AMERICAN UNIVERSITY OF BEIRUT

ASSESSMENT OF DIFFERENT TROPHOBLAST CELL LINES AS IN VITRO MODELS FOR TROPHOBLAST DEVELOPMENT

by
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"We must find time to stop and thank the people who make a difference in our lives." ― John F. Kennedy

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AN ABSTRACT OF THE THESIS OF

Joanna Nabil Barrak for Master of Science
Major: Physiology

Title: Assessment of different trophoblast cell lines as in vitro models for placental development

Background: The placenta is a temporary organ during pregnancy that is involved in insuring optimal growth and development of the fetus. Since the 1980s, there has been a growing interest in the isolation of villous trophoblasts from human placenta for primary culture. Although very interesting, isolated primary trophoblasts have the disadvantage of being extremely difficult to maintain in culture which motivated investigators to generate villous and extravillous trophoblastic cell lines. Nevertheless, when aspiring to extrapolate results they obtain in the trophoblastic cell lines, the scientific community must be cautious as to whether these cell lines are truly representative of the physiologic setting.

Aim: Our study raises questions regarding the validity of using the choriocarcinoma cell lines (BeWo, JEG-3, JAR) vs. the extravillous cell line (HTR-8/SVneo) as *in vitro* model systems for human trophoblast studies.

Methods: Immunofluorescence staining was used to investigate the expression of CK7, E-cadherin and vimentin in BeWo, JEG-3, JAR and HTR-8/SVneo cell lines. RT-PCR allowed the measurement of mRNA levels of CK7, EpCAM and vimentin in BeWo, JEG-3, JAR and HTR-8/SVneo while western blots assessed the protein expression of CK7, E-cadherin and vimentin in BeWo, JEG-3, JAR and HTR-8/SVneo.

Results: BeWo, JEG-3 and JAR cell lines all expressed CK7, E-cadherin and EpCAM and did not express vimentin while HTR-8/SVneo cell line showed lower expression of CK7 and E-cadherin and expressed vimentin. Additionally, two populations were observed in HTR-8/SVneo cell line: a CK7+/Vimentin- population vs. CK7-/Vimentin+ population.

Conclusion: Our study indicated that even though BeWo, JEG-3 and JAR cell lines have proved to be epithelial trophoblastic cell lines, HTR-8/SVneo has been disqualified in that regard and results based on this cell line should be controlled in primary trophoblast models.

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LIST OF ABBREVIATIONS

CTB:	Cytotrophoblast
STB:	Syncytiotrophoblast
EGF:	Epidermal Growth Factor
GM-CSF:	Granulocyte-Macrophage Stimulating Factor
LIF:	Leukemia Inhibitory Factor
TGFβ1:	Transforming Growth Factor Beta 1
hCG:	Human Chorionic Gonadotropin
HPL:	Human Placental Lactogen
SV40:	Simian Virus 40 large T antigen
HLA:	Human Leukocyte Antigen
evCTB:	Extravillous Cytotrophoblast
vCTB:	Villous Cytotrophoblast
CK:	Cytokeratin
PECAM:	Platelet Endothelial Cell Adhesion Molecule
NCAM:	Neural Cell Adhesion Molecule
EpCAM:	Epithelial Cell Adhesion Molecule
ECAD:	E-cadherin
VIM:	Vimentin
IF:	Immunofluorescence
EMT:	Epithelial-to-Mesenchymal Transition

CHAPTER I

INTRODUCTION

A. The placenta: Role and structure

The placenta is a temporary organ during pregnancy that is involved in insuring optimal growth and development of the fetus. It delivers oxygen and nutrients to the fetus, removes carbon dioxide and waste products and is also responsible for metabolizing many substances and protecting the fetus against many xenobiotics and maternal diseases. Being devoid of nerves, the placenta assumes an endocrine role to establish communication with the fetus. It releases hormones and growth factors such as estrogens, progesterone, chorionic gonadotropin, placental lactogen, placental growth hormone, among others, thus influencing the pregnancy or parturition and adapting to the metabolic needs of the developing fetus.

The placenta is composed of a fetal part called the chorionic plate and a maternal part called the basal plate (Gude et al., 2004)**.** Between these two plates lies the intervillous space containing the chorionic villi. Within the core of the villi runs the fetal blood, which is separated, from the maternal blood running in the intervillous space by only three or four cell layers called the placental membrane (Moore & Persaud, 2003). The cell layers of this membrane are the maternal facing syncytiotrophoblast, a layer of cytotrophoblast cells, connective tissue of the villus and the endothelium lining the fetal capillaries (Gude et al., 2004)**.**

B. The implantation process

To further understand the placenta, we must comprehend its development. Within 24 to 48 hours of ovulation, fertilization of the ovum occurs in the fallopian tube where the resulting zygote continues traveling and dividing mitotically to form the morula (which enters the uterine cavity 5 to 6 days after fertilization (Red-Horse et al., 2004). The hallmark of conversion from morula (16–32 fetal cells) to blastocyst (32–64 blastomeres) is the manifestation of a fluid-filled inner cavity known as the blastocoelic cavity (Figure 1) (Watari et al., 1996). At this stage, the differentiation of cells is occurring: Cells at the surface of the blastocyst are polarized and become the trophectoderm giving rise to the extraembryonic structures including the placenta whereas the inner cells which are unpolarized become the inner cell mass or embryoblast which gives rise to the embryo proper (Red-Horse et al., 2004).

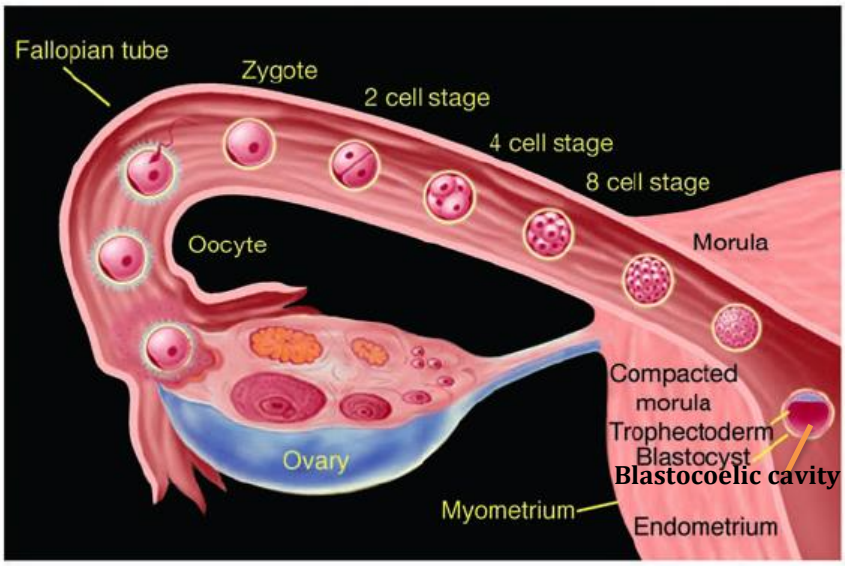


Figure 1: Stages of human development from fertilization to blastocyst formation. Figure modified from S.S. Gambir & J. Strommer, Stanford University (Stanford, California, USA).

Around 6 days after fertilization, the blastocyst emerges from the zona pellucida and implants into the uterine wall (Norwitz et al., 2001; Paria et al., 2002). As implantation of the blastocyst occurs, the endometrium witnesses a series of cellular and vascular changes known as the decidual reaction. Even though the function of the decidua is still uncertain, it has been suggested that its main role revolves around restraining the invasiveness and migration of trophoblasts (Loke & King, 1995). Implantation depends on trophoblast lineage differentiation (Figure 2) (Bischof & Irminger-Finger, 2005; Kunath et al., 2004). After attachment of the blastocyst, its trophoblast cell layer proliferates rapidly and differentiates to obtain an inner layer of cytotrophoblasts and an outer layer of multinucleated syncytiotrophoblasts (Gude et al., 2004) which are the only cells able to penetrate through the uterine epithelium and are formed by the intercellular fusion of cytotrophoblast cells (Gauster et al., 2013). The syncytiotrophoblasts have the capacity to sink below the endometrial surface and invade its connective tissue (Boyd & Hamilton, 1970). From this point on, the chorionic villi begin appearing due to the proliferation of the cytotrophoblasts, which create evaginations within the syncytiotrophoblasts (Moore & Persaud, 2003).

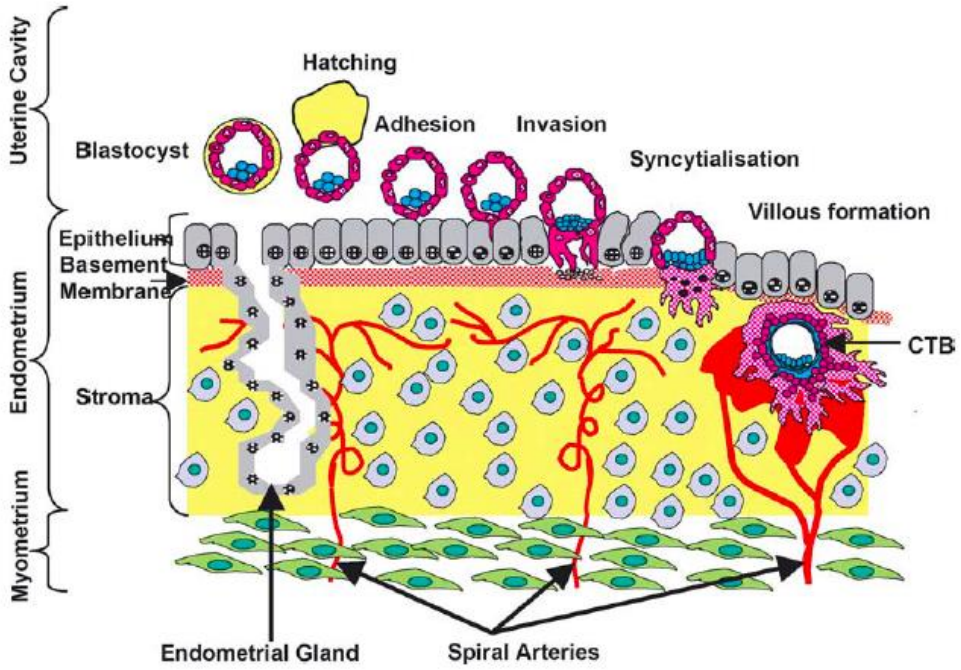


Figure 2: Implantation steps of the human blastocyst. Modified from: Bischof & Irminger-Finger, 2005.

