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DIFFERENTIAL EFFECTS OF IGF-I AND IGF-II ON THE FATE OF MOUSE TROPHOBLAST STEM CELLS

(Spine title: Differential Effects of IGF-I and IGF-II on the Fate of TS cells)

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by

Robert Stanley Porteous

Graduate Program In Biochemistry

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

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

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THE UNIVERSITY OF WESTERN ONTARIO
FACULTY OF GRADUATE STUDIES

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Differential Effects of IGF-I and IGF-II on the Fate of Mouse Trophoblast Stem Cells

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Master of Arts

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ABSTRACT

DIFFERENTIAL EFFECTS OF IGF-I AND IGF-II ON THE FATE OF MOUSE TROPHOBLAST STEM CELLS

The main objective of this thesis was to identify the role(s) of IGF-I and IGF-II on the fate of mouse trophoblast stem cells (TS cells) and their role in contributing to the stem cell fate, when grown in the absence of additional growth factors. By analyzing the expression of self-renewal and trophoblast-specific transcription factors, the addition of IGF-I and IGF-II restored nuclear OCT4 and CDX2 expression, with IGF-II inducing a longer effect, while the expression of SOX2 was not restored. IGF-I was found to induce the differentiation of TS cells based on expression and quantitative analysis of PL-1 expression. Annexin-V and TUNEL staining demonstrated that IGF-II protects TS cells from cell death. The addition of FGF-4 to the system did not have an additive effect of protection and induced further negative effect when combined with IGFs. These studies demonstrate that IGF-I and IGF-II, have different biological effects on the fate of mouse TS cells, likely through different signal transduction pathways.

Keywords: trophoblast stem (TS) cells, stem cell niche, IGF-I, IGF-II, FGF-4, self-renewal, differentiation, cell death, OCT4, SOX2, CDX2, PL-1, Annexin-V staining, TUNEL staining.

Dedicated to never ending curiosity.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
°C	Degrees Celcius
µgTS	microgramTrophoblast stem cell
µlTUNEL	microlitreTdT-mediated dUTP Nick End Labeling
^{32}P-dCTP	Radioactive phosphorous deoxy-cytidine triphosphate
ALS	Acid labile sub-unit
Amp	Amperes
Amp	Amperes
ANOVA	Analysis of variance
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
BMP	Bone morphogenic protein
C. elegans	Caenorhabditis elegans
Ca^{2+}	Calcium
CO_2	Carbon dioxide
cpm	Counts per minute
DAB	3,3'-Diaminobenzidine
dH_2O	Distilled water
DMEM	Eagle's minimal essential medium
DPBS	Dulbecco's Phosphate Buffered Saline
DTT	Dithiothreitol
E	Embryonic day
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetraacetic acid
EFCM	Embryonic fibroblast conditioned medium
EGF	Epidermal growth factor
EMFI	embryonic fibroblasts
ERK	Extracellular signal-regulated kinase

ES	Embryonic stem cell
FBS	Fetal bovine serum
FCM	Fibroblast conditioned medium
FGF-4	Fibroblast growth factor-4
FGFR	Fibroblast growth factor receptor
FITC	*Fluorescein isothiocyanate*
FRS2	Fibroblast growth factor receptor substrate 2
g	Gram
GFP	Green fluorescence protein
GH	Growth hormone
H&E	Hematoxylin and eosin
H_2O_2	Hydrogen peroxide
HCl	Hydrogen chloride
HGF	Hepatocyte growth factor
HMG	High mobility group
hr	hour
HRP	Horseradish Peroxidase
ICM	Inner cell mass
Ig	Immunoglobulin
Igf1	Murine insulin-like growth factor I gene
Igf2	Murine insulin-like growth factor II gene
IGFBP	Insulin-like growth factor binding protein
IGF-I	Insulin-like growth factor I
IGF-II	Insulin-like growth factor II
IGF-IIR	Insulin-like growth factor receptor II
IGF-IR	Insulin-like growth factor receptor I
IgG	Immunoglobulin G
IR	Insulin receptor
IR-A	Insulin receptor isoform A
IR-B	Insulin receptor isoform B
IRS	Insulin receptor substrate

JAK	Janus-associated tyrosine kinase
KCL	Potassium chloride
kDa	kilodalton
L	Flexible linker
LIF	Leukemia inhibitory factor
LIFR	Leukemia inhibitory factor receptor
M	molar
M6P	mannose-6-phosphate
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
MEK	MAP/ERK kinase
Mg^{2+}	Magnesium
$MgCl_2$	Magnesium chloride
min	minutes
ml	millilitre
mM	millimolar
MSC	Mesenchymal stem cell
NaCl	Sodium chloride
ng	nanogram
nm	nanometre
OH	hydroxyl
p	Significance value
PDGF	Platelet-derived growth factor
pH	*potentia hydrogenii* (potential of hydrogen)
PI	Propidium iodide
PI3K	Phosphatidyl-inositol-3-kinase
PL-1	Placental lactogen-1
PLCγ	Phospholipase C gamma
PMSF	phenylmethylsulphonyl fluoride
polydI/dC	Polydeoxyinosinic-deoxycytidylic acid
POU	Pituitary Octamer transcription factor

PRL	Placental prolactin
PRL3B1	Placental lactogen II
PS	Phosphatidylserine
PVDF	polyvinylidene fluoride
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RTK	Receptor tyrosine kinase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFK	Src family kinases
SH2	Src-homology 2
SOX2	SRY-related HMG box 2
SSEA	Stage specific embryonic antigen
STAT	Signal transducer and activator of transduction
TBE	Tris/Borate/EDTA solution
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline supplemented with 0.05% Tween-20
TdT	Terminal Deoxynucleotidyl Transferase
TE	Trophectoderm
TEMED	Tetramethylethylenediamine
TGC	Trophoblast giant cell
TGF	Transforming growth factor
TRITC	Tetramethylrhodamine isothiocyanate
V	volt
w/v	Weight per volume
WT	Wild type
XEN	Extraembryonic endoderm
$ZnSO_4$	Zinc sulfate

CHAPTER 1: INTRODUCTION

1.1. Impact of Stem Cells in Medical Research

In the past decade, there have been great advances in the study of stem cells, to the point where 'stem cell' has become a household term. However, despite these advances, a good deal is still unknown as to the mechanisms by which stem cells differentiate into the different cell lineages within the human body. By definition, a stem cell is a cell that can both self-renew (unlimited capability of cell division) and differentiate into a diverse range of cells with specialized functions (1). During cell division, one daughter cell will possess the same properties of the parent cell and remain a stem cell (symmetrical division), while the other daughter cell will differentiate and exhibit a different phenotype (asymmetrical division). Many stem cells possess the remarkable property of pluripotency, which allows them to give rise to every specialized tissue in the body (1). This highlights the importance of research on stem cells as they provide tremendous potential in regenerative medicine to repair or regenerate damaged tissues for the treatment of degenerative ailments. Stem cells that are derived from the early embryo stage are known as embryonic stem (ES) cells, while those that are isolated from various developed or adult tissues are known as adult or mesenchymal stem cells (MSCs) (2;3). MSCs have a limited potential in their ability to proliferate and differentiate with age and donor (4;5), while ES cells divide for numerous generations and have an unlimited potential for proliferation and self-renewal. This makes stem cells derived from the early embryo more vital and beneficial, as they help to provide a greater and vast variety of cell types for regenerative medicine (6-8).

Currently, stem cells are being used to understand the complex mechanisms and cellular events that occur during early development, and how alterations in these cellular events at any stage can lead to the progression of diseases (9). Since stem cells are the foundation to any cell type, understanding the mechanisms of self-renewal and proliferation of these cells will help provide valuable information in the ways by which alterations of these pathways would lead to disease states. Furthermore, continued study of stem cells will help develop novel therapeutic agents for the treatment and prevention of these diseases. The study of stem cells provides valuable information regarding developmental decisions and the various pathways by which stem cells differentiate. Diseases of particular interest that are currently of intense interest for regenerative medicine include Parkinson's disease, Alzheimer's disease, coronary heart disease and diabetes.

Another current application of stem cells is in their use for cell replacement therapies (10), as donated tissues and organs are often used to replace ailing and damaged tissue. The use of stem cell-based therapies can provide enormous possibilities for restoring damaged tissue, thereby providing a renewable source of replacement cells to treat many disorders. The shortage of organs for transplantation has resulted in the intensification and need for stem cell-based research. Before the use of stem cell-based therapies can be applied, there are still many hurdles to overcome. Therefore, detailed understanding of the precise mechanisms involved in controlling the self-renewal and proliferation of stem cells, as well as the signals involved in directing stem cell differentiation into the vast lineages need to be further investigated (11). Being able to maintain a large number of stem cells and direct differentiation without the spontaneous

formation of teratomas is a challenge (10). A specific balance is required and understanding the 'signal' that controls the decision to switch and direct a stem cell from proliferation to differentiation needs to be further explored, before the use of stem cells can be used to provide better strategies for therapeutic purposes.

1.2 Biology of Mouse Development

1.2.1 Mouse Blastocyst Development

After fertilization and three rounds of cell division, the embryo exists as a ball of cells known as the morula. Cells begin to compact against each other at the late 8-cell stage and each cell (also referred to as a blastomere) becomes polarized and possess a microvilli-dense apical pole and basolateral surface involved in cell-to-cell adhesions (12;13). Subsequent cell divisions are either symmetric in which two polar cells are formed, or asymmetric to produce one polar and one apolar cell. The 16-cell morula consists of two phenotypically different cell types of 10-12 outer polar cells surrounding 4-6 inner apolar cells (14). At this stage, cells are reversible in that inner apolar cells can become polarized and outer polarized cells can become apolar. The first differentiation event occurs at the late morula stage in which the outer polar cells form the trophectoderm, and the inner apolar cells form the inner cell mass (ICM) (15). The trophectoderm then seals off the inside of the blastocyst, facilitating formation and expansion of the blastocoele, resulting in the formation of the blastocyst. The second differentiation event occurs when cells of the ICM differentiate into epiblast and primitive endoderm. At this stage, by embryonic day (E) 3.5, the late blastocyst consists

of three distinct cells types - the epiblast, primitive endoderm, and the trophectoderm (16).

1.2.2 Mouse Blastocyst-derived Stem Cells

Mouse stem cells are an excellent model to study pluripotency and differentiation, as well as the mechanisms by which these processes are regulated and controlled (17). Since initially derived (18), there have been significant advances in the understanding of the signals that control pluripotency of stem cells, and the mechanisms by which these cells differentiate. Three different kinds of stem cells can be derived from the mouse blastocyst, or early mouse embryo: embryonic stem (ES) cells, trophoblast stem (TS) cells, and extraembryonic endoderm (XEN) cells (Fig. 1). These stem cells can give rise to three distinct tissue lineages: the epiblast, trophectoderm, and extraembryonic endoderm, respectively (19-21).

Figure 1. Cell types of the mouse blastocyst.

The mouse blastocyst is composed of three different stem cells: embryonic stem (ES) cells, trophoblast stem (TS) cells, and extraembryonic endoderm (XEN) cells. These stem cells can give rise to three distinct tissue lineages: the epiblast, trophectoderm, and extraembryonic endoderm. Based on *in vitro* cell culture, ES and TS cells assume a uniform morphology while XEN cells exhibit two morphologies of either round or stellate cells. The cell culture model is highly representative to their *in vivo* counterpart. The use of GFP-labeled ES, TS, and XEN cells injected into blastocysts to generate chimeras demonstrated that they contribute to the epiblast (embryo proper), trophoblast (placenta), or hypoblast (yolk sac) lineages, respectively.

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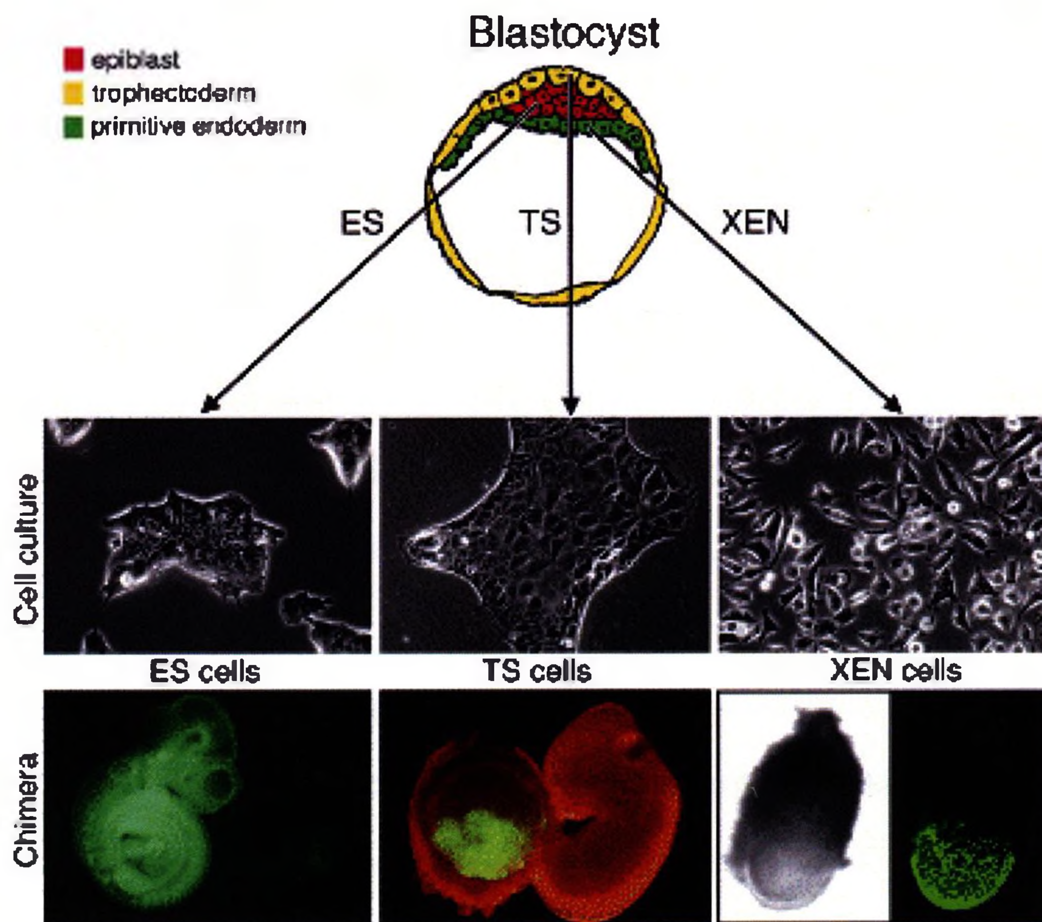
Figure 1.



1.2.2.1 Embryonic Stem (ES) Cells

Derived from the ICM of the mouse blastocyst, ES cells give rise to the epiblast (18;19), sometimes referred to as the primitive ectoderm (Fig. 1). Cells of the epiblast differentiate into all three embryonic germ layers and primordial germ cells (22). The developmental potential of ES cells has been demonstrated by use of chimeric embryos with labeled ES cells that had been introduced into a host blastocyst of a different strain of mouse. These ES cells are able to contribute to all parts of the embryo, including germ cells, but not extraembryonic and trophoblast lineages (22), thereby demonstrating the lineage-specific capability of ES cells. Mouse ES cells can be maintained *in vitro* in a proliferative and undifferentiated state in the presence of leukemia inhibitory factor (LIF) (23). LIF belongs to the interleukin-6 cytokine family and its binding to the LIFR-gp130 signaling complex leads to the activation of the JAK-STAT3 (Janus-associated tyrosine kinase, signal transducer and activator of transduction) pathway. Activation of STAT3 results in the induction of target genes that are essential for maintaining pluriopotency of mouse ES cells (24;25). LIF also stimulates the activation of the MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase) pathway, which antagonizes self-renewal and promotes differentiation of stem cells. The balance between LIF and ERK plays a pivotal role in regulating the self-renewal or differentiation of mouse ES cells (26). In the absence of LIF, ES cells undergo spontaneous differentiation. If maintained in the optimal culture conditions that inhibit differentiation, ES cells can proliferate indefinitely, maintain their pluripotency and be identified by the expression of cell surface markers such as stage-specific embryonic antigen (SSEA)-3 and -4 (27;28), and self-renewal markers OCT4 and SOX2 (29;30).

1.2.2.2 Extraembryonic Endoderm (XEN) Cells

A new class of stem cells derived from the blastocyst, extraembryonic endoderm (XEN) cells, gives rise to the primitive endoderm lineage, but not the epiblast or trophoblast lineages (Fig. 1). Studies in chimeras have demonstrated that XEN cells give rise to the yolk sac (20). The signaling required to maintain XEN cells in culture is not well understood, but the culture conditions to maintain these cells in an undifferentiated state are known. Unlike ES and TS cells which assume a uniform morphology, XEN cells can exhibit two morphologies of either round or stellate cells (20). XEN cells also have a different expression profile compared to ES cells and genes characteristic of XEN cells include *Gata4*, *Gata6*, *Sox7*, *Sox17* and *Disabled 2* (20).

1.2.2.3 Trophoblast Stem (TS) Cells

Trophoblast stem (TS) cells can be derived at the preimplantation stage from E3.5 blastocysts (Fig. 1), as well as from postimplantation embryos from the extraembryonic ectoderm at E6.5 and the chorionic ectoderm from E7.5 to E10 embryos (21;31). Most commonly, TS cells are derived at the preimplantation stage from the trophectoderm of the blastocyst, which is a single-cell layer of epithelial-like cells which give rise to the extraembryonic ectoderm and ectoplacental cone. For this, blastocysts must be obtained, isolated and cultured in order to harvest TS cells. Blastocysts are initially obtained from female mice on E3 by uterine flushing. Following initial seeding, and expansion of the blastocyst, TS cells can be harvested from the polar trophectoderm overlying the inner cell mass and expanded on their own.

Chimeric studies have demonstrated that TS cells can contribute to all trophectoderm derivatives (21). TS cells have different growth factor requirements than ES cells, and are maintained in an undifferentiated state in the presence of fibroblast growth factor-4 (FGF-4) (21) (discussed in detail later). TS cells also require mouse embryonic fibroblasts (EMFIs) as feeders or MEF-conditioned medium to be maintained in a proliferative state. Like ES cells, TS cells can also be maintained in an undifferentiated state for many generations, but upon the removal of either FGF-4 or EMIFs, TS cells differentiate into several trophoblast subtypes, including trophoblast giant cells (21). Markers for proliferating TS cells include CDX2 and EOMES (32;33), while placental lactogen-1 (PRL3D1) is used as a marker for trophoblast giant cells (21). *In vivo*, TS cells overlying the ICM (polar TE), receive FGF-4 from the embryo and are maintained in a proliferative state, while cells not in contact with the ICM (mural TE) stop dividing and differentiate into trophoblast giant cells (21).

1.2.3 Mouse Placental Development

Since the overall structures and molecular mechanisms in both mouse and human placental development are believed to be quite similar, the mouse is good model for the study of placental development (Fig. 2) (34). After formation of the mouse blastocyst at E3.5 (35), implantation of the embryo into the endometrium of the uterus occurs at E4.5 in which different trophoblast cell types begin to form from the TE. Cells of the mural TE (not in contact with the ICM) stop dividing and become polyploid by endoreduplication, forming trophoblast giant cells (34). In contrast, cells of the polar TE (in contact with the ICM) continue to divide and form extraembryonic ectoderm and

ectoplacental cone cell types by E6.0 (36). The extraembryonic ectoderm develops into cells of the chorion layer, an extraembryonic membrane consisting of mesothelium and ectoderm. The outer regions of the ectoplacental cone form more giant cells that surround the entire conceptus (36). The vascular portion of the placenta arises from the allantois, an extraembryonic mesoderm structure, which extends from the posterior end of the embryo at E8.0 and makes contact and fuses with the chorion in a process called chorioallantoic fusion at E8.5 (37). This creates the placental blood vessels and umbilical cord, which transfer nutrients and waste products between the growing fetus and the placenta.

Trophoblast cells in the chorion layer differentiate into the labyrinth, a layer consisting of maternal and fetal vessels involved the direct exchange of nutrients and gases (Fig. 2). This layer consists of syncytiotrophoblasts, a multinucleated cell that surrounds the endothelium of capillaries, and a mononuclear cell that lines maternal blood sinuses (34). The labyrinth is supported by the spongiotrophoblast layer, a compact structural layer of the placenta, which arises from the ectoplacental cone. Later in development, glycogen trophoblast cells differentiate within the spongiotrophoblast layer, and form the outer cell layer that invades the uterine wall (34).

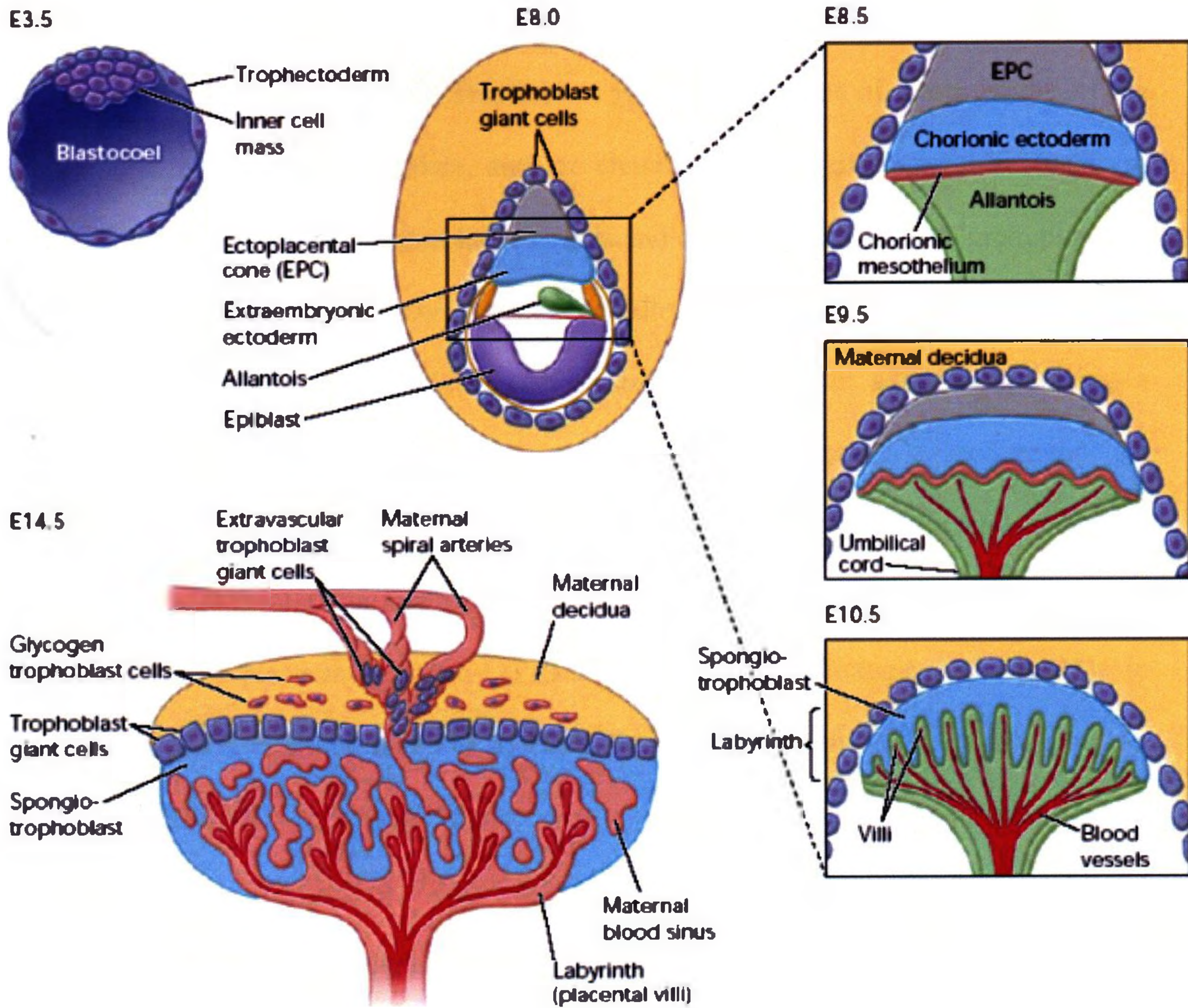
Since the placenta is vital during development, as it acts as the interface between the maternal and fetal compartments, defects at any stage during development of the placenta alter this 'communication' between mother and fetus. Understanding the signals essential in regulating development of the placenta at every stage can help elucidate the mechanisms by which these fetal disorders arise, either short or long term. The study of trophoblast stem cells can help decipher many of the answers to these questions.

Figure 2. Placental development in the mouse.

The trophoblast lineage forms at embryonic day (E) 3.5 with formation of the blastocyst. The vascular portion of the placenta arises at E8.0 from the allantois, which makes contact and fuses with the chorion in a process called chorioallantoic fusion at E8.5. By E14.5 the mature placenta consists of the labyrinth layer which is supported by the spongiotrophoblast layer, and the outer cell maternal decidua layer.

Image taken with permission from: Watson ED, Cross JC. (2005) Development of structures and transport functions in the mouse placenta. Physiology 20, 180-93.

Figure 2.



1.3 Trophoblast StemCell Differentiation

In vitro TS cells require the presence of FGF-4, heparin and embryonic fibroblast-conditioned medium (EFCM). Removal of these factors results in the differentiation into trophoblast giant cells (TGCs). In mice, four differentiated trophoblast cell types can be identified: (1) trophoblast giant cells, (2) spongiotrophoblast, (3) glycogen trophoblast, and (4) syncytiotrophoblast (38). Trophoblast giant cells are one of the first cells of the trophoblast lineage to differentiate, and are characterized by giant nuclei arising from continued DNA synthesis without karyokinesis and cytokinesis, called endoreduplication (39). Syncytiotrophoblast cells form the middle layer of the placenta between the outermost trophoblast giant cells and innermost labyrinth. In addition to having a structural role, syncytiotrophoblast cells are also a source of precursors of trophoblast giant cells and glycogen trophoblast cells (40). Trophoblast giant cells are located at the maternal-placental interface and express members of the placental prolactin (PRL) gene family, including placental lactogen-I (PRL3D1) and placental lactogen II (PRL3B1). *In vivo,* the PRL3D1 expression begins shortly after implantation and ends by mid-gestation (41), while PRL3B1 expression begins shortly after PRL3D1 and continues throughout gestation (42). The PRL3D1 expression serves as an early endocrine marker of trophoblast giant cell differentiation, while PRL3B1 serves as an intermediate-to-late endocrine marker of trophoblast giant cell differentiation. PRL3D1 and PRL3B1 function in the ovary, acting to maintain the corpus luteum of pregnancy and stimulate luteal progesterone production, as well as promote mammary gland development and lactation (43;44). Since development of the trophoblast cell lineage is essential for the establishment of an effective exchange and pathway of communication between maternal

and fetal tissues, understanding the signals and mechanisms that control differentiation of TS cells is crucial.

1.4. Receptor Tyrosine Kinase Signaling in TS Cells

1.4.1. Fibroblast Growth Factor (FGF) Signaling in TS Cells

The study of TS cells *in vitro* can help provide insight into the mechanisms of abnormal placental development. However, very little is known currently regarding the factors required for normal TS cell growth. The main signaling pathway associated with the self-renewal and maintenance of TS cells has been shown to be the fibroblast growth factor (FGF) signaling pathway. In this pathway, FGF-4, a ligand expressed in the ICM and early epiblast, is released from the ICM of the blastocyst and binds to its receptor, FGFR2, which is highly expressed in the polar trophectoderm. This interaction results in the self-renewal and proliferation of TS cells (21).

1.4.1.1. Fibroblast Growth Factor System

The fibroblast growth factor system is critical in early embryonic development and consists of 22 growth factors (FGF-1 to FGF-23) and four high-affinity ligand-dependent receptors (FGFR1 to FGFR4) in humans and mice; FGF-19 is the human ortholog of mouse FGF-15 (45). Two *Fgf* genes and one *Fgfr* gene are found in *C. elegans*, compared to that of humans and mice, indicating that *Fgf* and *Fgfr* gene families have greatly expanded during evolution from primitive metazoan to vertebrates (45;46).

