lower GJIC (Yamasaki, 1990a; Klaunig and Ruch, 1990; Mesnil and Yamasaki, 1993; Ruch, 1994) in comparison to their normal cellular counterparts, or in some cases, aberrant expression of connexins results in the formation of functionally disparate GJs (Yamasaki, 1990b; Wilgenbus et al. 1992). Besides various tumor promoters (Holder et al. 1993; Bager et al. 1994), oncogenes that are implicated in tumor development (Bignami et al. 1988; Holder et al. 1993), have also caused decreases in GJIC.

The inverse correlation of cell growth and GJIC in transformed cells has been observed in a number of studies. Hossain et al. (1989) found that retinoic acid reduced neoplastic transformation by elevating GJIC. Expression of Cx43 transfected into 3T3 cell line restored GJIC and decreased growth rate (Mehta et al. 1991). Using the C6 glioma cell line, Naus et al. (1992) found that C6 Cx43 transfectants had a reduced growth rate in vitro and in vivo. The reduction correlated with level of Cx43 expression. In a second study, C6 glioma cells transfected with Cx32 showed no decrease in their growth in vitro, but growth was reduced in vivo, and was also correlated with Cx32 expression level (Bond et al. 1994). A similar dichotomy between growth rate in culture and in vivo was also observed in SKHep1 Cx32 transfectants by Eghbali et al. (1991). These studies
demonstrate that the growth rate of a number of tumor cells is inversely correlated with the level of expression of connexins.

1.2.5 Tumor Progression and Metastasis

Progression of a benign tumor to malignancy is a multigenic and multifactorial phenomenon. Tumor progression may be characterized by changes in the genetic constitution of the cells (Nowell, 1986). Some of these changes result in metastasis, which involves the migration of malignant tumor cells from a primary site by blood or lymphatic vessels to distant sites where secondary foci or tumors develop (Frost and Fidler, 1986; Liotta et al., 1986; Liotta, 1988b). The process is complex and requires a series of events during the “metastatic cascade” (Liotta et al., 1986; Liotta, 1988b). One of the initial events is the invasion of the basement membrane (Liotta et al., 1986; Terranova et al., 1986) at the primary site, as well as that of the blood vessels or lymphatics. Although this ability is neither an exclusive characteristic of tumor cells nor sufficient for metastasis, metastatic tumor cells appear to produce increased levels of proteolytic enzymes (Ossowski et al., 1991b; Yu and Schultz, 1990; Gingras et al., 1990; see reviews, Stetler-Stevenson, 1990; Ray and Stetler-Stevenson, 1994), and are generally more invasive than their weakly metastatic or nonmetastatic counterparts (Liotta et al., 1986; Yagel et al., 1989c; see reviews, Liotta and Stetler-Stevenson, 1991; Liotta, 1992). Increased protease activity is likely due to the altered balance of production
of proteases and endogenous protease inhibitors. Metastatic tumors also show decreased level of ECM (Schalken et al. 1988; Liotta et al. 1984).

Many studies have shown the role of oncogenes in tumorigenesis (Dalgleish, 1989; Weinberg, 1989c; Seemayer and Cavenee, 1989) and metastasis (Liotta, 1988b; Greenberg et al. 1989). Oncogenes are viral or cellular genes that alone, or in combination with other genes, can impart characteristics associated with the transformed phenotype—increased growth rate, loss of contact inhibition growth, growth factor independence or autonomy, invasive behavior and tumorigenicity (reviewed by Varmus, 1989). Viral oncogene proteins, including SV40 Tag and adenovirus E1A and E1B, have been shown to both immortalize cells and transform them by inactivating cellular proteins involved in the regulation of cell growth, such as cellular protein p53 or the retinoblastoma gene, pRb. Oncogenes have provided investigators with the tools to examine the molecular underpinnings that contribute to tumors in both animals and humans. The ras oncogene family (K-ras, N-ras, Ha-ras) has been observed in a number of human cancers (McCormick, 1989) and the biology of ras has been recently reviewed (McCormick, 1989; Chambers and Tuck, 1993). The incidence of ras involvement in cancers varies with type of cancer and within any particular type of tumor, a particular ras oncogene predominates (McCormick, 1989). Transfection of ras oncogenes into immortalized Swiss 3T3 cells has led to the acquisition of
metastatic phenotype (Egan et al. 1987; Chambers et al. 1992), but in other cell lines ras expression did not induce malignant behavior (Tuck et al. 1991) in the cells (see reviews, Greenberg et al. 1989; Chambers and Tuck, 1993). Today, ras has been found to be a key player in an elaborate signal transduction pathway (see review by Pawson, 1995).

A number of oncosuppressor genes have also been identified and shown to reverse the malignant phenotype (Weinberg, 1988; Spandidos and Anderson, 1989; Green, 1989; Cavenee et al. 1989; Paul, 1989). These include cellular regulatory genes which play prominent roles in the regulation of the cell cycle. The prototypical tumor suppressor genes are p53 and pRb (Stanbridge and Cavenee, 1989). It was reported that ras transfected with mutated p53 resulted in transformation of rat embryo fibroblast cells, and when wild-type p53 was re-introduced, transformation was suppressed (Finlay et al. 1989). The role of p53 in vivo has been recently elucidated using p53 gene targeted disruption, which showed that it is not required for normal development; however, it is required in maintenance of regulated growth and proliferation as these mice showed increased propensity to develop tumors (Donehower et al. 1992). The importance of p53 as a key player in tumorigenesis may lie in its ability to function as a transcriptional activator (O'Rourke et al. 1990; Farmer et al. 1992). Recently, a new gene encoding a cell cycle inhibitor, p16/MTS-1 (multiple tumor suppressor gene), was
found to be deleted or mutated in a number of tumors (Kamb et al. 1994). p16 has been shown to act as a specific inhibitor of cell cycle progression through G1 of the cell cycle, and to inhibit cyclinD1/cdk4 kinase activity in vitro (Koh et al. 1995; Lukas et al. 1995). These studies also indicated that pRb is required for the inhibition of the cell cycle (Koh et al. 1995; Lukas et al. 1995). Since cdk4 kinase is required for cdk2/cyclinE phosphorylation of pRb (Okragly et al. 1994) allowing cells to proliferate, the lack of inhibition of cdk4 kinase by mutated p16 proteins would result in unchecked cdk4 function, leading to uncontrolled cell proliferation. Thus, it appears that there is a convergence of cell cycle regulation and genes associated with tumorigenesis.

Currently, only a few genes besides TIMP-1, e.g., as nm23 (Steeg et al. 1988a;b; Steeg, 1989; Backer et al. 1993), WDNM1 (Dear et al. 1988c), WDNM2 (Dear et al. 1988a;b), E-cadherin (Navarro et al. 1991; Chen and Obrink, 1991; Frixen et al. 1991; Vleminckx et al. 1991) and fibronectin (Schalken et al. 1988), have been inversely correlated with metastasis. nm23 (nm23-H1) codes for a nucleotide diphosphate kinase, and has been strongly correlated to the metastatic phenotype in certain human (Rosengard et al. 1989; Wang et al. 1993; Yamaguchi et al. 1994) and animal (Steeg et al. 1988a;b) cancers. In nm23 transfected K1735 TK melanoma cells, these cells were shown to have reduced responsiveness to cytokine signaling (Leone et al. 1993a), and reduced motility response to
chemotactic agents (Kantor et al. 1993). In addition, it has also been shown that
nm23 could play a role in the cell cycle, since anti-nm23 antibody injected into rat
embryo fibroblasts inhibited the ability of these cells to divide (Sorscher et al.
1993). Exceptions to, or even inversions of, this relationship have been reported
for certain malignancies (Hanash et al. 1991; Parker et al. 1991; Khoo et al. 1994),
indicating that nm23 is one of many genes that may be altered, leading to
metastatic phenotype. However, Leone and associates (Leone et al. 1993b) offered
another mechanism that may explain the exceptions; they found that mutations or
amplification of nm23 could contribute to the dysfunction of nm23, leading to
aggressive tumors.

Another metastasis suppressor candidate is the homophilic cell adhesion
molecule, E-cadherin, which has been noted to be altered in tumors (see reviews,
Edelman and Crossin, 1991; Honn and Tang, 1992; Albelda, 1993; Behrens,
1993). Reduction of intercellular adhesion may promote metastasis by facilitating
invasion, migration, and detachment from primary tumors. A number of studies
have demonstrated that following transformation, there was a loss of E-cadherin
leading to increased invasiveness (Behrens et al. 1989; Frixen et al. 1991;
Shimoyama and Hirohashi, 1991; Albelda, 1993). Moreover, in experiments in
which E-cadherin was reintroduced into highly invasive cells, there was a
demonstrated suppression of tumorigenicity, and decreased invasion and migration
of the transformed cells (Vleminckx et al. 1991; Navarro et al. 1991; Chen and Obrink, 1991).

Alterations in other well-characterized genes may also promote tumor progression in direct or indirect manners, e.g., changes in the production of growth factors, or responses to growth factors because of alteration is growth factor receptors, allowing cells either to maintain proliferation or escape from negative regulators of cell growth (Weinberg, 1989a; Gill, 1989; Herlyn and Malkowicz, 1991). Similarly, alterations in genes responsible for cell-cell or cell-extracellular matrix interactions, e.g., genes of the integrins family, have been implicated in tumorigenesis due to changes in cell growth, invasion, or migration (Juliano, 1987; Schreiner et al. 1991; Juliano, 1993; Albelda, 1993; Dedhar, 1995). However, such changes have usually been found to be tumor specific. Evidently, many more genes relevant for specific steps in tumor progression remain to be identified.
1.3 Hypothesis

The central hypothesis underlying the current project postulates that phenotypic changes in cellular behavior during the transition from the normal, nonmalignant, invasive stage to a "premalignant" stage can be explained on the basis of genetic changes involving known as well as novel genes. The hypothesis was tested by comparing the first trimester EVT cells, which show highly regulated proliferative, migratory, and invasive behavior, with SV40 Tag transformed trophoblasts which show a hyperproliferative and hyperinvasive, but nontumorigenic (i.e. premalignant) phenotype.

The first component of this thesis characterized the phenotypic changes in the EVT cells following SV40 Tag transformation including responses to growth factors and expression of invasion associated enzymes and their natural inhibitors. The second component of the thesis examined changes in gap junctional intercellular communication and connexin expression following SV40 Tag transformation of EVT cells. The third component of the thesis was devoted to identifying other known or novel genes differentially expressed by the parental or the transformed trophoblast.
1.4 Objectives

The objectives of the research were:

1. To characterize the phenotypic changes in a normal human invasive EVT cell line following SV40 Tag transformation.

2. To compare the response of these cells to a number of growth factor(s) known to regulate normal EVT cell proliferation and invasion.

3. To investigate whether there are any changes in the expression of certain invasion-associated enzymes (72 kDa type IV collagenase, 92 kDa type IV collagenase) or their inhibitors (TIMP-1 and -2) following SV40 Tag transformation and the effects of growth factors on the expression of these molecules.

4. To compare gap junctional intercellular communication (GJIC) and the expression of connexin(s) in the normal and SV40 Tag transformed cell lines.

5. To examine the expression of novel or known genes gained or lost following SV40 Tag transformation by r·RNA reverse transcription-polymerase chain reaction (RT-PCR) differential display (DD).

6. To isolate a full-length differentially expressed gene by screening a placental cDNA library and determine its identity by DNA sequencing.
2. **SV40 Tag Transformation of the Normal Invasive Trophoblast Results in a Premalignant Phenotype: Mechanisms Responsible for Hyperinvasiveness**

2.1 **Introduction**

Invasion is a property shared by normal embryonic cells during morphogenesis, where it is required for tissue remodeling, and by invasive tumor cells, where it is required for transgression of normal tissue barriers and metastasis. Degradation of the basement membrane and the extracellular matrix (ECM) components is one of the essential steps in cellular invasion (Terranova et al. 1986; Liotta, 1988a; 1992). This process is mediated by secreted proteolytic enzymes, including matrix metalloproteases (MMPs), serine and cysteine proteases, of which certain members of the MMP family, e.g., the 92 kDa and the 72 kDa type IV collagenases, appear to be key mediators (see reviews, Liotta and Stetler-Stevenson, 1991; Chen, 1992; Ray and Stetler-Stevenson, 1994). The net activity of the MMPs is determined by the balance between the levels of secreted MMPs and their natural inhibitors, the tissue inhibitor of metalloproteases (TIMP), in particular TIMP-1, TIMP-2, and, to a lesser extent, TIMP-3, which can bind to and
inactivate MMPs. A strong correlation exists between the production of matrix
degrading proteases and metastatic behavior (Bernhard et al. 1990; Gingras et al.
1990; Chambers et al. 1992). Highly invasive and metastatic tumor cells show
increased expression of a variety of matrix degrading enzymes, when compared to
their poorly metastatic counterparts (Ura et al. 1989; Yu and Schultz, 1990; Levy
et al. 1991; Ossowski et al. 1991b; Boag and Young, 1993). An engineered down-
regulation of endogenous TIMP-1 has been shown to impart a malignant
phenotype to immortal, nontumorigenic embryonic fibroblasts (Khokha et al.
1989), and endogenous TIMP levels have been shown to have an inverse
relationship to tumor cell aggressiveness (Hicks et al. 1984).

Invasiveness is not an exclusive property of malignant cells. For example,
normal placental development in certain species, including human, requires
invasion of the uterine lining and endometrium, inclusive of uterine blood vessels,
by a highly invasive population of placental trophoblast cells to allow for a proper
fetal-maternal exchange of nutrients, hormones and metabolite. (Yeh and Kurman,
1989; Lala and Graham, 1990; Kurman, 1992). However, unlike tumor invasion,
trophoblast invasion of the uterus is stringently controlled in a spatial and temporal
manner (Graham and Lala, 1991; 1992). This laboratory has succeeded in
maintaining pure first trimester normal human invasive trophoblast cells in long
term cultures (3-15 passages) by propagating migrant trophoblast cells from
placental chorionic villus explants (Yagel et al. 1989a; Graham et al. 1992; 1993b). These cells have been extensively characterized on the basis of a large number of markers to show that they belong to the invasive extravillous trophoblast (EVT) cells in situ (Graham et al. 1992; Irving et al. 1995). Using these cells in an in vitro invasion assay with de-epithelialized human amnion—composed of a thick basement membrane and an underlying stroma—as the substrate for invasion, it has been determined that mechanisms responsible for trophoblast cell invasiveness are identical to those of highly invasive tumor cells (Yagel et al. 1988). It was shown that invasion by both cell types is dependent on MMPs (in particular, type IV collagenases) and serine proteases e.g. urokinase-type plasminogen activator (uPA). Trophoblast cells made both enzymes (Graham and Lala, 1991; 1993b) and also expressed high affinity uPA receptors which bound endogenous uPA (Zini et al. 1992). Polarized expression of uPA receptors appeared to provide directionality for trophoblast invasion in situ in the human placenta (Multhaupt et al. 1994). This finding is in accord with the results suggesting that trophoblast cell activation of collagenase is plasmin-dependent (Yagel et al. 1988; Lala and Graham, 1990, Graham and Lala, 1992). This has also been shown for highly invasive tumor cells (Mignatti and Rifkin, 1993). Furthermore, the expression of sialylated β1-6 branched complex-type Asn-linked oligosaccharides is another property shared by invasive human trophoblast cells
and metastatic tumor cells, and is required for cellular detachment from the basement membrane and migration (Yagel et al. 1989b; 1990), a step required for their invasive function. In spite of sharing similar mechanisms with malignant tumor cells for motility and invasion, trophoblast cells were neither tumorigenic nor metastatic in nude mice, indicating that invasiveness is an insufficient, though essential, prerequisite for malignant or metastatic behavior (Lala and Graham, 1990).

In recent studies, using the normal first trimester EVT cells propagated in culture, some of the mechanisms regulating trophoblast invasion of the uterus *in situ* were elucidated. These studies have shown that trophoblast invasion is down-regulated primarily in a paracrine manner by the uterine decidua-derived transforming growth factor (TGF) β (Graham and Lala, 1991), and upregulated in an autocrine manner by trophoblast-derived insulin-like growth factor (IGF) II (Lala and Lysiak, 1994; 1995). TGFβ inhibited trophoblast invasion by an upregulation of TIMP-1 mRNA and protein (Graham and Lala, 1991), a downregulation of uPA (Graham et al. 1993a), and a reduction of cell migratory ability possibly due to an upregulation of integrins (Irving and Lala, 1995) making the trophoblast cells more adhesive to the ECM. Furthermore, two choriocarcinoma cell lines, JAR and JEG-3, were shown to be resistant to the anti-proliferative as well as anti-invasive effects of TGFβ exhibited by normal
trophoblast cells (Graham et al. 1994). Concurrent resistance of the malignant
trophoblast to both anti-proliferative and anti-invasive signals provided by local
mediators such as TGFβ may explain their malignant behavior *in situ*. However,
these cell lines may have drifted genetically from their tumor of origin, thus
requiring further studies with the normal trophoblast and its recently derived
malignant counterparts of the same genotype.