C. Cell types of the placenta

Placental villi are composed mainly of three layers, each with different cell types: Bathing in maternal blood is the syncytiotrophoblast/cytotrophoblast layer covering the entire surface of the villous tree. The core of the villi is formed by stromal or mesenchymal cells containing specific macrophages known as Hofbauer cells. Finally, fetal capillaries and vascular cells are found within the placenta and include vascular smooth muscle cells, perivascular cells and endothelial cells (Wang & Zhao, 2010).

1. The Trophoblast cell

At the end of the 19th century, A.A.W. Hubrecht, a Dutch scholar, introduced the term "trophoblast" (Hubrecht, 1899, cited by Boyd & Hamilton, 1970). The term "trophoblast" (from Greek trephein 'to feed' and blastos 'germinator') designates the cells that are derived from the blastocyst and which do not constitute any parts of the embryo but are necessary for its growth (John & Hemberger, 2012). Different types of trophoblasts, all derived from the trophectoderm layer of the blastocyst, are present in the placenta, the amniotic membranes as well as the decidua (Boyd & Hamilton, 1970). During implantation, the trophectodermal cells differentiate into cytotrophoblasts and syncytiotrophoblasts (Porter & Lane, 2003). Syncytiotrophoblasts do not undergo replication since they are the most differentiated and specialized type of trophoblast. Their store is replenished by the continuous differentiation and fusion of the cytotrophoblasts (Gauster et al., 2013).

2. The Syncytiotrophoblast

The syncytiotrophoblasts are mainly responsible of the metabolic, immunological and endocrine feto-maternal interactions. At 10 weeks of pregnancy, the developing embryo begins obtaining its nourishment from the maternal blood through the syncytiotrophoblasts. Maternal glucose, amino acids and lipids are transferred across the syncytiotrophoblasts towards the fetal capillary endothelial cells (Gude et al., 2004). Since the syncitiotrophoblasts are in direct contact with maternal blood, they contribute to the formation of biochemical barriers to protect the fetus. Furthermore, they function as an endocrine organ by secreting growth factors and hormones that regulate the fetal and maternal homeostasis and the activity

of the trophoblasts (Wang & Zhao, 2010).

The molecular mechanism behind CTB fusion was linked to phosphatidylserine since antibodies to phosphatidylserine inhibited STB formation (Adler et al., 1995). It has also been shown that the presence of serum (human or fetal bovine) in cultures (normoxic conditions) of isolated CTB induces syncytialization and lack of serum lead to poor or no syncytialization (Morrish et al., 1997; Kao et al., 1988). In addition, hormones such as hCG (Yang et al., 2003), estradiol (Cronier et al., 1999), glucocorticoids (Cronier et al., 1998) and cytokines such as EGF (Dakour et al, 1999) or GM-CSF (Garcia-Lloret et al., 1994) have been found to stimulate syncytialization. On the other hand, STB formation is blocked by LIF (Nachtigall et al., 1996), endothelins (Cronier et al., 1997) and TGFβ1 (Morrish et al., 1991).

3. The mesenchymal cells

The placental villi are formed mainly of a stromal core surrounded by one or two layers of trophoblast cells. The stromal core is formed of mesenchymal cells and fetal blood vessels. These mesenchymal cells are derived from the extraembryonic mesoderm and they are responsible of maintenance and structure of the placental villi (Haigh et al., 1999). Furthermore, the Hofbauer cells and the endothelial cells are derived from these mesenchymal cells (Ringler et al., 1990).

Two morphologies have been reported in the villous mesenchymal cells: they either resemble large fibroblasts or smaller reticular cells (Castellucci & Kaufmann, 1982, 1984; Jones & Fox, 1991; Benirschke & Kaufmann, 1995). These cells are responsible of a wide range of functions: they produce much of the villous extracellular matrix, generate

channels in the stroma that become the residence of the Hofbauer cells and are able to differentiate into myofibroblasts and vascular smooth muscle (Kohnen et al., 1996). Furthermore, they may have paracrine control over the trophoblastic epithelium (Garcia-Lloret et al., 1994; Aplin et al., 1999).

D. The villous and extravillous pathways

Once the implantation has succeeded and the placentation has begun, trophoblasts start to proliferate and differentiate along two pathways: villous and extravillous (Figure 3) (Bischof & Irminger-Finger, 2005; Gude et al., 2004). First, small intrasyncytial vacuoles appear within the syncitiotrophoblasts and form a lacunar system where each lacunae is separated from the other by trabeculae of syncytiotrophoblast which are invaded by cytotrophoblasts approximately 12 days after conception (Benirschke et al., 2006). At this point, the syncytiotrophoblasts dismantle the maternal capillary walls and one day later, the cytotrophoblast proliferation and fusion into syncytiotrophoblasts leads to longitudinal trabecular growth within the lacunar system and then forms a primitive villous tree thereby transforming the lacunar system into the intervillous space. The extraembryonic mesenchymal cells invade the primitive villi two days later and then the two-layered epithelium, the syncytiotrophoblast and underlying cytotrophoblasts, cover the placental villi and thus form the villous trophoblast population (Gauster et al., 2013). As mentioned earlier, the decidual cells undergo many changes as they enlarge and proliferate and become attached to the anchoring placental villi. In these villi, cell columns are formed due to the proliferation of the cytotrophoblasts within the syncytiotrophoblasts. The dynamics in these columns are such that the daughter cells of the cytotrophoblasts lying on the basal membrane will be pushed forward

by the new generation of cytotrophoblasts until they detach from the anchoring villi and lose their villous trademark to become extravillous trophoblasts. The extremely proximal parts of the cell columns consists of actively proliferating extravillous trophoblasts that are morphologically similar to the villous trophoblasts but express different markers. The extravillous trophoblasts are mostly located in the distal parts, which have stopped replicating and are acquiring an invasive phenotype allowing them to detach from the columns. They can either invade the decidual interstitium to become interstitial trophoblasts that replace the glandular epithelium (Moser et al., 2010) and anchor the fetus to the mother or they can invade the maternal spiral arteries on their way to the decidua and differentiate instead into endovascular trophoblasts dislocating the maternal endothelial cells in order to distend the arteries and guarantee appropriate blood flow to the fetus (Burton et al., 2009). The villous and extravillous trophoblasts are phenotypically different: the villous trophoblasts are epithelial stem cells characterized by their immobility, polarization and ability to differentiate into syncytiotrophoblasts while extravillous trophoblasts are characterized by being differentiated unpolarized, motile and invasive epithelial cells that either phenocopy endometrial fibroblasts in the case of interstitial trophoblasts or endothelial cells in the case of the endovascular trophoblasts (Bischof & Irminger-Finger, 2005).

E. Models of cell culture used in placental research

1. Isolation of cytotrophoblasts

In our laboratory and many other laboratories around the world, there is a growing interest in the isolation of villous trophoblasts from human placenta to study placental

development and function. The isolation of villous trophoblast was first established in the late seventies (Jurjus et al., 1979); it relies on subjecting the placental villi to consecutive trypsin digestions (Orendi et al., 2011).

Higher purity (80%) of villous trophoblasts was obtained when Kliman et al. further developed the isolation procedure by establishing the use of the Percoll gradient (Kliman et al., 1986). Later on, some laboratories began using magnetic beads for further purification purposes (Douglas & King, 1989).

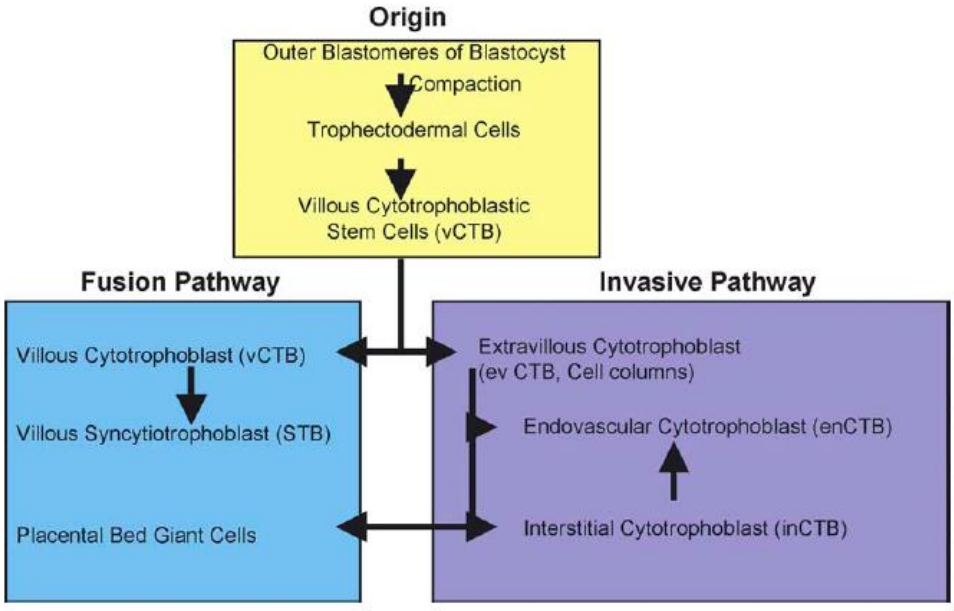


Figure 3: Trophoblast differentiation pathways. Modified from: Bischof and Irminger-Finger, 2005.

2. Trophoblastic cell lines

Although very interesting, isolated primary trophoblasts have the disadvantage of being extremely difficult to maintain in culture (Orendi et al., 2011). They no longer proliferate under normoxic conditions, divide poorly under hypoxic conditions (Bischof & Irminger-Finger, 2005) and tend to spontaneously syncytialize in culture. Thus, the study of syncytialization and the factors associated with the release of syncytial fragments became the most common use for these cells (Orendi et al., 2011). In order to overcome this handicap, investigators were motivated to generate stable and proliferating trophoblastic cell lines. However, one must keep in mind that these cell lines are not to be blindly considered as replicas of primary trophoblasts because they have been subjected to processes that extend their cellular lifespan (spontaneously or premeditatedly through transfection) and that may very well distort the patterns of gene expression in comparison to trophoblasts *in vivo* or *in vitro*.