1.4.1.2. Fibroblast Growth Factors

FGFs are widely expressed in developing and adult tissue. During embryonic development, FGFs are involved in a variety of biological processes including cell survival, growth, migration, differentiation, and apoptosis, while in the adult organism, FGFs are homeostatic factors involved in tissue repair and response to injury (47). FGFs range in molecular weight from 17 to 34 kDa and share 13-71% amino acid similarity. All FGFs contain a core region consisting of 28 highly conserved and six identical amino acid residues, which are important in the binding of FGFRs and heparin. Heparin is critical as it is necessary for stabilization and prevention of degradation, as well as aiding in the binding of ligand to its receptor. In addition, heparin has also been shown to increase the affinity and half-life of the FGF/FGFR complex (48). Based on structure, the majority of FGFs (FGF3-8, 10, 15, 17-19, and 21-23) contain an N-terminal signal sequence and are easily secreted from cells, while some FGFs (FGF-9, -16 and -20) lack a cleavable N-terminal signal sequence, yet are still secreted (49-51). On the other hand, FGF-1 and FGF-2 lack a signal sequence and are not secreted from cells but are in fact released from damaged cells (52), while FGF-11 to -14 also lack signal sequences, and remain in the intracellular environment and function in a receptor-independent manner (53). Each individual FGF has a unique site of expression, but the developmental expression pattern of FGFs are either exclusively during embryonic development (FGF-3, -4, -8, -15, -17 and -19), or are expressed in both embryonic and adult tissues (FGF-1, -2, -5to -7, -9 to -14, -16, -18, and -20 to -23) (45).

1.4.1.3. Fibroblast Growth Factor Receptors (FGFR)

The FGFRs are single-pass transmembrane proteins that consist of an intracellular tyrosine kinase domain and an extracellular ligand-binding domain. The extracellular region consists of three immunoglobulin-like (Ig) domains that are important in regulating FGF ligand affinity and specificity (54). Alternative splicing of the Ig domain results in different FGFR isoforms, which have different FGF ligand binding specificities; this splicing is tissue-specific. Alternative splicing of Ig domain III drastically changes ligand-receptor binding specificity, while in contrast, alternative splicing of Ig domain I has no effect on ligand specificity (55). Located between the Ig domains is an acidic box domain, a heparin-binding domain and cell adhesion homology domain, which are involved in the interaction with heparin sulfate proteoglycans and cell adhesion molecules (54). As mentioned, heparin is extremely vital and is required for FGF to effectively activate FGFR, as genetic studies have demonstrated that mutations in enzymes that synthesize heparin sulfate affect FGF signaling pathways during development (48). In addition to the tyrosine kinase domain, the intracellular domain consists of a juxtamembrane domain that contains protein binding and phosphorylation sites, including protein kinase C and FRS2 sites (54).

1.4.1.4. FGFR Signal Transduction in TS Cells

FGFRs exist as inactive monomers, and upon ligand binding, in which two FGF molecules connected by a heparin sulfate proteoglycan bind to the extracellular Ig domains, receptors dimerize leading to the trans-autophosphorylation of multiple tyrosine residues (56). These phosphorylated tyrosines serve as binding sites for SRC homology 2

(SH2) and phosphotyrosine binding domains, resulting in the assembly and recruitment of signaling complexes (57). FGFR signal transduction can proceed *via* three different pathways – Ras/MAPK pathway, PLCγ/Ca^{2+} pathway, and the PI3 kinase/Akt pathway. The main signaling pathway activated by FGFR is the Ras/MAPK pathway, which regulates the activity of downstream kinases and transcription factors(54).

In TS cells, FGF-4 binds and phosphorylates FGFR2 causing the formation of a Grb2/FGS2α/SHP2 complex (58;59). SHP2 is a ubiquitously expressed, non-receptor protein-tyrosine phosphatase containing two SH2 domains. SHP2 functions to dephosphorylate and activate SFK (Src family kinases) on specific tyrosine residues, thereby activating the Ras/Erk signaling pathway, leading to the self-renewal of TS cells (60;61). To date, the exact cellular and molecular mechanisms by which FGF-4 controls its effects on TS cells has not yet been thoroughly characterized. Over the past few years, more information regarding the major players involved in controlling the fate of TS cells is being revealed. In mouse and human TS cells, SHP2 is known to play a critical role in promoting stem cell survival and ensuring trophoblast survival as mouse embryos with a null mutation for *Shp2* die peri-implantation and fail to yield TS cells. To identify the exact effects of a loss of SHP2 in TS cells, the use of TS cells containing floxed alleles for SHP2 demonstrated that SHP2 functions to inhibit apoptosis of TS cells by activating the Erk pathway and destabilizing the pro-apoptotic protein Bim through phosphorylation (59); thereby demonstrating the importance of SHP2 in promoting normal TS cell self-renewal. It is not clear, however, if FGF-4 signaling through the Erk pathway is the only biological affect on TS cells, since the removal of FGF-4 promotes differentiation of TS cells as opposed to apoptosis (21). This suggests that other pathways, independent of

SHP2, are responsible for regulating the fate of TS cells and that SHP2 may be a critical factor controlling the fate of differentiation or apoptosis in TS cells. Furthermore, since FGF-4 alone can not maintain TS cells, as additional growth factors are required; it is possible that the effects of a *Shp2* mutation are a result of the activation of other signaling pathways (62). Many hypotheses have been developed, but the fact remains that the signaling pathways and factors that determine the fate of TS cells, whether it be self-renewal, differentiation, or apoptosis, have yet to be completely elucidated.

1.4.1.5. FGFs and Development

Gene targeting experiments in mice have demonstrated the importance of FGFs in several developmental processes. Loss of the *Fgf3* gene results in defects in the development of the inner ear and tail of mice, and are often unable to survive to adulthood (63). Loss of the *Fgf8* and *Fgf10* genes impair limb growth and patterning (64;65), while loss of either the *Fgf9* or *Fgf10* gene impairs lung development (65;66). The most severe effect is the loss of the *Fgf4* gene, in which *Fgf4* null embryos die shortly after implantation, demonstrating the importance of *Fgf4* in early mouse development (67). FGF-4 is the first FGF to be expressed during mammalian development, as its expression is observed as early as the four-cell stage, and is restricted to cells of the ICM by the blastocyst stage (68;69). FGFR2 is required for early post-implantation development as null mice for the *Fgfr2* gene resulted in peri-implantation lethal phenotypes (70), demonstrating the importance of FGF-4 and its receptor, FGFR2, in the maintenance of early mouse development.

1.4.2. Insulin-like Growth Factors

Little is known about how growth factors control the fate of TS cells via proliferation, differentiation or apoptosis. Currently, the role of insulin-like growth factors (IGFs) in the maintenance and differentiation of TS cells is unknown. The IGF system is comprised of a complex system of polypeptides (IGF-I, IGF-II), cell surface receptors (IGF-IR, IGF-IIR, insulin receptor), and circulating binding proteins (IGFBP-1 to -6) (Fig. 3). The IGF system plays a critical role in normal growth physiology, particularly in early fetal and placental development (71-75).

1.4.2.1. Insulin-like Growth Factor (IGF) -I and -II

IGF-I and IGF-II are 70 and 67 amino acid single-chain polypeptides which have a 62% homology in their amino acid sequence. The *Igf1* gene is located on chromosome 12, while the *Igf2* gene is located on the short arm of chromosome 12 (76). Both IGF-I and IGF-II share similar structural similarities to that of proinsulin (23) yet, despite their structural and high sequence similarity, IGF-I and IGF-II have distinct physiological roles in development and postnatal growth. Both are synthesized by almost all of the developing cells of the fetus, and are secreted into the fetal plasma and tissue fluids, where they act through endocrine as well and paracrine and autocrine signaling pathways (77).

In mice, both IGF-I (*Igf1*) and IGF-II (*Igf2*) are expressed early in fetal tissues, with *Igf2* expression occurring earlier at the 2-cell stage, whereas *Igf1* expression first occurs at E12.5 (78). IGF-I expression is more widespread and abundant in fetal tissues than that of IGF-I during development in both mice and humans (79). In the circulation,

plasma concentrations of IGF-II are 3- to 10-fold higher than that of IGF-I later in gestation (80). During prenatal development, the expression of both IGF-I and IGF-II are quite similar in humans and mice; however, their expression patterns begin to differ postnatally. After birth, expression of IGF-II in mice decreases dramatically with the exception of the brain, while in humans, although IGF-II is highly expressed *in utero*, its expression increases after birth and remains constant and higher than IGF-I expression throughout life (81;82). Expression of IGF-I in humans increases at a steady rate until adulthood, with a slight increase at puberty (82;83). This onset of IGF-I production in both mice and humans at birth is stimulated by the production of growth hormone (GH). In infants and children, GH is the main regulator of growth through the systemic production of IGF-I, however, during development, GH plays little or no role in growth of the fetus, as IGFs are the major regulators of growth (84). The primary regulator of IGF-II is unclear, as GH has no regulatory effect on its expression (85).

1.4.2.2. Regulation of IGFs

Expression of mouse *Igf2* is controlled by four promoters (P0-P3), which can be activated in a tissue-specific and development-dependent manner. Promoter P0 is a placenta-specific promoter in which *Igf2* transcripts are expressed only in the labyrinthine layer of mouse placenta. Transcripts P1-P3 are located throughout the developing mouse embryo and placenta (86;87). Expression of human IGF-II is also controlled in a tissue-specific and development-dependent manner by four promoters (P1-P4), but does not contain the placenta-specific murine P0 promoter (88-90). These four promoters produce five sizes of mRNAs, all of which encode for the same mature IGF-II protein.

Transcripts of P2-P4 are important in development and are found in the developing embryo and extra-embryonic structures, with P3 being the most active, while P1 is inactive. However, adult expression is limited to the liver-specific promoter P1, while activities of P2-P4 decrease or are lost (91;92).

Expression of *Igf2* is regulated also by genomic imprinting, a mechanism by which particular genes are expressed from only one parental allele (93). Imprinting is regulated by epigenetics in which DNA methylation, histone acetylation and use of specific promoter sites are mechanisms used to control allele-specific transcription (94-96). To date, approximately 50 human and 100 mouse imprinted genes have been identified (97;98), the majority of which have been shown to be critical in placental and/or fetal development. Imprinted genes are clustered together in chromosome domains. One domain of interest, human domain 11p15 and the distal portion of mouse chromosome 7, houses a large cluster of imprinted genes, including *Igf2* (99). The *Igf2* gene is a paternally imprinted gene, in which the paternal *Igf2* allele is active, while the maternal *Igf2* allele is silenced. Mouse *Igf2* contains four differentially methylated regions (DMRs), while human *IGF2* lacks DMR1. There have been many hypotheses for the role of imprinting, one being the genetic conflict hypothesis which states that paternally expressed genes acting in the placenta extract resources from the mother to enhance fetal growth, while maternally expressed genes act to restrict fetal growth and conserve resources to enhance the reproductive fitness of the mother.

As noted, growth hormone (GH) is the main regulator of IGF-I gene expression, while GH has no regulatory effect on IGF-II gene expression (100). In this system, GH is secreted from the pituitary and acts on the liver and other organs to produce local and

systemic IGF-I. Diet and nutrition have also been shown to have an effect on the production of circulating IGF-I levels. Additional hormones including estrogens, thyrotropin, and lutenizing hormone have a smaller role in the expression of IGFs, as well as other growth factors including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) (85).

1.4.2.3. IGF Signaling Effectors

The growth and metabolic effects of IGFs are mediated by binding to their receptors, IGF-I and IGF-II receptors (Fig. 3). Both IGF-IR and IGF-IIR are glycoproteins located on the cell membrane (101). Expression of both receptors has been shown to be ubiquitous and does not undergo significant alterations during development. IGFs act on almost every cell type through these receptors, with the exception of hepatocytes and mature B cells (102). Despite similar expression, these two receptors differ completely in structure and function.

a) IGF Type I Receptor

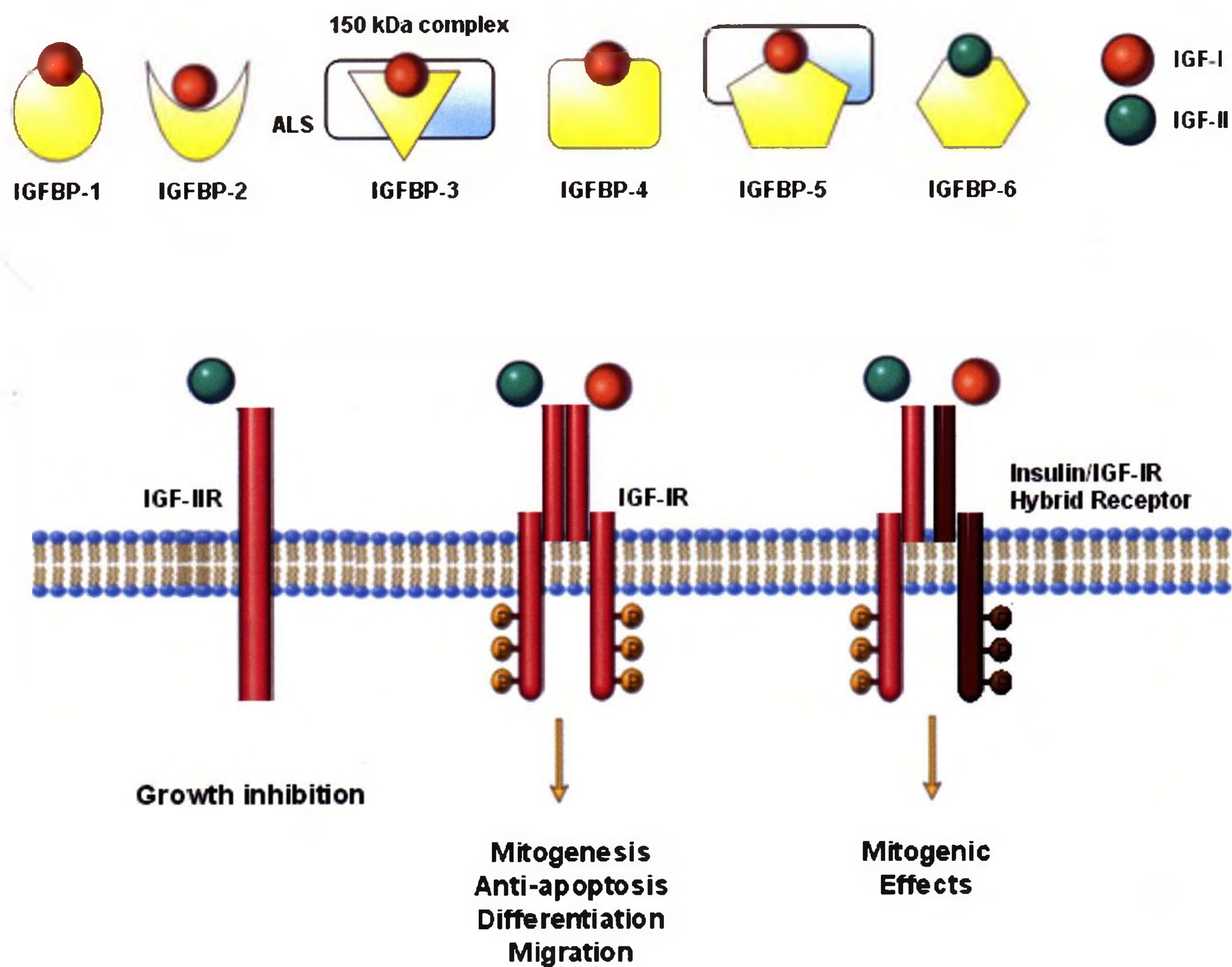
The IGF-IR is a tetramer composed of two identical cytoplasmic α-subunits involved in ligand binding, and two identical β-subunits which possess tyrosine kinase activity (103). Most of the mitogenic effects of IGFs are mediated by binding to the IGF-IR. IGF-I, IGF-II, and insulin all bind to IGF-IR with the highest affinity for IGF-I, followed by IGF-II (2- to 15-fold lower affinity) and insulin (100- to 1000-fold lower affinity) (Fig. 3) (100). Ligand binding to the extracellular α-subunit initiates a conformational change resulting in autophosphorylation of tyrosine residues (Tyr1131,

1135 and 1136) on the intracellular β-subunits, activating the intrinsic kinase activity of IGF-IR. Other tyrosine phosphorylated sites allow for the recruitment of specific adaptor molecules *including insulin receptor substrate (IRS)-1, Shc and 14-3-3 proteins* (104;105). Binding of these molecules leads to the activation of diverse signaling pathways (106-108). Shc is recruited to the phosphorylated tyrosine residue (Tyr950) and induces differentiation signals through the MAP/ERK kinase (MEK)-extracellular-regulated kinase (ERK) pathway (109). IRS-1 also binds to the same phosphorylated site (Try950) and results in the proliferation of cells through activation of the phosphatidyl-inositol-3-kinase (PI3K) pathway. It is thought that the decision to undergo either proliferation or differentiation depends on competition between these two adaptor proteins binding to this binding site (110;111), but further investigation into this hypothesis is still required. Alternate routes for activation of the MEK-ERK and PI3K pathway exist, demonstrating the complexity of the IGF-1R pathway, and that alternate mechanisms of controlling proliferation and differentiation likely exist (112-114). The IGF-IR also functions in the protection of apoptosis through the PI3K pathway, in which PI3K is activated by binding to phosphorylated IRS-1 causing a series of downstream effects leading to recruitment of Akt (104). This activated Akt inhibits the phosphorylation of pro-apoptotic factors (Bad, Caspase 9) and increases the expression of anti-apoptotic proteins (Bcl-2, Bcl-x) (115).

Figure 3. Schematic representation of the IGF system.

The IGF system consists of the peptides (IGF-I, IGF-II), receptors (IGF-IR, IGF-IIR, and IGF-1R/IR hybrids) and six high affinity binding proteins (IGFBPs -1 to -6). The IGF-IR is a tetramer composed of two identical cytoplasmic α-subunits involved in ligand binding, and two identical β-subunits which possess tyrosine kinase activity. Binding of IGF-I or IGF-II to the extracellular α-subunit initiates a conformational change resulting in autophosphorylation of tyrosine residues on the intracellular β-subunits, activating the intrinsic kinase activity of IGF-IR. The IGF-IIR is a monomeric transmembrane receptor which does not have intrinsic signaling transduction capability and binds to IGF-II with the highest affinity, resulting in its internalization and degradation. Hybrid receptors of the insulin receptor and IGF-IR can exist, which bind both IGF-I and IGF-II and are thought to function primarily as an IGF-1R, though its biological importance is not yet known.

Figure 3.



b) IGF Type II Receptor

The IGF-IIR is monomeric transmembrane receptor composed of three ligand-binding domains in the extracellular domain. One domain is involved in IGF-II binding, while the remaining two bind to proteins containing mannose-6-phosphate (M6P) including renin, proliferin and the latent form of TGF-β (100). Since IGF-IIR can bind both IGF-II and M6P-containing molecules, it is also called the IGF-II/M6P receptor. Unlike IGF-IR, IGF-IIR does not contain intrinsic signaling transduction capability and binds to IGF-II with high affinity, and a 500-fold lower affinity for IGF-I, and does not bind insulin (Fig. 3) (116-118). The binding of IGF-II results in the internalization and degradation of IGF-II, thereby providing a mechanism to control the levels of circulating IGF-II sequestering it from activating receptors (119). A unique feature of IGF-IIR is that it can exist as a circulating form. Upon proteolytic cleavage, the extracellular domain dissociates from the cell membrane as a soluble fragment that acts as a scavenger to bind and degrade IGF-II (120-122). In addition to its primary role in regulating levels of circulating IGF-II, the IGF-IIR has also been suggested to be involved in stimulating an intracellular signaling pathway using a G_i-coupled receptor (123;124), as well as play roles as a tumor suppressor (125). Since the IGF-IIR is not expressed until E13.5 in mice (126), it is believed that IGF-II acts through either the IGF-IR or the IR. Knockout of both the *Ir* and *Igf1r* in mice result in a growth retardation greater than mice lacking either receptor alone. Interestingly, this phenotype of mice lacking both *Ir* and *Igf1r* is similar to that of the phenotype of mice lacking *Igf1* or I*gf2*, suggesting that these two receptors account for the growth promoted by IGF-I and IGF-II (127;128).

c) Insulin Receptor

Structurally, the IGF-IR shares a high degree of similarity to the insulin receptor (IR), with their kinase domains possessing 84% homology to each other (129). Just like IGF-IR, the IR possesses intrinsic kinase activity and its activation leads to the activation of PI3 kinase, AKT and MAP-kinase pathways. Due to their significant similarity, there is cross-talk between the two systems (130). A hybrid receptor of the IR and IGF-IR exists, which has a higher affinity of binding for IGF-I than insulin (Fig. 3). The amount of hybrid receptor varies substantially from tissue to tissue, and though it is thought to function primarily as an IGF-1R, its biological importance is not yet known (100;101). There are two isoforms of the IR generated by alternative splicing, IR-A and IR-B, both of which are expressed in a developmental-specific manner, with IR-A being highly expressed in fetal tissues and IR-B in adult tissues (131). Besides binding to the IGF-IR and IGF-IIR, IGF-II also binds to IR-A, with high affinity, which has been shown to promote cell proliferation and protection from apoptosis (132).

1.4.2.4. Insulin-like Growth Factor Binding Proteins (IGFBPs)

IGF actions are controlled by six binding proteins, IGFBP-1 to -6, which function to transport IGFs, protect IGFs from degradation, and regulate the bioavailability of IGFs to interact with IGF-IR. IGFBP-1 to -6 are ~30 kDa proteins that share structural homology, consisting of IGF binding sites on their cysteine-rich N- and C-terminal domains, which are connected by a flexible linker (L-) region (133;134). The affinity of IGFBPs for IGFs is higher or similar to that of IGF-IR under normal physiological conditions; therefore binding of IGFBPs to IGFs blocks the interaction between IGFs and

IGF-IR thereby suppressing IGF action. Affinity of IGFBPs for IGFs can be altered by phosphorylation or glycosylation (135).

In serum, most IGFs can exist as a trimeric complex composed of IGFBP-3 and a liver-derived glycoprotein, acid labile sub-unit (ALS) which is a member of the leucine-rich repeat family of proteins, and functions to modulate both IGFs and IGFBPs. Few IGFs are bound to IGFBPs alone as a binary complex, and less than 1% exists as free circulating IGFs. An even smaller amount can exist as a ternary complex consisting of both IGF-I and IGF-II bound to IGFBP-5 (136). Binding of IGFs to IGFBPs increases the half-life from 10 minutes (free IGF) to 12 hours (ternary complex), thereby increasing its half-life and delivery of IGF to tissues (137). Once delivered to tissues, IGF actions can be both inhibited and potentiated by IGFBPs (135).

1.4.2.5. IGFs and Development

Gene deletion studies have demonstrated the importance of both IGFs and their receptors in fetal and placental growth. Of particular importance is IGF-II, which has been shown to be important in regulating placental growth, as loss of either *Igf2* or *Igf2r* has an affect on the placental phenotype. Both *Igf1* and *Igf2* null mice have a dramatic reduction in newborn pup sizes by 40% compared to wild-type littermates, whereas only *Igf2* null mice exhibited a reduction in placenta size (51% of wild type at E15, 80% of WT at E18); *Igf1* null mice exhibited normal placental sizes (71;138). Postnatally, *Igf2* null mice are viable and display normal growth and development, compared to *Igf1* null mice in which some dwarfs die shortly after birth (72). Null mutants for the *Igf1r* gene have a more severe growth deficiency (55% reduction compared to wild-type), and die

immediately after birth due to respiratory difficulties (73), while *Igf2r* knockout mice are 25-35% bigger than wild type mice and lethal in late embryonic stages. In addition to the larger fetal size, *Igf2r* deficient mice also have an increase in placental size (74). These effects are possibly due to the increase of extracellular IGF-II which cannot be degraded by the IGF-2R, resulting in an over-stimulation of growth.

The double knockout of *Igf1r* and *Igf2* leads to the smallest fetuses with a 70% reduction in size (138), compared to *Igf1/Igf1r* double knockout, which do not result in a further decrease in birth weight compared to *Igf1r* knockouts alone (73), demonstrating that IGF-I functions solely via the IGF-1R, and that the additional loss of *Igf2* suggests that IGF-II likely functions via another receptor during fetal growth (75). This other receptor is likely thought to be a spliced variant of the IR (IR-A), which as previously mentioned, binds to IGF-II with high affinity and is highly expressed in fetal tissues (131). Since mice lacking *Igf2r* are rescued from perinatal lethality when they carry null mutations for *Igf1r* (75), this demonstrates that IGF-II signaling through the IGF-IR is responsible for these abnormalities, but indicates that the IR is sufficient to compensate for lack of the IGF-IR. Since mice lacking all three receptors (*Ir, Igf1r, Igf2r*) are nonviable (139), these data provide evidence that the growth promoting actions of IGF-II are acting in part through the IR, to maintain growth of *Igf1r/Igf2r* knockout mice. Based on all this data, interactions among ligands and receptors in the IGF family demonstrate that insulin and IGF-I are acting through their own receptors (IR and IGF-IR) with high affinity. On the other hand, in addition to IGF-II binding to IGF-IIR to function in clearance of IGF-II, IGF-II also has the ability to also bind to the IR and IGF-IR with

comparably high affinities to induce a variety of cellular responses; this IR is likely the spliced variant IR-A.

1.4.2.6. Other Growth Factors

The process of early fetal and placental development requires the presence of other growth factors and signaling pathways besides the IGF family. It is clear that the FGF family is important in mediating the proliferation of TS cells, and this occurs either through interaction with other receptors or different intracellular signaling pathways (34). Although the IGF family has been implicated in early fetal and placental development, this process requires the presence of other growth factors and signaling pathways including the WNT signaling pathway, epidermal growth factor (EGF), bone morphogenetic protein (BMP), hepatocyte growth factor (HGF), activin and transforming growth factor (TGF)-β (140). Currently, it has been shown that the presence of activin and (TGF)-β can replace conditioned medium. Both TGF-β and activin are members of the TGF-β superfamily, which are important in regulating many developmental processes. An important role of TGF- β is in down-regulating the mRNA expression of c-*myc*, a short-lived transcription factor involved in cell-cycle progression. Work by Erlebacher A, *et al.* (141) demonstrated that FGF signaling in TS cells inhibits the ability of TGF- β to repress c-*myc* expression, thereby preventing cell-cycle arrest.

1.5 Transcriptional Regulation of Lineage Segregation

The most important cell types in the placenta are those of the trophoblast lineage, which give rise to the many cell types in the placenta providing the main structural and functional components to bring the fetal and maternal blood systems into close contact. Since the TE forms the embryonic portion of the placenta, the differentiation of TE can be regarded as a hallmark event in mammalian early development (34). Before the differentiation of ICM and TE, cells of the early blastocyst are reversible whereby inner apolar cells can become polarized, and outer polarized cells can become apolar. Once the two lineages of ICM and TE are organized, it is thought that this lineage segregation is reinforced by transcription factors (15). Understanding the signals involved in controlling the irreversibility of cellular polarization and the development of the two distinct cell lineages of TE and ICM are keys in early mouse development.

The Pituitary Octamer and Unc (POU) homeodomain transcription factor OCT4, also known as Pou5f1, plays a pivotal role in reinforcing lineage segregation of the TE and ICM. Initially, *Oct4* is expressed in all blastomeres of a developing embryo and is restricted to cells of the ICM upon formation of the blastocyst. Its expression is maintained in human and mouse stem cells and is down-regulated when cells differentiate and lose pluripotency (142). In post-implantation embryos, OCT4 is restricted to primordial germ cells (143). Mouse embryos with a homozygous *Oct4* mutation fail to form ICM and cells default to the TE lineage (144), while its repression in ES cell results in the differentiation towards the trophoblast lineage (145). Over-expression of *Oct4* induces the differentiation into extraembryonic endoderm (146), demonstrating that the dosage of OCT4 has an effect on lineage segregation. All these data implicate OCT4 as a

repressor of the TE lineage, as it has been shown to also repress the transcription of several trophoblast-specific genes (147;148). Although OCT4 is vital in lineage segregation and its down-regulation is required for TE formation, it is unlikely involved in initiating lineage segregation, as *Oct4* expression has been show to persist in the TE of developing wild type mouse blastocysts upon lineage segregation before being down-regulated (142). In other species however, such as human and bovine embryos, *Oct4* is expressed at low levels in the TE of developing embryos days after blastocyst formation, demonstrating that though OCT4 is not responsible for initiating lineage segregation, its dosage is important (149;150).