The long term objective of this laboratory is to induce tumorigenic and
metastatic phenotype in the normal invasive trophoblast, already expressing
invasion-associated genes, so that genetic events required to make invasive cells
tumorigenic and metastatic can be identified. As a first step, *SV40 T antigen (Tag)*
gene was introduced into a normal invasive first trimester human trophoblast cell
line, HTR8. The present study describes the phenotype of the transformed RSVT-2
cell line which exhibits an extended lifespan, and the immortalized RSVT2/C cell
line which was derived from RSVT-2 cells after forced cell crisis. The phenotypic
changes resulting from *SV40 Tag* transformation reveal that these cells have
reached a premalignant stage. We also show that their hyperinvasive behavior can
be explained by a reduction in the expression of invasion controlling genes TIMP-
1 and PAI-1.
2.2 Materials and Methods

2.2.1 Cell Culture

2.2.1.1 HTR8 cell line

The first trimester human trophoblast cell line, HTR8, was isolated in this laboratory as previously described (Yagel et al. 1989a) by propagating the outgrowth of adherent trophoblast cells from primary explants of mechanically derived chorionic villus fragments of a first trimester placenta. The cells were maintained in complete RPMI-1640 (10% FCS, 2% penicillin-streptomycin-fungizone, ICN Flow Laboratories, Toronto, ON) in 5% CO₂ humidified atmosphere. These cells have been extensively characterized for phenotype and function after 2-4 passages, indicating that they represent the invasive extravillous trophoblast in situ (Graham et al. 1992; Irving et al. 1995). The phenotypic profile at the time of SV40 Tag transfection was as follows: the cells were 100% positive for numerous cytokeratin chains, placental type alkaline phosphatase, HLA class I framework antigen (recognized by W6/32 antibody), uPA receptors of high density and numerous integrin chains (α1, α3, α5,αv, β1, and vitronectin receptor αv/β3/β5), cytoplasmic IGFII peptide and IGFII mRNA, and about 50% of the cells expressed NDOG-5 antigenic marker (Irving and Lala, 1995).
2.2.1.2 SV40 Tag transformed cell line, RSVT-2

HTR8 cells were passaged at 1:2 dilution on 100 mm plates (Corning™, Fisher Scientific, Mississauga, ON) in fresh medium for 24 h. Conditioned medium was transferred into new plates and placed on ice to be used for cell culture after electroporation of HTR8 cells (10th passage). Two million resuspended HTR8 cells were transferred to electrocuvettes (0.4 cm gap electrocuvette; BioRad, Mississauga, ON) and placed on ice for 10 mins with 15 μg of circular pRSV-T (Reddel et al. 1988). The SV40 Tag plasmid was obtained from Drs. B. I. Gerwin, NCI, Bethesda, MD with permission of Dr. J. Brady, NCI. The cells were electroporated (960 μF, 250 V), allowed to stand on ice for 10 mins and then returned into the conditioned medium with 5.0 ml of ice cold fresh medium. Plated cells were then placed into 37°C humidified incubator with 5% CO₂. Medium was changed the following day to remove cell debris, and cells were allowed to grow to semi-confluency and passaged. Since pRSV-T does not have a selection marker gene, RSVT-2 cells were selected on the basis of the ability of SV40 Tag to extend growth beyond the 12-15 passages at which HTR8 cells invariably died. The cell line is heteroclonal and the lifespan of the surviving RSVT-2 cells was greater than 36 passages. RSVT-2 cells were cultured in fetal calf serum supplemented with complete RPMI-1640, and were used between 20 to 24 passages post-transformation for phenotyping and functional studies.
2.2.1.3 Isolation of an immortal cell line, RSVT2/C, from RSVT-2 cells

RSVT-2 cells were grown to confluency in five 100 mm plates and with limited medium changes at a frequency of once a week over a period of several months in a forced crisis regimen. Full medium changes were done every third week. At first there were several colonies apparent in the plates. But only a single colony survived after three months in culture under the forced crisis regimen of limited medium changes; this was designated as RSVT2/C. Dead cells and debris were removed during the medium changes. The colony derived cells were expanded over a second period of three months for use in the present study. Choriocarcinoma cell lines, JAR and JEG-3, were also cultured in RPMI-1640 FCS-supplemented medium. These cells were used as positive controls for their colony forming ability in soft agar to test anchorage independent growth.

For the phenotypic and functional studies described below, both HTR8 (passage 8-11), RSVT-2 (passage 20-24), and RSVT2/C (passage 45-49) cell lines were passaged using 0.025% trypsin-PBS-EDTA solution. Trypsinized cells were centrifuged after neutralizing with complete medium, rinsed once with complete medium, re-pelleted by centrifugation, resuspended as single cell suspension, and replated onto 100 mm petri dishes at the desired densities. Cell numbers were obtained using a Coulter Counter (Coulter Electronics Inc., Haileah, FL). Cell viability was determined by Trypan Blue (0.4% w/v) dye exclusion.
2.2.2 Growth Factors and Antibodies

A number of growth factors shown to be produced at the fetomaternal interface (Lala and Lysiak, 1994) were employed in the present study to test the effects on cell proliferation or invasion as specified later. TGFα (Peninsula Laboratories, Belmont, CA), TGFβ1 (R & D Systems, Minneapolis, MN), and EGF (Upstate Biotechnology Inc. [UBI], Lake Placid, NY), were added at concentrations of 10 ng/ml (a concentration shown to be effective on early passage HTR8 cells in our earlier studies) to subconfluent cultures for a period of 24 h. Neutralizing antibodies (25 μg/ml) to TGFα (Berlex Bioscience, Alameda, CA; courtesy of Dr. B. Langton), EGF (UBI) and TGFβ (1D11.16.8, courtesy of Dr. H. Higley, Celltrix Laboratories, Collagen Corporation, Palo Alto, CA), as well as an EGF receptor blocking antibody (UBI), were also tested individually. Addition of the antibodies was designed to neutralize or block the possible effects of endogenous growth factors. The neutralizing antibodies to TGFα (Lysiak et al. 1993; 1994), TGF-β (Graham et al. 1992) and the EGFr blocking antibody (Lysiak et al. 1993; 1994) had already been tested to be functional in trophoblast proliferation assays in our laboratory at the concentrations used. Controls contained cells cultured without growth factors or antibodies.
2.2.3 Characterization of Cells

2.2.3.1 Indirect immunofluorescence.

Immunolabeling was carried out (in at least three separate experiments) with cells grown as monolayers. Cells (HTR8, RSVT-2, and RSVT2/C; 10⁴ cells) were passaged into Nunc 4 or 6 chamber glass slides, cultured for 48 h, and fixed in ice cold methanol. Slides were incubated with normal goat serum (1:10 dilution, Dimension Labs., Mississauga, ON) and 1% (w/v) of bovine serum albumin (BDH, Mississauga, ON) to block nonspecific binding sites.

The presence of cytokeratin and SV40 Tag was detected by indirect immunofluorescence using a rabbit polyclonal antibody (Ab) to cytokeratin (1:200 dilution (Dako); Dimension Labs.) and a mouse monoclonal Ab to SV40 Tag (1:200 dilution; Oncogene Science), respectively. Subsequently, fluorescein-labeled goat anti-rabbit immunoglobulin (Ig) secondary Ab and rhodamine-labeled goat anti-mouse Ig secondary Ab (1:250 dilution; Dimension Labs.) were used to visualize immunoreactivity for these respective markers. Replacement of the primary Abs with similar concentrations of rabbit Ig or mouse Ig served as negative controls.

Expression of HLA class I framework antigen and the human placental lactogen (hPL) by these cells was examined with the rabbit polyclonal W6/32 Ab
(1:200 dilution, Serolab, Sussex, UK) and rabbit polyclonal rabbit hPL Ab (1:200 dilution (Dako), Dimension Labs.), respectively. These slides were then incubated with biotinylated goat anti-rabbit Ab (1:200, Vector Laboratory Inc., Burlingame, CA) followed by rhodamine-conjugated streptavidin (1:250 dilution; Vector Laboratory Inc.) to identify the markers by immunoflourescent labeling. Negative controls were provided by replacement of the primary Ab with similar concentrations of rabbit Ig.

2.2.4 Cell Proliferation Assays

2.2.4.1 Tritiated thymidine ($^3$H-TdR) incorporation.

Five thousand cells in a volume of 50 µl were plated on a flat bottom 96 well (Corning) plate in triplicate in four separate experiments. Fifty µl of growth factors alone or antibodies alone in appropriate concentrations (as specified earlier in section 2.2.2) or medium alone (control) were added into the wells for 18 h; cells were then cultured for an additional 6 h with 50 µl of 0.3 µCi/ml of $^3$H-TdR (to give a final concentration of 2 µCi/ml). The medium was removed, the wells washed with PBS-EDTA and trypsin (0.25%)-PBS-EDTA was added for 15 mins. Wells were harvested with distilled water (hypertonic solution) onto filter paper using a Titertek™ cell harvester (ICN Ltd, St-Laurent, PQ). The amount of radioactivity incorporated into the cells in each well was determined using a scintillation counter (Beckman, Mississauga, ON).
2.2.4.2 Colorimetric assay for cellularity

Cells were plated as above and growth factor alone or antibodies alone were added as specified above. The colorimetric assay, which indicates the relative density of metabolically active cultured cells, was carried out on two separate occasions as described in the protocol (Promega Technical Bulletin No. 169) provided by the vendor (CellTiter 96™ AQueous (Promega), Fisher Scientific). To prepare the MTS/PMS solution, 2 ml of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; 2 mg/ml concentration) was mixed following addition of 100 μl of PMS (phenazine methosulfate; 0.92 mg/ml). At 21 h after incubation in the experiment, 20 μl of the MTS/PMS solution were pipetted into each well of the 96 well assay plate containing medium alone with no cells (for medium background control) and the wells containing the experimental conditions. The plate was then returned into the incubator for 3 h and then absorbance was measured in Titertek™ ELISA plate reader at the 492 nm wavelength. The MTS salt is converted to an aqueous soluble formazan product by endogenous dehydrogenases in the metabolically active cells. The absorbance measures the amount of formazan product and is reflective of the density of live cells in the culture.
2.2.5 Matrigel Transwell Invasion Assay

Invasiveness of HTR8, RSVT-2, and RSVT2/C cells was examined (in at least 3 separate experiments) using a Matrigel™ (Collaborative Biomedical Products, Bedford, MA) transwell invasion assay (Graham et al. 1993b) which measures the ability of cells prelabeled with \( ^3 \)H-TdR to transgress a Matrigel (reconstituted basement membrane) barrier. Transwells™ (6.5 mm filter, 8 \( \mu \)m pore size) were coated with 100 \( \mu \)l of Matrigel (1:20 dilution, 200 \( \mu \)g/ml), air dried overnight in a laminar flow hood and rehydrated with serum free RPMI-1640 for 90 mins on a shaker platform.

All three cell lines were incubated for 48 h after medium replenishment with 10 \( \mu \)Ci/ml \( ^3 \)H-TdR, and were lifted using 0.025% trypsin-PBS-EDTA. Cells were washed and resuspended in medium to a concentration of \( 2 \times 10^5 \) cells per ml. A volume of 200 \( \mu \)l of cell suspension was added with or without growth factors or with antibodies alone into the upper chambers, and 800 \( \mu \)l of complete medium was added to the lower chambers in triplicate wells. Following a 72 h incubation period, the medium from the lower chamber was placed into scintillation vials, 800 \( \mu \)l of 0.25% trypsin solution was added, and the upper well left in place for 20 mins. Adherent cells on the underside of the chamber were aspirated off, and the upper chamber, including the medium, was placed into a separate scintillation vial. The trypsin solution and washings from the lower chambers were then pooled
with medium. Ten mls of scintillation fluid were added and radioactivity of cells in each chamber was determined in a Beckman scintillation counter. Invasion index was calculated from the amount of radioactivity in the lower chamber, inclusive of cells adherent to the undersurface of the Millipore membrane divided by the total radioactivity in both chambers, and expressed as a percentage.

2.2.6 Tests for Tumorigenic Phenotype of SV40 Tag-Transformed Cells

2.2.6.1 In vivo tumorigenicity assay

Four or five athymic nude mice (Balb/c background, Harlan-Sprague Dawley), per group and per each cell line, were transplanted on two separate occasions: (i) subcutaneously \((2 \times 10^6)\) or intravenously \((10^5)\) with HTR8 and RSVT-2, and (ii) subcutaneously \(10^6\) and underneath the renal capsule \(10^6\) into the same mouse (by both routes), with HTR8, RSVT-2, or RSVT2/C cells, in order to determine tumorigenicity of the transformed cells. Mice were visually examined for tumor formation under the skin at regular biweekly intervals, and autopsied at three and eleven months in the first occasion and at three months in the second occasion for tumors in internal organs or under the renal capsule. A single nude mouse was transplanted with a mouse blastocyst under the kidney capsule and served as a control for the procedure in the second experiment.
2.2.6.2 Soft Agar Growth Assay

A modified soft agar assay was conducted as described previously by Ciardiello et al. (1991). One hundred thousand cells were suspended in 1.0 ml of 0.4% Difco Noble top agar (Difco, Detroit, MI) supplemented with RPMI-1640 medium containing 10% fetal bovine serum, and layered over a 0.8% agar-RPMI-1640 medium base in six well plates (Corning, Fisher Scientific). The ability of HTR8, RSVT-2, and RSVT2/C cells to survive and form colonies in soft agar was compared with that of known choriocarcinomas, JAR and JEG-3. Two separate experiments were conducted with six replicates in each experiment for a duration of three weeks in culture.

2.2.7 cDNA Probes

The plasmids containing human cDNA insert for the invasion-associated genes (72 kDa and 92 kDa type IV collagenases, and TIMP-2), as well as a 72 kDa type IV collagenase PCR fragment, (p320; bp 1467 to 1747) were all graciously provided by Dr. W. Stettler-Stevenson, NIH, Bethesda, MD. Human TIMP-1 plasmid cDNA (pUC9-F5) was obtained from Synergen Co. (Boulder, CO). Plasmid cDNAs for the human plasminogen activator inhibitors, PAI-1 and PAI-2, were kindly provided by Dr. K. McCrae, University of Pennsylvania, Philadelphia, PA.
PCR fragments for 92 kDa type IV collagenase (5'primer, 5'-CAA CAT CAC CTA TTG GAT CCA-3'; 3' primer, 5'- ACA AAC TGT ATC CTT GGT CCG-3'; bp 378 to bp 1205), TIMP-1 (5'primer, 5'- TTC TGC AAT TCC GAC CT-3'; 3'primer, 5'- GAA AGA TGG GAG TGG GAA-3'; bp 34 to 635), and TIMP-2 (5'primer, 5'- TGC AGA TGT AGT ATC A-3'; 3'primer, 5'- TCA GAG CTG GAC CAG T-3'; bp 64 to 676) were produced locally in this laboratory using human plasmid cDNA as templates. In the PCR reaction, the isolated cDNA template was incubated with 100 picomoles of each of the two primers, 250 μM dNTPs, 2 mM MgCl₂, 1X PCR buffer (GIBCO-BRL), and 2.5 U of Taq DNA polymerase (GIBCO-BRL). The reaction was brought up to 50 μl with sterile distilled water and overlayed with 50 μl of light paraffin oil (BDH). The PCR condition was: denatured at 95°C 30 secs, annealed at 55°C 45 secs and extended at 72°C 45 secs for 35 cycles followed by 10 mins incubation at 72°C (MJ Research Minicycler, Fisher Scientific, Mississauga). The PCR product was electrophoresed on a 0.8% acrylamide gel and electroeluted from the acrylamide gel in a dialysis tubing (Sambrook et al. 1987). The final product was then used for the synthesis of radiolabeled cDNA probes for northern blot analysis to examine mRNA expression in RSVT-2 and HTR8 cells.

The Eco RI digested inserts for PAI-1 and PAI-2 were separated on 1.2% low melting point (LMP) agarose (GIBCO-BRL) in 1X TAE buffer. The 1.9 kb bands
were cut from the LMP agarose, boiled, and 3 volumes of distilled water were added. The probe was made using 20-25 μl of cDNA solution as described previously.

An 18S rRNA probe (p100D9), a kind gift from Dr. A. F. Chambers, London Regional Cancer Centre, London, ON, was used to assess for differences in loading between lanes.

All the cDNA probes were labeled using the Megaprime labeling kit (Amersham, Mississauga, ON), 25 ng of DNA and 50 μCi (α- P; 3000 Ci/mmoll) dCTP (Amersham). Radiolabeled cDNA probes were passed through a Sephadex G50 nick column (Pharmacia, Baie d'Urfe, PQ) to remove unincorporated nucleotides. Specific activity of the probes were 1-2 X 10^6 dpm/μg of DNA.

2.2.8 Northern Blot Analysis

Total RNA was isolated from cells in culture using an alcohol-phenol method (Sambrook et al. 1987). The cells were lysed in 1% Nonidet P40 (BDH, Mississauga) diluted in low salt PBS (pH 7.4). The RNA was extracted by adding equal volume of Tris-saturated phenol (pH 8), and centrifuged at 10,000 x g for 20 mins at 4°C in a DuPont HB-4 rotor. The aqueous phase was then transferred to another tube and extracted with an equal volume of chloroform:isoamyl alcohol (24:1) to remove residual phenol. The RNA was precipitated with 100% ethanol
plus 0.1 volume of 3M sodium acetate (pH 5.2) at -80°C overnight. The RNA was then pelleted at 10,000 x g for 20 mins at 4°C (DuPont HB-4 rotor), resuspended in 0.5 ml of 1 mM EDTA (pH 8) and stored at -70°C. The RNA concentration was obtained by UV spectrophotometry using a Beckman DU50 spectrophotometer. Alternatively, a modified method of guanidium isothiocyanate-phenol of Chomczynski (1993) as described by Goldberg et al. (1993) was also used.

Ten μg of total RNA (from at least two separate experiments) were lyophilized and resuspended in 4.5 μl of DEPC-treated H₂O. A volume of 15.5 μl of sample denaturation solution (formamide (64.5% v/v), formaldehyde (37% v/v), 5X MOPS buffer (0.02M MOPS, 0.005M NaOAc, 0.5M EDTA (pH 7.0)) was added to the RNA samples and the samples were incubated at 65°C for 15 mins to denature the RNA. The solution was rapidly cooled in ice and 3 μl of formaldehyde loading buffer (37.5% formaldehyde, 30% glycerol, 0.4% bromophenol blue, 1 μg/μl of ethidium bromide) were added. Samples were then loaded and electrophoresed on a 1.0% formaldehyde-agarose gel (Lehrach et al. 1977) at 55 V for 3 h. The gels were transferred overnight to Nytran™ (Schleicher and Schuell, Keene, NH) nylon membrane by capillary action using 10X standard saline citrate (SSC) as the transfer buffer. After the transfer, the membranes were rinsed once in DEPC-treated dH₂O and baked at 80°C for 2 h in a vacuum oven.
The northern blots were hybridized to α- P-dCTP-labeled PCR fragments for 72 kDa type IV collagenase, 92 kDa type IV collagenase, TIMP-1 and TIMP-2, as well as PAI-1 and PAI-2. Prehybridization and hybridization of the membranes was performed in 50% formamide, 5X SSPE, 5X Denhardt's solution, 1.0% SDS and 100 µg/ml of salmon sperm DNA solution at 42°C overnight. After hybridization, the membranes were washed twice with 2X SSC, 0.1% SDS for 15 mins each, one time with 1X SSC, 0.1% SDS for 30 mins and once with 0.1X SSC, 0.1% SDS for 30 mins each. All of the prehybridizations, hybridizations, and washing of the blots were carried out in the roller bottles (Hybaid™ Mini-oven, Interscience Inc., Markham, ON). The membranes were exposed to XAR-5 or Biomax BMR x-ray film (Kodak) at -80°C, using intensifying screens (Hyperscreens, Amersham). Control 18S rRNA probing was also carried out under the same conditions. The densitometric measurements of total absorbance for each band were made with the JAVA for Windows™ image analysis software package (Jandel Scientific, Corte Madera, CA).