In this study, four cell lines were used: HTR-8/SVneo, BeWo, JEG-3 and JAR. BeWo, JEG-3 and JAR are choriocarcinoma cell lines. Choriocarcinoma is a malignant, trophoblastic and aggressive cancer of the placenta (Liu et al., 2011). In 1959, Hertz was able to isolate cells from an autopsy of a cerebral metastasis of a choriocarcinoma, which he then transplanted to the cheek pouch of a hamster. Over the period of 8 years, the choriocarcinoma was maintained through 304 serial transfers in hamsters (Hertz, 1959). The BeWo cell line was then established once cells from the tumors were removed from the cheek pouches and cocultured with decidual tissues (Pattillo & Gey, 1968). BeWo cell line became the most widely used cell culture model to mimic villous trophoblasts and mirror by excellence the majority of the villous trophoblast features. BeWo cells were found able

to form syncytiotrophoblasts by means of fusion (Gauster & Huppertz, 2010; Rote et al., 2010), regulate syncytin 1 and 2 (Vargas et al., 2009) and secrete hormones such as hCG, hPL, progesterone and estradiol (Wolfe, 2006). On the other hand, BeWo as well as other choriocarcinoma cell lines suffer the drawback of their long existence. Different strains of BeWo have been described with different fusion rates as well (Orendi et al., 2011). Due to serial cloning of BeWo, JEG-3 cell line was derived and thus shares the same DNA profile with BeWo. When the choriocarcinoma cells were implanted into the hamster cheek pouch, six clones including JEG-3 were derived from these cells. Then, irradiated feeder layers of human fibroblasts were used to propagate them. JEG-3 releases hCG and somatomammotrophin and progesterone and is also capable of transforming steroid precursors to oestrone and oestradiol (Pattillo & Gey, 1968). JAR cell line was also derived from a trophoblastic tumor of the placenta (Pattillo et al., 1971). The HTR-8/SVneo is an extravillous trophoblast cell line that was obtained when a physiologic extravillous trophoblast cell was transfected with a plasmid containing the simian virus 40 large T antigen (SV40) (Graham et al., 1993).

F. Trophoblast cell line markers

Trophoblastic cell lines fall within three categories according to their sources: They are either derived from normal tissue, from malignant tissue or from a fusion between primary cells and choriocarcinoma cell lines. In order to assess the trophoblastic nature of these cell lines, markers such as hCG, hPL, cytokeratins, vimentin and HLA class I molecules were used (King et al., 2001). Subsequently, investigators found that hCG and hPL were not reliable markers for trophoblasts since numerous cancerous cell lines, which

were not trophoblastic, expressed hCG and hPL. Next, a meeting of the European Placenta Group outlined the criteria that could be followed to determine whether a cell line was villous or extravillous (King et al., 2001). While evCTB should be positive for cytokeratin 7, human leukocyte antigen-G (HLA-G), and CD9, vCTB cell lines should be positive for CK7, but negative for HLA-class I and CD9 (Bischof & Irminger-Finger, 2005).

As mentioned earlier, the placenta is formed mainly of trophoblast cells and stromal cells. The trophoblast cells are of epithelial origin while the stromal cells are of mesenchymal origin. Both cell types express a specific set of markers such as adhesion molecules and cytoskeleton proteins. The expression of these markers in the different trophoblast cell types is summarized in table 1.

Table 1: Markers of trophoblasts vis-à-vis adhesion molecules and cytoskeleton markers. Modified from: Bischof & Irminger-Finger, 2005.

Category	Marker	vSTB	vCTB	evProximal	evDistal	evInterstitial	endCTB	References
Adhesion molecules	Vcadherin	-	-	-	+	+	+	Damsky & Fisher (1998)
	Ecadherin	+	+	+	-	-	-	Damsky & Fisher (1998)
	PECAM	-	-	-	+	+	+	Damsky & Fisher (1998)
	NCAM (CD56)	-	-	-	-	-	+	Damsky & Fisher (1998)
Cytoskeleton markers	CK 7	+	+	+	+	+	+	Blaschitz, Weiss, Dohr, & Desoye (2000)
	Vimentin	-	-	-	-	-	-	Shorter et al. (1993)

+: presence, -: absence.

Besides microfilaments and microtubules, keratins (cytokeratins, CKs) are intermediate filaments that represent a major constituent (80% of total protein content) of the cytoskeleton of epithelial cells thus impacting cellular polarity and shape (Fuchs, 1983; Pekny & Lane, 2007). Their expression is dependent on the cell type and the differentiation state of each cell. To date, 54 functional keratin genes are encoded in the human genome, in addition to 13 keratin pseudogenes. They are divided into epithelial and hair keratins (Schweizer et al., 2006). Epithelial keratins are formed of heterodimers of type I keratins, comprising 17 acidic keratins (CK9-CK28) and type II keratins including 20 basic keratins (CK1-CK8, CK71-CK80, CK81-CK86). (Er Rafik et al., 2004). Keratins are present in many maternal-fetal interface cells including trophoblasts (Khong et al., 1986; Beham et al., 1988; Neudeck et al., 1997; Haigh et al., 1999). However, unlike other keratins (table 2) and within the confines of the placenta, CK7 is only expressed in the trophoblast cells, which makes it a perfect marker to identify trophoblasts as a starting point (King et al., 2001). CK7 was chosen as a suitable marker over CK8/CK18 since the latter were detected also in mesenchymal cells isolated from human first trimester placenta (Haigh et al., 1999; Blaschitz et al., 2000).

Cell-cell adhesion is a major trademark of epithelial cells since it is directly related to cell polarity, which in turn influences cell migration, proliferation and differentiation (Schnell et al., 2013). Cell-cell adhesion is mediated by junctions where transmembrane glycoproteins interface with the cytoskeleton (Van Roy & Berx, 2008). One major cell-cell adhesion molecule is E-cadherin which is involved in the differentiation of trophoblasts and remodeling during pregnancy. It is believed that the loss of E-cadherin expression is linked to the achievement of an invasive profile (Liu et al., 2011).

Another epithelial marker is the cell adhesion molecule EpCAM. It is a homotypic calcium-independent cell adhesion molecule and a carcinoma-associated antigen that is also expressed on most normal epithelial cells (Schnell et al., 2013) and thus serves as a good epithelial marker.

On the other hand, vimentin is a ubiquitous marker of mesenchymal cells. It is a type III intermediate filament expressed in normal mesenchymal cells (Steinert et al., 1981) where it maintains the cell's integrity and resistance to stress (Satelli and Li, 2011).

Table 2: Keratin expression in different types of trophoblasts. Modified from: Gauster et al., 2013.

Type of trophoblast	Keratin expressed	Reference
Villous trophoblast		
Syncytiotrophoblast	7, 8, 13, 18, 19	Muhlhauser et al., 1995
Cytotrophoblast	1, 5, 7, 8, 13, 17, 18, 19	Muhlhauser et al., 1995; Ahenkorah et al., 2009
Extravillous trophoblast		
Cell column		
Proximal extravillous trophoblast	7, 8, 13, 18, 19	Muhlhauser et al., 1995
Distal extravillous trophoblast	7, 8, 18, 19	Muhlhauser et al., 1995
Interstitial trophoblast	5, 7, 8, 18, 19	Proll et al., 1997; Muhlhauser et al., 1995; Ahenkorah et al., 2009
Endovascular trophoblast		
Intramural trophoblast	8, 17, 18, 19	Proll et al., 1997
Intraarterial trophoblast	8, 18, 19	Proll et al., 1997
Endoglandular trophoblast	7	Moser et al., 2010

G. Aim of the study

The placenta is a vital organ for pregnancy maintenance and is responsible of insuring ideal growth and development of the fetus. In hopes of echoing placental physiology and mimicking its functions, trophoblast cell lines derived from normal or malignant cells were developed and became potent tools used in research to understand placental physiology and processes such as implantation, invasion and fusion. HTR-8/SVneo cell line has been largely used in placental implantation and invasion studies and considered as the closest model of trophoblast cells because it has been established by extending the lifespan of physiologic extravillous trophoblasts as opposed to other cell lines derived from choriocarcinomas.

Nevertheless, caution must be exercised when extrapolating results obtained in any trophoblastic cell line since alterations in gene expression has been expected in the trophoblastic cell lines upon *in vitro* cultivation (Bilban et al., 2010). Accordingly, it is essential to reach a clear verdict regarding suitable cell models to study human trophoblasts.

In this context, our study aims to validate the pure trophoblastic nature of the four cell lines: BeWo, JEG-3, JAR and HTR-8/SVneo cell lines.

CHAPTER II

MATERIALS AND METHODS

A. Cell line culture conditions

BeWo cells (ATCC, CCL-98) were a gift from Dr. Julie Lafond (University of Quebec at Montreal, Canada) and cultured in F-12K medium to which 10% heat inactivated Fetal Bovine Serum (FBS) (Gibco) and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich) were added. The cells were maintained in a T75 cm^2 flask. The cell line was incubated under standard conditions at a temperature of 37ºC, 5% CO2 and humidified atmosphere. At confluence, they were sub-cultured using a trypsin-EDTA solution to detach the cells.

JEG-3 (ATCC, HTB-36) and JAR (ATCC, HTB144) cell lines were obtained from Dr. Cathy Vaillancourt (INRS, Quebec, Canada) and cultured in Minimum Essential Medium Eagle (MEM) (Sigma-Aldrich, Steinheim, Germany) to which 10% heat inactivated FBS (Sigma), 1% Penicillin Streptomycin-P/S, 1% Non-essential Amino acids-NEN, 1% Sodium Pyruvate and 1% L-Glutamine were added. The cells were maintained in a T75 cm^2 flask. The cell line was incubated under standard conditions at a temperature of 37ºC, 5% CO2 and humidified atmosphere. At confluence, they were sub-cultured using a trypsin-EDTA solution to detach the cells.

HTR-8/SVneo cell line, first developed by Dr. Charles Graham (Queen's University, Kingston, ON, Canada) (Graham et al., 1993) was also obtained from Dr. Cathy Vaillancourt (INRS, Quebec, Canada) and cultured in DMEM-AQmedia to which 10% heat

inactivated FBS (Sigma), 1% Penicillin Streptomycin-P/S and 1% Sodium Pyruvate were added. The cells were maintained in a T75 cm^2 flask. The cell line was incubated under standard conditions at a temperature of 37ºC, 5% CO2 and humidified atmosphere. At confluence, they were sub-cultured using a trypsin-EDTA solution to detach the cells.

B. Reverse transcriptase- polymerase chain reaction RT-PCR

mRNA expression in BeWo, JEG-3, JAR and HTR-8/SVneo was analyzed by real-time RT-PCR using the $\Delta\Delta C_t$ method and the SYBR green system. Total RNA was extracted using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma) according to the manufacturer's instructions and then converted into cDNA using the RevertAid First strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer**'**s instructions.