OCT4 regulates expression of several target genes through the binding of octamer elements located in their regulatory region (151-153). One of these genes is the (High mobility group) HMG domain-containing transcription factor, SOX2 (SRY-related HMG box 2) (154). SOX2 plays a similar role to OCT4 in that it is essential in maintaining the self-renewal of ES cells as well as preventing trophoblast specification; however its expression is more complicated than that of OCT4 as its expression remains active in the proliferating trophoblast (29). *Sox2*-null embryos die and fail to implant, however blastocyst and TE formation occurs (29), while a knockdown of *Sox2* in mouse ES cells results in the differentiation of ES cells into multiple lineages, including cells of the trophoblast lineage (155).

Besides being regulated by OCT4, SOX2 also acts synergistically with OCT4 itself to regulate the expression of several OCT4 target genes involved in lineage segregation and maintaining pluripotency. One of these targets, the FGF-4 gene, encodes a growth factor released from the ICM which functions in the maintenance and

proliferation of TS cells, contains both SOX2 binding sox elements and OCT4 binding octamer elements in close proximity to each other within their regulatory regions (156). Interestingly, both OCT4 and SOX2 contain these OCT-SOX enhancers (157;158), and act cooperatively to activate several pluripotent-cell specific genes, including *Oct4* and *Sox2* themselves, through a positive feedback loop mechanism (159;160).

Until recently, the consequences of a complete loss of *Sox2* function has not been determined, making it difficult to understand the precise function and targets of SOX2. A recent study generated *Sox2*-null ES cells and identified that the essential function of SOX2 is to stabilize the pluripotent state of stem cells by maintaining the precise levels of OCT4 expression through the regulation of other transcription factors (161), since a slight increase or decrease in OCT4 expression results in differentiation. Therefore, though OCT4 is in the hierarchy of maintaining pluripotency of stem cells, its regulation is thought to be controlled downstream by SOX2, a transcription factor it collaborates with to reinforce lineage segregation.

Formation of the TE is not the result of a default pathway initiated by the down-regulation of OCT4, but requires expression of the caudal-related homeobox protein CDX2 (162). *Cdx2* is expressed in the outer polar blastomeres of the developing embryo, and its expression is reciprocally expressed to that of *Oct4* in the blastocyst, as it is expressed in the TE, not the ICM (163). In the absence of *Cdx2*, embryos cannot form the TE and die prior to implantation but *Oct4* is expressed in the TE region of the blastocyst (163). Since *Oct4* is expressed in the TE region of the blastocyst of *Cdx2-/-* embryos, this data suggest that CDX2 functions in the formation of TE by down-regulating *Oct4* expression. In addition, forced expression of *Cdx2* in ES cells promotes

differentiation into trophoblast-like cells, demonstrating the importance of CDX2 in formation of the TE (164). A recent study investigated the interaction between OCT4 and CDX2, and suggested that CDX2 upregulation is the primary trigger of lineage segregation and is initially unregulated in the inner cells to block OCT4 in TE commitment, and once this occurs, its expression in the inner cells is ceased (165).

RATIONALE

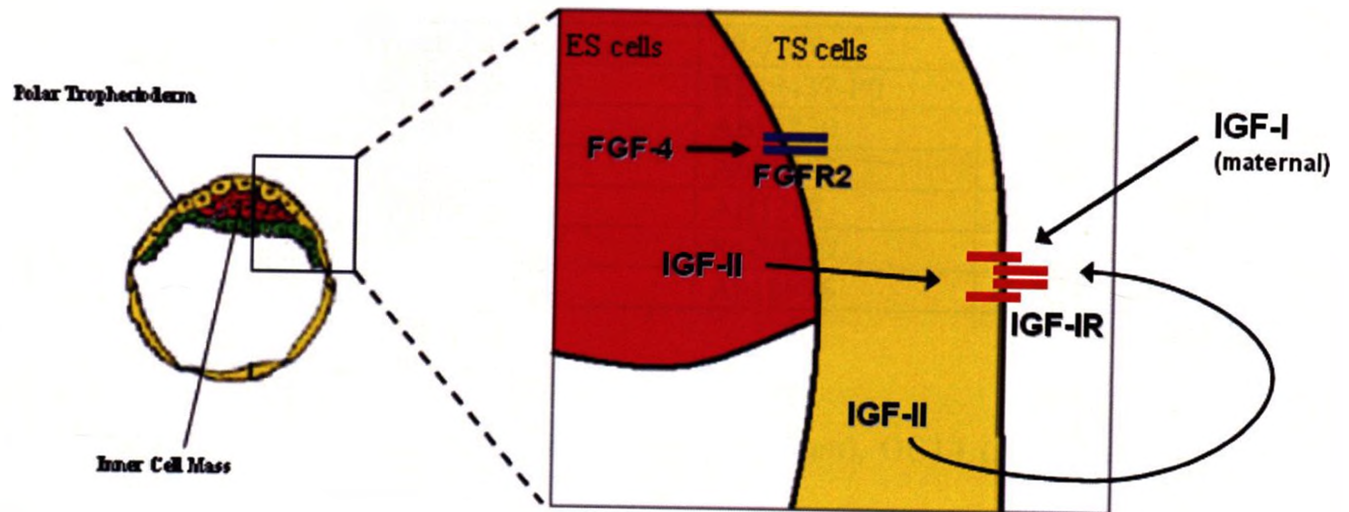
Development of the placenta requires the appropriate balance between self-renewal, proliferation, and differentiation. Understanding the mechanisms by which these different processes are controlled and regulated is of particular importance, as defects/irregularities in any of these processes can result in placental deficiencies, leading to fetal abnormalities. TS cells are derived from the trophectoderm of the blastocyst and contribute exclusively to extraembryonic structures. Mouse TS cells can be maintained *in vitro* in an undifferentiated state in the presence of fibroblast growth factor-4 (FGF-4) and fetal bovine serum (FBS). TS cells require additional, yet undetermined, growth factors and cytokines present in FBS for the proper maintenance of TS cells. Insulin-like growth factors (IGFs) are mitogenic and differentiative peptides that are important in regulating fetal and placental growth, but their role in the maintenance and differentiation of TS cells are unknown. Since FGF-4 alone is not sufficient in the maintenance of TS cells, and the fact that IGFs are highly expressed and important in early placental development, it is highly suggestive that IGFs contribute to the maintenance of TS cells. The *goal* of this project was to identify the individual effects of IGF-I and IGF-II on the fate of mouse TS cells (Fig. 4). We *hypothesized* that IGF-I stimulates differentiation while IGF-II promotes self-renewal of mouse TS cells under conditions of growth restriction. Based on this hypothesis the *objectives* of this project were to assess the effects of IGF-I and IGF-II on the self-renewal and differentiation of TS cells, as well as programmed cell death events.

Figure 4. Schematic representation of rationale.

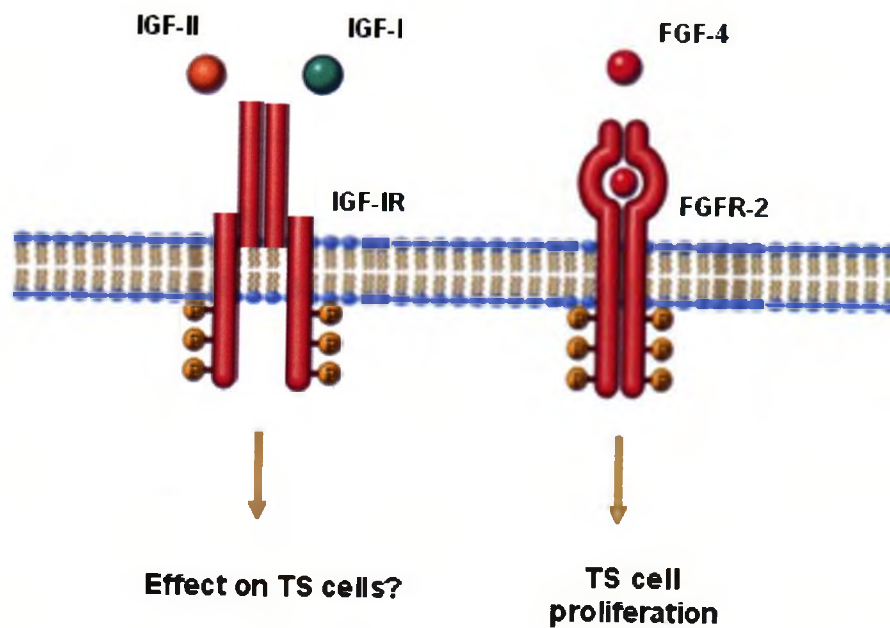
(A) Due to the expression pattern of IGF-II and its role in development of the placenta, it is thought that IGF-II has an important role in the maintenance of TS cells. IGF-II could be acting through either a paracrine or autocrine mechanism, or even as a result of endocrine signaling through maternal IGF-I. The capacity for TS cells to continually self-renew and proliferate depends on the microenvironment, whether that be from neighbouring cells of the epiblast or from the maternal environment. **(B)** *In vitro*, FGF-4 alone is not sufficient in the maintenance of TS cells, as additional growth factors present in FBS are required. Since IGF-I and IGF-II are highly expressed and important in early fetal and placental development and have a high affinity for IGF-IR, it is hypothesized that IGFs contribute to the maintenance of TS cells, either through self-renewal or differentiation.

Figure 4.

A



B



CHAPTER 2: MATERIALS AND METHODS

2.1. REAGENTS

2.1.1. Antibodies

Name	Origin	Company	Cat. #	Specificity*
CDX2	Mouse	Zymed Laboratories	39-7800	H, M, R
OCT3/4	Rabbit	Lab Vision	RB-9437-P0	H
SOX2	Rabbit	Abcam	Ab15830	H, M
CDX2	Mouse	Abcam	Ab15258	H. M
PL-1	Rabbit	Chemicon	AB1288	M, R
GAPDH	Goat	Santa Cruz	Sc-20357	H, M, R
OCT4	Rabbit	Abcam	Ab18976	H, M, R

* H=human, M= mouse, R= rat

Primary antibodies for immunocytochemistry: CDX2 (Abcam), OCT4 (Abcam), and SOX2 (Abcam)

Primary antibodies for western-blotting: CDX2 (Zymed), OCT3/4 (Lab Vision), SOX2 (Abcam), PL-1 (Chemicon), and GAPDH (Santa Cruz)

Secondary antibodies for immunocytochemistry: goat anti-mouse IgG TRITC, goat anti-rabbit IgG FITC were purchased from Cedarlane, West Grove, PA, USA. Secondary antibodies for western-blotting: goat anti-mouse IgG-HRP, goat anti-mouse IgG-HRP were purchased from BioRad, Mississauga, ON, CAN; donkey anti-goat IgG-HRP purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

2.1.2. Cell Culture Reagents

RPMI 1640 Medium, DMEM/F12, Dulbecco's Phosphate Buffered Saline (DPBS), Fetal Bovine Serum (FBS), 2-Mercaptoethanol, Sodium Pyruvate, L-Glutamine, Penicillin-

Streptomycin, 10X 0.5% Trypsin-EDTA were purchased from Gibco-Invitrogen, Burlington, ON, CAN.

2.2 MOUSE TS CELL CULTURE

2.2.1. Maintenance of Mouse TS cells

TS cell line (generously provided by Dr. Rossant, Samuel Lunenfeld Research Institute, Toronto, Canada) was cultured in TS culture medium consisting of RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100uM 2-Mercaptoethanol, 2 mM L-glutamine, 50 μg/ml penicillin-streptomycin, and 25 ng/ml FGF-4 and 1 μg/ml heparin, with 70% of the medium being fibroblast conditioned medium (FCM). TS cells were grown in a standard tissue culture incubator at 37°C, 5% CO_2. TS medium was replaced every 2-3 days and upon culture reaching 80% confluency, TS cells were passaged 1:2 to 1:3 (192).

2.2.1.1 Preparation of Fibroblast Conditioned Medium

Fibroblast conditioned medium (FCM) was used to culture TS cells in the absence of EMFIs. Mitomycin-treated EMFIs were cultured in TS medium for 72 hours and collected, filtered (0.45 μm) and stored in aliquots at -20°C. Each aliquot was thawed as needed and stored at 4°C; they were not re-frozen. EMFIs were only used up to 12 days after Mitomycin treatment (192).

2.2.1.2 Mitomycin Treatment

To the stock of 2 mg Mitomycin-C (Sigma, Oakville, ON, CAN), 2 ml PBS was added to make a 1 mg/ml solution, which was aliquoted and stored at 4°C. When EMFIs were confluent, media was removed and replaced with DMEM + 10% FBS supplemented with 100 μl Mitomycin solution (1mg/ml) for every 10 ml medium and incubated for 2.5 hr in 37°C incubator. Medium containing Mitomycin was removed and cells were washed 2x 5 min in PBS and replaced with TS medium.

2.2.1.3 1000x FGF-4 and 1000x Heparin

Human recombinant FGF4 (Sigma, Oakville, ON, CAN) was resuspended in its vial with 1.0 ml of PBS/0.1% w/v fraction V BSA and stored in aliquots at -20°C. Aliquots were thawed as needed and stored at 4°C. Ten ml PBS/0.1% w/v fraction BSA was prepared by dissolving BSA (Gibco-Invitrogen, Burlington, ON, CAN) in PBS without Ca^{2+}/Mg^{2+} (Gibco-Invitrogen, Burlington, ON, CAN) and stored in 1.0 ml aliquots at -20°C.

Heparin (Sigma, Oakville, ON, CAN) was resuspended in PBS to a final concentration of 1.0 mg/ml (1000x) and stored at -20°C. Aliquots were thawed as needed and stored at 4°C.

2.2.1.4 Freezing & Thawing TS cells

TS cells were trypsinized, pelleted and resuspended in TS medium and an equal volume of 2x freezing medium (50% FBS, 30% TS medium, 20% DMSO). Cells were allowed to freeze slowly at -20°C and then transferred and stored at -80°C. When

thawed, a vial was hand-thawed and resuspended in TS medium and pelleted. Cells were resuspended in TS medium + FGF-4 and Heparin (192).

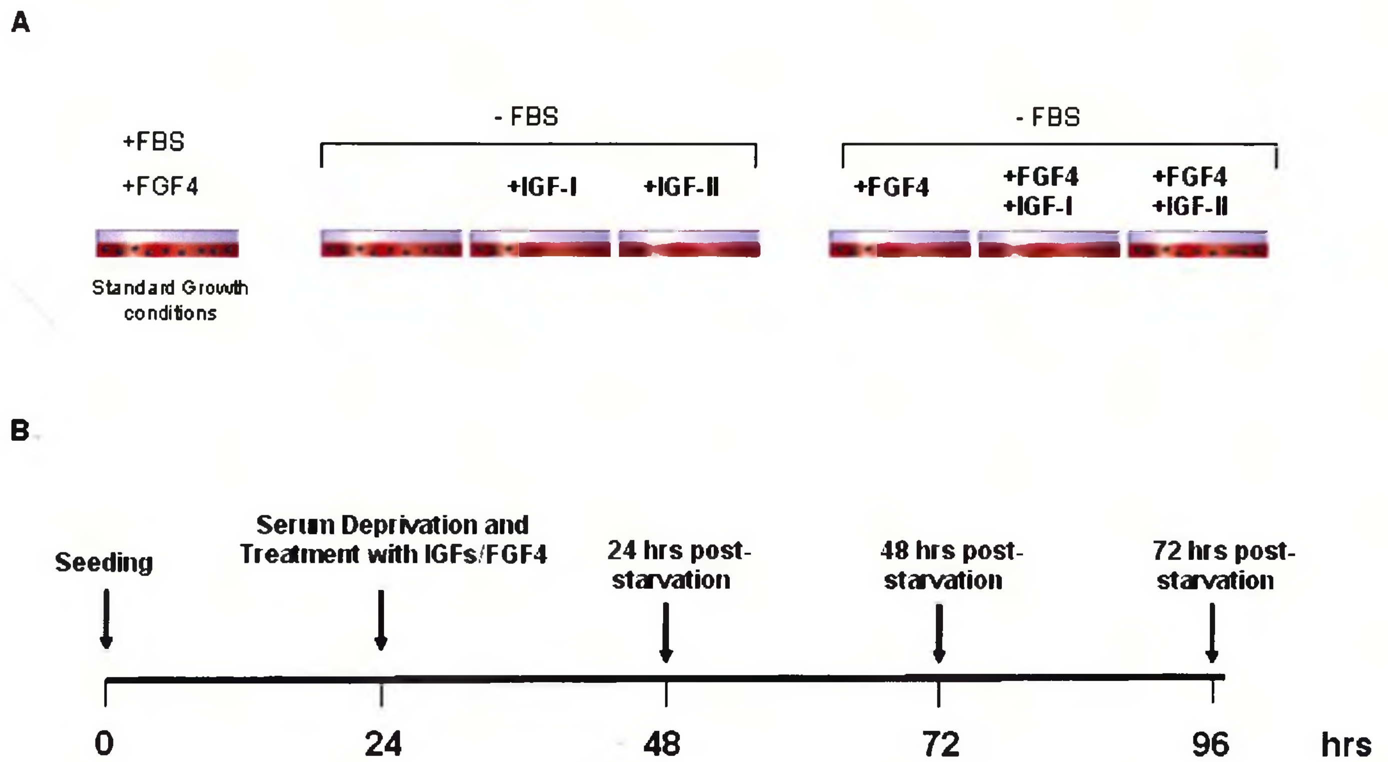
2.2.2. IGF Treatment & Experimental Conditions

TS cells were initially plated under standard TS cell culture conditions (time 0). Upon attachment of cells to culture flasks (~24 hours post-seeding), cells were washed in PBS and grown under serum deprived conditions in the presence of RMPI, lacking growth factors and cytokines present in FBS. RPMI medium was supplemented with either 100 ng/ml human recombinant IGF-I, or 100 ng/ml human recombinant IGF-II (Bachem Biosciences, King of Prussia, PA, USA). Since FGF-4 is an important growth factor in the maintenance of TS cells, cells were also grown in combination with FGF-4 in which RPMI medium was supplemented with 25 ng/ml FGF-4 + 100 ng/ml IGF-I, and 25 ng/ml FGF-4 + 100 ng/ml IGF-II. TS cells were also grown in RPMI medium supplemented with 25 ng/ml FGF-4 only (Fig. 5A). As a control, TS cells were grown in RMPI medium lacking any source of growth factors. To assess the individual effects of IGF-I and IGF-II, as well as in combination with FGF-4, cells were isolated and analyzed over three days at 24, 48, and 72 hours post-starvation (Fig. 5B).

Figure 5. Experimental Conditions.

(A) TS cells were grown under seven different conditions. Under standard growth conditions, TS cells were grown in the presence of fetal bovine serum (+FBS), FGF-4 and Heparin. TS cells were grown under serum deprived conditions in which FBS was removed and supplemented with either 100 ng/ml IGF-I or IGF-II. Since FGF-4 is an important growth factor in the maintenance of TS cells, cells were also grown under serum deprived conditions and supplemented with either IGF-I or IGF-II, in combination with FGF-4 and Heparin. **(B)** TS cells were initially plated under standard TS cell culture conditions (time 0). Upon attachment of cells to culture flasks (~24 hours post-seeding), cells were washed in PBS and grown under serum deprived conditions and treated with either IGF-I or IGF-II, in the presence or absence of FGF-4. The effects of IGF-I and IGF-II in the presence (+) or absence (-) of FGF-4 were analyzed by at 24, 48 and 72 hours post-starvation.

Figure 5.



2.3 MOUSE TS CELL CHARACTERIZATION

2.3.1. Hematoxylin & Eosin Staining

TS cells grown on cover slips were fixed in AAF fixative (83ml 95% alcohol, 5ml glacial acetic acid, 10ml 40% formaldehyde) for 1 min and immersed in Harris Modified Hematoxylin (Fisher) for 10 seconds and rinsed under running tap water. Coverslips then dipped once in 1% acid alcohol and rinsed well under running tap water and dipped in Eosin counterstain (1% Eosin Y in 95% alcohol with glacial acetic acid) three times. The cells were then dehydrated in ascending series of ethanol: 70% ethanol for 2x 30 sec, 90% ethanol 1x 1 min, 100% ethanol 2x 3 min, and immersed in xylene 3x 5 min and applied to slides containing Permount mounting media and stored at room temperature.

2.3.2. Sub-cellular Protein Extraction

Confluent monolayer of TS cells lifted by treatment with 1x Trypsin-EDTA, washed in PBS, centrifuged and PBS discarded. TS cell pellet was resuspended in five volumes of hypotonic buffer [10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 1 μM $ZnSO_4$] and incubated on ice for 10 min. Using Dounce-homogenizer B-pestle, performed 20 strokes and the lysate transferred to an Eppendorf tube and centrifuged 10 min at 3000 rpm at 4°C. Supernatant collected (cytoplasmic fraction) and pellet was resuspended and washed in 5 volumes hypotonic buffer and centrifuged 10 min at 3000 rpm at 4°C. Supernatant discarded and pellet resuspended in four volumes of the packed cell volume in nuclear extraction buffer [10 mM Hepes pH 7.9, 400 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 1 μM

$ZnSO_4$, 5% Glycerol] and incubated on ice for 30 min. Both cytoplasmic and nuclear fractions were centrifuged 45 min at 14,000 rpm at 4°C, and the supernatant was collected, aliquoted and stored at -80°C until used.

2.3.2.1 BCA Assay

Nuclear and cytoplasmic protein samples were quantified using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford IL, USA). BSA standards were prepared by diluting contents of one BSA ampoule to obtain concentrations of 2000, 1500, 1000, 750, 500, 250, 125, 25, 0 µg/ml. Triplicate 10 µl aliquots of each sample and standard were pipetted into a Thermo LabSystems Microtiter 96 well plate (Thermo LabSystems, Milford MA, USA) containing 200 µl BCA Assay Working Reagent. Working reagent was prepared by mixing 50 parts BCA Reagent A (sodium carbonate, sodium bicarbonate, bicinchoninc acid, sodium tartate in 0.1M sodium hydroxide) with 1 part BCA Reagent B (4% cupric sulfate). Each sample was mixed, covered, and incubated for 1 hr at 37°C. The plate was then placed in Thermo LabSystems Multiskan Ascent Plate Reader (Thermo LabSystems) to read protein concentration at absorbance of 560 nm. Results were analyzed using Multiskan Ascent analysis software (Thermo LabSystems).

2.3.3. Western Blotting Procedure

2.3.3.1 Sample Preparation

Nuclear and cytoplasmic proteins were examined for expression of self-renewal, differentiation and trophoblast-specific markers (Table 1) by Western-blotting analysis.

GAPDH was used as a loading control for the cytoplasmic cell fraction. Fifteen µg of nuclear and cytoplasmic proteins were prepared by adding 6x SDS gel-loading buffer containing [1% ß-mercaptoethanol, 6% SDS, 20% glycerol, 1.2 mg bromophenol blue, Tris HCl (0.5 M, pH 6.8)]. Samples were heated to 95°C for 10 min and quickly pulse spun.

2.3.3.2 SDS-PAGE

Protein samples were separated according to the molecular weight by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% polyacrylamide gels containing 0.375M Tris HCl pH 8.8, 0.125M Tris HCl pH 6.8, 30% Acrylamide/Bis Solution (29:1), 10% Ammonium Persulfate, and 0.01% TEMED. Protein samples were loaded onto polyacrylamide gel, with one well containing SeeBlue® Plus2 Prestained Standard (Invitrogen) and MagicMark™ XP Western Standard (Invitrogen). Samples were electrophoresed at 80V until the dye front passed through the stacking gel, and the voltage was raised to 100V for the remainder of the electrophoresis.

2.3.3.3 Protein Transfer & Antibody Probing

Protein gels were electrophorectically transferred to polyvinylidene fluoride (PVDF) membranes in Transfer buffer (25 mM Tris base, 190 mM Glycine, 0.05% SDS and 20% methanol) for 2 hr at 350 amperes (Amp) in 4°C cold room. After transfer, PVDF membranes were rinsed 5 min in distilled water to remove any traces of SDS, and blocked 2 hrs in 5% non-fat dried Carnation milk in Tris-buffered saline (TBS) pH 8.0 supplemented with 0.05% Tween-20 (TBS-T) on a platform shaker at room temperature.

Membranes were then rinsed with 1x TBS for 10 min and incubated overnight in 4°C cold room on a platform shaker in primary antibody [SOX2 (1:100 dilution blocked with 2.5% non-fat milk in TBS-T); OCT4 (1:100 dilution blocked with 2.5% non-fat milk in TBS-T); CDX2 (1:100 dilution in TBS-T); PL-1 (1:500 dilution blocked with 2.5% non-fat milk in TBS-T); GAPDH (1:500 dilution in TBS-T)].

Membranes were rinsed 3x 10 min in wash solution containing different concentrations of Tween-20 in 1x TBS (Table 1) and incubated 45 min at room temperature with Horseradish Peroxidase (HRP)-Conjugated secondary antibody [SOX2 (goat anti-rabbit IgG-HRP at 1:10,000 dilution blocked with 2.5% non-fat milk in TBS-T); OCT4 (goat anti-rabbit IgG-HRP at 1:10,000 dilution blocked with 2.5% non-fat milk in TBS-T); CDX2 (goat anti-mouse IgG-HRP at 1:10,000 dilution blocked with 2.5% non-fat milk in TBS-T); PL-1 (goat anti-rabbit at 1:10,000 dilution blocked with 2.5% non-fat milk in TBS-T); GAPDH (donkey anti-goat IgG-HRP at 1:10,000 dilution blocked with 10% non-fat milk in TBS-T)]. Membranes were washed 3x 10 min in 1x TBS containing different concentration of Tween-20 (Table 1) on a platform shaker at room temperature.

2.3.3.4 Chemiluminescence & Imaging

Membranes were rinsed in distilled water and the bands were visualized using the enhanced chemiluminescence (ECL) detection kit according to the manufacturer's protocol (Amersham, Piscataway, NY, USA) on Kodak-Biomax film (Rochester, NY, USA). A charged-coupled device camera, the Alpha Innotech FluorChem™ 80000

Imaging System (Alpha Innotech, San Leandro CA, USA), was used for imaging to enable densitometry analysis.

2.3.3.5 Stripping Blots

After chemiluminescence detection, membranes were rinsed in distilled water, placed in glycine buffer pH 2.3 [1.875 g glycine, 1.0 ml 0.5M EDTA in 500 ml dH_2O] and incubated for 30 min at room temperature on a platform shaker. Blots were removed from glycine stripping buffer and washed for 20 min in 1x TBS supplemented with 0.1% Tween-20 at room temperature on a platform shaker. Membranes were then either stored in 1x TBS at 4°C or re-blocked in 5% non-fat dried Carnation milk for the next immunoblotting experiment.

Table 1. Washing conditions and dilutions for each primary and secondary antibody used for Western-blotting analysis.

1° antibody	MW (kDa)	Dilution	Wash (Tween-20)	2° antibody	Dilution	Wash (Tween-20)
Trophoblast Marker						
CDX2	40	1:100	3x 0.05%	Anti-mouse	1:8000	2x 0.05% 1x 0.1%
Self-renewal Marker						
OCT4	38	1:100	3x 0.05%	Anti-rabbit	1:10000	2x 0.1% 1x 0.5%
SOX2	37	1:100	3x 0.1%	Anti-rabbit	1:10000	2x 0.1% 1x 0.5%
Differentiation Marker						
PL-1	36-40	1:500	3x 0.05%	Anti-rabbit	1:10000	3x 0.05%
Positive Marker						
GAPDH	37	1:500	3x 0.1%	Anti-donkey	1:10000	4x 0.5%

2.3.4. Immunocytochemistry

TS cells grown on Gold Seal glass cover slips (Becton Dickinson Labware, Lincoln Park, NJ, USA) were washed in PBS and fixed 15 min at room temperature in ice-cold ethanol. Samples were stored at -20°C until ready for staining. Samples were then rehydrated in PBS for 30 min, permeabilized in 0.1% Triton X-100 for 5 min at room temperature, and blocked 30 min in 2% BSA (GIBCO-Invitrogen, Burlington, ON, CAN) and washed 3x 5 min in PBS. Samples were incubated with primary antibody [CDX2 (1:10), SOX2 (1:100) and PL-1 (1:500)] overnight at 4°C in an incubation chamber and washed 3x 5 min in PBS and incubated with secondary antibody [FITC (1:50), TRITC (1:100)] 1 hour at room temperature in incubation chamber covered with aluminum foil. Samples were washed 3x 5 min in PBS and counterstained for chromatin with Hoechst dye (1:20,000) for 15 min at room temperature. Cover slips were mounted to glass slides with 70% glycerol and fluorescent images were captured using a Zeiss 410 confocal microscope.