2.2.9 Zymogram Analysis

2.2.9.1 Sample collection and protein preparation

For preparations of samples, cell lines (HTR8, RSVT-2, RSVT-2/C) were cultured in 24 well plates containing 0.5 ml of 1% BSA-supplemented RPMI 1640 medium with or without TGFβ (10 ng/ml) or antibody to TGFβ (25 µg/ml). After
four days, the medium from each well (500 µl) was collected, and lyophilized to concentrate samples. Lyophilized samples were resuspended in 100 µl of protein extraction buffer (0.1% SDS, 0.05M Tris-HCl, pH 5.2). Total protein concentration was measured by a modified Bradford method (Bradford, 1976) using BioRad™ Protein Assay Dye Reagent. A total of 15 µg of protein from each sample was incubated with 0.25X volume of 4X sample buffer (48% urea, 8% SDS, 0.5M Tris-HCl pH 6.8) for 10 mins at room temperature and loaded for the zymogram. Samples were collected from two separate experiments.

2.2.9.2 Zymogram Gels

Briefly, zymogram gels were prepared as described by Xuan et al. (1995), consisting of a 4% polyacrylamide/0.1% SDS stacking gel above a 10% polyacrylamide/0.1% SDS resolving gel containing gelatin (600 µg/ml; BDH). After electrophoresis the gels were washed twice (30 mins each time) in 2.5% Triton X-100 solution at room temperature on a shaker platform, and then incubated in 1X CAB (50mM Tris-HCl buffer, pH 8.0, 5mM CaCl₂, 0.02% (w/v) sodium azide) for 18-24 h at 37 °C. Gels were then stained in 0.05% Coomassie blue solution (40% methanol, 10.0% acetic acid) for 1 h, rinsed twice in distilled H₂O, and placed in destaining solution (40% methanol, 10% acetic acid) overnight. Gelatin degrading enzymes were visualized as clear bands, indicating proteolysis of the gelatin substrate.
2.2.10 Statistical Analysis

Possible differences amongst the means of proliferation and invasion indices measured under the different conditions were first analyzed by Kruskal-Wallis test (SchoolStat™). In the case of a significant difference identified by that test, differences between pairs were analyzed using Wilcoxon-Mann Whitney U test. A difference was considered significant at $p \leq 0.05$. These tests were independent of the distribution characteristic of the data.

2.3 Results

2.3.1 Parental HTR8 and $SV40$ Tag Expressing Transformants Express Extravillous Trophoblast Cell Markers

As presented in Figure 1, parental HTR8 cells show strong expression for cytokeratin, an epithelial cell marker, W6/32 (HLA framework antigen), and hPL (hormone produced by epithelial placental cells) as reported previously in this laboratory. No immunoreactivity for SV40 Tag was found. Both RSVT-2 (Figure 2) and RSVT2/C (Figure 3) cell lines also expressed CK, W6/32, and hPL. In addition, RSVT-2 (Figure 2) and RSVT2/C (Figure 3) cells also showed nuclear immunolocalization of SV40 Tag. Negative controls for all the cell types showed no immunostaining in any of the experiments.
Figure 1: Immunolabeling of normal HTR8 parental cells

HTR8 parental cells were examined for the expression of epithelial cell marker cytokeratin (CK; fluorescein, green fluorescence), SV40 large T antigen (SV40 Tag; rhodamine, red fluorescence), human leukocyte antigen framework (W6/32; rhodamine, red fluorescence), and human placental lactogen (hPL; rhodamine, red fluorescence). Photomicrographs were taken using 40X objective; bar represents ~100 μm. The cells were CK+, SV40 Tag-, W6/32+, and hPL+. 
Figure 2: Immunolabeling of extended lifespan RSVT-2 cells

RSVT-2 extended lifespan cells were characterized for the expression of epithelial cell marker cytokeratin (CK; fluorescein, green fluorescence), SV40 large T antigen (SV40 Tag; rhodamine, red fluorescence), human leukocyte antigen framework (W6/32; rhodamine, red fluorescence), and human placental lactogen (hPL; rhodamine, red fluorescence). Photomicrographs were taken using 40X objective; bar represents ~100 μm. The cells expressed all the above markers.
Figure 3: Immunolabeling of immortalized RSVT2/C cells

RSVT2/C immortal cells were characterized for the expression of epithelial cell marker cytokeratin (CK; fluorescein, green fluorescence), SV40 large T antigen (SV40 Tag; rhodamine, red fluorescence), human leukocyte antigen framework (W6/32; rhodamine, red fluorescence), and human placental lactogen (hPL; rhodamine, red fluorescence). Photomicrographs were taken using 40X objective, bar represents ~100 μm. The cells expressed all the above markers.
2.3.2 *In Vitro* Growth of RSVT-2 and RSVT2/C Cells as Compared to the Parental HTR8 Cells

The *in vitro* growth rate of *SV40* Tag transformed cell lines was compared with that of the parental HTR8 cells using \(^3\)H-TdR incorporation (Figure 4) as an index of cellular proliferation. RSVT-2 cells showed a greater \(^3\)H-TdR incorporation (~3 fold) than their parental counterpart, suggesting a higher proliferative rate (p=0.001; Figure 4). In Figure 5, both RSVT-2 (p=0.026) and RSVT2/C (p=0.025) cells in culture produced higher absorbance (at least 2.3 fold higher) than the parental HTR8 cells at 24 h after plating of an equivalent number of cells. Based on the \(^3\)H-TdR incorporation results of a higher proliferation of RSVT-2 cells than HTR8 cells, and equivalent absorbance (Figure 5) of RSVT-2 and RSVT2/C cells 24 h after plating, it appeared that both cell lines proliferated at a higher rate than the parental HTR8 cell line.

2.3.2.1 *Action of Growth Factors or their antibodies on proliferation of HTR8, RSVT-2 and RSVT2/C cell lines*

None of the EGFr ligands, TGF\(\alpha\) or EGF, nor the TGF\(\alpha\) neutralizing Ab or EGFr blocking Ab, at the concentrations employed, had any significant influence on the proliferation (\(^3\)H-TdR incorporation, Figure 4) of late passage HTR8 cells (at 11\(^{th}\) passage), although proliferation-stimulating effect of EGFr ligands has been documented on earlier passage of HTR8 cells from this laboratory (Lysiak *et al.* 1993). Similarly, RSVT-2 cell proliferation was also not influenced in the
Figure 4: $^3$H-TdR incorporation cell proliferation assay

Representative data on β-counts resulting from $^3$H-TdR incorporation of RSVT-2 (22nd passage) and HTR8 (11th passage) cells incubated with various growth factors or antibodies (added individually). Bars indicate means (± standard error (SE)) of quadruplicate determinations. Significant differences are given in the table below:

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Label and p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTR8 control:RSVT-2 control</td>
<td>A; p=0.001</td>
</tr>
<tr>
<td>HTR8 control:HTR8 TGF beta</td>
<td>B; p=0.002</td>
</tr>
<tr>
<td>RSVT-2 control:RSVT-2 TGF beta</td>
<td>C; p=0.001</td>
</tr>
</tbody>
</table>
Cell Proliferation Assay

3H-TdR Incorporation (mean dpm ± SE)

Conditions of Cell Culture

Control  TGF alpha  Anti-TGF alpha  Anti-EGFr  EGF  TGF beta  Anti-TGF beta

A  B  C
Figure 5: Colorimetric Cellularity Assay

Representative cellularity assay using MTS/PMS CellTiter AQueous™ measurement of cell proliferation. RSVT-2 (25\textsuperscript{th} passage), HTR8 (11\textsuperscript{th} passage) and RSVT2/C (46\textsuperscript{th} passage) cells were incubated with medium alone or TGF beta alone or anti-TGF beta alone in a 24 h assay. Bars indicate means (± standard error (SE)) of quadruplicate determinations. Significant differences are given in the table below:

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Label and p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTR8 control:RSVT-2 control</td>
<td>A; p=0.026</td>
</tr>
<tr>
<td>HTR8 control:RSVT2/C control</td>
<td>B; p=0.025</td>
</tr>
<tr>
<td>HTR8 control:HTR8 TGF beta</td>
<td>C; p=0.05</td>
</tr>
<tr>
<td>RSVT-2 control:RSVT-2 TGF beta</td>
<td>D; p=0.05</td>
</tr>
</tbody>
</table>
presence of TGFα, anti-TGFα Ab, or EGFr blocking Ab at similar concentrations (Figure 4).

TGFβ, however, significantly inhibited \(^{3}H\)-TdR incorporation (~1.8 fold inhibition) by HTR8 (p=0.002) as well as RSVT-2 (p=0.001) cells (Figure 4). A significant inhibiting effect was also noted on cell density of HTR8 (p=0.05) as well as RSVT-2 (p=0.05) but not RSVT-2/C (p=0.1) cells in the presence of TGFβ (Figure 5). Anti-TGFβ neutralizing Ab had no influence on the \(^{3}H\)-TdR incorporation (Figure 4) or on cell density (Figure 5) of any of the cell lines indicating that no significant level of active endogenous TGFβ was secreted by these cells.

2.3.3 Matrigel Invasion Assay

Figures 6 and 7 depict the results of the *in vitro* Matrigel invasion assay. The two cell lines RSVT-2 (Figures 6 [40.98% invasion index] and 7 [34.323%]; p < 0.05) and RSVT2/C (p < 0.05; Figure 7 [36.88%]) were more invasive than their parental HTR8 cells (Figure 6 [34.93%] and Figure 7 [29.19%]. None of the EGFr ligands had any significant effect on invasion by HTR8 or RSVT-2 cells (Figure 6). TGFβ caused significant inhibition (p=0.025) of invasion (29.4% for TGFβ treated vs. 34.9% for control) of the parental HTR8 cells but not in the SV40 Tag transformed RSVT-2 (Figure 6 and 7) and RSVT2/C (Figure 7) cells. Interestingly, in one experiment TGFβ significantly stimulated the invasion of RSVT-2 cells.
Figure 6: Matrigel invasion assay of HTR8 and RSVT-2 cells

Representative data for the Matrigel invasion assay are presented in this figure. Invasiveness of HTR8 and RSVT-2 cells was measured under various conditions including, growth factor alone, or antibody alone, or medium alone (control). Bar indicates means (± standard error (SE)) of triplicate determinations. Significant differences are given in the table below:

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Label and p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTR8 control: RSVT-2 control</td>
<td>A; p=0.05</td>
</tr>
<tr>
<td>HTR8 control: HTR8 TGF beta</td>
<td>B; p=0.025</td>
</tr>
<tr>
<td>RSVT-2 control: RSVT-2 TGF beta</td>
<td>C; p=0.025</td>
</tr>
</tbody>
</table>
Figure 7: Matrigel invasion assay with HTR8, RSVT-2 and RSVT2/C cells

Representative data for Matrigel invasion assay are shown. HTR8 (10\textsuperscript{th} passage), RSVT-2 (28\textsuperscript{th} passage) and RSVT2/C (44\textsuperscript{th} passage) were used in the invasion assay under the various culture conditions. TGF beta (10 ng/ml) and anti-TGF beta (25 μg/ml) were used in addition to medium only (control). Bar indicates means (± standard error (SE)) of triplicate determinations. Significant differences are given in the table below:

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Label and p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTR8 control:RSVT-2 control</td>
<td>A; p=0.05</td>
</tr>
<tr>
<td>HTR8 control:RSVT2/C control</td>
<td>B; p=0.05</td>
</tr>
<tr>
<td>HTR8 control:HTR8 TGF beta</td>
<td>C; p=0.05</td>
</tr>
</tbody>
</table>
INVASION ASSAY

□ HTR8  ■ RSVT-2  ■ RSVT2/C

INVASION INDEX (mean %) +/- SE

A
B
C

Control
TGF beta
Anti-TGF beta

CONDITIONS OF CELL CULTURE
(Figure 6 [51.24 % for TGFβ, p=0.025). In the second experiment, there was no
elevation of invasion by TGFβ (Figure 7, p=0.10). Anti-TGFβ antibody alone did
not influence invasion through the Matrigel by either HTR8 cells or RSVT-2 cells,
indicating low or no endogenous production of TGFβ by these cells under the
assay conditions used.

2.3.4 In Vivo Tumorigenicity Assay

Cell lines HTR8, RSVT-2 and RSVT2/C were injected subcutaneously,
intravenously, or under the kidney capsule of nude mice to assess their ability to
form tumors in two separate experiments (for details see section 2.2.6.1). None of
the cell lines produced detectable tumors on either occasion (data not presented),
as assessed by visual inspection of live mice (sc) or internal organs after autopsy at
three months (i.v., renal capsule) and eleven months (i.v). One nude mouse which
received an implant of a mouse blastocyst under the renal capsule revealed a
hemorrhagic mass at three months as earlier reported by Kirby (1960).

2.3.5 Soft Agar Assay

Soft agar assay was conducted to determine if HTR8, RSVT-2, and
RSVT2/C cells were capable of anchorage-independent growth as a measure of
transformation and progression towards a malignant phenotype. Two
choriocarcinomas (JAR and JEG-3; positive controls), the parental HTR8
(negative control) and the two SV40 Tag transformed cell lines were examined.
After three weeks, no HTR8, RSVT-2, or RSVT2/C colonies formed in any of the plates, whereas the two choriocarcinomas formed large colonies (results not presented). The top agar layer was passed through a pipette, and cultured on 100 mm plastic culture dish. Subsequently, a number of cell colonies were observed on the culture dish in the case of only the RSVT2/C cell line and choriocarcinomas (data not presented). Thus, although RSVT2/C cells did not form visible colonies in the agar, they survived in the soft agar for three weeks. This was not the case for HTR8 or RSVT-2 cells.

2.3.6 Expression of MMP and TIMP Genes: Effects of Growth Factors

Northern blot analysis of mRNA expression of the 72 kDa type IV collagenase and TIMP-1 in HTR8, RSVT-2 and RSVT2/C cells is presented in Figure 8. RSVT2/C cells are analyzed in a separate experiment conducted at a different time, hence an HTR8 control was included in the analysis. The picture is shown after digital image enhancement to enhance the weak TIMP-1 message in RSVT2/C lanes, hence the HTR8 control (similarly enhanced) is also included. Differences in expression were analyzed by densitometric measurement relative to 18S rRNA used as a control to standardize loading differences. Because of the conditions of the analysis, only differences greater than 1.5 fold were considered worth documenting. Key findings are listed as follows:
Figure 8: Northern autoradiograph for 72 kDa type IV collagenase and TIMP-1

Northern blot autoradiograph of 72 kDa type IV collagenase (~3.1 kb) and TIMP-1 (~1.0 kb) message are shown for the three cell lines. Control 18S rRNA was included to compare loading among lanes. HTR8 and RSVT-2 cells were examined at the same time, but RSVT-2 lanes were digitally image enhanced to enhance TIMP-1 images. A similarly enhanced HTR8 control lane (HTR8 Control*) was also included to allow comparisons of RSVT-2 to HTR8. Since RSVT2/C were done at a later time point, a control lane of HTR8 (untreated) total RNA was included (HTR8 Control**). Photographs of RSVT-2 and RSVT2/C were also image-enhanced to enable visualization of TIMP-1 message. Densitometric values are documented when changes were ≥ 1.5. Accurate measurement of TIMP-1 was not possible for RSVT-2 and RSVT2/C because of very low levels.

Arbitrary Densitometric Values (Normalized to 18S)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Treatment</th>
<th>72 kDa</th>
<th>Cell Line</th>
<th>Treatment</th>
<th>TIMP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTR8</td>
<td>Control</td>
<td>1</td>
<td>HTR8</td>
<td>Control</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>EGF</td>
<td>1.54</td>
<td></td>
<td>EGF</td>
<td>1.84</td>
</tr>
<tr>
<td>RSVT-2</td>
<td>Control</td>
<td>1</td>
<td></td>
<td>TGFβ</td>
<td>1.63</td>
</tr>
<tr>
<td></td>
<td>TGFβ</td>
<td>2.27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSVT2/C</td>
<td>Control</td>
<td>1</td>
<td></td>
<td>TGFβ</td>
<td>2.05</td>
</tr>
</tbody>
</table>

Difference of 72 kDa type IV collagenase expression between HTR8 and RSVT-2 is ~2 fold, and between HTR8 and RSVT2/C is ~5 fold. Difference of TIMP-1 expression between HTR8 and RSVT2/C is estimated to be ~5 fold and between HTR8 and RSVT2/C is estimated to be ~2 fold.
1. All cell lines expressed abundant 72 kDa type IV collagenase mRNA. TIMP-1 mRNA expression was low in both RSVT-2 and RSVT2/C cells, but appreciable in HTR8 cells. Relative abundance of both 72 kDa type IV collagenase mRNA as well as TIMP-1 mRNA was lower in the transformants as compared to HTR8 cells. However, the ratio of 72 kDa type IV collagenase to TIMP-1 mRNA (after standardization for loading) was still higher in the RSVT-2 cells than the HTR8 cells.

2. Exposure to TGFβ caused an upregulation of TIMP-1 in HTR8 as has been documented earlier (Graham and Lala, 1991); in contrast, TGFβ upregulated 72 kDa type IV collagenase in both RSVT-2 and RSVT2/C cells, but not TIMP-1.

3. Exposure to EGF caused an equivalent upregulation of 72 kDa type IV collagenase and TIMP-1 mRNA in HTR8 cells but not in the transformants.

4. Exposure to other agents (TGFα, anti-TGFα, anti-EGF, anti-EGFr, and anti-TGFβ) did not reveal any appreciable changes in the expression of mRNA molecules in any cell line.