Specific transcripts were quantified by real time RT-PCR (Bio-rad CFX™ manager). Experiments were performed using 2X SYBR Green master mix and human primers *for Vimentin, CK7 and EpCAM* (Table 3). The cycling conditions included a hot start at 95ºC for 10 min, followed by 30 cycles at 95ºC for 10 sec, 58ºC for 30 sec and 72ºC for 1 min. Fold change in gene expression was calculated according to the Relative quantitation method (ΔΔCt). Each sample was analyzed in duplicate. Specificity was verified by melting curve analysis. All the PCR reactions were normalized to the housekeeping gene GAPDH.

Table 3. Oligonucleotide primer sequences and conditions employed for RT.

Primers	Sequence	Annealing T (ºC)
GAPDH	**F: 5'- TGGTGCTCAGTGTAGCCCAG -3'** **R: 3'-GGACCTGACCTGCCGTCTAG-5'**	**58ºC**
CK7	**F: 5'- GGTTTCTGGAGCAGCAGAAC -3'** **R: 5'– AAGTCCTCCACCACATCCTG -3'**	**58ºC**
Vimentin	**F: 5'- ACCAACGACAAAGCCCGCGT-3'** **R: 5'- CAGAGACGCATTGTCAACATCCTGT-3'**	**58ºC**
EpCAM	**F: 5'-CCATGTGCTGGTGTGTGAAC-3'** **R: 5'-ACGCGTTGTGATCTCCTTCT-3'**	**58ºC**

C. Western Blot Analysis

BeWo, JEG-3, JAR and HTR-8/SVneo cells were lysed using RIPA buffer containing 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxylate, 150 mM sodium chloride, 50 mM Tris-hydrochloride, 100 mM EDTA, 1% Tergitol (NP40), and 1% of the protease and phosphatase inhibitors and 1 mM PMSF (100 µL of 100 mM). The lysates were then incubated on ice for 1 hour with intermittent mixing, then centrifuge at 4°C for 15 min at 13,600 rpm. Finally, the supernatant was collected and the pellet was discarded. Proteins concentration in the supernatant was measured using the Bradford Protein Assay. For immunoblotting, 50 µg of proteins were loaded and allowed to migrate by

electrophoresis on 10% Polyacrylamide gel and then transferred to nitrocellulose membranes (Bio-Rad Laboratory, CA, USA) for 2 hours on ice. The membranes were then blocked with 5% low fat milk and then incubated at 4 ºC overnight with primary antibodies: mouse EpCAM (1:1000, cat: 118 202 Biolegend), mouse e- cadherin (1:1000, cat: ab1416, abcam), rabbit E-cadherin (1:1000, cat: 24E10, Cell Signaling Technology), rabbit Vimentin (1:1000, cat: sc7557, Santa Cruz Biotechnology, CA) and mouse CK7 (1:1000, cat: ab9021, abcam). The primary antibodies were detected the second day using horseradish peroxidase–conjugated IgG: Goat anti-mouse (1:2000, sc2031, Santa Cruz biotechnology) and Goat anti-rabbit (1:2000, sc2030, Santa Cruz biotechnology). Finally, the bands were visualized by enhanced chemiluminescence (Roche).

D. Immunohistochemistry, immunofluorescence staining and confocal microscopy

1. Antibodies and Reagents

Antibodies from the indicated manufacturers used in this study were as follows: mouse EpCAM (1:100, cat: 118 202 Biolegend), mouse e- cadherin (1:100, cat: ab1416, abcam), rabbit E-cadherin (1:100, cat: 24E10, Cell Signaling Technology), rabbit Vimentin (1:100, cat: sc7557, Santa Cruz Biotechnology, CA) and mouse CK7 (1:100, cat: ab9021, abcam). Secondary antibodies used are the following: Alexa Fluor 488 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 568 goat anti-mouse and Alexa Fluor 568 goat anti-rabbit (1:100, Invitrogen, CA). Fluoro-gel II with DAPI was purchased from EMS (Electron Microscopy Sciences, PA).

2. Immunohistochemistry

Human term placentas were collected after delivery from 38-40 weeks pregnancies in accordance with the guidelines established by the Institutional Review Board (IRB) of the American university of Beirut Medical Center (AUBMC). Term placental tissues were then collected and fixed in 4% Formalin overnight, rinsed well in PBS and transferred to 70% ethanol before standard processing to obtain paraffin-embedded sections. Unstained tissue sections were deparaffinized, and antigen retrieval was performed in a citrate buffer in a steamer at 100°C for 60 min followed by 30 min incubation at room temperature. Slides were treated with Peroxidase block for 5 min and then blocking was performed with protein block from Leica biosystems (UK) for 5 min at room temperature. Primary antibody incubation with CK7 was performed overnight at 4^{0}C, followed by post primary block for 30 min. Slides were incubated then with Novolink polymer (Leica biosystems, UK) for 30 min followed by incubation with DAB chromogen prepared in Novolink DAB substrate buffer for 5 min. All slides were counterstained with hematoxylin.

3. Immunofluorescence

Double immunofluorescence was performed on tissue sections using the primary antibodies previously described. The secondary antibodies were Alexa Fluor 488 conjugated goat anti-mouse and goat ant-rabbit IgG and Alexa Fluor 568 conjugated goat anti-rabbit and goat anti-mouse IgG. Slides were mounted with the anti-fade Fluoro-gel II with DAPI. For immunofluorescence of cell lines grown *in vitro*, adherent cells were cultured in 12-well plates containing 15 mm cover slips and fixed in 4% paraformaldehyde (PFA) for 15 minutes, followed by permeabilization with 0.05% Triton X-100 for 5 min.

Non-specific sites were blocked by incubation in 0.1% BSA in PBS for 60 minutes. Cells were then incubated overnight at 4°C with the specified primary antibodies diluted in the blocking buffer. Cells were then washed, and incubated with either Alexa Fluor 488 or 568 conjugated goat anti-mouse and goat anti-rabbit IgG in blocking buffer for 60 minutes at room temperature, and finally washed and mounted using the anti-fade reagent Fluoro-gel II with DAPI.

Confocal microscopic analyses were performed using Zeiss LSM 710 confocal microscope and images were acquired and analyzed using the ZEN image software.

E. Statistical Analysis

All experiments are repeated at least 3 times. t-test was used to show significance in the real time PCR results.

CHAPTER III

RESULTS

A. Expression of Vimentin, E-cadherin and Keratin 7 in human term placental sections

The placental villus consists of two cell types: epithelial and mesenchymal. The outer layer of the villus is formed of epithelial trophoblast cells whereas the core of the villus is formed of mesenchymal cells in addition to fetal blood capillaries and vessels. E-cadherin and vimentin are widely used markers of epithelial and mesenchymal cells respectively. In addition, CK7 is an established marker of trophoblast cells (Daoud et al., 2005; Maldonado-Estrada et al., 2004). The aforementioned markers in addition to H&E staining were used to demarcate the different cell types and structures in placental cross-sections. The H&E staining reveals the normal shape of the placental villi containing blood vessels (Figure 4A) and surrounded by a layer of trophoblast cells as indicated by CK7 staining (Figure 4B). In line with the normal histology of the placental villi, the staining with E-cadherin showed that the latter was expressed in the outer layer of the villi whereas vimentin was expressed uniquely in the mesodermal core of the villi (Figure 4C and D).

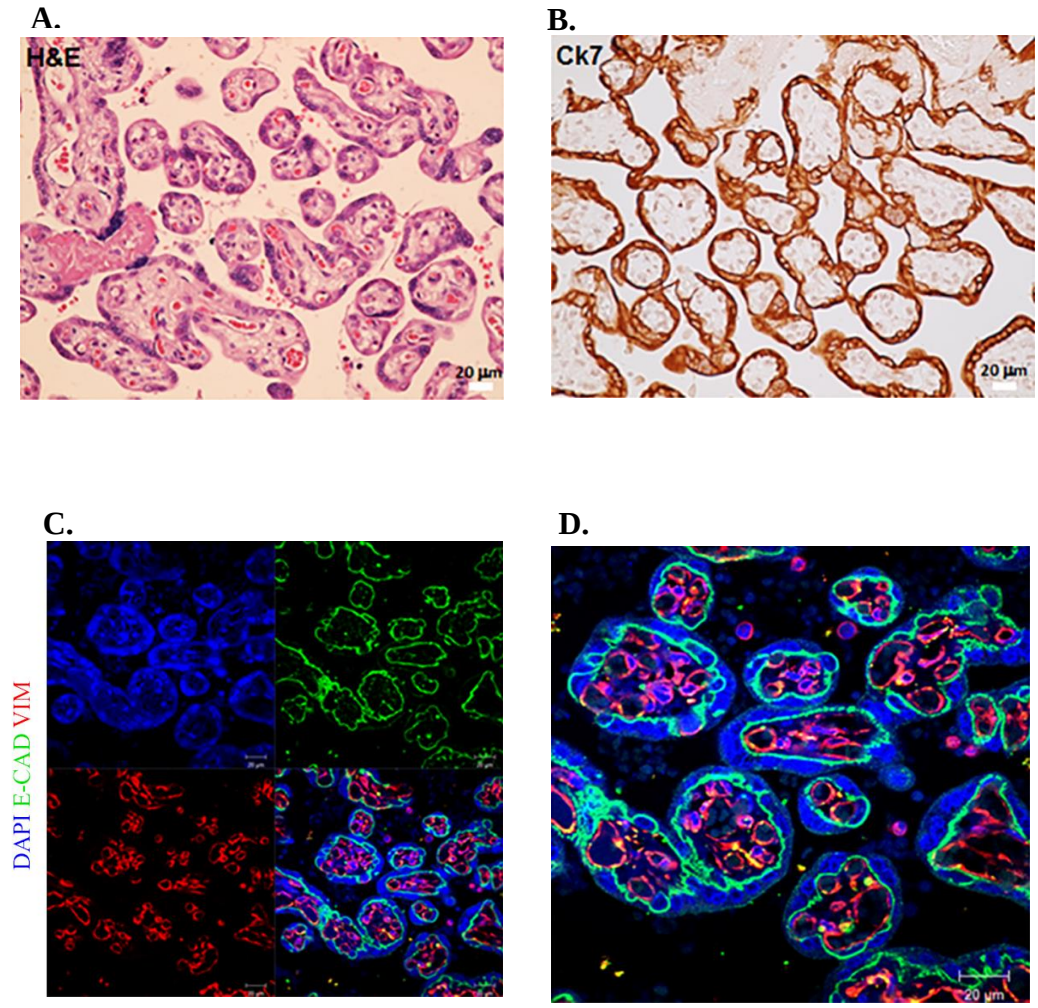
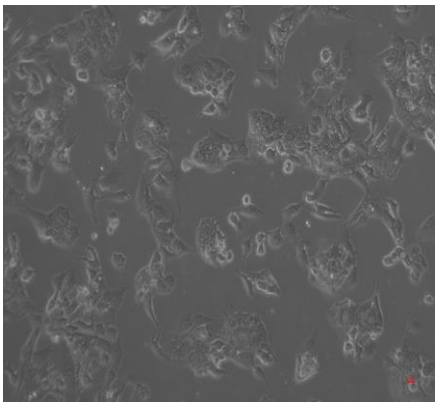


Figure 4. Histology of the human term placenta.
Paraffin-embedded human term placenta sections were stained as follows: H&E (A), immunohistochemistry staining showing positive CK7 expression (B), immunofluorescence with ECAD/VIM/DAPI showing positive ECAD and negative vimentin expression(C). (D) is a higher magnification of (C). Representative images are shown.