2.3.5. Electrophoretic Mobility Shift Assay (EMSA)

2.3.5.1 Probe Preparation & Labeling

A 1 μg linear DNA fragment containing the binding sequences of interest for OCT4 and SOX2 [5'- CAG ACA GCA GAG AGA TGC ATG ACA AAG GTG CCG TGA TGG TTC -3'] was hybridized to a 0.38 μg primer [5'- GAA CCA TCA C -3'] in 200 μl hybridization buffer [50 mM HEPES pH 7.9, 100 mM NaCl] by heating and cooling progressively: 5 min at 100°C, 15 min at 65°C, 15 min at 37°C, and 15 min at

room temperature. The probe was labeled with ^{32}P-dCTP using Megaprime Labeling Kit (GE Healthcare, Piscataway, NJ, USA) and purified using G-50 micro spin columns (GE Healthcare, Piscataway, NJ, USA) at 3,000 rpm for 2 min and cpm/μl calculated using scintillation counter. To ensure that binding was specific, a scrambled probe was developed [5'- GCT GCT GCT AAA GCT GCT AAA GCT GCT AAA GTA GCT AAA TG -3'].

2.3.5.2 Binding reaction mixture

The binding reaction mixture was performed by mixing 5 μg nuclear protein with 1mg/ml Polydeoxyinosinic-deoxycytidylic acid (polydI/dC) (Sigma, Oakville, ON, CAN) and 750,000 cpm ^{32}P -labeled probe made up to 80 μl in EMSA buffer [50 mM HEPES pH 7.9, 375 mM KCl, 12.5 mM $MgCl_2$, 0.5 mM EDTA, 5 mM DTT, 15% Ficoll] and incubated in 4°C cold room for 2 hrs shaking. For super-shift detection, antibodies were added before the initial incubation for 1 hr at 4°C: (1:100) anti-OCT4 (Abcam), (1:100) anti-SOX2 (Abcam), followed by the addition of radio-labeled probe for 2 hrs in 4°C cold room shaking.

2.3.5.3 Gel loading and drying

After incubation, 6x DNA loading dye was added to the reaction mixture and the samples were loaded using fine-point gel tips onto a 5% polyacrylamide gel in 1x TBE buffer. The samples were electrophoresed at 200V for 2 hr in 4°C cold room. The gel was then removed and placed on a sheet of Whatman paper and was dried using the gel

dryer for 1 hr at 80°C. The gel was then exposed to Kodak-Biomax MS film (Rochester, NY, USA) overnight at -80°C.

2.4 ASSESSMENT OF TS CELL APOPTOSIS & NECROSIS

2.4.1. Annexin-V Staining

In the early stages of apoptosis, changes occur at the cell surface in which phosphatidylserine (PS) is translocated from the internal plasma membrane to the external cell surface of the cell membrane and is exposed. Annexin-V, a Ca^{2+}-dependent phospholipid binding protein, has high affinity for PS and can be observed by fluorescence microscopy or flow cytometry. Since necrotic cells are also exposed to PS due to the loss of membrane integrity, propidium iodide is used to stain the DNA of leaky necrotic cells to discriminate from Annexin-V positive apoptotic cells.

The Annexin-V-FLUOS staining kit (Roche, Mississauga, ON, Canada) was used following manufacturer's instructions. TS cells were washed with PBS and centrifuged at 2,000x g for 5 min and resuspended in 100 μl of Annexin-V-FLUOS labeling solution for 15 min at room temperature. Annexin-V positive and PI-positive cells were then analyzed by flow cytometry.

2.4.2. TUNEL Staining

TUNEL staining performed using the ApoTag Peroxidase *In Situ* Apoptosis Detection Kit, according to the manufacturer's protocol (Chemicon International, Temecula, CA, USA). TS cells were fixed in 1% neutral-buffered formalin for 20 min at

room temperature and washed 2x 5 min in PBS and stored in fresh PBS at 4°C. Samples were permeabilized in 0.2% Triton X-100 for 5 min on ice and were washed 2x 5 min in PBS. Cells were quenched with 3% H_2O_2 [1 ml 30% hydrogen peroxide in 9 ml methanol] for 5 min at room temperature and washed well in distilled water. The excess liquid was removed and 75 µl equilibration buffer was added to each slide, and the slides were incubated in humidified chamber for 10-30 min. Working strength TdT enzyme was prepared during the equilibration step by mixing reagents in a ratio of 70% reaction buffer with 30% TdT enzyme in a fresh microcentrifuge tube. The reagent was mixed well and 55 µl TdT enzyme mix was added to each cover slip and covered with parafilm to distribute evenly, and incubated in humidified chamber at 37°C for 1 hr. The samples were transferred to working strength stop/wash buffer diluted 0.3% vol/vol stop/wash buffer in distilled water, agitated 15 sec and incubated for 10 min at room temperature. Samples were washed 3x 1min in PBS and 75 µl anti-DIG conjugate was applied to each sample and incubated in humidified chamber for 30 min at room temperature. Samples washed 4x 2 min in PBS and 100 µl Liquid Cardassian DAB was added to each sample, incubated at room temperature for 2 min, washed well and incubated for 5 min in distilled at room temperature. Samples were then counter-stained in filtered Carazzi's Hematoxylin for 2 min at room temperature and washed well in distilled water until clear. Samples were then dehydrated in ascending series of ethanol: 70% ethanol for 2x 30 sec, 90% ethanol 1x 1 min, 100% ethanol 2x 3 min, and then in xylene 3x 5 min, mounted using Permount and stored at room temperature.

2.5 Statistical Analysis

The significance of quantified data generated in this study was assessed by statistical analysis using either a One-way ANOVA or a Two-way repeated measures ANOVA using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California, USA. Significant difference was considered for $p<0.05$. Graphic representation shows means +/- standard deviation (as variance bars).

CHAPTER 3: RESULTS

3.1. Characterization of TS cells in standard cell culture conditions altered by IGFs

3.1.1. Optimization of TS cell maintenance

Initially, optimization and proper culturing of the TS cell line was performed before experimental analysis commenced. The initial isolation and expansion of TS cells from blastocysts was performed by members of Dr. Janet Rossant's laboratory (Samuel Lunenfeld Research Institute, Toronto, Canada). TS cells were initially cultured on embryonic mouse fibroblast (EMFI) feeders, which were mitotically inactivated by treatment with Mitomycin. EMFI feeders provide an additional source of growth factors and cytokines necessary for stem cell maintenance for ~10 days, after which TS cells have to be seeded onto fresh EMFI feeders (192). TS cells grew in tight colonies on top of the monolayer of EMFI feeders (Fig. 6a), which were grown in RPMI medium containing fetal bovine serum (FBS), sodium pyruvate, L-glutamine, β-mercaptoethanol and antibiotics (TS medium) supplemented with 25 ng/ml FGF-4 and 1.0 mg/ml Heparin. To perform experimental analysis on this cell line, TS cells were adapted to grow in the absence of EMFI feeders on tissue culture plastic (Fig. 6b). Since the EMFI feeders have been mitotically inactivated by Mitomycin treatment, they will eventually be removed through continued passages.

The mouse fibroblast feeder layer was removed from the TS cell culture based on the different adherence rates of EMFIs and TS cells. EMFIs adhere fast while TS cells adhere slowly, thereby allowing the isolation of a pure TS cell population. In addition,

since EMFI feeders were mitotically inactivated, any remaining EFMIs will be removed from the TS cell culture in subsequent passages (192). In the absence of EMFIs, TS cells were cultured in TS medium consisting of 70% EMFI feeder-conditioned medium (FCM) supplemented with FGF-4 and Heparin. FCM was collected from mitotically inactivated EMFI feeders grown in TS medium for 72 hours. EMFIs were only used up to 10-12 days after Mitomycin treatment to collect FCM, after which freshly mitotically inactivated EMFI feeders were prepared (192).

TS cells required the presence of FGF-4 to remain pluripotent, and upon its removal, differentiation of TS cells was initiated. Differentiation is associated with changes in cell morphology thereby leading to changes in gene expression (192). One of the first lineages to develop during differentiation was that of trophoblast giant cells, whereby cells change from tight epithelial TS cell colonies into multi-nucleated cells with a large cytoplasm (Fig. 6c). The presence of a small population of giant cells in standard TS cell culture conditions was normal as a small population of TS cells will undergo spontaneous differentiation even in standard culture conditions (192).

3.1.2. TS cell proliferation

TS cells were initially plated at a density of $7.5x10^5$ cells/35-mm dish. Following cultivation for 24 hours in TS cell medium supplemented with FGF-4 and Heparin (standard growth conditions), cells were subjected to serum deprivation (TS medium lacking FBS and FCM) and supplemented with either 100 ng/ml IGF-I or IGF-II in the presence and absence of 25 ng/ml FGF-4. TS cells were also grown in the presence of FGF-4 alone, as well as in serum deprived conditions, lacking all growth factors in the

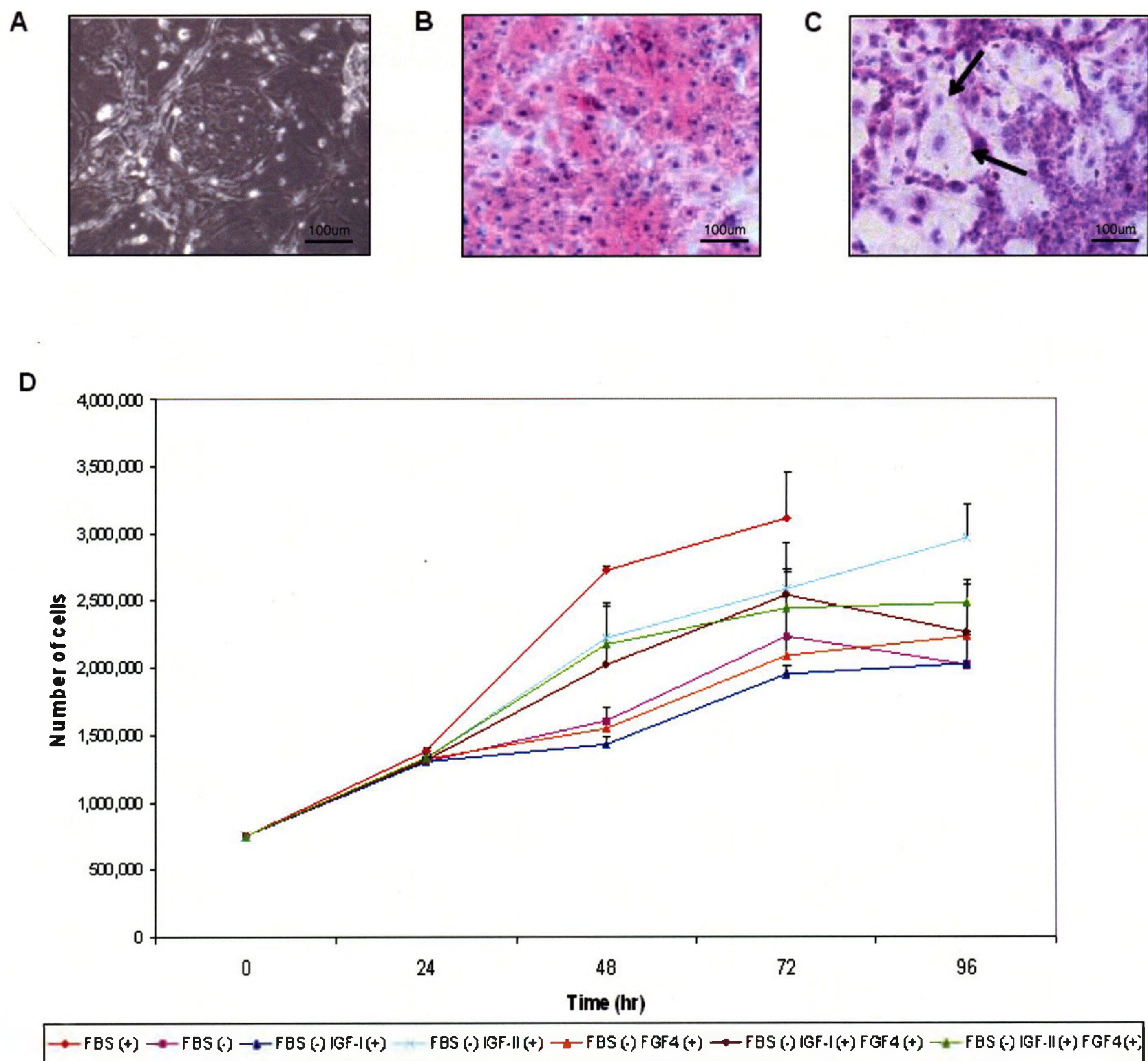
form of FBS and FCM This allowed characterization of the individual effects of IGF-I and IGF-II on TS cell proliferation (Fig. 6D).

Under standard growth conditions, TS cells doubled in population by 24 hours post-seeding, after which they continued to proliferate for another 24 hours. However, by 48 hours post-seeding, cell proliferation began to decrease, likely as a result of contact inhibition leading to the initiation of differentiation and/or cell death. Subjecting TS cells to serum deprivation resulted in a decrease in the rate of cell proliferation by 1.7-fold by 48 hours. While the addition of IGF-I or FGF-4 alone in serum deprived conditions did not cause an increase in cell proliferation, the addition of IGF-II alone compensated for the lack of growth factors and caused an initial increase in cell proliferation of 1.4-fold at 48 hours. Comparing the effects of IGF-I and IGF-II alone, addition of IGF-II caused a 1.5-fold increase in cell proliferation compared to the addition of IGF-I 48 hours; by 72 hours, the increase was reduced to 1.3-fold (Fig. 6D).

The addition of FGF-4 in the presence of IGF-I did not alter the rate of cell proliferation relative to cells grown in serum deprived conditions, nor did FGF-4 alone. However, the addition of FGF-4 in the presence of IGF-II caused a ~1.4 –fold increase in cell proliferation relative to cells grown in serum deprived conditions. This increase was resembled that observed in IGF-II treated cells, suggesting that the addition of FGF-4 does not have a significant effect on TS cell proliferation. Comparing the individual effects of IGF-I and IGF-II in the presence of FGF-4 under serum deprived conditions, the effects on cell proliferation were very similar to that of cells grown in the absence of FGF-4 and supplemented with either IGF-I or IGF-II only.

Figure 6. Characterization of TS cells in standard cell culture conditions and in the presence by IGFs. **(A)** TS cells were initially received and grown in the presence of embryonic mouse fibroblasts (EMFIs). **(B)** In the absence of EMFIs, TS cells were grown in the presence of TS medium supplemented with 70% EMFI-conditioned medium (FCM), adjusted with FGF-4 and heparin. **(C)** Removal of FGF-4 results in the differentiation of TS cells into trophoblast giant cells, characterized by large cytoplasm and a multi-nucleated cell (arrows). **(D)** The individual effects of IGF-I and IGF-II, as well as in combination with FGF-4 on TS cell proliferation were analyzed. TS cells were initially plated at a density of 7.5×10^5 and subjected to serum deprivation and supplemented with either 100 ng/ml IGF-I or IGF-II. Under conditions of standard growth (♦), TS cells doubled in population after 24 hours post-seeding and continued to proliferate by 48 hours. However, by 72 hours, the rate of cell proliferation began to decrease, likely a result of differentiation and/or cell death. In conditions of serum deprivation (■), in which FBS and FGF-4 were removed, cell proliferation decreased by 1.7-fold at 48 hours. Neither the addition of IGF-I (▲) nor FGF-4 only (▲) resulted in an increase in cell proliferation at either 48 or 72 hours. However, the addition of IGF-II only (✕) appeared to compensate for the lack of growth factors and promote cell proliferation, as a 1.4-fold increase was observed at 48 hours. The addition of FGF-4 in the presence of IGF-I (●) or IGF-II (■) did not have any combined effect on cell proliferation. Data are average of two separate experiments performed in quadruplicates, and bars represent standard deviations.

Figure 6.



3.2 Characterization of TS morphology in growth factor restriction conditions ameliorated by IGFs

Since FGF-4 alone is not sufficient in maintaining the self-renewing capacity of TS cells, additional yet undetermined growth factors present in FBS were required. The influence and effect of growth factors, IGF-I and IGF-II, on the proliferation and differentiation of TS cells was analyzed by examining changes in cell morphology every 24 hours over three days following hematoxylin and eosin (H&E) staining (Fig. 7). In addition to examining the individual effects of IGF-I and IGF-II, the effects of IGF-I and IGF-II in combination with FGF-4 on cell morphology were also examined. TS cells were grown under standard conditions of growth and then subjected to serum deprivation and treated with either IGF-I or IGF-II in the presence and absence of FGF-4 (see Fig. 5 in Materials and Methods section). Since a differentiated TS cell (giant cell) can be easily identified based on its morphology of a large cytoplasm and multi-nuclei, quantification of giant cell formation was performed to determine the influence of IGF-I and IGF-II on the rate of differentiation (Fig. 8). A cell was considered differentiated if its size was 3-4-fold larger than that of a standard TS cell.

3.2.1 Effects of Fetal Bovine Serum (FBS)

TS cells grown under standard growth conditions in the presence of FBS and FGF-4 (Fig. 7A-C) resembled cells of typical TS cell morphology, growing in tight epithelial-like colonies. Cells reached confluency by 48 hours post-seeding, and began the process of spontaneous differentiation into giant cells by 72 hours post-seeding.

Subjecting TS cells to serum deprivation (removal of FBS and FGF-4), caused a dramatic change in TS cell morphology (Fig. 7D-F). The nuclei of cells appeared smaller and shrunken, with larger and swollen cytoplasm near 48 and 72 hours, likely as a result of cells undergoing cell death by either apoptosis or necrosis. Analysis of the number of differentiated cells demonstrated that subjecting TS cells to serum deprivation resulted in a 2.7-fold increase in the number of differentiated cells at 24 hours, and a ~6-fold increase at 48 hours post-starvation relative to cells grown under standard conditions. By 72 hours however, only a 1.8-fold increase was observed in the number of differentiated cells (Fig. 8A). However, this decrease in giant cell formation at 72 hours was likely a result of cells in standard conditions undergoing a dramatic increase in differentiation at this time point, due to contact inhibition and lack of fresh TS medium. In addition, cells in serum deprived conditions were no longer undergoing differentiation, and were now undergoing cell death. During culture of TS cells grown strictly in serum deprived conditions, a higher level of floating cells was observed, indicative of cell death. Overall, the effects of subjecting TS cells to serum deprivation significantly induced differentiation into giant cells ($p < 0.0001$) (Fig. 8A).

3.2.2 Effects of IGF-I

Upon the addition of IGF-I only (Fig. 7J-L), the morphology of cells at 24 hours post-starvation closely resembled that of cells grown under serum deprived conditions (Fig. 7D-F), as larger number of multi-nucleated cells, characteristic of giant cells, was observed. The effects of IGF-I, however, became evident by 72 hours as the addition of IGF-I promoted the differentiation of TS cells (Fig. 7L), compared to cells grown strictly

in serum deprived conditions that underwent cell death by 72 hours (Fig. 7F). A 3.3-fold increase in giant cells was observed in IGF-I treated cells at 24 hours compared to serum deprived cells, which was reduced to 2-fold by 48 hours. By 72 hours, the number of giant cells was significantly increased in IGF-I treated cells (Fig. 8A) ($p < 0.001$). Although cell death possibly occurred in cells treated with IGF-I, the effects of differentiation were greater, suggesting that the addition of IGF-I promoted the differentiation of TS cells.

The addition of FGF-4 in the presence of IGF-I (Fig. 7M-O) did not alter the morphology of TS cells, relative to cells treated with IGF-I alone (Fig. 7J-L). However, the addition of FGF-4 in the presence of IGF-I reduced the number of giant cells by 3-fold at 24 hours, 2.5-fold at 48 hours, and no relevant change by 72 hours post-starvation compared to cells treated with IGF-I only (Fig. 8A), similar to serum deprived cells. The addition of FGF-4 significantly reduced the effects of differentiation induced by IGF-I ($p < 0.0001$) (Fig. 8B). Since cells grown in FGF-4 alone reduced the effects of giant cell formation induced by serum deprivation (Fig. 8A), these results suggest that FGF-4 in the presence of IGF-I inhibits the effects of differentiation induced by IGF-I.

3.2.3 Effects of IGF-II

The addition of IGF-II alone (Fig. 7P-R) improved the morphology of TS cells relative to serum deprived conditions (Fig. 7D-F), as cells resembled those grown in standard growth conditions and did not contain cells with shrunken nuclei, indicative of cell death. Analysis of giant cell numbers revealed that the presence of IGF-II did not induce giant cell formation relative to cells grown in serum deprived conditions (Fig.

8A). A slight increase observed may have resulted from serum deprived cells undergoing greater cell death compared to differentiation, thereby accounting for a higher number of differentiated cells observed in IGF-II treated cells. Difference in the effects of IGF-I and IGF-II on giant cell formation suggests a contrasting role between the two growth factors. The addition of IGF-II initially reduced the number of giant cells by 2-fold at 24 hours, 1.7-fold by 48 hours, and no change by 72 hours post-starvation, relative to IGF-I treated cells (Fig. 8A). The addition of IGF-I, but not IGF-II increased significantly the number of giant cells induced by serum deprivation ($p < 0.001$) (Fig. 8B). These results suggest that although IGF-I and IGF-II increased giant cell formation relative to serum deprived conditions, IGF-I influenced the differentiation of TS cells, while IGF-II promoted the maintenance of TS cell proliferation and self renewal.

The addition of FGF-4 in the presence of IGF-II (Fig. 7S-U) resembled cells grown strictly in FGF-4 alone (Fig. 7G-I). Further examination revealed that FGF-4, in combination with IGF-II, significantly reduced the number of giant cells, when compared to serum deprived cells ($p < 0.0001$) (Fig. 8A). Although IGF-II significantly reduced the effects of giant cell formation relative to IGF-I, the addition of FGF-4 in combination with IGF-II caused an even greater decrease in the number of giant cells (Fig. 8A), with a 1.6-fold decrease at 24 hours, and a 3.6-fold decrease at 48 hours post-starvation, relative to IGF-II treated cells ($p < 0.0003$) (Fig. 8B), suggesting that IGF-II in combination with FGF-4 reduced the effects of giant cell formation induced by serum deprivation. The addition of FGF-4 caused a significant decrease in giant cell formation compared to cells treated with IGF-I and IGF-II alone ($p < 0.0001$) (Fig. 8B). Interestingly, of all the treatments analyzed at 48 hours, the addition of IGF-II in combination with FGF-4

resulted in the lowest number of differentiated cells relative to cells grown in standard growth conditions (Fig. 8A).

Taken together, these results suggest that under conditions of serum deprivation, IGF-I induced the formation of giant cells, while IGF-II promoted TS cell maintenance, as opposed to differentiation of TS cells. The addition of FGF-4 promoted the reduction of differentiation induced by IGF-I, with greater effects observed in IGF-II and FGF-4 treated cells.

Figure 7. Changes in TS cell morphology induced by the addition of IGF-I or IGF-II, as well as in combination with FGF-4, under serum deprived conditions. (A-C) TS cells grown under standard growth conditions resembled cells of typical TS cell morphology, growing in tight epithelial-like colonies. By 48 hours, cells reached confluency and began the process of spontaneous differentiation (giant cell formation) by 72 hour post-seeding. **(D-F)** In the absence of FBS, TS cells undergo spontaneous differentiation by 24 hours post-starvation and appeared to undergo cell death in the absence of growth factors by 72 hours. **(G-I)** The addition of FGF-4 appeared to compensate for the lack of growth factors and promote proliferation of TS cells at 24 hours. By 48 and 72 hours post-starvation, morphology changed and cells appeared to undergo differentiation and/or cell death. **(J-L)** The addition of IGF-I appeared to promote differentiation, while **(P-R)** the addition of IGF-II seemed to promote proliferation of TS cells. **(M-O)** The addition of FGF-4 in the presence of IGF-I initially slowed the process of differentiation at 24 hours post-starvation, while **(S-U)** the addition of FGF-4 in the presence of IGF-II reduced the effects of differentiation.

Figure 7.

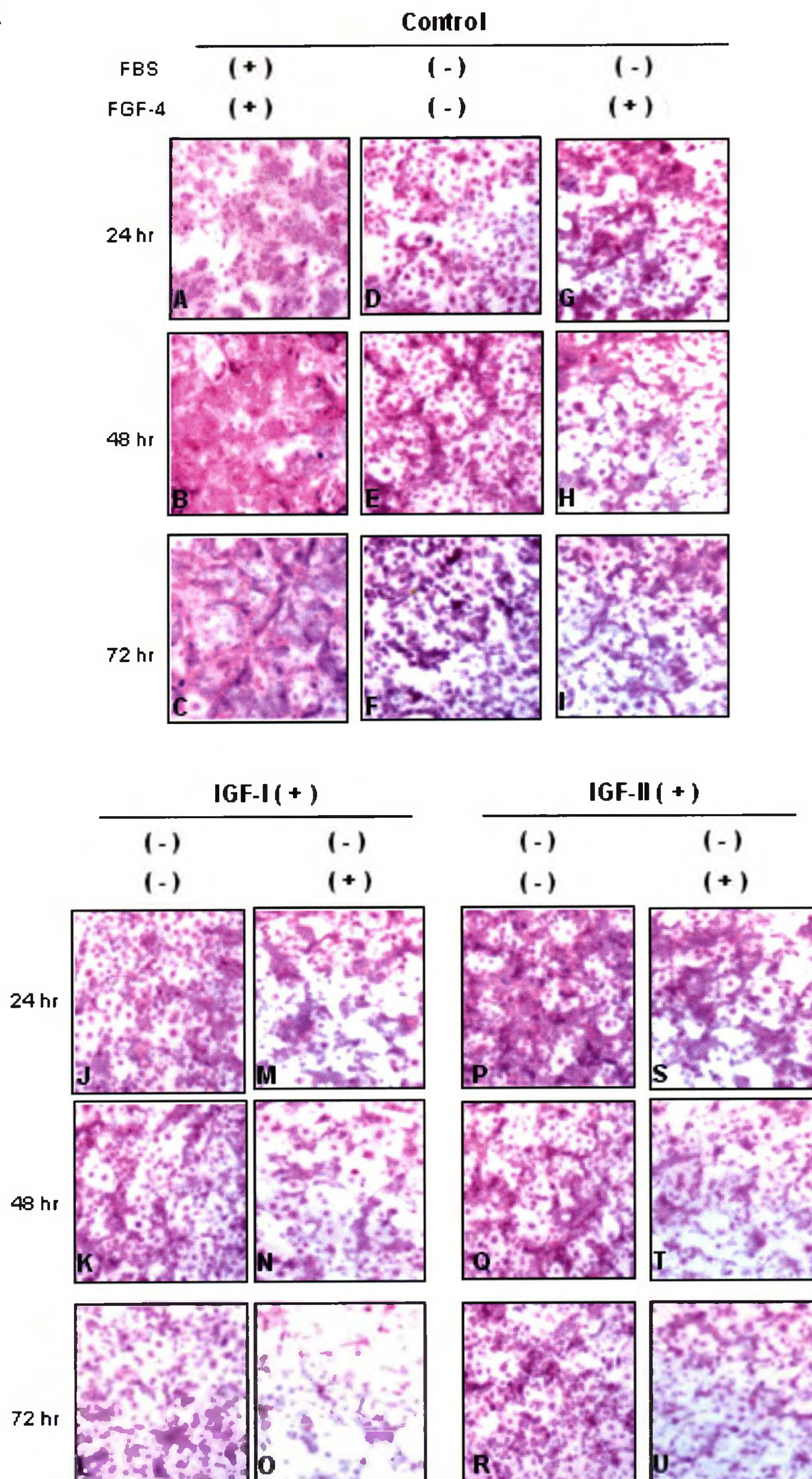
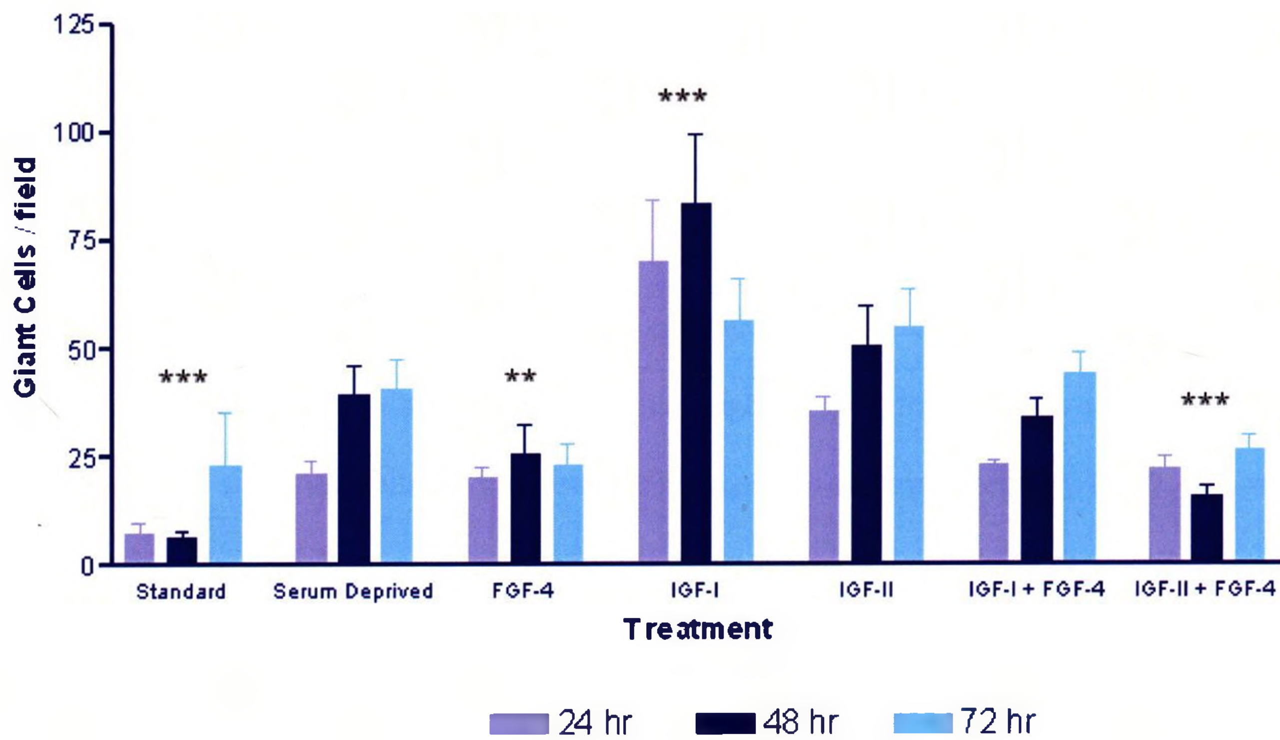


Figure 8. Quantification of giant cell formation induced by IGF-I and IGF-II.
(A) TS cells grown under standard conditions in the presence of FGF-4 and FBS were significantly better at reducing the effects of differentiation induced by serum deprivation ($p < 0.0001$). The addition of IGF-I significantly increased the number of giant cells relative to serum deprived conditions ($p < 0.001$), while IGF-II had an increase in the number of giant cells, but this was not significant. **(B)** Closer examination of the effects of IGF-I and IGF-II (data from **(A)**) demonstrate that there is a significant difference between the individual effects of IGF-I and IGF-II on the formation of giant cells ($p < 0.001$). Data demonstrate that IGF-I influenced the differentiation of TS cells into giant cells. The presence of FGF-4 reduced the effects of differentiation induced by serum deprivation, as a significant difference in the reduction of giant cells was observed between IGF-I in the presence and absence of FGF-4 ($p < 0.0001$), and between IGF-II in the presence and absence of FGF-4 ($p < 0.0003$). The effects of IGF-II in combination with FGF-4 were significant at reducing the number of giant cells relative to TS cells treated with IGF-I and FGF-4 ($p < 0.0001$). Statistical analysis was performed using a Two-way ANOVA comparing the effect of treatment over time (the signs *, ** and *** denote $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). The symbol * denotes treatments vs. serum deprived conditions; † denotes IGF-I vs. IGF-II (± FGF-4); • denotes IGF-I/-II vs. IGF-I/-II (+ FGF-4). For each individual treatment, six images were taken at each time point and the number of differentiated cells was counted per microscopic field (20X) and averaged expressing standard deviations (n=1).