Figure 9 presents the northern analysis of TIMP-2 mRNA expression in these cell lines under different conditions. The following were their notable features:
Figure 9: Northern autoradiograph of TIMP-2 message

Northern blot autoradiographs for TIMP-2 message are shown for HTR8, RSVT-2 and RSVT2/C cells. Control 18S rRNA was included to compare equal loading. As mentioned in Figure 8, a control lane for HTR8 (untreated) was included (HTR8 Control**). Figure photographs have been digitally enhanced to reveal the smaller TIMP-2 (1.0 kb) band. However, since the levels were low in RSVT-2 and RSVT2/C cells, some of the densitometry evaluations were inconclusive.

Arbitrary Densitometric Values for 3.5 kb band (Normalized to 18S)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Treatment</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTR8</td>
<td>Control</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>TGFα</td>
<td>1.74</td>
</tr>
<tr>
<td>RSVT-2</td>
<td>Control</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Anti-EGFr</td>
<td>2.0</td>
</tr>
</tbody>
</table>
1. Both 1.0 kb and 3.5 kb transcripts of TIMP-2 were detectable in all cell lines, however, these transcripts were only barely detectable in RSVT2/C cells.

2. While no appreciable influence of any agents was noted on the 1.0 kb transcript expression in any cell line, TGFα upregulated the 3.5 kb transcript in HTR8 cells. An upregulation of this transcript was noted with anti-EGFr antibody in the lane of RSVT-2 cells.

Although 92 kDa type IV collagenase was not detected by northern blot analysis of total RNA (data not presented), the message for 92 kDa type IV collagenase was detected by RT-PCR and slot blot hybridization (data not presented). In addition, it was clearly observed in the zymogram (see below).

2.3.7 Zymogram Analysis of MMPs (Gelatinases) In Normal Trophoblast Cells and Their Transformed Derivatives, RSVT-2 and RSVT2/C

Zymogram analysis of conditioned cell medium, collected 96 h after no treatment or treatment with TGFβ and anti-TGFβ, revealed that both 72kDa and 92kDa type IV collagenases are produced by HTR-8, RSVT-2 and RSVT2/C cells. It was also observed that all three cell lines produced the latent (~68 kDa band) as well as the active (~62 kDa band) forms of 72 kDa type IV collagenase. All three cell lines also produced latent (~92 kDa band) 92 kDa type IV collagenase. Only RSVT2/C cells produced the activated form (~80 kDa) of the 92 kDa type IV
Figure 10: Zymographic Analysis for 72 kDa and 92 kDa type IV collagenases

A representative zymogram is presented for HTR8, RSVT-2 and RSVT2/C cells under the various treatment conditions. The major metalloproteases appearing in the supernatants of the various cell lines correspond to 92 kDa type IV collagenase (migration of latent form appeared at ~ 92 kDa and its activated form appeared at ~ 80 kDa) and 72 kDa type IV collagenase (migration of latent form at ~ 68 kDa and its active form at ~62 kDa). These observations correlate well to published estimations of gelatinase (type IV collagenases) sizes in the literature (Albini et al. 1991; Kataoka et al. 1993; Lehtovirta and Vartio, 1994). A strong band corresponding to activated 92 kDa type IV collagenase appears only in the RSVT2/C lanes.
collagenase, which was barely detectable in the supernatants of HTR8 or RSVT-2 cells. Possible changes after exposure of cells to TGFβ or anti-TGFβ Ab (Figure 10) were not large enough for a quantitative evaluation.

2.3.8 Preliminary Analysis of the Expression of the mRNA of Plasminogen Activator Inhibitors (PAI-1 and PAI-2) in RSVT2/C and HTR8

As presented in Figure 11, PAI-1 mRNA expression appeared as two transcripts (3.2 kb and 2.4 kb) in both cell lines. The expression of both transcripts was lower in the RSVT2/C cell line when compared to the parental HTR8 cell line. Treatment with the EGF or TGFα did not appear to alter the expression of the PAI-1. In contrast, TGFβ treatment strongly upregulated the expression of PAI-1 mRNA in HTR8 cells, and possibly, to a minor extent, in RSVT2/C cells (Figure 11). However, since the natural level of expression was reduced, it was not possible to make a definitive evaluation by densitometry. Antibody to TGFβ did not cause any change in PAI-1 expression. PAI-2 message was not detectable in any of these cells (data not presented).
Figure 11: Northern autoradiograph for PAI-1

Northern blot autoradiograph for PAI-1 expression in HTR8 and RSVT2/C cells. Treatments with various growth factors or antibodies alone were also included. Control 18S rRNA was included to compare loading of the lanes. Evaluation of changes due to growth factors in RSVT2/C cells was not possible due to low natural expression and the relative background. This photograph has been overexposed to reveal bands in RSVT2/C lanes.

Arbitrary Densitometric Values for PAI-1 (both bands) (Normalized to 18S) in HTR8 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Upper</th>
<th>Lower</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TGFβ</td>
<td>1.74</td>
<td>2.23</td>
</tr>
<tr>
<td>RSVT2/C</td>
<td>HTR8</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>TGFα</td>
<td>Anti-EGF</td>
</tr>
<tr>
<td></td>
<td>Anti-EGF</td>
<td>EGF</td>
</tr>
</tbody>
</table>

PAI-1

~ 3.2 kb
~ 2.4 kb

18S
2.4 Discussion

2.4.1 Long Lived RSVT-2 and Immortal RSVT2/C Cells Share Trophoblastic Properties with the Original Parental HTR8 Cells

The oncogene SV40 Tag was successfully introduced into first trimester human trophoblast cells. Characterization of this cell line, designated as RSVT-2, and its subclone RSVT2/C, isolated after forced cell crisis, revealed strong nuclear immunoreactivity for SV40 Tag in 100% of these cells. The transformed cells also expressed HLA framework (W6/32) antigen, human placental lactogen and the epithelial cell marker, cytokeratin, similar to the parental HTR8 cells showing that the SV40 Tag transformants are trophoblastic in nature.

2.4.2 SV40 Tag Transfected Cells are Premalignant Trophoblasts

SV40 Tag transfection, followed by selection of cells for long life (RSVT-2) or immortality (RSVT2/C), revealed that these transfecants had higher proliferative and invasive ability in vitro than their parental counterpart. However, these cells failed to form distinct colonies in agar or produce tumors in nude mice indicating that they have not reached a malignant stage. RSVT2/C cells did however survive for an extended period in agar. Transformed cells also acquired variable resistance to the anti-proliferative and anti-invasive action of TGFβ exerted in the case of normal trophoblasts. These SV40 Tag transformed cell lines share characteristics of “preneoplastic” cells—hyperproliferative and non-tumorigenic; however these cell lines are also invasive, thus these properties taken
together indicate that the transfectants are best characterized as cells of the "premalignant" stage of tumor progression. It is also possible that RSVT2/C cells are at a more advanced stage than RSVT-2 cells along the path of tumor progression.

2.4.3 Possible Mechanism for a Long Life and for Hyperproliferative Behavior of SV40 Tag Transfected Trophoblasts

The oncoprotein SV40 Tag is shown to bind to and inactivate a number of cellular growth regulating genes such as p53 (Pipas, 1992; Ludlow, 1993; Perry and Levine, 1993) and the retinoblastoma gene, pRb (Ludlow et al. 1989; Pipas, 1992). When SV40 Tag binds to p53 and/or pRb, and inactivates them, it usually correlates with increased cell proliferation, and predisposes them to tumorigenic transformation (Ludlow, 1993). It has also been suggested that SV40 Tag binding to p53 is necessary for the extended lifespan (Lin and Simmons, 1991; Kuhar and Lehman, 1991) and for immortalization (Kuhar and Lehman, 1991) of SV40 Tag transformants. Aboagye-Mathiesen et al. (1995), using another SV40 Tag transformed cell line, HTR8/SVneo produced in this laboratory (Graham et al. 1993a), showed that SV40 Tag co-precipitated with p53 protein which was overexpressed in these cells. It was suggested that a complex formation with SV40 Tag inactivated p53, contributing to immortalization and increased proliferative capacity of these cells. Thus the extended lifespan of RSVT-2 and the acquired immortalization of RSVT2/C may also be due to p53 inactivation.
2.4.4 Lost Sensitivity to the Anti-Proliferative Effect of TGFβ in RSVT2/C Cells: Possible Mechanisms

Previous studies have shown that locally produced growth factors such as TGFα and EGF (Lysiak et al. 1994) stimulated proliferation whereas TGFβ (Graham et al. 1992; 1994) inhibited proliferation of early passage normal first trimester trophoblast cells. In the present study, late passage (10 to 11) HTR8 cells were refractory to the growth stimulating effects of TGFα or EGF but still retained the growth inhibiting effect of TGFβ. SV40 Tag-transfected RSVT-2 cells, but not RSVT2/C cells, were growth inhibited by TGFβ, indicating a dysregulation of growth inhibition by TGFβ in the latter cells. The proliferation inhibitory effects of TGFβ are lost in certain choriocarcinomas (Graham et al. 1994). This has been shown for other growth inhibiting molecules including IL-6 in the case of melanoma (Lu et al. 1992; Lu and Kerbel, 1993; see review by Shih and Herlyn, 1993). The present study reveals that an escape from negative growth regulation can occur at the premalignant stage of tumor progression.

The anti-proliferative action of TGFβ on certain cells appears to be mediated via inhibition of pRb phosphorylation indirectly through an intermediary, cyclin-dependent kinase 4 (cdk 4), thus blocking cell division. Moreover, p53 also acts on pRb, directly inhibiting pRb phosphorylation (reviewed by Okragly et al. 1994). Since SV40 Tag is known to bind to p53 as well as pRb (Ludlow, 1993), an inactivation of one or both products may explain variable resistance to the anti-
proliferative signal of TGFβ on SV40 Tag transformed trophoblast cells noted in this study.

2.4.5 Hyperinvasiveness of RSVT-2 and RSVT2/C Cells: Possible Mechanisms

In the Matrigel invasion assay, RSVT-2 and RSVT2/C cells were more invasive than the parental HTR8 cells. Similar hyperinvasive behavior was also documented in another immortalized SV40 Tag transfected trophoblast line, HTR8/SVneo from this laboratory (Graham et al. 1993a). The present study was extended to explore possible mechanism(s). The results showed an alteration in the balance of 72 kDa type IV collagenase mRNA relative to the MMP inhibitor, TIMP-1 in RSVT-2 and RSVT2/C cells. There were greatly reduced levels of both TIMP-1 and TIMP-2 mRNA expression, and similarly reduced levels of PAI-1 mRNA expression in RSVT2/C cells as compared to the parental HTR8 cells. Thus, a downregulation of natural inhibitors of invasion-associated enzymes could account for the increased invasive ability of the transformants. Further quantitative analysis of proteins secreted by these cell lines is needed to validate these possibilities. The molecular basis for the alteration in invasion associated enzymes/inhibitors following SV40 Tag transformation in the trophoblast remains unknown at present.
An alteration in the balance of matrix degrading enzymes and their inhibitors has been described for other cell systems using immortalized or tumorigenic cells (Khokha et al. 1989; Ponton et al. 1991; Chambers et al. 1992). Tuck et al. (1991) found that ras oncogene expression in NIH 3T3 cells induced increased expression of 72 kDa and 92 kDa type IV collagenases, and decreased expression of TIMP-1 and TIMP-2. Metastatic SP1 mammary adenocarcinoma cells were reported to show increased matrix-degrading activity resulting from the reduced expression of TIMP-1 (Ponton et al. 1991). Anti-sense TIMP-1 cDNA transfection studies clearly demonstrated that TIMP-1 downregulation resulted in tumorigenicity of Swiss 3T3 cells in vivo (Khokha et al. 1989) and an increase in their invasiveness in vitro (Khokha et al. 1992b). Present results provide the first documentation that TIMPs can be downregulated at a premalignant stage of highly invasive cells even prior to the acquisition of immortality, and reinforces the hypothesis that TIMPs may function as tumor suppressive genes. This hypothesis is further substantiated by the observations of Khokha and associates (Martin et al. 1995) who found that in SV40 Tag transgenic mice, crossed with TIMP-1 overexpressing transgenic mice, there was a resultant reduction in tumor size and an increased latency of tumor development in the SV40 Tag / TIMP-1 mice. The cross with anti-sense TIMP-1 transgenic resulted in higher tumor incidence and a shorter latent period of tumor development.
It has been shown that PAI-1 was exclusively expressed in EVT cells (Feinberg et al. 1989; Fazleabas et al. 1991). In this study, PAI-1 was found to be expressed in HTR8 cells, and was downregulated in SV40 Tag transformed cells. While downregulation of PAI-1 may provide another mechanism for hyperinvasiveness of these cells, its contributory role remains to be validated in relation to the expression of plasminogen activators, e.g. uPA and tPA.

2.4.6 Lost Sensitivity of SV40 Tag Transformants to the Anti-Invasive Action of TGFβ: Possible Mechanisms

The Tag-transfected RSVT-2, unlike the parental HTR8 cells, were completely unresponsive to the anti-invasive signal of TGFβ (discussed below). RSVT2/C, like the JAR and JEG-3 choriocarcinoma cells, were unresponsive to both anti-proliferative and anti-invasive signals provided by TGFβ (Graham et al. 1994). Another line of SV40 Tag transformed, immortalized HTR8/SVneo cells were also found to respond poorly to the anti-invasive signal provided by TGFβ (Graham et al. 1993a). Although the mechanism was not immediately clear, the present study confirmed the initial observations of Graham et al. (1993a) and extends them to RSVT-2 and RSVT2/C cells.

This laboratory has previously reported that TGFβ inhibited the secretion and activity of uPA and upregulated TIMP-1, thereby effectively blocking invasion in normal trophoblast cells (Graham and Lala, 1991; Graham et al. 1993a; 1994). The
present study has identified an additional mechanism, an upregulation of PAI-1 in the normal trophoblast cells by TGFβ. TGFβ mediated upregulation of PAI-1 has also been reported with mink lung cells (Zentella et al. 1991). In contrast to the upregulation of enzyme inhibitors by the normal trophoblast, both SV40 Tag transformed trophoblast lines responded to TGFβ by upregulation of 72 kDa MMP mRNA. An upregulation of TIMP-1 mRNA was either small or undetectable. This may be the reason for increased invasiveness of RSVT2/C cells in the presence of TGFβ. It is currently unknown whether there is an alteration in the expression of TGFβ receptors (i.e. aberrant receptor-mediated intracellular signalling) in the Tug transformed trophoblast cells.

EGFr ligands did not affect the invasion of the RSVT-2 and HTR8 cells in the Matrigel invasion assay, however, EGF was shown to increase the expression of both MMP and TIMP-1 in the HTR8 cells as was previously reported (Lysiak et al. 1994).

2.4.7 Possible Role of Other MMPs in Normal And SV40 Tag Transformed Trophoblast Cell Invasiveness

The 92 kDa type IV collagenase was not detected in our northern blots of total RNA (data not shown) in any of the cell lines. However, it was detected in a PCR reaction and slot blot analysis (data not shown). In the zymogram analysis, both 92 kDa type IV collagenase and 72 kDa type IV collagenase were detected in
the supernatants of all three cell lines. While both latent and activated forms of 72 kDa type IV collagenase, and the latent form of 92 kDa type IV collagenase were observed in all cases, the activated form of 92 kDa type IV collagenase was secreted only by RSVT2/C cells. It is unclear at present whether this was induced by a second activator not expressed in the other two cell lines.

The functional role of both 72 kDa and 92 kDa collagenase species has been demonstrated on trophoblast invasiveness by the application of anti-sense oligonucleotides in vitro (Lala and Connelly, 1994). Thus, in the case of RSVT2/C cells, a constitutive activation of 92 kDa type IV collagenase would also contribute to the increased invasiveness observed in the cells. Another possible contribution to increased invasiveness could be the increased expression of MT-MMP, which has been shown to be expressed by EVT cells (Nawrocki et al. 1995).
Chapter 3

3. Expression of Connexins in First Trimester Trophoblast and SV40 Tag Transformed Trophoblast Cells

3.1 Introduction

Gap junctions are aqueous channels in the cell membrane responsible for intercellular communication. They comprise hexameric arrays of connexin proteins (connexons) lining the aqueous channels which provide conduits for the passage of low molecular weight chemicals or electrical messages between cells (Waltzman and Spray, 1995). To date there are at least twelve mammalian connexins described and fully sequenced (Hotz-Wagenblatt and Shalloway, 1993; Wolburg and Rohlmann, 1995). Stringently controlled cell growth and differentiation are two key properties that separate normal cells from their malignant counterparts. It is believed that cell-cell communication by gap junctional complexes plays a significant role in regulating cellular proliferation and differentiation, as well as coordinating the biological activities of many cell types in normal tissues (see reviews, Loewenstein, 1979; Loewenstein and Rose, 1992) by the passage of regulatory molecules (< 1000 daltons) from a cell to its coupled neighbors (Loewenstein and Rose, 1992). In addition, various growth
factors including PDGF, EGF, TGFβ have been reported to modulate the expression of connexins (see review by Loewenstein and Rose, 1992).

Since cancer is generally considered as a disease of dysregulated cell growth and/or differentiation, many studies have sought to determine the roles of various connexins in the etiology of carcinogenesis (Yamasaki, 1987; 1990a; Klaunig and Ruch, 1990; Trosko et al. 1990b; Loewenstein and Rose, 1992; Hotz-Wagenblatt and Shalloway, 1993). Various investigators using chemically transformed cells (Holder et al. 1993; Bager et al. 1994) and oncogene transformed cells (Bignami et al. 1988; Holder et al. 1993; De Feijter et al. 1992; Fitzgerald et al. 1993) have reported a decrease in gap junctional intercellular communication (GJIC). There is also evidence for aberrant expression of functional or dysfunctional connexins in the tumorous tissue. For example, Oyamada et al. (1990) reported that human hepatocellular carcinomas had normal Cx32-mediated GJIC but aberrant Cx43-mediated GJIC, normally not seen in hepatocytes. Although GJIC is not decreased in all cancers, Loewenstein observed that all communication deficient cells have malignant properties (Loewenstein, 1979). The role of connexins in negative growth control, or as tumor suppressive genes, has been demonstrated by re-introducing connexins into both animal (Mehta et al. 1986; 1991; Zhu et al. 1991; Naus et al. 1992; Bond et al. 1994) and human (Wilgenbus et al. 1992; Fitzgerald
et al. 1993; Mesnil et al. 1995) tumor cells, resulting in the suppression of the
tumorigenic phenotype.