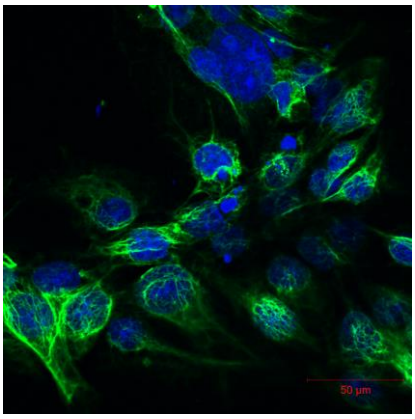
B. Expression of cytokeratin 7 in the trophoblastic cell lines

As previously mentioned, CK7 is an established marker of trophoblasts (Haigh et al., 1999) thus it was necessary to screen the different cell lines for CK7 expression. Our immunofluorescence staining showed that CK7 is expressed in BeWo (Figure 5C), JEG-3 (Figure 5F) and JAR cell lines (Figure 5I). Interestingly, HTR-8/SVneo cells (Figure 5L) were not uniformly positive for CK7 showing some islands of CK7 positive cells. Moreover, HTR-8/SVneo cells showed the presence of two cell types (Figure 5J): one fibroblast-like shape and one a cobblestone epithelial shape while all other cell lines showed a clear epithelial shape.

A.



B.



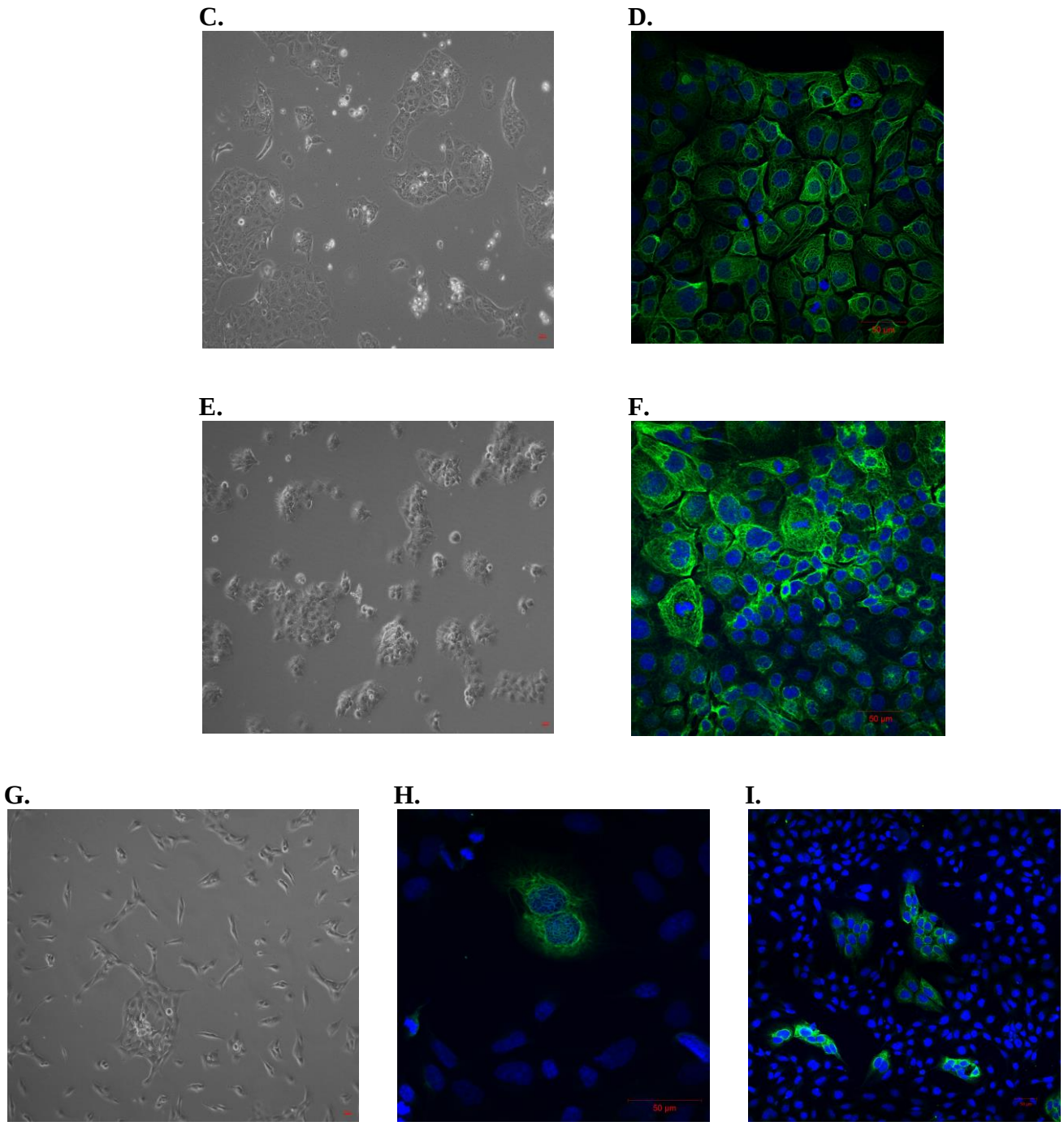


Figure 5. CK7 expression profile in the four trophoblastic cell lines.
Phase contrast images showing the cells in culture: BeWo (A), JEG-3 (C), JAR (E) and HTR-8/SVneo (G). DAPI was used in immunofluorescence staining to counterstain the nucleus of the cells. Immunofluorescence staining for CK7 was positive in BeWo (B), JEG-3 (D) and JAR (E) and HTR-8/SVneo (F). A tile scan of HTR-8/SVneo is shown (I).

C. Expression of trophoblast, epithelial and mesenchymal markers in BeWo, JEG-3, JAR and HTR-8/SVneo using immunofluorescence

Further investigation was required due to the novel result seen in HTR-8/SVneo cell line, which showed as mentioned above, that relatively only few HTR-8/SVneo cells were positive for CK7. We then intended to reassess the trophoblastic epithelial nature of the cell lines by using epithelial vs. mesenchymal markers to detect both cell types. Thus, cells from BeWo (Figure 6), JEG-3 (Figure 7), JAR (Figure 8) and HTR-8/SVneo (Figure 9) were each co-stained with either CK7/Vimentin, CK7/E-cadherin or E-cadherin/Vimentin and the nucleus was counterstained with DAPI. The immunofluorescence staining showed that BeWo (Figure 6A, 6B and 6C), JEG-3 (Figure 7A, 7B and 7C) and JAR cell lines (Figure 8A, 8B and 8C) were trophoblastic and epithelial by excellence and clearly expressed CK7 and E-cadherin with no expression of vimentin. Interestingly, two populations of cells appeared in the immunofluorescence imaging of HTR-8/SVneo cells (Figure 9C, 9D, 9E & 9F): CK7+/Vimentin- and E-cadherin+/Vimentin- cells vs. CK7-/Vimentin+ and E-cadherin-/Vimentin+ cells.