Figure 8.

A



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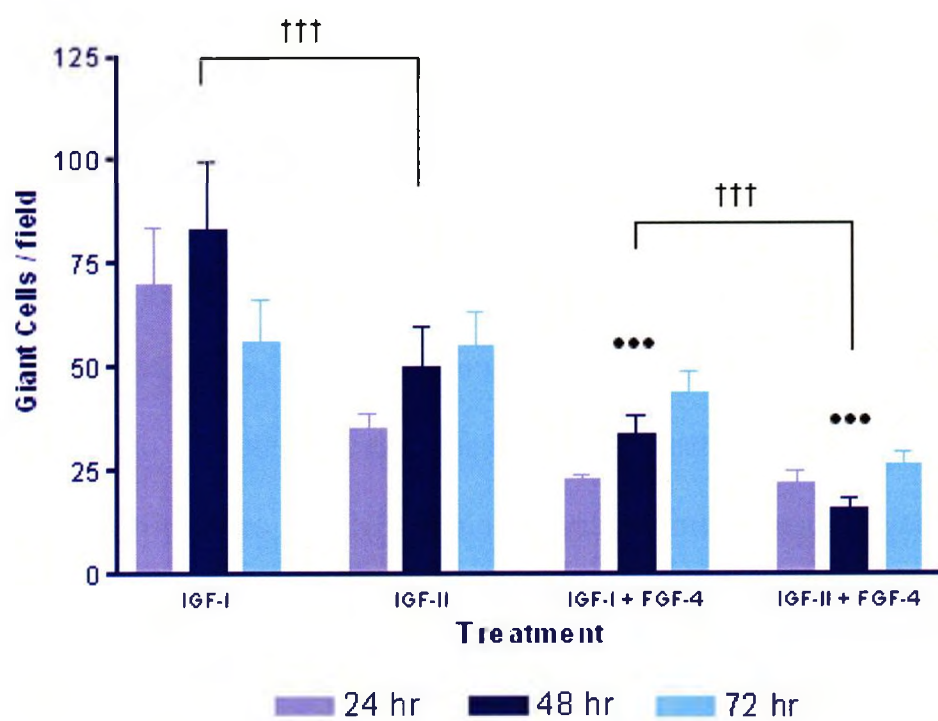


3.3 The effects of IGF in TS cell death

Based on morphological analysis, subjecting TS cells to serum deprivation and treatment with IGFs induced cell death, in addition to proliferation and differentiation. To further examine the individual effects of IGF-I and IGF-II on TS cell death, the effects on early and late stages of apoptosis were analyzed. In the early stages of apoptosis, changes occur at the cell surface in which phosphatidylserine (PS) is translocated from the internal cell membrane to the external cell surface of the cell. Annexin-V, a Ca^{2+}-dependent phospholipid binding protein, has high affinity for PS which permits the detection of apoptotic cells by fluorescence microscopy or flow cytometry (167). Since necrotic cells lose their membrane integrity, propidium iodide (PI) can then be used to stain DNA of leaky necrotic cells and discriminate from Annexin-V positively stained apoptotic cells. Later stages of apoptosis are characterized biochemically by the degradation of nuclear DNA. These DNA strand breaks can be detected enzymatically by labeling the free 3'-OH termini with polymerase terminal deoxnucleotidyl transferease (TdT) by the TUNEL assay (TdT-mediated dUTP Nick End Labeling).

To assess the individual effects of IGF-I and IGF-II, as well as in combination with FGF-4 on early and late stages of apoptosis, TS cells were analyzed by Annexin-V and TUNEL staining at 24 and 48 hours post-starvation (Fig. 9). Annexin-V staining was analyzed by flow cytometry, and the number of positive cells was expressed as a percentage relative to 10,000 cells examined for each treatment. Experiments were performed twice in triplicates. TUNEL staining was performed once by counting the number of positive cells for six images in each treatment at 24 and 48 hours, and

expressed as a percentage relative to the total population of cells in the field. A TUNEL-positive cell was characterized as a cell that was approximately the same size as a normal TS cell and stained brown. Cells that were smaller and more characteristic of apoptotic bodies were not counted as TUNEL-positive cells. The morphology of TUNEL staining for each treatment is presented (Figure 11).

3.3.1 Effects of Fetal Bovine Serum (FBS)

Subjecting TS cells to serum deprivation resulted in a 1.7–fold increase in the percentage of Annexin-V positive cells at both 24 and 48 hours, relative to cells grown under standard growth conditions (Fig. 9A); this was not statistically different. Analysis of later stages of apoptosis by TUNEL staining revealed that the serum deprivation resulted in a 2.4-fold increase in the percentage of TUNEL-positive cells at 24 hours, and a 1.9-fold increase by 48 hours post-starvation, relative to TS cells grown in the presence of FBS. This increase in TUNEL-positive cells was found to be statistically significant ($p < 0.0002$) (Fig. 9B), and morphological analysis confirmed the increase in TUNEL-positive cells upon serum deprivation of TS cells (Fig. 11A).

3.3.2 Effects of IGF-I

The addition of IGF-I in serum deprived conditions had no effect on reducing the percentage of Annexin-V positive cells at 24 hours, relative to cells grown in serum deprived conditions. However, by 48 hours post-starvation, a 2-fold decrease in the percentage of Annexin-V positive cells was observed. However, this was not statistically significant (Fig. 9A). During later stages of apoptosis, however, the addition of IGF-I

had an effect on the percentage of TUNEL-positive cells at either 24 or 48 hours post-starvation (Fig. 9B). Staining of TUNEL-positive cells and the morphology of the cells resembled more of those grown in serum deprived conditions (Fig. 11A). The data suggest that IGF-I alone did not alter the effects of apoptosis induced by serum deprivation.

The presence of both growth factors, FGF-4 and IGF-I, did not reduce the percentage of Annexin-V positive cells, relative to TS cells grown in the presence of IGF-I alone (Fig. 9A). Interestingly, analysis of the effects of IGF-I and FGF-4 at later stages of apoptosis revealed that the addition of both FGF-4 and IGF-I had a significant increase on the percentage of TUNEL-positive cells relative to cells grown in IGF-I alone ($p < 0.0045$) (Fig. 9B). The presence of both FGF-4 and IGF-I significantly increased the number of TUNEL-positive cells relative to serum deprived conditions ($p < 0.008$) (Fig. 9B), suggesting that IGF-I alone can inhibit the effects of apoptosis independent of FGF-4. Morphological analysis of TUNEL staining also demonstrated that there was a change in morphology that resembled serum deprived conditions with the highest levels of TUNEL-positive cells, in the presence of both FGF-4 and IGF-I, (Fig. 11A). Since the addition of FGF-4 alone had similar effects to IGF-I alone at early and late stages of apoptosis, and the addition of both caused a significant increase in the percentage of TUNEL-positive cells, the data suggest that IGF-I alone decreased apoptosis and in combination with FGF-4, increased apoptosis of TS cells.

3.3.3 Effects of IGF-II

Addition of IGF-II in serum deprived conditions had a slight reduction on the percentage of Annexin-V positive cells, but was not statiscally significant relative to cells grown in serum deprived conditions (Fig. 9A). Analysis of later stages of apoptosis by TUNEL staining revealed that the addition of IGF-II significantly decreased the percentage of TUNEL positive cells ($p < 0.0025$) (Fig. 9B). Comparing the individual effects of IGF-I and IGF-II, IGF-II was more effective at reducing the effects of apoptosis at later stages, as TUNEL staining demonstrated that the addition of IGF-II significantly reduced the percentage of TUNEL-positive cells relative to cells treated with IGF-I alone ($p < 0.03$). These data suggest that under conditions of serum deprivation, IGF-II reduced apoptosis significantly relative to IGF-I.

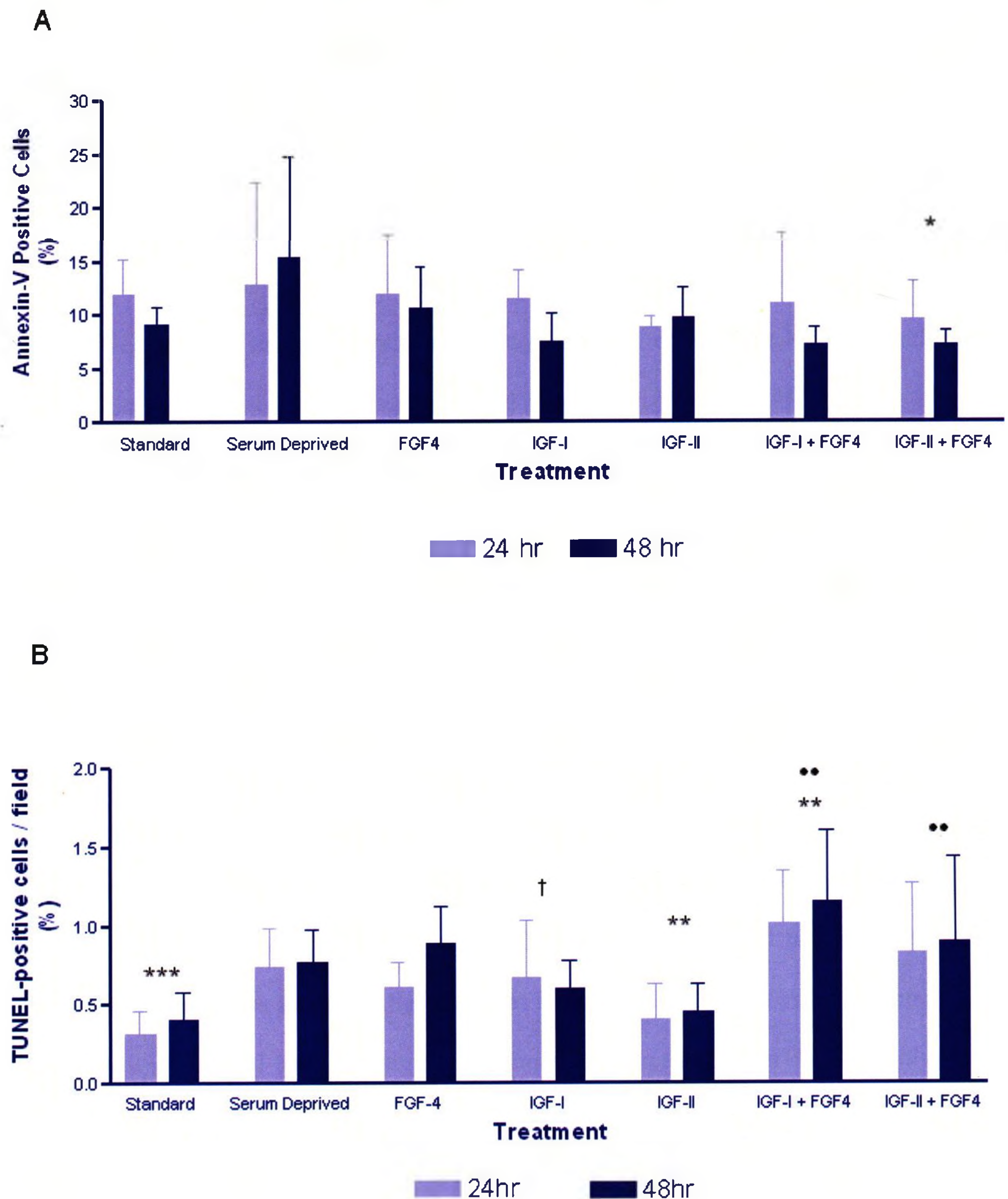
The addition of FGF-4 in the presence of IGF-II did not reduce further the percentage of Annexin-positive cells relative to cells grown with IGF-II only. The effects of IGF-II alone and in combination with FGF-4 were similar between the two treatments. However, at later stages, the addition of FGF-4 in the presence of IGF-II had a significant increase on the percentage of TUNEL-positive cells compared to cells treated with IGF-II alone ($p < 0.007$) (Fig. 9B). The increase in number of TUNEL-positive cells was similar that observed in serum deprived conditions, suggesting that the anti-apoptotic effect of IGF-II was disrupted by the addition of FGF-4, and that both growth factors may act in antagonism to each other. Morphological analysis of TUNEL staining also demonstrated that IGF-II had better effect against apoptosis when acting independently (Fig. 11A).

Taken together, analysis of the effects of IGF-I and IGF-II on apoptosis of TS cells indicate that IGF-II is better than IGF-I in protecting TS cells from apoptosis, and

that the addition of FGF-4 in combination with either IGF-I or IGF-II increases the rate of apoptosis, with IGF-I and FGF-4 having the greatest cell death.

Figure 9. Effects of IGF-I and IGF-II, as well as in combination with FGF-4, on TS cell apoptosis, measured by Annexin-V and TUNEL staining. **(A)** Annexin-V analysis did not reveal any significant differences upon the addition of either IGF-I or IGF-II alone. In the presence of FGF-4, the addition of IGF-II resulted in the lowest percentage of positive cells, which was found to be significant ($p < 0.03$). Data shown are standard deviations for two separate experiments performed in triplicates. **(B)** TUNEL staining revealed a significant increase in TUNEL-positive cells, relative to cells grown under serum deprived conditions ($p < 0.0002$). The addition of IGF-I had no effect on the percentage of TUNEL-positive cells, while the addition of IGF-II had a significant effect at reducing the effects of apoptosis ($p < 0.0025$). IGF-II significantly reduced the percentage of TUNEL-positive cells relative to IGF-I ($p < 0.03$). Both IGF-I and IGF-II significantly increased the percentage of TUNEL-positive cells when grown in combination with FGF-4 ($p < 0.0045$; < 0.007) relative to cells grown in the presence of IGF-I or IGF-II alone. Data shown are standard deviations for the number of TUNEL-positive cells for six images in each treatment at 24 and 48 hours, and expressed as a percentage relative to the total population of cells in the field. Statistical analysis was performed using a Two-way ANOVA comparing the effect of treatment over time (the signs *, ** and *** denote $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). The symbol * denotes treatments vs. serum deprived conditions; † denotes IGF-I vs. IGF-II (± FGF-4); • denotes IGF-I/-II vs. IGF-I/-II (+ FGF-4).

Figure 9.



3.3.4 Effects of Dosage of IGF-I/IGF-II

Since the half-life of IGFs is ~12-16 hours (137), the individual effects of IGF-I and IGF-II on apoptosis were examined further by replenishing TS cells with an additional dose of either 100 ng/ml IGF-I or IGF-II, 24 hours after initial IGF treatment. TS cells were grown under the same conditions in which they were subjected to serum deprivation 24 hours post-seeding and treated with either 100 ng/ml IGF-I or IGF-II (see Figure 5 in Materials and Methods). Following 24 hours after initial IGF treatment, TS cells were then treated with an additional dose of 100 ng/ml IGF-I or IGF-II and the effects of replenishing the system with an additional dosage of IGF-I or IGF-II at early and late stages of apoptosis were then examined by Annexin-V and TUNEL staining 24 hours later (48 hours post-starvation) (Fig. 10). The dosage of FGF-4 remained constant, as the specific effects of IGF-I and IGF-II were being examined.

Replenishing the TS cell system with an additional dose of IGF-I reduced the percentage of Annexin-positive cells by only 1.3-fold relative to cells treated with a single dose of IGF-I (Fig. 10A). On the other hand, an additional dose of IGF-II reduced the percentage of Annexin-positive cells by 2.0-fold relative to cells treated with only one dose (Fig. 10A). The effects of IGF dosage were not found to be statistically significant at reducing the percentage of Annexin-V positive cells relative to each other. Examination of later stages of apoptosis by TUNEL staining also did not reveal any significant differences as a result of the dosage of IGF-I or IGF-II (Fig. 10B). Treatment with two doses of IGF-I caused a slight reduction in the percentage of TUNEL-positive cells, while treatment with two doses of IGF-II resulted in a slight increase in the percentage of TUNEL-positive cells by 1.2-fold. Since this increase was not found to be

significant, replenishing the system with an additional dose of IGF-II did not result in a further decrease in the effects of apoptosis, and that one treatment may be adequate to reduce the effects of apoptosis induced by serum deprivation. Although the effects of IGF-II were evident at early stages of apoptosis (Fig. 10A), the effects were not observed in later stages as examined by TUNEL staining (Fig. 10B). Furthermore, morphological examination of TUNEL staining demonstrated that replenishing the system with an additional dose of either IGF-I or IGF-II did not change the morphology or number of TUNEL positive cells (Fig. 11B).

In the presence of FGF-4, replenishing the system with an additional dose of IGF-I caused a slight decrease in the percentage of Annexin-V positive cells by 1.4-fold compared to cells treated with only one dose of IGF-I in the presence of FGF-4 (Fig. 10A). This effect in the reduction of Annexin-positive cells was similar to that observed in cells treated with two doses of IGF-I, in the absence of FGF-4. In the presence of two doses of IGF-II, a small increase in the percentage of Annexin-positive cells by 1.4-fold was observed (Fig. 10A). Neither treatments were significant, indicating that in the presence or absence of FGF-4, replenishing the system with an additional dose of either IGF-I or IGF-II did not cause a significant decrease in the effects of apoptosis induced by serum deprivation. Examination at later stages of apoptosis by TUNEL staining revealed that the addition of FGF-4 in the presence of either an additional dose of IGF-I or IGF-II also did not have any significant differences in reducing the percentage of TUNEL-positive cells (Fig. 10B). An additional dose of IGF-I in the presence of FGF-4 caused a 1.2-fold decrease, while an additional dose of IGF-II resulted in a 1.4-fold decrease in

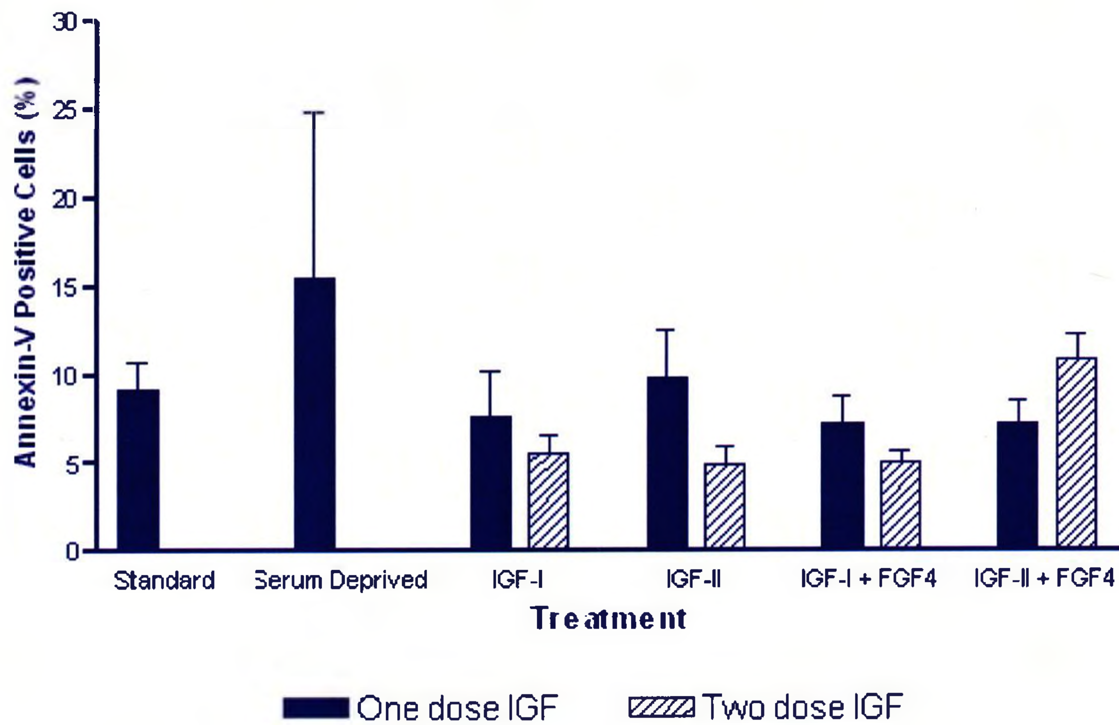
TUNEL-positive cells. Morphological analysis of TUNEL staining confirms this data (Fig. 11B).

These results demonstrate that although IGF-I and IGF-II have a half-life of less than 24 hours, replenishing the system with an additional treatment in the presence or absence of FGF-4 did not result in any significant differences in reducing the effects of apoptosis induced by serum deprivation at either early or later stages.

Figure 10. The effect of replenishing the TS cell system with an additional dosage of IGF-I or IGF-II. Since IGFs have a half-life of ~12-16 hrs, IGF effects on Annexin-V and TUNEL staining were examined at 48 hours post-starvation. **(A)** At early stages of apoptosis, replenishing the system with an additional dose of IGFs had no effect for any treatment group. Data shown are standard deviations for two independent experiments performed in triplicates. **(B)** At later stages of apoptosis, treatment with two doses of IGF-I caused a slight reduction in the percentage of TUNEL-positive cells, while treatment with two doses of IGF-II had no additional effect at reducing the percentage of TUNEL positive cells. The addition of FGF-4 in the presence of either an additional dose of IGF-I or IGF-II also did not reduce the percentage of TUNEL-positive cells. Data shown are standard deviations for the number of TUNEL-positive cells for six images in each treatment, expressed as a percentage relative to the total population of cells in the field. Statistical analysis was performed using a One-way repeated measures ANOVA, comparing the effect of two treatments of IGF with one treatment; no significant differences were found.

Figure 10.