Extravillous trophoblast (EVT) cells of the human placenta have been shown
to share many characteristics with tumor cells (Lala and Graham, 1990; Strickland
and Richards, 1992), including invasive ability (Yagel et al. 1988). However,
unlike tumor cells, trophoblast cell proliferation and migration are stringently
regulated in situ by locally produced factors in the trophoblast microenvironment.
For example, decidua-derived growth factor TGFβ (Graham and Lala, 1991)
inhibits invasion and migration (Irving and Lala, 1995), whereas trophoblast-
derived IGFII stimulates invasion (Lala and Lysiak, 1995) as well as migration
(Irving and Lala, 1995). In addition, it was shown that EGFr ligands, TGFα and
EGF (derived from the trophoblast or the decidua), stimulate proliferation of early
passage EVT cells (Lysiak et al. 1994), but had no appreciable effect on their
invasive ability (Lysiak et al. 1994). Earlier studies presented in this thesis
(Chapter 2) have also shown that normal EVT cells in culture respond to the anti-
proliferative and anti-invasive signal of TGFβ. However, responses to TGFβ were
altered in SV40 Tag transformed premalignant derivatives of EVT cells, RSVT-2
(long lived) and RSVT2/C (immortal clone derived from RSVT-2) cells. RSVT-2
cells lost the anti-invasive response to TGFβ signals, whereas RSVT2/C cells lost
both anti-proliferative and anti-invasive responses to TGFβ signals (Chapter 2).
Gap junctions are expressed by placental trophoblast cells in the mouse (Pauken and Lo, 1995; Dahl et al. 1995), the rat (Risek and Gilula, 1991; Grummer et al. 1995; Reuss et al. 1995), and the human (Cronier et al. 1995a;b; Hellmann et al. 1995). It was found that cultured human term trophoblast cells express Cx43 (Cronier et al. 1995a;b), and dye transfer, an indicator of gap junctional intercellular communication, was reduced following TGFβ treatment. Hellmann and associates (1995) reported the immunoreactivity of human trophoblast cells for Cx43 and Cx40 in situ. In addition, they reported that BeWo and JAR choriocarcinoma cell lines expressed Cx40, but did not express Cx26 or Cx43, whereas a third choriocarcinoma cell line, JEG-3, showed no connexin expression.

The present study examined the possible changes in gap junctional communication during the transition of normal EVT cells to the premalignant stage represented by SV40 Tag transformed EVT cells propagated in culture. This was achieved by a comparison of the expression of connexin proteins (by immunofluorescent labeling) and mRNA (by northern blot analysis), and GJIC (by dye transfer test) in normal parental EVT cells and their SV40 Tag transformed derivatives. GJIC was also examined in cells treated with TGFβ.
3.2 Materials and Methods

3.2.1 Trophoblast Cell Lines and Cell Culture

Details of the normal EVT cell line, HTR8, and its SV40 Tag transformants, RSVT-2 and RSVT2/C lines are presented in Chapter 2. Cell lines were cultured as previously described (see section 2.2.1). For dye coupling described below (see section 3.2.5), cells were cultured on 60 mm petri dishes (Corning).

3.2.2 Connexin Probes

Probes for the various connexins were obtained through collaborations with members of Dr. C. C. G. Naus's laboratory in this department. Connexin probes were obtained from the following original sources: [1] rat Cx43 (1.4 kb insert; Dr. E. Beyer, Washington University School of Medicine, St. Louis, MO), [2] rat Cx32 (1.2 kb insert; Dr. D. Paul, Harvard Medical School, Boston, MA), [3] rat Cx26 (0.68 kb insert; Dr. B. Nicholson, State University of New York at Buffalo, NY), and [4] rat Cx33 (0.476 kb PCR product; K. Orlando-Mathur and Dr. T. Kennedy, University Hospital, London, ON). The cDNA probes were made as described in section 2.2.8.

Probes for Cx32, Cx26, and Cx43 were previously used to detect the human connexins (Willecke et al. 1990). The PCR product for Cx33 has not been
previously used with human RNA, but the probe detects Cx33 message in rat testes RNA (Orlando-Mathur and Kennedy, personal communication).

3.2.3 Connexin Antibodies and Immunolabeling

Connexin antibodies were also obtained in collaboration with members of Dr. Naus's laboratory. The following antibodies were used in the study: [1] Cx43, rabbit polyclonal antibody (40-5) to amino acid (aa) sequence 368-382, 1:400 dilution (Dr. A. Lau, University of Hawaii, Manoa, HA), [2] Cx32, rabbit polyclonal antibody to aa sequence 223-244, 1:50 dilution (Dr. B. Nicholson, State University of New York [SUNY], Buffalo, NY), [3] Cx26, rabbit polyclonal antibody to aa sequence 101-119, 1:25 dilution (Dr. B. Nicholson, SUNY), and Cx40, rabbit polyclonal antibody to aa sequence 335-336, 1:400 dilution (Dr. Robert Gourdie, Medical University of South Carolina, Charleston, SC). All antibodies were made using rat oligopeptides as the antigen. The antibodies to Cx32, Cx43 (human glioma cells; Bond et al. 1993), Cx26 (human liver, Yamasaki and Naus, personal communication), and Cx40 (human cardiac cells; van Kempen et al. 1995) have been successfully used on human tissues.

Immunolabeling was carried out as described previously (section 2.2.5) by exposure of cells to primary antibody, followed by biotin conjugated goat anti-rabbit secondary antibody (Vector). Fluorescein-conjugated streptavidin (1:250
dilution) was used to visualize immunoreactivity for connexins. Negative control was included using normal rabbit serum in place of primary antibodies.

3.2.4 RNA Isolation and Northern Blot Analysis

Total RNA was isolated as previously described in section 2.2.8. northern blot analysis was carried out as outlined in section 2.2.8.

3.2.5 Dye Coupling

Dye coupling was conducted as described previously using dye transfer from preloaded cells to unlabeled cells (Goldberg et al. 1995) with a minor modification. Briefly, calcein acetoxyethyl methyl (calcein AM; Molecular Probes, Eugene, OR) and 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbo-cyaine perchlorate (Di-I; Sigma Chemicals) were dissolved in DMSO at 10 mM stock concentrations. Di-I is a lipophilic dye which marks the cell membrane of preloaded cells. Calcein AM is a membrane permeable cytoplasmic dye which is cleaved by endogenous esterases to yield a membrane impermeable calcein, a molecule that is small enough to pass through gap junctions, and thus, calcein transfer to other cells indicates possible communication present between cells. Solutions were stored at -20°C. Since, in preliminary experiments, the normal EVT cell line HTR8 did not label well in 0.3 M glucose-PBS solution, the two fluorescent labels were diluted in RPMI-1640 medium to working concentrations
of 10 μM calcein AM and 10 μM Di-I. Cells cultured on 60 mm petri dishes were incubated at 37°C with 2 ml volume of labeling mixture for 20 mins. Cells were washed twice with RPMI-1640, trypsinized with 0.025% trypsin-PBS-EDTA solution, centrifuged, and resuspended in RPMI-1640 medium to give an approximate cell concentration of 10^5 cells/ml. Fifty μl of resuspended Di-I labeled donor cells (containing calcein AM) were plated on top of receiving cells grown as attached monolayer cultures of the same (homologous coupling, *i.e.*, HTR8 to HTR8) or different cell lines (heterologous coupling, *i.e.*, HTR8 to RSVT-2). Receiving cells (monolayer) were examined with or without prior TGFβ treatment for 24 h. After 2 h of co-incubation with the receiving cells to allow Di-I labeled cells to settle, at least ten representative fields with cells labeled with Di-I were examined, and the number of cells receiving calcein from donor cells was determined. Photomicrographs were taken with rhodamine filter set to visualize Di-I, and with fluorescein filter set to visualize calcein dye transfer. Dye coupling is only an indicator of the possible gap junctional communication between cells. The number of cells receiving calcein per Di-I membrane labeled cell was then recorded under each condition.
3.2.6 Statistical Analysis

Statistical analysis to determine differences in the dye coupling assay was carried out as follows. The Kruskal-Wallis test was first conducted to determine if there were differences in the experiments. If a significance was detected then differences between pairs were analyzed using the Wilcoxon-Mann Whitney-U test.

3.3 Results

3.3.1 Immunolabeling for Connexin Proteins

Immunofluorescent staining was performed using normal parental HTR8 and SV40 Tag transformed, RSVT-2, and RSVT2/C cell lines. None of the cell lines showed any immunoreactivity to Cx26, Cx32, or Cx40 antibodies (data not shown). Immunostaining for Cx43 was abundant in HTR8 cells (Figure 12). Lower levels of immunostaining were detected in the RSVT-2 cells, whereas no detectable immunoreactivity for Cx43 was observed in RSVT2/C cells (Figure 12). Immunoreactivity for Cx33 was not performed as no antibody is available.

3.3.2 Gap Junctional Communication in the HTR8, RSVT-2 and RSVT2/C Cells

Fluorescent photomicrographs of the three cell lines representative of dye transfer (indicative of GJIC) under control (medium only) or TGFβ treatments are
Figure 12: Immunostaining of Cx43 in HTR8, RSVT-2 and RSVT2/C cells

Immunofluorescence of connexin43 and phase contrast images are taken from representative samples. Images were taken with a 40X objective, and bar represents ~100 μm. Punctate cell membrane staining indicates the presence of abundant Cx43 in HTR8 cells and a comparatively low level in RSVT-2 cells. No staining is seen in RSVT2/C cells.
presented in Figures 13-15. Homologous coupling of the three cell lines showed a relative reduction of dye transfer in RSVT-2 cells as compared to parental HTR8 cells and minimal dye transfer in RSVT2/C cells (Figure 16A). The reductions were significant when compared in pairs: RSVT2/C (p=0.001) < RSVT-2 (p=0.001) < HTR8.

When compared to homologous HTR8 (medium only) coupling, treating HTR8 with TGFβ resulted in a reduction (p=0.001) in the number of cells receiving calcein, suggesting a decrease of GJIC. Homologous dye coupling of RSVT-2 cells after TGFβ treatment showed that an increase (p=0.001) in the number of cells receiving calcein compared to homologous RSVT-2 to RSVT-2 coupling under control (medium only) conditions, thus indicating an increase of GJIC. No change was observed for RSVT2/C to RSVT2/C cells coupling after TGFβ treatment of RSVT2/C cells (p=0.055) (Control, Figure 16A).

The extent of heterologous of both HTR8:RSVT-2 when compared with heterologous coupling of HTR8:RSVT2/C cells was also significantly reduced (p=0.0001; Figure 16B). Heterologous dye coupling of HTR8 to either RSVT-2 or RSVT2/C cell lines following treatment of the recipient cells with TGFβ for 24 h was compared to HTR8 heterologous dye coupling prior to TGFβ treatment (Figure 16B). Treating RSVT-2 with TGFβ also caused an increase (p=0.001) in
Figure 13: Representative dye coupling of HTR8 to HTR8 cells

Representative images of homologous coupling of donor HTR8 to HTR8 receiving cells under control or TGFβ treatment. Membrane staining by Di-I (red) marks the preloaded donor cells, and dye transfer of calcein (green) is seen spreading from labeled donor cells to the receiving cells of the monolayer. Arrowhead indicates the position of preloaded cell, in photograph with 16X objective; bar represents ~75 μm.
Figure 14: Representative dye coupling of HTR8 to RSVT-2 cells

Representative images of heterologous coupling of donor HTR8 cells to RSVT-2 receiving cells under control or TGFβ treatment. Membrane staining by Di-I (red) marks the preloaded donor cells, and dye transfer of calcein (green) is seen spreading from labeled donor cells to the receiving cells of the monolayer. Arrowhead indicates the position of preloaded cell, in photograph with 16X objective; bar represents ~75 μm.
Figure 15: Representative dye coupling of HTR8 to RSVT2/C

Representative images of heterologous dye coupling of donor HTR8 cells to RSVT2/C receiving cells under control or TGFβ treatments. Membrane staining by Di-I (red) marks the preloaded donor cells, and calcein (green) is seen from labeled donor cells. Arrowhead indicates the position of preloaded cell, in photograph with 16X objective; bar represents ~75 μm.
Figure 16: Dye coupling quantitation of homologous and heterologous coupling

In panel A, homologous coupling of HTR8, RSVT-2, and RSVT2/C cells is presented as the mean (±SE) number of cells receiving dye per donor Di-I labeled cell for each cell line with or without prior treatment with TGFβ for 24 h. Statistical significance of differences is given below:

<table>
<thead>
<tr>
<th>Comparisons of couplings</th>
<th>Label; p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTR8 (homologous):RSVT-2 (homologous)</td>
<td>A; p=0.001</td>
</tr>
<tr>
<td>HTR8 (homologous):RSVT2/C (homologous)</td>
<td>AA; p=0.001</td>
</tr>
<tr>
<td>RSVT-2 (homologous):RSVT2/C (homologous)</td>
<td>A'; p=0.001</td>
</tr>
<tr>
<td>HTR8 (homologous):HTR8 (homologous, TGFβ)</td>
<td>B; p=0.001</td>
</tr>
<tr>
<td>RSVT-2 (homologous):RSVT-2 (homologous, TGFβ)</td>
<td>BB; p=0.001</td>
</tr>
</tbody>
</table>

In panel B, heterologous coupling of HTR8 to RSVT-2 or RSVT2/C receiving cells untreated or treated with TGFβ are graphically represented. Statistical significance of differences is given below:

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Label; p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTR8 to RSVT-2 (heterologous, control): HTR8 to RSVT2/C (heterologous, control)</td>
<td>A; p=0.001</td>
</tr>
<tr>
<td>HTR8 to RSVT-2 (heterologous, control):HTR8 to RSVT-2 (heterologous, TGFβ)</td>
<td>B; p=0.001</td>
</tr>
<tr>
<td>HTR8 to RSVT2/C (heterologous, control): HTR8 to RSVT2/C (heterologous, TGFβ)</td>
<td>BB; p=0.053</td>
</tr>
</tbody>
</table>
the number of cells receiving calcein compared to heterologous HTR8 to RSVT-2 coupling under control conditions, thus indicating an increase of GJIC. No change was observed for HTR8 to RSVT2/C cells coupling after TGFβ treatment of RSVT2/C cells (p=0.053).

3.3.3 Northern Blot Analysis of Cx43

Using total RNA in the northern blot analysis, no message for Cx32, Cx26, or Cx33 was detected (data not presented) in any of the three cell lines used in the study. In contrast, Cx43 message was detected in the normal HTR8 cell line; the level of message was drastically lower in the RSVT-2 cell line (comparison of control lanes in Figure 17A and 17B); the signal was weak and could not be seen in the photograph but could be seen in the original autoradiogram (Figure 17A). Longer exposure of another autoradiogram shows clearly that RSVT-2 cells do express Cx43 message and an apparent decrease of ~15 fold (in comparison with HTR8 cells) was noted in this autoradiogram (Figure 17B). No message was detectable in the RSVT2/C cells; this is another significant finding of the study.

Treatment with growth factors yielded various results. HTR8 cells treated with anti-EGFr and anti-TGFα, but not anti-EGF antibodies, showed an apparent reduction of Cx43 (Figure 17A) mRNA expression, suggesting that possibly TGFα or another EGFr ligand may have an autocrine upregulatory effect on Cx43
Figure 17: Northern blot analysis for Cx43 expression

Photograph of the autoradiogram showing the hybridization signal of Cx43 mRNA on the northern blots with HTR8, RSVT-2, and RSVT2/C cell lines under various conditions (panel A). Panel B was included to illustrate the low level of Cx43 mRNA expressed in RSVT-2 cells, since the panel A photographic image did not clearly show a band in RSVT-2 control lane. Given below are densitometric values which showed greater than 50% difference (values were normalized to 18S rRNA; controls (value set at 1.0) for growth factor responses):

<table>
<thead>
<tr>
<th>Cell line; Treatments</th>
<th>Arbitrary densitometric units</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTR8; Anti-TGFα treated</td>
<td>0.49</td>
</tr>
<tr>
<td>HTR8; Anti-EGFt treated</td>
<td>0.28</td>
</tr>
<tr>
<td>HTR8; EGF treated</td>
<td>1.67</td>
</tr>
<tr>
<td>HTR8; TGFβ treated</td>
<td>0.38</td>
</tr>
</tbody>
</table>

In panel B, the difference of Cx43 mRNA expression in RSVT-2 in comparison with HTR8 cells was found to be approximately 15 fold based on normalized values relative to 18S rRNA, used as a control for RNA loading.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Controls</th>
<th>Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTR8</td>
<td>TGFα</td>
<td>Anti-TGFα</td>
</tr>
<tr>
<td></td>
<td>Anti-EGF Fr</td>
<td>EGF</td>
</tr>
<tr>
<td></td>
<td>Anti-TGFβ</td>
<td>TGFβ</td>
</tr>
<tr>
<td></td>
<td>Anti-TGFβ</td>
<td></td>
</tr>
<tr>
<td>RSVT-2</td>
<td>TGFα</td>
<td>Anti-TGFα</td>
</tr>
<tr>
<td></td>
<td>Anti-EGF Fr</td>
<td>EGF</td>
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<tr>
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<td>Anti-TGFβ</td>
<td>TGFβ</td>
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<tr>
<td></td>
<td>Anti-TGFβ</td>
<td></td>
</tr>
<tr>
<td>RSVT2/C</td>
<td>TGFα</td>
<td>Anti-EGF Fr</td>
</tr>
<tr>
<td></td>
<td>EGF</td>
<td>TGFβ</td>
</tr>
<tr>
<td></td>
<td>Anti-TGFβ</td>
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18S

CX43

<table>
<thead>
<tr>
<th>Control</th>
<th>HTR8</th>
<th>RSVT-2</th>
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B

Control
expression. No change in Cx43 expression in HTR8 was observed with TGFα at
the concentration (10 ng/ml) used in the study. An apparent increase was,
however, noted in EGF treated HTR8 cells. Treatment of RSVT-2 or RSVT2/C
cells with either EGFr ligand or the anti-EGF and anti-TGFα antibody did not
cause a detectable change in the expression of Cx43 mRNA.