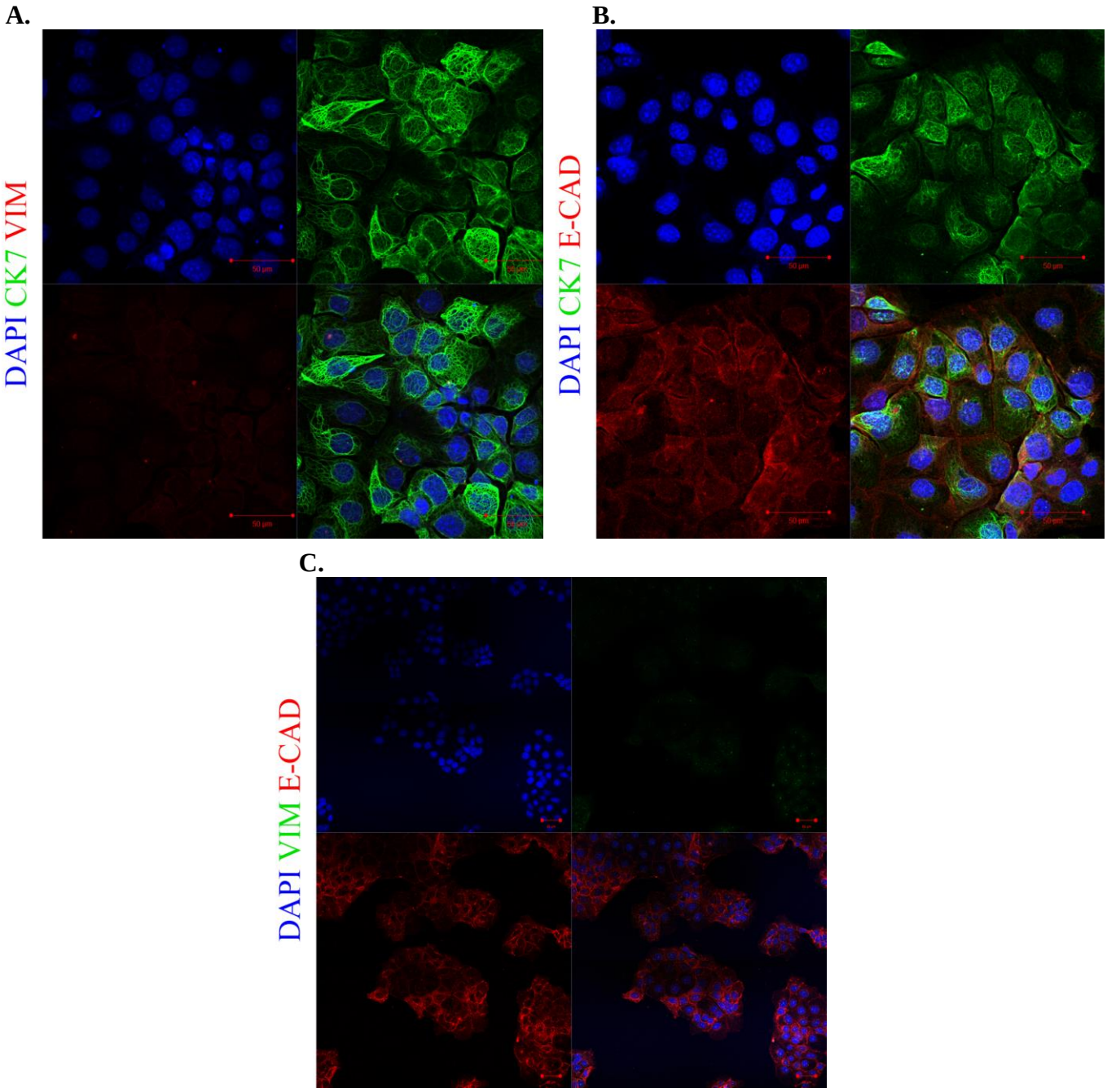


Figure 6. JEG-3 cells express epithelial and trophoblast markers.
Dual immunofluorescence staining of JEG-3 cells shows positive expression of CK7 and negative expression of Vimentin (A), positive expression of CK7 and e-cadherin (B) and positive e-cadherin expression with negative Vimentin expression (C).

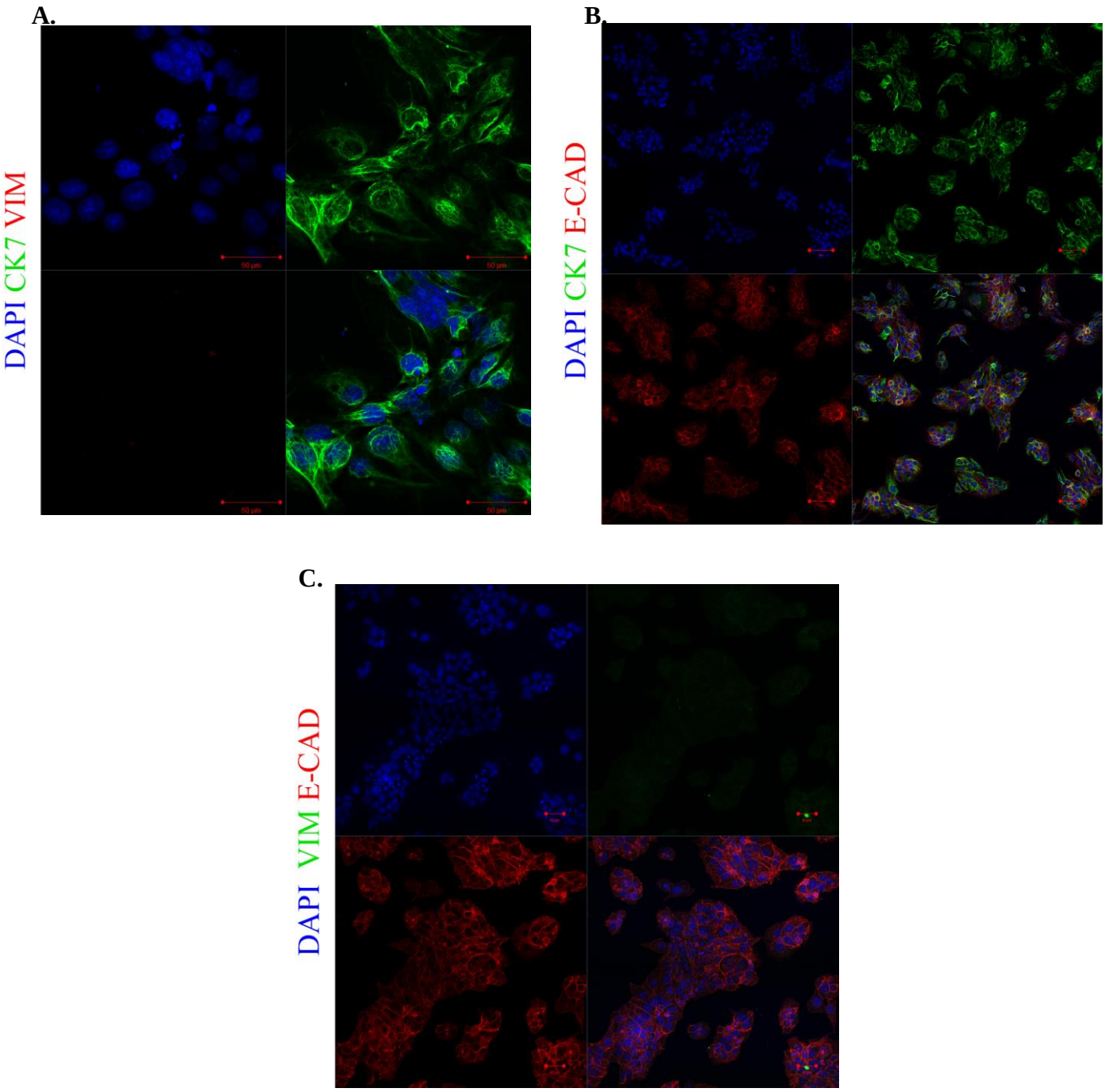


Figure 7. BeWo cells express epithelial and trophoblast markers.
Dual immunofluorescence staining of BeWo cells shows positive expression of CK7 and negative expression of Vimentin (A), positive expression of CK7 and e-cadherin (B) and positive e-cadherin expression with negative Vimentin expression (C).

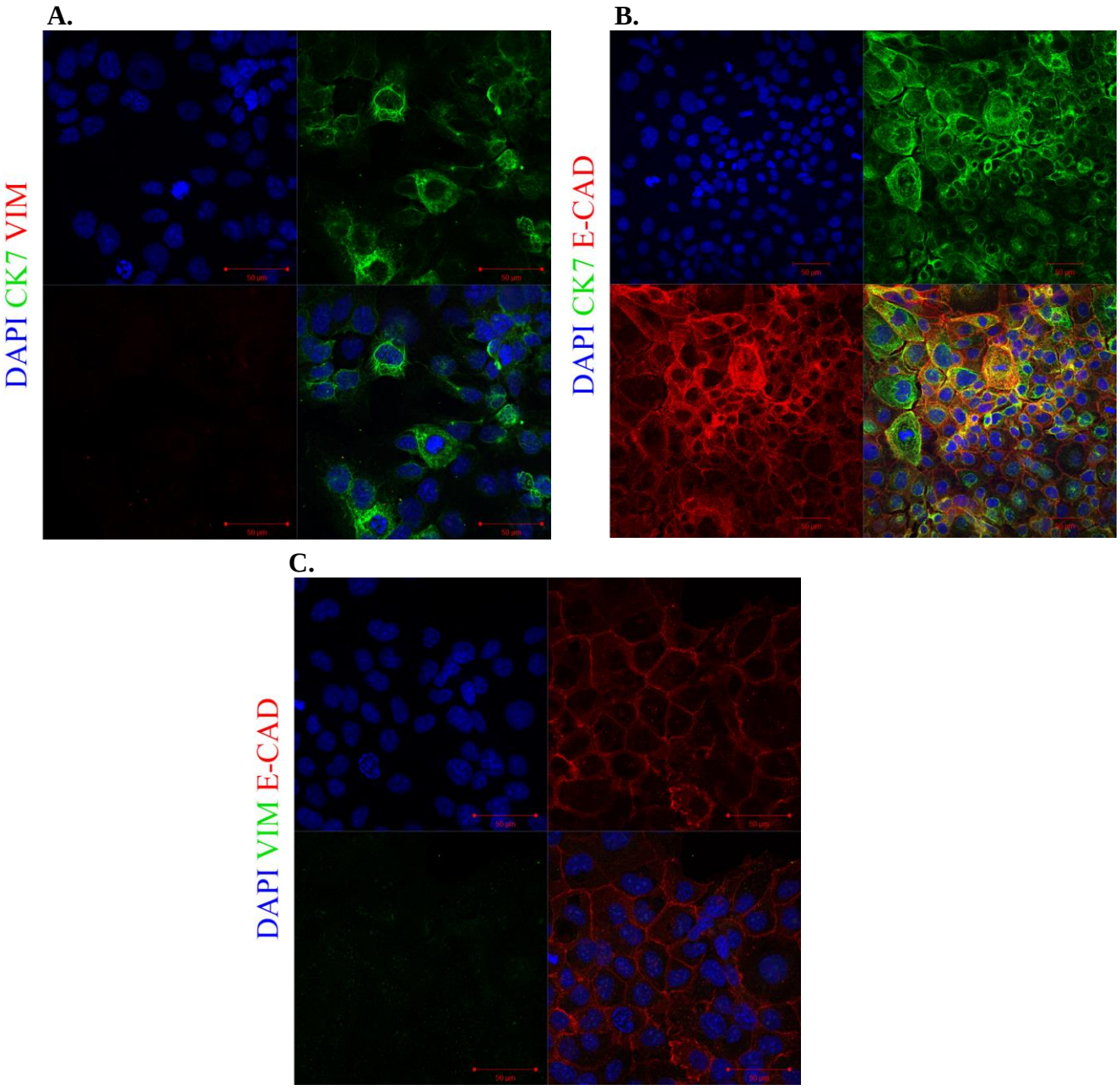


Figure 8. JAR cells express epithelial and trophoblast markers.
Dual immunofluorescence staining of JAR cells shows positive expression of CK7 and negative expression of Vimentin (A), positive expression of CK7 and e-cadherin (B) and positive e-cadherin expression with negative Vimentin expression (C).