A



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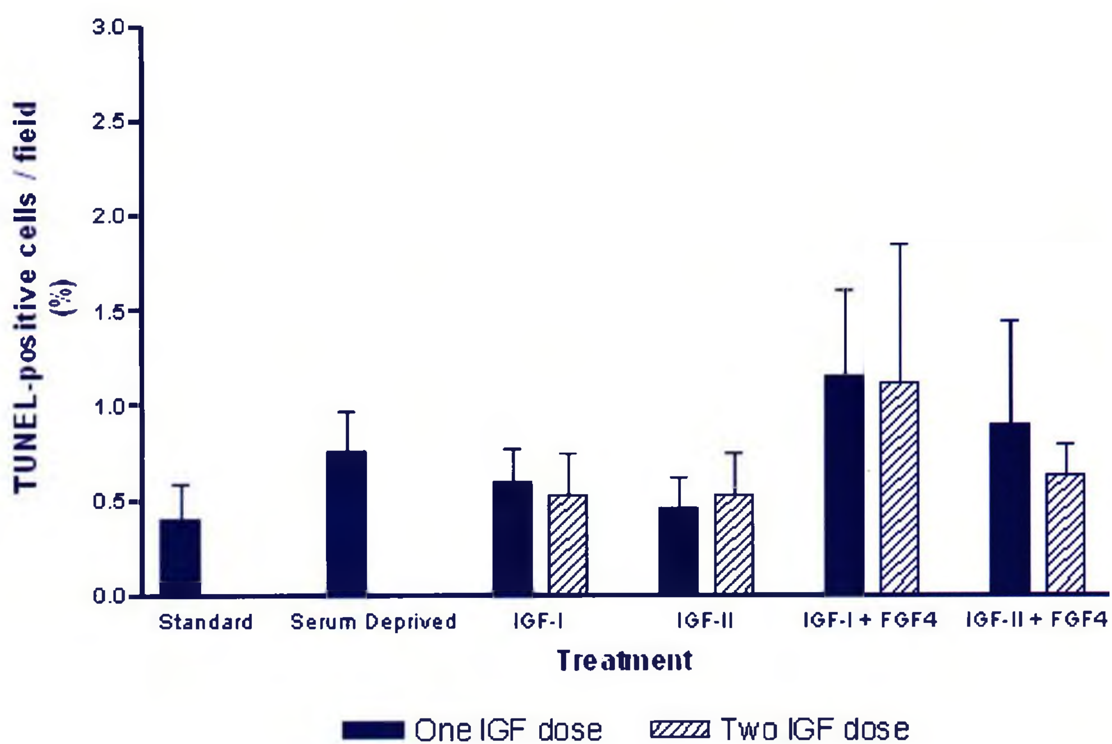
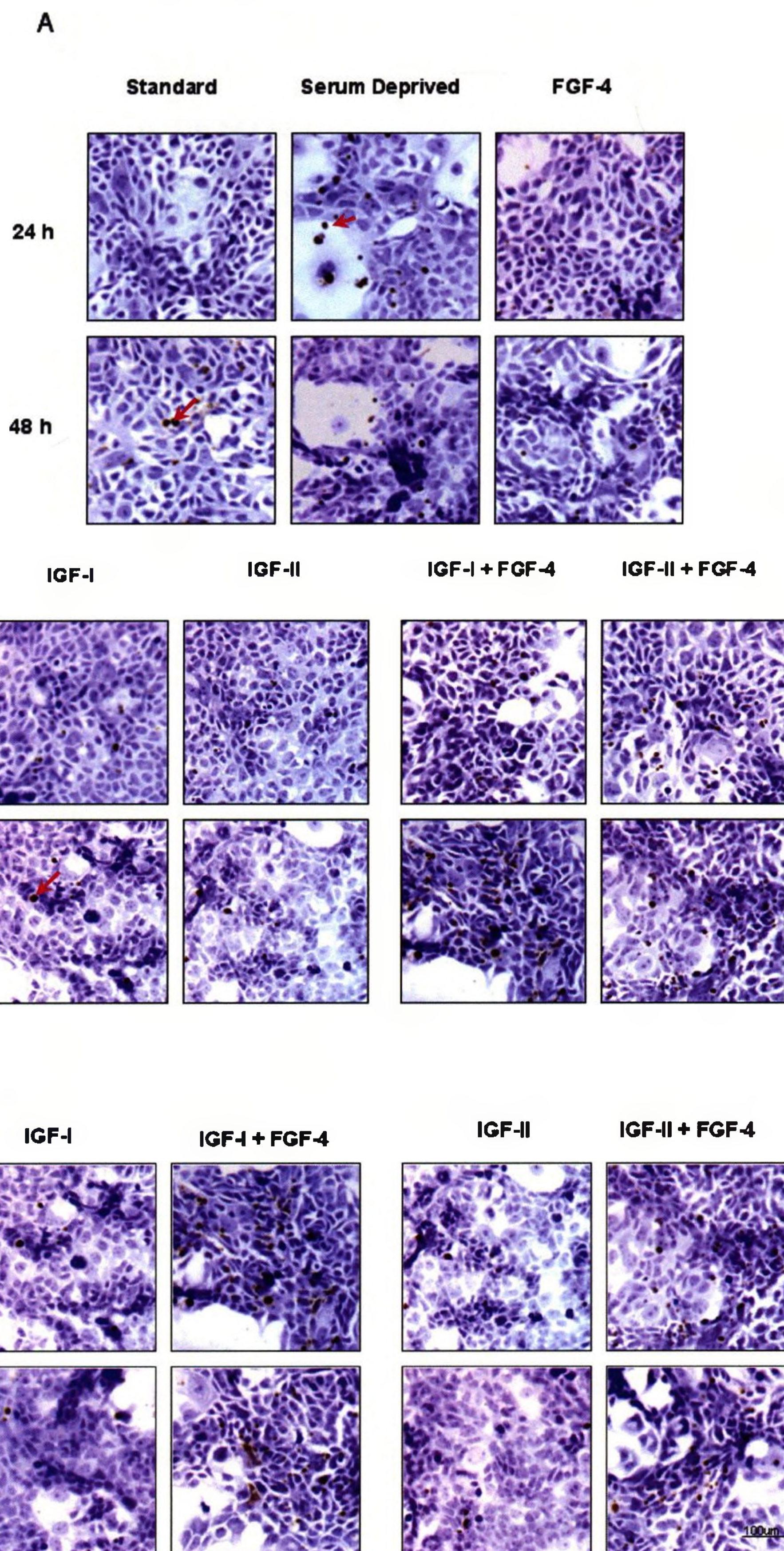


Figure 11. Changes in TUNEL staining induced by the addition and dosage of IGF-I or IGF-II, in the presence and absence of FGF-4. A TUNEL-positive cell was characterized as a cell that was approximately the same size as a normal TS cell and stained brown (indicated by red arrows). **(A)** The removal of serum caused a significant increase in the number of TUNEL-positive cells relative to cells grown in standard growth conditions. The addition of IGF-II decreased the number of TUNEL-positive cells relative to serum deprived conditions. The addition of FGF-4 in the presence of either IGF-I or IGF-II resulted in a change in morphology resembling that of serum deprived conditions, with IGF-I in combination with FGF-4 possessing higher numbers of TUNEL-positive cells. **(B)** Replenishing the TS cell system with an additional dosage of either IGF-I or IGF-II in the presence or absence of FGF-4, 24 hours after initial IGF treatment, does not have any effect on cell morphology or number of TUNEL-positive cells observed.

Figure 11.



3.4. IGF Effects on TS Cell Renewal and its Master Switch Mechanisms

3.4.1 Immunodetection of OCT4, SOX2 and CDX2 markers

TS cells were maintained in an undifferentiated state in the presence of FGF-4 and FBS, which contains a variety of growth factors and cytokines. To determine whether the addition of IGF-I or IGF-II could compensate for the lack of growth factors present in FBS, the effects of IGF-I and IGF-II on the self-renewal of TS cells were analyzed by monitoring changes in the nuclear and cytoplasmic expression of self-renewal markers OCT4 and SOX2, as well as a trophoblast-specific marker CDX2, by Western-blotting analysis and immunocytochemistry (Fig. 12-15). OCT4 and SOX2 factors are two important players of the transcriptional apparatus responsible for the self-renewal of stem cells in general, while CDX2 mediates the trophoblast.

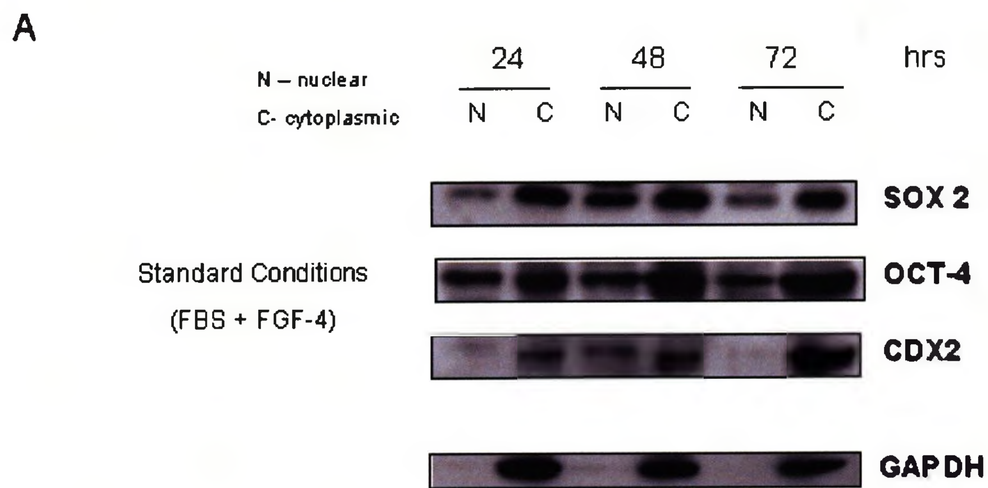
3.4.1.1 Effects of Fetal Bovine Serum

At 24 hours post-seeding, under standard growth conditions in the presence of FGF-4 and FBS, TS cells expressed both OCT4 and SOX2 self-renewal markers, in the nuclear compartment as observed by Western-blotting analysis, demonstrating that TS cells are self-renewing. (Fig. 12A). By 72 hours however, OCT-4 and SOX2 nuclear expression decrease, while its expression in the cytoplasmic compartment increased. The translocation of self-renewal factors from the nuclear to cytoplasmic compartment suggests that under normal growth conditions, TS cell are no longer in a self-renewing capacity and are likely undergoing spontaneous differentiation. Immunocytochemistry confirmed this data, demonstrating that at 24 hours, SOX2 expression was localized to

the nuclear and perinuclear regions, and by 72 hours post-seeding SOX2 expression had translocated to the cytoplasmic region (Fig. 12B). Expression of the trophoblast-specific marker, CDX2, was evident in the nuclear compartments at both 24 and 48 hours post-seeding, but absent by 72 hours post-seeding (Fig. 12A). This corresponded with an increase in cytoplasmic CDX2 expression, with the highest levels observed at 72 hours-post-seeding (Fig. 12A). Immunocytochemistry confirmed this result by demonstrating that nuclear expression of CDX2 was localized to the nuclear compartment at 24 hours demonstrating cells were in a trophoblast fate. By 48 hours, CDX2 expression was localized to the nuclear and perinuclear region, and by 72 hours was seen in the cytoplasmic compartment (Fig 12B), demonstrating TS cells were no longer in a stem cell fate and had begun the process of differentiation.

Figure 12. Expression analysis of self-renewal (OCT4, SOX2) and trophoblast markers (CDX2) in TS cells grown under standard growth conditions. (A) Based on western-blotting analysis, TS cells expressed both SOX2 and OCT4 self-renewal markers in the nuclear compartment at 24 hours, and by 72 hours, expression translocated from the nuclear to cytoplasmic compartment demonstrating TS cells were no longer undergoing a self-renewing capacity. CDX2 expression decreased in the nuclear compartment and increased in the cytoplasmic compartment by 72 hours. GAPDH was used as a loading control to determine that there was no contamination between compartments. **(B)** Immunocytochemistry of SOX2 (FITC) revealed nuclear and perinuclear localization at 24 and 48 hours. By 72 hours, SOX2 was localized to the cytoplasmic compartment. Expression of CDX2 (TRITC) was localized to the nuclear compartment at 24 and 48 hours and translocated to the perinuclear and cytoplasmic compartment by 72 hours post-seeding. DAPI was used to stain cell nuclei.

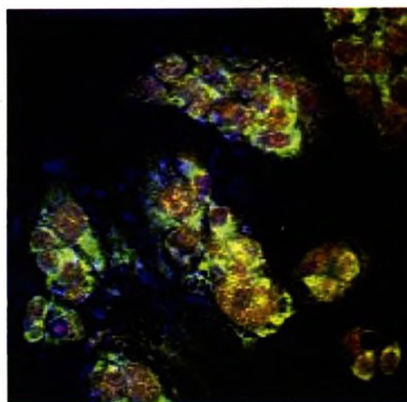
Figure 12

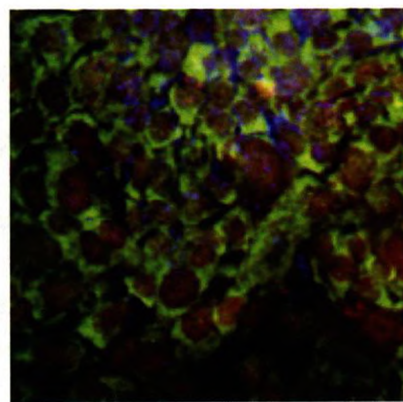


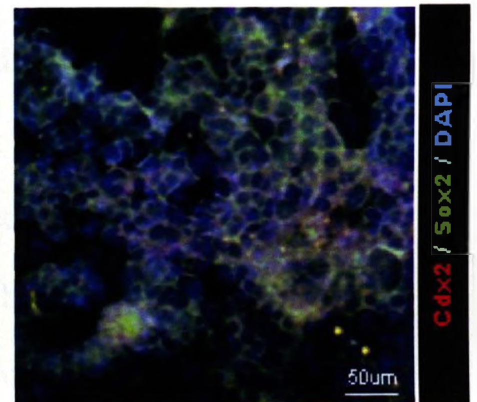
B

24 hr **48 hr** **72 hr**

Standard Conditions







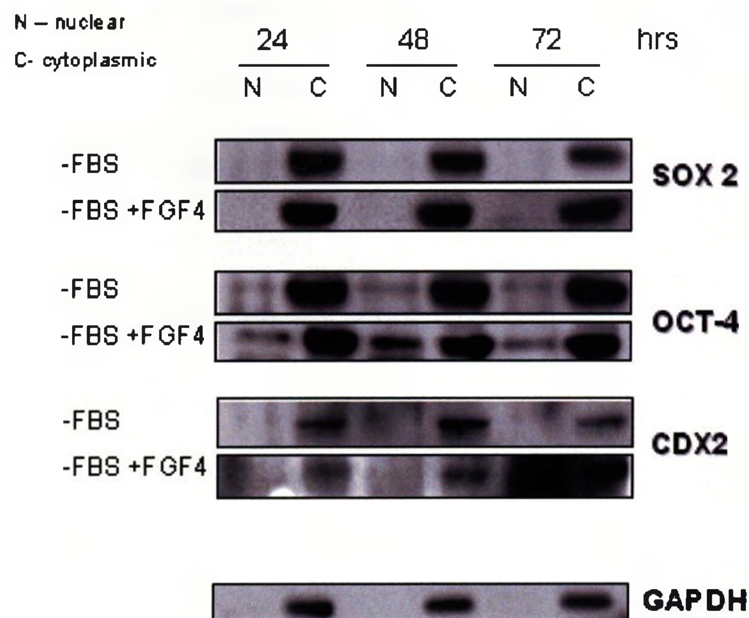
3.4.1.2 Effects of FGF-4

Under serum deprived conditions, the nuclear expression of self-renewal markers OCT4 and SOX2 dramatically decreased relative to cells grown under standard TS cell conditions (Fig. 13A). The addition of FGF-4 under serum deprived conditions restored nuclear OCT4 activity at 48 hours post-starvation. However, the nuclear activity of SOX2 was not restored upon the addition of FGF-4 under serum deprived conditions at any time point (Fig. 13A). This was unexpected, as both SOX2 and OCT4 are markers of self-renewal and pluripotency in stem cells, and are known to act synergistically to regulate the expression of several pluripotent specific genes (161). Immunocytochemistry confirmed SOX2 expression observed by western-blotting, demonstrating that SOX2 expression (FITC) was localized to the cytoplasmic compartment in the presence and absence of FGF-4 under serum deprived conditions (Fig. 13B). CDX2 expression by western-blotting was not altered upon the addition of FGF-4 in serum deprived conditions, as CDX2 was localized in the cytoplasmic compartment of cells (Fig. 13A). Immunocytochemistry provided a clearer understanding to the role of FGF-4, as its addition in serum deprived cells restored CDX2 expression in the nucleus at 24 hours post-starvation. By 48 hours, nuclear CDX2 expression was reduced and translocated into the cytoplasmic compartment by 72 hours post-starvation (Fig. 13B).

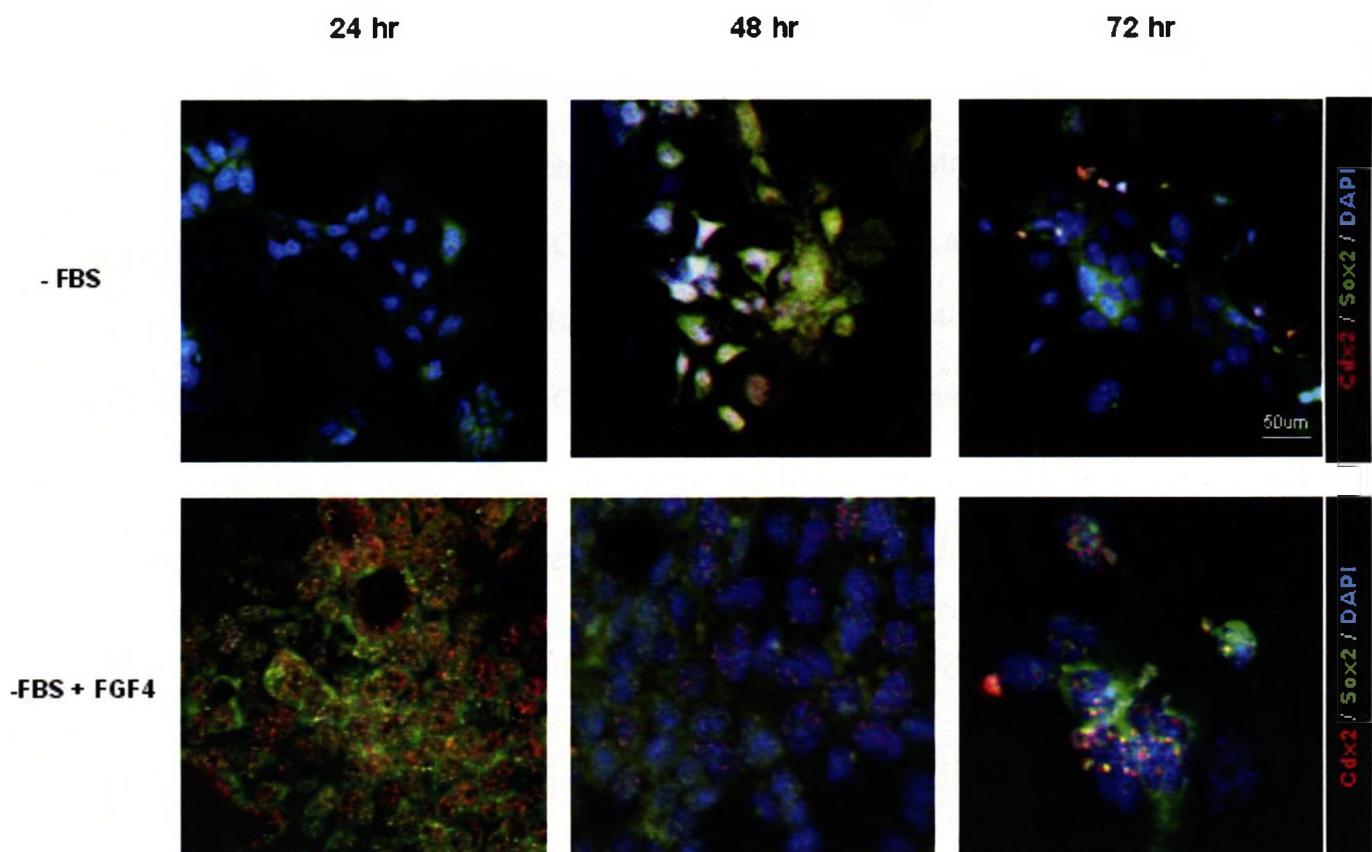
Figure 13. Expression analysis of self-renewal (OCT4, SOX2) and trophoblast markers (CDX2) in TS cells grown in the presence of FGF-4. (A) In the absence of fetal bovine serum (-FBS), CDX2, SOX2 and OCT4 localized to the cytoplasmic compartment based on western-blotting analysis. The addition of FGF-4 increased nuclear OCT4 activity at 48 hours post-starvation; while having no affect on the expression of SOX2. GAPDH was used as a loading control to ensure no contamination between compartments. **(B)** The addition of FGF-4 increased nuclear CDX2 expression (TRITC) at 24 hours post-starvation. By 72 hours, its expression translocated to the cytoplasmic compartment. Addition of FGF-4 did not change the expression pattern of SOX2 (FITC), which was expressed in the cytoplasm at all three time points, confirming western-blotting data.

Figure 13.

A



B

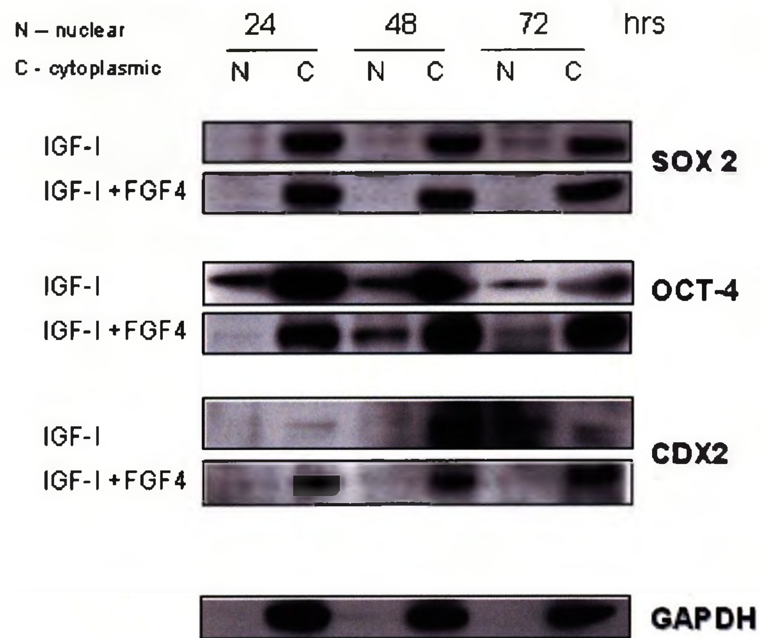


3.4.1.3. ***Effects of IGF-I***

In the presence of IGF-I only, OCT4 nuclear expression was restored at 24 hours post-starvation relative to cells grown in the absence of FBS, and its expression decreased by 48 hours with the lowest expression levels observed at 72 hours post-starvation (Fig. 14A). In comparison, SOX2 nuclear expression was not restored upon the addition of IGF-I (Fig. 14A). This was confirmed by immunocytochemistry which demonstrated that SOX2 expression was restricted to the cytoplasmic compartment upon the addition of IGF-I (Fig. 14B). This finding suggests that OCT4 and SOX2 may not work synergistically in early committed mouse TS cells. As seen upon the addition of FGF-4 alone (Fig 13A), the addition of FGF-4 in the presence of IGF-I restored the nuclear expression of OCT4 at 48 hours post-starvation, in contrast to 24 hours (Fig. 14A). This result confirmed earlier data that demonstrated the highest level of differentiated cells at 48 hours during growth factor deprivation (Fig. 7). Neither the addition of IGF-I alone, nor IGF-I in the presence of FGF-4 appeared to restore nuclear expression of both SOX2 and CDX2 (Fig. 14A). However, based on immunocytochemistry, the addition of IGF-I only restored nuclear expression of CDX2 at 24 hours post-starvation, which translocated to the cytoplasm by 48 hours (Fig. 14B). The addition of FGF-4 in the presence of IGF-I also restored nuclear expression of CDX2 at 24 hours post-starvation, with a decrease in nuclear expression by 48 hours post-starvation. In the presence of both IGF-I and FGF-4, there was no increased nuclear expression of CDX2 (Fig. 14B).

Figure 14. Expression analysis of self-renewal (OCT4, SOX2) and trophoblast markers (CDX2) in TS cells grown in IGF-I in the presence and absence of FGF-4. **(A)** Based on western-blotting, the addition of IGF-I restored nuclear OCT4 activity at 24 and 48 hours post-starvation, but SOX2 and CDX2 expression was not restored. In the presence of FGF-4 and IGF-I, OCT4 expression was restored at 48 hours post-starvation. GAPDH was used as a loading control to ensure no contamination between compartments. **(B)** Unlike CDX2 expression data by western-blotting, the addition of IGF-I increased nuclear CDX2 (TRITC) localization at 24 hours post-starvation, which translocated to the cytoplasm by 48 hours. SOX2 (FITC) localized to the cytoplasm upon the addition of IGF-I, supporting western-blotting data. The addition of FGF-4 in the presence of IGF-I appeared did not have a combined effect to increase SOX2 and CDX2 nuclear expression.

Figure 14.



B

24 hr

48 hr

72 hr

IGF-I

IGF-I +FGF4

Cdx2 / Sox2 / DAPI

50um

Cdx2 / Sox2 / DAPI

3.4.1.4 Effects of IGF-II

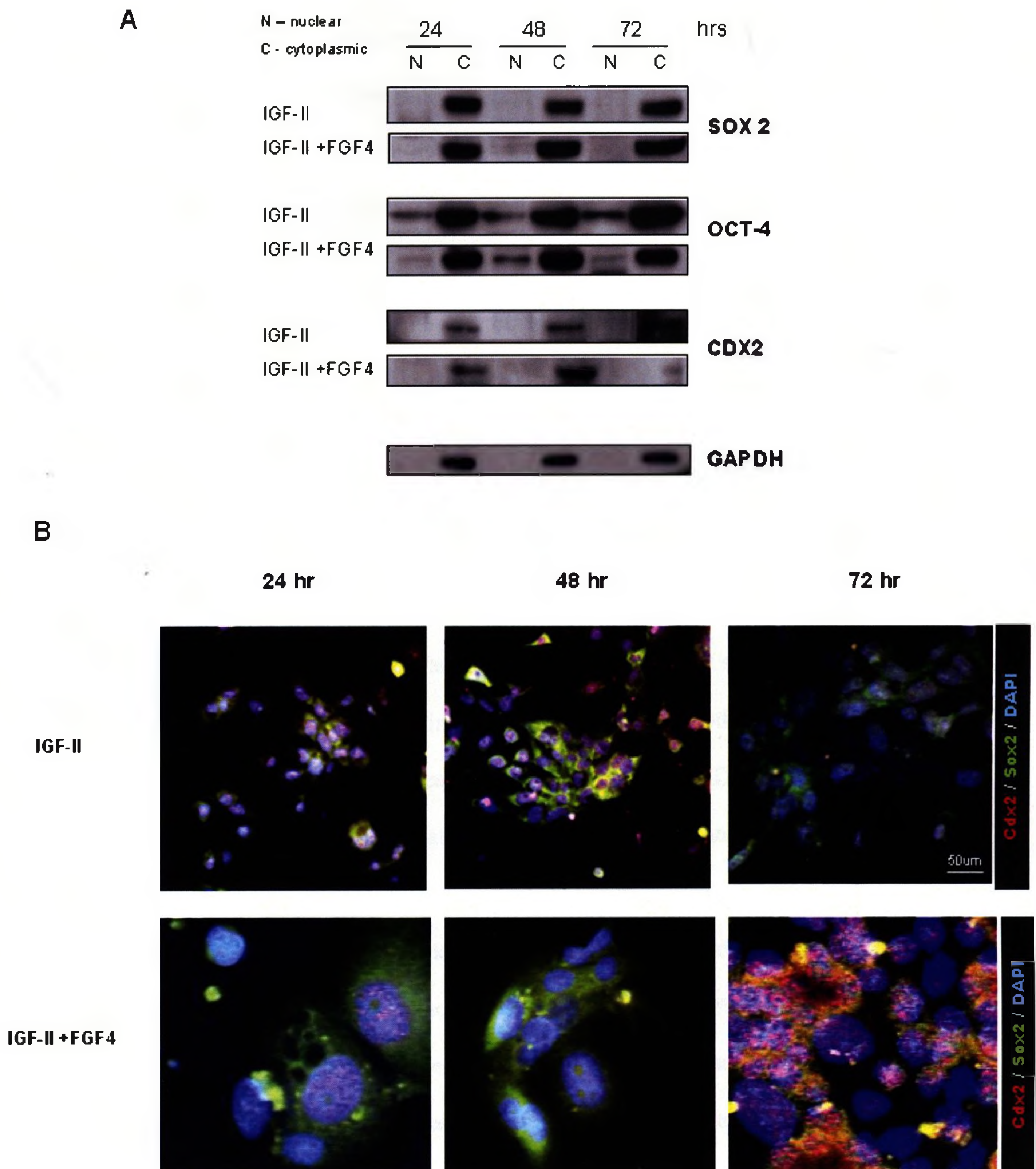
Since IGF-II has a critical role in early development, particularly in early fetal and placental development (71;138), it is expected that IGF-II will have an effect on the self-renewal of TS cells under serum deprived conditions. The addition of IGF-II restored nuclear OCT4 expression at 24 hours post-starvation relative to cells grown in serum deprived conditions (Fig. 15A). Interestingly, this restored Oct-4 expression was extended up to 72 hours post-starvation, compared to that of cells treated with IGF-I only (Fig. 15A). As in the case with IGF-I treated cells, the addition of IGF-II did not restore SOX2 nuclear expression (Fig. 15A). This was confirmed by immunocytochemistry analysis which demonstrated SOX2 was restricted within the cytoplasmic compartment (Fig. 15B). Unlike western-blotting analysis in which CDX2 expression was restricted to the cytoplasm (Fig. 15A), immunocytochemistry analysis revealed that the addition of IGF-II restored CDX2 localization within the nuclear and perinuclear compartments (Fig. 15B) compared to cells grown in serum deprived conditions (Fig. 13B) The CDX2 antibody did not prove to be sensitive enough to be used in western-blotting analysis.

The addition of FGF-4 in the presence of IGF-II restored OCT4 nuclear expression at 48 hours post-starvation (Fig. 15A). This was an interesting phenomenon that occurred in all samples treated with FGF-4. However, unlike in cells treated with IGF-I, the addition of IGF-II in the presence of FGF-4 prolonged the nuclear expression of Oct-4 at 72 hours post-starvation (Fig. 7). Neither the addition of IGF-II alone nor a combination of IGF-II and FGF-4 restored nuclear expression of the self-renewal marker, SOX2 as well as the trophoblast-specific marker, CDX2 (Fig. 15A). However, by immunocytochemistry, CDX2 was localized to the nuclear and perinuclear compartments

upon treatment with IGF-II and FGF-4 (Fig. 15B). There was no additive effect on the expression of CDX2 in the presence of both IGF-II and FGF-4. Regardless of whether treated with IGF-II or in the presence of both IGF-II and FGF-4, SOX2 was predominantly localized in the cytoplasm (Fig. 15B), confirming the western-blotting data.