Transforming growth factor β was observed to decrease the expression of
Cx43 mRNA only in HTR8 cells, whereas no influence of anti-TGFβ antibody
was noted, indicating that little if any endogenous TGFβ was produced by these
cells. Neither TGFβ nor anti-TGFβ antibody treatment caused a detectable effect
on Cx43 mRNA expression by RSVT-2 or RSVT2/C cells.

3.4 Discussion

This study used normal extravillous trophoblast cell line HTR8 and its SV40
Tag transformed derivatives, i.e., extended lifespan RSVT-2, and immortal
RSVT2/C cell lines to investigate possible changes in the expression of connexins
during the early stages of tumor progression. It was already shown that both
RSVT-2 and RSVT2/C cells were nontumorigenic but acquired a premalignant
phenotype as considered by their hyperproliferative and hyperinvasive properties
and altered responses to proliferation and invasion-regulating growth factors. Of
the panel of connexins examined in this study, Cx43 was the only detectable
connexin expressed by the cultured first trimester human EVT cells both at mRNA and protein levels. The expression was reduced in the presence of TGFβ. In the RSVT-2 cell line, Cx43 mRNA and protein were both reduced in comparison to HTR8, whereas the expression was not detectable at all in RSVT2/C cells. Dye coupling studies of these cell lines, both homologous (e.g. HTR8 to HTR8 cells) and heterologous (HTR8 to RSVT-2 or RSVT2/C cells), revealed that (i) homologous dye coupling was abundant in HTR8 cells, reduced in RSVT-2 cells, and minimal or absent in RSVT2/C cells; (ii) heterologous dye coupling of HTR8 cells to the transformed cells was small; (iii) TGFβ-treatment of the recipient cell reduced HTR8 to HTR8 coupling, but increased homologous RSVT-2 to RSVT-2 dye coupling as well as heterologous HTR8 to RSVT-2 dye coupling.

A concurrent reduction in Cx43 protein, Cx43 mRNA expression, and homologous dye coupling following SV40 Tag transformation of normal EVT cells suggests that the transformation was associated with a downregulation of the Cx43 gene with the resultant reduction of Cx43 protein and GJIC. These findings are in agreement with the hypothesis of connexin mediated growth control and the observations that GJIC is reduced during tumor progression (Loewenstein and Rose, 1992). Other studies have shown that homologous coupling of cells to their neighbors is reduced within preneoplastic or tumorigenic foci as compared to the normal tissue of origin (Yamasaki, 1987; Trosko et al. 1990a; Krutovskikh et al.
1991; Holder et al. 1993). Our findings reinforces the concept. This reduction is a feature appearing at the premalignant stage. Loss of Cx43 expression in the immortalized RSVT2/C cells indicate they are at a more advanced stage in tumor progression pathway.

It has been postulated that a reduction or breakdown in gap junctional intercellular communication in situ between transformed cells and neighboring normal cells allows autonomous growth of transformed foci due to an escape from normal regulatory controls mediated by molecules that pass through gap junctions from surrounding normal cells (Loewenstein, 1979; Yamasaki, 1987; 1990a). This postulate is supported by our findings that heterologous dye coupling of normal HTR8 cells to SV40 Tag transformed cells was reduced (RSVT-2) or minimal (RSVT2/C). Fitzgerald et al. (1993) also showed a reduced communication between normal and SV40 Tag transformed hepatocytes.

A decreased level of homologous dye coupling noted after TGFβ treatment of HTR8 cells can be explained on the basis of TGFβ-mediated downregulation of Cx43 mRNA, as documented in our study, leading to a reduction in the Cx43 protein produced by these cells. Functional significance of this finding remains unclear at present. TGFβ-mediated reduction in GJIC has also been reported in human bronchial epithelial cells (Vleminkx et al. 1991) as well as human term
trophoblast cells (Cronier et al. 1995a;b) indicating that this may be a general TGFβ effect on epithelial cells, independent of anti-proliferative function of TGFβ, since term trophoblast cells are nonproliferative.

The reasons for an increase in heterologous coupling of HTR8 cells with RSVT-2 cells when the latter cells were treated with TGFβ, remains undetermined. This finding is somewhat similar to those of Albright and associates (1991) showing that TGFβ treatment of SV40 Tag transformed BEAS-2B cells caused an increase in dye transfer. There are two possible explanations for our findings. (1) TGFβ may cause an upregulation in Cx43 mRNA expression in RSVT-2 cells, but this explanation is inconclusive since the message level on the northern blot was too low to quantitate precisely. (2) A different connexin other than Cx43 was upregulated by TGFβ in RSVT-2 cells. Evidently, the effect of SV40 Tag transformation on TGFβ regulation of connexin expression requires further investigation.

Treatment with anti-TGFα and anti-EGFr, but not anti-EGF, antibodies resulted in decreased expression of Cx43 mRNA in normal HTR8 cells. It appears that TGFα and/or an EGFr ligand, other than EGF, may be required for an autocrine upregulation of Cx43 expression in HTR8 cells. It has already been shown that normal trophoblast cells can produce TGFα (Filla et al. 1993). It is
possible that addition of exogenous TGFα (10 ng/ml) did not have an additional effect because of an abundance of the endogenous ligand. A small upregulation noted in the presence of exogenous EGF is consistent with the role of EGFr in Cx43 upregulation in these cells. This finding is in contrast to other studies (reviewed by Loewenstein and Rose, 1992) showing that EGF treatment of keratinocytes, NRK and Balb/c 3T3 cells reduced the GJIC. TGFβ-mediated reduction of GJIC was also believed to be dependent on functional EGFr, since the presence of EGF with TGFβ synergistically reduced GJIC in NRK cells, and the TGFβ response was aborted in an EGFr negative NIH 3T3 cell line (reviewed by Loewenstein and Rose, 1992).

Consistent with our results, other studies using various oncogenes have demonstrated that oncogenes downregulate expression of connexins (Loewenstein and Rose, 1992), suggesting a common mechanism(s) for modulating expression of connexins. Further studies to analyze the promoter regions of connexins could provide insights into whether the promoter contains elements that are bound to the oncogene protein or oncogene induced secondary products.

In conclusion, the present results, combined with the data in the literature, suggest that a down regulation of connexins with a resultant decrease in intercellular communication is an early event in tumor progression, noted at the premalignant stage.
Chapter 4

4. Differential Gene Expression in the Premalignant Trophoblast Cell Lines Produced by SV40 Tag Transformation of Normal Invasive EVT Cells

4.1 Introduction

Genetic changes responsible for the transition of a normal cell to a tumor cell, and eventually a tumor cell capable of metastatic spread to a distant site, have been a major focus of cancer research in recent years. These studies have led to the discovery of two classes of events: (i) overexpression of one or more “oncogenes”, e.g. ras, myc, fos, the normal counterparts of which are responsible for many normal cellular functions (reviewed by Varmus, 1989; Weinberg, 1989b; Eisenman, 1989); and (ii) loss/inactivation of one or more “tumor suppressor” genes, many of which have been recognized as normal regulators of cell proliferation and differentiation e.g. p53 and pRb (reviewed by Weinberg, 1989c; Stanbridge and Cavenee, 1989; Culotta and Koshland, 1993). Despite the identification of a large number of genes belonging to both classes, our understanding of the genetic events responsible for specific stages in tumor progression, e.g. the transition of a normal cell to a premalignant cell, and a malignant cell to a metastatic cell, remains incomplete.
Tumorigenesis is demarcated by discrete changes in a cell's ability to proliferate, migrate, and invade. Cancer cells proliferate *in situ* as they override normal regulatory controls. This phenotypic alteration can result from changes in one or many genes which can directly or indirectly control various steps in the cell cycle, *e.g.* inactivation or loss of p53 or pRb (Green, 1989; Marshall, 1991). Tumor cells are also highly invasive so that they defy the normal geographical restraints imposed by tissue barriers such as the basement membrane. High invasive ability of the cells may result from an upregulation of matrix degrading enzymes *e.g.* 72 kD type IV collagenase (reviewed by Khokha and Denhardt, 1989; Stetler-Stevenson *et al.* 1993) or a down regulation of the natural inhibitors of such enzymes *e.g.* TIMP-1 (Khokha *et al.* 1989), thus shifting the balance of enzymes to their inhibitors (Ray and Stetler-Stevenson, 1994). Acquisition of metastatic ability, a key step in tumor progression, is a poorly understood process. Invasive and migratory abilities are also essential but are not always sufficient as prerequisites for metastasis. Some of the recently identified genes reported to suppress metastasis are *nm23* (Steeg *et al.* 1988a;b; Leone *et al.* 1992), *E-cadherin* (Navarro *et al.* 1991; Chen and Obrink, 1991; Frixen *et al.* 1991; Vleminckx *et al.* 1991), *WDNMI* (Dear *et al.* 1988c) and *WDNM2* (Dear *et al.* 1988a;b). In addition, there are other factors *e.g.*, autocrine motility factor (Nabi *et al.* 1992)
and hepatocyte growth factor (Rong et al. 1994) that could promote metastasis. Possibly many more remain unidentified in both categories.

The human placenta is a highly invasive structure and placental invasion of the uterus is essential for the establishment of proper fetal-maternal exchange. This function is executed in situ by a highly invasive, migratory, and proliferative subpopulation of placental trophoblast cells known as the extravillous trophoblast (EVT) cells (reviewed by Graham and Lala, 1992). Using in vitro propagated first trimester EVT cells, it has been demonstrated in this laboratory that they share some of the same mechanisms of invasion as malignant tumor cells (Yagel et al. 1988). However, it has also been shown that EVT cell proliferation (Graham et al. 1992; Lysiak et al. 1993; 1994; 1995a;b; Lala and Lysiak, 1994; 1995), invasion (Graham and Lala, 1991; 1992), and migration (Irving et al. 1995) are stringently regulated by a number of growth factors or growth factor binding proteins produced normally at the fetal-maternal interface in situ, whereas choriocarcinoma (trophoblastic cancer) cells are resistant to many of their regulatory signals (Graham et al. 1994). Furthermore, normal EVT cells propagated in culture are nor immortal, tumorigenic, or metastatic, as has been documented earlier in this thesis (Chapter 2).
In the present study, normal and premalignant EVT cell lines, and the
differential mRNA display method (Liang and Pardee, 1992; Liang et al. 1994)
were used in order to identify genetic events associated with the transition of a
normal invasive cell to a premalignant stage. As documented earlier (Chapter 2),
"premalignant" (hyperinvasive and hyperproliferative, but nontumorigenic and
nonmetastatic) phenotype was achieved by SV40 Tag transformation of the normal
EVT cells (HTR8 cell line).

Since normal trophoblast cells can be maintained in culture for a limited
number of passages (12-15) before senescence, they were transformed with SV40
Tag to develop cell lines with extended lifespans. Following transfection of a
normal HTR8 EVT cell line with SV40 Tag, two lines were isolated; RSVT-2
which is a long lived line selected on the basis of extended lifespan alone, and
HTR8/SVneo, an immortalized cell line (Graham et al. 1993a) selected on the
basis of G418/neomycin resistance. Both cell lines were transformed at the same
time, however, HTR8/SVneo, with its greater selection pressure derived from the
neomycin resistance gene, yielded immortalized cells much earlier. A post-crisis
immortal line, RSVT2/C, was derived later from extended lifespan RSVT-2 cells.
All SV40 Tag transformed lines exhibited a premalignant phenotype. There were
two reasons for using these cells from the same genetic lineage: (1) to reduce the
number of irrelevant differentially expressed genes that may be encountered with
the differential display method used in the present study; and (2) to use *SV40 Tag* transformed cells in the future for eventual malignant transformation and identification of genetic changes associated with transition from a premalignant to a malignant stage.

Differential display allows the investigator to compare gene expression in various cell types concurrently and without previous knowledge of the gene sequence or structure or possible alterations in their gene expression. Messenger RNA is isolated from the cells, reversed transcribed, and then PCR-amplified using an anchored degenerate 3' primer and a short (10-mer) 5' primer. At low stringency, this nonspecific PCR reaction produces a large number of PCR cDNA products for each primer set. Approximately 20 different 5' primers with the 4 degenerate anchored 3' primers are believed to allow amplification of a majority of the cellular mRNAs in the representative product pool (Liang and Pardee, 1992). The PCR products are radiolabeled with $^{35}$S-dATP during the PCR reaction. These products are then separated by size on a denaturing polyacrylamide gel, allowing for visualization of the products *i.e.* amplified fragments of cDNA. When the PCR products of different cell types are run simultaneously in adjacent lanes, a large number of bands are seen including the segments of differentially expressed genes which are either “turned on” or “turned off”. Since differential display is a PCR based technique, subtle changes in mRNA expression levels may not be readily
visible. However, the advantage of this technique is that only small quantities of total RNA are necessary and, in addition, rare genes are still represented in the product pool which does not always occur when subtractive libraries are used.

4.2 Materials and Methods

4.2.1 Cell Culture

HTR8, RSVT-2, RSVT2/C (described in Chapter 2), and HTR8/SVneo (Graham et al. 1993a) cell lines were cultured as described previously. See chapter 2: section 2.2.1.

4.2.2 Total RNA and mRNA Isolation

4.2.2.1 Total RNA isolation

Total RNA from HTR8, RSVT-2, RSVT2/C (immortalized clone derived from RSVT-2), HTR8/SVneo, D8 and G10 (both clonal derivatives of HTR8/SVneo), as well as JAR choriocarcinoma cells was isolated as outlined in Chapter 2, section 2.2.8.

4.2.2.2 mRNA isolation

Messenger RNA was isolated using MAP™ paper (Amersham, Oakville, ON) selection as per manufacturer's instructions. Briefly, a volume containing 100 μg of total RNA was transferred to 1 cm² MAP™ prewetted with 10 mM NaCl
solution. The mixture of total RNA and MAP™ paper was allowed to stand for 45 mins at room temperature. The solution was then removed, rinsed with 10 mM NaCl solution, and drained. The MAP™ paper with mRNA was then soaked in 300 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.8). The MAP™ paper and TE solution was heated to 65°C for 10 mins to elute the mRNA and then centrifuged at 12000 rpm to collect mRNA in solution.

4.2.3 Differential Display

Differential display was performed as described by the protocol provided in the mRNAmap™ kit (Genehunter Corporation, Brookline, MA). Briefly, the mRNA (0.5 μg) was reverse transcribed with a degenerate anchored 3' primer (T12MG; 10 μM) to form a cDNA template in 20 μl reaction volume. This cDNA template was then amplified in the presence of 10 μCi of α-[35S]-dATP (1000 Ci/mmol, Amersham), 4μM of dNTPs and 2.0 units of Amplitaq™ DNA polymerase (5 units/μl; Perkin-Elmer Corp., Norwalk, CT) using the same 3' primer and a 5' primer (random 10-mer; AP-2 (5'-GACCGCTTGT-3', 10 μM)). The PCR cycle parameters were: 30 secs at 94°C, 2 mins at 40°C, 30 secs at 72°C for 40 cycles, and followed by 5 mins extension at 72°C. Controls were run with mRNA only which had not been reverse-transcribed.
The resulting amplified cDNAs were then separated on a 6% denaturing polyacrylamide-urea DNA sequencing gel. The sequencing gel was run at constant power of 70 W in Tris borate-EDTA buffer for approximately 3.25 h.

4.2.3.1 Isolation and reamplification of cDNA

The DNA sequencing gel was transferred from the glass plate onto Whatman 3MM filter paper and dried, without methanol/acetic acid fixation, at 80°C for 2 h on a BioRad gel vacuum dry apparatus (BioRad Model 583, BioRad, Mississauga, ON). The dried gel was then exposed to a BioMax™ BMR X-ray film for 72 h at room temperature. The dried gel was marked with a luminescent paint to allow re-orientation of the gel to x-ray film at a later time point. After developing the film, it was examined for displayed cDNA bands present in the one cell type and not in the others (see Figure 19). The film was then re-aligned with the dried gel; the band(s) of interest cut from the gel, and transferred to microfuge tube. The cDNA band was eluted from the gel slices after incubation in 100 μl of dH₂O for 10 mins, and boiling for 15 mins in a microfuge tube as described by Genehunter™. After elution, the cDNA was recovered by ethanol precipitation in the presence of 0.3M sodium acetate (pH5.2), 5 μl of 10 mg/ml glycogen carrier, and redissolved in 10 μl of dH₂O. A 4 μl aliquot of the sample was then reamplified in a 40 μl reaction volume using the same primer set and PCR conditions with the following exceptions: 20 μM of dNTPs were used instead of 4 μM and no ³⁵S-dATP was
incorporated. The amplified cDNA (20 μl) was run on a 1.5% agarose gel and stained with ethidium bromide. The remainder was used to make probes for probing northern blots as described in section 2.2.8.

4.2.4 Bacteriophage Libraries

4.2.4.1 Plating and transferring of bacteriophage libraries

A human placental λZAPII™ cDNA library (male Caucasian fetus, Stratagene, La Jolla, CA) was used to screen for the differentially expressed gene. All techniques for growing, transferring and hybridizing bacteriophage libraries were according to Short Protocols in Molecular Biology (Ausubel et al. 1989). Host bacteria (XL-1 Blue, Stratagene) were grown overnight in LB media (1% tryptone, 0.5% yeast extract, 0.5% NaCl pH 7.0) with 0.2% maltose and 10 mM MgSO₄. The next day 500 μl of the host bacteria were mixed with titered phage from the cDNA library, incubated at room temperature for 20 mins, then 37°C for 10 mins, and plated on 150 mm plates (LB containing 15% agar) with 3 ml of 0.7% top agarose (1% tryptone, 0.8% NaCl, 0.7% agarose) to give approximately 25,000 plaques/ 150 mm plate. The cDNA plates were incubated at 37°C for 5 to 9 hours until sufficient lysis had occurred.