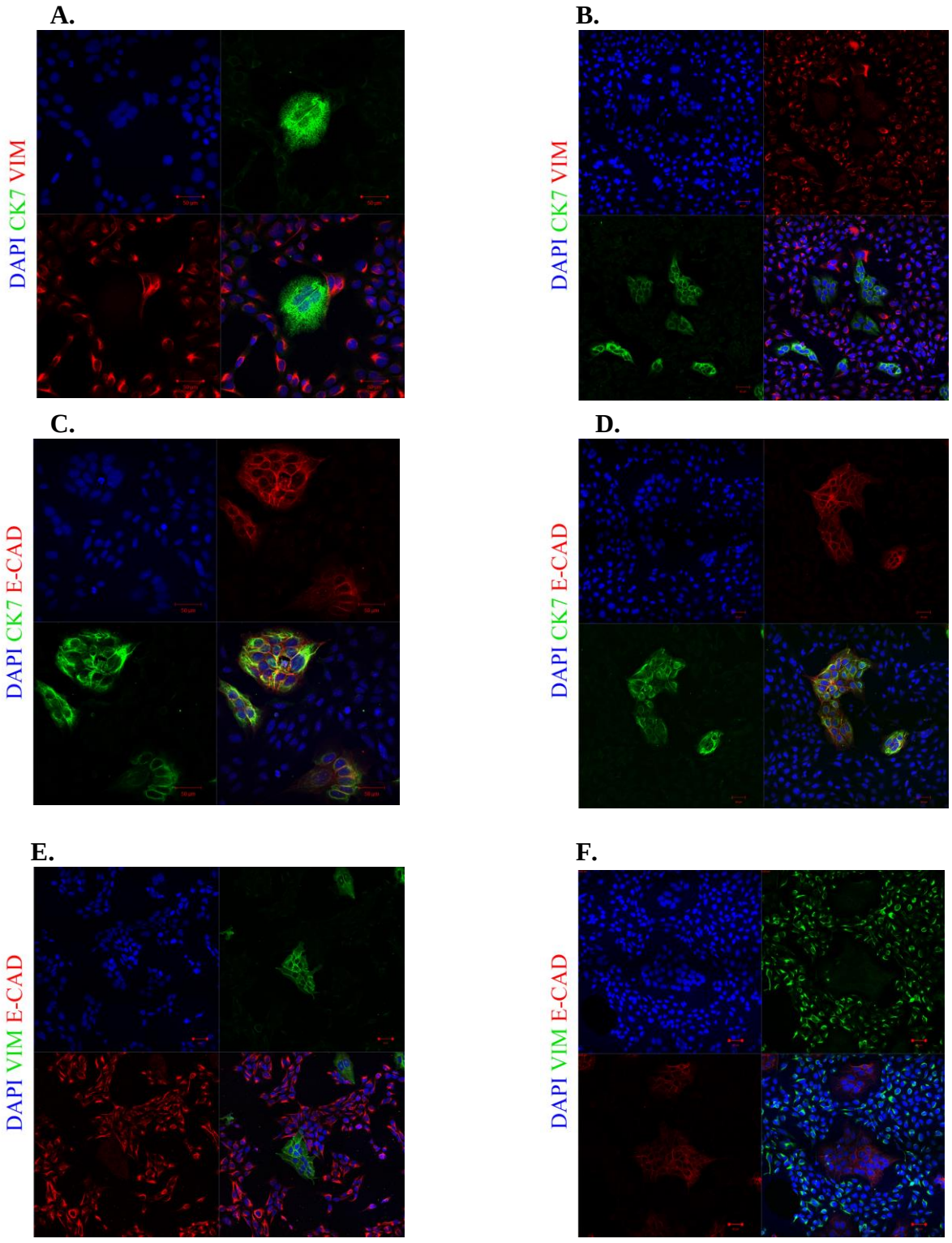


Figure 9. HTR-8/SVneo cells express epithelial and mesenchymal markers.
Dual immunofluorescence staining of HTR-8/SVneo cells shows cells are positive for CK7 and e-cadherin (A & B). Two populations of cells appear in HTR-8/SVneo cells: CK7+/Vimentin- (C & D) and e-cadherin+/Vimentin- cells (E & F) vs. CK7-/Vimentin+ (C & D) and e-cadherin-/Vimentin+ cells (E & F).

D. Expression of CK7, EpCAM and Vimentin in BeWo, JEG-3, JAR and HTR-8/SVneo was evaluated by real time PCR

To further validate our immunostaining results, the RNA expression of CK7, Vimentin and EpCAM was evaluated in all cell lines. Our RT-PCR results showed lower expression of CK7 (Figure 10A) and EpCAM (Figure 10B) in HTR-8/SVneo in comparison with BeWo, JEG-3 and JAR. Interestingly, Vimentin (Figure 10C) was only expressed in HTR-8/SVneo.

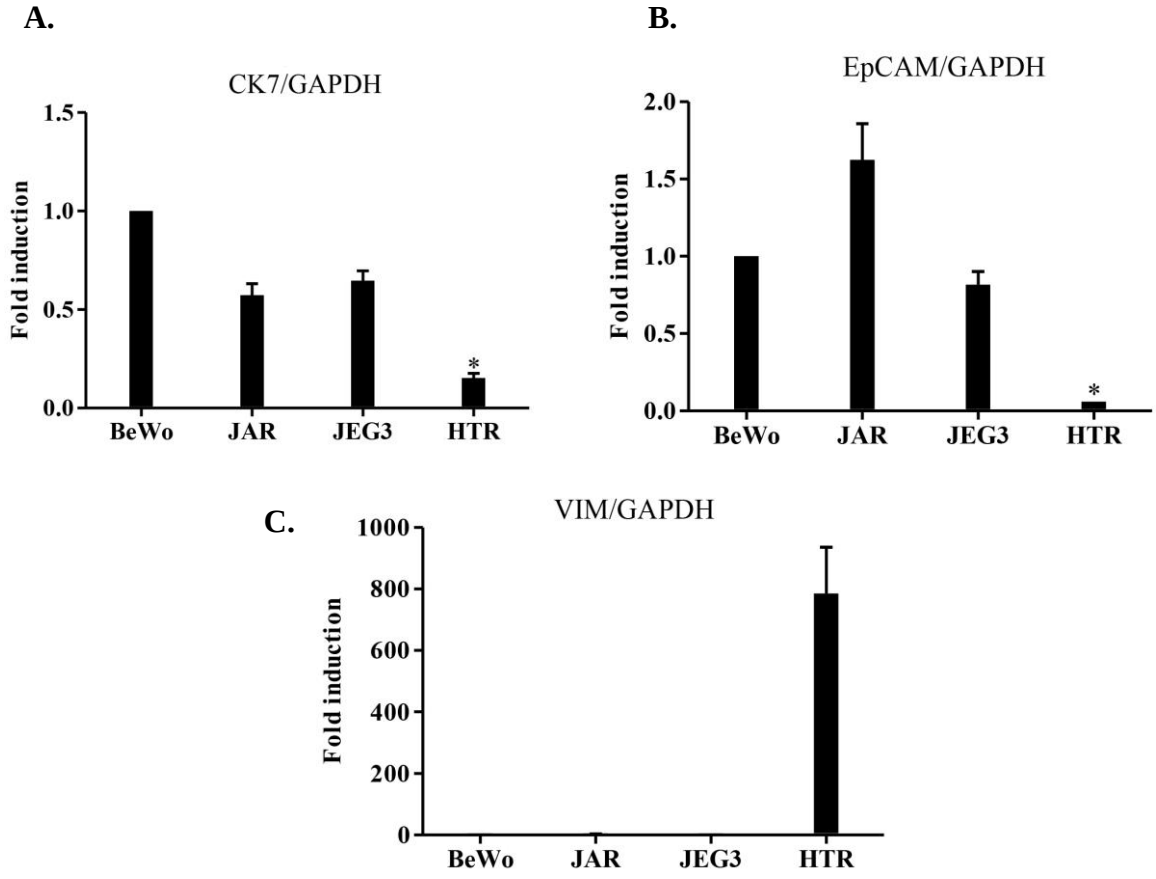
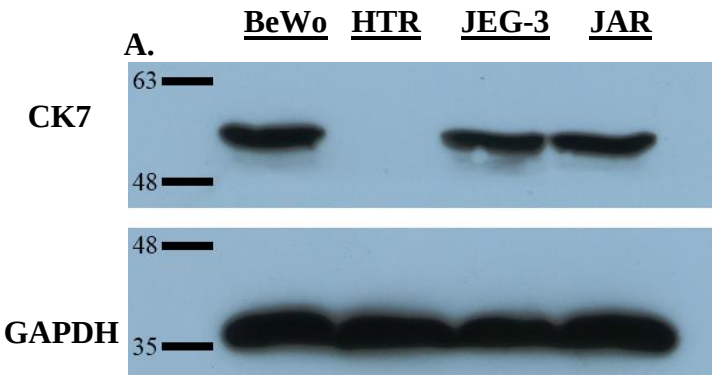
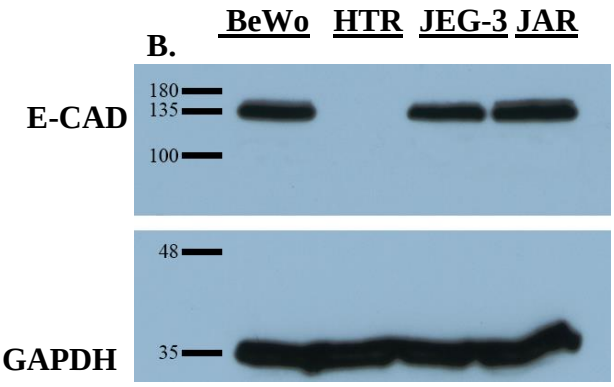


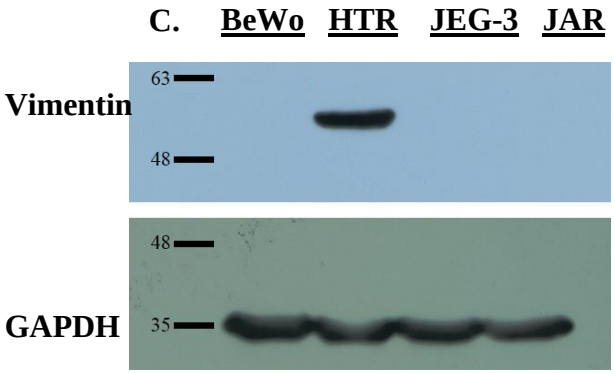
Figure 10. Expression of vimentin, CK7 and EpCAM in HTR-8/SVneo, BeWo, JEG-3 and JAR cell lines was evaluated by real-time RT-PCR analysis.
Fold changes of mRNA level in HTR-8/SVneo cells in comparison with BeWo, JEG-3 and JAR on each transcript are shown: CK7 (A), EpCAM (B) and Vimentin (C). The messenger RNA levels were normalized to that of GAPDH and error bars show standard deviations. *P<0.05 HTR-8/SVneo vs. BeWo, JEG-3 and JAR.