Figure 15. Expression analysis of self-renewal (OCT4, SOX2) and trophoblast markers (CDX2) in TS cells grown in IGF-II in the presence and absence of FGF-4. **(A)** Based on western-blotting, the addition of IGF-II restored nuclear OCT4 expression, while expression of CDX2 and SOX2 was restricted to the cytoplasmic compartment. Upon the addition of FGF-4 in the presence of IGF-II, OCT4 nuclear expression was restored at 48 hours post-starvation, and extended up to 72 hours. The addition of FGF-4 had no effect at restoring the expression of SOX2 and CDX2. GAPDH was used as a loading control to ensure no contamination between compartments. **(B)** SOX2 (FITC) was predominately localized in the cytoplasmic compartment, regardless of the presence or absence of FGF-4 in cells treated with IGF-II, confirming western-blotting data. However, CDX2 (TRITC) expression was restored in the nuclear and perinuclear compartments of TS cells treated with IGF-I in the presence and absence of FGF-4.

Figure 15.



3.4.2. DNA-binding capacity of OCT4 during TS cell renewal

OCT4 is thought to be the main regulator in controlling the switch between self-renewal and differentiation of stem cells. Its expression in the nucleus indicates that cells are self-renewing, while its absence indicates that stem cells have begun the process of commitment and differentiation (147;148). While OCT4 has been shown to be expressed in human and bovine TS cells at detectable levels, in mouse models this transcription factor hasn't yet been reported as being expressed. However, mouse models show high levels of SOX2 expression, which in stem cells is known to work synergistically with OCT4 (168;169).

To test if IGF-I and IGF-II are able to change the capacity of OCT4/SOX2 in binding DNA, thus being functional within the nucleus, the OCT4 protein-DNA interaction was analyzed by electrophoretic mobility shift assay (EMSA) using a previously published DNA probe containing overlapping binding sites for both OCT4 and SOX2 (Fig. 16) (170). Summarizing the data on the immuno-detection of OCT4 in TS cells, it was evident that the nuclear expression of OCT4 in TS cells grown under standard growth conditions decreased at 72 hours post-starvation and corresponded with significant spontaneous differentiation (Fig. 16A). The removal of serum resulted in a decrease in OCT4 expression by 48 hours post-starvation, with low expression by 72 hours, as observed by decrease in the OCT4-DNA complex band on the gel. The addition of FGF-4 alone in serum deprived conditions compensated for the lack of growth factors and prolonged nuclear expression of OCT4 at 72 hours post-starvation. In the presence of IGF-I alone, there was an unexpected increase in OCT4 expression from

24 to 72 hours post-starvation. The addition of FGF-4 in the presence of IGF-I increased OCT4 expression with the highest levels at 48 hours, followed by a decrease by 72 hours post-starvation. In the presence of IGF-II only, OCT4 expression increased from 24 to 72 hours, while the addition of FGF-4 in the presence of IGF-II had an additive effect on the increased expression of nuclear OCT4.

To confirm that the DNA probe was specific for the binding of OCT4, a scrambled DNA probe was designed and incubated with nuclear lysates to detect non-specific binding (Fig. 16B). Results indicated that the DNA probe specific. Since the DNA probe contains overlapping binding sites for OCT4 and SOX2, the identity of the DNA-bound protein was analyzed using increasing antibody concentrations. Antibodies against OCT4 were separately added and incubated in the EMSA reaction mixture to bind the OCT4 proteins, which will generate super shift-complexes (DNA probe-OCT4-Ab). The probe was specific for detecting the presence of OCT4, and the addition of an OCT4 antibody demonstrated that shifts indeed contain OCT4 protein. Moreover, this test re-confirmed the presence of OCT4 in the nuclear samples and its capacity of binding DNA. Therefore, we concluded that the immuno-detected form of OCT4 was capable of binding its cognate site. Regarding the specificity test, two different commercially available antibodies, both previously used for immuno-precipitation, were used to identify OCT4. Only one of them was shown to be effective in super-shifting in EMSA assays. As a consequence, a decrease in the OCT4 DNA-protein complex was observed, indicating that antibody was binding to the radio-labeled complex, decreasing its relative mobility (Fig. 16B). In the presence of SOX2 antibodies, no super-shifted complex was detected,

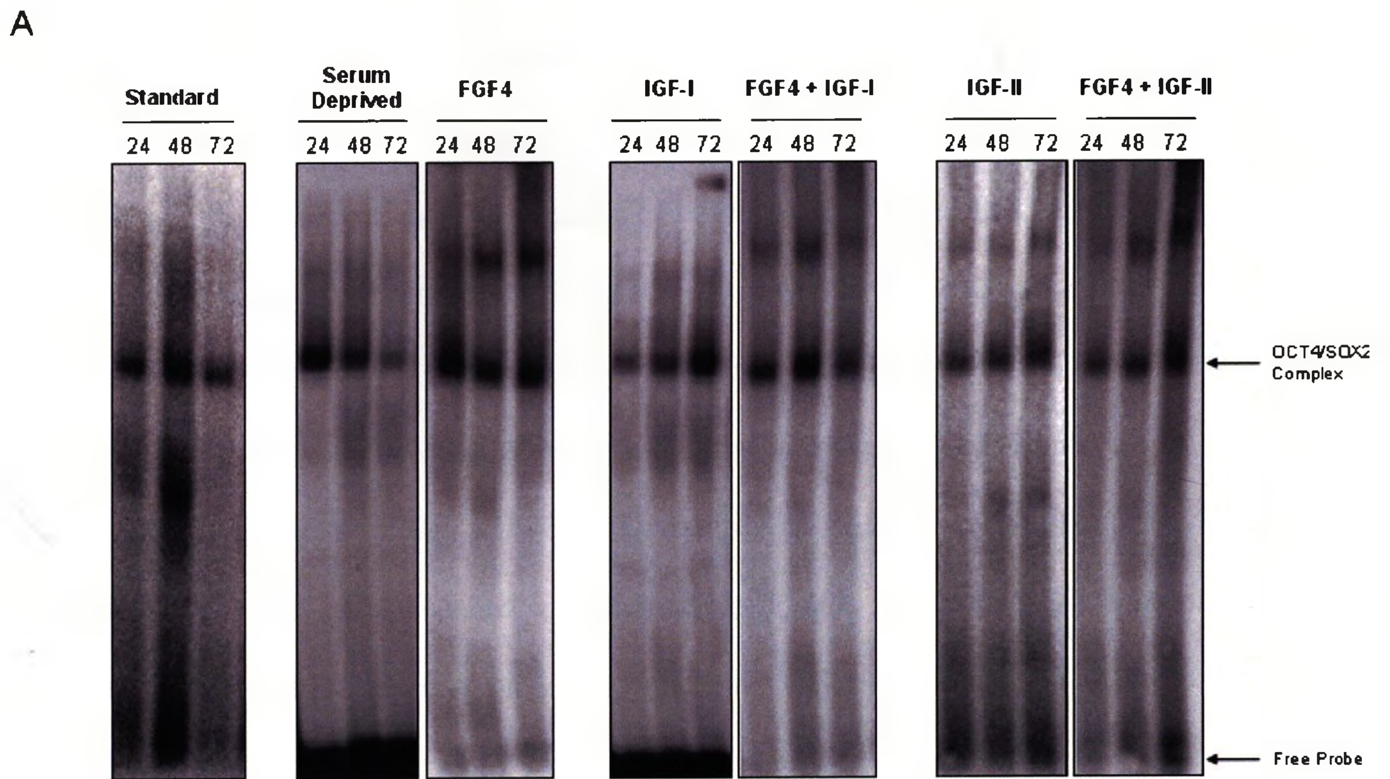
demonstrating that SOX2 was not a part of the DNA-protein complex, and that the DNA probe was specific for binding OCT4 protein and no other proteins (Fig. 16B).

Figure 16. Analysis of IGF effects on OCT4 DNA-binding capacity by EMSA.

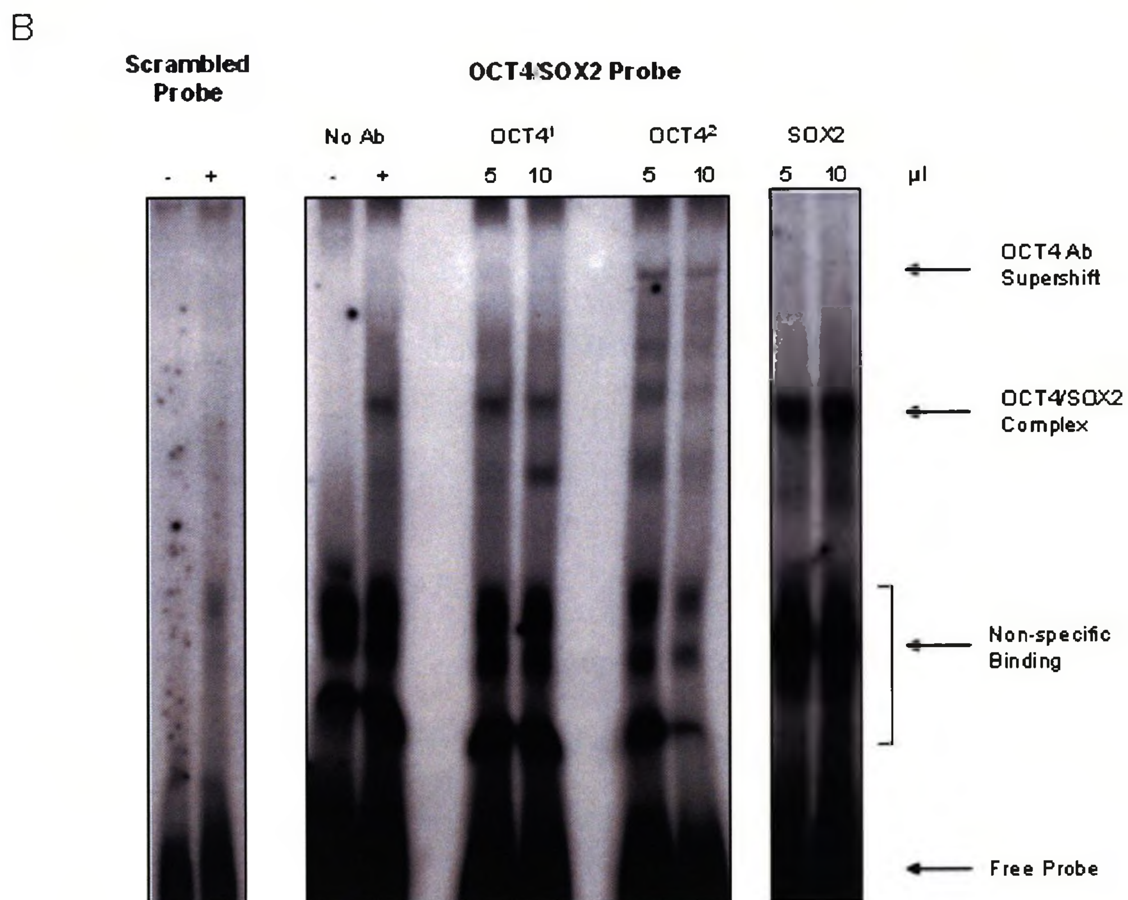
(A) The removal of serum resulted in a drastic decrease in OCT4 expression by 48 hours post-starvation with low expression by 72 hours. In the presence of IGF-I only, there was an unexpected increase in OCT4 expression from 24 to 72 hours post-starvation. The addition of FGF-4 in the presence of IGF-I increased OCT4 expression with the highest levels at 48 hours, followed by a decrease by 72 hours post-starvation. In the presence of IGF-II only, OCT4 expression increased from 24 to 72 hours, while the addition of FGF-4 in the presence of IGF-II had an additive effect on the increased expression of nuclear OCT4. **(B)** To test the specificity of the probe, a scrambled probe used in EMSA revealed no DNA-protein shift. Since the DNA probe contains binding sites overlapping binding sites for OCT4 and SOX2, the identity of the bound protein was analyzed using increasing concentrations of OCT4 and SOX2 antibodies. Two different commercial antibodies both known to be used for immuno-precipitation, were used to determine OCT4. Only one of them proved effective in super-shifting EMSA complexes. As a consequence, a decrease in the OCT4 DNA-protein complex was observed, indicating that antibody was binding to the radio-labeled complex, decreasing its relative mobility. In the presence of SOX2 antibodies, no super-shifted complex was detected, demonstrating that SOX2 was not apart of the DNA-protein complex, and that the DNA probe was specific for binding OCT4 protein.

Figure 16.

A



B



[1]Abcam
[2]Neomarkers

3.5. IGF effects on TS cell differentiation

As TS cells differentiate, changes in marker expression occur in which cells begin to express markers of later cell types of the trophectoderm lineage and a decrease in markers of the blastocyst, extra-embryonic ectoderm, and ectoplacental cone cell types. To assess the potential effects of IGF-I and IGF-II, as well as in combination with FGF-4 on the differentiation of TS cells, the expression of an early giant cell marker, placental lactogen-1 (PL-1), was analyzed by western-blotting in cytoplasmic fractions, as well as by immuncytochemistry (Fig. 17A-B). Two types of positive cells were identified: mononucleated and polynucleated. Mononucleated cells localized PL-1 within the cytoplasm, while PL-1 expression in polynucleated cells was localized more to the perinuclear region. This might be indicative of differentiation into two separate trophoblast lineages. The percentage of PL-1 positive cells was quantified to provide an indication as to the effects of IGF-I and IGF-II on differentiation of TS cells (Fig. 17C). For each treatment, three images were taken at 24, 48 and 72 hours, and the number of PL-1 positive cells was counted and expressed as a percentage relative to the total number of cells in the field. Data shown are standard deviations for two separate experiments performed.

3.5.1 Effects of Fetal Bovine Serum (FBS)

Subjecting TS cells to serum deprivation upon the removal of FBS and FGF-4 caused a slightly increased PL-1 expression based on Western-blotting analysis, relative to cells grown under standard conditions of growth (Fig. 17A); this increase was not

significant as revealed by densitometry analysis. Based on morphology, immunocytochemistry analysis of PL-1 expression demonstrated by 72 hours post-starvation, there were fewer numbers of total cells present in serum deprived conditions, and the overall morphology of cells by this time point changed as cellular debris was present, suggesting cell death was likely occurring (Fig. 17B). Closer examination of the percentage of PL-1 positive cells in serum deprived conditions revealed a significant increase in PL-1 expression over time relative to cells grown in standard growth conditions (Fig. 17C).

3.5.2 Effects of IGF-I

The addition of IGF-I increased the expression of the giant cell marker, PL-1, relative to cells grown in standard growth conditions (Fig. 17A). Although this increase was not substantial, densitometry revealed that this increase was greater than TS cells grown in standard conditions. By 72 hours post-starvation, expression of PL-1 in IGF-I treated cells was similar to that of cells grown in serum deprived conditions. Immunocytochemistry demonstrated that the addition of IGF-I alone resulted in an overall increase in PL-1 expression relative to cells grown in serum deprived conditions (Fig. 17B). Closer examination at the percentage of PL-1 positive cells confirmed that the addition of IGF-I had a significant increase at inducing differentiation of TS cells, relative to serum deprived conditions ($p < 0.04$) (Fig. 17C). Interestingly, the highest percentage of PL-1 positive cells was observed at 48 and 72 hours post-starvation, which coincided with earlier morphological data demonstrating that the addition of IGF-I alone induced the highest number of giant cells at this time (Fig. 8A).

The majority of PL-1 positive cells in IGF-I treated cells were polynucleated with perinuclear expression of PL-1, while the majority of PL-1 positive cells grown in serum deprived conditions were mononucleated (Fig. 17B).

The addition of FGF-4 in the presence of IGF-I resulted in a slight increase in PL-1 expression relative to serum deprived conditions, as revealed by Western-blotting analysis (Fig. 17A). Compared to cells treated with IGF-I only, levels of PL-1 expression where quite similar upon the addition of FGF-4 when analyzed by densitometry (Fig. 17A). Immunocytochemistry revealed that the majority of differentiated cells grown in the presence of both IGF-I and FGF-4 were polynucleated with perinuclear expression of PL-1; similar to that observed with IGF-I treated cells (Fig. 17B). Unlike IGF-I treated cells, based on the morphology, there was a higher degree of cellular debris and artifact staining in cells treated with IGF-I and FGF-4, suggesting a higher level of cell death. Interestingly, this confirmed the results obtained by TUNEL staining which demonstrated a higher percentage of TUNEL-positive cells treated with IGF-I and FGF-4 (Fig. 9B). Closer examination of the percentage of PL-1 positive cells revealed that the addition of IGF-I and FGF-4 caused a decrease relative to serum deprived conditions, but was not significant. However, compared to IGF-I treated cells, the addition of FGF-4 resulted in a significant decrease in the percentage of PL-1 positive cells ($p < 0.0001$) (Fig. 17C). Furthermore, this decrease in PL-1 staining observed corresponds with earlier morphological data that demonstrated a significant reduction in the number of giant cells upon the addition of FGF-4 in the presence of IGF-I (Fig. 8B).

3.5.3 Effects of IGF-II

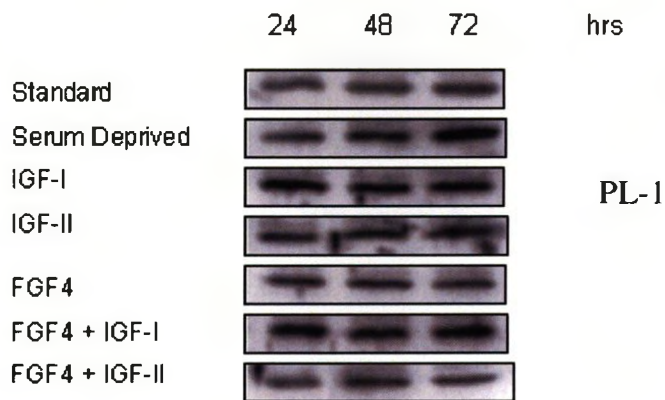
Western-blotting analysis did not reveal significant differences between the addition of IGF-II relative to serum deprived conditions (Fig. 17A). Differences in PL-1 expression between IGF-I and IGF-II treated cells were minor, with IGF-I having a slightly increased expression, but not significant (Fig. 17A). Immunocytochemical analysis provided a clearer indication of the effects of IGF-I and IGF-II on the differentiation of TS cells. The morphology and levels of PL-1 positive cells in IGF-I and IGF-II treated cells were similar to each other at 24 hours post-starvation (Fig. 17B). However, by 48 and 72 hours the morphology of cells grown in IGF-II was improved compared to IGF-I treated cells, with fewer PL-1 positive cells. The majority of cells positive for PL-1 expression in the presence of IGF-II were mononucleated with PL-1 localized to the cytoplasmic compartment (Fig. 17B); similar to the expression localization observed in serum deprived conditions. This effect was in contrast to cells treated with IGF-I only, in which the majority of positive cells were polynucleated with PL-1 localized to the perinuclear region. Analysis of the percentage of PL-1 positive cells demonstrated a differential role between IGF-I and IGF-II on their effects on differentiation of TS cells. Compared to IGF-I, the addition of IGF-II significantly decrease in the percentage of PL-1 positive cells ($p < 0.0001$) (Fig. 17C). This result is supported by earlier morphological analysis (Fig. 8B), which demonstrated that the addition of IGF-II caused a significant decrease in giant cell formation compared to IGF-I treated cells, suggesting that IGF-I influences the differentiation of TS cells, while IGF-II functions to inhibit the effects of differentiation induced by serum deprivation.

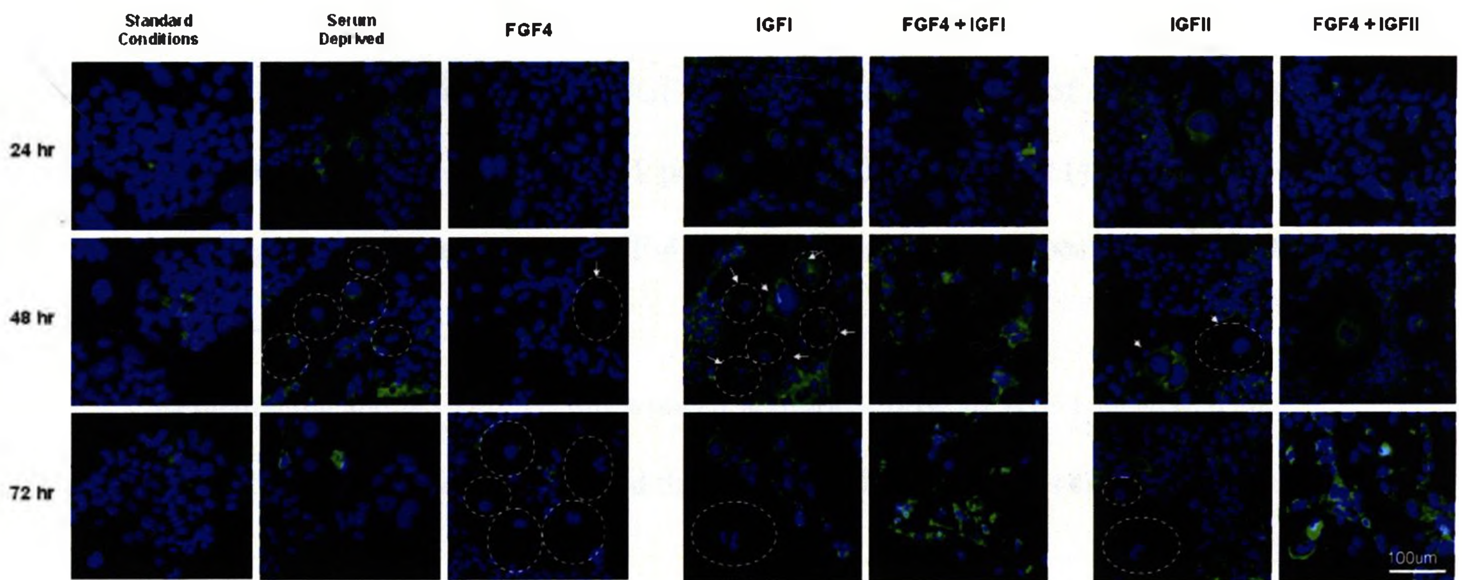
In the presence of FGF-4, the addition of IGF-II as opposed to IGF-I decreased PL-1 expression slightly; however these differences were not significant according to densitometry of Western-blotting analysis (Fig. 17A). Relative to cells grown in FGF-4 alone, the addition of IGF-II also did not appear to have a major effect on the expression of PL-1 as assessed by western blot. Based on morphology, immunocytochemistry analysis did not show any drastic differences between the addition of IGF-II in the presence and absence of FGF-4 (Fig. 17B). Further examination at the percentage of PL-1 positive cells confirmed this, demonstrating that the addition of FGF-4 did not cause a significant difference (Fig. 17C). Comparing the effects of IGF-I and IGF-II in the presence of FGF-4, the addition of IGF-I as opposed to IGF-II resulted in more cellular debris and cellular destruction, but the levels of PL-1 positive cells appeared to be similar (Fig. 17B). Closer examination at the percentage of PL-1 positive cells confirmed this data as levels of PL-1 positive cells were quite similar between IGF-I and IGF-II treated cells grown in the presence of FGF-4, with no significant differences (Fig. 17C).

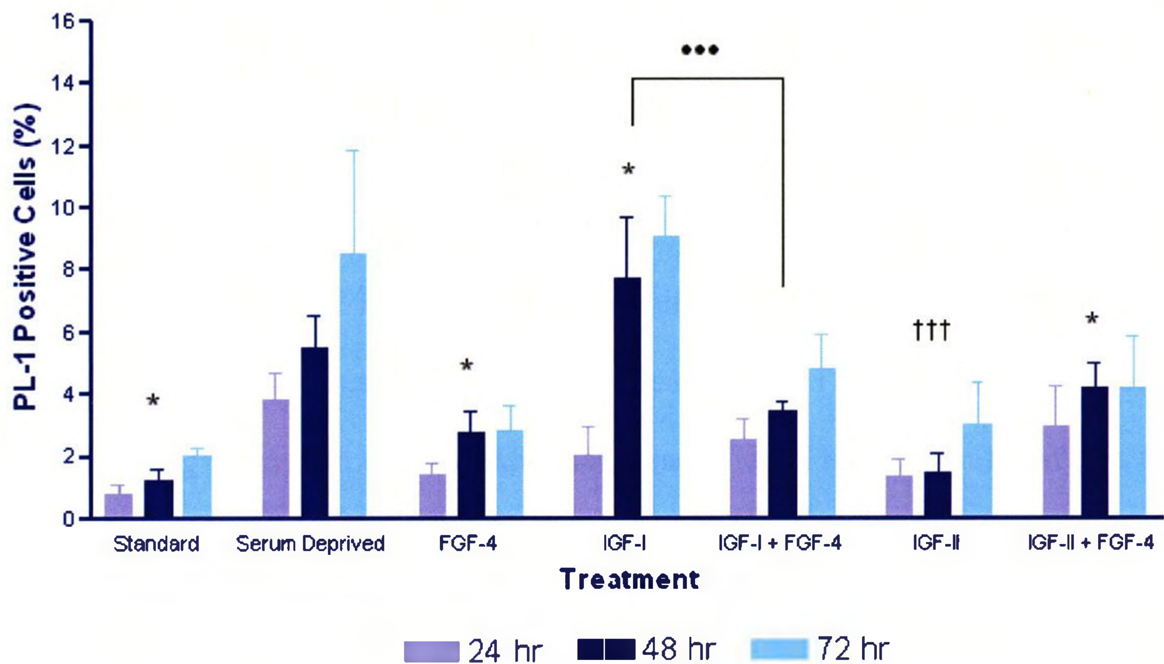
Overall, all these results suggest that IGF-I alone influenced the differentiation of TS cells, and when grown in the presence of FGF-4, a significant decrease in differentiation is observed, which may be the result of cells undergoing apoptosis as opposed to differentiation. IGF-II however, appeared to decrease the effects of differentiation and performed better independently of FGF-4.

Figure 17. Effects of IGF-I and IGF-II on the differentiation of TS cells.

(A) Western-blotting analysis revealed a slight increase in PL-1 expression in those TS cells treated with IGF-I, and IGF-I + FGF-4 based on densitometry. **(B)** The addition of IGF-I resulted in a high number of PL-1 positive polynucleated cells with perinculear expression compared to serum deprived cells with mononucleated cytoplasmic expression. The addition of FGF-4 in the presence of IGF-I resulted in similar perinuclear expression of PL-1, with a higher degree of cellular debris by 72 hours. In the presence of IGF-II, PL-1 positive cells were mononucleated positive cells containing cytoplasmic expression. The addition of FGF-4 in the presence of IGF-II reduced the appearance of cellular debris. Arrows indicated PL-1 positive cells **(C)** Subjecting TS cells to serum deprivation caused a significant difference in the formation of PL-1 positive cells, relative to cells grown under standard growth conditions ($p < 0.03$). The addition of IGF-I caused a significant increase relative to serum deprived conditions ($p < 0.04$). The addition of IGF-II caused a significant decrease in the percentage of PL-1 positive cells relative to cells treated with IGF-I ($p < 0.0001$). In the presence of IGF-I, the addition of FGF-4 caused a significant decrease in PL-1 positive cells relative to cells grown strictly in IGF-I ($p < 0.0001$), while in the presence of IGF-II, the addition of FGF-4 caused no significant decrease. Data shown are standard deviations two experiments performed in triplicate. Statistical analysis was performed using a Two-way ANOVA comparing the effects of both treatment and time on the formation of giant cells (the signs *, ** and *** denote $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). The symbol * denotes treatments vs. serum deprived conditions; † denotes IGF-I vs. IGF-II (± FGF-4); • denotes IGF-I/-II vs. IGF-I/-II (+ FGF-4).


A
24
48
72
hrs
Standard
Serum Deprived
IGF-I
IGF-II
FGF4
FGF4 + IGF-I
FGF4 + IGF-II
PL-1


B
Standard Conditions
Serum Deprived
FGF4
IGFI
FGF4 + IGFI
IGFII
FGF4 + IGFII
24 hr
48 hr
72 hr
100um


C
PL-1 Positive Cells (%)
0
2
4
6
8
10
12
14
16
Standard
Serum Deprived
FGF-4
IGF-I
IGF-I + FGF-4
IGF-II
IGF-II + FGF-4
Treatment
24 hr
48 hr
72 hr
*
•••
†††

3.5.4 Effects of Dosage of IGF-I/II

Since the half-life of IGFs is ~12-16 hours (137), the individual effects of IGF-I and IGF-II on differentiation were examined more closely by replenishing TS cells with an additional dose of either 100 ng/ml IGF-I or IGF-II, 24 hours after initial IGF dose. TS cells were grown under the same conditions in which they were subjected to serum deprivation 24 hours post-seeding and treated with either 100 ng/ml IGF-I or IGF-II. Following 24 hours after initial IGF treatment, TS cells were then treated with an additional dose of 100 ng/ml IGF-I or IGF-II and the effects of replenishing the system with an additional dosage of IGF-I or IGF-II on the differentiation of TS cells was analyzed by examining the levels of PL-1 positive cells 24 hours later (48 hours post-starvation) (Fig. 18). The dosage of FGF-4 was not altered, as the specific effects of IGF-I and IGF-II were being examined.