Following lysis the plates were stored at 4°C overnight. Nitrocellulose filters (Gelman Sciences, Ann Arbor, MI) labeled with ballpoint pen were placed on the
agarose surface for 2 mins to allow transfer of the phage particles. The filters were marked with India ink for later realignment on the plates before removal. After air drying for 15 mins, the phage DNA was denatured by saturating the filters with 0.2 M NaOH/1.5 M NaCl for 1.5 mins. The filters were neutralized by soaking in 0.4 M Tris-Cl (pH 7.6)/2 X SSC followed by 2 X SSC for 1.5 mins each. The filters were then baked in a vacuum oven for 90 mins at 80°C.

4.2.4.2 Hybridization of plaque lifts

The nitrocellulose filters were prehybridized in heat-sealable bags in 48% formamide, 4.8 X SSC, 20 mM Tris-Cl pH 7.6, 1 X Denhardt's solution, 10% dextran sulfate, and 0.1% SDS at 42°C for 1 hour. The cDNA PCR probe was random prime labeled as previously described (section 2.2.8), boiled for 10 mins with 1 ml of 2 mg/ml sonicated salmon sperm DNA solution, and rapidly cooled on ice for 5 mins before being added to the filters. The hybridization proceeded overnight in a shaker bath set at 42°C.

The next day the filters were removed from the bags, and washed twice in 2X SSC/0.1% SDS, 15 mins each at room temperature. Then the filters were washed in a high stringency wash buffer 0.2X SSC (0.03 M NaCl, 3 mM Na₃C₆H₅O₇·2H₂O pH 7.2)/0.1% SDS at 55°C for 20 mins. The filters were then wrapped in Saran wrap and exposed to Biomax™ BMR film (Kodak) overnight at -70°C, using a
Hyperscreen™ intensifier screen. The films were developed as mentioned previously through an autodeveloper.

4.2.4.3 Purification of bacteriophage clones

Positively hybridizing plaques on the primary plates of the cDNA library were identified following alignment of the orientation markers on the filters, film and primary plates. The plaques were isolated by inserting sterile toothpicks first into the area on the primary plate, and then transferring them onto a grid-marked secondary plate. Secondary plates were made by plating 200 µl of XL-1 Blue host bacteria in 0.7% top agarose onto an 82 mm petri dish with 25 ml of LB agar, and allowed to incubate for 1 h at 37°C. For each positive primary clone a number of stabs were picked. The plates were grown overnight at 37°C. The phage plaques were then transferred to nitrocellulose filters and hybridized using the same conditions as the primary screening. Positive plaques from the secondary screen were further purified by inserting a sterile toothpick into the positive plaque and placing it in 500 µl of SM (0.58% NaCl, 0.2% MgSO₄·7H₂O, 50 mM Tris-Cl pH 7.5, 0.1% gelatin) for 5 mins. Serial dilutions of this stock were then plated. Plates with 10 to 50 well isolated plaques were used for transfer and hybridization, as described above. Single plaques which gave a strong signal on this hybridization were then used to make pure phage stocks. A sterile toothpick was inserted into the plaque and placed in SM for 5 mins. Approximately 300 µl of XL-1 Blue host
bacteria grown overnight were incubated with the 500 μl pure phage stock at room
temperature for 20 mins, 37°C for 10 mins and then plated onto 82 mm LB agar
plates with 0.7% top agarose. The plates were grown at the 37°C until confluent
lysis was achieved. The agar was then covered with 3 ml of SM buffer containing
100 μl chloroform and incubated overnight at 4°C. The next morning the SM
buffer was harvested from the plates and stored in a sterile glass tube at 4°C over
chloroform. This phage stock was then stored.

4.2.4.4 DNA isolation from cDNA λZAPII clones

The insert of interest was isolated following in vivo excision using helper
virus as described in the protocol provided by Stratagene. Briefly, 200 μl of XL-1
Blue were incubated in a 50 ml tube with 250 μl of phage stock and 1 μl of
ExAssist™ helper phage for 15 mins at 37°C. Three ml of LB were added after 15
mins and bacteria were allowed to grow for 2 h at 37°C in a shaker incubator at
225 rpm. Bacteria were pelleted by centrifugation at 2000X g for 15 mins. The
supernatant containing pBluescript phagemid was heat treated for 15 mins at 70°C
to kill XL-1 Blue bacteria. Subsequently, the supernatant was centrifuged at
4000X g for 15 mins and transferred to another 50 ml tube. Phagemid was added
to two tubes containing SOLR cells at two different concentrations (100 μl and 10
μl), mixed gently, incubated at 37°C, then 10 μl from each tube was plated onto
LB-ampicillin (50 μg/ml) plates, and incubated at 37°C. The plates were removed the next day, and stored at 4°C.

4.2.4.5 Preparation of plasmid DNA

Plasmid DNA was isolated by the standard procedure of alkaline lysis outlined by Sambrook et al. (1987) with some modifications. Mini-preps of plasmid DNA were isolated from 3 ml of overnight cultures and no lysozyme was used to lyse the bacteria. Plasmid DNA isolated for sequencing reactions was extracted once with phenol saturated with 50 mM sodium acetate (NaOAc) pH 4.0, then once with chloroform:isoamyl alcohol (24:1) (Sambrook et al. 1987). Bluescript vector DNA and the SOLR host bacteria were obtained from Stratagene (La Jolla, California).

4.2.4.6 Restriction enzyme digestion

Plasmid DNA (5 μg) of relevant clones was digested at 37°C for 2 h in 2X One-Phor-All Plus (Pharmacia) buffer and the restriction enzyme, EcoRI, to excise the insert from the pBluescript vector. The digestion was stopped by adding 0.2 volume of gel loading buffer (50 mM EDTA, pH 8.0, 0.125% bromophenol blue, 12.5% Ficoll), and the samples loaded onto a 1% agarose gel made with 1X TAE buffer, 1 μg/ml of ethidium bromide and electrophoresed at 80 V for 2 h. The gel was photographed, the insert size was determined, and clones containing the insert
of interest were selected for further analysis including sequencing and northern blot analysis (described later).

4.2.5 DNA Sequencing

Plasmid DNA (2.0 μg) containing the gene insert of interest was denatured in a total volume 50 μl of 0.4 M NaOH solution in a 0.5 ml microfuge tube for 10 mins at room temperature, ethanol precipitated (0.1 volume of 3.0 M NaOAc (pH 5.2) and 3 volumes of absolute ethanol were then added) at -80°C for 30 mins and then centrifuged at 12,000 x g for 10 mins at 4°C. Plasmid DNA pellet was washed in 500 μl of 80% ethanol, re-centrifuged and dried.

The DNA pellet was resuspended in 10 μl of sterile water and 2 μl (0.80 μM) of the appropriate sequencing primer (M13 universal sequencing primer; Pharmacia) or T3 Bluescript sequencing primer (Stratagene) and 2 μl of annealing buffer were added. The DNA and appropriate primer were denatured together at 65°C, annealing was allowed to proceed for 20 mins. at 37°C, then the reaction was cooled to room temperature for 30 mins. Sequencing by dideoxynucleotide termination reaction was carried out as described in the protocol provided using the T7 DNA polymerase sequencing kit (Pharmacia) α-35S dATP (Amersham, Oakville, ON).
Sequencing reactions were separated on 6% polyacrylamide 8M urea gels using an IBI (Interscience, Mississauga, ON) sequencing system (0.4 mm X 35 cm X 45 cm) and a Biorad 3000V power pack (Biorad, Mississauga, ON). The gels were run at 50°C for 3 or 5 h. Extended sequencing reactions using the 'read-long' reactions from the T7 sequencing kit were performed and run on a 6% polyacrylamide 8M urea gel. After electrophoresis, the gels were transferred onto Whatman 3MM paper (without fixation), covered by Saran plastic wrap and dried for 4 h in a BioRad Model 583 gel drier under house vacuum. Biomax BMR X-ray film (Kodak) was placed next to the dried gel in a film cassette and exposed 18 - 72 h at room temperature. The film was then developed as described previously.

4.2.6 Northern Blot Analysis

4.2.6.1 Probe labeling and hybridization

The relevant DNA fragments of isolated clones (determined above) were isolated by digesting the entire plasmid with Eco RI, subsequently running on a 0.8% 1X TAE low melting point gel containing 1 μg/ml of ethidium bromide in 1X TAE running buffer, and then the desired band was excised. The agarose containing the gel fragment was then melted at 70°C for 10 mins and diluted with three volumes of sterile water. Radiolabeled probes were made and used for hybridization to northern blots as described previously in section 2.2.8.
4.3 Results

4.3.1 Detection of Uniquely Expressed mRNAs by Differential Display

Poly A\(^+\) selected RNA from 3 different cell lines (i) HTR8 parental trophoblast cells, (ii) the immortal and neomycin resistant HTR8/SVneo cells and (iii) the extended lifespan RSVT-2 cells was used to isolate uniquely expressed mRNAs. The poly A\(^+\) selected mRNA was first reverse transcribed using T\(_{12}\)MG as the degenerate anchored oligo-(dt) primer and then amplified with T\(_{12}\)MG and AP-2 primers in the presence of \(^{35}\)S-dATP.

As seen in Figure 18, a number of bands were detected, with a majority of bands being found in more than one cell line. Many bands were shared by the two SV40 Tag transformed cell lines, which are absent in the HTR8 cell line. One band (band 7, Figure 18) was expressed in the immortal HTR8/SVneo cell line, but not in the extended lifespan RSVT-2 cells or in the parental HTR8 cells. At the initial stages of this study, we chose to characterize the gene(s) that are uniquely represented within a single cell line. This band was cut from the gel and re-amplified, as previously described, for further analysis.

4.3.2 Library Screening

The re-amplified PCR product of band 7 (indicated in Figure 18) was radiolabeled with \(^{32}\)P-dCTP as previously described (section 2.2.8) and used as a
Figure 18: Differential display autoradiograph of HTR8, RSVT-2 and HTR8/SVneo

The differential display autoradiograph showing band 7 (indicated by arrow). The primers were T12MG and AP-2.
Differential Display Gel

Band 7
(HTR8/Svneo)
probe to screen a human fetus male Caucasian placenta cDNA library (Stratagene) in λZAPII. A large number of clones were obtained on the primary screening. Five positives were selected for the secondary screening. These five positives yielded three positives on the tertiary and final screening. The desired insert plus the pBluescript cDNA cloning vector was excised from the λZAPII phagemid by in vivo excision and the recombinant plasmid DNA was isolated, digested with Eco RI and analyzed by gel electrophoresis. Two clones, 7-3 and 7-4, were used to probe the northern blots. When clone 7-3 (approximately 3.2 kb insert) was used, the hybridization signal was poor with high background, and was not consistently expressed by the transformed cells (data not presented). Thus it was not pursued further. In Figure 19, clone 7-4 (approximately 1.8 kb) was found to contain an internal Eco RI site which produced two fragments of approximately 1.2 kb and 0.6 kb in size. The 600 bp fragment was isolated from a low melting point agarose gel and used to make a radiolabeled probe for northern blot analysis.

4.3.3 Differentially Expressed mRNAs Detected by Northern Blot Analysis

The radiolabeled clone 7-4 (0.6 kb fragment) probe was used to screen a northern blot of total RNA from parental HTR8, extended lifespan RSVT-2, immortal RSVT2/C, immortal and G418/neomycin resistant HTR8/SVneo, and its subclones, D8 and G10, as well as JAR, a choriocarcinoma cell line. The clone 7-4
Figure 19: Agarose separation and sizing of clones 7-3 and 7-4

The figure shows the clones 7-3 and 7-4 digested with Eco RI run on a 1.2% agarose gel stained with ethidium bromide. Sizing was based on calculations using the pBluescript vector (2.97 kb) as a size marker. Estimated size of clone 7-3 is ~3 kb and clone 7-4 is ~1.8 kb (~1.2 kb for the larger insert Eco RI fragment and ~600 bp for the smaller fragment). The smaller fragment was used as a probe on northern blot.
was found to be differentially expressed (Figure 20). A strong signal at approximately 3 kb was apparent in all of the immortal cell lines (RSVT/C, HTR8/SVneo, D8, G10, and JAR). A very faint signal is apparent at 3.0 kb in the parental HTR8 cells, but this was negligible, and no detectable signal is found in the extended lifespan RSVT-2 cells. A faint band at 2.0 kb is also seen in the immortal cell lines, except G10 cells, and may represent a differentially spliced or a degradative product. The northern blot was re-probed with 18S rRNA to compare RNA loading of the lanes (Figure 20).

4.3.4 DNA Sequencing and Comparison with Known Sequences

Clone 7-4 was partially sequenced - 267 nucleotides at the T3 primer end (Figure 21) and 357 nucleotides at the M13 primer end (Figure 22). A computer search using BLAST™ against Genbank™ and EMBL™ DNA data bases indicated a high degree (98%) of partial homology (150/153 matched nucleotides) of the 276 queried nucleotides at the T3 end to yh70b11.r1 Homo Sapiens cDNA clone 135069, in the antisense orientation (data not shown). Only 202 nucleotides of clone 135069 have been sequenced and its identity and function have not yet been determined (Genbank; submission by UWash-Merck EST project). The possible relationship between 7-4 and clone 135069 has also not been established, since only a partial match has been observed.
Figure 20: Northern blot with the clone 7-4 gene fragment

Northern blot of the normal HTR8, RSVT-2, RSVT2/C, HTR8/SVneo, D8, G10 and JAR choriocarcinoma cells. The 18s rRNA control was used to assess loading variations.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTR8</td>
<td>0.02</td>
</tr>
<tr>
<td>RSVT-2</td>
<td>0.00</td>
</tr>
<tr>
<td>RSVT2/C</td>
<td>0.40</td>
</tr>
<tr>
<td>HTR8/SVneo</td>
<td>1.00</td>
</tr>
<tr>
<td>D8</td>
<td>0.78</td>
</tr>
<tr>
<td>G10</td>
<td>0.85</td>
</tr>
<tr>
<td>JAR</td>
<td>0.40</td>
</tr>
</tbody>
</table>

* Densitometric value for HTR8/SVneo (normalized to 18S) is set to one. Relative abundance of clone 7-4 message in the other cell lines are given in relation to HTR8/SVneo.
Figure 21: Sequence data for the clone 7-4 using T3 primer

Sequence data of 267 bp using the T3 primer.
atcagtgcat  aaatBBBBBBBBtctttttcatttt  cagcagatgg
acaBBBaaacagat  ggactctaca  gctaatggataatcaaaagg
BBAgggggtg  attctgtgaa  gactgatagg  cctgactatt
Bcctcaattctc  BBBBBcccaactgcag  ttgggccagaacgca  acctctgaa
Btataggggtct  ccttaaaacac  atctgacgcctgacat
Bgaacggtgcac  ttgggagacc  ttgggtacagg  cacaaaagc
atatagcgg  ctagcctcat  acaggggag
**Figure 22: Sequence data for clone 7-4 using M13 universal primer**

Sequence data of 357 bp using M13 universal primer.
Analysis of the sequenced 357 nucleotides of 7-4 from the M13 primer end indicated a high degree (97%) of partial homology (145/149 nucleotides) with 379 bp EST29905 *Homo sapiens* cDNA. EST29905 is an expressed sequence tag, of an RNA message known to be expressed in humans (Genbank, submission by Venter, The Institute for Genomic Research). The function and identity of EST29905 and its possible relationship to 7-4 have also not been determined.

4.4 Discussion

Introduction of a number of viral transforming genes, like *SV40 Tag* or *EIA*, has been shown to extend the lifespan and immortalize normal cells (Reddel *et al.* 1988). Subsequently, some of the transformants have been reported to acquire a tumorigenic phenotype. For example, *SV40 Tag* transformed human bronchial epithelial cells originally observed to be nontumorigenic (Reddel *et al.* 1988) acquired tumorigenic phenotype after transformation with *SV40 Tag* (Reddel *et al.* 1993). It is likely that cells evolved through a premalignant stage, which has been identified in the case of *SV40 Tag* transformed trophoblast cells.

Earlier results presented in this thesis have documented certain phenotypic changes in the *SV40 Tag* transformed EVT cells *e.g.* long life or immortality, increased proliferative and invasive ability, as well as reduced or lost responsiveness to negative regulators of proliferation and invasiveness (TGFβ).
These changes, combined with the findings that the transformed cells failed to exhibit anchorage-independent growth \textit{in vitro} or tumorigenicity in nude mice, indicated that they reached a "premalignant" stage in tumor progression. It was also shown that these phenotypic changes are associated with alterations in the expression of invasion associated genes (Chapter 2) as well as connexins (Chapter 3). The present study was undertaken in an effort to identify changes in other gene(s) during this transition using the differential display method. Simultaneous display of RT-PCR products from parental, extended lifespan and immortal cell lines with the same genetic background permitted detection, isolation and partial identification of a differentially expressed clone. While the functional significance of the gene as well as its possible relevance to tumor biology remain unknown, it is possible that this gene may have a contributory role in the process of immortalization.

It has been reported that \textit{SV40 Tag} transforms cells at a very low frequency and that the majority of cells in the population undergo cell crisis (Shay and Wright, 1989; Shay \textit{et al.} 1991; 1993). It has been proposed that \textit{SV40 Tag} functions first, to alter cellular senescence resulting in the extended lifespan of transfectants, and second, to override, or render dysfunctional, the mechanisms responsible for cellular crisis (Wright \textit{et al.} 1989). The molecules involved in the
immortalization of a transformed cell and their role in the tumorigenic process have not been defined.

The speculation that the gene represented by clone 7-4, which was initially isolated using the immortal HTR8/SVneo cell line in the differential display, may have a role in the immortalization of the transformed cells is based on two observations. (i) The message for clone 7-4 was extremely low in the normal HTR8 cells which are mortal, and was nondetectable in the extended lifespan RSVT-2 cell lines. (ii) This gene was not only strongly expressed in the immortal HTR8/SVneo cells and its immortal subclones D8 and G10, it was also expressed in RSVT2/C cells, a recently isolated immortal subclone of extended lifespan RSVT-2 cells. even though it was derived at a much later time point than the immortal HTR8/SVneo cells. In addition, this gene is also expressed in the JAR choriocarcinoma, which is an immortal trophoblastic cell line. However, additional studies with this gene as well as other immortal cell lines are necessary to substantiate this possibility.