E. Expression of CK7, E-cadherin and Vimentin in BeWo, JEG-3, JAR and HTR-8/SVneo was evaluated by Western blot

The protein levels of vimentin (50 kDa), CK7 (51 kDa) and E-cadherin (135 kDa) were evaluated by western blot. Our results confirmed the expression of the CK7 (Figure 11A) and E-cadherin (Figure 11B) in BeWo, JEG-3 and JAR and lack of expression of vimentin (Figure 11C). On the other hand, HTR-8/SVneo was clearly the only cell line to express vimentin (Figure 11C) and lower CK7 expression in comparison with the other cell lines (Figure 11A). Furthermore, HTR-8/SVneo lacked any E-cadherin expression (Figure 11B). Since E-cadherin signal might have been masked by the strong signal present in the other cell lines proteins, western blot analysis was conducted on HTR-8/SVneo protein samples alone. Results showed positive CK7 expression (Figure 11D) that a faint E-cadherin expression in HTR-8/SVneo (Figure 11E).







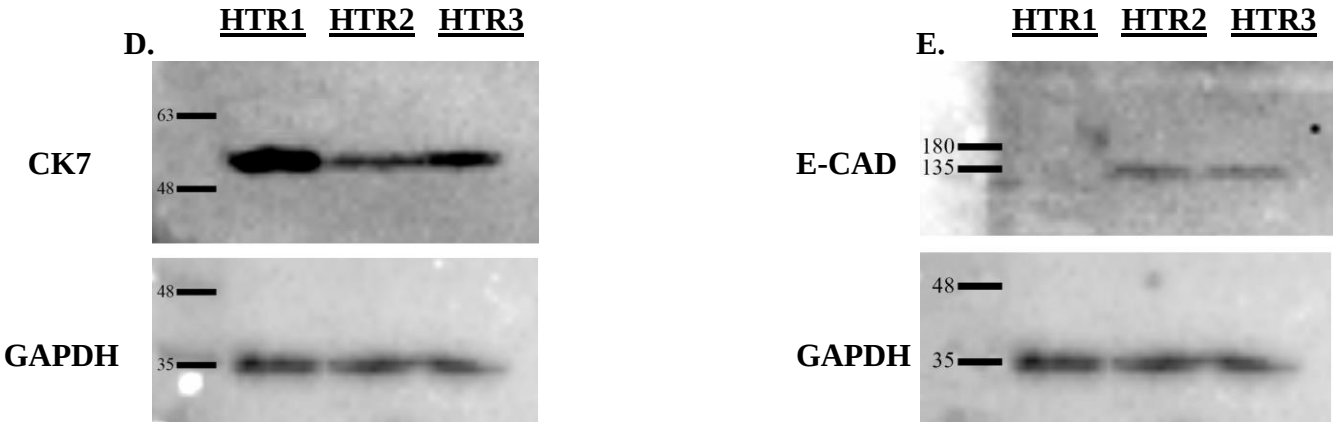


Figure 11. Protein expression of CK7, vimentin and E-cadherin in HTR-8/SVneo, BeWo, JEG-3 and JAR cell lines.

Illustrative western blots of CK7 (A), E-cadherin (B), Vimentin (C) protein expression in BeWo, HTR-8/SVneo, JEG-3 and JAR cell lines (left to right) and repeated western blot of CK7 (D) and E-cadherin (E) protein expression in HTR-8/SVneo. GAPDH was used as a loading control.

CHAPTER IV

DISCUSSION

This study intended to evaluate the adequacy of using the placental cell models (BeWo, JEG-3, JAR and HTR-8/SVneo) to mimic the human trophoblast in the placenta. Originally, HTR-8/SVneo was designed as a first trimester trophoblast cell line with extended lifespan due to its transfection with the simian virus large T antigen. Both parental HTR-8 and transfected HTR-8/SVneo were identical in morphology and characterized as epithelial by cytokeratin 8/18 staining (Graham et al., 1993). While our results showed that BeWo, JEG-3 and JAR, which are all choriocarcinoma-derived cell lines, were epithelial in morphology and gene expression, we found controversial evidence that HTR-8/SVneo cell line, which was derived from physiologic extravillous trophoblasts, was not purely epithelial as was originally believed. Our RT-PCR, Western blot and IF results indicated that even though HTR-8/SVneo cell line expressed CK7, it also expressed vimentin. Interestingly, our IF images clearly exhibited the presence of two populations in the HTR-8/SVneo cell line: a CK7+/Vimentin- population and a CK7-/Vimentin+ population. Thus, this cell line is not homogeneous in nature: both epithelial and mesenchymal cell types are present which might indicate the presence of both trophoblasts and mesenchymal stromal cells. The presence of these heterogeneous populations might be due to multiple factors.

One plausible explanation relates to the possibility that HTR-8/SVneo cells were originally heterogeneous when first isolated. Graham et al. believed that the cell line they established was comprised of transformed pure trophoblast cells from the first trimester

placenta (Graham et al., 1993). Our results suggest the possibility of contamination of the original trophoblast cells with mesenchymal cells during the isolation process. We must point out that Graham et al. originally relied on CK8/CK18 expression to characterize the HTR-8/SVneo cells as epithelial. Subsequently, it was shown that CK8/CK18 were not reliable markers of epithelial cells nor trophoblasts since they were also detected in mesenchymal cells of the human first trimester placenta (Haigh et al., 1999; Blaschitz et al., 2000). As mentioned earlier, HLA-G is a typical differentiation marker of extravillous trophoblasts (Morrish et al., 2002). It was shown that HTR-8/SVneo slightly expressed HLA-G or not at all (Takao et al., 2011) and have less in common with the primary extravillous trophoblast than JEG-3 cells (Morales-Prieto et al., 2012) which further underlines the possibility of HTR-8/SVneo being a heterogeneous cell line of trophoblast and mesenchymal cells. On the other hand, primary trophoblasts are characterized by negative vimentin expression (Blaschitz et al., 2000; Beham et al., 1988; Frank et al., 2001). Thus it becomes clear, based on our results, that HTR-8/SVneo is not a purely trophoblastic cell line.

The second plausible explanation to the characteristics described in HTR-8/SVneo in our investigation is that transfection of this cell line with the simian virus large T antigen has lead to the induction of a mesenchymal phenotype in addition to the epithelial phenotype. Some primary epithelial cultures have been shown to express mesenchymal markers when they were subjected to extension of their lifespans (Davies et al., 2003). While HTR-8/SVneo might have been originally trophoblastic in nature, it might have been subjected to Epithelial-to-Mesenchymal Transition (EMT) upon culture. During EMT, epithelial cells differentiate into mesenchymal cells and thus undergo radical

transformations in shape and motility; they lose their apical-basal polarity and their cell-cell and cell-substrate adhesions (Liu et al., 2011). Morphologically, the transitioning epithelial cells adopt an elongated flattened shape. Additionally, at the level of the cytoskeleton, they change from expressing exclusively cytokeratins to expressing vimentin, which has become the ultimate marker of EMT (Jiang et al., 1997). When expressed within the same cell, keratins and vimentin do not copolymerize but form two separate networks (Moggs & Orphanides, 2001). EMT occurs during normal embryogenesis when cells detach and migrate from site to site (El Shalakany et al., 2006) or during tumorigenesis, metastasis and fibrosis (Fujimoto et al., 2005; Taylor et al., 2009). Interestingly, a study found that vimentin-knockout mice exhibited reduced migratory capacity of epithelial cells (Baulida & Garcia, 2000), which proves the central role of vimentin in EMT in terms of migration and invasiveness of the cells. Interestingly, HTR-8/SVneo express N-cadherin (Bulmer et al., 2012) in addition to vimentin, which was shown in all assays in our study. Thus, it is safe to conclude that HTR-8/SVneo expresses at least two known EMT markers (Pannuti et al., 2010). On the other hand, the lower expression of CK7 in HTR-8/SVneo in comparison to BeWo might be explained by the transition from epithelial to the mesenchymal phenotype due to EMT. It has been shown that extravillous trophoblasts highly express CK7 but this expression is decreased during migration and invasion assays until its expression completely disappears (Muhlhauser et al., 1995). This is consistent with our results and EMT hypothesis whereby CK7 levels are very low compared to BeWo possibly due to the invasive mesenchymal phenotype acquired by HTR-8/SVneo cells. Moreover, the low E-cadherin expression in HTR-8/SVneo in comparison with BeWo cells is possibly due to EMT since it has been shown that in both extravillous trophoblasts and

choriocarcinomas, reduced E-cadherin expression correlates with increased invasiveness (Hohn et al., 1996; Zhao et al., 2010).

Our results showed that HTR-8/SVneo contained two different populations of cells. Therefore, it would be interesting to assess whether HTR-8/SVneo was originally contaminated with mesenchymal cells during the isolation process or whether it is undergoing EMT in culture. Future studies are required whereby cell sorting could be used to separate the two populations in HTR-8/SVneo. The two populations, which are identical in DNA, express different proteins: CK7 and Vimentin. Therefore, it would be very useful to separate them phenotypically by cell sorting or subcloning and to quantify them based on protein expression. Fluorescence Activated Cell Sorting (FACS) is a method that can achieve these goals. By tagging our cells with fluorescent-linked antibodies for CK7 and Vimentin or E-cadherin and Vimentin, we would be able to separate the cells into: epithelial cells that are CK7+/Vimentin- cells and mesenchymal cells that are CK7-/Vimentin+ cells. These two populations of cells can be cultured separately and monitored for EMT. The cells would then be subjected to IF, RT-PCR and western blot to verify whether the epithelial and mesenchymal cells retained their respective phenotype or whether they underwent EMT again. If the cells switch phenotypes, that would suggest that they underwent EMT and that the cells in our study have underwent EMT as well. On the other hand, if these two populations of cells retain their respective phenotypes, it would indicate that they were accidentally isolated in conjunction from the origin.

In conclusion, due to the genetic diversity seen in cell lines in general, any results obtained using placental cell lines are to be verified and controlled in primary models of trophoblast cultures. In particular, using HTR-8/SVneo cell line as an extravillous trophoblast model

should be reconsidered since it contains both epithelial and mesenchymal cells. Finally, with all the drawbacks of using cell lines, they are still very valuable models to study different cellular and physiological events in the placenta. More importantly, the use of these cell lines allows us to better understand the pathogenesis of pregnancy-related diseases such as preeclampsia and gestational trophoblastic disease among others. Studies of key events of placentation and implantation mechanisms including cell differentiation, migration, and invasion are hindered *in vivo* by ethical concerns thus requiring the use of *in vitro* culture and proving once more the cell lines in this study indispensable.

CHAPTER V

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