Replenishing the TS cell system with an additional dose of IGF-I improved the morphology of TS cells, as well as reduced the number of PL-1 positive cells (Fig. 18A). Closer examination of the effect of two treatments compared to a single treatment of IGF-I on the percentage of PL-1 positive cells at 48 hours post-starvation showed a 2.6–fold decrease; however this decrease was not statistically significant (Fig. 18B). On the other hand, treatment with an additional dose of IGF-II did not have an effect on cell morphology, as well as the relative number of PL-1 positive cells, compared to cells treated with one dose of IGF-II (Fig. 18A). Examination of the percentage of PL-1 positive cells confirmed this result, demonstrating that treating TS cells with either one dose or replenishing the system with an additional dosage did not have a significant effect at reducing the percentage of PL-1 positive cells (Fig. 18B), suggesting that one

treatment of IGF-II is sufficient enough to inhibit the effects of differentiation induced by serum deprivation.

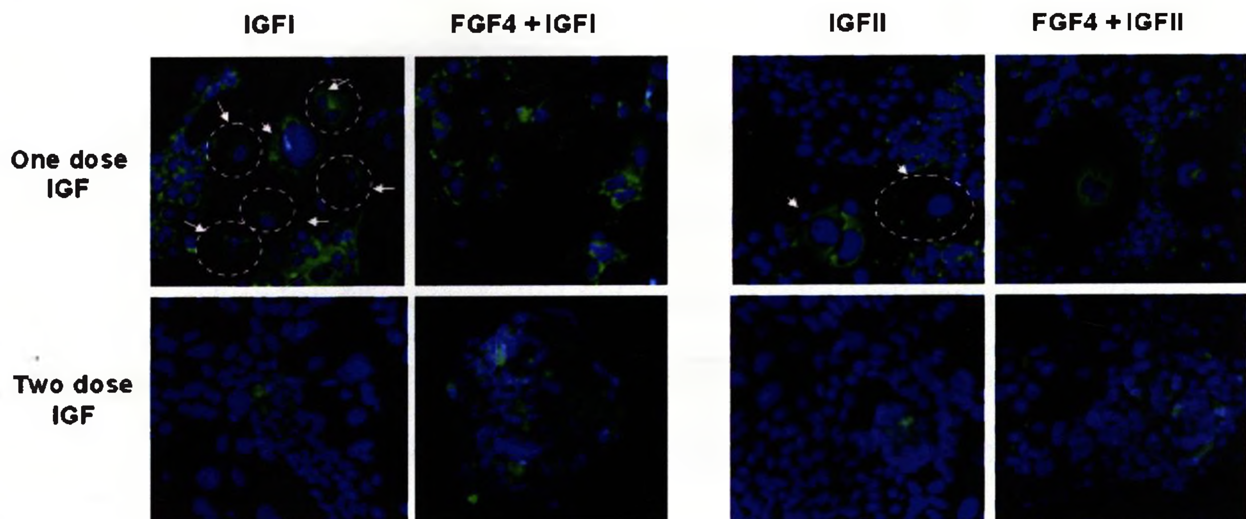
In the presence of FGF-4, replenishing the TS cell system with an additional dose of either IGF-I or IGF-II did not effect the morphology of TS cells or PL-1 positive cells (Fig. 18A). Closer examination of the percentage of PL-1 positive cells revealed that the addition of two doses of IGF-I as opposed to one dose, caused a slight 1.4-fold increase in PL-1 positive cells (Fig. 18B). On the other hand, the addition of two doses of IGF-II, as opposed to one dose, in the presence of FGF-4 caused a 2.5-fold decrease in PL-1 positive cells (Fig. 18B). Interestingly, the effect of replenishing the system with two doses of IGF-II in the presence of FGF-4 reduced levels of PL-1 positive cells similar to that of treatment of cells with IGF-II only, but was not statistically significant.

These results suggest that IGF-I promotes differentiation of TS cells, and replenishing the system with an additional dose of IGF-I reduces the effects of differentiation. IGF-II inhibits the effects of differentiation, and replenishing the system with more IGF-II does not have added benefit. In the presence of FGF-4 however, IGF-I decreased differentiation and was not affected by replenishing the system. Although IGF-II appears to function efficiently independent of FGF-4 at reducing the effects of differentiation induced by serum deprivation, replenishing the system with an additional dose of IGF-II, when grown in the presence of FGF-4, seems to restore the inhibition of differentiation.

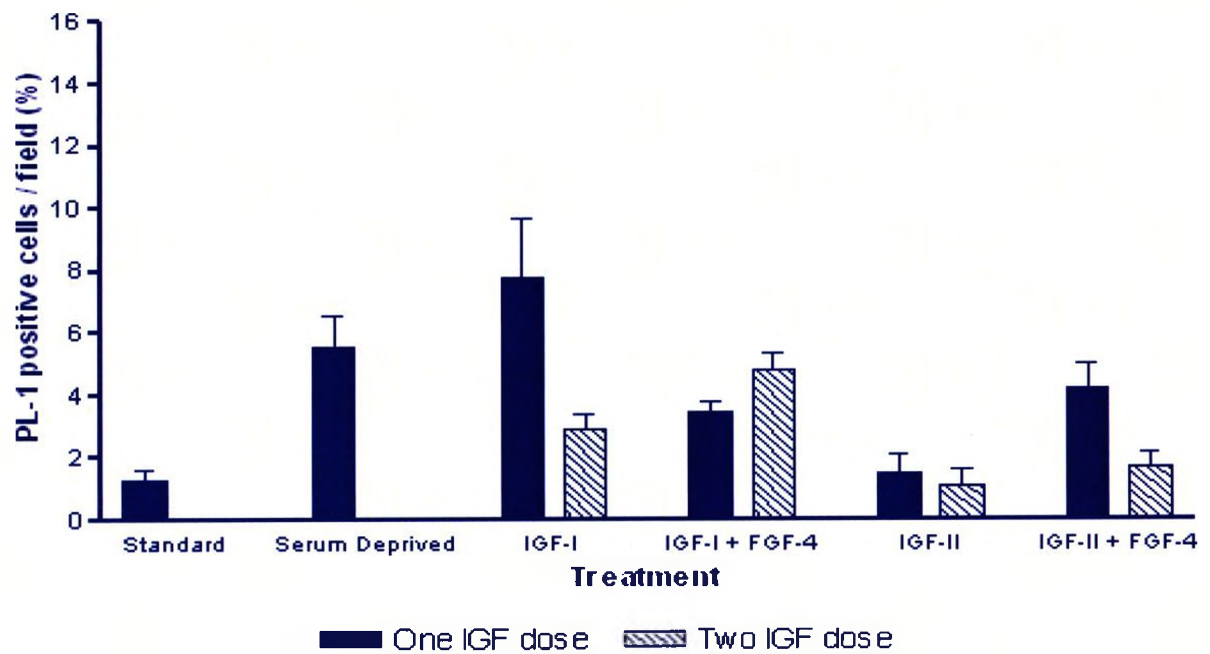
Figure 18. Effect of IGF-I and IGF-II dosage on TS cell differentiation at 48 hours. Since IGFs have a half-life of ~12-16h, the effects of replenishing the TS cell system with an additional dosage of IGF-I or IGF-II, 24 hours after initial IGF treatment, were examined. **(A)** The addition of two doses of IGF-I improved the morphology of TS cells and there appeared to be a reduction in PL-1 positive cells, while the addition of IGF-II had no apparent effect on cell morphology. In the presence of FGF-4, no drastic changes were observed in either IGF-I or IGF-II treated cells. Arrows indicated PL-1 positive cells. **(B)** Analysis of the percentage of PL-1 positive cells at 48 hours post-starvation, suggested that IGF-II, regardless of the dosage, is effective at inhibiting the process of differentiation of TS cells with a 2.8–fold decrease in PL-1 positive cells relative to serum deprived cells. On the other hand, IGF-I appeared to be dosage dependent, as the addition of two doses of IGF-I caused a 2.4-fold decrease relative to cells treated with one dose of IGF-I. In the presence of FGF-4, the addition of two doses of IGF-I as opposed to one dose of IGF-I caused an increase in the percentage of PL-1 positive cells by 2.2–fold. On the other hand, the addition of two doses of IGF-II in the presence of FGF-4 resulted in a decrease in PL-1 positive cells by 2.6–fold. Statistical analysis was performed using a one-way repeated measures ANOVA, comparing the effect of two treatments of IGF with one treatment. (n=2).

Figure 18.

A



B



CHAPTER 4: DISCUSSION

4.1 Current Status and Future Directions

4.1.1. Growth Factors as Part of the TS Cell Niche

Stem cells can undergo a variety of cell fates including self-renewal, differentiation and cell death. How these different processes are regulated, and the mechanisms by which cell fate is controlled and regulated is not well understood. Recent evidence suggests that the fate of stem cells is not autonomously achieved, but requires a certain level of external control from the micro-environment. Theoretically, a typical stem cell niche consists of a variety of growth factors and cytokines acting in an autocrine, paracrine and/or endocrine manner on a variety of cell-surface receptors. A recent study provided the first evidence that human ES cells possess the ability to autonomously generate a stem cell niche *in vitro*, despite removal from their *in vivo* microenvironment (171). This was a remarkable finding, which demonstrated that IGF and FGF can cooperatively work to establish the regulatory stem cell niche in human ES cells. This previous study, together with my current observations, suggests that the IGF and FGF family also work together to regulate the stem cell niche of trophoblast-derived stem cells.

In this study, the fate of TS cells was investigated by adding external growth factors in the medium (IGF-I, IGF-II and FGF-4), either individually or in combination, and examining their effects on the stem cell niche. In the present TS cell model, FCM and FBS were added directly to the system to provide an array of growth factors and

cytokines; and upon its removal, differentiation and/or cell death, by either apoptosis or necrosis, occurred. FGF-4 signaling is essential for the maintenance of TS cells (21); however, FGF-4 alone was not sufficient, since TS cells usually require additional growth factors and cytokines present in FCM for their maintenance. Members of the TGF-β superfamily have been implicated as important components in FCM that contribute to the maintenance of TS cells, particularly Nodal and activin (141). However, the fact remains that other growth factors present in FCM play a critical role in contributing to the maintenance of TS cell proliferation. Since IGF-I and IGF-II play an important role during early mouse development (71;138), I investigated their role on the fate of TS cells by growing cells in the absence of FCM and FBS, as well as examining the individual effects of IGF-I and IGF-II, and in combination with FGF-4, on self-renewal, differentiation, and apoptosis of TS cells.

4.1.2. IGFs and Apoptosis

Formation of the placenta requires a proper balance between cell proliferation, differentiation and cell death, and this balance is thought to be regulated by growth factors. The maintenance of undifferentiated and proliferating TS cells requires the presence of FGF-4, which binds to FGFR2, activating the Ras/Erk signaling pathway, resulting in the proliferation and self-renewal of TS cells (60;61). Since its removal promotes the differentiation of TS cells as opposed to apoptosis, it is not clear whether FGF-4 signaling through the Erk pathway is the only biological effect on TS cells (21), suggesting that other signaling pathways and growth factors may be responsible for regulating the fate of TS cells. Closer examination at the individual effects of IGF-I and

IGF-II on TS cells suggests that there are in fact, other growth factors responsible for controlling the fate of stem cells, and that IGF-I and IGF-II are likely critical candidates.

In the present study, the initial investigation of the morphology of TS cells as revealed by H&E staining demonstrated that IGF-I and IGF-II induced a varying degree of both differentiation and cell death. IGF-I induced greater cell death and differentiation compared to that of the effects of IGF-II (Fig. 7). IGF-I has been demonstrated to act in an anti-apoptotic manner in a variety of cell types (172;173), as well as in a variety of stem cells (174;175), however, its role in protection against apoptosis in stem cells of the placental origin has not been well characterized. Since IGF-I has not been shown to be important in regulating early mouse placental development (138), the effects induced by the addition of IGF-I to our placental stem cell system have to be questioned, as IGF-II is the prominent growth factor regulating early placental development in mice (71). Results from Annexin-V and TUNEL staining assays demonstrated that IGF-I and IGF-II had differential effects on TS cell apoptosis. IGF-II induced a greater level of protection against apoptosis compared to that of IGF-I, which was found to be significant ($p < 0.03$) (Fig. 9). This effect was not dependent on replenishing the system with an additional dose of either IGF-I or IGF-II 24 hours later after initial IGF treatment (Fig. 10). Due to these biological differences, it is highly suggestive that IGF-I and IGF-II are acting through different adaptor molecules at the IGF-IR level, in order to induce these different cellular responses. Since IGF-I had neither a dramatic increase nor decrease in the effects of apoptosis relative to serum deprived cells, it is highly suggestive that IGF-I alone does not have a substantial effect on either protecting or inducing cellular death this early in development.

4.1.3. IGFs and TS Cell Differentiation

Upon closer examination of the effects of IGF-I and IGF-II on differentiation of TS cells, IGF-I induced a significant increase in the percentage of differentiated cells as assessed by placental lactogen-1 (PL-1) expression, compared to IGF-II ($p < 0.0001$) (Fig. 17), indicating that IGF-I and IGF-II have differential effects on TS cell differentiation. Since IGF-II is a prominent growth factor regulating early placental development (71), and is expressed earlier than IGF-I (78), it is likely that during this stage of development, IGF-II is acting to promote the maintenance of TS cells by inhibiting the effects of differentiation and apoptosis, and the actions of IGF-I are not required until later in development. However, since the addition of IGF-I at a physiological dose to our stem cell system induced the onset of both differentiation in TS cells, this is highly suggestive that IGF-I functions to control this 'switch', meaning that IGF-I is likely the primary regulator of differentiation in TS cells. The mechanism by which this 'switch' is controlled requires further examination. The switch from a TS cell in a mitotic cell cycle to a giant cell in an endoreduplicative cell cycle is controlled by a shift in the expression of several cell cycle regulators. Cyclins E1 and E2 have been shown to be essential for the endoreduplicative cell cycle of trophoblast giant cells (176;177). In addition, absence of the cell cycle regulator Geminin induces endoreduplication within cells, allowing them to become polyploidy and incapable of further cell division; characteristics of trophoblast giant cells (178). Analysis of the effects of IGF-I and IGF-II on the differentiation of TS cells at the level of the cell cycle may provide further information regarding the roles of IGF-I and IGF-II on the differentiation of TS cells.

In the current study, differentiation was determined by analyzing levels of the early giant cell marker, PL-1. Further analysis, however could be performed by examining the expression levels of specific transcription factors, Mash-2 and Hand1. Both are basic helix-loop-helix (bHLH) transcription factors which are essential regulators of giant cell differentiation with opposing functions. Mash-2 functions to block giant cell differentiation (179), while Hand1 induces differentiation into giant cells (180). Examining the expression levels of these transcriptions factors upon the addition of IGF-I and IGF-II to TS cells may provide further information regarding the roles of IGF-I and IGF-II on the differentiation of TS cells.

4.1.4. IGFs and FGF-4: Cumulative or Opposing Effects?

Since FGF-4 alone is not sufficient in the maintenance of TS cells, and additional growth factors and cytokines present in CM are required, the effects of IGF-I and IGF-II in combination with FGF-4 were examined to see if there was an additive or inhibitory effect on either differentiation or cell death in TS cells. The presence of FGF-4 in combination with either IGF-I or IGF-II, did not have an additive effect at reducing the effects of apoptosis induced by serum deprivation. In fact, the opposite effect was observed with increased levels of apoptosis induced at later stages. These increases induced by the presence of FGF-4 were found to be significant [$p < 0.005$ (IGF-I + FGF-4); $p < 0.01$ (IGF-II + FGF-4)] compared to cells grown in the presence of either IGF-I or IGF-II alone (Fig. 9). Although IGF-I induced higher levels of apoptosis relative to IGF-II, both growth factors worked better independently of FGF-4, with IGF-II alone inducing the greatest level of protection against apoptosis amongst the growth factors. These data

suggest that the presence of FGF-4 in combination with either IGF-I or IGF-II, induced stress to the system, meaning that these growth factors do not work together in an additive manner.

Such a stress may have been generated by the onset of serum deprivation combined with intense proliferation (due to the high concentrations of growth factors) followed by rapid consumption of nutrients and metabolites. A recent study examined the effects of stress on TS cells by analyzing stress-induced stress-activated protein kinase/jun kinase (SAPK/JNK) activation, and found that SAPK/JNK was completely responsible for mediating cell cycle arrest and apoptosis in TS cells (181). Analyzing levels of activation of SAPK/JNK in TS cells grown under these different growth conditions may help to elucidate the response of the cell to these varying treatments. As mentioned, analysis of the receptors involved and activated downstream signaling pathways will provide more information on the increased apoptosis induced by the addition of either IGF-I or IGF-II with FGF-4. Furthermore, the effects of different growth factors (IGFs, EGF, and insulin) on cell proliferation and apoptosis were recently analyzed in corneal epithelium cells. Addition of IGF-I, IGF-II and insulin were found to increase cell proliferation and inhibit the process of apoptosis by activating the Akt pathway (182). Although IGF-I has a differential effect relative to our data, this may be a result of the cell type involved, as IGF-I is not vital in the development of early placental-type cells in mice. Therefore, we propose to explore the dynamics and the turn-over of the IGF-IR and other RTKs in TS cells in the future.

Upon analysis of differentiation in TS cells in the presence of FGF-4, the addition of IGF-I caused a significant decrease in the percentage of differentiated cells relative to

cells grown strictly in IGF-I (Fig. 17). This may also be a result of stress induced by the presence of both growth factors, thereby disrupting the differentiation of TS cells into giant cells induced by IGF-I. Based on morphological analysis, this stress induced by the presence of both FGF-4 and IGF-I seems to result in cell death as opposed to differentiation of TS cells (Fig. 17C). This is supported by TUNEL-staining analysis which demonstrated that TS cells grown in both IGF-I and FGF-4 conditions had the highest levels of TUNEL-positive cells (Fig. 9B). Furthermore, this hypothesis was supported by experiments in which the TS cell system was replenished with an additional dose of IGF-I, 24 hours after initial treatment with both IGF-I and FGF-4, and a 2.2–fold increase in PL-1 positive cells was observed (Fig. 18B), thereby demonstrating that IGF-I alone functions to influence the differentiation of TS cells, and when grown in combination with FGF-4, stress was induced which could be reduced by replenishing the system with an additional dose of IGF-I.

4.1.5. IGFs and Self-Renewal of TS Cells

OCT4 is a POU family transcription factor responsible for the maintenance of pluripotency in stem cells (144). It has been implicated in the formation of TS cells from human ES cells, as its downregulation in human ES cells yields spontaneous formation of TS cells, suggesting that OCT4 inhibits formation of trophoblast development (146). Although this may be the case in human ES cells, recent studies on mouse ES cells demonstrated that long-term culture of mouse ES cells can allow for reprogramming of the developmental potential by which cells form blastocyst-like structures express trophoblast-specific genes (183). This was thought to be caused by cells harboring "pre-

TS cell" markers which are precursors of TS cells and have the potential to develop into the trophoblast lineage (184). This result suggests that mouse ES cells can serve as progenitors for TS cell and that mouse ES and TS cells may not be as developmentally programmed and directed as previously thought. Since OCT4 has been shown to be expressed in TS cells of the bovine and human origin (149;150), and our TS cell line was shown to express OCT4 under conditions of standard growth conditions (Fig. 12), this suggests that our cell line may possibly be an early committed line, not yet showing a full TS-phenotype.

Investigation into the effects of IGF-I and IGF-II on the self-renewal of TS cells by examining levels of OCT4 and SOX2 markers, and the trophoblast-specific marker, CDX2, demonstrated that the addition of either IGF-I or IGF-II alone restored self-renewal capacity to TS cells, with IGF-II inducing a stronger effect. OCT4 and SOX2 are known to work synergistically in order to regulate the expression of a variety of pluripotent-specific genes (156). Interestingly, our data showed an unbalanced expression of self-renewal markers (OCT4 and SOX2) in TS cells treated with either IGF-I or IGF-II. Both the IGF-I and IGF-II restored nuclear OCT4 expression, with IGF-II inducing a longer effect, but neither restored nuclear expression of SOX2. Although unusual, this observation was confirmed by immunocytochemistry analysis, which showed the presence of SOX2 predominantly within the cytoplasm, regardless of the treatment. The analysis of CDX2 expression, a notable transcription factor which is critical in formation of TS cells (163), demonstrated that IGF-II alone prolonged the expression of CDX2, while IGF-I did not. Immunocytochemistry demonstrated that IGF-II maintained TS cells in the trophoblast lineage longer than IGF-I.

An interesting observation was evident at 48 hours post-starvation, upon the addition of FGF-4, in either the presence of IGF-I or IGF-II: nuclear OCT4 expression was restored (Fig. 12-15). This event was unusual since the expression was not restored until 48 hours post-starvation. Since the addition of FGF-4 in the presence of either IGF-I or IGF-II induced the highest level of cell death by apoptosis (Fig. 9), this upregulation of OCT4 expression at 48 hours post-starvation may be an ultimate self-defense mechanism induced by TS cells before cell death. Interestingly, IGF-II in the presence of FGF-4 induced lower levels of cell death compared to IGF-I, and this corresponded with an extended expression of OCT4 up to 72 hours post-starvation (Fig. 12-15). Since this effect was different from IGF-I, it suggests that IGF-II can better protect TS cells against cell death. Closer examination by EMSA showed that the highest OCT4 DNA-binding activity was detected at 48 hours in both cells treated with IGF-I or IGF-II in combination with FGF-4 (Fig. 16A). The addition of FGF-4 did not, however, have an effect on the expression of either CDX2 or SOX2, as no increase in nuclear CDX2 or SOX2 expression was observed in either IGF-I or IGF-II treated cells (Fig. 13-15). Based on our data, OCT4 expression is stronger in TS cells and more sensitive to the IGF treatment. FGF-4 does not appear to have a direct interconnected or additive effect, and the upregulation of OCT4 in cells treated with both IGFs and FGF-4 may be a result of a defense mechanism induced by the onset of cell death.

4.1.6. Role of IGF-I/II in TS Cell Maintenance and Development

IGF-II appeared to be well-defined and more specific in determining TS cell fate when compared to IGF-I, which was significantly influenced by the presence of FGF-4. In the presence of IGF-II and FGF-4, a small increase was observed in the percentage of PL-1 positive cells compared to IGF-II alone (Fig. 17), suggesting that IGF-II, with or without FGF-4, plays a role in the maintenance of TS cells. Replenishing the TS cells system with an additional dose of IGF-II, 24 hours after initial treatment, resulted in a 2.6-fold decrease in PL-1 positive cell numbers (Fig. 18), demonstrating that IGF-II is more fundamental in the maintenance of TS cells. The outcome of IGFs on the fate of stem cells appears to depend on its temporal and spatial expression, especially IGF-I. IGF-I alone influenced the differentiation of TS cells into trophoblast giant cells, while FGF-4 induced cell death. Interestingly, it appears that the cell may induce a self-defense mechanism by upregulating the expression of OCT4 just prior to the onset of cell death. Whether this is a result of stress, or a regulated process remains unknown at present. Further investigation into the downstream signaling pathways activated by IGF-I and IGF-II in TS cells will help in delineating the mechanisms by which different IGFs trigger different cellular fates.

4.1.7. Future Directions

Numerous published reports, like this study, were focused on identifying the growth factors and the conditions that control the self-renewal or the differentiation of stem cells. However, little is known about the cell-surface receptors involved in self-renewal control by growth factors. A recent study showed that human ES cells grown in

mouse fibroblasts conditioned medium required IGF-1R signaling for their self renewal (185). Though this was not studied in stem cells of the placental-origin, this study provided direct evidence demonstrating that IGF-IR is important in maintaining self-renewal in stem cells, suggesting that IGF-I and IGF-II, are likely candidate ligands controlling this fate. Nevertheless, IGF-IR is the major receptor used by IGF-I and IGF-II during mouse development, however several studies have suggested that an additional receptor, besides IGF-IR and IGF-IIR, mediates the actions of IGF-II in the placenta (73;139;186). Most likely, the IR or an IGF-IR/IR hybrid receptor may induce the differential effects to the TS cell system. Investigation into the mRNA levels of IGF-IR, IGF-IIR, and IGF-IR/IR hybrids could provide a better understanding of the effects of IGFs on the fate of TS cells.

The effects of IGF-I and IGF-II on the fate of TS cells can be studied at many different levels: the cell cycle effectors, cell surface receptors, gene expression and transcription factor activity. The key IGF-IR downstream effectors that regulate the self-renewal of TS cells are most likely important. A recent study suggests that a transcription factor, ETS2, is essential for TS cell self-renewal (187). ETS2 is a direct target of CDX2, which contributes to the regulation of multiple genes important in maintaining the undifferentiated state of TS cells. Another downstream target responsible for regulating differentiation within the placental trophoblast lineage is *Alkbh1*, a member of the AlkB gene family. This factor is important in regulating epigenetic events during early development by methylating and ethylating DNA and RNA substrates (188-190). Another study on *Alkbh1* shows that this molecule plays an important role in the differentiation of TS cells into all cell types of the trophoblast

lineage, particularly trophoblast giant cells (191). Analysis of Alkbh1 levels in our TS cell system may provide further information regarding the roles of IGF-I and IGF-II on the differentiation of TS cells.

This study utilized a variety of different experiments to investigate the effects of both IGF-I and IGF-II on the fate of TS cells. We conclude that IGFs indeed have an important role in TS development. Further work is required to better understand how the effects of IGFs influence differentiation, as well as lineage specification into the ICM and TE. A full understanding of the timing, molecular interactions and pathways responsible for the formation of the trophoblast lineage will help to decipher many of the questions that arose from this study. This study has hopefully provided further information regarding the effects of the IGF and FGF system on the fate of TS cells, as well as their role on the TS cell niche.

SUMMARY AND CONCLUSIONS

1. Based on data from Annexin-V and TUNEL staining which examined the effects of IGF-I and IGF-II on TS cell death, we conclude that:

   i) IGF-II had a greater role in protecting TS cells from cell death compared to IGF-I, which was found to be significantly different ($p < 0.03$)

   ii) The addition of FGF-4 in the presence of either IGF-I or IGF-II significantly increased the percentage of TUNEL-positive cells

   iii) Both growth factors work better independently of FGF-4, with IGF-II alone inducing the greatest level of protection against apoptosis

   iv) The addition of FGF-4 likely induced a stress to the TS cell system.

2. Based on expression and quantitative analysis of the giant cell marker, PL-1, which examined the effects of IGF-I and IGF-II on TS cell differentiation, we conclude that:

   i) IGF-I induced a significant increase in the percentage of PL-1 positive cells , relative to IGF-II treated cells ($p < 0.0001$)

   ii) In the presence of FGF-4, the addition of IGF-I caused a significant decrease in the percentage of differentiated cells relative to cells grown strictly in IGF-I

   iii) Replenishing cells with an additional dose of IGF-I (24 hr after initial treatment with IGF-I and FGF-4) caused an increase in PL-1 positive cells

   iv) IGF-I likely controls the switch between differentiation and apoptosis of TS cells, as IGF-I alone induces differentiation, while the addition of FGF-4 induces cell death, which can be reduced by replenishing the system with an additional dose of IGF-I.

3. Based on self-renewal and trophoblast marker expression, which examined the effects of IGF-I and IGF-II on the self-renewal of TS cells, we conclude that:

   i) Both the addition of IGF-I and IGF-II restored nuclear OCT4 expression, with IGF-II inducing a longer effect, while the expression of SOX2 was not restored

   ii) IGF-II alone prolonged the expression of CDX2

   iii) OCT4 expression is stronger in TS cells and more sensitive to IGF treatment

   iv) Treatment with FGF-4 did not appear to have a direct interconnected or additive effect, and the upregulation of OCT4 at 48 hours may be a result of a defense mechanism induced by the onset of cell death.

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