Partial sequencing of clone 7-4 revealed a high degree of homology to previously sequenced human cDNA fragments, 135069 and EST29905. The relationship to these fragments has yet to be fully determined. Complete sequencing of clone 7-4 will be necessary to establish its identity or to determine if it is a novel gene. In addition, the complete nucleotide sequence may provide
further insights into the possible role of clone 7-4 gene *i.e.* the structural motifs or regions as well as protein characteristics (based on the amino acid predictions derived from sequencing data). This information may provide direction and impetus for future experiments with this gene. Once the gene has been sequenced completely, its functional role for cellular immortality or premalignant behavior can be tested by transfection studies: (a) overexpressed in the HTR8 and RSVT-2 cell lines to determine whether it leads to immortality and (b) by downregulation in HTR8/SVneo and JAR choriocarcinoma cell lines to determine if reduced expression leads to a loss of cellular immortality and/or the transformed phenotype. It is also important to explore in the future, whether this gene is expressed in other tumor models inclusive of preneoplastic cells, for its potential use as a diagnostic or functional marker. Finally, future functional studies will be aided by the development of a GST fusion protein for this gene and antibodies to its bioactive regions.
Chapter 5

5. General Summary, Discussion, and Conclusions

Trophoblast cells of the human placenta provide a unique in vitro model for studies of genetic changes relevant to tumor progression. Normal first trimester extravillous trophoblast cells propagated in culture share similar mechanisms of invasiveness with malignant cells (Yagel et al. 1988), however, unlike malignant cells, their proliferative (Graham and Lala, 1992; Lala and Lysiak, 1995), invasive (Graham and Lala, 1991) and migratory (Irving and Lala, 1995) abilities are exquisitely regulated by a number of factors produced in situ. These functions, shared by the normal trophoblast and malignant tumor cells, are only one of many mechanisms necessary for malignancy and metastasis. The fact that trophoblast cells already express many of the genes responsible for these shared functions makes them uniquely suited for further genetic manipulation in order to induce sequential changes in phenotype, i.e., to generate premalignant, malignant, and finally, metastatic properties in these cells.

Development of cell lines representing these stages has been a long term objective of our study in order to identify genetic changes relevant to these transitions. The present study successfully induced a premalignant phenotype by SV40 Tag transfection of the normal EVT cell line, HTR8. Further experiments
were then designed to identify changes in known and novel genes which could account for the premalignant behavior of SV40 Tag transformed trophoblast cells. Most experiments compared the parental HTR8 and two SV40 Tag transformants, the long lived RSVT-2 cell line and the immortal RSVT2/C cell line. Other experiments also utilized another SV40 Tag immortalized line, HTR8/SVneo, previously produced in this laboratory from the HTR8 lineage. First, a series of experiments compared the proliferative and invasive behavior of the cells as well as the cellular responses to growth factors, previously shown to alter normal trophoblast cell proliferation and invasiveness. A second series of experiments examined the expression of connexins and dye coupling (an indicator of cell-cell communication) in normal HTR8, and transformed premalignant RSVT-2 and RSVT2/C cell lines. In addition, the influence of growth factors on connexins was examined. In the third series of experiments, attempts were made, using the differential display mRNA method, to identify gene(s) gained or lost during the transition to the premalignant stage. A comparison of cells of the same genetic background assured the exclusion of many irrelevant genes in the differential display.

The first part of the study reported that RSVT-2 and RSVT2/C cells were more proliferative than their parental HTR8 cells. Based on several observations on the biology of SV40 Tag (discussed in Chapters 1 and 2), it was postulated that
increased proliferation is presumably a result of the inactivation of a cellular regulator of cell growth, p53 and or pRb (Chapter 2). In fact, a complexing of p53 with SV40 Tag protein (which results in p53 inactivation) has been shown in the case of HTR8/SVneo cells (Aboagye-Mathiesen et al. 1995). Although the proliferation of HTR8 and RSVT-2 cells was inhibited by TGFβ, the immortal RSVT2/C cells showed a resistance to the anti-proliferative signals of TGFβ. These findings are in agreement with the postulation that the ability to evade negative growth controls can provide an advantage to premalignant cells which later would lead to the acquisition of a malignant phenotype (Nicolson, 1987; Herlyn and Malkowicz, 1991; Shih and Herlyn, 1993).

It was also shown that RSVT-2 and RSVT2/C cells are more invasive than the parental HTR8 cells. This had also been shown previously for another SV40 Tag cell line (HTR8/SVneo), also derived from the HTR8 cells. However, the mechanism involved in increased invasion was not clear in that study. The present study identified several possible mechanisms in increased invasiveness. It was found that the mRNA expression of the natural inhibitor of metalloproteases, TIMP-1, was reduced in the extended lifespan RSVT-2 cells, early in this model of tumor progression. In addition to TIMP-1, immortalized RSVT2/C cells showed a reduction in TIMP-2 as well as PAI-1 mRNA expression (PAI-2 was not detected). Since uPA is known to activate collagenases (reviewed in Chapter 1), a reduction
in its inhibitor would permit uPA-mediated activation of collagenases to be unchecked. Furthermore, this study found that 92 kDa type IV collagenase was activated by RSVT2/C cells. Together, these results indicate that the hyperinvasive behavior of the transformed cell lines is, at least in part, due to an increase in the matrix degrading ability of these cells owing to a downregulation of natural enzyme inhibitors. The precise role of these enzymes on the invasiveness of RSVT-2 and RSVT2/C remains to be defined through functional assays using neutralizing antibodies.

The present study also confirmed the previous findings that TGFβ inhibits invasion of normal trophoblast cells, at least partly by upregulating the expression of TIMP-1 message. We also found that TGFβ upregulated the PAI-1 message in HTR8 cells, which provided a further mechanism by which TGFβ can control the invasion of trophoblast cells. When RSVT-2 and RSVT2/C cell lines were treated with TGFβ, no inhibition of invasion was observed. We found that in RSVT-2 and RSVT2/C cells, TGFβ actually increased the expression of 72 kDa type IV collagenase. The message for TIMP-1 was low in both the RSVT-2 and RSVT2/C cells, and was not upregulated by TGFβ to the same extent as observed in HTR8 cells. Thus, the balance between the 72 kDa type IV collagenase and its inhibitor TIMP-1 was either unchanged or altered in favor of the enzyme. Thus, the premalignant cells had altered responsiveness to TGFβ, the molecular mechanisms
for which (e.g. alteration in TGFβ receptors or receptor-mediated signal transduction) remain to be investigated.

In the second part of this study, connexin-mediated intercellular communication was examined in normal HTR8 and SV40 Tag transformed cells. Connexins are the basic subunits that form the functional gap junction complex involved in cell to cell communication. There is evidence to suggest that they play a role in cell growth regulation, and that their altered or aberrant expression tends to decrease intercellular communication in a number of tumor models as well as in preneoplastic foci (Chapters 1 and 3). Dye transfer was used in this part of the study as an indicator of cell to cell communication. Homologous (between same cell types) and heterologous (between two different cell types) communication were studied using the preloading method for dye transfer. In both assays, we found that normal HTR8 cells were communication efficient, while RSVT-2 cells showed reduced communication, and RSVT2/C cells showed poor or no communication. These functional studies concurred with the results obtained from immunostaining and northern blot analysis. Cx43, which was abundantly expressed in HTR8 cells, was found to be poorly expressed in RSVT-2 cells and undetectable in RSVT2/C cells. These results are consistent with the hypothesis that connexins play a role in tumorigenesis, probably through their ability to control cell growth. In HTR8 cells, TGFβ reduced the level of communication, as
measured by the homologous dye transfer assay, and caused a parallel reduction in the level of Cx43 mRNA expression. Both post-transcriptional and post-translational events in gap junction formation can account for this finding. In contrast, exposure of RSVT-2 cells to TGFβ increased heterologous dye transfer from HTR8 to RSVT-2 cells, but no conclusion could be drawn from the northern analysis as to whether there was a change in the Cx43 message. Taken together, these results in our trophoblast model suggest that a reduced level of connexin expression and intercellular communication appear to precede the acquisition of a malignant phenotype during the tumor progression pathway.

In the third part of this study (Chapter 4), mRNA differential display was used to identify gene(s) differentially expressed in the normal HTR8, extended lifespan RSVT-2, and immortalized HTR8/SVneo cell lines. We chose to select a cDNA band exhibited only by the immortal HTR8/SVneo cell line and not by others. A gene fragment, designated 7-4, was isolated from the placental cDNA library. It was found to be expressed in all of the immortal cell lines used in this study; that is, in RSVT2/C, HTR8/SVneo (as well as its subclones) and JAR choriocarcinoma cells. However, it was not found to be expressed in the normal HTR8 or extended lifespan RSVT-2 cells. Therefore, it was speculated that this gene may have a functional role in cell immortality and/or premalignant behavior. A complete sequencing of this gene, as well as functional studies, following
engineered overexpression or downregulation of this gene remain to be accomplished in the future.

The transformed trophoblast cell lines described here may be offered as valuable tools for further studies of genetic changes responsible for phenotypic changes during tumor progression. An earlier study (Chou, 1978) produced temperature sensitive, immortalized trophoblast cell line by SV40 Tag transformation. Although these cells expressed similar markers as normal trophoblast cells (Logan et al. 1992), they shed viral particles which could infect other cells and are, thus, not suitable for in vivo studies. Furthermore, the commercially available choriocarcinomas are far removed from their original hosts, no genetic comparison can be made with the normal trophoblasts of the same host in order to detect genetic changes. Since this laboratory has normal parental cells of the same genotype, it is believed that RSVT-2 and RSVT2/C cells will provide a good tool in the examination of genetic alterations necessary for malignant changes induced by the introduction of a secondary oncogene, e.g. \textit{H-ras} or \textit{v-myc}. This should be feasible in the near future using a selectable marker since the present premalignant lines RSVT-2 and RSVT2/C were had been produced without a selection marker.
5.1 *Original Contributions*

1. Successful isolation and development of two premalignant trophoblast cell lines: an extended lifespan cell line, RSVT-2, and immortalized cell line, RSVT2/C, which are hyperinvasive and hyperproliferative, variably resistant to anti-proliferative and anti-invasive actions of TGFβ, and are nontumorigenic in nude mice.

2. Demonstration that a reduced expression of natural enzyme inhibitors, TIMP-1, TIMP-2, and PAI-1, can account for the hyperinvasive behavior of premalignant cells.

3. Demonstration that refractoriness to the anti-invasive action of TGFβ in premalignant cells can be explained by the inability of these cells to upregulate enzyme inhibitors, TIMP-1 and PAI-1, when exposed to TGFβ. Further studies are required to identify the molecular basis of these findings e.g. alterations in the nature of TGFβ receptors or receptor mediated signal transduction.

4. Demonstration that a reduction in gap junctional intercellular communication due to a downregulation of connexin expression is a property which may occur at the premalignant stage *i.e.* prior to malignant transformation.
5. Demonstration that premalignant cells are resistant to TGFβ mediated downregulation of connexins, and gap junctional intercellular communication. The molecular basis for this finding remains to be investigated.

6. Isolation of a potentially novel gene fragment (7-4) differentially expressed in immortal, premalignant as well as malignant trophoblasts. We speculate that this gene may have a functional role in conferring immortality. Further studies are needed to completely sequence this gene and test its function by transfection experiments.
5.2 Future Directions

1. RSVT2/C showed the capacity to survive in soft agar, while HTR8 and RSVT-2 cells could not. Can RSVT2/C form colonies in soft agar if allowed to grow longer, or if supplemented with growth factors, such as TGFα, CSF-1 or VEGF?

2. Recently, RSVT2/C cells were transfected with *Harvey ras* oncogene, and several clones selected on the basis of G418/neo resistance have been isolated (data not presented). What are the phenotypes of these clones? Are they capable of anchorage-independent growth (forming colonies in soft agar)? Are they tumorigenic or metastatic? What are the relevant genetic changes that have occurred in these cells? In addition, the following questions also apply to the analysis of the *H-ras* transformed RSVT2/C cells.

3. This study has shown that 92 kDa type IV collagenase is expressed and the enzyme is present in the zymogram, but the active form is secreted only by RSVT2/C cells. What regulates the 92 kDa type IV collagenase expression by these cells? What is activating the 92 kDa type IV collagenase in RSVT2/C cells?
4. What is the contribution of the uPA/plasmin system to the increased invasiveness of RSVT-2 and RSVT2/C cells? Is uPA uninhibited, since PAI-1 is reduced in RSVT2/C, contributing to invasion by activating 92 kDa type IV collagenase in these cells?

5. Membrane-type metalloproteinase (MT-MMP) has been demonstrated on EVT cells in situ (Nawrocki et al. 1995). Is it present on the EVT cell lines, and if so, what is the role of membrane type metalloproteinase (MT-MMP) in these cells? What growth factors regulate its activity? Does TGFβ or IGFII have an effect on MT-MMP expression these cell lines?

6. What is the molecular basis for reduced or lost sensitivity of SV40 Tag transformed trophoblast cells to anti-proliferative and anti-invasive action of TGFβ? Is there a change in the nature of the TGFβ receptors in these cell lines? Or has there been a change in the TGFβ-mediated signal transduction pathway?

7. Integrin expression is important in normal development, and has been shown to affect the behavior of trophoblast cells and also the behavior of tumor cells. Studies of the integrin profile of RSVT-2 and RSVT2/C cells have shown gain of α6 and β4 integrin subunits, and
the loss of α2 integrin subunit in RSVT-2 and RSVT2/C cells (Lala et al. 1995; data not presented). What is the functional significance of these findings?

8. What is the molecular basis for Cx43 downregulation by TGFβ in the normal trophoblast cells?

9. What is the molecular basis for downregulation of Cx43 expression in SV40 Tag transformed trophoblast cells? Is there binding of SV40 Tag to a key molecule that regulates the expression of connexin(s) or connexin promoter resulting in its inactivation? Is the decrease in connexin expression due to changes in cell adhesion molecules? For example, E-cadherin (a homophilic cell adhesion molecule shown to be expressed in EVT cells (Fisher et al. 1989a)) has been reported to play a role in formation of gap junctions (Jongen et al. 1991; Kamibayashi et al. 1995).

10. Do connexins play a direct role in the proliferative and invasive behavior of EVT cells? Can the upregulation of Cx43 in the SV40 Tag transformed cells reduce their proliferative/invasive ability?

11. What is the role of gene 7-4 in cellular immortality or tumorigenicity or both? What are the consensus sequences, motifs or protein domains
that may provide clues to its function? To answer these questions, a full-length gene is required. What is the protein that this gene 7-4 coding for? Is it an important member of a signaling pathway? Is it important for cell growth or differentiation? Is it expressed during embryogenesis?

12. Can overexpression of gene 7-4 cause transformation or can its downregulation reverse the transformed phenotype?

13. Is the gene 7-4 expressed in other tumor models? If so, how frequently does it appear in these tumors?

14. What are the other genes gained or lost or altered in this model that includes the normal HTR8, RSVT-2, RSVT2/C, and most recently, H-ras transfected RSVT2/C cells? Do these genes contribute to the malignant phenotype?
5.3 References


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Appendix A: Plasmid map of pRSV-Tag

LEGEND:

Plasmid map of pRSV-Tag (Reddel et al. 1988) from Drs. Curtis Harris and Brenda Gerwin (Laboratory of Molecular Virology, Division of Cancer Etiology, National Cancer Institute, NIH, Bethesda, MD). The size of plasmid is 6.033 kb with ampicillin resistance for bacterial selection. It contains no eukaryotic cell selection marker. The SV40 oncogene insert is comprised of early genes (large T and small t antigens) under the Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter.
Vita

Name: Nelson K.S. Khoo
Year of Birth: 1963
Birthplace: Penang Island, Malaysia

EDUCATION:
1990- Nov, 1995 Ph.D. Program, Anatomy, University of Western Ontario, London, ON
1984-1987 B.Sc. (Hons) Program, Biology, Wilfrid Laurier University, Waterloo, ON

RESEARCH AND RELATED EXPERIENCE:

Research Experience:
1996 Postdoctoral position. UCMP, Umea University, Umea, Sweden. Supervisor: Dr. Charles Sentman. Accepted.

Related Experience:
1993-1994 Two lectures in Medical Gross Anatomy. Department of Anatomy, University of Western Ontario, London, ON.
1993-1994 Laboratory Teaching Assistant in Medical Gross Anatomy, Department of Anatomy, University of Western Ontario,
1992  Accepted for the National Cancer Institute of Canada sponsored *Eighth Course in Oncology*, McMaster University, Hamilton. June 21-26, 1992.

1991-1992  Laboratory Teaching Assistant in Medical Gross Anatomy. Department of Anatomy, University of Western Ontario, London, ON.


1987-1988


Seminars or Presentations

Nov., 1994  Human placental trophoblast cells: A model for tumor progression. Amgen Institute, Princess Margaret Hospital, Toronto, ON.


Supervisory and Administrative Responsibilities:

1990-1995  Supervised work study students in various aspects of the molecular projects in the laboratory.
1993-1994  Graduate Student Representative at the departmental faculty meetings and Graduate Representative in the Graduate Affairs Committee for the Department of Anatomy.

**SCHOLARSHIPS AND AWARDS:**

**Scholarships**

1995  Special University Scholarship, Faculty of Graduate Studies, University of Western Ontario, London, Ontario.

1988 - 1989  Special University Scholarship, Faculty of Graduate Studies, University of Western Ontario, London, Ontario.

1987 - 1988  Special University Scholarship, Faculty of Graduate Studies, University of Western Ontario, London, Ontario.

1986 - 1987  WLU Incentive Scholarship, Faculty of Science, Wilfrid Laurier University, Waterloo, Ontario.

1981 Summer  Grade 12 Early Admission Scholarship, Wilfrid Laurier University, Waterloo, Ontario.

**Awards**

1992  Young Investigator Travel Award, 12th Rochester Trophoblast Conference, Rochester, NY.

1991  Travel Award, Canadian Association of Anatomists, Canadian Federation of Biological Sciences, Kingston, ON.

1986  Dean's Honour List for 1985-1986, Faculty of Science, Wilfrid Laurier University, Waterloo, ON.
THESES AND PUBLICATIONS:

Theses


Publications


Submitted Paper(s) or Manuscripts in Preparation


Abstracts


